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Alteration in Enzymatic Function of Human Cytochrome P450 by Silver Nanoparticles

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ABSTRACT

Silver nanoparticles (AgNPs) have been recently used for a wide range of applications including health and household products even though an understanding of their mechanistic action in human is not fully explored. In this study, AgNPs were chemically synthesized, characterized and evaluated for *in vitro* biological effect on human cytochrome P450 (P450) metabolizing enzymes, namely CYPs 1A2, 2C9, 2C19 and 3A4. The findings indicated that the particle size diameter of AgNPs was 12.42±2.48 nm with negative surface charges, -43.6±0.7 mV. AgNPs could alter the function of P450 by decreasing the enzymatic activities in a concentration dependent manner. AgNPs exhibited the greatest inhibition on CYP3A4 with an IC_{50} value of 13.52±0.01 μM, followed by CYP2C19 (IC_{50} = 14.31±0.01 μM), CYP2C9 (IC_{50} = 26.46±0.01 μM) and CYP1A2 (IC_{50} = 43.51±0.03 μM), respectively. Thus, an awareness of the exposure to AgNPs is suggested due to their ability to interfere with metabolic pathway in human.

Key words: Biotransformation, cytochrome P450, metabolism, nanotechnology, silver nanoparticles

INTRODUCTION

Nanotechnology becomes eminent in the present world according to the powerful applicability of nanometric scale materials. Metallic silver nanoparticles (AgNPs) have recently attracted interest from many applications due to unique physicochemical properties such as high resistance to oxidation (Maria *et al*., 2010). Furthermore, the antimicrobial capability of AgNPs allows them to be suitably employed in numerous household products such as textiles, food storage containers, home appliances and in medical devices (Navarro *et al*., 2008; Marambio-Jones and Hoek, 2010). Recently, the finding on growth inhibition of HIV-1 virus by AgNPs has enlightened the extensive research on the use of AgNPs for the health products (Elechiguerra *et al*., 2005). Because numerous commercial products containing AgNPs are currently available, the safety issue regarding the effect of any exposure to AgNPs on biological function in the human body is urgently required. However, scarce reports of the potential impact of AgNPs on human health have been published. Since, the metabolic process is the main chemical process in which the substances are either catabolized to give energy or therapeutic effect or anabolized to yield the molecules for life maintenance, the effect of AgNPs on metabolizing enzymes is perhaps worth findings.

Cytochrome P450 (P450, CYP450) is a group of metabolizing enzyme involved in the oxidative biotransformation of chemical and xenobiotic substrates (Coleman, 2005). The reaction between substrate and P450 enzyme initiated by NADPH-CYP450 reductase results in an oxidized and more water soluble metabolite (Scheme 1) which can be easily eliminated (Glue and Clement, 1999).

$$\text{Substrate (RH)} + O_2 + \text{NADPH} + H^+ \xrightarrow{\text{CYP450}} \text{Product (ROH)} + H_2O + \text{NADP}^+$$

Scheme 1: Typical reaction by CYP450 enzyme

Most of P450s are expressed mainly in liver microsomes and can be classified into numerous isozymes. Although the role of P450 in bioactivation of some compounds is not clearly understood, the risk of environment toxicants and carcinogens is known to be minimized by metabolic process in the liver (Guengerich, 2008). However, the function of P450 can be inhibited by particular xenobiotics as called inhibitors which can sustain the compound metabolized by specific P450 isozymes (Goshman *et al.,* 1999; Coleman, 2005). The preliminary study of inhibitory effect of metallic AgNPs on cDNA expressed human P450 was reported by our group (Sereemaspun *et al.*, 2008), however, an extent of alteration in P450 activities of specific isozymes by AgNPs is still undiscovered.

Accordingly, in order to systemically compare the potential of AgNPs on oxidative metabolism, the AgNPs were synthesized and investigated for their effect on dose-related enzymatic activities of four dominant human CYP isozymes, CYPs 1A2, 2C9, 2C19 and 3A4.

MATERIALS AND METHODS

Materials: Silver nitrate ($AgNO_3$) and Tris(hydroxymethyl)aminomethane (Tris base) ($C_4H_{11}NO_3$) were purchased from Sigma-Aldrich (St. Louis, USA). Sodium borohydride ($NaBH_4$) was supplied from Merck, Darmstadt, Germany. Vivid® CYP450 blue screening kit (Invitrogen, Madison, USA) comprises of Baculosome® reagent (microsomes from baculovirus-infected cells containing cDNA-expressed human CYPs 1A2, 2C9, 2C19 and 3A4 and incorporating with NADPH-cytochrome P450 reductase), $NADP^+$ solution, potassium phosphate buffer (reaction buffer) solution, NADPH regeneration system containing glucose-6-phosphate dehydrogenase (G-6-PD) and glucose-6-phosphate (G-6-P) and finally, fluorogenic Vivid® substrate, which are either 7-ethyloxymethyloxy-3-cyanocoumarin (EOMCC) for CYPs 1A2 and 2C19 or 7-benzyloxymethyloxy-3-cyanocoumarin (BOMCC) for CYPs 2C9 and 3A4.

