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Overview of Multidrug-Resistant *Pseudomonas aeruginosa* and Novel Therapeutic Approaches

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ABSTRACT

Gram-negative bacilli *Pseudomonas aeruginosa* is an important pathogen in hospitalized patients, contributing to their morbidity and mortality due to its multiple resistance mechanisms. Therefore, as therapeutic options become restricted, the search for new agents is a priority. Latterly an accelerated increase in frequency of multidrug-resistant clinical strains has severely limited the availability of therapeutic options. Several *in vitro* and *in vivo* studies evaluating the efficacy of different antimicrobials agents and development of vaccines against *P. aeruginosa* have been reported as novel approaches, such as inhibition of virulence factor expression or inhibition of their metabolic pathways.

Keywords: Bacilli; Gram-Negative; *Pseudomonas aeruginosa*; Multidrug Resistance; Pathogen; Resistance Mechanisms

1. Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that may cause severe invasive diseases in critically ill patients. The frequency of infections caused by them is increasing and multidrug-resistant (MDR) strains, resistant to almost all available antimicrobials, are emerging in hospitalized patients.

Because of its ubiquitous nature, ability to survive in moist environments, and innate resistance to many antibiotics and antiseptics, *P. aeruginosa* is a common pathogen in hospitals and particularly in intensive care units. It has become increasingly clear that resistance development in *P. aeruginosa* is multifactorial, with mutations in genes encoding porins, efflux pumps, penicillin-binding proteins, and chromosomal β-lactamase, all contributing to resistance to β-lactams, carbapenems, aminoglycosides, and fluoroquinolones [1]. Strains of *P. aeruginosa* are the cause of several diseases in nosocomial environments, predominantly pneumonia, bacteremia, meningitis, urinary tract infections, as well as skin and soft-tissue infections [2]. Due to the emergence of MDR pathogens, it is of ultimate importance to develop new antimicrobial drugs.

P. aeruginosa has been characterized as one of the most versatile microbial organisms, with a wide span of habitats including soil, disinfectant solution and jet plane fuel [3]. Low permeability of its outer membrane by a complex set of efflux pump systems and secretion of alginate during biofilm formation are major factors that allow the pathogen to become highly virulent and resistant to multiple antibiotic agents. Adding to these factors, other baterial exoproducts such as lipopolysaccharides and elastase induce harmful pathogenesis resulting in tissue destruction.

Flagellins in *P. aeruginosa* perform several functions during host infection [4]. Apart from enabling motility, the flagellum of *P. aeruginosa* plays an indirect role in membrane permeabilization and surfactant protein-mediated bacterial clearance [5]. Similarly, pili is involved during inflammation due to glycosylation in the interface between pili and host cells. Flagellins are classified into two types: Type-a (polymorphic glycosylated) and type-b (non-glycosylated).

2. Pathogenesis and Colonization

Pili, flagella, exoenzyme S, and mucoid exopolysaccharide are recognized as major adhesins in P. aeruginosa. Invading pathogens are recognized by Toll-like receptors (TLRs) on epithelial cells and innate immunocytes, both of which are then activated to express inflammatory mediators. Thereafter, defense systems such as mucociliary clearance, phagocytosis and humoral immunity are promoted to neutralize the danger [3].

Invading organisms are first trapped by the mucus layer coating the airway epithelial cells. Airway mucin, the main component of mucus, is a large heterogeneous

glycoprotein with carbohydrate side chains consisting of *N*-acetylglucosamine (GlcNAc), GalNAc, D-mannose, L-fucose, and *N*-acetylneuraminic acid (NeuAc) which may promote colonization, whereas binding to the glycolipids may cause an inflammatory response [6]. These exposed oligosaccharide residues become adhesive receptors for *P. aeruginosa* and others microbes.

The diversity of oligosaccharide side chains on glycoproteins or glycolipids in mucin may determine which organisms will effectively bind to it [7]. *P. aeruginosa* has a variety of lectin-like adhesions (**Figure 1**), including pili, mucoid exopolysaccharide, and non-pilus adhesins, represented by exoenzyme S, that have binding domains similar to that of the pilus. Flagella motility and pili, which mediate twitching motility in *P. aeruginosa*, are thought to be the prevailing adhesins for the initial attachment required for colonization of the airway tract [3].

