Journal of Trace Elements in Medicine and Biology 29 (2015) 235–241

Contents lists available at ScienceDirect

Journal of Trace Elements in Medicine and Biology

journal homepage: www.elsevier.de/jtemb

PHARMACOLOGY, Pharmacology

Anti-biofilm activity of biogenic selenium nanoparticles and selenium dioxide against clinical isolates of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Proteus mirabilis*

Mojtaba Shakibaie[a], Hamid Forootanfar[b], Yaser Golkari[c], Tayebe Mohammadi-Khorsand[a], Mohammad Reza Shakibaie[c,d,e,*]

[a] *Pharmaceutics Research Center, Institute of Neuropharmacology, Kerman University of Medical Sciences, Kerman, Iran*
[b] *Herbal and Traditional Medicines Research Center, Kerman University of Medical Sciences, Kerman, Iran*
[c] *Department of Microbiology and Immunology, Kerman University of Medical Sciences, Kerman, Iran*
[d] *Research Center for Tropical and Infectious Diseases, Kerman University of Medical Sciences, Kerman, Iran*
[e] *Environmental Health Engineering Research Centers, Kerman University of Medical Sciences, Kerman, Iran*

ARTICLE INFO

Article history:
Received 3 March 2014
Accepted 22 July 2014

Keywords:
Selenium nanoparticle
Biosynthesis
Biofilm
Selenium dioxide

ABSTRACT

The aim of the present study was to investigate the anti-biofilm activity of biologically synthesized selenium nanoparticles (Se NPs) against the biofilm produced by clinically isolated bacterial strains compared to that of selenium dioxide. Thirty strains of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Proteus mirabilis* were isolated from various specimens of the patients hospitalized in different hospitals (Kerman, Iran). Quantification of the biofilm using microtiter plate assay method introduced 30% of *S. aureus*, 13% of *P. aeruginosa* and 17% of *P. mirabilis* isolates as severely adherent strains. Transmission electron micrograph (TEM) of the purified Se NPs (produced by *Bacillus* sp. MSh-1) showed individual and spherical nano-structure in the size range of 80–220 nm. Obtained results of the biofilm formation revealed that selenium nanoparticles inhibited the biofilm of *S. aureus*, *P. aeruginosa*, and *P. mirabilis* by 42%, 34.3%, and 53.4%, respectively, compared to that of the non-treated samples. Effect of temperature and pH on the biofilm formation in the presence of Se NPs and SeO_2 was also evaluated.

Introduction

Biofilm is currently defined as structured bacterial communities enclosed in a self-produced extracellular polymeric substance (EPS) such as exopolysaccharide, extracellular DNA (eDNA), and proteins adhered to abiotic or biological surfaces [1,2]. Bacterial cells in biofilm are better protected, less subject to mutation, become more resistant to antibiotics, and represent lower metabolic activity [3]. There are several reports on relation between biofilm and antibiotic resistance which made this problem rarely resolved [4,5]. In this regard, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Proteus mirabilis* are among the leading nosocomial pathogens capable of producing severe biofilm-related infections such as colonization on central venous catheters (CVCs), lower respiratory tract infections (due to contaminated ventilators), and catheter-related ascending urinary tract infections [6,7]. Furthermore, biofilm formation is important, because this mode of growth is associated with the chronic nature of the subsequent infections, and colonizing bacteria can resist against phagocytosis and evade the body's defense system [3]. Biofilm-associated infections have affected millions of people in both developed and developing countries and consequently caused death in their victims [4,8]. So, investigations on biofilm inhibitory activity of even natural or synthetic compounds have received more attention in recent decades [2].

Nanotechnology concerns the arrangement of materials at the atomic stage to achieve nanoscale materials with unique physicochemical and biological characteristics [9,10]. The ability of nanostructures for the inhibition or disruption of microbial-derived biofilm has been recently reported. For example, Naik and Kowshik [11] investigated the effect of sol–gel coatings of AgCl–TiO_2 nanoparticles for the inhibition of biofilm formed by *Escherichia coli*,

* Corresponding author at: Department of Microbiology and Immunology, Kerman University of Medical Sciences, Kerman, Iran. Tel.: +98 3403221660; fax: +98 3403221671.
E-mail addresses: mohammadreza.shakibaie@gmail.com, mr_shakibaei@kmu.ac.ir (M.R. Shakibaie).

http://dx.doi.org/10.1016/j.jtemb.2014.07.020
0946-672X/

S. epidermidis, and *P. aeruginosa*. Anti-biofilm activity of chemically synthesized ZnO and CuO nanoparticles was reported by Tabrez Khan et al. [12]. Kalishwaralal et al. (2010) [13] studied the inhibition effect of silver nanoparticles on the biofilm formation of *P. aeruginosa* and *S. epidermidis*.

Selenium (Se) is a micronutrient metalloid incorporated (in the form of selenomethionine, selenocysteine) in the structure of several enzymes such as glutathione peroxidases (GPx), iodothyronine deiodinases, and thioredoxin reductase (TrxR), which are involved in antioxidant defense, detoxification, and metabolism, respectively [14,15]. Excellent biological properties of selenium nanoparticles (Se NPs) such as antibacterial, antiviral, and antioxidant activity together with their lower toxicity have introduced it as an interesting subject in the field of nanotechnology [16,17]. Behind the physicochemical techniques applied for the synthesis of Se NPs [18,19], biological methods, (synthesis of nanostructures using some bacterial and fungal strains as well as several plant extracts) [20,21] supply novel, clean, non-toxic, and eco-friendly method for the production of Se NPs. To the best of our knowledge and according to a survey of the literature, there is no report on the anti-biofilm effect of the biogenic Se NPs. In the present study, the biogenic Se NPs was purified from the whole cell lysate of *Bacillus* sp. MSh-1 and their effects on the biofilm formation by three bacterial strains (*P. mirabilis*, *S. aureus*, and *P. aeruginosa*) isolated from clinical specimens compared to selenium dioxide were also studied.

