2 LITERATURE REVIEW

2.1 Antimicrobial resistance

Antimicrobial resistance has emerged as one of the major public health problems of the 21st century that threatens the effective prevention and treatment of an ever-increasing range of infections caused by bacteria, parasites, viruses, and fungi that are no longer susceptible to the common medicines used to treat them. Antimicrobials - including antibiotics, antivirals, antifungals, and antiparasitics- are medicines used to prevent and treat infections in humans, animals, and plants. Microorganisms that develop AMR to all antimicrobial agents are referred to as "superbugs". The problem of AMR is especially urgent regarding antibiotic resistance in bacteria. Over several decades, to varying degrees, bacteria causing common or severe infections have developed resistance to each new antibiotic coming to the clinic (Prestinaci *et al.*, 2015).

The most common terminology used to represent the level of resistance are multidrug resistance (MDR), extensive drug resistance (XDR), and pan drug resistance (PDR). The term MDR refers to the condition when an organism develops resistance to at least one antimicrobial agent in three or more categories. The term XDR is used to describe bacterial isolates that demonstrate resistance to all but two or fewer antimicrobial categories, meaning they remain sensitive to just one or two categories of antimicrobial agents. The term PDR refers to the condition of being resistant to all antimicrobial drugs across all categories, indicating a lack of sensitivity (Basak *et al.*, 2016).

WHO stated, "Combat drug resistance: no action today, no cure tomorrow". According to the 2019 antibiotic resistance threat report issued by the CDC, there were about 5 million fatalities linked to AMR. Out of these deaths, 1.27 million were specifically ascribed to bacterial AMR. In the United States, an annual incidence of over 2.8 million illnesses caused by AMR pathogens has been documented, leading to a consequential mortality rate of over 35,000 persons (Loh *et al.*, 2021). As per the report, AMR

will affect the lives of 700,000 people annually, and an additional 10 million are expected to die from it by 2050. India has one of the highest rates of resistance to antimicrobial agents used both in humans and animals (Taneja *et al.*, 2019).

2.2 Factors contributing to the emergence of antibiotic resistance

Antimicrobial resistance spreads rapidly between environments, driven by a multitude of factors, including human and animal movement, surface water run-off, and the exchange of agricultural products. Antimicrobial resistance is an inherent phenomenon that occurs in microbes as a means of survival. However, it is unfortunate that human factors also contribute to the promotion of AMR (Byarugaba, 2010).

2.2.1 Natural factor

Selective pressure: Microorganisms are either destroyed or, in the case of those with resistance genes, can survive when exposed to antimicrobial agents. The survivors will engage in replication, leading to the rapid proliferation of their offspring, ultimately resulting in the establishment of the prevailing variant among the microbial community.

Mutation: The majority of microorganisms engage in a process of reproduction by division at regular intervals, often occurring every few hours. This rapid reproductive capability enables them to undergo evolutionary changes at an accelerated pace and promptly adapt to novel environmental circumstances. During the process of replication, genetic changes occur, and certain alterations have the potential to confer a survival advantage to individual microorganisms when they are exposed to antimicrobial agents. The mutation is a widespread mechanism of resistance to various synthetic antimicrobial agents, such as oxazolidinones and fluoroquinolones, although resistance to these classes develops less frequently (Munita *et al.*, 2016).

Gene transfer: Horizontal gene transfer facilitates the transmission of genetic components (carried by transposons or plasmids), particularly antibiotic-resistance genes, across different bacterial species. This process plays a crucial role in

promoting interaction among bacterial populations and contributes significantly to the development of multidrug resistance. Recent research has brought to light the rise of "superbugs" that possess a multitude of horizontally transmitted antibiotic resistance genes on plasmids, rendering them highly tolerant against approximately all antibiotics (Sun *et al.*, 2019). Antibiotics may promote the conjugation of a gene encoding antibiotic resistance. For example, the conjugative transposon CTnDOT transmits resistance to the antibiotic tetracycline (Salyers *et al.*, 2006).

2.2.2 Human factors

Misuse or overuse of antimicrobials by a patient: Patient compliance is an important driver in the emergence of AMR. Inadvertently or intentionally, patients skip antibiotic doses, have inconsistent vaccinations, do not complete antibiotic courses, share medications, overuse antibiotics, and consume alcohol during therapy. These practices expose surviving organisms to subtherapeutic concentrations of the drug, which increases the probability of resistance. Due to poverty, many unhealthy patients from developing nations seek their first-line treatment from retail shopkeepers who offer an unsuitable selection of over-the-counter antibiotics and antimicrobial medications, as well as inappropriate dosages and treatment durations. In addition, they seek out traditional healers who provide them with unproven herbal concoctions for their management of infections. These chemicals' effects are uncertain, although they may improve pathogen health, which in turn contributes to AMR (Ayukekbong *et al.*, 2017).

Incorrect antibiotic prescribing: Healthcare professionals are crucial in the diagnosis, treatment, and prevention of illnesses, but if their methods are not supported by research, they run the risk of jeopardizing these efforts. For instance, different doctors in different nations have different prescription procedures for antibiotics. Sometimes, doctors in underdeveloped nations have a lot of patients to see and not enough time to properly educate them about antimicrobial prescriptions. This can lead to mistakes like

prescribing the wrong drug or dose, or even giving antibiotics when they're not needed. It's a result of the high patient-doctor ratio and the lack of meaningful communication about following the guidelines for taking these medications. Sometimes, when it comes to treatment, doctors might give patients broad-spectrum antibiotics even if they don't have a clear diagnosis or specific reason for needing antimicrobial treatment (Ayukekbong *et al.*, 2017). According to research conducted in Lebanon, findings revealed that a significant proportion of patients (52%) exhibited erroneous prescription dosages, while a considerable number of physicians (63.7%) prescribed antibiotics with incorrect treatment durations (Saleh *et al.*, 2015).

Non-human use of antimicrobials: Antibiotics are widely used in several domains such as animal husbandry, fisheries, aquaculture, honey production, industry ethanol production, agriculture, antifouling coatings, preservation of food, and residential applications. These circumstances present several prospects for the emergence and dissemination of microorganisms that are resistant to antibiotics (Ayukekbong *et al.*, 2017).

2.3 Mechanism of antibiotic resistance

Most pathogenic microbes can acquire resistance to antimicrobial agents. Resistance mechanisms may be inherent or may have been acquired from other microorganisms. Four major classes of AMR mechanisms (**Fig. 2.1**) have been identified

Altering drug target: Numerous bacterial cell elements can serve as targets for antimicrobial agents, along with as many targets that can be altered by bacteria to confer drug resistance. Several classes of antibiotics, including -lactams, macrolides, glycopeptides, lincosamides, aminoglycosides, and streptogramins, are resistant to target modification. The strong penicillin resistance of the Gram-positive Streptomyces species is caused by either excessive PBPs synthesis or the formation of low-affinity PBPs (Peterson *et al.*, 2018).

