Antimicrobial Peptides-Silver Nanoparticles for Methicillin-Resistance Staphylococcus aureus

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Antibiotics are regarded as a miracle in the medical field as it prevents disease caused by pathogenic bacteria. Since the discovery of penicillin, antibiotics have become the foundation for modern medical discoveries. However, bacteria soon became resistant to antibiotics, which puts a burden on the healthcare system. Methicillin-resistant Staphylococcus aureus (MRSA) has become one of the most prominent antibiotic-resistant bacteria in the world since 1961. MRSA primarily developed resistance to beta-lactamases antibiotics and can be easily spread in the healthcare system. Thus, alternatives to combat MRSA are urgently required. Antimicrobial peptides (AMPs), an innate host immune agent and silver nanoparticles (AgNPs), are gaining interest as alternative treatments against MRSA. Both agents have broad-spectrum properties which are suitable candidates for controlling MRSA. Although both agents can exhibit antimicrobial effects independently, the combination of both can be synergistic and complementary to each other to exhibit stronger antimicrobial activity. The combination of AMPs and AgNPs also reduces their own weaknesses as their own, which can be developed as a potential agent to combat antibiotic resistance especially towards MRSA.

Keywords: antibiotic resistance ; antimicrobial peptides ; MRSA ; silver nanoparticles

1. Introduction

Antibiotics are one of the outstanding discoveries in the medical field in treating infectious diseases caused by pathogenic bacteria. Before the antibiotic discovery era, the lethality and death rate caused by pathogenic microorganisms was high until the accidental rediscovery of penicillin in 1928 by Alexander Fleming [1]. This rediscovery grants the exploration of other types of antibiotics such as sulphonamides, lipopeptides, aminoglycosides, fluoroquinolones, and many more [1][2]. Antibiotics also allow modern medical technology to exist as it aids in preventing infection in chemotherapy and various surgical wounds.

Although antibiotics give significant advantages in treating diseases caused by pathogenic bacteria, Alexander Fleming warns of the danger of uncontrolled antibiotic usage where resistance can be developed. The warning appeared to be true as *Escherichia coli* started to exhibit antibiotic resistance (AR) towards penicillin in 1940 [3]. Up until this day, antibiotic resistance has been a significant threat in the healthcare system as more bacteria developed resistance towards various classes of antibiotics. It is predicted that, by 2050, AR related death may reach 10 million per year [4][5].

A recent comprehensive report released in The Lancet [6] stated that 4.95 million AR associated death and 1.27 million AR attributed death were estimated from 204 countries in 2019. Highest AR related death can be found in Western Sub-Saharan Africa with estimated 27.3 AR attributed death per 100,000 and 114.8 AR associated death per 100,000. Meanwhile, the lowest death can be found in Australasia where only 6.5 AR attributed deaths per 100,000 and 28 AR associated deaths per 100,000. The same report also lists out six pathogenic bacteria that cause the most death in 2019 [6]. In order of the number of deaths, *E. coli*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa* caused 929,000 AR attributed deaths and 3.57 million AR associated deaths.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an antibiotic-resistant type of *S. aureus* that is generally resistant towards beta-lactam antibiotics such as penicillin (methicillin and oxacillin) and cephalosporin [7][8][9]. Beta-lactam inhibits the bacterial growth by halting the cell wall synthesis process [10][11][12]. MRSA generally overcomes the beta-lactam effects by producing beta-lactamase and altering the binding site for cell wall synthesis [7][8][9][13]. The current clinically approved method to treat MRSA infection involves different antibiotic classes such as vancomycin and teicoplanin [14][15]. These glycopeptide antibiotics act on the bacterial cell wall similar to beta-lactam, but it utilises different target by binding to the peptidoglycan side chain, which prevents peptidoglycan crosslinking [13][14][15]. However, the newer MRSA strain started to exhibit resistance towards glycopeptide antibiotics, which makes it difficult to treat the infection [13][14]. Other types of antibiotics such as mupirocin, clindamycin, fusidic acid, and co-trimoxazole also used a second line option in

treating MRSA [16]. However, these antibiotics can only be prescribed when there is no other alternative available due to the risk of resistance [16][17]. Thus, alternatives to treat MRSA without the use of different classes of antibiotics are greatly needed.

