

Egg white-mediated green synthesis of silver nanoparticles with excellent biocompatibility and enhanced radiation effects on cancer cells

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Abstract: A simple, cost-effective, and environmentally friendly approach to the aqueous-phase synthesis of silver (Ag) nanoparticles was demonstrated using silver nitrate (AgNO₃) and freshly extracted egg white. The bio-conjugates were characterized by UV-visible spectroscopy, transmission electron microscopy, Fourier transform infrared spectrometry, and dynamic light scattering. These results indicated that biomolecule-coated Ag nanoparticles are predominantly spherical in shape with an average size of 20 nm. The proteins of egg white, which have different functional groups, played important roles in reducing Ag⁺ and maintaining product attributes such as stability and dispersity. In vitro cytotoxicity assays showed that these Ag-protein bio-conjugates showed good biocompatibility with mouse fibroblast cell lines 3T3. Furthermore, X-ray irradiation tests on 231 tumor cells suggested that the biocompatible Ag-protein bio-conjugates enhanced the efficacy of irradiation, and thus may be promising candidates for use during cancer radiation therapy.

Keywords: green chemistry, biosynthesis, egg white, Ag nanoparticles, X-ray irradiation

Introduction

A key aim in X-ray irradiation-aided cancer therapy is to achieve a dose that avoids damage to healthy tissues and organs while maintaining an efficient therapy outcome.¹ Nanobiotechnology may address this issue by improving the effectiveness and selectivity of X-ray irradiation therapy.

In recent years, bio-conjugated nanomaterials have shown significant potential for application in biological/biomedical fields through their use in luminescence tagging, labeling, drug delivery, and imaging.²⁻⁵ To fabricate an available bio-conjugate for such applications, it is important to select an appropriate synergism between the nanoparticle surface and biological molecules. Noble metal nanoparticles have attracted extensive attention, particularly in the biomedical field, due to their intriguing physical-chemical properties.

Many studies have examined the controlled synthesis of noble metal nanomaterials of various sizes and morphologies through the use of biomolecules as templates.^{6,7} Extracts from plants such as tea, aloe vera, apple, and pimiento have been successfully used to synthesize gold (Au) and silver (Ag) nanoparticles.⁸⁻¹² Naturally occurring small molecules such as vitamins B₂, C, and E have been used to prepare Au, platinum, and Ag nanomaterials.¹³⁻¹⁵ Proteins and peptides such as apoferritin, bovine serum albumin (BSA), lysozyme, and tryptophan-based peptides have also been used in the synthesis of Au and Ag nanoparticles.¹⁶⁻²⁰

Compared with traditional chemical syntheses, biomolecule-assisted syntheses of noble metal nanomaterials have a number of advantages.^{7,21} Since biomolecule-assisted

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syntheses are carried out at room temperature and under aqueous conditions, energy input is reduced and the solvents or agents used are nontoxic – factors that minimize environmental damage and enhance human health. The structural diversity of biomolecules results in nanoparticles with a wide range of sizes, shapes, and polymorphisms that determine their physical-chemical properties. As is well known, biomolecules can carry on slow kinetics to self-reduce metal precursors and develop stable coating layers to avoid particle aggregation. In particular, proteins with different functional groups as capping shells will facilitate post-surface modification for further biomedical applications.

Egg white or albumen is the liquid that surrounds the yolk of an egg. There are approximately 40 types of protein present in a chicken's egg white, all of which are beneficial to the human body. Besides their high nutritional quality, egg white proteins (ovalbumin) also have various functional properties such as gelling, foaming, emulsification, heat setting, and binding adhesion.²² Another advantage is their solubility in water and tendency to associate with metal ions in solution. Metal ions such as Mn^{2+}/Mn^{3+} , Fe^{2+}/Fe^{3+} , Cu^{2+} , Zn^{2+} , and Ni^{2+} have been combined with egg white to obtain novel nanomaterials with interesting properties.^{23–28}

However, to date, it appears that the use of egg white as a template for the synthesis of noble metal nanoparticles has not been investigated, let alone these nanoparticles' application in cancer therapy. In the current paper, we report a simple, nontoxic, and eco-friendly green pathway to prepare stable spherical and spheroidal Ag-protein bio-conjugates by using egg white as a stabilizer and reductant.