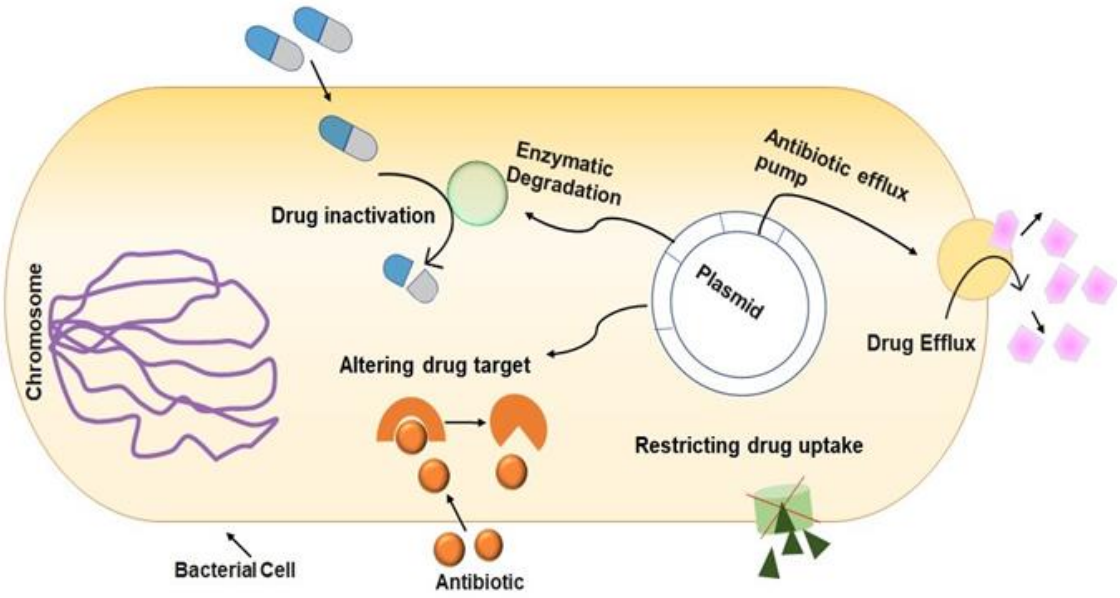

Figure 2.1 Mechanism of antimicrobial resistance

Restricting drug uptake: The outer membrane of Gram-negative bacteria acts as a barrier to the passage of many substances, including antibiotics. Some Gram-negative bacteria are intrinsically resistant to specific antibiotics due to the outer membrane's limited permeability to those compounds. Changes in the permeability of the outer membrane can also contribute to the development of acquired resistance (Ogawara, 2015). OprD, the specific channel porin found in *P. aeruginosa* strains, is responsible for drug absorption. Porins serve as the primary means by which hydrophilic antibiotics, including β-lactams, chloramphenicol, fluoroquinolones, and tetracyclines, gain access to the bacterial outer membrane. The sensitivity of bacterial cells to hydrophilic antibiotics is influenced by the quantity and kind of porins present on the outer membrane. Acquired antibiotic resistance may be caused by mutations that alter the expression or function of porins. These mutations may cause changes in porin size, porin loss, conductance, or decreased porin expression (Fernández *et al.*, 2012).

Drug inactivation: A wide variety of bacteria, including Gram-positive and Gram-negative strains, are capable of producing antibiotic-degrading enzymes. Antibiotics

(penicillins, cephalosporins, carbapenems, etc.) that are resistant to beta-lactamases are the best example of antibiotic resistance that is mediated by the breakdown of the antibiotic molecule. These enzymes break the amide link in the beta-lactam ring, which effectively renders the antibacterial agent useless against the microbes (Christaki *et al.*, 2020). In addition, the presence of aminoglycoside modifying enzymes (AMEs) that covalently modify the hydroxyl or amino groups of the aminoglycoside molecule is indicative of resistance via modification of the drug (Munita *et al.*, 2016).

Drug efflux: Toxic chemicals may be pumped out of the cell using efflux pumps, which are energy-dependent complicated bacterial systems located on the cytoplasmic membrane. The study of fluoroquinolone resistance in *S. aureus* by Costa *et al.*,2015 demonstrated that an intermediate resistance phenotype (via upregulation of efflux pump expression) appears first and serves as a platform for higher-level resistance mutations to occur by guaranteeing a sub-lethal intracellular fluoroquinolone concentration (Costa *et al.*, 2015). Various Gram-positive and Gram-negative bacterial efflux systems implicated in antibiotic resistance have been reported. Although there are examples of drug-specific efflux pumps, many efflux systems can transport multiple unrelated substances, which can lead to multidrug resistance. Zhang *et al.*, 2008 described a novel efflux pump, PA1874-1877, in *P. aeruginosa*, the expression of which was higher in biofilm than during planktonic development. This pump appears to be implicated in gentamicin, ciprofloxacin, and tobramycin resistance in biofilms (Zhang *et al.*, 2008).

The formation of biofilm is an essential mechanism that contributes to the development of antibiotic resistance. This is achieved by the inhibition of antibiotic penetration, deactivation caused by the biofilm environment (characterized by low oxygen levels and pH changes), and the development of persister cells (Abebe, 2020).

2.4 Biofilm

Biofilms are defined as "aggregates of micro-organisms in which the associated cells are frequently embedded in a self-produced matrix of extracellular polymeric substances (EPS) that are adherent to each other and/or a surface." The EPS matrix not only provides microorganisms with a multilayered scaffold in which most cells experience cell-to-cell contact, either in flocs or in surface-attached biofilms, but also creates a microenvironment that is different from other sites in terms of key environmental inputs known to affect microbial behaviors, including pH, redox, and nutrient availability. Compared with planktonic microorganisms, the microorganisms in mature biofilm show increased tolerance to antimicrobial agents.

2.4.1 Components of biofilm

The cells of a biofilm are immersed in an extracellular matrix that is secreted by the microbial organisms themselves. The biofilm's three-dimensional structure is supported by a complex network of biopolymers called EPS. Biofilms shield cells from harsh conditions and help them survive. The following are the key components of the biofilm matrix:

Exopolysaccharides: Polysaccharides are essential elements of the extracellular matrix and are found extensively in biofilms generated in many habitats, including fresh water, salt water, soils, human infections, laboratory cultures, and others. Most of these molecules have elongated or branching structures of sugars, with a molecular mass of approximately 106 Daltons. For example, one of the most common exopolysaccharides is alginate, which is made of L-guluronic acids and D-manuric and is found in Pseudomonas aeruginosa biofilms (Ryder *et al.*, 2007).

Extracellular DNA: Extracellular DNA (eDNA) is a crucial structural component of biofilms that shields resident bacteria from the antimicrobial agents and host immune

system. To comprehend the growth of bacterial biofilm communities, it is crucial to define the structure of the eDNA (Montanaro *et al.*, 2011).

Extracellular proteins: The proteins in the extracellular matrix perform several tasks, such as supplying the enclosed cells with nutrients to grow and live and controlling the structural stability and integrity of the biofilm. Proteins in the biofilm matrix are involved in both the formation and destruction of biofilms. Some bacteria, such as the Haemophilus influenzae biofilm, have been shown to include matrix-associated proteins. This biofilm has approximately 200 proteins involved in cell movement and secretion (Gallaher *et al.*, 2006). Matrix-associated proteins are thought to play an important role in biofilm formation, however, their dynamics during biofilm development have not been thoroughly explored (Zhang *et al.*, 2015).

Surfactants and lipids: EPS containing surfactants like viscosin, surfactin, and emulsan can disperse hydrophobic substances and increase their accessibility. Biosurfactants promote the initial development of microcolonies, thereby facilitating the migration of associated bacteria to the surface and the formation of mushroom-shaped structures, preventing the colonization of channels, and facilitating the spreading of the biofilm (Karygianni *et al.*, 2020). The biofilms of *C. albicans* showed an extensive amount of lipid rafts, with higher quantities of sphingolipids observed in the biofilm cells as compared to the planktonic cells (Lattif *et al.*, 2011).

