Enzyme and Microbial Technology 95 (2016) 192–200

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

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

Anti-neoplastic selenium nanoparticles from *Idiomarina* sp. PR58-8

Pallavee Srivastava, Meenal Kowshik*

Department of Biological Sciences, Birla Institute of Technology and Science Pilani, K K Birla Goa Campus, NH-17B, Zuarinagar, Goa, 403 726, India

ARTICLE INFO

Article history:
Received 19 March 2016
Received in revised form 18 July 2016
Accepted 1 August 2016
Available online 2 August 2016

Keywords:
Idiomarina
Biosynthesis
Selenium nanoparticles
Thiols
Anti-neoplastic
Reactive oxygen species
Apoptosis

ABSTRACT

Selenium nanoparticles (SeNPs) with novel biological activities, cancer cell selectivity, and low toxicity towards normal cells have gained attention for chemo-therapeutic and chemo-preventive applications. These nanoparticles may be synthesized using micro-organisms, which is the green alternative of nanofabrication. Here we report the intracellular synthesis of SeNPs by the moderate halophilic bacterium, *Idiomarina* sp. PR58-8 using sodium selenite as the precursor. Characterization of SeNPs by XRD exhibited the characteristic Bragg's peak of hexagonal selenium with a crystallite domain size of 34 nm. Morphological characterization by TEM exhibited spherical nanoparticles with a size distribution of 150–350 nm. The non-protein thiols were found to be involved in resistance/reduction of sodium selenite. The SeNPs exhibited selectivity in exerting cytotoxicity towards human cervical cancer cell line, HeLa, while being non-toxic towards model normal cell line, HaCaT. The SeNPs induced a caspase-dependent apoptosis in HeLa cell lines as exhibited by the ROS assay, apoptotic index assay, and western blot analysis. These results suggest the application of SeNPs synthesized by *Idiomarina* sp. PR58-8 as potential anti-neoplastic agents.

1. Introduction

Selenium (Se) is an essential micronutrient, with 55 μg/day of recommended dietary allowance (RDA) for adults and children above 14 years of age. Se gets incorporated in various proteins such as glutathione peroxidase, thioredoxin reductase (TR), selenoprotein P, and other enzymes as selenocysteine (Se-Cys), and/or Seleno-methionine (Se-Met) in place of methionine [1]. Thus, Se at nutritional doses, supports cell cycle progression, prevents cell death, and is essential for optimum immune response [2]. Adequate Se supplementation is co-related to a reduced risk of cancer, cardiovascular diseases, diabetes and male fertility, while Se deficiency results in an increased risk of mortality, poor immune function and cognitive decline [3,4].

Selenium compounds, both organic (Se-Met, methylselenocysteine, etc.) and inorganic (sodium selenite, selenous acid, etc.) are known to exhibit anti-cancer properties [5]. However, the doses required to achieve cell death/anti-cancer activity using these compounds are higher than the RDA and result in systemic toxicity [6]. Previous studies suggest that the chemical form and the dosage are important determinants of anti-cancer activities of selenocompounds [7]. Elemental Se nanoparticles (SeNPs) present an excellent alternative to selenocompounds for cancer therapy and prevention. They exhibit low toxicity and unique biological activity towards non-cancerous cell, while being cytotoxic towards cancer cells at lower concentrations [8,9]. SeNPs have shown efficient anti-proliferative properties against various cancer cell lines like A375 (human melanoma cell line), HepG2 (hepatocellular carcinoma cell line), MCF-7 (breast adenocarcinoma cell line), MDA-MB-231 (human breast carcinoma), HeLa (human cervical carcinoma cell line), and HK-2 (human kidney cell line) [8,10,11]. These SeNPs are known to enhance/augment the anti-neoplastic properties of chemotherapeutic drugs such as adriamycin, doxorubicin, and irinotecan [12–14].

