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Macrocycles as a Tool: A Facile and One-Pot Synthesis of Silver Nanoparticles Using Cucurbituril Designed for Cancer Therapeutics

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Cucurbituril (CB), a macrocycle comprised of glycoluril units, forms stable host–guest complexes with various guest molecules.[1–3] Cucurbiturils have gained wide attention and been studied extensively due to their unique structure and outstanding multiple recognition properties, as well as their potential applications for constructing sensors, drug delivery and biomimetic systems, and so on.[1–4] Through several studies it was proved that CB is appropriate for application in host–guest processes. However, to the best of our knowledge, no work has described its suitability for the synthesis and stabilization of metal nanoparticles in general and, in particular, silver nanoparticles (AgNPs) in aqueous solution at room temperature without using conventional reducing agents and/or external energy. In our preceding studies, we have established a supramolecular approach to synthesize copper oxide nanoparticles by using macrocycles such as cucurbit[7]uril (CB[7])[5] and β-cyclodextrin (β-CD).[6] In addition, we also introduced new techniques to fabricate surfactant-based gold nanoparticles and AgNPs.[7,8] Hence, with this experience and expertise in supramolecular strategies,[3,5,6] macrocyclic materials,[3,9] and nanoparticles,[5–8,10,11] we thought of combining them all together to form a new supramolecular macrocycle-based metal nanoparticle in pure aqueous medium that can be used for diverse pivotal biomedical applications. This fundamental thinking directs us to prepare well-dispersed AgNPs by using a macrocycle, namely cucurbit[7]uril, in the presence of sodium hydroxide in aqueous medium at ambient experimental conditions. This is the first report on the preparation of AgNPs by using CB[7].

Recently, there has been significant interest towards AgNPs in biomedical applications, predominantly due to their antibacterial activity.[12,13] The biomedical applications of AgNPs are also very interesting due to their size-dependent interaction with the HIV-1 virus.[14] Though a considerable amount of work has been carried out to evaluate the antimicrobial potential of AgNPs, the therapeutic applications, such as the anticancer activity of AgNPs, have not yet been well documented. Thus, it is challenging for chemists and materials scientists to introduce a facile and green strategy for the preparation of AgNPs and explore their biomedical application.

Herein, we report a facile, green, one-pot synthesis of well-dispersed AgNPs by the reaction of an aqueous silver nitrate solution with CB[7] in the presence of NaOH at room temperature. Furthermore, we have investigated the cytotoxic properties of the as-prepared AgNPs against two different human cancer cells, namely human breast adenocarcinoma (MCF-7) and human lung bronchoalveolar (NCI-H358) cells in vitro. We demonstrated that the as-prepared CB[7]-protected AgNPs, with an average size of about 5 nm, could be a suitable candidate for cancer therapy applications.

The reaction of an equivalent aqueous solutions of $AgNO_3$ and CB[7] in the presence of NaOH at room temperature (≈25 °C) led to the manifestation of a brownish-black colored solution after a reaction time of about 15 min, visually indicating the formation of AgNPs. The UV/Vis absorption spectra recorded from this solution shows the characteristic surface plasmon resonance band of AgNPs centered at 439 nm,[15] substantiating the formation of AgNPs. The constant increase of the surface plasmon band intensity at 439 nm with time indicates the continuous formation of AgNPs. This band evolved with time, finally reaching a constant absorbance after 40 min and only a slight increase in absorption occurred thereafter. The solution of the silver salt in the absence of any of the other reactants (CB[7] or NaOH) did not show the characteristic surface plasmon

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band of AgNPs at around 439 nm, which principally offered a clue that CB[7] may play a role in the reduction of silver salts to AgNPs in the presence of NaOH.

