2. Literature Review

2.1. Infection

Microbial infections may be either in the form of planktonic cells or the close association of the microbial colonies (biofilm). Planktonic cells are highly motile free living microbial cells with high metabolic rate and higher susceptibility for antibiotics. On contrary, biofilm is a close association of microbes which exhibit low metabolism and are highly recalcitrant to the antibiotic therapy also capable of evading human immune response (Spoering and Lewis 2001).

2.2. Biofilm

Biofilm is the dense architecture of the microbial colonies adhered to biotic or abiotic surface in the self-secreted dense extrapolymeric substance (EPS). The biofilm associated microbes imposes the significant resistance, attenuate the inhibitory activity of the antibiotics, severely aggravate the diseased condition and produce the chronic situation. National Institutes of Health (NIH) reported that about 65% microbial infections and 80% of chronic infections are biofilm induced (Jamal, Ahmad et al. 2018).

2.2.1. Biofilm formation

Biofilm formation is complex process that involves the several steps to establish biofilm from the planktonic culture. The biofilm growth may be stimulated by several stimuli such as the expression of the gene, specifically responsible for the biofilm production, in response to the host physiological conditions such as pH, hypoxia, and availability of nutrient and presence of the ions or antibiotics concentration. The various steps leading to the growth of the mature biofilm adaptive to diverse nutritional and environmental conditions are as following;

a) Surface attachment

The planktonic microbes invade the host body and interact with tissue macromolecule by self-secreted substance to initiate the adherence on the tissue surface and thus start the biofilm formation. Also, the solid-liquid interface is perfect condition for the progress and growth that ensures the favorable environment for micro-organism to bind and grow (e.g. blood, water) (McKenney, Hübner et al. 1998).

b) Reversible adhesion

In this step, bacteria start adhesion and prepare for the multiplication by intracellular communication also called the quorum sensing. At the same time, EPS secretion also triggers simultaneously (Soto 2014).

c) Irreversible adhesion

During this step, each cell firmly attach to the surface with stronger bond and multiply fast to increase the microbial cell count and form the microcolonies with parallel substantial production of the EPS to cover the newly formed microbial colonies. The genetic mechanism of exopolysaccharide production is activated when intensity of the signal cross certain threshold. Apart from this, microbes initiate the differentiation of the cells and strengthen the biofilm. Post initial adhesion to the surface, the cells that still have weak bonds with the surface consolidate the adhesion process by high EPS production that leads to an increase in their reproduction rate (Danese, Pratt et al. 2000).

d) Maturation

The maturation step or biofilm maintenance represents the dynamics between the microbial cells and the biofilm architecture. In this step, replication of the microorganisms occurs resulting in the generation of intense communication via

signaling molecules (quorum sensing, QS). Such a mechanism enables cells to communicate and coordinate their actions by the production and detection of extracellular signaling molecules that are called the auto-inducers. Auto-inducers are responsible for the uptake of the substrate into the biofilm. Further, in this step, other microorganisms may also get adhered to form a multispecies biofilm (Danese, Pratt et al. 2000).

e) Dispersal

After biofilm formation, the researchers have often noticed that bacteria leave the biofilms itself on regular basis. By doing this the bacteria can undergo rapid multiplication and dispersal. Detachment of planktonic bacterial cells from the biofilm is a programmed detachment, having a natural pattern. Sometime bacteria are detached from the colony into the surrounding due to some mechanical stress but in most of the cases bacteria stop EPS production and detach thereafter. Dispersion of biofilm cells occurs either by detachment of newly formed cells from growing cells or due to quorum- sensing. The cells which are dispersed form biofilm, as a result of growth, may return quickly to their normal planktonic phenotype.

2.2.2. Quorum sensing

The process of communication between two bacterium by means of the biochemical signaling molecules (auto inducers) is known as quorum sensing. It induces the gene expression and facilitates intra-species and interspecies communication, which further coordinate the cell aggregation, biofilm propagation, bacterial colonization and EPS formation during the nutritional deficiency or the environmental stress conditions (such as disinfectants, antibiotics, metal ions, pH, oxygen free radicles etc.) (Ji, Beavis et al. 1995, Miller and Bassler 2001, Flemming and Wingender 2010).

2.2.3. Biofilm's components

Biofilm is a close aggregation of the microorganisms along with self-produced extracellular polymeric substances (EPS). The proteins, DNA, RNA, polysaccharides (e.g. Alginate), enzymes and water channels are the major components of the EPS. The water channels are responsible for the transport of nutrients and removal of the toxic substances from the micro colonies (Flemming and Wingender 2010).

The EPS matrix is a reservoir which selectively retains the lysed cells and its components including the DNA. This also acts as a nutrient's source and provides a shield to the microbial cells against the host physiological condition including drying, oxidation, exposure to biocides, antibiotics, certain metallic cations, ultraviolet radiations, and immune responses. The production of the matrix represents the successful formation of biofilm communities, and propagation and survival of the cells in their local environment (Lebeaux, Ghigo et al. 2014).

The dense architecture and complex composition of the biofilm restrict the entry of the antibiotics and also escape the host immune response. At the same time, the biofilm producing bacteria are more resistance to antimicrobial therapy as compared to the normal strain due to modification in the cell density.