Chemical synthesis of silver nanoparticles: Synthesis of AuNPs was performed by the chemical reduction method as previously described by Solomon *et al.* (2007) with some modifications. The 10 mL volume of $AgNO_3$ was added dropwise into 40 mL of chilled $NaBH_4$ solution with vigorously stirring for at least 30 min. The synthesized AgNPs were stored in a closed container at room temperature.

Characterization of silver nanoparticles: UV-Vis measurement: The UV-Vis spectroscopy of AgNPs was carried out on an Evolution 300 UV-Visible spectrophotometer (Thermo Scientific Ltd., USA). The sample solution was pipetted into a quartz cell with a path length of 10 mm and deionized water was used as a reference standard.

TEM measurement: Transmission Electron Microscopy (TEM) was used to determine the particle size, morphology and size distribution of AgNPs on a JEM2100 (Jeol, Japan). For sample preparation, 10 μL of sample solution was dropped into a carbon-coated 200-mesh copper grid and air-dried before TEM measurement.

Zeta potential measurement: Zeta potential represents the surface charge of a particle. The Zetasizer NanoZS (Malvern, UK) was used to measure the zeta potential by using laser Doppler electrophoresis technique. The AgNP solution was added into a disposable zeta potential cell and deionized water was used as a reference standard.

Assay of human cytochrome P450 enzymatic activity: The *in vitro* activity of AgNPs against four isozymes of cytochrome P450 was performed according to the procedure described elsewhere (Sereemaspun *et al.*, 2008). Briefly, Costar® black, flat-bottom, 96-well plates (Corning Life Sciences, USA) were used to acquire a total reaction volume of 100 μL per well. For assay of CYPs 1A2 and 2C19, a 50 μL buffer solution (pH 8.0), containing P450 Baculosomes® at assay concentration of 5 nM P450, 0.3 U mL^{-1} G-6-PD and 3.33 mM G-6-P, was added to 40 μL of various concentrations (10-80 μM) of AgNPs. For the control wells, 40 μL of deionized water was used instead of AgNPs. The plate was pre-incubated for 20 min at room temperature prior to adding a 10 μL solution containing EOMCC substrate (3 μM for CYP1A2 or 10 μM for CYP2C19) and 100 μM $NADP^+$. After 30 min incubation at room temperature, the enzymatic reaction was stopped by an addition of 5 μL of 0.5 M Tris base (pH 10.5) to all wells. The similar assay procedure was applied for determination of CYPs 2C9 and 3A4 activities. However, assay concentrations of 10 μM BOMCC substrate, 10 nM CYP2C9 and 5 nM CYP3A4 were used. The relative fluorescence unit (RFU) was read on a Victor^3 V fluorescence plate reader (PerkinElmer, USA) using excitation and emission wavelengths of 409 and 460 nm, respectively. The background fluorescence was established by wells containing only reaction buffer and sample solution (or deionized water for control wells) and was subtracted prior to analysis.

Data analysis: The percentage of loss of enzymatic activity was calculated by comparisons of RFU obtained from sample and control wells. The median inhibition concentration (IC_{50}) was then determined by non-linear regression analysis using Graph Pad Prism 4.03 (evaluation version) software program (GraphPad Inc., San Diago, USA). The IC_{50} values expressed as mean±SD were statistically analyzed by one-way Analysis of Variance (ANOVA), followed by Bonferroni post hoc test for a pairwise comparison.

RESULTS

Synthesis and characterization of AgNPs: The chemical reduction of $AgNO_3$ by $NaBH_4$ using a molar ratio of 2:1 $NaBH_4$ to $AgNO_3$ yielded yellow solution of AgNPs containing 500 μM Ag atom. The synthesized AgNPs were found to be stable for at least several months. The characteristic of UV-Vis spectrum of AgNPs showed the absorption maxima of around 420 nm (Fig. 1a). In fact, the light absorption of AgNPs represents the Surface Plasmon Resonance (SPR) of the metallic nanoparticles. The SPR phenomena are due to the oscillation of electrons on the surface of nanoparticles induced by electromagnetic radiation (Link and El-Sayed, 1999). The result from TEM analysis revealed that the AgNPs were spherical in shape with an average diameter of 12.42±2.48 nm (Fig. 1b); similar to the result previously obtained using the same synthesis method