Alginate expression is observed afterward the initial attachment of *P. aeruginosa* to a solid surface. Alginate may be involved in cementing the primary adherence of the non-pilus adhesion found on the surface of *P. aeruginosa* so it can bind to respiratory cells or mucin in the absence of other adhesions [3]. When the organisms trapped in mucus multiply faster than the removal rate the production of exoproducts increases, most of which are virulent and result in decreased mucociliary transport and airway epithelial cell function. The latter results in enhanced mucus inactivity and cell surface colonization.

Following, quorum sensing and biofilm formation start. Intracellular communication is involved in *P. aeruginosa* biofilm development [8]. The phenomenon of quorum sensing or cell-to-cell communication requires self-generated signal molecules, named autoinducers. As cell density increases, there is a proportional increase in autoinducer production. *P. aeruginosa* has at least two quorum-sensing systems. Each system includes a gene encoding a transcriptional activator, LasR or RhlR and a gene encoding an autoinducer *lasI* or *rhlI*. These systems contribute to the development of the biofilm. In addition,

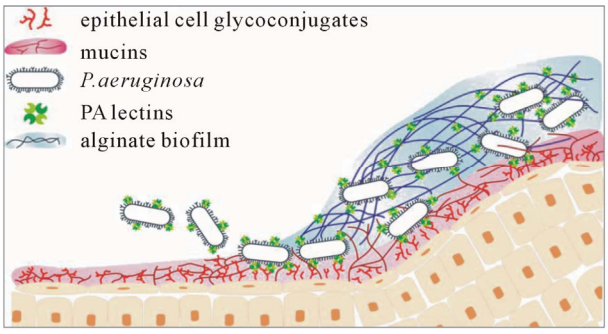

Figure 1. Schematic interactions of the possible roles of *P. aeruginosa* lectins during host recognition and biofilm formation [10].

the LasR-*lasI* and RhlR-*rhlI* quorum-sensing systems regulate the expression of various virulence genes in a density-dependent fashion [3].

In animal models of acute and chronic infections with *P. aeruginosa* containing a mutation in quorum-sensing genes, less tissue destruction was induced and less mortality was observed compared with findings in wild-type strains [9]. Bacteria inside a mature biofilm exhibit increased resistance to antibacterials and phagocytic cells, and are less stimulatory to the mucosa; these facts are found in bacterial colonization grown particularly in inappropriate environments. It can be said that one of the ways the pathogen and host coexist is established at the site of colonization.

In a chronic colonization with *P. aeruginosa* biofilms, the lungs show chronic inflammation that is associated with the development of lymphocyte follicles around respiratory bronchioles and with the influx of PMNs into airway lumens [3].

3. General Mechanisms against Multiple Drugs

3.1. Multidrug Resistance

P. aeruginosa is naturally resistant to a significant number of antimicrobials (**Table 1**). Furthermore, they easily acquire resistance to new antibacterial agents by mutational changes or acquisition of genetic material. In a study, *P. aeruginosa* strains isolated presented resistance to carbenicillin and gentamicin. *P. aeruginosa* is intrinsically less susceptible to the fluoroquinolones and usually it is moderately susceptible or resistant [11].

Resistance of *P. aeruginosa* to commonly used therapeutic agents has increased in recent years. MDR can be defined as resistance to at least four classes of antibiotics used during treatment of these infections: third-generation cephalosporins, fluoroquinolones, aminoglycosides, and carbapenems [12].

Emergence of MDR strains is often due to selective pressure of antimicrobial therapy. Genetic studies confirm the selection of resistant mutants and their subsequent spread. Outbreaks caused by MDR *P. aeruginosa*

Table 1. Natural resistance of *P. aeruginosa* to antibiotics [2].

Bacteria	Natural resistance
P. aeruginosa	ampicillin amoxicillin amoxicillin/clavulanate first-generation cephalosporins second-generation cefotaxime ceftriaxone nalidixic acid trimethoprim

may follow an increased use of third-generation cephalosporins or carbapenems for therapy of infections caused by other resistant bacteria [2].