Tables 2.1: List of biofilm components and their % composition

S. no.	Component	% amount
1	Microbial cells	2-5%
2	DNA and RNA	<1-2%
3	Polysaccharides	1-2%
4	Proteins (enzymes)	<1-2%
5	Water	Up to 97%

a) Role of the DNA and the Alginate in the biofilm

Each biofilm component plays a specific role in the growth and maturation of the biofilm. Amongst the various components, DNA and alginate play a crucial role in the biofilm growth and disease progression. DNA is a most common component of all the bacterial biofilm however, alginate is abundantly found in the biofilm formed by the mucoid *P. aeruginosa* strain specifically found in the cystic fibrosis (Okshevsky, Regina et al. 2015). The main functions of DNA and alginate include:

- **Surface attachment:** Interact with the tissue surface and initiate the microbial surface attachment.
- **Microbial cell aggregation:** Facilitate the accumulation of the microbial cells to increase the cell density in the biofilm.
- **Cohesion of biofilms:** These components often provide the mechanical strength and the rigidity to the biofilm matrix in conjunction with multivalent cations.
- **Protective barrier:** Prevent the penetration or entry of the antimicrobials and the host immune cells deep inside the biofilm, resulting in eliciting the significant antimicrobial tolerance and avoiding the nonspecific and specific host immune response.
- **Horizontal gene transfer:** Responsible for genetic information transfer, which induces the gene expression in other microbial cell or microbial species.
- **Binding of enzymes:** Results in the accumulation, retention and stabilization of enzymes through their interaction with different components.

2.2.4. Implication of the Biofilm

Biofilm formation is responsible for the several complications in the disease, which lead to the severe clinical manifestations. The following are the biofilm mediated

complications related to the various infections (Spoering and Lewis 2001, Lebeaux, Ghigo et al. 2014):

a) Imposes the high resistance to the antibiotic therapy which increases the minimum inhibitory concentration even up to 100-1000 times
b) Develop the chronic disease condition
c) Responsible for inducing the septicemia at the affected site (e.g. wounds)
d) Facilitate the low susceptibility against the host immune response
e) Generate the low grade persistent inflammatory condition due to host immune mediator such as polymorphic nucleocytes.

2.2.5. Mechanism of biofilm resistance for the antibiotics

Several mechanisms have been proposed which are responsible for the high antibiotic resistance of biofilms. These mechanisms include impaired penetration, antibiotic hydrolyzing enzymes, persister cells limited diffusion, heterogeneous functions, biofilm phenotype such as adaptive mechanisms e.g. efflux pump and membrane alteration .

a) Low penetration

Primarily, biofilm matrix is a major physical obstacle for the diffusion of antibiotics to deeper layers of biofilm. The matrix impedes the distribution of the antibiotics in the deep layers of biofilm, leading to the sub therapeutic concentration near bacterial colonies and resulting in antibiotic resistance (Mah and O'Toole 2001).

b) Presence of drug hydrolyzing enzyme

Matrix also contains the enzyme responsible for the cleavage of the drug (example pinicillinase), which inactivates the antibiotics and produces the drug resistance. Additionally, the enzyme may also induce the resistance by antibiotics modification due different biochemical interactions (Anderl, Franklin et al. 2000).

c) Heterogeneous nature

The biofilm embedded bacteria are highly heterogeneous, metabolically as well as structurally, due to different levels of the oxygen in various parts of the biofilm. Microbes present on the biofilm surface are highly susceptible for the antibiotics, while the same microbes within the deep layer of the biofilm imposes high tolerance, perhaps due to low oxygen level, slow microbial growth and different pH conditions.

d) Slow growth rate of microbes

Limited availability of the nutrients and the oxygen generate the slow growth rate in the microbes resulting in forming the microbial mutant having less susceptibility to the antimicrobial drugs (Stewart 2002).

e) Existence of persistent cells

Few microbial cells are metabolically inactive within the biofilm and irresponsive for the antibiotics as most of the antimicrobial agents act on the metabolic pathways. This is an adaptive mechanism for the survival of the microbes under physiological stress and pressure of the host body (Stewart 2002).

f) Biofilm phenotype

Microbes associated with biofilm have different phenotype depending on the host condition. The bacteria synthesize some signaling molecules also called secondary metabolites responsible for enhancing the biofilm formation. Apart from that, depending on the stimuli and gene expression the components in the biofilm matric vary. Predominantly, DNA is the main component of biofilm matrix responsible for the biofilm propagation in most of the microbial biofilm matrix. However, *P. aeruginosa* discharge alginate as a key component of the EPS in cystic fibrosis, specifically after

genetic mutation under certain stressed physiological conditions (Mah and O'Toole 2001).

g) Efflux pumps

Efflux pumps (protein structures expressed either constitutively or intermittently) are responsible for the elimination of the antibiotics from inside the bacteria thereby eliciting the antibiotic resistance. Efflux pumps are responsible for the multidrug resistance including tetracycline, macrolides, fluoroquinolones, β-lactam and thus help in maintaining the concentration up to the low toxic level (Grkovic, Brown et al. 2002).

h) Membrane proteins alterations

Bacterial membrane is highly permeable for the low molecular weight hydrophilic molecules through outer membrane channel proteins (porins), mostly expressed in Gram-negative bacteria. Under the stress or presence of the antibiotics mutation in porins encoding genes result into the production of non-functional or altered proteins leading to the impermeability of the cell membrane (Hancock 1997).

i) Immune response to biofilms

The biofilm also weakens the host immune activity by attenuating the immune cells entry in the biofilm structure and restricting the reach of immune cells to the microbes (Yamada and Kielian 2019).

2.2.6. Biofilm forming bacteria

Almost all the microorganisms have the ability to produce the biofilm over the biological and inert surfaces. The most frequently observed biofilm producing bacterial species includes *Pseudomonas aeruginosa*, *Staphylococcus epidermidis, Escher coli*, *Staphylococcus aureus, Enterobactor cloacae*, *Klebsiella pneumonia, Actenomyces israelii, Haemophilus influenza* and *Burkholderia cepacia.*

2.2.7. Biofilm associated diseases

There are number of diseases associated with the biofilm formation. These may include device related or the tissue related. Various biofilm associated infections are listed below:

a) Device related

- Ventricular derivation
- Contact lenses
- Endotracheal tubes
- Central vascular catheter
- Prosthetic cardiac valve and pace makers
- Breast implants
- Urinary catheters
- Peripheral vascular catheters
- Prosthetic joints

b) Tissue related infections

- Chronic otitis media
- Endocarditis
- Chronic wounds
- Lung infection in cystic fibrosis
- Dental plaque
- Kidney stones
- Osteomyelitis
- Urinary tract infections

2.3. Biofilm prevention and treatment strategies

Microbial biofilm infection's prevention and treatment seem to be insufficient with antibiotic treatment alone, as biofilm enforces number of hurdles for the antibiotic therapy. Furthermore, high dose of antibiotics is required to meet the elevated MIC, which may further result into dose dependent toxicity. Several strategies have been investigated to prevent the biofilm development and more importantly for the eradication of the established biofilm infections.

