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Self-assembly of biogenic gold nanoparticles and their use to enhance drug delivery into cells

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ABSTRACT

Integration of the principles of green chemistry into nanotechnology is one of the key issues in nanobioscience research. There is a growing need for development of a synthesis method for producing environmentally harmless nanoparticles in order to avoid adverse effects in medical applications. Here, we report the use of a simple and rapid *in vivo* biosynthesis method for the preparation of gold nanoparticles (AuNPs) using heavy metal binding proteins (HMBPs) in recombinant *Escherichia coli*. The HMBPs were found to act as reducing, stabilizing, and capping agents to form the spherical nanoparticles with 5–20 nm in diameter. The size and the shape of AuNPs were modulated by varying the concentration ratio of recombinant proteins in the medium. Only 20 min was required to form AuNPs at room temperature, suggesting that the reaction rate of the proposed method is faster than that of the chemical methods commonly used for nanoparticle synthesis. The AuNPs could be applied as drug carriers in therapeutic applications to improve drug delivery, since they exhibit higher biocompatibility and less toxic effects than chemically synthesized materials. To achieve high cytotoxicity for cancer chemotherapy, doxorubicin (Dox) was released from AuNPs, which can be a more efficient anti-cancer agent than free Dox.

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1. Introduction

Synthesis of nanoparticles is one of major integral parts of nanotechnology and its application. Synthesis of nanoparticles is usually carried out by several methods founded on concepts of physical and chemical sciences due to its simple and rapid means [1,2]. However, the synthesis of nanoparticles using physical methods requires expensive experimental equipment, and toxic reducing and stabilizing agents are needed for chemical methods that can lead to environmental pollution [2–4]. To overcome such problems, biological ways of synthesizing nanoparticles have become an effective alternative. In recent years, biological entities were shown to have excellent capability for the synthesis of metallic nanoparticles. Biological synthesis of nanoparticles involves clean, non-toxic, non-hazardous, and environmentally friendly sources, which are sustainable, economic, and easy to handle [2–8]. The synthesis of nanoparticles of desired shape and size is the emerging challenge faced by biosynthesis researchers. Several research groups reported successful biosynthesis of metallic nanoparticles comprising gold (Au), silver (Ag), platinum (Pt), selenium (Se), and many more [9]. Among all the nanoparticles, gold nanoparticles (AuNPs) have attracted the highest amount of interest from nanotechnology researchers, owing to their dynamic properties at the nanoscale in spite of their bulk form [10]. The unique optical and electronic properties of AuNPs make them suitable for various applications in biomedical research, medical sciences, bio-imaging, cosmetics, pharmaceuticals, drug delivery, genetic engineering, conjugation with genomic DNA, and cancer treatment [10–15]. Although gold is recognized as a non-toxic metal, a number of researchers have reported the toxicity of AuNPs. This phenomenon is considered to be associated with the use of toxic chemicals in the process of their synthesis [14,16].

Green chemistry currently focuses on the exploration of cost-effective, environmental friendly, and bio-compatible reducing agents for the synthesis of AuNPs [17]. Various biological systems such as bacteria, yeast, fungi, algae, and plants are recently undergoing investigations for potential use in the synthesis of AuNPs [18,19]. Among them, proteins from recombinant *E. coli* have been of particular interest to the scientific community due to easy expression system, their eco-friendly nature and advantages over other biological processes, including elimination of the elaborate process of maintaining cell structures and the ability for large-scale synthesis of nanoparticles.

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Our current study developed a general strategy of *in vivo* synthesis of AuNPs using recombinant *E. coli* expressing *Arabidopsis thaliana* phytochelatin synthase (AtPCS) and *Pseudomonas putida* metallothionein (PpMT). Phytochelatin (PC) and metallothionein (MT) are recognized as cysteine and tyrosine-rich heavy metal binding proteins (HMBPs). PCs are enzymatically synthesized peptides and MTs are gene-encoded polypeptides having high metal-binding affinity and capacity for accumulation inside the cells. Among them, *E. coli* was genetically designed and engineered to express a PC synthase (PCS) of *A. thaliana* along with MT of *P. putida* to obtain the synergetic effect of the PpMT-AtPCS fusion protein on the assembly of metal components for nanoparticle synthesis [20].

In this paper, we report a green approach for the synthesis of AuNPs that reduces aqueous Au ions using the recombinant proteins in *E. coli*. The bioactive constituents of recombinant *E. coli* responsible for the biosynthesis were identified. The expected reaction mechanism involved in the biosynthesis of nanoparticles is also reported. The procedure described in this study is simple in steps, affordable and easy to functionalize the synthesized AuNPs which can be applied in large-scale synthesis of nanoparticles according to its need in therapeutic fields as efficient biosensors and drug carriers.