Synthesis of these chemo-therapeutic and/or chemo-preventive SeNPs has been achieved by numerous physical, chemical, and biological methods [15–19]. Biological methods of nanoparticle syntheses entail the use of either micro-organisms or plant extracts [18,19,36]. These methods are advantageous over the chemical and physical methods, as the synthesis of nanoparticles does not

Abbreviations: SeNPs, selenium nanoparticles; XRD, X-ray diffraction; TEM, transmission electron microscope; ZMB, Zobell's marine broth; MIC, minimum inhibitory concentration; 2,3 DAN, 2,3 diaminonaphthalene; EDS, energy dispersive spectroscopy; CFL, cell free lysate; TSH, total thiol; NP-SH, non-protein thiol; PB-SH, protein-bound thiol; DTNB, 5,5′dithio-bis (2-nitrobenzoic acid); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; DCFH-DA, 2′7′- dichlorofluorescein diacetate; AI, apoptotic index; AO/EB, acridine orange/ethidium bromide; GSH, glutathione.

* Corresponding author.
E-mail addresses: meenal@goa.bits-pilani.ac.in, meenalkowshik@gmail.com (M. Kowshik).

http://dx.doi.org/10.1016/j.enzmictec.2016.08.002
0141-0229/

require extreme conditions. The nanoparticles synthesized using microbes are usually capped by cellular metabolites contributed by the organism [20]. In view of these advantages, numerous bacteria, fungi, and archaea have been used for SeNPs synthesis [18,19,21]. Here we report the intracellular synthesis and characterization of SeNPs by a moderate halophilic marine bacterium, *Idiomarina* sp. PR58-8. The anti-proliferative properties of these SeNPs against human cervical cancer cell line, HeLa and its mechanism is also reported.

2. Materials and methods

2.1. Materials

All the materials purchased from HiMedia (India), unless specified, were of certified A.R. grade and used without any further purification.

2.2. Organism and culture maintenance

Idiomarina sp. PR58-8 was isolated in our laboratory previously by Seshadri et al. [22], from the soil samples of the banks of Mandovi Estuary, Goa, India. The culture was stored as glycerol stocks at −80 °C. Zobell marine broth 2216 (ZMB; Himdeia, India) was used for revival, enrichment, growth and sub-culturing.

2.3. MIC determination and growth kinetics studies

Idioamrina sp. PR58-8 was grown in ZMB 2216 medium in the presence of sodium selenite (Na_2SeO_3; 0.05- 12 mM) to determine the minimum inhibitory concentration (MIC). Na_2SeO_3 was prepared as a 1 M stock in sterile distilled water (SDW) and filter sterilized. The cultures were incubated at 37 °C, for 72 h under agitation (110 rpm). The minimum concentration of Na_2SeO_3 at which no growth was observed was designated as the MIC. Appropriate negative controls were also run simultaneously. Growth was indicated by brick-red coloration. Further, the growth kinetics of *Idiomarina* sp. PR58-8 was determined in presence of 4 and 8 mM Na_2SeO_3. In brief, ZMB 2216 was inoculated with 1% culture, supplemented with the requisite amount of Na_2SeO_3 and the flasks were incubated at 37 °C, 110 rpm. An aliquot of 1 ml was withdrawn every 4 h, till 60th h, and the total protein content of the cells was estimated by Bradford's method using bovine serum albumin (BSA) as the standard [23]. The growth kinetic parameters such as specific growth rate (μ; h^{-1}), lag time (t_l; mins) and doubling time (t_d; mins) were determined according to Berney et al. [24], and Breidt et al. [25]. The growth kinetic parameters of *Idiomarina* sp. PR58-8 grown in presence of Na_2SeO_3, were compared with that of control (culture grown in the absence of Na_2SeO_3). The experiment was performed in triplicates on different days.

