Study DNA Damage after Photodynamic Therapy Using Silver Nanoparticles with A549 Cell Line

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Abstract

Lung Cancer is one of the major health problems worldwide. Nanomedicine is considered as one of the promising research applications nowadays. This is due to the unique physical and chemical properties of the nanoscale elements. Silver nanoparticles (Ag NPs) in specific has been extensively studied recently in many biomedical applications especially in cancers, since they possess multifunctional effects that make these nanostructures ideal candidates for biomedical applications. Ag NPs were proved to have anti-tumor activity with apoptotic cell death pathway. The goal of the current work was to investigate the degree of DNA damage that results from the usage of Ag NPs as a photosensitizer (PS) in photodynamic therapy (PDT), besides to evaluate the reactive oxygen species (ROS) yield that was associated. The results showed the occurrence of DNA damage in lung cancer cells (A549) through the generation of ROS via the detection of the mitochondrial membrane potential changes.

Keywords: Silver nanoparticles; Reactive Oxygen species; DNA damage; Mitochondria membrane potential

Introduction

Lung cancer is considered one of the main causes of death all over the world. It is believed to be the major account for about 1.6 million deaths, 20% of the total cancer deaths [1]. The effect of this disease has increased lately in Africa due to the tobacco epidemic that resulted in its vast dispersion of this disease [2]. According to the international agency for research on cancer (IARC), smoking alone is attributing for about 65% of lung cancer. Their estimations extend to the appearance of 1.8 million new cases in 2012 where about 60% of this number is only in the developing countries [3]. Eighty five percent of lung cancer patients are diagnosed with either metastasis or advanced local tumors or both. Surgery could be one of the first approaches, but unfortunately it is only valid for 15% of the patients. Bronchoscopic therapy could be the quickest choice and even the only possible choice in treating superficial tumors that are found intraluminally and for the treatment of tumors in the main airways as it can cause a rapid symptomatic relief for those patients and hence their overall conditions. This can give the chance for the chemo and/or radio therapeutic treatments to maintain or improve local patients’ conditions [4]. The blockage of the main airways by tumors is the main cause of deaths due to dyspnea and obstructive pneumonia. However, this approach fails with deep tumors and those who are extension distal to the segmental bronchi. The term tumor resistance is very familiar to maintain or improve local patients' conditions [4]. The blockage of the main airways by tumors is the main cause of deaths due to dyspnea and obstructive pneumonia. However, this approach fails with deep tumors and those who are extension distal to the segmental bronchi. The term tumor resistance is very familiar to
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The aim of the present work was to study closely how Ag NPs mediate their cytotoxicity and if they affect the cells' DNA or not. Hence a better understanding of their way of action can help in future development in such nanostructures and their applications in PDT alone and/or with combined chemotherapy.

Materials and Methods

Cell Culture

Lung cancer (A549) cell lines were originated from the American type Cell Culture (ATCC; Manassas, VA, USA) (ATCC HTB-53). Cell culture growth media were manufactured by Gibco (Thermo Fisher Scientific, Waltham, MA, USA). A549 cancerous cells were cultured in Roswell Park Memorial Institute media that was supplemented with 10% Fetal Bovine Serum and 1% penicillin/streptomycin/fungizone (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) with 5% CO₂ and at 37°C. Cells were washed by Hank’s balanced salt solution (HBSS; Thermo Fisher Scientific) upon reaching confluency and trypsinized by TrypLE™ Express (Gibco®; Thermo Fisher Scientific). Cells were seeded with a concentration of 3×10⁴ in culture dishes of 3.3 cm diameter where they were left 4 hours to attach. Then 3.23 mg/ml of Ag NPs were introduced to those attached cells and left overnight. Laser irradiations with 20 J/cm² were performed at dark through an optical fiber at room temperature where the laser spot size covered exactly the cells monolayer in the culture plates. The parameters of the used laser were summarized in our previous work [10] and summarized in table 1. The Ag NPs were synthesized at the Institute
of Photonic Technology in Jena, Germany. The used Ag NPs have been described in an earlier work [10] where the stock solution was of a concentration 80.884 mg/ml.

**Laser Induced Breakdown Spectroscopy**

The Ag NPs that were used in the current study have an absorption peak at 630 nm which is not in the usual absorption spectrum of silver. Laser induced breakdown spectroscopic (LIBS) technique was used to confer more validation about the identity of the used nanoparticles. It is an elemental quazi invasive tool with minimal sample preparation [11]. The setup is described before by Ahmed et al. [12].

**Experimental Setup**

The current experiment had the setup where cell cultures were divided into four study groups: The first one was used as a control where cells were cultured normally in its nourishing media and otherwise reversibly change its color into red. The assay can be described briefly as follow; The cells from the four experimental groups were plated in 96 well plates at 1×10⁵ cells/ml. 100 µl/ml of JC-1 staining solution is added to each well and mixed gently. The plates were then incubated for 15-30 minutes at 37°C and 5% CO₂. The plates were then read using excitation /emission 540/570 nm for healthy cells or 485/535 nm for apoptotic conditions and imaged using Zeiss multi lasers confocal microscope.

**Comet assay**

Single cell gel electrophoresis was used for the estimation of DNA damages that may be caused through the usage of Ag NPs as a PS in PDT treatment of A549 cell line. The assay was explained in details in an earlier work [13] where a visual assessment to the relative tail intensity using Zeiss upright microscope associated with a CCD camera was done, by sorting 100 comets into classes from 0 (no detectable tail) to 4 (large tail, minimal head), giving an overall damage score of 0–400. All sets of experiments were repeated four times with duplicate replications of each assay. Origin pro 8 was used as software for performing the one way statistical analysis to get all the mean, standard deviation, and standard error values. P-values were designated for the statistical significance between the control groups and the PDT treated groups with Ag NPs.

