An effective strategy for the synthesis of biocompatible gold nanoparticles using danshensu antioxidant: prevention of cytotoxicity *via* attenuation of free radical formation

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To suppress the cytotoxicity of gold nanoparticles (AuNPs), danshensu, a naturally occurring polyphenol antioxidant isolated from Chinese herb, was used to provide a fundamental protection layer for AuNPs, to alleviate oxidative stress and as a reducing agent to react with chloroauric acid. Besides danshensu, gum arabic was chosen as an auxiliary stabilizing agent to improve the stability of AuNPs against aggregation. As expected, the prepared GA–DS–AuNPs (Ggum Aarabic–Ddanshensu–gold nanoparticle) was remarkably stable in various buffer solutions. More interestingly, the GA–DS–AuNPs not only did not show any appreciable cytotoxicity, but also could alleviate the oxidative damage induced by

AuNPs. Meanwhile, the ROS/RNS scavenging activities of GA–DS–AuNPs was evaluated by electron spin resonance spectroscopy (ESR), potentiometric nitric oxide (NO) sensor and cell confocal imaging. The results suggest that GA–DS–AuNPs might have effectively reduced the AuNPs-induced cytotoxicity and oxidative stress by down-regulation of ROS/NOS production. The GA–DS–AuNPs may provide potential opportunities for the application in nanomedicine and nanobiology.

Keywords: nanoparticles, oxidative stress, antioxidant, free radicals, cell toxicity

Introduction

Gold nanoparticles (AuNPs) have attracted increasing attention due to a wide variety of potential applications in chemical, biomedical and diagnostic techniques (Paciotti et al. 2004; Villiers et al. 2010; Nie et al. 2006; Jahnen-Dechent & Simon 2008). However, the unique properties of AuNPs may also lead to oxidative stress and consequent cell toxicity (Mironava et al. 2010; Khlebstov et al. 2011). For example, it was found that oxidative stress induced by AuNPs played an important role in the cytotoxicity of AuNPs in Hela cells (Pan et al. 2009); Moreover, AuNPs could inhibit cell proliferation by downregulating cell cycle genes and affect genes associated with genomic stability and DNA repair (Li et al. 2008; 2011). Our recent studies have also demonstrated that citrate-encapped gold nanoparticles (CT–AuNPs) could catalyzse nitric oxide (NO) production from endogenous RSNOs in blood serum (Jia et al. 2009). It is known that NO may further react with superoxide anion ($O_2^{\bullet-}$) to produce a harmful peroxynitrite (ONOO-) species (Yang et al. 2006; Stamler 2004), which is a very powerful oxidant that causes oxidative stress and cytotoxicity (Arteel et al. 1999).

It has been reported that danshensu, a major component of salvia miltiorrhiza, was a good candidate for protecting the cellular component from oxidative stress through directly scavenging ROS, NO and peroxynitrite (ONOO-) (Zhao et al. 2008; Kuang et al. 1996; Zhao et al. 1996). Therefore, if the nanoparticles were encapped with danshensu, the oxidative stress and cytotoxicity induced by AuNPs would probably be alleviated. Further considering the structural similarity between danshensu and some other polyphenol antioxidants, such as epigallocatechin gallate (EGCG), danshensu was supposed to be used as reducing as well as stabilizsing agents for the synthesis of AuNPs (Liu et al. 2003; Naeini et al. 2010). After the reducing reaction, the remaining danshensu may still maintain its antioxidative activity, and consequently inhibit the oxidative stress caused by AuNPs.

Although AuNPs synthesizsed by the green chemistry approach using antioxidant phytochemicals haves been previously reported, little attention has been paid to the antioxidant activity and protective effects of the plants extracts against the oxidative stress and cytotoxicity caused by AuNPs (Smitha et al. 2009; Nune et al. 2009; Shukla et al. 2008). All of them focused on performing the reduction capabilities of antioxidant phytochemicals to chloroauric acid.