Materials and Methods

Materials

Fresh eggs were bought from a local supermarket. Silver nitrate ($AgNO_3$, analytical grade) was purchased from Sinopharm Chemical Reagent Co, Ltd (Shanghai, People's Republic of China). Purified water (18.2 mΩ-cm) was made using a Purelab Classic DI MK2 system (Veolia Water, Paris, France). Mouse fibroblast 3T3 cell line was kindly provided by Dr Jianbing Liu (School of Medicine, Shanghai Jiaotong University, People's Republic of China). Human breast adenocarcinoma cell line was kindly donated by Dr Jianming Luo (Fudan University Shanghai Cancer Center, People's Republic of China).

Synthesis of Ag nanoparticles in the presence of egg white

In a typical procedure, 1.0 mL egg white extract was fully dissolved in 97.0 mL water with a strong magnetic stirrer for

30 minutes. The cloudy white solution was filtered through two layers of gauze and a clear solution was obtained. Then, 2.0 mL 10.0 mM $AgNO_3$ solution was added rapidly under vigorous stirring, bringing the total reaction volume to 100.0 mL. The reaction was carried out at room temperature for 72 hours, and it was observed that the solution gradually changed from white to yellow within this time. The collected solution was centrifuged at 15,000 rpm for 15 minutes then washed with purified water. This was repeated twice, and the final samples were dried under vacuum for further characterization.

Characterization

UV-vis spectra were measured using a Shimadzu UV-2450 UV-visible spectrophotometer (Shimadzu Scientific Instruments, Kyoto, Japan). Infrared spectra in the range of 400–4000 cm^{-1} were recorded using a Nicolet 870 Fourier-transform infrared (FTIR) spectrometer (Thermo Nicolet, Madison, WI). A small quantity of the sample was blended with dry potassium bromide for analysis. The size of the particles in the resulting mixtures was analyzed using a Beckman Coulter DELSA Nano C-Nano Particle Size Analyzer (Beckman Coulter Inc, Brea, CA). Transmission electron microscopy (TEM) measurements were performed at an accelerated voltage of 120 kV (JEM-2010; JEOL Ltd, Tokyo, Japan).

In vitro cytotoxicity assay

Cell Counting Kit-8 (CCK-8; Dojindo Lab, Kumamoto, Japan) was employed in this experiment to evaluate the cytotoxicity of the Ag-protein bio-conjugates.

One hundred microliters of 3T3 cell suspension was dispersed in a 96-well plate, giving a concentration of 5000 cells/well. The plate was pre-incubated for 24 hours in a humidified incubator (37°C, 5% CO_2), after which 10 μL of various concentrations of Ag-protein bio-conjugates were added into the culture media in the plate. After the plate was incubated for a further 24 hours, 10 μL of CCK-8 reagent was added to each well of the plate. The plate was then incubated for another 4 hours and then the plate's absorbance at 450 nm was measured using a microplate reader.

X-ray irradiation enhancement test

Ionizing radiation treatments were carried out using a standard radio-oncology linear accelerator (Siemens Oncor Avant-Garde, Siemens Medical Solutions, Los Angeles, CA) with a 6 meV beam irradiator at a dose rate of 1.0 Gy min^{-1} . A sample of 231 cells was irradiated with different concentrations of

Ag-protein bio-conjugates at room temperature, after which these cells were incubated for another 48 hours. Cell viability tests were determined by CCK-8. The same experiment was also performed with radio-resistant 231 cells.

Results and discussion

The general process for the synthesis of nanomaterials in egg white extract involves the electrostatic complexation of Ag ions with oppositely charged proteins, followed by the foam generation and subsequent in situ chemical reaction.¹⁸ Biomolecules with carboxyl, hydroxyl, and amine functional groups have the potential to reduce metal ion and cap the newly formed nanoparticles,^{29–31} which has allowed Au and Ag nanoplates to be obtained in green algal solutions. Similarly, various types of proteins with a large number of hydroxyl groups in egg white may be expected to attach to the different faces of Ag nanocrystals, leading to isotropic growth and the subsequent formation of stable spherical Ag nanoparticles.

