Journal of Photochemistry & Photobiology, B: Biology 166 (2017) 202–211

Contents lists available at ScienceDirect

Journal of Photochemistry & Photobiology, B: Biology

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

Photo-induced biosynthesis of silver nanoparticles from aqueous extract of *Dunaliella salina* and their anticancer potential

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ARTICLE INFO

Article history:
Received 17 October 2016
Accepted 26 November 2016
Available online 5 December 2016

Keywords:
Dunaliella salina
Photo-induced
Green route
Silver nanoparticles
Anticancer activity
Apoptosis

ABSTRACT

The synthesis of silver nanoparticles (AgNPs) *via* green route, using biological entities is an area of interest, because one of the potential applications in the nanomedicine. In the present study, we have developed photo-induced, ecofriendly, low cost method for biosynthesis of the stable silver nanoparticles using aqueous extract of *Dunaliella salina* (AED) which act as both reducing as well as stabilizing agent. Biosynthesis of the AgNPs was optimized as: sunlight exposure (30 min), AED (5% (v/v)) and $AgNO_3$ (4 mM). Biosynthesis of AgNPs was monitored by using UV–Vis spectroscopy which exhibited sharp SPR band at 430 nm after 30 min of bright sunlight exposure. SEM and TEM analyses confirmed the presence of spherical AgNPs with average size of 15.26 nm. Crystalline nature of AgNPs was confirmed by SAED and XRD analyses where Braggs reflection pattern at (111), (200), (220) and (311) corresponded to face centered cubic crystal lattice of metallic silver. FTIR analysis revealed the involvement of various functional groups present in AED. AFM analysis confirmed the average surface roughness of synthesized AgNPs as 8.48 nm. AgNPs were also screened for anticancer potential using assay of calcein AM/PI, Annexin/PI and cancer biomarkers against cancer cell line (MCF-7), while normal cell line (MCF-10A) were kept as control. Interestingly, anticancer potential was comparable to the known anticancer drug (Cisplatin), and was not detrimental to the normal cell line. Therefore, such green synthesized AgNPs may be explored as anticancer agent.

1. Introduction

Cancer is havoc to human health, worldwide. It is on the top among the diseases causing deaths in the developed countries in contrast to the developing countries, where this is still at second level [1]. The cancer risk is magnified through changes in the human lifestyle, mainly due to modernization. More than 70% of the cancer deaths occur in Africa, Asia, Central and South America [2]. Treatment strategies such as, classical chemotherapy and radiotherapy are not only costly but have side effects on the normal cells in addition to the cancer ones [3]. However, many chemotherapeutic agents are derived from natural resources [4]. Emerging area of nanomedicine indicated, that among the various nanoparticles, AgNPs can act as the better agent for assessing their anticancer potential, owing to their relative safety for use in the medicine [5,6]. Silver nanoparticles may be synthesized by various methods such as chemical, physical and biological ones. Synthesis of nanoparticles with various chemicals may lead capping of toxic chemical species on nanoparticles surface, affecting adversely in the biological applications [7]. Rapid (15 min) synthesis of AgNPs (4–8 nm in size) was obtained at relatively low temperature using hydroxypropyl starch (HPS) in a chemical reduction method (without any organic solvent or other reducing agent) [8]. In another strategy, green and rapid synthesis of gold and core-shell silver – gold nanoparticles were synthesized with microwave assisted irradiation, using curdlan (a neutral exoploysaccharide) [9]. There was already report of light controlled growth of silver nanoparticles in terms of shape and size [10]. Synthesis of AgNPs using loading of HPS on the fabrics have shown effective antibacterial activity [11]. *In situ* AgNPs loaded on cellulose substrate has also been demonstrated as antibacterial in nature [12]. However, green synthesized AgNPs from the flower extract of *Achillea biebersteinii* [13], and leaf extract of *Couroupita guianensis* [14] against cancer cell line MCF-7 cells and fruit extract of *Piper nigrum* towards MCF-7 and Hep-2 cells [15] as well as chemically

Abbreviations: AgNPs, silver nanoparticles; AED, aqueous extract of *D. salina*; FTIR, Fourier transform infrared spectroscopy; XRD, X-ray diffraction; SEM, scanning electron microscopy; TEM, transmission electron microscopy; AFM, atomic force microscopy; TGA, thermo gravimetric analysis; MJM, modified Johnson's medium; SPR, surface Plasmon resonance; MFI, mean Fluorescence intensity; SD, standard deviation; EDX, energy dispersive X-ray detector; SAED, selected area electron diffraction; JCPDS, Joint Committee on Powder Diffraction Standards; FCC, face centered cubic.