2.3.1. Preventive approaches

a) Early removal of an unnecessary device

The implanted device with non-significant importance should be removed shortly after their insertion to prevent the microbial contamination and biofilm adherence (Meddings, Rogers et al. 2010).

b) Systemic antibiotic prophylaxis during device insertion

Once the medical devise inserted in the body a prophylactic dosing of the systemic antibiotics should be started as a preventive measure and for reducing the risk of the biofilm growth (Del Pozo and Patel 2009).

c) Antibiotic coating of implanted devices

Prior to the device implantation in the human body, antibiotics can be coated to prevent the microbial attachment and contamination. Darouiche et al. 1999 conducted a potential randomized clinical trial in hospitals in which they coated either minocycline or rifampin, chlorhexidine or silver sulfadiazine on central venous catheters (polyurethane, triple-lumen catheters) to evaluate the comparative microbial adherence. The findings revealed that minocycline and rifampin impregnated catheters efficiently prevented the microbial growth with least infection rate (Darouiche, Raad et al. 1999).

Similarly, use of rifampicin and minocycline impregnated central venous catheters successfully attenuated the infection probability (Falagas, Fragoulis et al. 2007).

d) Inhibiting microbial adhesion

Microbial cell adhesion to the surface is an initial stage of the biofilm growth, therefore inhibiting the cell adhesion may prevent the biofilm growth. The extensive research to prevent the microbial cell adherence has been performed. Some of them are described below;

- The selection of material type and surface play the key role in the biofilm growth. Hence, selection of appropriate material and its surface modification may help in preventing the biofilm adherence. For instances, a study suggested that the susceptibility of the Zirconium oxide surface for microbial infection was significantly lower compared to the pure Titanium (Scarano, Piattelli et al. 2004). Additionally, a study recommended that coating of silicone with material comprising of ester and cyclic hydrocarbon moieties significantly reduced the risks of *in vitro* and *in vivo* attachment of the *S. aureus* and *E. coli (Hook, Chang et al. 2012).*
- A surface coated with antibody releasing polyurethane significantly impeded the microbial adhesion (Rojas, Slunt et al. 2000).
- Microbes produce the adhesins (e.g. curli subunit proteins and type 1 pilus) to interact with surface for starting the colonization and form the biofilm. Interestingly, a molecule called FN075 blocked the production of the curli and type 1 pilus and shown to exhibit the inhibitory activity against the biofilm formation, and also attenuated virulence in a mouse model of urinary tract infection (UTI) (Cegelski, Pinkner et al. 2009).

- Low-molecular-weight mannose derivative known as mannosides effectively prevented the UTI by competitively blocking the adhesins binding receptors (Cusumano, Pinkner et al. 2011).
- Lactoferrin found to have excellent activity to reduce the bacterial surface irreversible adhesion by iron chelation (Ammons, Ward et al. 2011).

e) Quorum sensing inhibitor

Quorum sensing have prominent role in the microbial production, biofilm progression, maturation and the differentiation of the cells by enabling the cell to cell or cell to biofilm communication. Blockage of this communication using certain quenching agent prevented biofilm growth or even facilitated the biofilm treatment. For instance, RNAIII-inhibiting peptide (RIP) reportedly prevented the biofilm formation alone and along with antibiotics also by inhibiting the quorum sensing in the *S. aureus* and *S. epidermidis* (Cirioni, Giacometti et al. 2006). Similarly, N- acyl-homoserine lactones hydrolase potentially diminished the biofilm development in the *P. aeruginosa* infection by inhibiting the molecule acyl-homoserine lactones (AHLs), which is a major quorum sensing molecule and responsible for biofilm growth (Bijtenhoorn, Schipper et al. 2011).

Numerous potential quorum quenching agents have been identified that considerably declined the chances of the biofilm development by inhibiting the quorum sensing. Some of the identified compound includes garlic extract, 1-isothiocyanato- 3-(methylsulfinyl) propane (iberin, from Horseradish), 4,5,9-trithiadodeca-1,6,11-triene-9-oxide, and green tea epigallocatechin gallate (Lebeaux, Ghigo et al. 2014).

f) Vaccination

Vaccination is one of the biofilm preventive measures, which induces the formation of antibodies against bacterial biofilm antigens (such as structures involved in adhesion or

biofilm maturation). To produce the antibiofilm antibody, the predefined group of patients was exposed to the biofilm component for facilitating the immune response and obtains the antibody. Fascinatingly, FimH or components of the P pilus competently induced the in vitro or in vivo immunization in the UTI and found to have the least in vivo colonization in immunized bladder mucosa (Lebeaux, Ghigo et al. 2014).

2.3.2. Treatment strategies to established biofilms

Biofilm matrix is a foremost cause of the complex pathogenicity, chronic disease progression and extreme recalcitrance for antibiotics. Recently, several compounds having the potential to erode the biofilm have been identified. These substances degrade the EPS and clear the physical barrier for penetration of the antibiotics and also for the host immune response, thereby prominently improve the antimicrobial efficacy of the antibiotics. Though the agents efficiently eliminate the matrix, antibiotics are needed to incorporate antimicrobial activity.