All bacteria rely on a heavily cross-linked peptidoglycan layer for cell shape and morphological stability [13]. The formation of this layer depends on the catalytic activity of transpeptidase enzymes, which utilize an active site serine and perform their catalytic cycle by way of an acylation/deacylation pathway. Beta-lactam antibiotics inhibit the action of transpeptidases, effectively blocking the transpeptidation reaction and therefore leaving bacteria susceptible to cell lysis. Beta-lactamases confer significant antibiotic resistance to their bacterial hosts by hydrolysis of the amide bond of the four-membered beta-lactam ring. Their mechanism depends heavily on the concentration of zinc ions, required by the four different classes of beta-lactamases during hydrolisis [14].

Bacteria exhibit two control mechanisms when in presence of metallic compounds: One based on sensing of the environment and, in proportion to what is detected, one of regulatory response [15].

The process occurs in the context of chemical gradients, which activate several metabolic pathways acquired by bacteria along their evolutionary history. Enthalpy plays a determinant role in this case: All non-deleterious mutations attempt to minimize metabolic costs. Toxicity of metals in the cellular medium can be either independent of concentration or regulated by it, in which case metals at appropriate concentration levels become catalytics. In the case of *P. aeruginosa* zinc is not only required for metabolic functions but fulfills a definitive role in resistance to beta-lactams, also dependent on chemical gradients. A similar case occurs with iron acquisition, allowing the pathogen to degrade host iron binding proteins and act as an extracellular protein, bringing the host cell into metabolic stress [16].

Apart from the two general mechanisms described above, the complexity in the genome of *P. aeruginosa* provides precise binding mechanisms in different ways. As in the case of beta-lactamases which hydrolize beta-lactams by attacking their moieties, microcalorimetry studies have shown that paralogous genes in *P. putida* have the capacity to bind in different ways depending on subtle changes in enthalpy [17]. Evidence suggests that selective pressure induced by both natural and clinical factors has forced adaptations based on both general mechanisms (extracellular and within the cell) for molecular recognition as well as specific binding strategies for novel antimicrobials based on physicochemical parameters.

3.2. Efflux Pumps

The resistance of MDR strains may be mediated by the active export of the antibiotics out of the bacterial cell by efflux pumps [18]. Evidence from diverse bacterial ge- nomes indicates that approximately 5% - 10% of genes are involved in transport, with a large proportion of them encoding efflux pumps [19]. These can be organized into five superfamilies: small multidrug resistance (SMR), multidrug endosomal transporter (MET), major facilita- tor superfamily (MFS), resistance nodulation division (RND) and multi antimicrobial resistance (MAR) [20]. The latter indicates the mechanism has evolved long ago and is shared amongst all Gram-positive and Gramnegative bacteria. *P. aeruginosa* exhibits several efflux pump systems that allow it to be resistant to several antimicrobial agents [21]. **Table 2** summarizes some of these systems and their effect on resistance to different agents.

3.3. Other Resistance Mechanisms

Another mechanism present in *P. aeruginosa* is the formation of permeability barriers (OM) [18]. Impaired penetration of different substances through the membrane (e.g. imipenem) is due to diminished expression of specific OM protein. It has been shown that OM permeabilizers such as EDTA increase susceptibility to antibiotics, indicating that the lack of OprD protein leads to a reduction of active antibiotic molecules capable of reaching the target penicillin-binding-proteins.

Two-component systems (2CS) are common molecular mechanisms that allow diverse bacteria to have adaptive regulation in response to complex environments, often composed by a sensor histidine kinase and a response regulator [22]. The sensor kinase is composed of

Table 2. Efflux pump systems associated to antibiotics resistance in *P. aeruginosa* [21].

Efflux pump system	Antimicrobial agents
MexAB-OprM	fluoroquinolones, beta-lactams, tetracyclines, macrolides, chloramphenicol, novobiocin, trimethoprim, sulphonamides
MexEF-OprN	fluoroquinolones, chloramphenicol, trimethoprim, imipenem
MexXY-OprM	fluoroquinolones, aminoglycosides, tetracyclines, erythromycin
MFP-RND-OMF multidrug efflux pump systems	fluoroquinolones, tetracyclines

at least one signal recognition domain coupled to an autokinase domain in an input-transmitter arrangement. Two hypothesis exist regarding the evolution of 2CS. The co-evolution model proposes that 2CS genes have appeared as a result of duplication and further differentiation of these in bacterial genomes. The recruitment model on the other hand proposes that some of the 2CS operons have appeared as a result of an assembly of a sensor gene and a regulator gene from heterologous 2CS genes. Both are supported by evidence from phylogenetic analysis and gene regulatory network modeling in the *P. aeruginosa* PA01 strain.