To investigate the influence of reactant concentration and reaction temperature on particle size and shape, we carried out several experiments with different experimental conditions (see Table 1). The TEM images of the nanoparticles formed under different concentrations of NaOH are shown

Table 1. Synthesis of AgNPs and average size at different experimental conditions.

c Ag salt/CB[7] [mM]	*c* NaOH [mM]	*T* [°C]	Average diameter [nm]	Number of AgNPs for statistical analysis
5/5	10	25	5.53±0.52	672
5/5	40	25	5.62±0.83	301
5/5	60	25	5.60±1.13	260
5/2.5	40	25	5.61±0.76	284
2.5/5	40	25	6.66±2.86	426
5/5	40	4	5.62±0.76	433
5/5	40	45	6.10±0.47	359
5/5	40	65	5.28±0.83	212

in Figure 1 a–c. As can be clearly seen from these images, the nanoparticles formed are almost spherical in shape, well dispersed with a minimal polydispersity in size and are non-agglomerated. Further, the cautious examination of the particles by a higher resolution of TEM, showing the lattice fringes of the nanoparticles, reveals the crystalline nature of the nanoparticles. Furthermore, the powder XRD pattern (Figure 1 d) and an electron diffraction (ED) pattern (Figure 1 d, inset) from a selected area of image 2b of the as-prepared AgNPs, demonstrate that the AgNPs feature a face-centered cubic (fcc) structure.[15] A typical high magnification TEM image (Figure 1 e) shows that the average size of the AgNPs (obtained from 10 mM NaOH concentration) was 5.53±0.52 nm, as calculated from a statistical study on 672 nanoparticles (Figure 1 f). Interestingly, the image analyses of the TEM (see Supporting Information, Figure S1) show that the average particle size of the AgNPs obtained from 40, and 60 mM NaOH concentration at room temperature were 5.62±0.83 and 5.60±1.13, respectively. Further, the average particles size of the AgNPs observed at 4, 45, and 65 °C were 5.62±0.76, 6.10±0.47, and 5.28±0.83, respectively, (Figure S2) as analyzed from the typical TEM micrographs and their size distribution plots.

In contrast to our previous observations,[7] surprisingly it was observed that changing the concentration of reactants and the reaction temperature may produce no significant change in size and shape of the AgNPs. This exceptional behavior may be related to the structure of CB[7], which is a unique macrocyclic molecule and has a larger diameter (0.73 nm) cavity in the center than the two identical carbonyl-laced portals (0.54 nm). Due to the rigid structure of this molecule all donor atoms are preorganized and situated inside the cavity. Hence, we believe that the rigid and symmetrical cyclic structural arrangement of CB[7] plays an es-

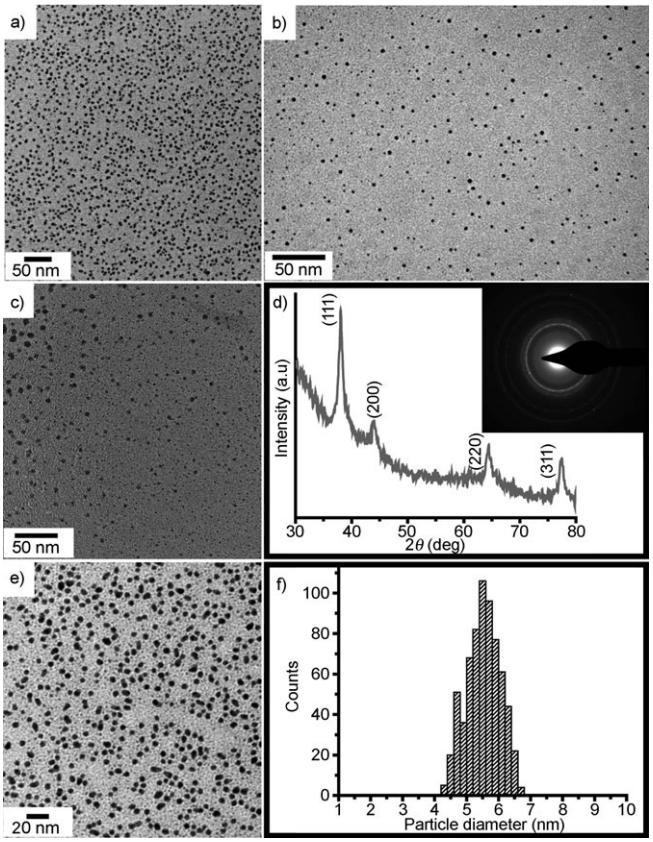

Figure 1. TEM micrographs of AgNPs obtained with a) 10, b) 40, and c) 60 mM NaOH concentration at room temperature. d) The XRD and ED (inset) patterns of sample b. e) High magnification TEM image of the well-dispersed AgNPs synthesized with 10 mM NaOH at room temperature, and f) the related histogram.

sential role on the surface of the AgNPs (CB[7] surrounding and interacting with the outer surface of the nanoparticles) and may prevent the growth of nanoparticles, irrespective of the reaction temperature and reactant concentration.