These substances include;

a) Enzymes

Enzymes specifically target the crucial components of the EPS and destabilize the biofilm, which enable the faster and effective antimicrobial activity. For example the DNase-I hydrolyze the extracellular DNA essential for the development of the biofilm in many microbial species *viz. S. aureus*, *P. aeruginosa*, *S. epidermidis*, *E. coli* and reduces the cohesiveness of the biofilm (Hymes, Randis et al. 2013). Similarly, the Dispersin B, *a* hexosaminidase produced by *Aggregatibacter actinomycetemcomitans* hydrolyzes poly-*N*-acetylglucosamine abundantly found in the EPS matrix of *the S. epidermidis* and improves the antimicrobial potency of the drugs (Chaignon,

Sadovskaya et al. 2007). Moreover, alginate lyase found to be effective along with the antibiotics for the removal of alginate stabilized mucoid *P. aeruginosa* biofilm explicitly found in the cystic fibrosis (Boyd and Chakrabarty 1994).

I. DNase-I

Deoxyribonuclease I (DNase-I) belongs to the endonuclease family, which predominantly degrades both single-stranded DNA as well as double stranded DNA. This nuclease appears to account for the major nucleolytic activity on DNA in serum and is responsible for the degradation of the majority of circulating DNA derived from apoptotic and necrotic cell death and from neutrophil extracellular trap (Tamkovich, Cherepanova et al. 2006).

- **Mechanism of hydrolysis**

DNase-I preferentially cleave the phosphodiester linkages at pyrimidine nucleotide, producing 5'-phosphate-terminated polynucleotides with a free hydroxyl group on position 3'.

- **DNase as a tool in biofilm control**

DNA hydrolytic efficiency caught the attention of researchers working on the biofilm infection to dismantle the biofilm matrix by hydrolyzing the structural and functional component (eDNA) found abundantly in biofilm. Several studies performed on the biofilm dispersal activity of DNase-I suggested the DNase-I as potential adjunct therapy along with antibiotics for preventing and treating the biofilm infection. Main points of some of the studies investigated the antibiofilm potential of DNase-I are discussed below;

- Swarjes et al. 2013, demonstrated that DNase I enzyme coated on the polymethylmethacrylate surface with the help of linking agent reduced the S.

aureus biofilm adherence up to 95%. While *P. aeruginosa* biofilm formation was declined up to 99% (Swartjes, Das et al. 2013).

- Kaplan et al. 2012 evaluated the antibiofilm efficacy of recombinant human DNase I (rhDNase) against *S. aureus* and *S. epidermidis* and concluded that rhDNase exhibits strong antibiofilm and antimicrobial-sensitizing (increase 4-5 log) activities at clinically attainable concentrations (Kaplan, LoVetri et al. 2012).
- Martins et al. 2011 proved that addition of the DNase-I augmented the susceptibility of amphotericin and fluconazole against the biofilm protected candida albicans (Martins, Henriques et al. 2012).
- Alipour et al. 2009 suggested that co-administration of the DNase-I along with aminoglycosides is essential to prevent biofilm growth and also for eradicating the biofilm of *P. aeruginosa* (Alipour, Suntres et al. 2009)
- The outcomes of the phase II double-blind randomized placebo-controlled trial on 71 patients conducted by Ranasihna et al. *1993* reported that short term aerosolized rhDNase administration in lungs of cystic fibrosis patients significantly improved the impaired pulmonary function keeping lungs physiological structure unaffected (no toxicity) (Ranasinha, Assoufi et al. 1993).
- Similarly, in vitro study performed by Shak et al. 1990 proved that DNase-I reduces the viscosity of the cystic fibrosis patient's sputum containing the *P. aeruginosa* biofilm (Shak, Capon et al. 1990).

II. Alginate Lyase

Alginate lyase is abundantly isolated from various source including algae, marine mollusks, terrestrial bacteria, and even from some viruses and fungi.

- **Mechanism**

Alginate lyase cleaves the *β*- glycosidic bonds of alginate and yield unsaturated oligosaccharides with double bonds between the C4 and C5 carbons of the sugar rings (Wong, Preston et al. 2000).

- **Role in biofilm dispersal**

Alginate is a crucial component and plays a leading role in biofilm cohesion and development mostly in cystic fibrosis. Therefore, to disperse the biofilm, reduce its cohesiveness and dense architecture and consequentially potentiating the antibiotics response on biofilm infection, alginate lyase has been investigated stupendously in conjunction with drugs (Boyd and Chakrabarty 1994).

- Austafa et al. 2006 empirically proved that co-administration of alginate lyase with gentamicin and ceftazidime in mucoid *P. aeruginsa* biofilm significantly enhanced the antimicrobial response (Alkawash, Soothill et al. 2006).
- Maiorana et al. 2015 investigated the effect of alginate lyase on production of EPS of *H. pylori* and suggested that enzyme restricted the matrix production (Maiorana, Bugli et al. 2015).
- The findings of the study conducted by Richard & Schiller 1998 suggested that alginate lyase can be the alternative adjunct therapy to improve the distribution of gentamycin and tobramycin in the mucoid *P. aeruginoa* biofilm, which resulted in enhanced antimicrobial response (Hatch and Schiller 1998).
- Bugli et al. 2012 reported alginate lyase as a potential adjunct therapy along with Amphotericin B for the elimination of the *Aspergillus fumigatus* biofilm (Bugli, Posteraro et al. 2013).

b) Cation chelator

Divalent ions, such as calcium, stabilize the biofilm by providing the strength and cohesiveness to the biofilm. Calcium ions cross link the alginate and extracellular DNA molecules to incorporate the stable 3D structure to the biofilm. Therefore, calcium concentration acts as a limiting factor for the formation of the more stable biofilm. Similarly, iron also performs the role in biofilm integrity and stability. Interestingly, chelating agents such as EDTA and citrate limit the concentration of these ions in the microbial colonies and hampers the formation of the stable structure (Turakhia and Characklis 1989). A study stated that calcium specific chelating agent EGTA instantly removed the biofilm grown on the reactor tube's walls (Lebeaux, Ghigo et al. 2014).