Finally, it has been shown that cytotoxicty is an important mechanism that contributes to high morbidity and mortality in *P. aeruginosa* infections, particularly in cystic fibrosis [23]. Along with mucoidy resultant from the release of alginate, *P. aeruginosa* synthesizes a secretory apparatus (Type III) that allows it to inject toxins from their cytoplasm into the target cell. The latter mechanism allows mucoid bacteria to lyse the host's macrophages and overcome various defense such as in the case of cystic fibrosis lung infection.

3.4. Genomic Profile of *P. aeruginosa*

Complete sequencing of the *P. aeruginosa* PA01 strain (**Figure 2**) revealed a complex organism, comparable in terms of genome length (6.3 Mb) and amount of open reading frames (5570 ORFs) to *Saccharomyces cerevisiae* [24]. Comparison with *E. coli* revealed a strong evolutionary link between both. This fact is based on evidence of almost one half of the ORFs in *P. aeruginosa* having an E value of 10e-5 in BLASTP alignments with *E. coli* segments despite only 40% of amino-acid identity in the common coding regions. Also, low gene replication across the genome indicates that such a comparatively large genome has vast potential of functional diversity, which may support rich mechanisms responsible for multidrug resistance thanks to environmental versatility. Available genome data supports the hypothesis of the existence of evolutionary paths where resistance to naturally available antibiotics was acquired.

The wide collection of genes in *P. aeruginosa* can be mapped to diverse functions. **Table 3** shows some relevant data regarding the relation between the gene pool and mechanisms ranging from cell regulation to chemosensing and chemotaxing. It is interesting to note that the limited ability to grow on sugars has forced *P. aeruginosa* down to an evolutionary path where it adapted to

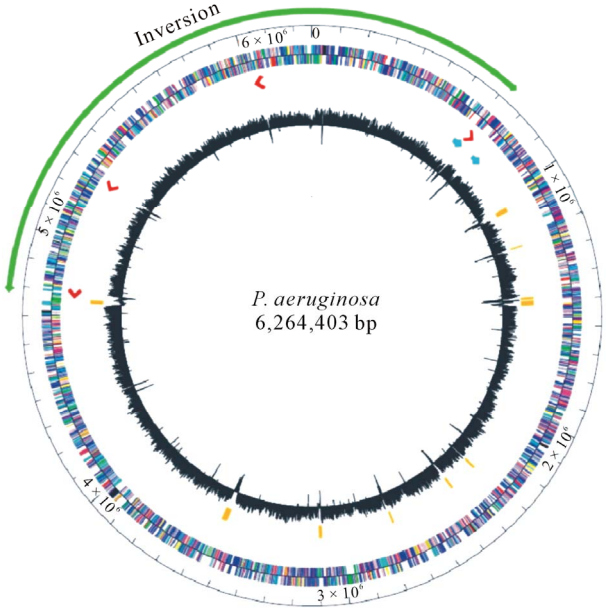

Figure 2. Circular representation of the *P. aeruginosa* PA01 genome [24].

Table 3. Number of genes associated to functions related to multidrug resistance in *P. aeruginosa* [24].

Function	No. of genes	Cellular activities and products
Cell regulation	521	Transcriptional regulators, two-component regulatory system proteins
Cell-surface exposure	71	OprD family of specific porins, TonB family of gated porins (metal-related uptake), OprM family of outer membrane proteins (efflux and secretion)
Import of nutrients	555	300 cytoplasmic membrane transport systems
Metabolism	626	Amino acid biosynthesis and metabolism, carbon compound catabolism, central intermediary metabolism, energy metabolism, fatty acid and phospholipid metabolism, nucleotide biosynthesis and metabolism
Intrinsic drug resistance and efflux systems	34	Resistance/nodulation/cell division, major facilitator superfamily, small multidrug resistance family, multidrug and toxic compound extrusion family, ATP-binding cassette family
Protein secretion	83	Secretion of alkaline protease, general secretion pathway, contact-dependent delivery of proteins into the cytoplasm of host cells, exoenzymes S, T and Y
Chemotaxis	43	Flagella-mediated swimming towards chemoattractors, attraction to sugars, amino acids and inorganic phosphate, repulsion to thiocyanic and isothiacyanic esters

use an ample range of carbon compounds.

