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NANO-STRATAGIES TO COMBAT MULTI DRUG RESISTANT BACTERIA

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ABSTRACT

The antibiotic resistance is one of the most pressing issues in global public health. Associated with the rise in antibiotic resistance is the lack of new antimicrobials. This has triggered initiatives worldwide to develop novel and more effective antimicrobial compounds as well as to develop novel delivery and targeting strategies. Bacteria have developed many ways by which they become resistant to antimicrobials. Among those are enzyme inactivation, decreased cell permeability, target protection, target overproduction, altered target site/enzyme, increased efflux due to over-expression of efflux pumps, among others. Other more complex phenotypes, such as biofilm formation and quorum sensing do not appear as a result of the exposure of bacteria to antibiotics although, it is known that biofilm formation can be induced by antibiotics. These phenotypes are related to tolerance to antibiotics in bacteria. Different strategies, such as the use of nanostructured materials, are being developed to overcome these and other types of resistance. Nanostructured materials can be used to convey antimicrobials, to assist in the delivery of novel drugs.Nanoparticles (e.g., metallic, organic, carbon nanotubes, etc.) may overcome the drug resistance mechanisms in bacteria and, associated with their antimicrobial potential, inhibit biofilm formation or other important processes.

Other strategies, including the combined use of plant-based antimicrobials and nanoparticles to overcome toxicity issues, are also being investigated. Coupling nanoparticles and natural-based antimicrobials to inhibit the activity of bacterial efflux pumps; formation of biofilms; interference of quorum sensing; and possibly plasmid curing, are just some of the strategies to combat multidrug resistant bacteria.

INTRODUCTION

The numbers of infections produced by Multi drug resistant (MDR) bacteria are increasing globally.This acquired resistance of pathogens presents a key challenge for many antimicrobial drugs. Recent advances in nanotechnology offer new way to develop novel formulations based on distinct types of nanoparticles (NPs) with different sizes and shapes and flexible antimicrobial properties. NPs may offer a promising solution as they can not only combat bacteria themselves but can also act as carriers for antibiotics and natural antimicrobial compounds (Wang et al., 2017a). While various materials have been explored from liposomal to polymer based .

Nano- drug carriers, metallic vectors, such as gold NPs, are attractive as core materials due to their essentially inert and nontoxic nature (Burygin et al., 2009). The most attractive aspect of NPs drug delivery systems is their ability to introduce a wide range of therapeutics, either bound to their large surface area or contained within the structure, to the site of infection effectively and safely by having a controlled rate of targeted delivery (Pissuwan et al., 2011; Gholipourmalekabadi et al., 2017). By improving the pharmacokinetic profile and therapeutic index of encapsulated drugs compared to free drug equivalents, the dose required to achieve clinical effects can be dramatically decreased (Gao et al., 2018). This in turn, can reduce the toxicity and the adverse side effects associated with high systemic drug concentrations and frequent dosing (Liu et al., 2009). This review covers the latest approaches in the development of new nanobiotechnology approaches that may challenge the medical practice to fight bacteria and particularly MDR bacteria.

ANTIBACTERIAL MECHANISM OF NPS

The antibacterial effect of NPs against MDR bacteria and biofilms depends on a number of factors, namely, their large surface area in

contact with bacteria through electrostatic attraction, van der walls forces or hydrophobic interactions; on the nanoparticle size and stability; together with the drug concentration (Chen et al., 2014; Gao et al., 2014; Li et al., 2015). The interaction of NPs with bacteria generally triggers oxidative stress mechanisms, enzymatic inhibition, protein deactivation and changes in gene expression. Still, the most common antibacterial mechanisms are related to oxidative stress, metal ion release, and non-oxidative mechanisms (Wang etal, 2017a; Zaidi et al., 2017). Oxidative stress induced by ROS is one of the most important mechanisms assisting the antibacterial activity of NPs (Dwivedi et al., 2014; Rudramurthy et al., 2016). ROS are natural byproducts of cellular oxidative metabolism and have significant important roles in the modulation of cell survival and death, differentiation. In bacteria, ROS are formed from aerobic respiration, and their production is balanced by the cell antioxidant machinery but oxidation of biomolecules, and cell components result in severe cellular damage (Li et al., 2012b). The excessive production of ROS leads to a disturbed redox homeostasis resulting in oxidative stress,affecting the structureofDNAandproteins(Dwivedietal., 2014). It has been shown that while O_2 and H_2O_2 can be neutralized by endogenous antioxidants, ·OH and singlet oxygen lead to acute microbial death (Zaidi et al., 2017). Different NPs may generate distinctive ROS, such as superoxide or hydroxyl radical (·OH), hydrogen peroxide (H_2O_2), and O_2 (Wang et al., 2017a). Metallic NPs is currently being considered to overcome bacterial infections due to their high surfaceto-volume ratio. An increase ratio is usually accompanied by increased production of ROS. ROS generation and metal ion release significantly enhanced the antibacterial activity through uncoated AuNPs in aqueous suspension under UV irradiation (365nm).The antibacterial activity of AuNPs against E. coli, Salmonella Typhi, Pseudomonas aeruginosa and Klebsiella pneumoniae were due to oxidative stress caused by increased intracellular ROS. A recent study (Zhang et al., 2013) evaluated AuNPs and AuNPs - laser combined therapy against C. pseudotuberculosis and suggested that the mechanism of action is related with ROS production, that causes an increase of oxidative stress of microbial cells in the form of vacuole formation as an indication of potent activity. Several other studies have addressed the role of metal NPs to induce