Recent scientific development showed some promising potential in inhibiting MRSA through the usage of antimicrobial peptides (AMPs) and silver nanoparticles (AgNPs). These two agents exhibit broad-spectrum antimicrobial properties, which makes them the suitable candidates to combat MRSA threat [18][19][20][21]. AMPs are naturally occurring molecules that can be found in all types of life, which are involved in innate immunity defense [20][21]. AMP mainly takes action on the bacterial membrane, and it can be simplified into two mechanisms of action: membranolytic and non-membranolytic action [21][22][23]. Membranolytic action can be defined as direct AMP action on the bacterial membrane, which greatly alters its structural integrity [23][24][25]. Meanwhile, non-membranolytic action is when AMPs were internalised into the cells without causing major damage to the membrane, but it targets the vital intracellular components [26][27][28]. AgNPs are metallic nanoparticles that have unique physicochemical properties including optical, thermal, electrical and high electrical conductivity in comparison to its bulk form due to its nano size [29][30]. Their enhanced antimicrobial properties mainly contributed with their large surface area per volume area, which allows more antibacterial contact with the pathogenic bacteria [31],[32],[33]. Despite their excellent antimicrobial properties, AMPs are susceptible to proteolytic degradation, their production and purification can be costly sometimes. AgNPs also tend to aggregate and oxidise which limits their antibacterial properties. Thus, the combination of AMPs and AgNPs overcome these limitations by enhancing their antibacterial properties and covering each agent weaknesses.

2. Methicillin-Resistant *Staphylococcus aureus*

Staphylococcus aureus is Gram-positive bacteria with round shape morphology that commonly can be found in the body as a part of its microbiota. Despite it acting commensally on the human body, it can be opportunistic bacteria since it can cause skin infections and food poisoning. Methicillin-resistant *Staphylococcus aureus* (MRSA) is an antibiotic-resistant strain of *S. aureus* that are mainly resistant to beta-lactam antibiotics. MRSA was first identified in 1961 in United Kingdom just a year after methicillin was introduced to treat *S. aureus* infection [34][35]. Despite methicillin no longer being used clinically, the term methicillin-resistant is still used to reflect *S. aureus* resistance towards commercial antibiotics such as beta-lactams antibiotics including oxacillin. According to World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC), MRSA has been a big and serious threat on the pathogenic bacteria watch list respectively [36][37]. According to recent systematic analysis in the Lancet in 2019, MRSA alone caused more than 100,000 deaths [6]. Originally, MRSA are common in the healthcare setting, and this type of MRSA is often dubbed as healthcare-associated or hospital-acquired MRSA (HA-MRSA) [38]. The infection can be spread through direct contact with an infected wound or contaminated hands. Untreated infection can cause serious bloodstream infections, surgical site infections, sepsis and pneumonia [7][39]. Other types of MRSA are community-associated (CA-MRSA) and livestock-associated MRSA (LA-MRSA) [35][39].

Beta-lactam antibiotics act on the bacterial cell wall by binding to the penicillin-binding protein (PBP), which is responsible for the crosslinking of N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc) [10][11]. This crosslinking will form a cell wall that protects the bacteria from external threats. MurNAc subunits have pentapeptide chains attached to it, typically with a sequence of l-Ala-γ-d-Glu-l-lysine (or -meso-diaminopimelic acid)-d-Ala-d-Ala [11]. Beta-lactam antibiotics such as penicillin, cephalosporin, carbapenem and monobactams have a beta-lactam ring which shared similar structural homology to d-Ala-d-Ala of the pentapeptide chain [10][40]. The d-Ala-d-Ala substrate is responsible for the PBP binding site for crosslinking, and this similarity causes beta-lactam antibiotics bind to PBP, causing the crosslinking between the glycan stands to be halted [11]. The binding between beta-lactam and PBP causes the build-up of peptidoglycan precursors which trigger autolytic digestion of old peptidoglycan by hydrolase [10]. Without the production of new peptidoglycan, the structural integrity of the cell wall is significantly disrupted and led to cell damage due to high internal osmotic pressure [11][12].

MRSA overcomes this detrimental effect by producing beta-lactamase, an enzyme to break down the antibacterial effect of beta-lactam antibiotics and production of the *mecA* gene, which changes the penicillin-binding protein (PBP) confirmation. Beta-lactamase is an enzyme produced by bacteria to counteract the effects of beta-lactam antibiotics. This enzyme hydrolyses beta-lactam in the periplasmic space, thus deactivating it before PBP interaction [4]. Beta-lactamase production in staphylococci is controlled by the repressor BlaI and the sensor protein BlaR1 [40]. The genes encoding beta-lactamase, the *blaZ-blaR1-blaI* genes, are repressed by BlaI is from transcribing beta-lactamase when beta-lactam is absent [10][12]. Once beta-lactam is presented, the transmembrane sensor, BlaR1, covalently binds to it and irreversibly acylated at its active site serine. This will activate the intracellular zinc metalloprotease domain of BlaR1 and cause BlaI that are bound to *blaI-blaRI* operator to proteolytically cleave and dissociate from its binding site [12]. The dissociation

allows *blaZ* to be upregulated and transcribed beta-lactamase enzyme. The produced beta-lactamase enzyme later hydrolyses beta-lactam antibiotic by hindering it from binding with PBP [10][12]. Thus, the peptidoglycan synthesis of the bacteria can be initiated as usual.