Recent advances in *in silico* genomic approaches have provided an opportunity to specifically highlight potential drug targets and has facilitated a paradigm shift from direct antimicrobial screening programs toward rational target-based strategies, where drug discovery starts at the level of the gene [25].

According to the generally used definition, essential genes cannot be deleted from an organism without a lethal effect, being more evolutionarily conserved than nonessential genes. If genes are grouped by their functional classification, variation is lowest for genes involved in transcription, RNA processing, degradation, translation, post-translational modification, degradation and cell division, which are enriched for essential genes (**Figure 3**). In addition to gene essentiality, a high gene expression rate has previously been shown to correlate with low sequence variation, and it was proposed that the underlying driving force for the slower evolution of essential genes is that most of the highly expressed genes are also indispensable in general [25].

4. Current Approaches against Multidrug Resistance

4.1. New Strategies with Known Antimicrobials

Polymyxin B agents were used in the therapy of infections in the 1970s, but due to reported toxicity and the subsequent development of less toxic drugs such as nephrotoxicity, ototoxicity and neuromuscular blockade, their use has been discontinued. Polymyxin B is a polypeptide antibiotic produced by a strain of *Bacillus polymyxa* and is primarily used for resistant Gram-negative infections. Now, with the emergence of MDR strains, their clinical use is being reconsidered [2]. Several reports in the past five years showed that colistin toxicity is not as frequent as previously reported [26]. Renal failure was rare and usually reversible, while neurotoxicity was not reported.

Furthermore, colistin (polymyxin E) has been used in several cases as a salvage agent during therapy of infections caused by strains resistant to all available antimicrobials [26]. However, clinical strains with reduced susceptibility to polymyxin B have been reported [27]. Colistin, in combination with antibiotics from other classes, may be a useful agent for the treatment of infections caused by pandrug-resistant *P. aeruginosa* [28]. Aztreonam may be used in the therapy of infections caused by *P. aeruginosa*. Combination therapy of aztreonam with other antimicrobials may be effective. A two-drug (aztreonam and amikacin) and a three-drug combination (aztreonam, ceftazidime, and amikacin) were very active against MDR strains of *P. aeruginosa* in an *in vitro* study [29].

Imipenem and meropenem are carbapenems commonly used in hospital practice. Many reports confirm their usefulness in the therapy of nosocomial infections caused by MDR Gram-negative bacilli. Apart from imipenem and meropenem, new carbapenems are being evaluated for their efficacy against MDR pathogens [2]. Carbapenems may be administered as monotherapy, but with the emergence of MDR *P. aeruginosa*, combination

