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Comparative Toxicity Studies of Ultra-Pure Ag, Au, Co, and Cu Nanoparticles Generated by Laser Ablation in Biocompatible Aqueous Solution

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Nanoparticles (NPs) are increasingly used in consumer products, which have aroused many concerns and debates regarding their fate in biological systems from a point of their safety/toxicity. Although a number of studies on the biological effects of NPs have been published, these are often complicated by the possible toxicity of conventional NPs, caused by contamination with chemical precursors or additives during their synthesis and/or purification procedures. To explicitly understand the toxicity basis of NPs, it is necessary to directly address a main problem related to their intrinsic/inherent toxicity and/or incompatibility with biological objects. The present study is designed to take advantage of a novel laser-assisted method called laser ablation to generate Ag, Au, Co, and Cu NPs in biocompatible aqueous solution, and to evaluate the toxicity of the resulting ultra-pure NPs. Our results show that the ultra-pure NPs with nascent surfaces possess moderate cytotoxicity to human cells in a cell-dependent manner.

Key Words : Nanoparticle, Toxicity, Laser ablation

Introduction

Recent progress clearly and promisingly shows that inorganic nanoparticles (NPs) exhibit superior properties compared to traditional organic materials and bring the enhanced magnetic, electrical, optical, mechanical, and structural characteristics for many applications in the novel methods development in biosensing, medical diagnostics and therapeutics, cosmetics coating, *etc*.[1] The production and use of inorganic NPs, however, have aroused many concerns and debates regarding their fate in biological systems from a point of their safety/toxicity because their use in many applications (especially *in vivo*) remains under scrutiny, of which the main problem is related to their possible toxicity and/or incompatibility with biological objects.[2] Although a number of studies on the *in vitro* and *in vivo* effects of NPs have been published to date, these are often complicated by the possible toxicity of conventional NPs, caused by contamination with chemical precursors or additives during their synthesis and/or purification procedures.[3]

A laser-assisted method called laser ablation has proven itself as an alternative physical nanofabrication method.[4] It consists in the ablation of a target (mostly solid) by intense laser radiation and leads to an ejection of its constituents and the formation of NPs.[4] In particular, the ablation in a liquid ambient (for example, in neat water or in 7 mM NaCl solution) causes a release of nanoclusters to the environment to form a colloidal solution of NPs.[4] Laser ablation in liquid has several advantages over the traditional chemical reduction methods for NPs, such as technical simplicity and chemical pureness, because this method when performed in a clean and well-controlled environment enables us to avoid the use of toxic chemical reduction agents to control the growth of the colloidal NPs, resulting in production of ultra-pure NPs with nascent surfaces.[4] Thus, laser ablation is expected to offer novel opportunities to provide insight into the origin of NP toxicity. This study presents the evaluation of the intrinsic/inherent toxicity of ultra-pure Ag, Au, Co, and Cu NPs generated by laser ablation in a liquid ambient, revealing that the chemical nature of metallic NPs strongly influences the toxicity and cellular uptake into the model cells.

Experiments

Chemicals and Materials. Unless additionally noted, reagents were obtained from commercial suppliers and were used without further purification. All experiments were performed in triplicate.

Experimental Setup for Laser Ablation and Nanoparticle Preparation. The experimental setup for laser ablation and the synthetic methods for Ag, Au, Co, and Cu NPs are described elsewhere.[5] In brief, Ag, Au, Co, and Cu NPs were produced by laser ablation of silver, gold, cobalt and copper targets (all 99.9%), placed in a Pyrex cell filled with 7 mM NaCl solution for Ag and Au NPs and with neat water for Co and Cu NPs, respectively. The targets were vertically irradiated by a Q-switched Nd:YAG laser (Surelite III) equipped with PLCX-25.4-103.0-UV lens and BSNP-1064-50-1025 beam-splitter, while the cell was rotated continuously to minimize the target aging effect and ensure a fresh surface of the targets in addition to a stirring effect during the formation of NPs. The laser beam was focused to a beam diameter of 0.5 nm. The absorbance data of the