Ag nanoparticles were first characterized using a UV-vis spectrophotometer. As shown in Figure 1, the surface plasmon resonance peak occurred at 425 nm, indicating the formation of spherical Ag nanoparticles (reddish-yellow color, inset). Additionally, the peak at 325 nm is ascribed to the protein characteristic.

FTIR has become an important tool in understanding the involvement of functional groups in interactions between metal particles and biomolecules.³² In the current study, FTIR measurements were performed to identify the biomolecules responsible for capping and stabilizing the Ag nanoparticles. As is shown in Figure 2, the spectra record was carried out in the range of 400–4000 cm^{-1} . The very strong peak at 3442 cm^{-1} was assigned as –OH stretch of the proteins in the egg white extract. A peak was observed around 2950 cm^{-1}

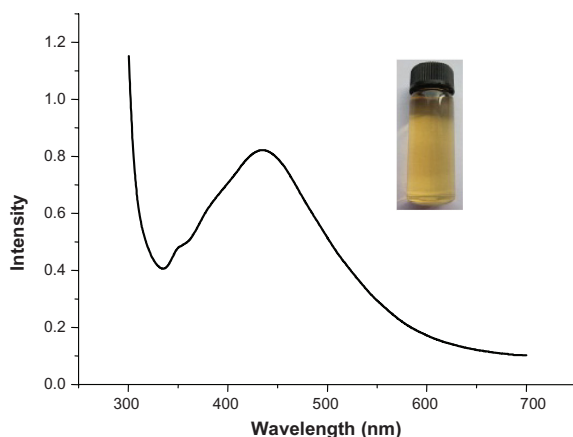


Figure 1 UV-vis spectrum of purified Ag egg-white nanoparticles.
Note: Inset shows a typical optical image of the resulting product.

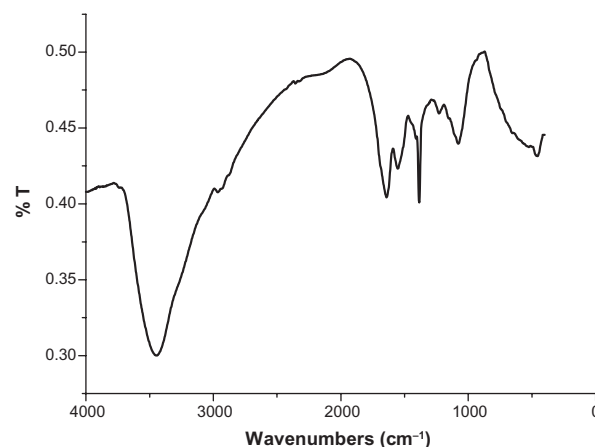


Figure 2 Fourier-transform infrared spectrum recorded from the powder of Ag nanoparticles synthesized through the use of an egg-white template.

that could be assigned to the C–H stretching vibrations of methyl, methylene, and methoxy groups. The peak located at 1643 cm^{-1} was assigned to the C=O stretching in carboxyl or C=N bending in the amide group. The absorption band at 1539 cm^{-1} was characteristic of amide II (N=H) bonds of proteins. The peak at 1072 cm^{-1} was assigned to the stretch of the C–O bond.

TEM and dynamic light scattering (DLS) were used to evaluate the size, morphology, and hydrodynamic size of the Ag nanoparticles. As can be seen in Figure 3A, the nanoparticles are homogeneous and spherical with an average diameter of 12 nm. It was observed that the edges of the particles were lighter than the centers (Figure 3B), showing that biomolecules (such as proteins in egg white) coated the surface of the Ag nanoparticles. This is consistent with the FTIR results.

Given the importance of the hydrodynamic volume of nanoparticles for their biomedical implications, a DLS experiment was performed to study the polydispersity of the Ag nanoparticles in aqueous solution. The particle size distribution was fitted by a Gaussian curve as shown in Figure 4. It was ascertained that the particles were 154.2 nm in diameter, larger than was indicated by TEM. This was attributed to the