c) Quorum sensing inhibitors

Quorum sensing actively involves in the biofilm growth and its dispersion after maturation. The biochemical substances that carry the information and transduce to the other bacterial population are called as auto inducers. Specifically, *S. aureus* abundantly express the accessory gene regulator during the dispersion. A study has proved that the use of auto inducing peptide induced the biofilm dispersion by initiating the artificial dispersion in *S. aureus* biofilm (Boles and Horswill 2008). Likewise, the addition of the short fatty acid chain (*cis*-2-decenoic acid) associated with quorum sensing of microbes proved to facilitate the dismantling of the established biofilm of gram positive as well as gram negative microbes (Davies and Marques 2009).

d) Eradication of persister cells

Killing the persister cells can be another additional approach to combat the biofilm infection and augment the antibiotics efficacy. Therefore, compounds having the activity against these cells can be identified and tested. Recently, a study suggested that

combination of mannitol or fructose along with aminoglycoside enhanced the uptake of aminoglycoside and its activity by initiating the glycolysis in the persister cells (Allison, Brynildsen et al. 2011). Likewise, a group demonstrated the improved activity against the biofilm by enhancing the silver mediated reactive oxygen species (Morones-Ramirez, Winkler et al. 2013).

e) Bacteriophages

Bacteriophages are well known for their lytic activity against the bacteria. Interestingly, some of the study proposed that bacteriophage can be the better alternative for the treatment of multidrug resistant biofilm. For instances, bacteriophage named PT-6 mediated the *P. aeruginosa* biofilm eradication by enhancing the alginate lyase production (Glonti, Chanishvili et al. 2010).

2.4. Nanoparticles

Nanotechnology, a multifunctional tool with broader prospect, has fascinated the worldwide researchers and extensive research conducted has led us to find the broader and multiple application of nanotechnology in the drug delivery. Delivery system with improved pharmaceutical aspects encapsulating the drug within the nanostructured matrix ranging from 50 -500 nm is generally considered as nano drug delivery carrier. Nano drug delivery system commendably overcomes the limitation of conventional dosage forms (Tablets, capsules, powders, gels etc.) such as low solubility, low bioavailability, frequent dosing, high dose, low stability, higher toxicity, low ophthalmic and skin permeation and many more (Agrawal, Harde et al. 2013, Jain, Sharma et al. 2013, Jain, Harde et al. 2014, Kaler, Mittal et al. 2014, Uchechi, Ogbonna et al. 2014, Agrawal, Aqil et al. 2017).

Advantages

- It improves the solubility of the drugs
- Enhance absorption from gut mucosa and thus the bioavailability
- Provide the sustained drug release
- Reduces the dosing frequency and dose size as well
- Attenuates the systemic toxicity of the drugs
- Targeted drug delivery to the affected site
- Offers higher surface area for attaching the ligands or for surface modification
- Prevents the physiological and environmental degradation of the active constituent
- Have several applications in the regenerative medicine
- Can be linked with diagnostic imaging system

2.4.1. Types of the nanoparticle

Based on the origin the nanoparticles are classified as:

❖ **Organic**

- Polymeric nanoparticles
- Lipid nanoparticles

❖ **Inorganic**

- Metallic nanoparticles (gold, silver and silica nanoparticles etc.)

a) Solid lipid nanoparticles (SLNs)

SLNs are colloidal drug delivery systems in which the matrix is formed by solid lipids, which usually consists of physiologically well-tolerated substances with low systemic toxicity. The structure of SLNs is very similar to nanoemulsions, but their core is formed by lipids that are solid at room temperature. This structural rigidity provides

less mobility to the incorporated drug, which is displayed in terms of a better control during the release of drugs. SLNs comprised of one or more solid lipids and their structure is stabilized by the presence of surfactants. SLNs exhibit the properties of different colloidal carriers. For instance, these are physiologically acceptable compounds like emulsions and liposomes and capable of yielding a controlled release of drugs from lipid matrices such as polymeric nanoparticles. The mentioned advantages include the incorporation of lipophilic as well as hydrophilic drugs, enhance physical stability, demonstrate controlled drug release, better biocompatibility, potential for site-specific drug delivery, better formulation stability, ability to freeze-dry and reconstitute, high drug payload, controllable particle size, avoidance of carrier toxicity, low production cost, and easy scale-up and manufacturing (Müller, Mäder et al. 2000, Müller, Radtke et al. 2002).

b) Chitosan nanoparticles

Chitosan is a biodegradable, biocompatible polymer regarded as safe for human dietary use and approved for wound dressing applications. Chitosan has been used as a carrier in designing of polymeric nanoparticles for drug delivery through various routes of administration. Additionally, chitosan has chemical functional groups that can be modified to achieve specific goals and making it a polymer with tremendous potential applications. Nanoparticles prepared with chitosan and chitosan derivatives typically possess a positive surface charge and mucoadhesive properties and help in adhering in larger proportion to the mucus membranes and release the drug payload in a sustained release manner. Chitosan-based nanoparticles have various applications in non-parenteral drug delivery for the treatment of cancer, gastrointestinal diseases, pulmonary diseases, drug delivery to the brain and ocular infections. Chitosan shows low toxicity both in vitro and some in vivo models. Additionally, the chitosan itself has

antimicrobial activity against the various microbes (Qi, Xu et al. 2004, Grenha, Seijo et al. 2005).