Water: Biofilms include 70% water either bound or free form, bound water is absorbed into bacterial surfaces or biofilm (matrix) structures. The presence of water is essential for the survival of bacterial life inside a biofilm. Water plays a crucial role in multiple biological processes, including the maintenance of osmotic fluid pressure, the dissolution of essential nutrients, and the facilitation of macromolecular transportation (Quan *et al.*, 2022).

2.4.2 Biofilm lifecycle

Biofilm formation is generally considered a cyclic process comprising phenotypically distinct stages (**Fig.2.2**). Despite the diversity of biofilm-forming species and biofilm architectures, numerous studies using single species have revealed biofilm formation coincides with distinct developmental stages and general features regardless of the species.

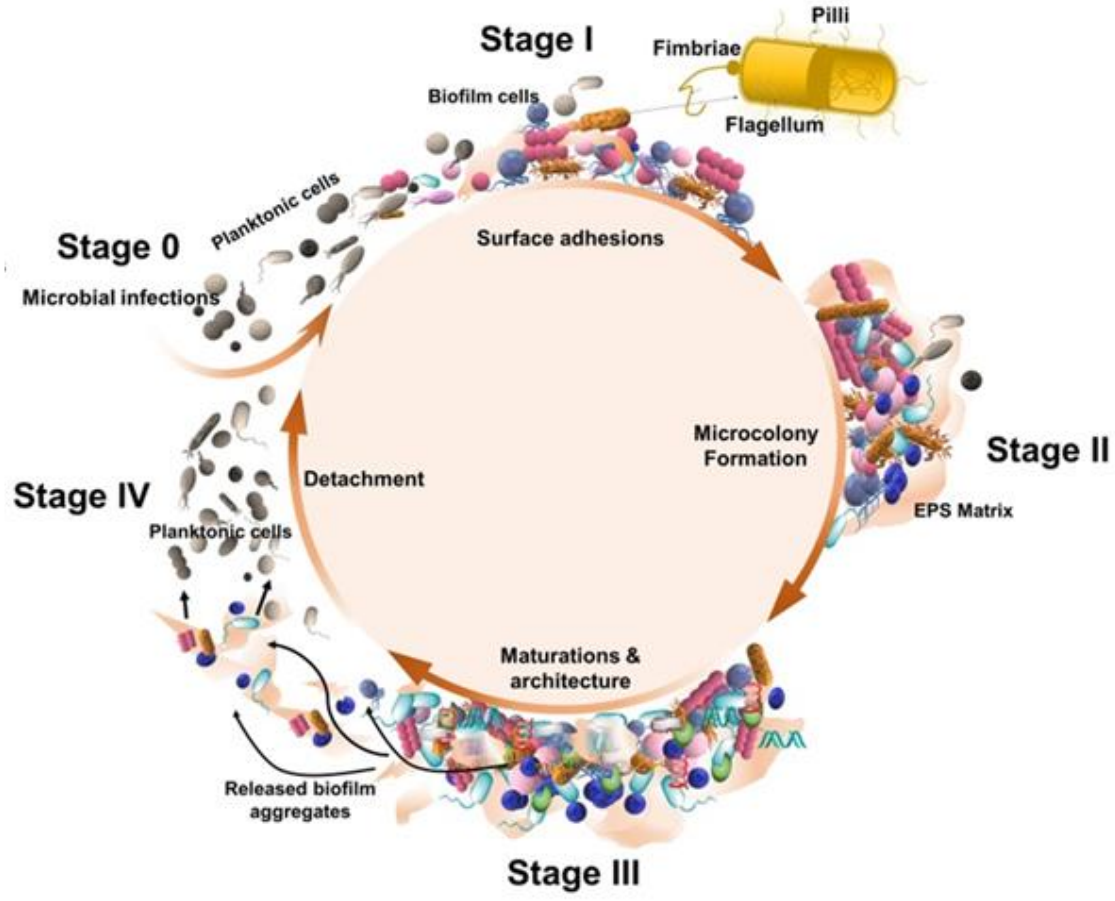

Figure 2.2 Life cycle of biofilm

<u>Stage 0: Planktonic cell interaction</u>

Bacterial infections are caused by the deposition and multiplication of microorganisms on the surgical site of a susceptible host, originating either from the skin, other parts of the body, or the outside environment (nosocomial). The deposition of bacteria promotes biofilm formation which can be minimized by maintaining proper sanitation. Moreover, polymer coating, antimicrobial coating, nanostructured coating, and biosurfactant are

used as preventive methods for microbial biofilm formation. Various strategies are made to produce anti-biofilm coatings using natural and synthetic materials.

Stage 1: Microbial cell attached to the surface

Stage 1 can be reversible and irreversible, firstly microbial cell attaches to the surface through physical forces like van der Waal's forces, electrostatic attraction, hydrophobic interaction, solid liquid interaction, and polarity produces reversible surface attachment but their appendages like pilli, flagella, fimbriae show irreversible attachments. Pili, fimbriae, and flagella are made up of the pilin protein which is glycoprotein or glycolipid receptor.

Stage 2: Multiplication/micro-colony formation

Irreversible attachment of microorganisms to a biotic or an abiotic surface occurs, and this attachment becomes stable, a process of multiplication and division of microbial cells starts, initiated through specific chemical signaling within the EPS. This is followed by clonal growth into more complex multicellular structures during the maturation stages of biofilm.

Stage 3: Maturation and architecture

In this stage of biofilm formation microbial cells communicate with each other through auto-inducer signals. This leads to the secretion of signaling molecules, known as autoinducers that facilitate quorum sensing. At this stage of maturation, certain gene products are expressed, that are considered important for the formation of extracellular polysaccharides (EPS). Since EPS is the main material in the biofilm's three-dimensional structure, interstitial voids are then produced in the matrix. Various mature biofilm structures have been reported, including irregular shape, overall flat biofilms, and mushroom-like or pillar-like structures referred to as 'microcolonies' or 'microcolonies' that are interspersed with fluid-filled channels. These micro-communities coordinate with one another in multiple aspects. This coordination plays a

crucial role in the exchange of substrate, distribution of important metabolic products, and excretion of metabolic end-products. These exchanges fulfill essentials (water, nutrients, energy) for the subsistence of bacteria. Acyl-homoserine lactones (acyl-HSLs) are important intercellular signaling molecules used by many bacteria to monitor their population density in quorum sensing control of gene expression (regulate virulent gene). Identification of inhibitors of signal synthesis has implications for the development of quorum sensing-targeted antivirulence molecules. Incorporation of Acylase I of porcine kidney grade performs deacylation of acylHSL inhibit signal transduction. Extracellular DNA (eDNA) is a matrix component of most biofilms (gram-positive and gram-negative) and is, therefore, an attractive target. Enzymatic degradation of eDNA can sensitize biofilm to antimicrobials (Amankwah *et al.*, 2021; Karygianni *et al.*, 2020; Parsek *et al.*, 1999).

Stage 4: Detachment

Detachment is a naturally occurring process in which microbial communities within the biofilm produce different saccharolytic enzymes that help to release the surface of the microbes into a new area for colonization. For instance, *Escherichia coli* produces N-acetyl-heparosan lyase, *Pseudomonas aeruginosa,* and *Pseudomonas fluorescens* produce alginate lyase, and *Streptococcus equi.* produces hyaluronidase for the lysis of the EPS matrix and subsequent detachment. These saccharolytic enzymes can also be used for breaking polysaccharide matrix in stages 2 and 3 and can be helpful for the eradication of biofilm. A glycoside hydrolase works by cleaving poly-β-1,6-N-acetyl-d-glucosamine, an extracellular polysaccharide substance that facilitates the aggregation of bacteria. Also, it can disperse EPS layers present on medical devices. These biofilm-dispersing enzymes are more efficient when administered in combination with antimicrobial agents in killing the bacteria embedded in the EPS (Roy *et al.*, 2018).