We anticipate that CB[7] at a particular concentration in the alkaline reaction medium preferentially allows the adsorption, reduction, and growth of a metal, resulting in nanoscale structures. As CB[7] has two identical polar carbonyl-laced portals, the net negative surface charges that appear around carbonyl portals may play an important role in the reduction of silver salt to AgNPs. As the metallic particles shrink into the nanoscale region, either electrostatic or steric stabilization for the particles must take place to account for the high surface energies of the particles in the nanometer size range. CB[7] molecules serve this purpose well. Alkaline conditions may facilitate the kinetic evolution of nanoparticles. To offset the van der Waals forces responsible for particle agglomeration, the highly electronegative oxygen atoms of carbonyl groups with net negative charges may offer a coating on the surfaces of the AgNPs and may provide stabilization. A schematic illustration of the formation and stabilization of well-dispersed AgNPs is shown in Figure 2 a. Furthermore, it was observed from molecular model studies that the silver atom (diameter: 288 pm) may

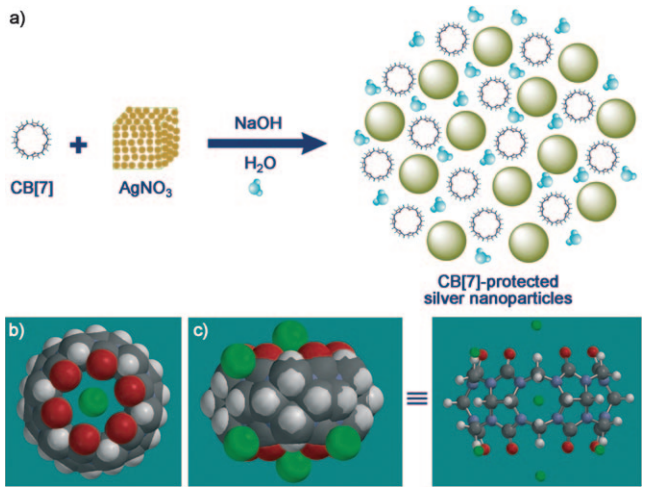

Figure 2. a) Schematic illustration of the formation and stabilization of well-dispersed AgNPs. b) and c) Energy-minimized space-filling molecular model of the CB[7] and silver atoms (bottom right: ball and spoke model).

be encapsulated in the cavity of the CB[7] (diameter: 730 pm), as shown in the model (Figure 2b), when the size of the nanoparticles is smaller than the cavity size,[16] which was dismissed in our case because the average AgNPs size is about 5 nm. On the other hand, the model suggests that the particles are stabilized by CB[7] with the possible interaction between surface of the nanoparticles and carbonyl-laced portals as shown in Figure 2c. This theoretical prediction using the energy minimized molecular model was experimentally substantiated by FTIR results. As can be clearly observed from the spectra, the characteristic carbonyl peak intensity of CB[7] decreased noticeably in the spectrum of the CB[7]-stabilized nanoparticles in addition to a significant high frequency shift from 1728 to 1735 cm^{-1} revealing the supramolecular interaction between carbonyl groups and the surface of the nanoparticles.[17]

Furthermore, from thermogravimetric analysis (TGA), it was determined that the CB[7] and metallic silver content was around 54.5 and 45.5%, respectively. Most importantly, the CB[7]-protected AgNPs may be isolated and redispersed in aqueous solution. In addition, we investigated the efficiency of the synthetic strategy to prepare other metal nanoparticles and the initial results observed for gold and palladium nanoparticles are encouraging.