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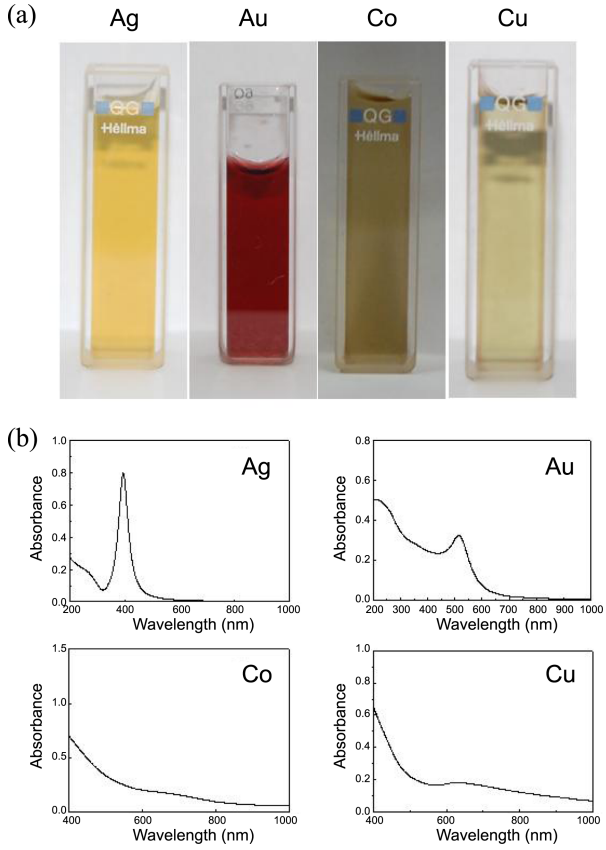

Figure 1. (a) Photograph comparing the appearance of Ag, Au, Co, Cu nanoparticles (NPs) generated by laser ablation. (b) Absorption spectra of Ag, Au, Co, Cu NPs.

prepared solutions were recorded using a spectrometer (Shimadzu UV-1800), as shown in Figure 1.

Cell Culture and Viability Assays. HeLa human cervical cancer cells, PC3 human prostate cancer cells, and MCF-7 human breast cancer cells were obtained from American Type Culture Collection and were maintained in tissue culture plates at 37 °C in an atmosphere of 5% CO_2 in MEM/EBSS (with Earle's salts) media for HeLa cells and in RPMI-1640 media for PC3 and MCF-7 cells, all supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 100 IU/mL penicillin and 0.1 mg/mL streptomycin. In order to keep cells in log phase, the cultures were re-fed with fresh media two or three times/week.

Cell viability was assessed using the standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay according to the literature.[6] In brief, exponentially growing cells were seeded in 96-well flat-bottomed microplates (100 μL/well) at a density of 1×10^5 cell per mL and after 24-h incubation at 37 °C, followed by exposure for 24 h to various concentrations of Ag, Au, Co, and Cu NPs, respectively. For each concentration at least 8 wells were used. After incubation, MTT solution (5 mg/mL in PBS) was added and the microplates were further incubated for 4 h at 37 °C. The quantity of formazan product obtained was determined at 570 nm. The cell survival fractions were calculated as percentage of the untreated control. The experimental data were transformed to sigmoidal dose-response curves using nonlinear regression analysis (SigmaPlot) to calculate the corresponding IC_{50} values.

Results and Discussion

The high promise of NPs for biological applications is tempered by a lack of knowledge concerning the unintended consequences of the NPs or the byproducts associated with their synthesis. In this regard, there have been an increasing number of studies to examine the potential toxicity of NPs prior to widespread clinical application.[3] Importantly, however, the reported data are highly dependent upon the synthesis methods and resulting NP size, shape, surface chemistry, and surface charge.[3] Indeed, one can distinguish two potential toxicity problems. The first one is related to the inherent toxicity of NPs, which is a concern of this study. The second toxicity is related to the use of toxic or non-biocompatible compounds during the chemical synthesis procedure (for example, surfactants) or released from NPs interacting with the biological environment. In fact, conventional methods for the fabrication of colloidal metal nanostructures (NPs, nanorods, core-shells) are based on a chemical reduction process,[7] which generally gives rise to the surface contamination by residual anions and reducing agents.[4(d),7] The resulting toxicity of such nanostructures complicates their applications in *in vivo* biosensing and imaging.[2]

In the present study, to exclude any second toxicity of NPs described and thus to provide insight into the origins of toxicity of metallic NPs, laser radiation is used to ablate a solid target (Ag, Au, Co and Cu) placed on the bottom of a glass vial filled with biocompatible aqueous solution (7 mM NaCl solution or neat water). Among a variety of metal-based NPs, Ag, Au, Co and Cu NPs were chosen because these NPs are receiving considerable attention as additives in consumer and industrial products.