MDR bacteria death via oxidative stress.Titanium dioxide NPs were shown to adhere to the surface ofthe bacterial cell and activate the production of ROS, which lead to damage of the structure of cellular components and consequent cell death (Foster et al., 2011). Different metal NPs, AgNPs were shown to generate superoxide radicals and hydroxyl radicals, whereas Au, Ni, and Si NPs generated only singlet oxygen, which upon entering the cell produced an antibacterial effect (Zhang et al., 2013). Metal oxides slowly release metal ions that are up taken by the cell, reaching the intracellular compartment where they can interact with functional groups of proteins and nucleic acids, such as amino (–NH), mercapto (–SH), and carboxyl (–COOH) groups (Wang et al., 2017a).

BIOFILM FORMATION AND QUORUM-SENSING

Biofilm formation plays an important role in bacterial resistance, protecting bacteria and allowing then to evade the action of antibiotics (Lebeaux et al., 2014; Khameneh et al., 2016). The most active fractions of bacteria are now recognized to occur as biofilms.Bacterial cells are adhered to each other on surfaces within a self-produced matrix of extracellular polymeric substance (EPS). EPS provide a barrier allowing inhibiting the penetration of antibiotics and further promoting antibiotic resistance leading to a serious health threat.Biofilms are resistant to antibiotics penetration and escape innate immune system by phagocytes (Hall-Stoodley et al., 2004; Bjarnsholt, 2013). Numerous experimental evidence show that NPs are capable of disrupting the bacterial membranes and can hinder biofilm formation thus reducing the survival of the microorganism (Peulen and Wilkinson, 2011; Leuba et al., 2013; Pelgrift and Friedman, 2013; Slomberg et al., 2013; Chen et al., 2014; Miao et al., 2016; Yu et al., 2016; Kulshrestha et al., 2017). This way, NPs provide an alternative strategy to target bacterial biofilms with potential to use both antibiotic-free and antibiotic-coated approaches (Gu et al., 2003; Li et al., 2012a; Sathyanarayanan et al., 2013). NPs are able to interfere with biofilm integrity by interacting with EPS and with the bacterial communication - quorum sensing (QS). The use of NPs demonstrates an exclusive approach to penetrate infectious biofilms and target bacterial communication, overcoming this major health issue related with biofilm infections.

The multi-target action of NPs may overcome multidrug resistance by circumventing several obstacles encountered by traditional antibiotics (Chen et al., 2014; Hemeg, 2017; Jagtap et al., 2017; Rai et al., 2017; Zaidi et al., 2017).

Nanoparticles against MDR pathogens and their mechanisms of action.

Type sof nanoparticles	Targeted bacteria	Antibiotic resistance type	Mechanisms of antibacterial action
AgNPs	Enterococcus faecalis, S. aureus	Vancomycin-resistant	Combination with vancomycin. Bacterial cell death.
	S. aureus	Methicillin-resist	
	E. coli, P. aeruginosa	Ampicillin-resistant	Combination with antibiotics. Combination with ampicillin leads to entry into the bacterial cell. Inhibition of cell wall synthesis, protein synthesis and nucleic acid synthesis.
	S.aureus,E.coli, P.aeruginosa,K.pneumoniae, E.faecalis,	Erythromycin-resistant	Cell surface damage and loss of the chain integrity.