2.4.2. Role of nanoparticles in the infection and biofilm

The nanoparticles deliver the several advantages and subside the pathophysiological manifestation of the biofilm mediated chronic infection when compared to the conventional therapeutics (Cheow, Chang et al. 2011). The nanoparticles;

- Increase the drug uptake by fusing with the bacterial cell membrane
- Improve the antimicrobial activity
- Prolonged the microbial inhibitory activity
- Prevent the drug resistance
- Propel the penetration of the of drug in the biofilm matrix, thus restore the antimicrobial response
- Bypass the efflux pump

2.4.3. Research

- Gnanadhas et al. 2012 unraveled that ciprofloxacin equipped chitosan-dextran nanocapsules proved to be the effective therapeutic tool against the intraphagosomal pathogens, especially Salmonella infections (Gnanadhas, Ben Thomas et al. 2013).
- Abdelghany et al. 2012 enhanced the in vitro and in vivo antimicrobial effects of gentamicin on planktonic- and biofilm based *P. aeruginosa* by fabricating gentamycin laden PLGA nanoparticles (Abdelghany, Quinn et al. 2012).
- Likewise, the findings of the similar study performed by Sabaeifard et al. 2017 recommended the amikacin enriched poly(D,L-lactide-co-glycolide)

nanoparticles as a potential candidate for treatment of planktonic *P. aeruginosa* and biofilm cells as well (Sabaeifard, Abdi-Ali et al. 2017).

- Maya et al. 2012 summarized that tetracycline encapsulated *O*-carboxymethyl chitosan nanoparticles were more effective in killing the intracellular *S. aureus* in HEK-293 and differentiated THP1 macrophage cells compared to tetracycline alone (Maya, Indulekha et al. 2012).
- Ghaffari et al. 2012 published that incorporation of amikacin in solid lipid nanoparticles significantly enhanced the stability and antimicrobial activity.

2.5. Burn wound

A burn is a severe trauma to the skin or other body tissue principally caused by fire, heat, radiation, electricity, or chemicals. The basic symptoms associated with burns are redness, swelling, pain, blistering, scarring, infections and in severe cases, sepsis (https://medlineplus.gov/burns.html). Based on the severity burns are classified as; First-degree or superficial burn restricted to the epidermal layer; Second-degree or partial-thickness burn damages the epidermal layer and part of the inner dermis; third-degree or full-thickness burn damage or destroy the epidermis and dermis of skin and fourth degree or subdermal burn involves destruction of skin layers and extends into the tissue below including fat, tendons, muscle, and bone (DeSanti 2005). In patients with severe burns beyond 40% of the total body surface area (TBSA), sepsis is the main reason for death (Church, Elsayed et al. 2006). The severity of the burns and its recovery depends on the cause of the burn, burn site, patient age, depth of the wound, and total body surface area it covers (DeSanti 2005).

Burns are highly susceptible to infection for numerous reasons such as damage to the epidermis, cutaneous barriers, vascular damage of the skin and impaired immune system. The burned skin is with denatured proteins and lipids provide nutrients and

fertile environment for microbial growth (Sevgi, Toklu et al. 2013). Burn injury persuades several organ dysfunctions results in morbidity and mortality. Hypermetabolism and sepsis are the two systemic problems in burn patients. The hypermetabolic state appears as glucose metabolism with insulin resistance encouraging the hyperglycemic condition and lipid metabolism with an increased lipolysis. Moreover, sepsis is a life-threatening systemic inflammatory response to infection (Abdullahi, Amini-Nik et al. 2014).

Burn wounds are infected with Gram-positive bacteria like *S. aureus*, including methicillin-resistant *S. aureus* (MRSA); *Enterococcus* species, including Vancomycin-resistant species; and Gram-negative bacteria like *E. Coli***,** *P. aeruginosa***,** *Klebsiella* species, *Enterobacter* species, and *Proteus* species, yeasts such as *Candida albicans*. Infection of burnt surfaces with microorganisms causes delay or nonhealing of the wound. Principally, 60% percent of wound infections are associated with biofilms formation. *S. aureus* and *P. aeruginosa* are frequently observed microbes in biofilms and are mainly involved in burn wound infection also (Ramos-Gallardo 2016).

2.5.1. Role of the biofilm in the wounds

Around 60% of the burn wounds are associated with biofilm mediated infections which leads to severe traumatic condition and prevent the effective healing of the wounds. Biofilm develops;

a) Chronic wound condition ascribed to ineffective antimicrobial therapy raised due to high resistance
b) Low grade persistent inflammatory condition due to local immune response or polymorphic nucleocyte
c) Develop the septicemia and pus formation leading to the low antimicrobial response

2.5.2. Stages of burn wound healing

Wound healing is a complicated process consisting of three phase *viz.* inflammation, proliferation, and remodeling.

a) Inflammatory phase

Immune response is initiated by migration of neutrophils and monocytes to the damaged site by vasodilation and fluid extravasation which is further sustained by the collection of macrophages by cytokines. During this phase, pro-inflammatory factors like serotonin, bradykinin, prostaglandins, prostacyclins, thromboxane, and histamine are released into the local wound site. This phase serves by clearing bacteria, dead and damaged cells (Velnar, Bailey et al. 2009).

b) Proliferative phase

In this phase, angiogenesis, collagen deposition, granulation tissue formation, epithelialization, and wound contraction occurs. Platelet-derived growth factors, cytokines are released and cause activation of keratinocytes and fibroblasts. Keratinocytes and fibroblasts migrate over the wound. Fibroblasts further grow and form a new extracellular matrix (ECM) (Kirsner and Eaglstein 1993).

c) Remodeling Phase

Myofibroblasts secretes collagen and elastin along and responsible for initiating the wound contracture. In addition to fibroblast conversion, apoptosis of keratinocytes and inflammatory cells are the key steps in the termination of wound healing and the overall final appearance of the wound closure (Rowan, Cancio et al. 2015).

2.6. Cystic fibrosis

Cystic fibrosis is a life threatening genetic disease primarily characterized by fluid retention, compromised mucociliary clearance and impaired respiratory function leading to the thick, viscous, and sticky mucus. Additionally, it also affects the other body organs including gastrointestinal tract and endocrine glands etc. The cystic fibrosis predominantly occur in the children and reduce the life expectancy significantly (Quinton 1983).

2.6.1. Cause

Cystic fibrosis is caused due to the mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene responsible for the translation of the CFTR protein and the mutation induces expression of nonfunctional protein. The protein, present at the outer membrane of cells in the sweat glands, lungs, pancreas and all other remaining exocrine glands in the body is responsible for movement of halogen ions from the cell cytoplasm to the surface. The impaired chloride transport across the membrane facilitates the production and retention of the thick and sticky mucous in the body cavities.