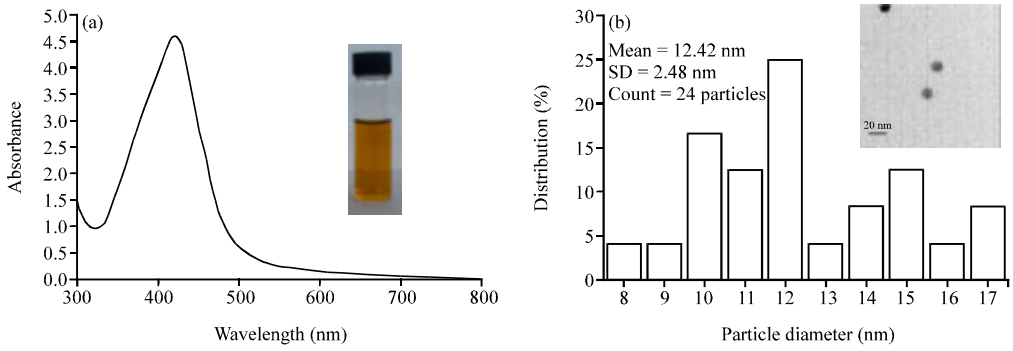

Fig. 1: (a) The UV absorbance peak and (b) size analysis of AgNPs

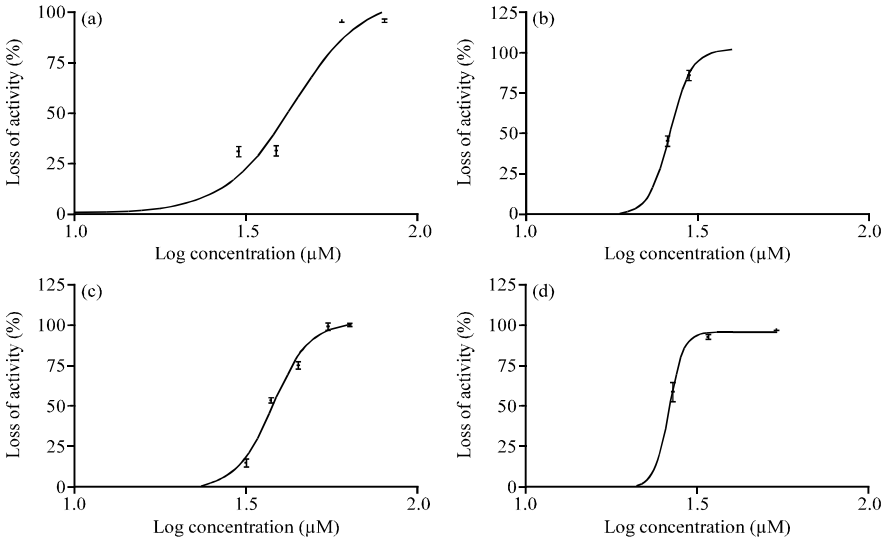

Fig. 2: Effect of AgNPs on activities of (a) CYP1A2, (b) CYP2C9, (c) CYP2C19 and (d) CYP3A4

(Solomon *et al*., 2007). The surface potential of AgNPs, -43.6±0.7 mV (n=3), indicated the negative charges of tetrahydridoborate (III) ions (BH_4^-) as particle stabilizers.

P450 activity studies: The fluoreogenic Vivid® substrate which is initially non-fluorescent converses into a fluorescent product upon oxidation reaction by specific P450 isozymes (Cohen *et al*., 2003). A degree of loss in human P450 activity can be measured by a decrease in fluorescent intensity and is used to forecast the isozyme-specific metabolism inhibition. Figure 2a-d shows the dose-dependent curves which were used for calculating the IC_{50} (Mean±SD, n=4) of AgNPs on CYPs 1A2, 2C9, 2C19 and 3A4. The AgNPs inhibited CYP3A4 the most with an IC_{50} value of 13.52±0.01 μM. For CYPs 2C subfamily, the higher in residual activity of CYP2C9 than CYP2C19 was obtained when compared at the same concentration of AgNPs. Correspondingly, the IC_{50} value of CYP2C9, 26.46±0.01 μM, was twice that of CYP2C19, IC_{50} = 14.31±0.01 μM. Surprisingly, the effect of AgNPs on an alteration of CYP1A2 activity was the least with an IC_{50} value of 43.51±0.03 μM. It was noteworthy that the differences among the IC_{50} values of all P450s were statistically significant ($p \leq 0.05$). In addition, AgNPs at concentrations of 80 μM or less were able to absolutely inhibit the P450 activities.