In MRSA, the PBP responsible for the peptidoglycan cross-linking is altered to novel penicillin-binding protein 2a (PBP2a), which has a lower binding affinity to beta-lactam antibiotics [35]. The resistance arose from the *mecA* gene located in the staphylococcal cassette chromosome *mec* (*SCCmec*), and this resistance gene can be passed to other populations through horizontal gene transfer [12]. Upon acquiring the *mecA* gene, it will be localized in the *S. aureus* chromosome. The production of PBP2a is controlled by MecI repressor and transmembrane MecR1 sensor protein[10]. In the absence of beta-lactam antibiotics, MecI represses *mecA* gene expression by binding to the promoter region of *mec* operon [10][35]. In the presence of beta-lactam antibiotics, the antibiotic binds to the MecR1 sensor protein. It triggers autolytic activation of the metalloproteinase domain in the cytoplasm part of MecR1, causing signal transduction to be activated [12]. The latter caused the MecI repressor to be proteolytically cleaved from its binding site, and this allows the expression *mecA* to produce PBP2a [10]. The PBP2a production allows the peptidoglycan wall synthesis to continue without the interaction of beta-lactam antibiotics due to its low binding affinity to the antibiotic [7][35]. Interestingly, the *mec* operon shared a similar structure and function with the *bla* operon, which produces beta-lactamase [7][12]. This similarity allows the Blal repressor to bind to the *mec* operon to repress *mecA* transcription[10].

3. Antimicrobial Peptides (AMPs) and Silver Nanoparticles (AgNPs) Combination on MRSA or MSSA

Despite AMPs and AgNPs having their own weaknesses on their own, the combination of these two, or sometimes with the addition of polymer, enhances its antibacterial properties while greatly reducing their toxicity effects. Synergistic effect in terms of stronger antibacterial activity of these two agents can also be observed once they are administered together.

A study by Jin et al. utlises AMPs, Tet-213 and AgNPs that are loaded onto porous silicon microparticles [41]. Tet-213 is a 10 amino acid peptide (sequence: KRWWKWWRRC) that possesses broad spectrum activity due to the presence of thiol group and, with the combination of AgNPs, the antimicrobial effect increases drastically. The presence of porous silicon microparticles (PSiMPs) acts as a carrier for effective delivery of the antimicrobial agent to the infected site [41][42]. PSiMPs was chosen due to its tunable pore size, biocompatibility and decompatibility. However, PSiMPs only dissociate in an alkaline condition as it is normally acidic during the early stage of infection [43]. Despite the carrier only being able to dissociate in alkaline conditions, the presence of ROS also allows PSiMPs be to be dissociated easily. When ROS is high during the wound infection, it allows the carrier to be disintegrated and releases silver ions from AgNPs together with Tet-213. The acidic condition also allows a gradual release of AgNPs-AMPs, which allows more effective and stable antimicrobial action. For the combination of these agents, the MIC value was greatly reduced to 2 mg/mL in comparison to AgNPs-PsiMPs (2.5 mg/mL) and AMPs-PsiMPs (>5 mg/mL) on *S. aureus* [41]. In-vitro testing on mouse fibroblast (NIH3T3) cells and human immortal keratinocyte (HaCaT) showed low toxicity effects as this complex does not affect the cells' proliferation. This AgNPs-AMPs-PSiMPs combination also exhibits low toxicity and faster wound healing on rats infected with *S. aureus* [41]. The faster wound healing contributed with the release of silicon ions in the complex, with the help of AgNPs and AMPs to reduce the bacterial infection in the wound. Note that silicon ions promote wound healing by activating the epidermal growth factor receptor (EGFR), epidermal growth factor (EGF) and extracellular signal-related kinase (ERK) signaling pathway [41][44][45].

A star conjugated PCL-*b*-AMPs nanocomposite was also used in stabilising AgNPs and enhancing antimicrobial activity of it with the help of AMPs [46]. Star conjugated PCL-*b*-AMPs consist of polycaprolactone (PCL) and polypeptide (Phe_8-*stat*-Lys_{32}), which are later loaded with AgNPs. This complex is relatively stable at room temperature for three months with any sign of aggregations. In this case, PCL-*b*-AMPs penetrate the negatively charged membrane since this complex is positively charged. This penetration allows AgNPs to be released in the cytoplasm and the deactivating of vital cellular components. This complex managed to exhibit enhanced inhibition on *S. aureus* (27.6 mm) when compared to the combination of PCL-*b*-AMPs (19.1 mm) and AgNPs (12.7 mm) alone. A low MIC value (4 µg/mL) is also observed when PCL-*b*-AMPs with AgNPs is tested on MRSA [46]. This suggests that a synergistic effect of AMPs and AgNPs allows higher inhibition on the bacterial growth. A damaged membrane was also observed on MRSA, which later led to cell death [46][47]. This complex also showed no sign of resistance even after 21 passage exposure with a sub-lethal MIC value of the complex when tested on the wild type *S. aureus* [46]. It also showed low cytotoxicity towards normal mouse fibroblast cells (L929) as it managed to retain up to 80% of cell viability after 48 h. The PCL-*b*-AMPs managed to reduce AgNPs toxicity by only releasing it to the target site besides from their biocompatibility.