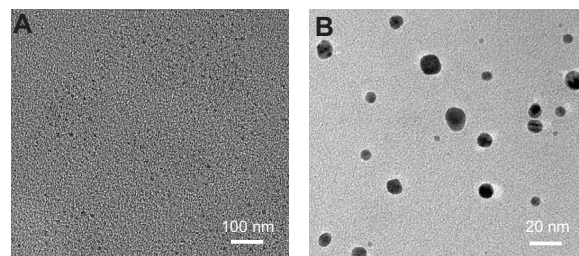
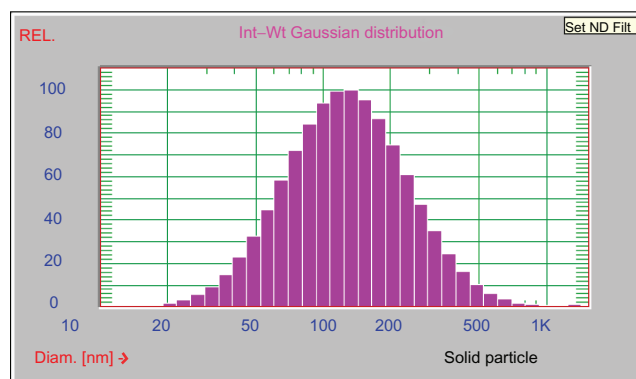


Figure 3 Transmission electron microscopy images of Ag nanoparticles at different magnifications, (A) the lower image and (B) the higher image.



Intensity weighting:

Mean diameter = 154.2 nm
Std deviation = 97.8 nm [63.40%]

Cumulative result: Int-Wt Vol-Wt

25% of deviation < 82.3 nm < 32.5 nm
50% of deviation < 126.2 nm < 47.7 nm
75% of deviation < 193.4 nm < 71.6 nm

Figure 4 Size distribution of the egg white and Ag ensemble in aqueous solution according to dynamic light scattering measurements.

fact that the DLS is sensitive to the size of the entire protein-nanoparticle ensemble as well as the water associated with this, while only the metallic crystal lattice is visible under the electron beam in bright-field TEM observations.³³ Both the TEM and DLS exhibited a narrow particle size distribution, a finding which has important biomedical implications.

Potential biomedical applications of nanoparticles also depend greatly on their biocompatibility. Therefore, the cytotoxicity of Ag-protein bio-conjugates was examined under in vitro conditions in the mouse fibroblast cell line 3T3. This was assessed in terms of the effect of Ag-protein bio-conjugates on cell viability, determined by CCK-8 assay. After treatment with Ag-protein bio-conjugates for 24 hours, it was demonstrated that viability remains above 80% when the Ag-protein bio-conjugate concentration remained below 12 $\mu\text{g/mL}$ (Figure 5). It has been reported that some Ag nanoparticles produce significant toxicity in cell culture media,³⁴ while protein-coated Ag nanoparticles show minimal or no toxicity in a 3T3 cell culture, which is consistent with the results of cinnamon phytochemical-coated Au nanoparticles.³⁵ A possible explanation is that both of these are naturally occurring biomolecules that have evolved and have been proven safe in the human body.^{36,37} They supply a nontoxic coating for Ag nanoparticles, which provides biocompatibility for in vivo administrations. The lack of any noticeable toxicity of Ag-protein bio-conjugates thus presents new opportunities for safe delivery and applications of such nanopharmaceuticals in molecular therapy.

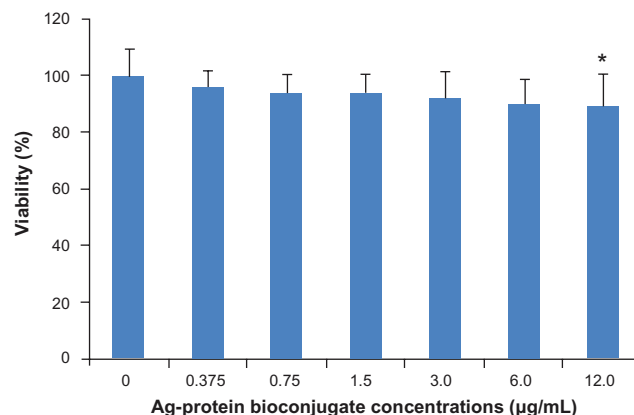


Figure 5 Cell viability of mouse fibroblast 3T3 cells after 24 hours' incubation with increasing amounts of Ag-protein bio-conjugates showing nontoxic profiles.