We used a Q-switched Nd:YAG laser (Surelite III) emitting 6 ns pulses centered with a maximum energy of 100 mJ/pulse at a repetition rate of 10 Hz.[5] The laser energy was controlled by a set of fixed and variable attenuators and the spot size was adjusted by a motorized microscope objective. The target is moved in the focusing plane using a motorized stage to obtain identical surface condition during the ablation process. In the case of Ag or Au target, such ablation process leads to a coloration of the solution in the vial within a few seconds after the beginning of the ablation process (the solution becomes yellow and red in the case of Ag and Au, respectively), as demonstrated in Figure 1(a). Such coloration is related to the excitation of surface plasmons of newly formed colloidal metal NPs. It should be noted that ablation of Ag target in NaCl solution may lead to the formation of AgCl that is not soluble in water and forms white sediment, which needs to be considered for quantitation of Ag NPs. Figure 1(b) shows the absorbance data of the colloidal solutions prepared. It is also important to note that Cu NPs are characterized by the typical plasmon resonance

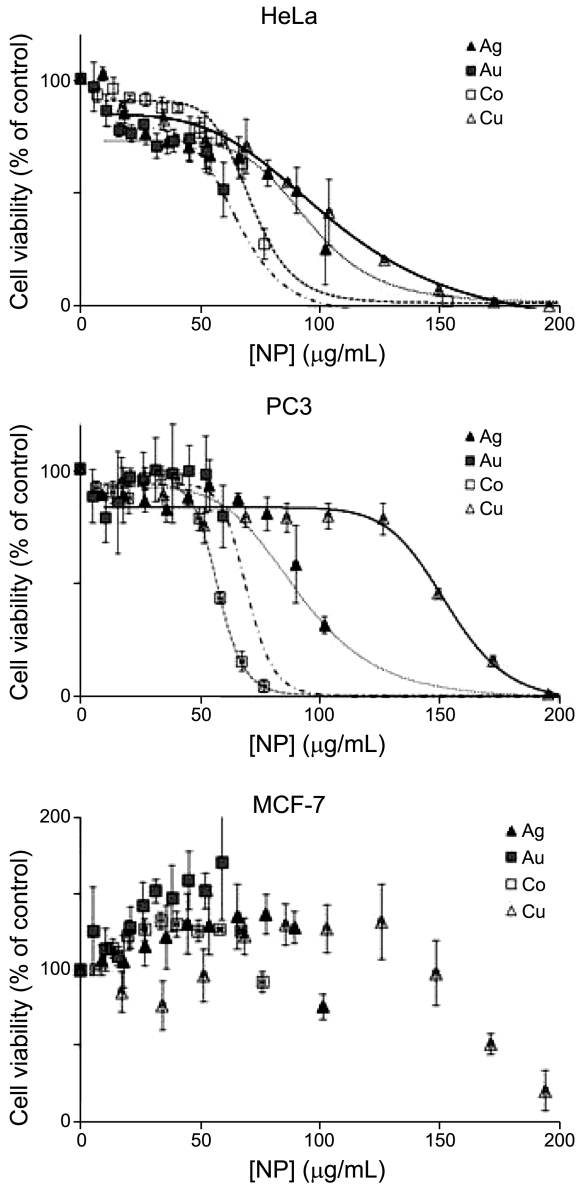

Figure 2. Dose-dependent viability of HeLa human cervical cancer cells, PC3 human prostate cancer cells, and MCF-7 human breast cancer cells, exposed to Ag, Au, Co, Cu NPs, respectively. The experimental data were transformed to sigmoidal dose-response curves using nonlinear regression analysis (SigmaPlot) to obtain the theoretical curves and to calculate the corresponding IC_{50} values.

at 590 nm.[4] The physicochemical characterization of the obtained NPs, such as particle size and distribution, zeta potential, and degree of aggregation, is in progress in this laboratory.

With the produced ultra-pure NPs with nascent surfaces, MTT assays were performed using HeLa human cervical cancer cells, PC3 human prostate cancer cells, and MCF-7 human breast cancer cells, to address the inherent toxicity of the prepared NPs, as a pre-screening tool for NP's biological effects. Figure 2 clearly indicates that the bio-effects of NPs are drastically different in a dose-response manner. Au NPs showed the lowest cytotoxic effect in HeLa cells compared

Table 1. IC_{50} values (μg/mL) of Ag, Au, Co, Cu NPs in human cancer cell lines. IC_{50} values were defined as the NP concentrations at which cell growth was inhibited by 50% compared with NP-free controls. Values are the mean ± SD of three experiments. IC_{50} values for MCF-7 cells could not be determined (*n.d.* = not determined)