Biofilm associated infections

Biofilm-associated infections can be broadly divided into two types: infections associated with indwelling medical devices and native biofilm infections of host tissues (Sun *et al.*, 2013). Biofilm-based infections have been identified in virtually all tissue and organ systems of the human body, including oropharyngeal soft tissues, teeth and dental implants, the middle ear, eye, endobronchial and pulmonary parenchymal tissues, cardiac valves, the gastrointestinal tract, and the urogenital tract. Similarly, biofilm-producer microorganisms can also cause medical device-related infections such as endocarditis, intravenous catheters, prosthetic heart valves, joint prostheses, peritoneal dialysis catheters, cardiac pacemakers, cerebrospinal fluid shunts, urinary tract infections, septic arthritis, chronic rhinosinusitis, ocular infections, wound infections, etc. The results of biofilm produced on indwelling medical devices are recurrent, untreatable infections and failure of medical devices. Biofilm makes infections harder to treat, with an increased risk of disease spread leading to death.

2.5 Burn wound

One of the most widespread and life-altering types of traumas is burns. Approximately 1.2 million persons in the United States have burn injuries each year, according to data collected by the National Center for Injury Prevention and Control (Church *et al.*, 2006b). According to WHO, approximately 3,00,000 deaths are caused each year mainly due to fire burns, with additional deaths attributed to heat and other causes of burns, *viz.*, electric devices, chemical materials, radioactive rays, etc.

Anatomically, it was observed that during a burn wound, the epithelial integrity of the skin gets disrupted, which further disrupts the structure and function of normal tissues underlying the damaged epithelial cells (Masson-Meyers *et al.*, 2020). As the skin gets disrupted, the bacteria gain access to the underlying tissues, providing a suitable condition for colonizing the bacteria, leading to severe local and systemic diseases (Shariati *et al.*, 2021).

2.5.1 Classification of burn wound

The degree of burn injuries varies tremendously from case to case. The severity of the burn has a direct correlation to the morbidity and death that result. Classifying a burn correctly is important since it may affect the prognosis and guide early treatment. A burn's severity is determined by its position on the body, the intensity of the heat, and the length of time it was exposed to it. Burns are categorized based on their degrees of severity. The look, blanching to pressure, pain, and feeling of a burn are the four aspects necessary to evaluate its severity. Those four factors are used in the American Burn Criteria to classify burns based on their severity.

2.5.1.1 Partial thickness burn

Superficial (first-degree) burn affects just the outermost layer of skin. It's dry and looks pink or crimson, without any blisters. In 5-10 days, a superficial burn will heal completely without leaving any scars.

Superficial partial thickness (second degree) affects the superficial dermis layer of the skin. It is moist and has a crimson, blistery appearance. Applying pressure causes the erythema to fade. Extreme discomfort is typical with superficial partial thickness injuries. Within three weeks, most wounds are completely healed with little scarring.

Deep partial thickness (second-degree) involves the deeper dermis. It has a dry, yellow, or white appearance and becomes red when squeezed. Reduced sensitivity means the discomfort is mild. Scarring is visible after the healing process is complete (3-8 weeks).

2.5.1.2 Full-thickness/third-degree burn

The term "full-thickness" refers to a condition or injury that affects all layers of tissue. Third-degree burns are characterized by the involvement of both the whole thickness of the skin and the underlying subcutaneous tissues. The coloration of the object is seen to be either white or black/brown. Blanching does not occur under pressure. The burn

exhibits characteristics of being parched and having a texture like leather. The presence of diminished feeling results in a reduced or absent perception of pain. Full-thickness burns undergo the process of contracture throughout the healing phase, which often exceeds 8 weeks (Warby *et al.*, 2019).

The fourth degree of burn injuries is characterized by the presence of charred skin, perhaps accompanied by the exposure of underlying bone tissue.

The fifth degree of burn is characterized by the presence of burned, pale skin, with visible *bone exposure.*

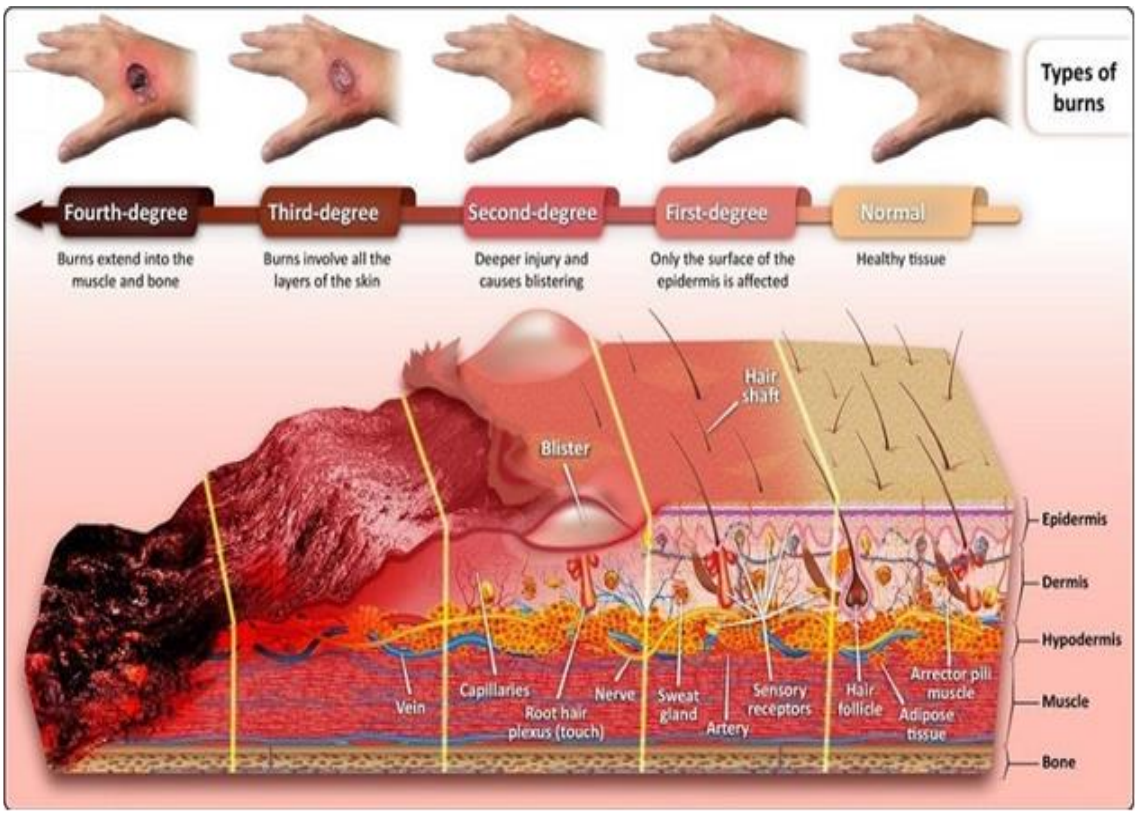

Figure 2.3 Classification of burn (Source fig. phoenix society for burn survivors)

2.6 Burn wound infection

Infection of the wound caused by a burn is a potentially fatal consequence. Patients with burns are more likely to get pneumonia than any other infection, yet burn wound infection is still a major problem. In burn patients, in particular, the leading cause of mortality is an infection, with 75% of all deaths from burns resulting from infection