2. Materials and methods

2.1. Preparation of HMBPs

The DNA fragment encoding PpMT was amplified using the genomic DNA of *P. putida* KT2440 as a template, and AtPCS was amplified by the polymerase chain reaction (PCR) using the cDNA library of *A. thaliana* ecotype Columbia (leaves) as a template. These two fragments were digested with restriction enzymes and ligated into pTac99A plasmid to make pTJ1-PpMT-AtPCS. This vector was then transformed into *E. coli* BL21(DE3) strain. A 100 μL of the *E. coli* BL21(DE3) harboring pTJ1-PpMT-AtPCS was incubated in 10 mL of LB medium containing ampicillin (50 μg/mL, Sigma–Aldrich, St. Louis, MO) for 18 h at 37 °C with 200 rpm stirring rate. After incubation, 1 mL of the incubated *E. coli* solution was incubated again with a 100 mL of Luria-Bertani (LB) medium for 2 h for proliferation of the cells. Then, the concentration of the cell solution was measured by a UV/vis spectrophotometer (Optizen Pop, Mecasys, Daejeon, Korea). When the absorbance value at 600 nm (OD_{600}) was reached at 0.4, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma–Aldrich) was added for induction of PCS and MT HMBPs [20]. After further incubation for 7 h, the cells were centrifuged at 3500 rpm for 10 min, and cell pellet was then obtained after removal of supernatant. This cell pellet was resuspended in phosphate-buffered saline (PBS, pH 7.4, Sigma–Aldrich) solution and then ultrasonicated centrifuged at 13,000 rpm to collect soluble fraction of proteins (see Fig. S1 in the Supplementary data). The collected HMBPs were stored at 4 °C until used.

2.2. Synthesis of AuNPs@HMBP complex

AuNPs@HMBPs were synthesized according to the following procedures. HMBPs obtained from ultrasonicated recombinant *E. coli* were diluted in PBS solution (pH 7.4) and stirred for 15 min. The resultant solution (5 mL) was stirred with aqueous gold (III) chloride hydrate 99.999% (100 mM, 50 μL of $HAuCl_4 \cdot xH_2O$; Sigma–Aldrich) for 5 min. Aqueous NaOH (2 M, 100 μL) was added drop-wise to the mixture under constant stirring for 5 min. The mixture was then transilluminated by UV-irradiation. The resultant AuNPs@HMBPs were centrifuged at 17,000 rpm (rotor radius: 85 mm) for 30 min to remove large particles and subsequently purified using an Amicon Ultra-4 centrifugal filter with a cut-off of 30 kDa by centrifugation at 3500 rpm for 20 min to remove the unreacted species. Finally, the fraction retained on the filter membrane was subjected to freeze–drying to obtain AuNPs@HMBPs powder used for further characterization. Mili-Q (Millipore, Billerica, MA) deionized (DI) water was used in all synthesis and experimental procedures.

2.3. UV–visible spectrometric analysis

Visible color change was observed in the reaction mixture containing gold (III) chloride hydrate and HMBPs. Periodic sampling of solution was carried out to monitor the bio-reduction of AuNPs. The UV–visible spectrum of sampled solution was measured using a UV–visible spectrophotometer (Optizen Pop, Mecasys, Daejeon, Korea) at wavelengths in the 400–700 nm range. The stability of AuNPs was evaluated over the 3-month time period. Dynamic light scattering (DLS) analysis was performed to measure the hydrodynamic diameter of biologically synthesized nanoparticles. DLS particle size analyzer (ELSZ-1000, Otsuka, Japan) was used to determine the size distribution and zeta potential of AuNPs in aqueous phase.

2.4. Characterization of AuNPs@HMBP complex

The characterization of synthesized AuNPs@HMBPs was conducted using particle size analysis, X-ray diffraction (XRD), transmission electron microscopy (TEM), scanning electron microscopy (SEM), energy dispersive X-ray spectroscopy (EDS), and a multimode microplate reader (BioTek Synergy H1, Winooski, VT) to measure absorbance and fluorescence. The size and morphology of biogenic AuNPs@HMBPs were characterized by TEM (Tecnai G2 F30 S-TWIN, FEI, Hillsboro, OR) at accelerating voltage of 300 kV with 0.20 nm point resolution and SEM (UHR FEG-SEM, FEI) at accelerating voltage of 15 kV with 1-nm point resolution. The sample for TEM analysis was prepared by dispersing the nanoparticles in 1 M PBS and placing a droplet of nanoparticle dispersion on a copper grid with mesh size of 300 with a 3-nm diameter. The sample for SEM analysis was dried overnight in a vacuum drier at room temperature and coated with Pt using a sputter coater (Cressington 208C HR, Hertfordshire, UK) to achieve a specific coating thickness. A film thickness monitor (MIM-10, Ted Pella Inc., Redding, CA) with a resolution of 0.1 nm was used to monitor the coating thickness during the coating process. The excess solvent was allowed to evaporate at room temperature. The elemental analysis of nanoparticles was carried out by XRD and EDS analyzer associated with TEM, at an accelerating voltage of 20 kV.

2.5. Evaluation of cytotoxicity of AuNPs@HMBPs in HeLa cells

The effect of a range of concentrations of chemically synthesized AuNPs [21] and AuNPs@HMBPs on cell viability were assessed in HeLa cells using 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) analysis. Chemically synthesized AuNPs were prepared using a citrate reduction of $HAuCl_4$ and cleaned up with PBS (pH 7.4) before experiments. HeLa cells, a human cell line, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and non-essential amino acids at 37 °C in humidified conditions with 5% (w/v) CO_2. Effect of a range of each AuNPs concentrations (0.625–10 nM) on viability of HeLa cells (1×10^6 cells/well in 24-well plates) was determined by the conventional MTT reduction assay [22], allowing the cytotoxic effects to be determined. After treatment with each AuNPs, the medium was changed and the cells were washed twice with PBS (pH 7.4) to remove the dead cells. The cells were subsequently incubated with 300 μL (1 mg/mL) of

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