Materials and methods

Chemicals

Selenium dioxide (SeO_2), nutrient broth, *n*-octyl alcohol, sodium dodecyl sulfate (SDS) and Tris-base were purchased from Merck Chemicals (Darmstadt, Germany). All other chemicals and solvents were of analytical grade.

Biosynthesis and purification of the Se NPs

Bacterial strain of *Bacillus* sp. MSh-1 which was previously isolated from the Caspian Sea (located in the northern part of Iran) and identified by 16S rDNA gene analysis technique was applied for the biosynthesis of Se NPs based on the method described by Shakibaie et al. [21]. In brief, 100 mL sterile nutrient broth (NB) medium containing SeO_2 (final concentration of 1.26 mM) was inoculated with 1 mL of the fresh inoculums (OD_{600}, 0.1) of *Bacillus* sp. MSh-1 and incubated in a shaker incubator (150 rpm) at 30 °C for 14 h. Thereafter, the bacterial cells were harvested by centrifugation (4000 × *g* for 10 min) followed by washing the obtained biomass with the sterile NaCl solution (0.9%) for three times. The bacterial pellets were then disrupted by grinding the frozen cells in liquid nitrogen using a mortar and pestle. The resulting slurry was consequently ultrasonicated at 100 W for 5 min using ultrasonic processor (Sonics Vibra Cell VC-505/220, Newtown, USA) over three 15 s periods, with an interval of 45 s between the periods. The sonicated extract was then washed for three times by the sequential centrifugation (14,000 × *g*, 5 min) with a 1.5 M Tris–HCl buffer (pH 8.3) containing SDS (1%) and deionized water, respectively. Subsequently, Se NPs were extracted and purified by organic–aqueous partitioning system (*n*-octanol–water), as previously described [21]. Surface morphology of the prepared biogenic Se NPs was examined by transmission electron microscope (Zeiss Supra 55 VP TEM, operated at 100 kV) equipped with an EDX (energy dispersive X-ray) microanalyzer. The related size distribution pattern of biologically synthesized Se NPs was plotted by manually counting of 400 individual particles from different TEM images.

Isolation and identification of biofilm-producing bacteria

Clinical specimens of wounds, urine, cerebrospinal fluid (CSF), and blood as well as lung and nasal secretion were collected from the patients hospitalized in different hospital wards like burn, ICU, pediatric, and surgery from August 2012 to March 2013 in Kerman, Iran. All the collected samples were aseptically transported to the nutrient broth within 45 min of sample collection. Each sample was then diluted (10^{-2}) using sterile normal saline solution (0.9%) and streaked onto MacConkey and sheep blood agar medium followed by incubation at 37 °C for 24 h and identification of isolated microorganisms based on the standard procedures [22]. The identified isolates were then mixed with 2 mL sterile Trypticase Soy Broth (TSB) containing glycerol (15%) and preserved at −70 °C.

Antibiotic susceptibility testing and determining the MIC for Se NPs

Susceptibility of each isolate to the antibiotics of methicillin, tetracycline, amikacin, gentamicin, ciprofloxacin, ceftazidime, vancomycin, erythromycin, chloramphenicol, amoxicillin + clavulanic acid, and imipenem was evaluated using disk diffusion method based on the protocol described by Clinical and Laboratory Standards Institute (CLSI 2006) [23]. All the applied antibiotic disks were purchased from Oxoid Inc. (Mumbai, India). Reference strains *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 were included as controls. Zone of inhibition surrounding each disk was measured and labeled as resistance, intermediate, and sensitive according to CLSI protocol. In order to determine the minimum inhibitory concentration (MIC) of biogenic Se NPs (concentration range of 0–100 $\mu g\,mL^{-1}$) on the isolated strains, the agar dilution method was applied according to the method by Zare et al. [24].

Biofilm formation assay

The biofilm formed by the above isolates was quantified by microtiter method as described previously [25] with some modification. Briefly, one loopful from each isolated colony was inoculated into a sterile TSB medium (2 mL) containing glucose (1% W/V) to optimize biofilm production. Optical density (OD_{650}) was then adjusted to 0.13 to reach 0.5 McFarland standard (1.5×10^8 CFU mL^{-1}) followed by further dilution of the prepared bacterial suspension to reach $\sim 10^6$ CFU mL^{-1} and addition of 100 μL of each prepared inoculum to 96-well flat bottom tissue culture microplate. Similarly, 100 μL of the TSB medium without any bacterium (negative control) was added to the related well and the microtiter plate was then incubated at 37 °C under static condition. To evaluate the quality of the method, standard strain of *E. coli* (DH5α) was used as control for no biofilm microorganism. After 24 h incubation at 37 °C, non-adherent cell suspensions were aseptically aspirated, washed, and replaced with 10 μL of sterile phosphate buffered solution (pH 7.2) to remove any remaining suspended cells. In order to fix the biofilm, 150 μL of methanol was added to each well and kept at room temperature (25 °C) for 20 min. Methanol was then removed and replaced with 200 μL of crystal violet solution (1% W/V). The wells containing biofilm matrix were slowly washed with sterile deionized water and kept at room temperature until drying. Thereafter, 200 μL of glacial acetic acid (33% V/V) was added to each well and the optical density of each well was measured at 570 nm using Synergy 2 multi-mode microplate reader (BioTek, USA). The isolates were then classified into strongly adherent, moderately adherent, weakly adherent, and non-adherent strains based on the formula given by Stepanovic et al. [25]. All the mentioned experiments were performed in