(Branski *et al.*, 2009). Most of the bacteria that settled in the burn wounds of new patients came from the burn wound surfaces and the patient's gastrointestinal (GI) systems. Furthermore, healthcare workers' hands, feces, and hydrotherapy water can all spread bacteria. The size of the burn wound expressed as a percentage of the total body surface area (TBSA) affected, and the length of hospitalization are two important risk factors linked to burn site colonization or infection (Mayhall, 2003). Opportunistic bacteria, including *P. aeruginosa*, *A. baumannii*, *K. pneumoniae*, *S. aureus*, and *E. coli* are well-known for their tendency to colonize in burn wounds. Also, *P. aeruginosa* and *S. aureus* are most commonly co-isolate from chronic burn wound infections (Alves *et al.*, 2018; DeLeon *et al.*, 2014). These bacteria, specifically, are often seen within the hospital environment and have been responsible for epidemics in burn units worldwide. Additionally, biofilms are thought to be present in most wounds and are known to be comprised of multiple bacterial species which further delays the wound-healing process (DeLeon *et al.*, 2014; Ferry *et al.*, 2022; Aghaee *et al.*, 2022). Medical procedures may introduce bacterial biofilms via healthcare-provider interaction, patient skin bacteria, and more. This exposure causes implant deterioration and mechanical instability, which may lead to implant failure and revision surgery. A systematic review of the available data on chronic wounds shows that biofilms are linked to almost 80% of wound infections. *P. aeruginosa* is an opportunistic Gram-negative bacterium that can cause acute and chronic infections. It is recognized as a critical cause of mortality and morbidity among burn patients with studies showing it can be responsible for as high as 77% of burn wound mortalities.

2.6.1 *P. aeruginosa* biofilm in burn wound infection

P. aeruginosa can cause cross-transmission and outbreaks within hospitals by circulating through contaminated areas leading to localized outbreaks in burn treatment centers. The ability of *P. aeruginosa* to form biofilms is well-known as a key virulence

trait that is central to its pathogenic success. By forming a biofilm *P. aeruginosa* decreases antibiotic treatment efficacy resulting in more chronic infections and prolonged hospital stays (Trøstrup *et al.*, 2017). Alginate, *Pel*, and *Psl* are the three polysaccharides that can make up the *P. aeruginosa* EPS matrix, but the role of each in biofilm formation is strain dependent. These polysaccharides facilitate immune evasion and antibiotic tolerance by protecting the cells within the biofilm from these external insults.

2.6.2 *A. baumannii* biofilm in burn wound infection

A. baumannii is an aerobic, Gram-negative, opportunistic coccobacillus. This pathogen tends to target areas of the exposed layer of burn. Since its proliferation among U.S. military treatment facilities during the Iraq–Afghanistan conflicts, *A. baumannii* has been a leading cause of severe infections among personnel. In Latin America and the Middle East, multidrug-resistant *A. baumannii* isolates account for up to 70% of the total population. Thus, the spread of MDR *A. baumannii*, which can cause localized epidemics in burn and intensive care units, is one of the most significant global public health challenges. Due to its ability to survive on both biotic and abiotic surfaces under desiccated conditions, *A. baumannii* has a remarkable capacity for survival and spread in hospital environments. *A. baumannii* QS plays a significant role in modulating biofilm formation, which is mediated by an AHL-based system, AbaI/AbaR (Longo *et al.*, 2014).

2.6.3 *K. pneumoniae* biofilm in burn wound infection

Klebsiella are bacteria in the family *Enterobacteriaceae* that are Gram-negative, have a protective shell, and are nonmotile bacteria. They can be found in a variety of places, such as water and dirt, and sediment in both plants and animals. Also, *Klebsiella* can be found in the gut tract of humans. However, some species, like *K. pneumoniae*, have been known to cause illnesses in the lungs, urinary tract, wound, and bloodstream.

Klebsiella pneumoniae is one of the causes of nosocomial illnesses that are found most often. Virulence factors like capsular polysaccharides, pili, and adhesins are major contributing factors to infections. In addition, one of its virulence factors enables this opportunistic pathogen to form a dense biofilm on wound surfaces. Which contributes to drug resistance and prolongs wound healing. The increased use of antibiotics has promoted the emergence of carbapenem-resistant and extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae* strains (Paterson *et al.*, 2005).

2.6.4. *S. aureus* biofilm in burn wound infection

Some Gram-positive bacteria including *Staphylococci* are commonly found on healthy skin. This localized reservoir enables colonization of burn wounds within the first 48 h, making S. aureus one of the most common pathogens isolated from burn wounds. The estimated cost for infections due to *S. aureus* is $450 million annually. Like *P. aeruginosa*, *S. aureus* can adopt a planktonic or a biofilm mode of growth. Biofilm formation acts as a significant pathogenicity factor for *Staphylococcus spp.* particularly in burn wounds (Vautor *et al.*, 2008).

2.6.5. Polybacterial biofilms infection in burn wound

Biofilms, which comprise a variety of bacterial species and impede healing, are present in most wounds. Bacterial species use co-aggregation and co-localization processes to facilitate interactions with other species within a biofilm. Several reports found that mixed microbial communities withstand disinfectants and antimicrobials better than mono-species biofilm. Mixed populations acquire AMR via many processes (DeLeon *et al.*, 2014; Ferry *et al.*, 2022; Aghaee *et al.*, 2022). The most frequent polymicrobial infections in chronic wounds are caused by *P. aeruginosa* and *S. aureus*. Evidence shows that *P. aeruginosa* and *S. aureus* coinfections are more virulent than either species infection in isolation; however, the mechanisms remain unclear (DeLeon *et al.*,

2014). The occurrence of a polymicrobial infection led to an elevated level of microbial resistance against antibiotic therapy and subsequent clearance from the host.

Nature's revenge—the unforeseen result that cancels the worst—is upon us. Miracle drugs are losing their magic, but for every problem, there is a solution in nature, and bacteriophage is one of them

2.7 Bacteriophage

Bacteriophages (BPs) are ubiquitous bacterial viruses that infect and kill bacteria through cell lysis but are otherwise harmless to human cells. Several studies have demonstrated the ability of bacteriophages to treat infectious diseases in plants, animals, and humans, including those caused by multidrug-resistant bacterial strains (Lin *et al.*, 2017).

Since ancient times, there have been first documented reports of river waters having the ability to cure infectious diseases such as leprosy. But, in 1896, the British bacteriologist Ernest Hankin reported antibacterial activity against Vibrio cholera, which he observed in the Gangas and Jamuna rivers in India. A French-Canadian microbiologist, Felix d'Herelle, first observed in 1910 the bacteriophage, Six years later, he proposed the name "bacteriophage" or "bacterium-eater". The use of bacteriophage therapy was employed to treat full-thickness wounds infected with Klebsiella pneumoniae infection. This treatment approach included the topical administration of silver nitrate, gentamicin, and phage Kpn5. The results of the study indicated that Kpn5 exhibited a higher level of antibacterial effectiveness compared to silver nitrate (0.5%) and gentamicin (1000 mg) (Kumari *et al.*, 2011).

2.7.1 Mechanism of action

Antibiotics are made of chemicals, but phages are parasites that live on bacteria and have complicated, co-evolving relationships with the host. Based on their development cycles, phages can be classified as virulent or benign: lytic (destruction of the bacterial

cell by the phage) or lysogenic (insertion of the phage's genetic material into the bacterial DNA, conferring immunity against infection by an identical phage).