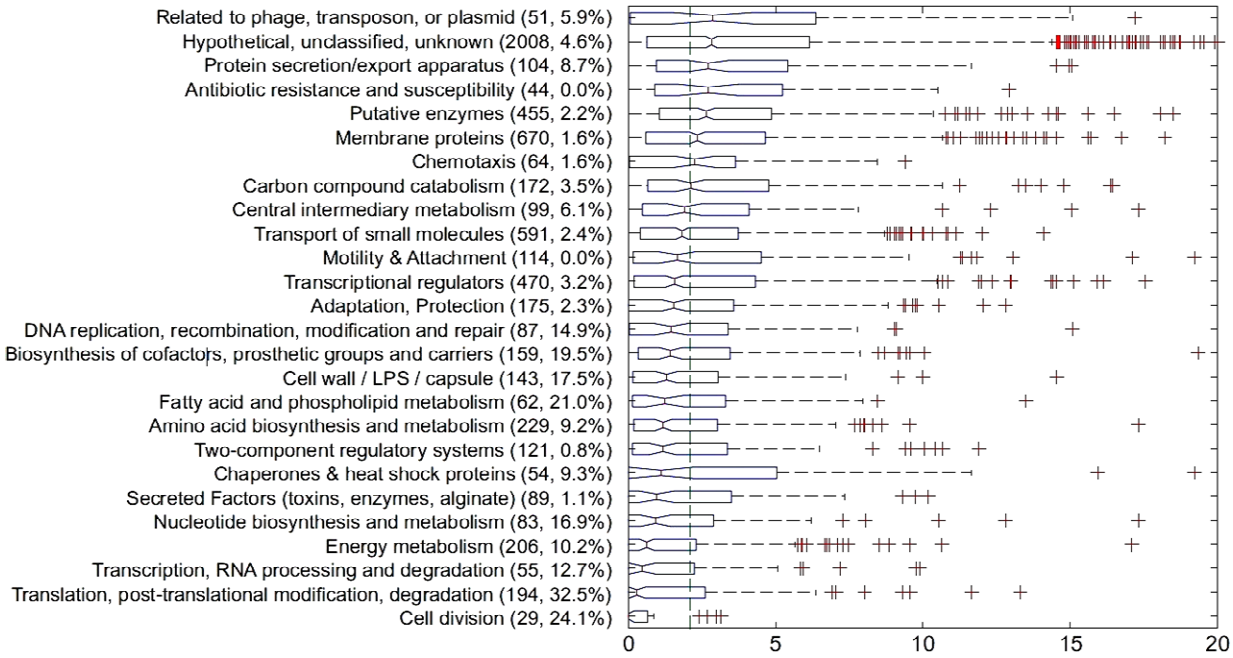

Figure 3. Protein evolution rates in genes of different functional categories [25].

therapies are being evaluated.

4.2. Novel Antimicrobials

Efflux pump inhibitors are under development for use in therapy of infections with resistant strains. In *P. aeruginosa*, two enzymes are involved: the enoyl-acyl carrier protein (ACP) reductase FabI and the alternative enoyl-ACP reductase FabK. Triclosan and other novel FabI- and FabK-directed inhibitors could prove to be broad-spectrum antibacterial agents, particularly for the therapy of infections caused by MDR pathogens [30].

Bacteriophage therapy of bacterial infections has also been investigated for many years. It has now received renewed attention as a result of the emergence of MDR strains of pathogenic bacteria. Several studies have shown the efficacy of bacteriophages in the treatment of experimental infections caused by *P. aeruginosa* in animals [31]. These studies indicate bacteriophages might also be useful in the therapy of infections caused by MDR bacterial strains in humans. Bacteriophages may be administered alone or in combination with antibiotics, and can be given prophylactically or as a therapy of infection. They offer several advantages, as they are very specific, replicate at the site of infection, and no serious adverse effects of their administration have been described. However further studies are needed in order to assess their therapeutic use in humans.

4.3. Nanomedicine

Use of nanotechnology in in the treatment of infections consist in designing, delivering antimicrobial drugs, and diagnosis and control of infections, in particular in overcoming multidrug-resistant microorganisms, has been explored as a good alternative to the current antibiotics.

The recent development of nanotechnology has allowed the study of the effect of nanostructures in the biomedical area, and has promoted studies around the use of nanomaterials and nanoparticles as antimicrobial agents. Nanomaterials can be useful for *in vivo* and in *vitro* biomedical research and applications. The integration of nanomaterials with biology has led to the development of diagnostic devices, contrast agents, analytical tools, physical therapy applications, molecular sensors and drug delivery vehicles. From all nanomaterials with antibacterial properties, metallic nanoparticles provide the best results.

The importance of studying and developing bactericidal nanomaterials is given by the increase of new bacteria strains resistant against most potent antibiotics available and antimicrobial nanoparticles board multiple biological pathways (**Figure 4**), found in broad species of microbes and many concurrent mutations would have to occur in order to develop resistance against nanopartciles antimicrobial activities [28].

The latter has promoted research in the well-known activity of silver ions and silver-based compounds, including silver nanoparticles. Their effect was shown to be size and dose dependent, and was more pronounced against gram-negative bacteria than gram-positive organisms [32].