	S. pneumoniae	Teicoplanin-resistant	ROS generation, cellular uptake of silver ions
	P. aeruginosa	Ofloxacin-resistant	Evade multidrug efflux pumps
AuNPs	S. aureus	Vancomycin-resistant	Combination with vancomycin.
	S. aureus	Methicillin-resistant	ROS generation
	E. coli, K. pneumoniae	Cefotaxime-resistant	Disruption of the bacterial cell wall, DNA damage.
ZnONPs	K. pneumonia	Ampicillin carbenicillin-resistant	ROS generation and disruption of bacterial cell wall.
	S. aureus	Methicillin-resistant	Enzyme inhibition.
CuNPs	S. aureus.	Methicillin-resistant	Copper ions release and subsequently bind with DNA

SILVER NANOPARTICLES (AGNPS)

Since the ancient times, silver has been recognized as having antimicrobial effects (Rai et al., 2009; Reidy et al., 2013). `Several mechanisms have been proposed to understand how AgNPs mediate cell death, including cell wall disruption (Lok et al., 2007; Bondarenko et al., 2013), oxidation of

cellular components, inactivation of the respiratory chain enzymes, production of ROS, and decomposition of the cellular components (Chen et al., 2014; Rizzello and Pompa, 2014; Dakal et al., 2016). The permeability of the membrane increases after incorporation of AgNPs into the cell membrane. The adsorption of the NPs leads to the depolarization of the cell wall, altering the negative charge of the cell wall to become more permeable. It was demonstrated disruption of the cell wall with subsequent penetration of the NPs. The entry of AgNPs induces ROS that will inhibit ATP production and DNA replication (Zhang et al., 2013; Dakal et al., 2016; Durán et al., 2016; Ramalingam et al., 2016). However, there is evidence that AgNPs can release Ag+, known to exhibit antimicrobial activity, when interacting with thiol-containing proteins, which weaken their functions (Durán et al., 2010). All the existing data indicates that AgNPs exert several bactericidal mechanisms (Karimietal, 2016).Concerns regarding the cytotoxicity and genotoxicity of AgNPs havebeen raised (Chopra, 2007) but various authors have conducted clinical trials based on AgNPs and no important clinical alterations have been detected (Munger et al., 2014a,b; Smock et al., 2014). Interestingly, AgNPs have been found to exhibit higher antimicrobial activity than antibiotics like gentamicin or vancomycin against P. aeruginosa and MRSA (Saeb et al., 2014). Lara et al. showed the potential bactericidal effect of AgNPs against MDR P. aeruginosa, ampicillin-resistant E. coli O157:H7 and erythromycin-resistantStreptococcus pyogenes (Lara et al) E. coli via the up-regulation of the expression of several antioxidant genes and ATPase pumps (Nagy et al., 2011). Nanocrystalline Ag-containing dressing are effective agents against antibiotic sensitive Gram-negative and -positive bacteria as well as antibiotic resistant bacteria, such as MRSA, Vancomycinresistant Enterococci (VRE) and Serratia marcescens, avoiding the formation of biofilms on biomaterials (Percival et al., 2007). An emerging practice is to combine AgNPs with antibiotics to enhance antimicrobial potency. Recently, Katya and collaborators showed that the combination of gentamicin and chloramphenicol with AgNPs has a better antibacterial effect in MDR E. faecalis than both antibiotics alone (Katva et al., 2018). McShan et al. described that AgNPs combined with either one of two-different class of antibiotics (tetracycline and neomycin) can exhibit a synergistic effect,

showing an enhanced antibacterial activity at concentrations below the MIC of either the NPs or the antibiotic (McShan et al., 2015). Polymyxin B is the most used AMP and exhibits antibacterial activity via interaction with the endotoxin LPS in the outer membrane of Gram-negative bacteria (Morrison and Jacobs, 1976; Lambadi et al., 2015). It was proved that AgNPs functionalized with polymyxin-B removed almost completely endotoxins from solutions and hindered the formation of biofilm onto surgical blades (Jaiswal et al., 2015; Lambadi et al., 2015. A recent study by Pal et al. describes a system consisting of a cysteine containing AMP conjugated with AgNPs, which demonstrated that the Ag-S bonds increased stability and enhanced antimicrobial activity than conjugation using electrostatic interactions (Pal et al., 2016).