Polymersomes, which are polymeric biocompatible vesicle, were also used for an effective synergistic antimicrobial effect of AMPs and AgNPs [47]. PR-39 peptide was utilised in the polymeric compound as it is effective towards inhibiting bacterial growth. Originally, porcine PR-39 peptide could not translocate across the bacterial membrane as MRSA produces protease which degrades the AMPs. For the addition of polymersomes and AgNPs, the MRSA growth was totally eradicated under 23 h [47]. Polymersomes and AgNPs allow the complex to translocate the cells and release the antimicrobial agent to inhibit the bacterial growth. From the scanning electron microscopy, apparent damage on MRSA membrane can be observed, which led to cell death [27][47]. A low toxicity level toward CCL-110 human dermal fibroblast (HDF) cell lines can be observed since the coating reduces the toxicity effects of AgNPs and stabilises AMPs [46][47].

A combination of protegrin-1 AMPs and gelatinized coated AgNPs also greatly enhances its antimicrobial properties as it exhibits low MIC value (6 µg/mL) in comparison to AgNPs (48 µg/mL) and AMPs (8.5 µg/mL) treatment alone [48]. It is said that this complex limits MRSA growth by membrane permeabilisation (possibly through the toroidal pores model) [28][48]. The same study also combines AgNPs with another type of AMPs, Indolicidin [48]. This combination also exhibits excellent antimicrobial properties as its MIC value to inhibit MRSA is 12 µg/mL. The MIC value for indolicidin alone on MRSA 40 µg/mL is relatively high in comparison to the AgNPs-Indolicidin complex. This complex acted on MRSA by self-translocating into the cells by forming an apparent pore on the membrane and interacting with nucleic acid, which halts the DNA synthesis [22][27]. Low haemolytic activity can be observed when the complex was tested with human erythrocytes. However, more optimisations are required as they showed a cytotoxicity effect towards cancerous and normal cell lines, which grants in vivo assessment to elucidate the actual toxicity.

A novel composite of AgNPs and designed AMPs P-13 (amino acid sequence: KRWWKWWRRCECG) were tested against *S. aureus* (ATCC 25923) [49]. Based on the MIC values, this composite manages to inhibit bacteria effectively at lower concentration (7.8 ± 0.05 µg/mL) compared to AgNPs and AMPs alone with 7.8 ± 0.05 µg/mL and >500 ± 0.04 µg/mL, respectively. Interestingly, with the addition of P-13 to AgNPs, a drastic toxicity reduction can be observed on mouse fibroblast cells (NIH-3T3) [49]. This addition allows AMPs to stabilise AgNPs and reduce its cytotoxicity effect in comparison to AgNPs alone. It is proposed that this complex inhibits bacteria growth by adhesion to the bacteria through electrostatic force and was internalised into the cell reacting with vital cellular components. This causes cellular leakage out of the cell, which led to cell death [27][49].

Another study by Li et al. developed multifunctional peptide (MFP)-coated silver nanoparticles as an alternative to combat antibiotic resistance [50]. AMPs tachyplesin-1 and target peptide N-ac-PGP-PEG were combined to adsorb AgNPs through electrostatic interaction. This complex was proven to be effective at inhibiting *S. aureus* and MRSA growth with MIC values of 8 µg/mL and 16 µg/mL, respectively [50]. Despite the MIC for vancomycin, an antibiotic control in this experiment is much lower than the complex (2 µg/mL); this complex was proven to be a promising agent to inhibit the bacterial growth with future optimisations.

The AMP@PDA@AgNPs nanocomposite was created through polymerisation to inhibit biofilm formation by *S. aureus* [51]. PDA was added as a delivery agent, which allows more effective AMPs and AgNPs delivery to the target site. This allows more effective internalisation into the cell to exhibit its antimicrobial activity. This nanocomposite showed no cytotoxicity effect even at a high concentration (400 µg/mL) when tested on human embryonic kidney (HEK293T) cells. To inhibit *S. aureus* growth, only a concentration of 25 µg/mL was required, which is much lower than the concentration used in the cytotoxicity assessments. This complex also managed to reduce biofilm formed by the bacteria by reducing the expression of biofilm forming genes (las I and rh II, fim H) [51].

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