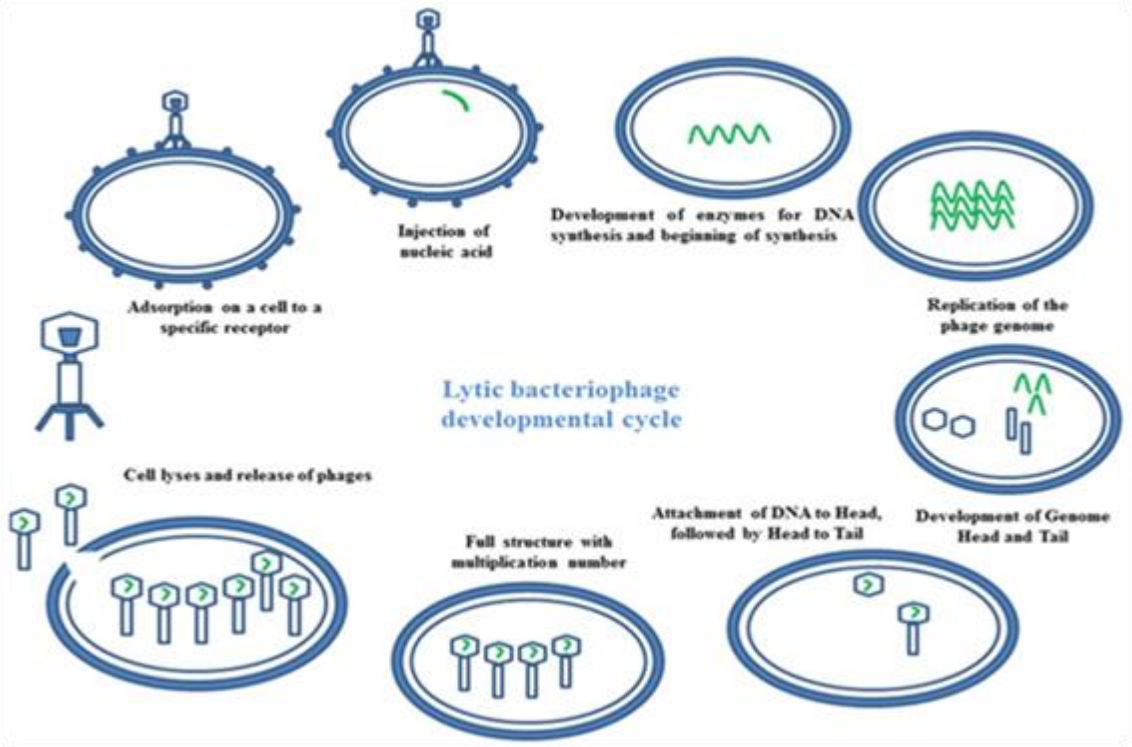

Figure 2.4 Bacteriophage life cycle with bacteriolytic mechanism. The provided image depicts the schematic diagram illustrating the developmental cycle of a lytic bacteriophage (Mandal *et al.*, 2014)

The therapeutic application requires virulent phages, which can only undergo lytic cycles. The lytic cycle of phages is shown in **Fig 2.4**. In the binding phase, the phage sticks to the membrane of the bacteria. This step is very specific; a given phage can usually only connect to a given species of bacteria, and sometimes only to some strains (genetic variations) of that species. The virus then puts its DNA into the bacterium, where it will be copied by bacterial enzymes. The enzymes will also make the proteins and lipids that are needed to make capsids. After the different parts of the virions are put together, the bacteria will be killed. This will release new phages, which can attach themselves to new bacteria and start the cycle over again (Brives *et al.*, 2020).

2.7.2 Bacteriophage as personalized therapy for bacterial wound infection

Personalized or precision medicine is a developing method for enhancing patient care by deploying the most appropriate intervention at the optimal time. The concept of personalized treatment encompasses several aspects, such as determining the appropriate recipients of certain therapies or the optimal dosage for a particular therapy. Additionally, it involves identifying people who should be subject to closer monitoring owing to their susceptibility to specific safety concerns and the potential influence of these factors on their response to medicines. Bacteriophages and formulations containing bacteriophages are effective against bacterial infectious diseases.

Loganathan *et al.*, 2021 conducted an in-depth review of the treatment of infections caused by gram-positive bacteria, focusing on the use of phage-based personalized therapy. The researchers concluded that the need of using phages as a viable alternative therapy is evident in forthcoming times. They propose that by employing a particular phage sourced from a repository known as a phage bank, it will be possible to effectively address the issue of AMR (Loganathan *et al.*, 2021).

Schooley et al., 2022, describe a method for creating a personalized bacteriophage-based treatment for a diabetic patient with necrotizing pancreatitis complicated by an MDR *A. baumannii* infection. In the absence of potent antibiotics, they identified nine distinct bacteriophages with lytic activity against the patient's *A. baumannii* isolate. The administration of these bacteriophages intravenously and percutaneously into the abscess cavities was associated with the recovery of the patient's clinical symptoms, elimination of the *A. baumannii* infection, and restoration of health was observed (Schooley *et al.*, 2017).

Similarly, Ferry *et al.*, 2022, utilized personalized bacteriophage therapy for spinal infections caused by PDR *P. aeruginosa*. Despite bacterial persistence and the expression of small colony variants, the patient was cured by local and intravenous

administrations of purified phages as adjuvant therapy. They believed that personalized phage therapy could be used as adjuvant therapy for complex bone joint infections, particularly those caused by pan drug resistant *P. aeruginosa (Ferry et al., 2022)*.

Furthermore, firms such as Adaptive Phage Therapeutical, USA, have devised a methodology including the use of individualized phages sourced from a phage bank. This approach aims to ascertain the host range and thereafter formulate a pre-prepared phage cocktail for therapeutic purposes (Duplessis *et al.*, 2019).

Eliava Phage Institute, Georgia also focuses on phage-based personalized treatment by isolating phage against a specific bacterial strain identified from a patient's sample. They have isolated and stored broad-spectrum phages (Staphylococcal, INTESTI, FERSISI, SES, and PYO Bacteriophages) for immediate treatment of bacterial infections (Zaldastanishvili *et al.*, 2021).

2.7.3 National status

Anti-Microbial resistance is already an epidemic for health services in India. An additional 58,000 newborn children suffer each year from sepsis when antibiotics struggle to control diseases of the bacteria (Mathew, 2016). India and the United Kingdom signed a EUR 13 million contract in 2016 to study this issue and help solve it. Consequently, the Department of Biotechnology (DBT), the Government of India, and the United Kingdom Research Councils (RCUK) performed a comprehensive study on AMR and collected surprising figures (DBT (with Research Councils United Kingdom) Scoping report on AMR in India). In April 2017, the National Action Plan on Antimicrobial Resistance (2017-21) was launched along with the Delhi Declaration on Anti-Microbial Resistance, a collective statement supported by 12 ministries to pursue a coordinated approach to antibiotic resistance prevention and containment within the world. In India, bacteriophage treatment is going on in progressive developing conditions, where suspension of bacteriophage is used for the treatment of acute and

chronic burn wounds, urinary tract infections, sinusitis, gastritis, and many more bacterial infections.

In India Vital Phages Therapy New Delhi which runs under the partnership of Eliava phage therapy centre (EPTC) Georgia serves phage treatment. Vitalis Phage Therapy has built programs for overcoming the barriers confronting Indian patients who want to receive phage therapy at EPTC. In the last year, more than 20 patients were treated by phage therapy at EPTC via Vitalis.