GOLD NANOPARTICLES (AuNPs)

Cationic and hydrophobic functionalized AuNPs were shown to be effective against both Gram-negative and positive pathogens, including MRSA. This AuNP sexhibited low toxicity to mammalian cells (biocompatibility) and the development of resistance to these NPs was very low (Li et al., 2014). Vinoj et al. demonstrated that coating AuNPs with N-acylated homoserine lactonase proteins (AiiA AuNPs) resulted in a nanocomposite with activity against MDR species compared with AiiA proteins alone (Vinoj et al., 2015). Other approaches were also studied, as adsorbing AuNPs to PVAlysozyme micro bubbles potentiate the antibacterial activity due to the interaction of AuNPs with cells membranes causing bacterial lysis (Mahalingam et al., 2015).

METAL OXIDES

Metal oxides NPs are among one of the most explored and studied family of NPs and are known to effectively inhibit the growth of a wide range of sensitive and resistant Gram positive and gram negative bacteria, emerging as hopeful candidates to challenge antimicrobial resistance (Raghunath and Perumal, 2017; Reshma et al., 2017; Kadiyala et al., 2018). Iron oxide (Fe3O4), Zinc oxide (ZnO), and Copper oxide (CuO) possess antimicrobial properties and can be applied in clinical care.

ZINC OXIDE (ZnO)

ZnO releases Zn2+ in liquid medium and is adsorbed on the surface of bacteria or may entry the cell, where it interacts with functional groups in proteins and nucleic acids, normal physiological processes(Yuetal.,2014). Aswathanarayan

and Vittal described the antimicrobial effect of ZnO NPs against MDR Gram-positive and -negative pathogens. ZnONPs are also known for inhibiting biofilm formation and production of quorum-sensing-dependent virulence factors in P. aeruginosa (Lee et al., 2014; García-Lara et al., 2015).

COPPER OXIDE (CuO)

Copper containing NPs have been shown effective against animal and plant pathogens (Lewis Oscar et al., 2015), impeding formation of MDR biofilms, and showing the potential to serve as antimicrobial coating agents (Lewis Oscar et al., 2015). Kruk et al. and Zhang et al. showed that copper NPs are capable of inhibiting the growth of MDR bacteria, namely, P. aeruginosa and MRSA (Zhang et al., 2014, 2015b; Kruk et al., 2015) When CuONPs entry into bacteria metabolic functions are affected, such as active transport, electron transfer, and nitrogen metabolism (Su et al., 2015).

Genomic effect of silver nanoparticle in staphylococcus *aureus* bacteria

Staphylococcusaureus is a gram-positive bacterium that can cause human infections as skin ulcers and lethal diseases such as bacteremia, endocarditis, pneumonia, septicaemia. More than 90% of *S. aureus* have become resistant to antibiotics such as penicillin, methicillin, aminoglycoside, macrolides, and lincosamides. The silver nanoparticles are bound with antibiotics such as penicillin G, amoxicillin, erythromycin and vancomycin; this cooperation leads to increase antimicrobial effect against gram-positive (*S. aureus*) and gram-negative (*E. coli*) bacteria]. Usually, heavy metal ions (Ag^+ and Cu^+) have been recognized as fatal substances. However, their lethal doses for human cells are relatively high. Typically, Ag^+ ions at low concentrations (0.001-0.050 ppm) are effective bactericide, so it could also affect other organisms and eukaryotic cells during actions as antibacterial substances. Therefore, there are needs to conduct further studies in order to obtain effective antibacterial concentrations and its genotoxic effect as a suitable alternative for antibiotics and harmless dis infectants.This study was conducted to evaluate the effect of different dosages and duration of treatments with silver nanoparticles on the genome of the *S. aureus* as a bacterial model. For this purpose, RAPD-PCR technique was used in this study.

MATERIALS AND METHODS

In the present study, the *S. aureus* bacterium was cultured on eosin methylene blue (EMB) and blood agar media and then was passaged to 5 ml of brain heart broth (BHB) broth in order to assess the inhibitory properties of nanoparticles and DNA extraction.

Assessment of antimicrobial properties of silver nanoparticles

Phosphate-buffered saline (pH=7.4) was applied as a solvent for silver nanoparticles(silver nanoparticles less than 20 nm in diameter) in order to prepare a stock solution for silver nanoparticles.To investigate the effects of nanoparticles on bacteria, the concentrations of 100 and 150µg/ml of silver nanoparticles were inoculated into each test tube. The test tubes were placed in the shaking incubator at 37 °C overnight with a speed of 200 rpm and the optical density of each test tubes was measured at a wavelength of 600 nm during intervals of 2, 4 and 24 hours in order to evaluate bacterial growth. Before treatment, the test tubes without nanoparticles were used as a control as above method.