2.6.2. Sign and symptom

The most common symptoms associated with cystic fibrosis patients are:

- Salty-tasting skin
- Persistent coughing, at times with phlegm
- Frequent lung infections including pneumonia or bronchitis
- Wheezing or shortness of breath
- Poor growth or weight gain in spite of a good appetite

- Frequent greasy, bulky stools or difficulty with bowel movements
- Male infertility

2.6.3. Complications

Cystic fibrosis severely affects many body parts. People who have cystic fibrosis produce thick, sticky mucus that causes problems in the lungs and digestive system. The buildup of mucus in the lungs makes it easy for bacteria to grow and often leads to serious lung infections. People who have cystic fibrosis often have problems with nutrition too because their pancreas does not work properly (Lyczak, Cannon et al. 2002, Flume, Mogayzel Jr et al. 2010).

Frequently observed complications of cystic fibrosis include;

a) **Allergic bronchopulmonary aspergillosis (ABPA)** is an allergic reaction in the lungs to the fungus *Aspergillus*.
b) **Bronchiectasis,** a widening of the airways in the lungs caused by chronic inflammation or obstruction of the airways.
c) **Cancers of the digestive tract,** including the esophagus, stomach, small bowel, large bowel, liver, and pancreas.
d) **Collapsed lung** called pneumothorax, resulting in presence of air in space between lung and chest wall.
e) **Diabetes** due to damage to the pancreas.
f) **Fertility problems:** Leads to the infertility some times.
g) **Gastrointestinal complications,** such as distal intestinal obstruction syndrome (DIOS), in which intestine becomes blocked by very thick intestinal contents.

Another possible complication is rectal prolapse, in which part of the rectum sticks out through the anus.

h) Heart failure because of lung damage.

i) Hemoptysis

j) Kidney problems due to diabetes and some antibiotics, or kidney stones

k) Liver disease or failure caused by blockage of the bile ducts in the liver, which leads to the damaged liver. This may further lead to cirrhosis and a need for a liver transplant.

l) Malnutrition because the pancreas may not make enough enzymes to help digest and absorb nutrients from food.

m) Mental health problems, such as depression and anxiety.

n) Muscle and bone complications, including low bone density and osteoporosis, joint pain and arthritis, and muscle pains.

o) Pancreatitis and low levels of pancreatic enzymes leading to nutritional deficiencies, including low levels of vitamins A, D, E, and K.

p) Pulmonary exacerbations, which are episodes of worsening cough, shortness of breath, and mucus production caused by airway inflammation and blockage from an increase in bacteria in the airways and lungs. These episodes may also cause fatigue, loss of appetite, and weight loss.

q) Salt loss syndrome, in which body quickly loses salt, or sodium and chloride, causing electrolyte and other imbalances.

r) Urinary incontinence, or loss of bladder control.

s) Lung infection and biofilm

Persistent *P. aeruginosa* infection in CF, in association with inherent complication of CF including chronic pulmonary inflammation and progressive impairment to the

pulmonary function, is an underlying reason of morbidity and mortality. Mostly, it is associated with copious biofilm formation in the CF patient's lung and inflicts bigger obstacle to the antibiotic therapy. Moreover, the ability to adopt different phenotypes either as non-mucoid (non-alginate producing strain) or mucoid (hyper alginate producing strain) during biofilm production makes the *P. aeruginosa* infection more resistant to the antibiotic therapy (Oliver, Cantón et al. 2000, Sauer, Camper et al. 2002, Kidd, Canton et al. 2018). Biofilms facilitated bacterial infection, a physical modification in the living environment, has been accepted as most frequent and aggressive mechanism of resistance. Biofilm is a highly antibiotic and immune resistant surface attached (biotic or abiotic) well-organized consortium of microbial colonies that confined within the dense architecture of self-secreted extracellular matrix (ECM). Consequently, antibiotics fails to produce the sufficient concentration (minimum inhibitory concentration) needed in close vicinity of microbial colonies surrounded by ECM (Del Pozo and Patel 2007, T Rybtke, O Jensen et al. 2011, Pang, Raudonis et al. 2018).

2.7. Silver Sulfadiazine

Silver sulfadiazine is wide spectrum antibiotic active against Gram positive as well as Gram negative microbes responsible for the severe infection. It is silver salt of the sulfonamide drug called sulfadiazine (Fox 1968).

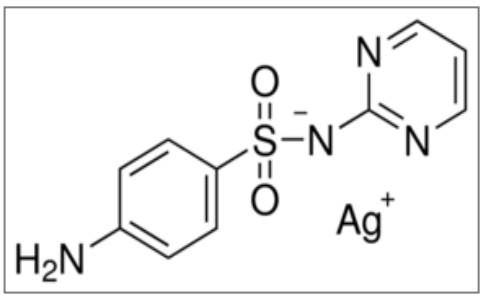

Figure 2.1: Chemical structure of Silver Sulfadiazine

2.7.1. Molecular formula: $C_{10}H_9AgN_4O_2S$

2.7.2. Molecular Weight: 357.136 g/mol

2.7.3. Solubility: Sparingly soluble in water and freely soluble in nitric acid and ammonia

2.7.4. Log P: 0.19

2.7.5. Dose: Marketed as 1% cream, aqueous suspension, and ointment.

2.7.6. Mechanism of Action

Silver products are thought to interact with microbial thiol, carboxylates, phosphates, hydroxyls, amines, imidazoles, and indoles groups in proteins causing protein denaturation and enzyme inhibition. Moreover, binding of silver to base pairs in DNA results in blockage of protein transcription. At the same time, sulfadiazine is a competitive inhibitor of bacterial para-aminobenzoic acid (PABA), a substrate of the enzyme dihydropteroate synthetase, thereby resulting in disruption of folic acid metabolism and ultimately block DNA synthesis (Fox and Modak 1974).

