Journal of Drug Delivery Science and Technology 37 (2017) 20–27

Contents lists available at ScienceDirect

Journal of Drug Delivery Science and Technology

journal homepage: www.elsevier.com/locate/jddst

Nisin gold nanoparticles assemble as potent antimicrobial agent against *Enterococcus faecalis* and *Staphylococcus aureus* clinical isolates

Pradeepa [a], K. Udaya Bhat [b], S.M. Vidya [a, *]

[a] Department of Biotechnology, N.M.A.M. Institute of Technology, Nitte, 574110, India
[b] Department of Metallurgical and Materials Engineering, National Institute of Technology Karnataka, Mangalore, 575025, India

ARTICLE INFO

Article history:
Received 13 September 2016
Received in revised form
7 October 2016
Accepted 6 November 2016
Available online 8 November 2016

Keywords:
Gold nanoparticles
Nisin
Enterococci
Staphylococci
Drug delivery
Multidrug resistant

ABSTRACT

Enterococci and staphylococci have the potency to acquire resistant to antibiotics and have emerged as serious nosocomial pathogens responsible for various diseases. The continuous seeking of new antimicrobials against these pathogens is the only way to avoid the rapid spreading of diseases. Controlled fabrication of existing antimicrobials with nanoparticles offers an alternative strategy to combat against these pathogens with an effective manner. In the present study, gold nanoparticles (AuNPs) were functionalized with nisin to kill a wide range of clinically isolated *Enterococcus faecalis* and *Staphylococcus aureus* strains. Nisin functionalized gold nanoparticles (NAuNPs) exhibited good inhibitory activity against all seven multidrug resistant (MDR) and eight non-MDR *E. faecalis* and *S. aureus* strains. Minimum inhibitory concentration of NAuNPs was >8–32 fold lower than nisin. Interestingly, antibiotic resistant was not observed by these pathogens up to 8 generation. TEM and AFM investigation revealed that, the antimicrobial action of NAuNPs appears to act in three sequential stages: membrane destabilization, pore formation, followed by intracellular fluid leakage. In addition, NAuNPs were non toxic and showed less hemolytic activity. These findings indicated that, the NAuNPs can be served as an alternative antimicrobial agent to treat a wide range of enterococcal and staphylococcal infections.

1. Introduction

The emergence of drug-resistant infections has presented a serious challenge to antimicrobial therapies [1]. Bacteria such as *Staphylococcus aureus*, *Enterococcus faecalis* and *Enterococcus faecium* are the most frequent Gram positive infecting agents [2] and also causing agents of high human mortality rate worldwide. Staphylococci and enterococci are considered as the normal flora, but have emerged as serious nosocomial pathogens responsible for skin and soft-tissue infections, necrotizing pneumonia, endocarditis, urinary tract infections, bloodstream, biliary tract infections and meninges [3,4]. The extensive use of antimicrobials against these infections promotes the development of multidrug resistance (MDR), which made the situation harsher. Infections caused by MDR staphylococci and enterococci have reached epidemic proportions globally [5]. In addition to antimicrobial resistance, the acquisition of virulence factors and the ability to form biofilms have also contributed to the rise in nosocomial prevalence [6]. Drugs such as vancomycin, linezolid and daptomycin against MDR *S. aureus* and daptomycin dalbavancin, oritavancin, linezolid against *E. faecalis* are the preferred choice of treatment [7,8]; however, high rates of microbiological and clinical failures, increasing prevalence of non-susceptible strains and drug toxicity limit their effectiveness [9,10]. Hence, the identification of new antimicrobials with broad-spectrum activity and reduced toxicity became the global challenge.

The design and discovery of new antimicrobials or antimicrobial assembly is one of the most important priorities in infectious diseases control. One such assembly is drug delivery using nanoparticles. Nanoparticles provide a versatile platform to incorporate a variety of antibiotics, proteins, nucleic acids and anticancer molecules for effective drug delivery [11]. The most studied nanoparticles for drug delivery is gold nanoparticles (AuNPs); due to their low cytotoxicity, small size and greater delivery efficiencies [12–14]. AuNPs is recognized as potent agents to solve many pharmacological issues such as antibiotic resistance, cellular toxicity and reduced bioavailability of drugs. The high surface ratio and presence of capping agent on AuNPs allow incorporating a variety of ligands for effective delivery [15,16]. Cationic property of

* Corresponding author.
E-mail address: drvidyasm@nitte.edu.in (S.M. Vidya).

http://dx.doi.org/10.1016/j.jddst.2016.11.002
1773-2247/

most AuNPs is another important criterion to target negatively charged bacteria; since it improves the interaction with the bacterial membrane [17]. Further criterion of AuNPs is able to enhance the internalization of antibiotics or any biomolecules into the bacterial cell. Taking this in account, in the present study, a well known antimicrobial peptide-nisin was fabricated with AuNPs to improve the killing potential towards a wide range of nosocomial pathogens. The reason behind the selection of nisin is due to GRAS status (generally regarded as safe) and non toxicity [18]. AuNPs was first synthesized by a lactobacillus exopolysaccharide (EPS) to avoid potential toxic issues. Functionalized AuNPs were tested against various nosocomial pathogenic strains of *E. faecalis* and *S. aureus* using agar well diffusion assay, minimum inhibitory concentration (MIC) and minimum bactericidal concentration assay (MBC). Transmission electron microscopy (TEM) and atomic force microscopy (AFM) were performed to understand the bactericidal mechanism of nisin functionalized AuNPs (NAuNPs). Cytotoxicity of NAuNPs was evaluated using mammalian cell lines. Finally, biocompatibility of NAuNPs was tested in human red blood cells (RBC) to evaluate the efficacy for *in vivo* application and biosafety.