DNA extraction and RAPD-PCR

DNA extraction of treatment and control bacteria was carried out using a DNA extraction kit based on the instruction. Its quality and quantity were analyzed on 1% agarose gel electrophoresis and spectrophotometry.

Eleven 10-nucleotide random primers were used to examine genomes of control and treated bacteria using RAPD-PCR. Primers sequences and properties are given in Table 1. Each component of the polymerase chain reaction to perform RAPD-PCR techniques included 1 ml of extracted DNA samples, 2.5 µl (x10) of PCR buffer, 3 µl of $MgCl_2$, 1 µl of dNTP mix, 1µl of primers and 0.3 µl of DNA Taq polymerase. The volume of PCR reaction solution was brought to the volume of 25 µl with deionized distilled water. PCR reaction was carried out in Thermal Cycler device under a program, including five-minute cycle at 95 °C for initial denaturation of DNA template and then 40 cycles involving denaturation of DNA template strands at 95 °C for 35 seconds, primer annealing at 30 °C for 45 seconds, the extension phase at 72 °C for 45 seconds and finally a 7 minute cycle at 72 °C to complete the final extension. After optimizing the PCR conditions, the

combinations and the thermal profile were used for all 11 primers.

The proliferated products on 2% agarose gel containing red safe were electrophoresed in the TBE buffer (1x) for 5 hours with a voltage of 100 V. DNA ladder marker with a size of 100-bp was used to determine the size of the product and was imaged by Gel Doc. Scoring the bands obtained from RAPD analysis was conducted. Then the data were inserted in software based on the molecular weight.

RESULTS

Assessment of antimicrobial properties of silver nanoparticles

The results of evaluating of antibacterial effects of silver nanoparticles at different concentrations and times on *S. aureus*. Adding silver nanoparticles to the culture medium of bacteria after 2, 4 and 24 hours caused significant changes in the reduction in bacterial growth at the concentrations of 100 and 150 µg/ml, proving the effectiveness of these particles as suitable antimicrobial compounds.

Analysis of RAPD- PCR products

Electrophoretic bands obtained from amplification of 11 primers by RAPD- PCR. Primers from left to right are respectively related to the control sample, first treated sample with the concentration of 100 µg/ml and the second treated sample with the concentration of 150 µg/ml.Then the sizes of the bands obtained from RAPD analysis were determined using markers. Samples were scored as one or zero based on the presence or absence of bands. The basis of conclusion was a difference among bands, for each primer forms for control samples and treated samples. 11 respective primers produced 44 bands, among which 26 bands were different between the control and treated samples.

Based on data from RAPD, a similarity matrix was calculated for control and treated samples by DIC method. The genetic distance between samples was ranged from 0.6333333 to 1.000 in which the numbers closer to one shows the more genetic similarity between samples.

The dendrogram drawn by UPGMA method in NTSYS-PC software to compare the genetic variations between the control and the samples treated with silver nanoparticles. The control sample and treated samples were allocated separately in two main

branches that explain the genetic differences.

REPORT

The results of the present study indicated the efficacy of silver nanoparticles at the lowest dose and shortest time as an antibacterial agent. In addition, genetic diversity between control and treated bacteria demonstrated the silver nanoparticles effect on the genome of *S. aureus* as a model for gram-positive bacteria.

Genomic effect of copper nanoparticle in staphylococcus *aureus* bacteria

Copper oxide nanoparticle (<20nm) have been used in this study. The bacteria were first treated with 30 and 60 mg/ml copper oxide nanoparticle at the intervals of 2,4,24 hrs, and then DNA were extracted. In order to investigate the effects of copper oxide nanoparticle on the genome RAPD-PCR was employed.

Report

This study revealed that copper oxide nanoparticle not only effect thegrowth of bacteria but also affect the sequencing of genomic DNA

CONCLUSIONS

The nanoparticles are antibacterial compounds and can be an appropriate alternate to antibiotics. Given the irvast the rapeuticpotential, it is becoming in creasingly important to understand the mechanisms by which NPs complexes can impact bacterial viability. While one of the beneficial aspects of NPs drug carriers involves “macro targeting” Their impact of cell functions such as cell wall permeability, etux activity, formation of reactive species, and inhibition of essential cellular metabolism and reproduction is of utmost importance.

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