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http://dx.doi.org/10.1016/j.jphotobiol.2016.11.020
1011-1344/

synthesized AgNPs [16], showed cytotoxicity and induction of apoptosis including cell cycle inhibition. Although, AgNPs have already been synthesized by bacteria [17], cyanobacteria [18], green algae [19,20], fungi [21] and plants [22,23], therefore, exploration of natural drug resources including microbes is well emphasized [24]. Three microalgae, *Nannochloropsis oculata*, *Dunaliella salina* and *Chlorella vulgaris* in addition to *Lactobacilli acidophilus*, *L. casei* and *L. reuteri* were used to biosynthesize AgNPs by adding $AgNO_3$ in the culture medium itself but AgNPs failed to be synthesized in *D.* salina culture [25]. Thus, we have selected *Dunaliella salina,* a wall less chlorophyte (isolated from hyper saline lake), not known to have any toxicity to human cells. It is already established a rich source of the antioxidant, as β-carotene and other compounds [26]. Ethanolic extract of the organism was reported to induce cell cycle arrest and apoptosis in A-549 human lung cancer cell line [27] and skin carcinoma cell line A431 [28]. The protective effect of *D. salina* (Lyophilized powder) was also reported against the experimentally induced fibro sarcoma on Wistar rats [29]. Invasive methods such as radiation and prevailing toxic chemicals as anticancer agents creating another facet problem, therefore we intended to incorporate nanotechnology with eco-friendly green chemistry adopting extract of *D. salina*. However, photo induced green synthesis of AgNPs using *D. salina* is not reported till date to the best of our knowledge. Extremophiles like *D. salina* may be a source of bioactive molecule(s) having strong anticancer potential, if coupled with the AgNPs, may act synergistically to offer strong anticancer efficacy with minimal side effects.

In the present paper, we have biosynthesized AgNPs using AED under exposure of bright sunlight and optimized duration of exposure, concentration of $AgNO_3$ and the AED inoculum dose. The biosynthesized AgNPs were characterized using various spectroscopic methods (UV–Vis, FTIR, XRD) microscopic (SEM, TEM, AFM) and TGA. The optimally synthesized AgNPs were tested for their impact on cellular proliferation, apoptosis and the expression of cancer biomarkers against cancer cell line (MCF-7) in comparison to well known anticancer drug (Cisplatin), keeping normal cell line (MCF-10A) as control.

2. Materials and Methodology

2.1. Chemicals

The chemicals ($AgNO_3$, Cisplatin, propidium iodide, Annexin V-FITC; Sigma Aldrich) and Calcein-AM (Thermo Fischer Scientific, USA) used in the present study were of analytical grade.

2.2. Culture Conditions

D. salina, was cultured in MJM [30]. Micro algal sample from the axenic stock culture was inoculated in Erlenmeyer flasks (1000 mL) containing MJM (500 mL) with NaCl (2.93%) and kept at 28 ± 2 °C under a cool white fluorescent and tungsten light (14.4 Wm^{-2}) with the light/dark regime of 16/8 h. The glasswares and culture media were sterilized at 1.0546 kg/cm^2 (15 lb) and 121 °C (30 min). The transfer and maintenance of culture was done aseptically under a laminar flow hood (INSTECH, India).

2.3. Preparation of D. salina Extract

Algal biomass was harvested by centrifugation (6500 rpm, 4 min) and washed three times with deionized water. The fresh algal biomass (5 g) was taken and boiled (15 min at 80 °C) in the deionized water (30 mL). This extract of *Dunaliella* was filtered through Whatmann filter paper No. 1 and stored (4 °C) for further use.