2.7.7. Uses

The SSD is extensively prescribed for the severe burn wound to prevent the risk of infection and also treat existed infections for preventing the septicemia and augmenting the wound healing.

2.7.8. Adverse effects

Fibroblast and keratinocyte toxicity are the major dose dependent toxicity responsible for the delayed wound healing (Khorasani, Hosseinimehr et al. 2009). Moreover, burning, painful sensations, hypersensitivity (rashes or erythema multiforme) and local argyria (discoloration of the skin due to silver) are observed rarely. However, excessive

long term application may cause generalized argyria due to deposition of silver accumulation in kidneys, liver, and retina.

2.7.9. Absorption

The SSD do not show transdermal permeation on topical application except when applied on the large open burn wounds.

2.7.10. Assay Methods

SSD can be readily analyzed using the UV spectrophotometry, Diazotization titration, and HPLC.

2.7.11. Interactions

Proteases such as trypsin and clostridia peptidase, which are contained in ointments used for the removal of dead skin on wounds, can be inhibited by silver ions if applied simultaneously. When silver sulfadiazine is absorbed in significant amounts, it can increase effects and side effects of some drugs such as vitamin K antagonists.

2.7.12. Pregnancy Warning

Topical application of drugs merely have the chance to cause fetus toxicity though, FDA restricted the topical use of SSD in the pregnancy. Recommended the use in special conditions when there are no alternatives and potential benefits may warrant use.

2.7.13. Recent studies on silver sulfadiazine

a) Aloe vera gel containing SSD nanosuspension was developed and proved to have fibroblast cytoprotective action and accelerated wound healing (Barkat, Ahmad et al. 2017).

b) Similarly, another study reported that SSD loaded silk fibroin nanofibers were able to reduce the fibroblast toxicity and accelerate the wound healing (Jeong, Kim et al. 2014).

c) Azevedo *et al.* 2006 has developed potential wound dressing of chitosan film impregnated with SSD with good mechanical properties and controlled release and concluded as effective in severe burn cases (Azevedo, Saldanha et al. 2006).

d) Lichtenstein *et al.* 1995 has developed Liposomes encapsulating SSD, for the topical treatment of infected burns, having potential benefits over treatment with free SSD, as an improved delivery system that could act as a locally targeted sustained-release drug depot (Lichtenstein and Margalit 1995).

e) Venkataraman *et al.* 2013 has developed stable SSD Nanosuspensions using the Microprecipitation–high-pressure homogenization technique and Nanogels suitable for topical delivery with a view to increasing bactericidal activity in burn therapy (Venkataraman and Nagarsenker 2013).

2.8. Ciprofloxacin

Ciprofloxacin is a second generation fluoroquinolone commonly administered by oral, intravenous, intratympanic, ophthalmic, and otic route to eliminate the number of infections (Campoli-Richards, Monk et al. 1988) .

2.8.1. IUPAC Name: 1-cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid

2.8.2. Chemical Formula: C17H18FN3O3

2.8.3. Molecular Weight: 331.34

2.8.4. Mechanism of action

Ciprofloxacin block the DNA replication in bacteria by inhibiting the DNA gyrase (Topoisomerase II and Topoisomerase IV) responsible for supercoiling (Hooper, Wolfson et al. 1987).

2.8.5. Indication

Ciprofloxacin is available in market as tablets, oral suspension and injections for the treatment of skin structure infections, bone and joint infections, complicated intra-abdominal infections, nosocomial pneumonia, febrile neutropenia, adults who have inhaled anthrax, plague, chronic bacterial prostatitis, lower respiratory tract infections including acute exacerbations of chronic bronchitis, urinary tract infections, complicated urinary tract infections in pediatrics, complicated pyelonephritis in pediatrics, and acute sinusitis. Moreover, ciprofloxacin eye drop and ointments are prescribed in bacterial corneal ulcer and conjunctivitis. Ciprofloxacin is also used in the treatment of uncomplicated urinary tract infections, complicated urinary tract infections, and acute uncomplicated pyelonephritis.

2.8.6. Absorption

Oral administration of the ciprofloxacin achieves approximately 64-85% systemic concentration depending on the patient's condition.

2.8.7. Volume of distribution

On oral administration, ciprofloxacin shows three compartmental model. The central compartment achieves 0.161L/kg volume of distribution however, total volume of distribution varies from 2-3 L/kg

2.8.8. **Protein binding:** 20-40%

2.8.9. Metabolism:

Enzyme CYP1A2 metabolize the ciprofloxacin into two main metabolites oxociprofloxacin and sulociprofloxacin comprising around 3-8% of dose. Apart from that, desethylene ciprofloxacin and formylciprofloxacin are the minor metabolites.

2.8.10. Elimination

Most of the dose administered eliminated by urinary elimination.

2.8.11. Half life

The half-life of the ciprofloxacin for orally administered dose is 4.7 h. However, the half-life for intravenous administration is reported 3.6 h.

2.8.12. Adverse effects

Nausea, vomiting, abdominal pain, crystalluria, nephrotoxicity, oliguria, acute renal failure, anuria and elevated creatinine level are the commonly observed side effect of overdose ciprofloxacin.

2.8.13. Research

Several studies have been conducted to improve the ciprofloxacin therapy associated limitations and to enhance its antimicrobial activity. Summary of some the studies are discussed below;

Recently, Raveendran et al. 2019 revealed that ciprofloxacin and fluconazole loaded fibrin nanoparticles significantly reduced the microbial burden in infected rat wound models (Thattaruparambil Raveendran, Mohandas et al. 2018).

Similarly, a study conducted by Obaidi et al. 2018 proved that ciprofloxacin hybrid nanoparticles were 3–4 folds more effective in inhibiting growth and biofilm formation of P. aeruginosa then ciprofloxacin (Al-Obaidi, Kalgudi et al. 2018).