Indian medical institutions like the Department of Microbiology, Institute of Medical Science (IMS), Banaras Hindu University, Varanasi, Department of Microbiology (Punjab University) Punjab, Department of Microbiology (Pondicherry Institute of Medical Sciences) Puducherry, School of Biosciences and Technology (Vellore Institute of Technology) Vellore, Department of Biology (Indian Institute of Science Education and Research) Tirupati, India, and numerous institutes are extensively working on phage therapy.

Gangagen Biotechnologies Pvt Ltd. Bangalore, India is developing a phage product as an alternative to antibiotics in animal health including Salmonella infections in poultry. The company retains significant expertise and intellectual property related to phage biology and related technologies.

2.7.4 International status

Commercially available Eastern European phages and phage formulations are an alternative phage source used both in clinical trials and in humanitarian therapies in licensed and Western countries (McCallin *et al.*, 2019). Even Poland and Georgia can be considered hubs for bacteriophage therapy. The best example of it the Phage Therapy Unit (PTU), which performs phage therapy under the national program, was established there in 2005 in Poland, and since 2000 scientists have released summaries and case

studies for up to 1,500 patients (Letkiewicz *et al.*, 2009; Weber-Dąbrowska *et al.*, 2001; Zhvania *et al.*, 2017).

Phage International Inc., a US corporation, is engaged in many of the multiple facets of phage therapy, from strain acquisition, and treatment of the most difficult/drug-resistant/chronic infections, to consulting services.

- **ListShield™ and LISTEX™ P100**, **Netherlands** is an anti-*Listeria* Phage cocktail approved by the US Food Drug Administration as food additives for Poultry products and meat (Knoll *et al.*, 2014).
- **Eliava International Phage Therapy Center,** Georgia is a day outpatient clinic that uses phage therapy in its practice. Phage therapy is a specific approach the clinic uses to treat infectious diseases including those caused by bacteria resistant to antibiotics. This aims, by using the current long-term research experience starting from 1923, to create and maintain Eliava products and services for human, animal, plant, and environmental protection, and to comply with the modern quality and standards requirements (Kutateladze, 2015).
- **HIIET. The Hirszfeld Institute (http://surfer.iitd.pan.wroc.pl/index1.htm)** was founded in 1952, and its staff has been actively involved in phage therapy research since 1957 when therapeutic phages were used to treat *Shigella* infections. The bacteriophage laboratory of the Institute was instrumental in developing and producing phages for the treatment of septicemia, furunculosis, and pulmonary and urinary tract infections and for the prophylaxis or treatment of postoperative and posttraumatic infections (Slopek *et al.*, 1983).

2.8 Challenges associated with phage therapy

Despite all the successful cases of patients treated with phages documented to date, the introduction of phage therapy in Western countries still faces major obstacles, especially

regulatory issues (Manohar *et al.*, 2022). Major challenges to the widespread clinical implementation of phage therapy for biofilm-mediated wound infections are:

Quality and safety requirements: For broad medical applications, phages need to be produced on a large scale under Good Manufacturing Practices (GMP) approved by regulatory agencies. Although the production of phages for therapy must comply with the strict regulations that are usually applied for pharmaceutical products to ensure high-quality standards appropriate for their intended use. The absence of clear guidelines is a major challenge.

Stability of phage preparations: The stability of phage preparations is a key requirement for successful treatment and the regulation of phages as pharmaceuticals. The protection of phages is important for certain combined therapies that can inactivate phages when applied together and impair the outcome of the treatment. As an example, burn wound care products and their active ingredients usually exhibit high acidity that can negatively affect the activity of phages in wounds.

Another issue of phage stability is the occurrence of spontaneous mutations in phage stocks stored for long periods or accumulated during phage production and manufacturing, which can impair viral fitness.

Fast phage screening methods: Due to the high specificity of phage activity, finding a phage that targets a particular strain often requires the screening of large phage collections. The most traditional method to detect phage activity against a strain is the double layer agar (DLA) method, in which different phages are spotted on top of a lawn of the bacteria of interest but it is laborious and time-consuming.

In the future, a simple and fast high-throughput method for phage screening should be established and implemented in clinical settings and phage banks, if phage therapy is to be widely used as a treatment option.

Efficacy of phages against biofilms: In biofilms, bacterial cells closely collaborate as a strategy for survival and persistence in harsh environments. The spatial organization of the biofilm is one of the determinant factors for phage infection. To form a biofilm, cells organize so that localized niches are created with distinct nutrient availability and consequently with bacteria of distinct motility, metabolic state, and gene expression, all of which affect the capacity of phages to infect biofilm cells.

Evolution of bacterial resistance to phages: One of the major concerns in phage therapy is the possible emergence of bacteriophage-insensitive mutants (BIMs) that could hamper the success of this therapy.

Regulatory framework of phage therapy: Regulatory authorities have classified phages as biological substances and, as such, phages fall within the scope of the pharmaceutical legislation. The regulatory framework in the European Union and the United States stipulates that marketing authorization is required for medicinal products prepared industrially or manufactured by a method involving an industrial process. As such, marketing a phage product requires proof of both safety and efficacy and quality by the manufacturer under GMP. GMP compliance requires extensive financial resources and is, therefore, a critical obstacle for hospitals or not-for-profit phage therapy centers.

The current regulations need to undergo serious modifications before a fully practicable regulation is implemented for phage therapy, as well as other customized medicinal products meant to be tailored to an individual patient.

2.9 Bacteriophage formulation

Phages are effective at targeting and killing bacterial strains of interest and have yielded encouraging results when administered as part of a tailored treatment for severely ill patients as a last resort. Despite this, success in clinical trials has not always been as forthcoming, with several high-profile trials failing to demonstrate the efficacy of phage

preparations in curing diseases of interest. As with other protein-based macromolecules, bacteriophages are prone to the effects of protein misfolding and aggregation as well as denaturalization, resulting in subsequent loss of functionality when exposed to adverse conditions (Olofsson *et al.*, 2001; Silva *et al.*, 2014). Previous studies have reported on the sensitivity of phages to organic solvents, pH, temperature, and salinity. Several protocols for the long-term storage of free phage have been established by researchers. In general, phages that commonly exist at ambient temperatures can be stored at 4 °C for extended periods with only limited drops in titer observed in most cases. Additional preservation is often observed by freezing at -80 °C (Jończyk *et al.*, 2011a).

This can be achieved using bacteriophage formulations instead of purely liquid preparations. Several encapsulation-based strategies can be applied to produce phage formulations and encouraging results have been observed for efficacy as well as long-term phage stability. Additionally, the actual process by which a given formulation is produced can result in bacteriophage degradation, as exemplified by the processes of freeze-drying and spray-drying. All of these variables must be taken into account when creating stable phage formulations, with the development process typically focusing on evaluating phage delivery to the target bacteria, determining the level of stability a formulation offers under various conditions, and enhancing phage survival throughout formulation production. Common methods used to produce phage formulations typically rely on some form of encapsulation. This is a broad term that is used to describe various techniques including emulsification, freeze-drying, spray-drying, liposome encapsulation, and electrospinning, in which bacteriophages are coated/surrounded by certain stabilizing agents, protecting the external environment.

Among all methods, encapsulation methodologies allow the effective protection of bacteriophages for overcoming critical environmental conditions. Moreover, they

improve the stability and the controlled delivery of bacteriophages which is of great value in bacteriophage therapy (Ma *et al.*, 2008b).