To evaluate the anticancer activity of AgNPs, a control experiment was performed with AgNPs (≈5 nm) prepared at room temperature ($AgNO_3$; 5 mM, CB[7]; 5 mM, and NaOH; 40 mM) and CB[7] using two different cancer cell lines, namely MCF-7 and NCI-H358 as in vitro cell models. The cytotoxicity induced by the CB[7]-protected AgNPs and CB[7] was assessed by a standard MTT (thiazoyl blue tetrazolium bromide) assay and the results are presented in Figure 3. As can clearly be seen from the analysis, there was a significant enhancement in the cytotoxic capability of the CB[7]-protected AgNPs compared with that of the CB[7] alone after 24 h. Interestingly, a dose-dependent decrease of

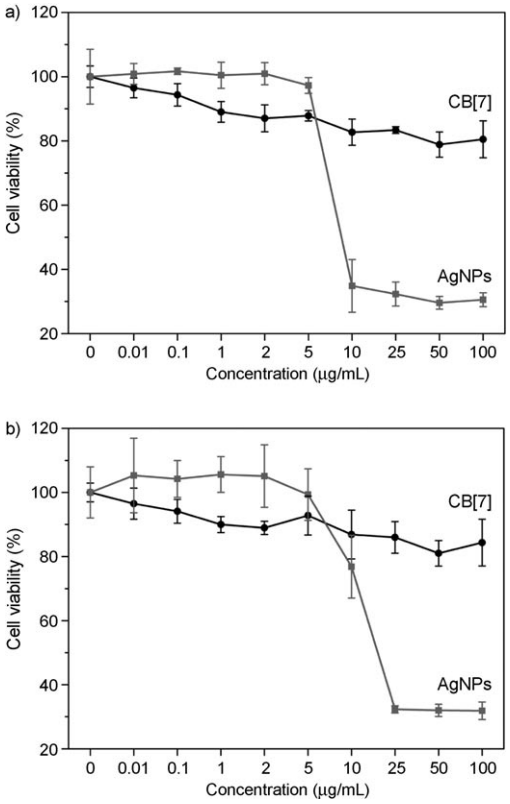

Figure 3. Cytotoxic activity of AgNPs and CB[7] on MCF-7 and NCI-H358 cells in vitro. The cell viability of a) MCF-7 cells and b) NCI-H358 cells after 24 h incubation at different concentrations of AgNPs and CB[7]. Untreated cells were used as the control.

cell viability of MCF-7 and NCI-H358 cells from about 100 to 30.4% and about 100 to 31.9%, respectively, compared with the control was observed while increasing the AgNPs concentration from 0.01 to 100 $\mu g\,mL^{-1}$ after 24 h incubation of the cells with as-prepared AgNPs (Figure 3). Importantly, as shown, the ability to kill the cancer cells induced by CB[7] alone was significantly lower than that caused by CB[7]-protected AgNPs. Therefore, the enhanced cytotoxicity obtained with the CB[7]-protected AgNPs against MCF-7 and NCI-H358 cells in vitro suggests that AgNPs may possess an inherent capacity to interact within cancer cells and may effectively kill the MCF-7 and NCI-H358 cancer cells. However, the exact contributing mechanisms are not yet fully elucidated and, therefore, need to be further investigated. Nevertheless, in the present study, it is pertinent to mention that the cytotoxicity caused by as-prepared CB[7]-protected AgNPs against MCF-7 and NCI-H358 cells in vitro was considerably higher (≈2.04 and 2.06 times greater, respectively, when compared at a concentration of 25 $\mu g\,mL^{-1}$ after 24 h incubation) than that of the recently reported glutathione-coated AgNPs against a human leukemic K562 cell line in vitro.[18]

In conclusion, a simple, effective, and one-pot method toward the synthesis of a defined macrocycle–silver nanoparticle system in an aqueous medium has been described. To the best of our knowledge, this report is the first for the

preparation of AgNPs by using CB[7] in an aqueous medium by chemical reduction exclusive of employing conventional reducing agents and/or external energy. Interestingly, a significantly increased cytotoxicity of the as-prepared CB[7]-protected AgNPs against MCF-7 and NCI-H358 cells as model cell lines in vitro has also been demonstrated. Taken together, the green approach for the synthesis of AgNPs by using a biocompatible material (CB[7]) in water at room temperature and the demonstration of their remarkable anticancer potential makes this system a novel platform for biomedical applications in general and cancer therapy in particular. A mechanism for the reduction and stabilization of AgNPs by CB[7] in the presence of NaOH has not been well understood and further studies are in progress.