Notes: Means \pm standard deviation; $n = 5$; * $P < 0.05$.

To further explore their potential for biomedical application, an experiment was performed to assess the effects of Ag-protein bio-conjugates on 231 tumor cells when these were treated with X-ray irradiation. Radiotherapy is widely used in the clinical treatment of cancer and it is hoped that the rapid development of nanomedicine will allow nanomaterials to be combined with radiation therapy to produce a novel and effective strategy for the treatment of this disease.³⁸ Besides preserving the advantages of standard radiotherapy, such as high penetration, the presence of nanoparticles can enhance radiotherapy's cancer-killing effect. However, this is dependent on nanoparticles becoming concentrated in cancerous regions and interacting strongly with the irradiating beam.

Although the detailed mechanism remains largely unexplored, it is clear that the presence of strongly absorbing elements in the cells can increase the production of photoelectrons or free radicals and lead to the damage of organelles and/or nuclei. It has been noted that Au nanoparticles can enhance the efficacy of radiation therapy on a murine squamous cell carcinoma,³⁹ while glucose-capped Au nanoparticles can enhance radiation sensitivity in radiation-resistant human prostate cancer cells through regulation of the cell cycle.⁴⁰ Other researchers have found that polyethylene glycol-modified Au nanoparticles accumulate at tumor sites and enhance the response of CT26 cells to X-ray irradiation¹ and that fetal bovine serum-modified Ag nanoparticles increased the sensitivity of glioma cells to ionizing radiation (IR) treatment.⁴¹

The current study employed Ag-protein bio-conjugates as novel radiosensitizers. The Ag-protein bio-conjugates were applied to human breast adenocarcinoma 231 cells at different concentrations (0 $\mu\text{g/mL}$, 0.375 $\mu\text{g/mL}$, 0.75 $\mu\text{g/mL}$, 1.5 $\mu\text{g/mL}$, 3 $\mu\text{g/mL}$, 6 $\mu\text{g/mL}$, and 12 $\mu\text{g/mL}$) and 4 Gy X-ray irradiation was applied. Figure 6A–H shows the

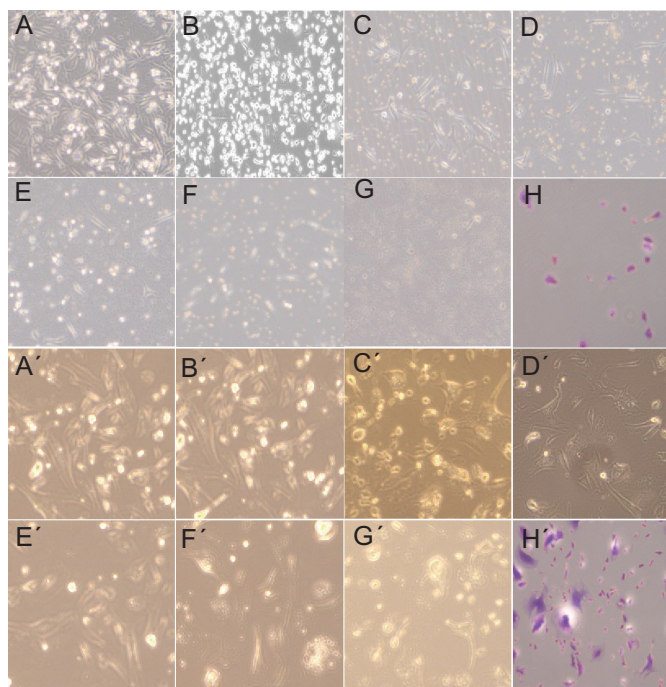


Figure 6 Optical pictures of 231 colonies. **A–H** are images of 4 Gy X-ray-irradiated 231 cells with the addition of Ag-protein bio-conjugate concentrations at 0, 0.375, 0.75, 1.5, 3.0, 6.0, and 12.0 µg/mL respectively. **A'–H'** are images of 10 Gy X-ray-irradiated 231 cells (note that these cells were 4 Gy X-ray resistant) with the addition of Ag-protein bio-conjugate concentrations at 0, 0.375, 0.75, 1.5, 3.0, 6.0, and 12.0 µg/mL respectively. **Note:** **H** and **H'** are the crystal violet-stained results corresponding **G** and **G'**, respectively.

number of changes and morphology of these changes to cancer cells following 24 hours' incubation. It is clear that the cell numbers decrease as nanoparticle concentration increases, and morphological changes are also observed with the addition of Ag-protein bio-conjugates. The spindles of treated cells become spherical in the presence of Ag nanoparticles and X-ray (Figure 6B–H, in contrast to the control, Figure 6A). It was concluded that the combined use of nanoparticles and X-ray treatment caused significant damage to the cells' cycle.