2.4. Selenite uptake studies

The selenite concentration of the supernatant and cell pellet of *Idiomarina* sp. PR58-8 grown in presence of Na_2SeO_3 was estimated to determine selenite uptake by the cells. The selenite concentration in the supernatant was determined according to Watkinson [26] using 2,3-diaminonapthalene (2,3-DAN) with minor modifications. In brief, the culture was grown in the presence of 4 mM and 8 mM Na_2SeO_3 and an aliquot of 1 ml was withdrawn every 4 h starting from 0 h. The aliquots were centrifuged and the supernatants were used for determination of selenite content, while the cell pellets were used to determine the selenite uptake. Sample supernatant (250 μl) was added to a mixture of 10 ml 0.1 M HCl, 0.5 ml 0.1 M EDTA, 0.5 ml 0.1 M NaF, and 0.5 ml 0.1 M disodium oxalate. Subsequently 2.5 ml of 0.1% 2,3-DAN was added and the tubes were incubated at 40 °C for 40 mins. After cooling the mixture, the selenium-2,3-DAN complex was extracted with 6 ml cyclohexane by shaking the tubes vigorously for 1 min. To further accelerate the phase separation, the tubes were centrifuged at 3,000 × *g* for 10 mins. Absorbance of the organic phase was measured at 377 nm on UV–vis double beam spectrophotometer (Shimadzu, Japan, UV-2450) using a 1-cm-path length cuvette. Appropriate positive and negative controls were run simultaneously. All the experiments were carried out in triplicates. Calibration curves were obtained using 0–10 mM selenite solution.

The cell pellets obtained at various time points were lysed by sonication (Microson^{TM} Sonicator) at 0 °C for three cycles of 1 min each at three RPS (40 W). The selenium obtained after lysis was converted to selenite by oxidation according to Kessi and Hanselmann [27] and the selenite was quantified according to Watkinson [26] as described above. Any loss of selenite was estimated by determining the difference between the selenite uptake by the cells, and the unutilized selenite in the medium at the end of the experiment.

2.5. Selenium nanoparticles (SeNPs) synthesis and characterization

Idiomarina sp. PR58-8 was grown in ZMB 2216 in presence of 8 mM Na_2SeO_3 at 37 °C, 110 rpm for 48 h. The selenium nanoparticles synthesis was indicated by brick-red coloration of the medium. Culture supernatant and medium controls were also incubated with 8 mM Na_2SeO_3 and acted as negative controls. The SeNPs were extracted from the cells by wet heat sterilization process in a laboratory autoclave at 121 °C, 15 psi for 20 mins. The pellet obtained on centrifugation (10,000 × *g*, 20 mins), was further purified by acid wash, and then dialyzed against deionized water for 12 h with change of water every 2 h. The dialyzed samples were dried in a hot air oven at 70 °C overnight, and the powder obtained was ground in a mortar and pestle. The nanopowder was used for characterization studies.

The SeNPs were dispersed in deionized water and their excitation spectrum was recorded in the range of 200- 800 nm using a UV–vis double beam spectrophotometer. Rigaku Mini-Flex II powder X-Ray −diffractometer operated at 30 kV/15 mA with Cu Kα (1.54 Å) as radiation source and scanning mode of 2θ/θ continuous scanning was used for crystallographic characterization of the SeNPs. Debye-Scherrer formula: $D = k\lambda/\beta \cos\theta$, where λ = wavelength of X-ray applied (1.54 Å), k = numerical constant with a value of 0.94, $\beta_{1/2}$ = full width (radians) at half maximum of the signal (1 0 1) and θ = Bragg angle of signal (1 0 1), was used for determining the crystallite domain size. The lattice parameters were determined using the formula: $\frac{1}{d^2} = \frac{4}{3}\left(\frac{h^2+hk+k^2}{a^2}\right) + \frac{l^2}{c^2}$; where (*hkl*) are Braggs diffraction planes. Morphological characterization of the SeNPs was achieved by transmission electron microscopy. The colloidal suspension of SeNPs was drop-coated on carbon-coated TEM grids and the images were obtained using Philips (Model- CM200) transmission electron microscope (TEM; resolution 2.4 Å) operated at an accelerating voltage of 190 keV. Field emission gun- scanning electron microscope (FEG-SEM; JSM-7600F) equipped with energy dispersive spectroscope (EDS) operated at 20 keV was used for determining the elemental composition of the SeNPs.

2.6. Thiol assay

Idiomarina sp. PR58-8 grown in the presence of 0 mM (control), 4 mM, and 8 mM Na_2SeO_3, was harvested by centrifugation at 10,000 × *g* for 20 mins and the cell pellet was washed twice with 25 mM Tris-HCl buffer (pH 7.5). The pellet obtained was re-suspended in the same buffer and subjected to sonication at 0 °C for