Silver nanoparticles (AgNP) are intrinsically antibacterial, whereas gold nanoparticles (AuNP) have antimicrobial effect only when ampicillin was bound to their surface. Both AuNP and AgNP functionalized with ampicillin are bactericides against Gram-negative and

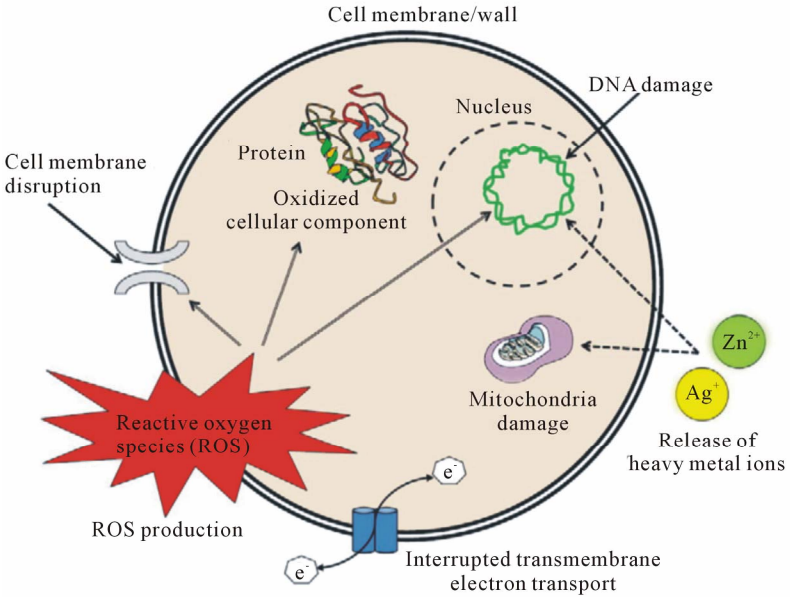

Figure 4. Various antimicrobial mechanisms of nanomaterials [28].

Gram-positive bacteria. Most importantly, when AuNP and AgNP are functionalized with ampicillin they became potent bactericidal agents with unique properties that subverted antibiotic resistance mechanisms of multiple-drug-resistant bacteria as *P. aeruginosa* [33].

Currently nanoparticles such as chitosan nanoparticles, quantum dots, dendrimers and liposomes are under study as antimicrobial agents. Polymyxin B-loaded liposomes represent a successful example of liposomal antimicrobial drug delivery [34]. As mentioned before, polymyxin B has been recognized as a viable treatment for P. aeruginosa related infections. However, its systemic use has been limited due to toxic side effects. It has been reported that liposomal encapsulation of polymyxin B dramatically diminishes side effects and improves its antimicrobial activity against resistant strains of P. aeruginosa [35]. The action mechanism of liposomal po- lymyxin B against bacteria has been identified as membrane fusion. Membrane fusion between liposomes and bacteria is a rapid and spontaneous process driven by non-covalent forces such as van der Waals force and hy- drophobic interactions that minimize the free energy within the system. Antibiotic efflux is a widely accepted mechanism of microbial drug resistance, in which pro- teinaceous transports located in bacterial membranes preferentially pump antimicrobial drugs out of the cells. When liposomes fuse with cell membranes, a high dos- age of drug contents is immediately delivered to the bac- teria, potentially suppressing the antimicrobial resistance of the bacteria by overwhelming the efflux pumps, thereby improving drug's antimicrobial activity [34].

5. Concluding Remarks

The pathogenesis of chronic airway infection of *P. aeruginosa* has been discussed, and the factors affecting the progress of the pathogenic manifestations described in detail. This review focuses at first on bacterial adherence, biofilm formation, immunological disorders and current and novel therapeutic treatment.

Resistance to antibiotics exhibited by some strains of pathogenic bacteria pose a serious challenge in combating infectious diseases. As it is known, application of more powerful antibiotics can lead to limited and temporary advances and eventually contribute to developing greater resistance. Resources against multidrug-resistant pathogenic infections are now limited.

Due to their promising antimicrobial properties, nanomaterials and nanoparticles are currently being studied as potential, highly potent antimicrobial agents for a variety of medical applications. Different kinds of nanoparticles have been investigated for carrying and delivering anti- biotics. Also, nanoparticles enable combining multiple approaches in order to enhance antimicrobial activity and overcome the various resistance mechanisms in *P. aeruginosa*.

Further investigation is required indeed. In particular, the performance of nanomaterials and nanoparticles is an interdisciplinary endeavor. Tools from pathology, immunology, biocompatible materials, polymers, nanotoxicology, pharmacology and nanotechnology are essential for such task.

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