	HeLa	PC3	MCF-7
Ag	78.9 ± 8.2	88.6 ± 8.0	*n.d.*
Au	66.4 ± 6.5	82.9 ± 9.0	*n.d.*
Co	71.8 ± 7.6	77.5 ± 5.9	*n.d.*
Cu	85.5 ± 4.1	91.7 ± 10.3	*n.d.*

to the other NPs, and Cu NPs were found to be the most toxic NPs among the four examined in PC3 and MCF-7 cells. The obtained IC_{50} values of Ag, Au, Co, and Cu NPs were calculated to be 78.9, 66.4, 71.8, and 85.5 μg/mL (or 731, 337, 1130, and 1450 μM) for HeLa cells, and 88.6, 82.9, 77.5, and 91.7 μg/mL (or 821, 420, 1220, and 1560 μM) for PC3 cells, respectively, as summarized in Table 1. Although size-dependent toxicity of NPs needs to be further investigated, these results demonstrate that the uncontaminated and surfactant-free NPs themselves possess moderate cytotoxicity to human cells in a cell-dependent manner.

It is worthwhile to compare the obtained results to the literature values, since there are many contrary reports on the cytotoxicity of NPs. As well reviewed in the literature,[3] NPs have been shown to interfere with the antioxidant defense mechanism leading to reactive oxygen species generation, the initiation of an inflammatory response and perturbation and destruction of the mitochondria causing apoptosis or necrosis in different mammalian cells *in vitro*.[8] Ag NPs are reported to induce apoptosis, as effectively as well-known oxidative stress-related genes such as *ho-1* and *mt-2A*, in HeLa cells with the IC_{50} value ~92 μg/mL (or 850 μM).[9] This is consistent with our MTT assay results, inferring that there may be no cytotoxic difference between the conventional and NPs with nascent surface. This consistency, however, is attributed to the capability of Ag NPs to bind to sulfur-based groups such as thiols in mammalian cells, which may diminish the difference in surface chemistry of the NPs. Importantly, Au NPs have been reported to be inherently non-toxic to human cells,[10] in contrast to our results. While there are still big concerns about the adverse biological effects of Au NPs in terms of the synthesis methods and the resulting Au NP size, shape, surface chemistry and surface charge,[3] Alkilany *et al.* reported that the apparent cytotoxicity of surfactant-coated Au nanorod solutions with human colon carcinoma cells (HT-29) might be caused by free surfactant in solution and that overcoating the nanorods with polymers could substantially reduce cytotoxicity,[11] indicating that the molecular desorption or residual contamination of the starting NPs might be the major cause of cytotoxicity. Taken together with our results, Au NPs have intrinsic and moderate cytotoxicity as much as Ag NPs do, but theirs may be controlled by the surface modification. Cu NPs, similar to Ag NPs, have been shown

to induce oxidative stress and interact with SH groups leading to protein denaturation,[12] and the oral LD_{50} in mice is known to be 413 mg/kg, which is considered moderate toxicity similar to Zn powder.[13] Cu NPs, however, are known to display a dual capacity to act as a required cofactor and biocatalyst with a critical balance for proper intracellular metal homeostasis and metabolism,[14] presumably explaining why Cu NPs in our study have the lowest cytotoxicity in PC3 and MCF-7 cells. In consideration that Co NPs can be internalized with a 50- to 100-fold increased uptake compared to cobalt chloride, resulting in the IC_{50} value of 0.589-1.18 μg/mL (or 10-20 μM) for Balb/3T3 mouse fibroblasts,[15] our data indicate that Co NPs with nascent surface can significantly (almost 100-fold) decrease the cytotoxicity probably due to the exclusion of any contaminants, although the possibility that the bigger-sized Co NPs may not produce any cytotoxic effects as the case of Co NPs or that Balb/3T3 mouse fibroblasts may have higher sensitivity to Co NPs than the human cells used in our study cannot be ignored.[3(b)]

Conclusion

To summarize, we have made an attempt to evaluate and discuss the intrinsic toxicity of ultra-pure Ag, Au, Co, and Cu NPs using three human cancer cell lines as a model system. The NPs produced by the laser-assisted method are stable, free of contamination and have been found to have inherent surface chemistry with the potential for cytotoxicity in the case of high concentration exposure. This important approach is unique in avoiding the secondary toxicity related to the traditional chemical synthesis procedure, because incomplete characterization of NPs may hinder attempts to find a correlation between a variety of biological effects and NP properties.

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