DISCUSSION

The wide spread use of nanotechnology especially in household and other commercial products can inevitably result in the diffusion of nanomaterials into the environment. The implication of plausible exposure risk of nanomaterials would provide the useful statement for preventive healthcare and development of novel materials in nanoscale. The popularity of AgNP-composed nanoproducts has encouraged us to postulate their imminent impact on one of the main biological functions in human, the activity of P450 metabolizing enzymes. It was notably that although our previous report indicated the inhibition of P450 by AgNPs, only a specific concentration of AgNPs was used and no IC_{50} values for P450 were compared (Sereemaspun *et al.*, 2008).

According to the criterion for classes of inhibitors, the compound having IC_{50} value of 10-50 μM is classified into a moderate P450 inhibitor whilst that having IC_{50} value of ≤10 μM is known to be a potent inhibitor (Zou *et al.*, 2002). From the results, AgNPs are perhaps considered to be moderate inhibitors for CYPs 1A2, 2C9, 2C19 and 3A4. The greater inhibition of AgNPs was observed on CYP3A4 followed by CYPs 2C19, 2C9 and 1A2, consecutively. Since the role of P450s involves the oxidative metabolism of drugs and xenobiotics, a decrease in enzymatic activities would delay the elimination of drugs and cause drug toxicity or drug-drug interaction (Glue and Clement, 1999; Coleman, 2005). In human, CYP3A4 is responsible for metabolism of a wide variety of drugs including antiarrhythmics, calcium antagonists, psychotropics, opioid analgesics, antihistamines, benzodiazepines, antimicrobial agents, antiretroviral agents, antiulcer agents, anticonvulsants and immunosuppressants. For CYP2C9, it metabolizes several common drugs, including phenytoin, warfarin, losartan, tolbutamide, plipizide and non-steroidal anti-inflammatory drugs. Antidepressants, anticonvulsants, anxiolytics and benzodiazepines are oxidized by CYP2C19. Finally, CYP1A2 metabolizes drugs that resemble aromatic amines, including caffeine, β-naphthylamine, theophylline and tricyclic antidepressants. It also oxidizes estrogen and this series of hormones. Although CYP1A2 seems to be significant for the bioactivation of procarcinogens such as polycyclic hydrocarbon, their metabolism in the liver was able to avoid the distribution of the toxic compounds to other tissues (Guengerich, 2008).

The appraisal regarding the alteration of P450-mediated metabolism by AgNPs is how the nanoparticles can enter into the cell membrane and generate their effects. The potential of nanoscale AgNPs to interact with the cell membrane has been recently reported in bacterial (Sondi and Salopek-Sondi, 2004), viral (Elechiguerra *et al.*, 2005), animal (Bar-llan *et al.*, 2009) and human cells (Chung *et al.*, 2008). In general, positively charged molecules provide the better penetration into the anionic phospholipid membrane due to electrostatic attraction (Aillon *et al.*, 2009). The greater interaction with cell membrane of positive surface charges of nanoparticles than neutral or negative surface charges have been reported in several nanoparticulate systems including magnetite nanoparticles (Osaka *et al.*, 2009), liposomes (Miller *et al.*, 1998) and gold nanoparticles (Goodman *et al.*, 2004). For AgNPs, it was the negative charges around the particle surface that promoted the particle stability by preventing the aggregation of nanoparticles due to electrostatic repulsion (Song *et al.*, 2009).

The capability of cell penetration of AgNPs is however unlikely to depend upon surface charges. Indeed, the nanosize particles cause greater cell uptake and readily distribution of particles to almost all tissues than the larger particles (Foged *et al.*, 2005; Aillon *et al.*, 2009). In previous literatures, the negatively charged AgNPs (13.4 nm in diameter) showed the antimicrobial effect on *Escherichia coli* and *Staphylococcus aureus* with the minimum inhibitory concentrations (MICs) of 0.4-0.7 ng mL^{-1} for *E. coli* and >3.6 ng mL^{-1} for *S. aureus* (Kim *et al.*, 2007). Contrarily,

chitosan-stabilized AgNPs (20-25 nm in diameter) containing positive charges exhibited the MIC of 5.6 μg mL^{-1} for both *E. coli* and *S. aureus* (Yoksan and Chirachanchai, 2010). Thus, the effect of surface charge is oblique and there might be some specific mechanism for the interaction between AgNPs and cell membrane. From the experiments so far, it could be drawn that AgNPs can accumulate and permeate through the membrane, leading to the binding of intracellular protein with AgNPs and finally the protein inactivation (Sondi and Salopek-Sondi, 2004; Kim *et al.,* 2007).

In summary, the alteration of biotransformation of compounds metabolized by P450 enzymes is obviously caused by AgNPs. Any exposure to AgNPs should be addressed the possibility of AgNPs to pass through the microsomal membrane and affect the enzymatic function.

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