**Results and Discussion**

LIBS technique confirmed the elemental constituent of the synthesized nanoparticles to be silver. According to the national institute of standards and technology (NISTA), the spectral line which is at 328.2 nm should be to silver element as shown in figure 1.

**Table 1:** Parameters of the lasers used in the study

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Diode Laser</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manufacturer</td>
<td>Oriel Corporation</td>
</tr>
<tr>
<td>Wavelength</td>
<td>636 nm</td>
</tr>
<tr>
<td>Wave Emission</td>
<td>Continuous</td>
</tr>
<tr>
<td>Spot size</td>
<td>9.1 cm²</td>
</tr>
<tr>
<td>Output power</td>
<td>88 mW</td>
</tr>
<tr>
<td>Power Density</td>
<td>1.0288 mW/cm²</td>
</tr>
<tr>
<td>Fluences</td>
<td>10 J/cm²</td>
</tr>
<tr>
<td></td>
<td>15 J/cm²</td>
</tr>
<tr>
<td></td>
<td>20 J/cm²</td>
</tr>
<tr>
<td>Irradiation times</td>
<td>16 minutes, 11 seconds</td>
</tr>
<tr>
<td></td>
<td>24 minutes, 17 seconds</td>
</tr>
<tr>
<td></td>
<td>32 minutes, 23 seconds</td>
</tr>
</tbody>
</table>

**Figure 1:** LIBS Spectra showing the silver spectral line with high intensity for the synthesized nanoparticles confirming the existence of silver.
The current study proves that PDT mediated cytotoxicity using Ag NPs was found to be via the generation of intracellular ROS as shown in figure 2. Yet the dosage and exposure time are needed to be further studied for their importance and complexity. It is apparent from the figure that the Ag NPs and the laser irradiation alone resulted in non-significant amount of ROS compared to the control group. On the other hand the PDT group generated intracellular biphasic ROS in a significant yield compared to the control group where $p < 0.00002$.

Many studies like Mukherjee et al. [14] and Bhattacharya et al. [15] have reported the generation of such ROS in many cell lines due to the introduction of nanoparticles. Ag NPs can be one of the most prominent candidates in nanomedicine since they are believed to have a big role in the cellular oxidative stress which consequently results in ROS production. The cytotoxicity of Ag NPs has shown by many research centers to be size and shape dependent [16-18]. As long as the size of the Ag NPs decreases, their exposed surface area that can be involved in the biochemical interactions with the cells is increased and hence their cellular effects are elevated by several folds post laser irradiation. The size of the used Ag NPs in the current study was to be 27 nm and of spherical shape that rendered them to be very relevant and preferable in such kind of applications.

A significant increase in the mitochondrial membrane potential ($\Delta \Psi_m$) in the PDT group was observed when compared to the control group ($p < 0.00001$) as shown in figure 3 during the tracing of ($\Delta \Psi_m$) in the different experimental groups. Noting that there was no such drastic increase in the mitochondrial membrane potential for the Ag NPs group only or the laser light only confirming the viability study that has been shown in our previous study [10], where either laser light alone or Ag NPs alone have any toxic effects on A549 cells with the used light intensity and concentration respectively.

Confocal microscopic imaging added strength to the above results by showing the green monomer form of the JC-1 fluorescent dye in the case of the PDT experimental group where the other experimental groups have shown the healthy red J aggregates as a result of the complex formation in the mitochondria as shown in figure 4.

Despite the existence of many pathways for the generation of ROS, one can adopt the mitochondrial generation of ROS pathway via the electron transport chain mechanism [19]. This can be attributed to the correlation between the significance increase in the intracellular ROS and the mitochondrial membrane potential of the PDT group compared to the controls.

Studying the genotoxicity that may be caused by Ag NPs in...
the PDT treatment of A549 cells using the comet assay showed a significant DNA fragmentation in the PDT group compared to the control group where \( p < 0.0001 \) as shown in figure 5. The increased DNA damage in the PDT group matches perfectly with the previously published results [10], where the viability percentage and cellular proliferation significantly dropped in the PDT treated cells as well as increased levels of Lactate dehydrogenase (LDH) which is an indication of increased cytotoxicity.

There is not yet a confirmation about the sole pathway of the mitochondrial ROS generation in the Ag NPs mediating cytotoxicity. As there might be other cell organelles and pathways that could be involved in these reactions with other possibilities the presence of increased post oxidative stress after intracellular ROS generation and DNA fragmentation. O’Rourke has investigated the relationship between the change of the mitochondrial membrane potential and ROS generation, when he concluded that the rate of activation of the K+ ion for the oxidative phosphorylation is elevated by increasing the mitochondrial membrane potential and hence increased level of ROS [20]. One proposed mechanism could be estimated from the overall data and from other recent researches [21] that the silver ions which were generated after laser irradiation can intervene with the DNA replication after the induction of ROS and eventually cell death. The produced silver cations may act as oxidative entities by capturing electrons and hence can reduce cellular ATP contents and consequently can elevate the PDT treatment of A549 cells using the comet assay showed a significant DNA fragmentation in the PDT group compared to the control group where \( p < 0.0001 \) as shown in figure 5. The increased DNA damage in the PDT group matches perfectly with the previously published results [10], where the viability percentage and cellular proliferation significantly dropped in the PDT treated cells as well as increased levels of Lactate dehydrogenase (LDH) which is an indication of increased cytotoxicity.

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References


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