Experimental Section

Synthesis of silver nanoparticles: In a typical experiment, an aqueous solution of $AgNO_3$ (5 mM; 2 mL) was mixed with an equivalent amount of aqueous CB[7] (5 mM; 2 mL) at room temperature. After the addition of NaOH (4 mL; 40 mM) to the resulting solution, a brownish/black-colored solution was observed in 15 min, indicating the formation of AgNPs. The reaction finally was completed after 40 min, which has been demonstrated by UV/Vis spectroscopy. No stirring was necessary after the solution was shaken gently for homogenization.

Cell culture and assessment of anticancer activity: The MCF-7 and NCI-H358 cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), and 1% antibiotic–antimycotic in a humidified incubator at 37°C, 95% room air, and 5% CO_2.

For the MTT cell survival assay, a suspension (200 μL) of MCF-7 and NCI-H358 cells were seeded in an independent new 96-well plate at a density of 1×10^4 cells well^{-1}. Cells grew for 20 h after seeding and were then treated with AgNPs at different concentrations, namely 0.01, 0.1, 1, 2, 5, 10, 25, 50, and 100 μg mL^{-1} and were incubated for 24 h at 37°C. For comparison, the same experiment was also performed with CB[7] (0.01, 0.1, 1, 2, 5, 10, 25, 50, and 100 μg mL^{-1}) to evaluate the cytotoxicity of CB[7] alone against these two cell lines. Untreated cells were used as a control. After the treatment the cells were rinsed three times with PBS (phosphate-buffered saline). Subsequently, MTT (20 μL of a 5 mg mL^{-1} solution) was added to each well, and incubated for an additional 4 h at 37°C. After incubation with the MTT reagent, all the media were gently removed by an aspirator using a syringe and purple formazan crystals formed by live cells were dissolved with 200 μL of dimethyl sulfoxide and the solution was gently shaken for 10 min. UV absorption of the solution was recorded at 570 nm by means of a FL600, microplate fluorescence reader, Bio-Tek. Each experiment was performed at least three times. The solid CB[7]-protected AgNPs obtained were collected by centrifugation of the silver colloids at 4000 rpm for 15 min and the residue was washed with ethanol several times by replacing the supernatant and then dried well. The resulting solid CB[7]-protected AgNPs were used for the cell toxicity tests.

Materials: The human breast adenocarcinoma (MCF-7) and human lung bronchoalveolar (NCI-H358) cell lines were obtained from the Laboratory of Molecular Biology, Department of Biotechnology, College of Natural Science, Chosun University, South Korea and the Korean cell line bank (KCLB), respectively. RPMI 1640 medium (Gibco, Invitrogen), FBS (WelGENE Inc.), antibiotic–antimycotic solution (Gibco, Invitrogen), MTT (≥97.5% TLC, Sigma–Aldrich), and dimethyl sulfoxide (DMSO, Junsei Chemical Co., Ltd.) were purchased and used as obtained. Cucurbit[7]uril (Australia), $AgNO_3$ (Aldrich), and NaOH (DC Chemical, Korea), were purchased and used without further purification. Deionized water was used throughout the experiments.

Instruments: The UV/Vis absorption spectra were recorded on a Varian Cary 500 spectrophotometer. The morphology, the crystal size, and the size distribution were studied by means of a Philips T20ST TEM. The TEM specimens were prepared by placing a few drops of sample solution on a copper mesh covered with a carbon film and allowing the solvent to evaporate at room temperature overnight. The particle size distribution was calculated by image analysis. The FTIR spectra were taken at room temperature by using a Perkin–Elmer System 2000 under nitrogen in KBr pellets in the range 4000–400 cm^{-1}. The XRD pattern was measured on a Rigaku diffractometer with copper radiation ($\lambda=0.15406$ nm) at 40 kV and 40 mA. TGA was recorded on a TA-2050 thermal analyzer. The measurements were carried out under nitrogen with platinum cups as sample holders with 2–3 mg of the samples at a heating rate of 10°C min^{-1}.

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