2.4. Biosynthesis of AgNPs

AED inoculum dose (2% (v/v)) was added to 1 mM $AgNO_3$ (100 mL) solution and kept under bright sunlight (69,000 lx), pH of the reaction mixture was 7 and temperature 38 °C. Bright sunlight exposed reaction mixture showed an instant color change from watery yellow to reddish brown with the sharp SPR band within 10 min, whereas the reaction mixture kept in the dark, neither attained the same coloration nor produced the sharp SPR band even up to 10 h [S1]. Therefore, all further experiments for the synthesis of AgNPs were performed in bright sunlight, and the process variables optimized using one factor at a time, approach. The process variables *i.e.*, exposure to sunlight duration (0–35 min) $AgNO_3$ concentration (1–5 mM) and AED inoculum dose (1.0–7.0%), were screened for optimizing biosynthesis of AgNPs. AgNPs synthesized at optima were purified by centrifugation (15,000 rpm, 30 min) and re-dispersed in de-ionized water to eliminate the water soluble biological residues. The process was repeated four times, and dried at room temperature for final mass of AgNPs to be used in future.

2.5. Characterization of AgNPs

The optical property of AgNPs was checked in 300–700 nm using UV–Visible spectrophotometer (Evolution 201, Thermo Scientific). For SEM analysis, a drop of ultrasonicated colloidal AgNPs was dried over thin aluminum foil (under table lamp) for 2 h and gold coated. The exact size and morphology of the AED synthesized AgNPs were further confirmed by TEM EM-CM 12 (PHILIPS). For TEM analysis, a drop of AED - AgNPs was placed on the carbon coated copper grid, dried at room temperature for 2 h and loaded onto the specimen holder. The structure or phase of AgNPs crystals was determined by X-ray Diffractometer (Rigaku Miniflex II) having Cu Kα radiation source and Ni filter in the range of 20–80° at the scanning rate of 2° min^{-1}. To identify the possible biomolecules responsible for the reduction of the Ag^+ ions and capping of the phycofabricated AgNPs, FTIR analysis was performed using (PerkinElmer Spectrum 100) in the range of 4000–400 cm^{-1}. TGA was conducted using a thermo gravimetric analyzer Pyris 1 TGA (PerkinElmer) at the constant heating rate of 10 °C min^{-1} from (47–925 °C) under nitrogen atmosphere. The surface texture of AED–AgNPs synthesized was studied using AFM with NT-MDT in the contact mode, and the images processed using NOVA software.

2.6. Cell Line Culture and Maintenance

MCF-7 and MCF-10A cells were grown in 75cm^2 cell culture flasks at 37 °C in 5% CO_2 atmosphere. DMEM/F12 media (supplemented with 5% horse serum, EGF (10 ng/mL), hydrocortisone (0.5 μg/mL), cholera toxin (100 ng/mL), insulin (10 μg/mL) and 1× antibiotic-antimycotic) was used to grow MCF-10A cell line. For MCF-7, EMEM media supplemented with 10% FBS and 1× antibiotic-antimycotic were used. Both the cell lines were trypsinized at 80% confluency and plated in 24 and 96 well cell culture plates at the density of 10^5 cells/mL. After 24 h incubation, cells were treated with different concentrations of AgNPs and Cisplatin (25 and 50 μg/mL).

2.7. Calcein/PI Assay

Confirmation of anticancer properties of the synthesized AgNPs, was performed Calcein AM/propidium iodide (PI) dual fluorescent labeling. The assay is based on the principle that hydrophobic dye calcein AM easily penetrates in live cells and gets converted to fluorescent calcein compound by the esterases, and retained in the cytoplasm. PI is the cell impermeable nucleic acid intercalating dye and PI can enter the cells with damaged membranes where it intercalates with the nucleic acid and shows red fluorescence after excitation [31]. For calcein/PI assay, both MCF-7 and MCF-10A cells were seeded in 24 well plates (4×10^4 cells/mL) and allowed to adhere for 24 h under optimum culture conditions. Subsequently the cells were treated with different concentrations of AgNP and Cisplatin (25 and 50 μg/mL) for 24 and 48 h. This was followed by the addition of 1 μM calcein AM (1 μm) and PI