Since some solid tumors are radio resistant, 4 Gy-resistant 231 cells were selected and the irradiation dose was increased to 10 Gy to determine whether Ag-protein bio-conjugates are also available to radio-resistant cells. As is shown in Figure 6A'–H', the cell nuclei are larger than the corresponding cells exposed to 4 Gy. This change in the nucleus could retard its division and lead to death.

A detailed study of cell survival is shown in Figure 7. These results will be discussed based on normal 231 cells and X-ray irradiated 231 cells with different Ag-protein bio-conjugates concentrations. In the case of cells that received no X-ray irradiation, the survival of normal cells and the irradiated group remain very similar as Ag-protein bio-conjugates increase, indicating that the Ag-protein bio-conjugates possess good biocompatibility within the experimental concentrations.

When cells are exposed to a certain dosage of X-ray irradiation, the survival of both the normal and the irradiated cell groups begin to decrease as the Ag-protein bio-conjugate concentration increases. It was noted that the survival of the normal cells decreased more quickly than those that were irradiated, indicating that X-ray treatment kills more normal 231 cells

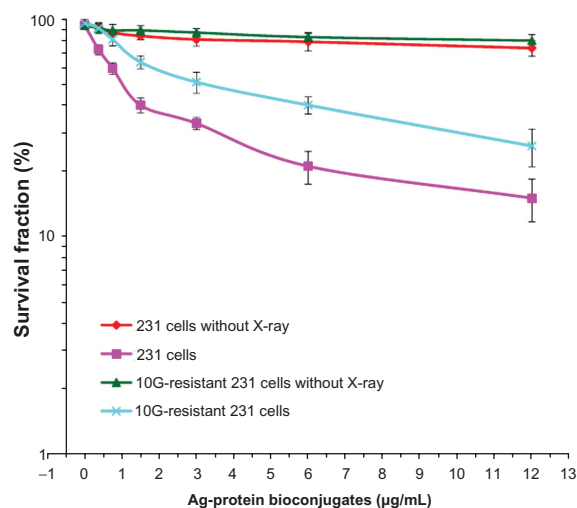


Figure 7 Survival curves of different strains of 231 cells at different Ag-protein bio-conjugate concentrations, contrasting those with/without X-ray irradiation. Clearly, the combined used of both Ag-protein bio-conjugates and X-ray did enhance the irradiation to cancer cells.

Notes: Means \pm standard deviation; $n = 3$.

when these have been treated with Ag-protein bio-conjugates. Both the irradiation dosage and Ag-proteins bio-conjugate concentration affected cell survival. These results suggest that Ag-protein bio-conjugates may function as a radio-therapeutic sensitizer in anticancer therapy.⁴²

The practical clinical application of treatments such as this will ultimately depend on in vivo biological absorption, distribution, and metabolism of Ag-protein bio-conjugates. The researchers behind the current study will report on the mechanism behind the interactions between Ag-protein bio-conjugates and cancer cells in the near future. This work will include detail across molecular, organ, and animal levels.

Conclusion

The current study has illustrated that egg white may be used as an active template for the spontaneous reduction of Ag ions and the consequent “one-pot” formation of protein-conjugated Ag nanoparticles. This synthesis method is very simple, cost-effective, and environmentally friendly, and the nanoparticles produced were shown to be biocompatible through in vitro cell arrays. Further work is needed to investigate the mechanism of metal-ion uptake and bioreduction by egg white. This study also showed that Ag-protein bio-conjugates can strongly enhance the cell damage induced by X-ray irradiation, which suggests that protein-conjugated Ag nanoparticles are a promising candidate for the development of green nanomaterials for biomedical and health applications.⁴³

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Disclosure

The authors declare no conflicts of interest in this work.

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