Alginate is one of the most often utilized biomaterials for bacteriophage encapsulation due to its high viscosity which confers mucoadhesive properties. Moreover, microcapsules of alginate in combination with other materials (chitosan, CaCO3, pectin, whey protein) can be obtained to improve their mucoadhesive properties and also to modify the release kinetics of bacteriophages. Alginate is a polymer that allows encapsulation by ionotropic gelation technique. Alginate hydrogel is gradually formed through the reaction between divalent cations (i.e., Ca^{2+}) and guluronate blocks of the alginate chains, giving a structure termed as egg-box suitable to entrap molecules higher than 10,000 Da as lower mass molecules would diffuse through the capsule wall. The study conducted by Batalha *et al.,* 2021 reported the encapsulation of the UFV-AREG1 bacteriophage using alginate polymers, resulting in enhanced stability and controlled release. Bacteriophages were encapsulated in alginate-carrageenan beads, as well as alginate-chitosan as a bead-forming hydrocolloid. The assembled system showed efficiency in the encapsulation of UFV-AREG1 bacteriophages using different hydrocolloids and has the potential to be used for the entrapment of a variety of bioactive compounds (Batalha *et al.*, 2021).

2.10 Chitosan

Chitosan is a biopolymer macromolecule that is derived from crustaceans. The degree of acetylation and deacetylation, as well as surface modification and molecular weight, collectively determine the biological and chemical properties of chitosan (Aranaz *et al.*, 2021). Furthermore, chitosan possesses antimicrobial properties, biocompatibility, and nontoxicity, which makes it a suitable drug delivery carrier (Yan *et al.*, 2021). The versatility of chitosan makes it a suitable polymeric material for wound dressings and drug delivery systems (Liu *et al.*, 2018; Meng *et al.*, 2021). The use of chitosan as a

biomaterial for microencapsulating bacteriophages has been demonstrated in previous publications (Abdelsattar *et al.*, 2019; Ma *et al.*, 2008a; Rahimzadeh *et al.*, 2021; Rotman *et al.*, 2023). There are some grades of chitosan (low degree of deacetylation), which has low water solubility, that require 0.1-2 % acetic acid or formic acid for solubilization. In such an acidic environment, a bacteriophage cannot survive. In this research, we have used chitosan oligosaccharide (water-soluble grade, extra pure, >90% degree of acylation (**Fig. 2.5**), which is biodegradable, biocompatible, and non-toxic with potential for pharmaceutical applications (Mehata *et al.*, 2019).

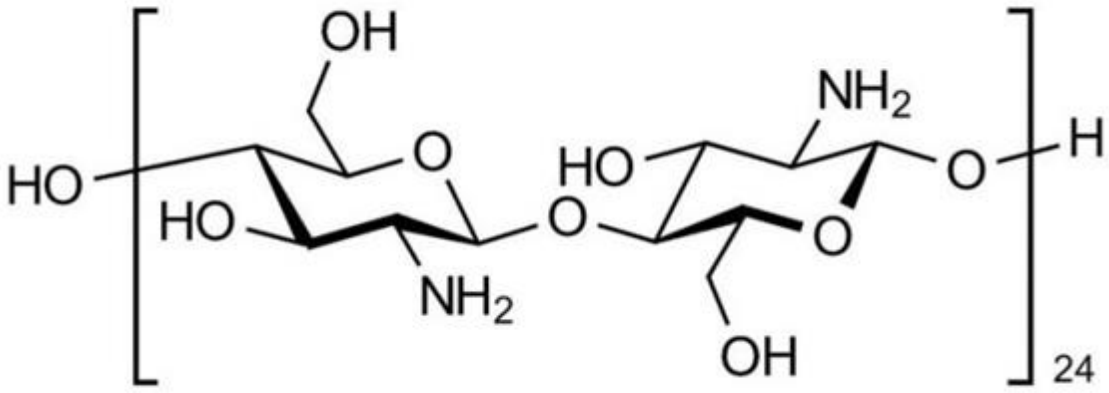

Figure 2.5 Chitosan oligosaccharide

COS (Chitosan oligosaccharide) has a low molecular weight (Mw), a higher degree of deacetylation (DD), a higher degree of polymerization (DP), and less viscous and complete water solubility, which endowed it with significant biological properties like antimicrobial, antioxidant, anti-inflammatory and antihypertensive, as well as drug/DNA delivery ability. Chitosan has been often used for the encapsulation of bacteriophage in several research studies. However, the dissolution of chitosan necessitates the use of acetic acid (1% v/v), which has an impact on the survivability of the bacteriophage (Ma *et al.*, 2008b). Here we are using water-soluble grade chitosan (chitosan oligosaccharide **Fig. 2.5**) which does not require acetic acid for solubilization. Furthermore, chitosan and its derivatives, especially N,N,N-trimethyl-chitosan (TMC),

N,O-carboxymethyl-chitosan (CMC), and O-carboxymethyl-N,N,N-trimethyl-chitosan (CMTMC), used to accelerate wound healing. A chitin material, Beschitin®, was used for human application as dressing for skin and nasal wounds (Azad *et al.*, 2004).

2.11 Bacteriophage microparticle

Microparticles pose numerous advantages over nanoparticles as a carrier for bacteriophages. Microparticles are generally easier to prepare, handle, and characterize. Their larger size allows for the controlled release of bacteriophage over an extended period, providing sustained therapeutic effects. Microparticles are generally more stable than nanoparticles (Daly *et al.*, 2020; Hong *et al.*, 2017). Several studies have shown the efficacy of bacteriophage microparticle preparations as follows.

- Emilie *et al*., 2023 developed eudragit polymeric microparticles to entrap the bacteriophage by spray drying mechanism for delivery to the colon. The prepared microparticles serve the dual purpose of preserving the activity of LUZ19 phage throughout the production process and shielding it from the harmful effects of very acidic environments. Additionally, the stability experiments indicated that the microparticles exhibited stability for a minimum duration of six months when kept at a temperature of +5 °C (Tabare *et al.*, 2023).
- Bacteriophage-loaded Poly (lactic-co-glycolic acid) microparticles for the treatment of bacterial (*S. aureus* and *P. aeruginosa*) infections were developed by Kalelkar *et al.,* 2022. The phage-loaded microparticles (phage-MPs) exhibit potent antimicrobial activity against multiple strains of *S. aureus* both *in vitro* and *in vivo* and inhibit the growth of a clinical isolate of *S. aureus* in the presence of sputum supernatant from cystic fibrosis patients (Kalelkar *et al.*, 2022).

- Using spray drying, Matinkhoo and colleagues successfully prepared bacteriophages into a dry powder inhalation for the treatment of pulmonary infections. They incorporated bacteriophage into microparticles consisting of L-leucine, trehalose, and casein sodium salt or surfactant, resulting in 2.5–2.8 mm MMAD microparticles. Their research demonstrates the viability of administering bacteriophage microparticles made respirable by spray drying at low temperatures (Jain *et al.*, 2020; Wu *et al.*, 2013).
- To enhance the biological stability of spray-dried phages, tri leucine, and pullulan were compared to leucine and a polymeric surfactant in the study performed by Nicholas et al., 2020. This was examined using anti-Campylobacter phage CP30A as a model biological agent and trehalose as a bulk stabilizing agent. The inactivation during processing and the short-term biological and physical storage stability of the powder were evaluated without refrigeration. They observed that the use of processing modeling, microparticle engineering, and a supplemented phase diagram facilitated the rapid design of a physically stable spray-dried powder capable of stabilizing biological material, without the need for iterations to determine optimal processing conditions (Carrigy *et al.*, 2020).