2. Materials and methods

2.1. Chemical compounds and microorganisms

Gold (III) chloride trihydrate was purchased from Sigma Chemicals, USA. Nisin (1 mg = ~1000 U) and Muller Hinton (MH) broth were procured from HiMedia Laboratory, India. MDR and other bacteria were obtained from local hospitals, Mangalore, India. *Staphylococcus aureus* 9144 was purchased from American Type Culture Collection (ATCC), USA. All bacteria were maintained in Luria Broth.

2.2. AuNPs preparation and characterization

AuNPs was prepared by exopolysaccharide (EPS) as a reducing agent which is initially isolated from fish derived *L. rhamnosus* RVP1. In an Erlenmeyer flask, a mixture of aqueous gold (III) chloride trihydrate (1 mM) and EPS (1%) was stirred at 100 °C until colour of the mixture turned pink. The mixture was then centrifuged at 20,000 rpm for 30 min at 4 °C, washed three times with distilled water, resuspended in same (10 ml) and stored at 4 °C. For functionalization of AuNPs, 1 ml of AuNPs solution was mixed with 500 μg/ml nisin and incubated at room temperature for 2 h with constant shaking. Later, the mixture was centrifuged at 18,000 rpm for 30 min at 4 °C, washed three times with distilled water and resuspended in the same. Concentration of functionalized nisin was measured by the Bradford protein assay [19].

UV visible spectra of AuNPs and NAuNPs were recorded in UV spectrophotometer at 400–600 nm scale (UV-3600, UV-VIS-NIR spectrophotometer, Shimadzu). FTIR spectra were recorded using a Shimadzu FTIR-8300 spectrometer at 4000-400 cm^{-1} frequency range using the KBr pellet method. TEM images were recorded using JEOL model 1200EX, coupled with energy dispersive X-ray spectroscopy (EDX), and operated at an accelerating voltage of 200 kV. Zeta potential was measured using Zetasizer Nano-ZS (Malvern Instruments, UK).

2.3. Antibacterial activity

Inhibitory activity of nisin alone and NAuNPs was evaluated against various pathogenic bacteria listed in Table 1 by well diffusion method. Approximately 100 μl of 10^6 CFU/ml of the indicator bacteria were seeded on the surface of MH media containing 1% agar and then wells were made with a sterile cork borer. In each plate, 30 μl free nisin at 250 U/ml, NAuNPs (250 U/ml nisin equivalent) and AuNPs were loaded to the wells and incubated at 37 °C for 24 h. After incubation, plates were observed for the presence of a zone of inhibition around the well.

The effect of nisin and NAuNPs on bacterial growth was further determined by a broth dilution method. Here, the initial concentration of NAuNPs and nisin alone was maintained to 2500 U/ml. In aseptic condition, 100 μl of MH broth was distributed to all the wells of microtiter plates and 100 μl NAuNPs or nisin was added to the wells of the first column and mixed. Then 100 μl of mixed sample from the first column was transferred to wells of the second column and similar transfer was repeated so that each wells of the column received half of the drug concentration of the previous column. Finally, 20 μl of the freshly prepared indicator bacterial strains (10^6 CFU/ml) were added and incubated for overnight at 37 °C. At the end of the incubation, the result was recorded by observing the visual sign of bacterial growth and the lowest concentration of the test sample that inhibited the bacterial growth was considered as MIC. To determine the MBC, 10 μl of the sample from MIC microtiter plate was directly transferred to agar plates and incubated for 24 h at 37 °C. The concentration at which no bacterial colony appeared was considered as the MBC.

Resistance development assay was performed according to the method of Li et al. [20]. MDR *S. aureus* and *E. faecalis* were inoculated in MH broth with 104.1 U/ml NAuNPs (2/3 of 156.2 U/ml, MIC) at 37 °C for overnight (first generation). The cultures were then harvested and tested for MIC as describe above (second generation). Similar repetitions were performed for up to 8 generation.

2.4. TEM and AFM

For microscopic evaluation, *S. aureus* was incubated with NAuNPs with corresponding MIC (156.2 U/ml) for 6 h at 37 °C, harvested by centrifugation and washed with sterile distilled water. For TEM imaging, NAuNPs treated bacteria were prepared by slow

Table 1
Antimicrobial activity of NAuNPs and nisin against various *S. aureus* and *E. faecalis* strains.

Indicator bacteria (number of strains)	Well diffusion (mm)		AuNPs	MIC (U/ml)		AuNPs	MBC (U/ml)		AuNPs
	Nisin (250 U/ml)	NAuNPs (250 U/ml)		Nisin	NAuNPs		Nisin	NAuNPs	
E. faecalis (4)[a]	<1	2.6–5.5	<1	>2500	156.2–312.5[b]	NA	>2500	312.5–625[b]	NA
E. faecalis (3)	<1	6.8–7.5	<1	>2500	156.2–312.5[b]	NA	>2500	312.5–625[b]	NA
S. aureus (5)[c]	<1	6.3–11.8	<1	>1250–2500	39.0–78.1[b]	NA	>2500	78.1–312.5[b]	NA
S. aureus (3)[a]	<1	2.3–6.5	<1	>2500	156.2–312.5[b]	NA	>2500	312.5–625[b]	NA
S. aureus 9144	3.6 ± 0.3	8.2 ± 2.0	<1	312.5	39.0[b]	NA	625	78.1[b]	NA

NA-no inhibitory activity.
[a] Multidrug resistant bacteria. MDR bacteria were resistant to erythromycin, levofloxacin, ampicillin and ceftriaxone.
[b] Significant difference ($p < 0.05$) between NAuNPs and nisin alone.
[c] One *S. aureus* strain (out of five) showed 3 mm inhibitory zone against nisin at 250 U/ml.