In this study, to further improve the stability of AuNPs against aggregation, besides danshensu, gum arabic was chosen as an auxiliary agent for stabilizsing the nanoparticles. After the preparation, the stability of AuNPs was evaluated by monitoring the plasmon wavelength and bandwidth in various solutions. Meanwhile, the cellular toxicity and

oxidative stress induced by AuNPs were examined by using 3-(4, 5-dimethylthiazol-2-~~u~~yl)-2, 5-diphenyltetrazolium bromide (MTT) assay and thiobarbituric acid reactive substance assay (TBARS) *in vitro*. The free radical-~~-~~scavenging ability and the inhibition effect of GA-~~-~~DS-~~-~~AuNPs on the LPS-induced intracellular NO production were determined by using ESR (electron spin resonance)-spin trapping, potentiometric nitric oxide (NO) sensor and confocal laser scanning microscope, respectively.

Methods

Synthesis of gum arabic-~~-~~danshensu coated gold nanoparticle

To a 50 mL round-bottom flask was added 4 mg of gum arabic, 7 mg danshensu~~,~~ and 20 mL of ultra-pure water~~,~~; the mixture was stirred continuously at room temperature for 5 min. Then to the stirring mixture was added 900 µL of 50 mM chloroauric acid ($HAuCl_4$) solution (in DI water). The colour of the mixture turned purple red from pale yellow within 15 minutes of the addition, indicating the formation of gold nanoparticles. The reaction mixture was stirred for an additional 30 minutes (Figure 1). The free gum arabic and danshensu were separated using dialysis method. The gold nanoparticles were characteri~~z~~sed by UV-~~-~~Vis absorption spectroscopy and transmission electron microscopy.

Synthesis of citrate-coated gold nanoparticles

Gold nanoparticles were prepared *via* the common technique of citrate reduction, which has been described in reference (Katherine et al. 1995). Briefly[L3], 50 mL of aqueous $HAuCl_4$

solution (0.01 wt%) in a 100 mL round-bottom flask equipped with a condenser was heated to boil with vigorous stirring, and then sodium citrate solution was added. After the solution turned brilliant red, the solution was kept boiling for another 10 min, then the heating mantle was removed, and stirring continued for another 30 min.

In vitro stability studies of gold nanoparticles

In vitro stabilities of the AuNPs were tested in the presence of NaCl, histidine, BSA and different pH phosphate buffer solutions. Typically, 1 mL of gold nanoparticles solution was added to glass round-bottom flask containing 0.5 mL of 10% NaCl, 0.2 M histidine, 0.5% BSA solutions and in pH 5.5, 7.0, 8.5 phosphate buffer solutions respectively and incubated for 60 min. The stability of gold nanoparticles was measured by recording UV–Vis absorbance after one 1 day and 15 days. The plasmon resonance band at 530 nm confirmed the retention of gold nanoparticles in the above experiments.

Cell culture and cytotoxicity assays

RAW 264.7 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM). Media contained 10% foetal calf serum, L-glutamine (2.9 mg mL^{-1}), streptomycin (1 mg mL^{-1}), and penicillin (1000 units mL^{-1}). All cells were cultured at 37°C in water-saturated air supplemented with 5% CO_2. For the cytotoxicity evaluation of GA–DS–AuNPs, an MTT assay was performed as described by the manufacturer (ATCC, USA). Briefly, 2×10^4 cells at logarithmic phase were seeded in each well of a 96-well polystyrene-coated plate and were incubated for 24 h in a CO_2 incubator at 5% CO_2 and 37°C.

Series of dilutions with 40, 80, 120, 160 μM of GA–DS–AuNPs were prepared in the medium. After 24 h of incubation, 10 μL per well of MTT (5 mg mL^{-1} PBS) was added for 24 h. The water-insoluble formazan was dissolved in a solvent mixture (100 μL) consisting of isopropanol (80 μL) with hydrochloric acid (0.04 μM) and 3% sodium dodecyl sulfphate (20 μL). Absorption of the samples was measured with a spectrophotometer at 584 nm. The amount of formazan produced was directly proportional to the number of living cells in the well. All experiments were done in triplicate.

Oxidative stress assay

Malondialdehyde (MDA) measurement was used to indicate the oxidative damage caused by nanoparticles. RAW 264.7 cells per well in 2 mL culture medium[AuQ4] and allowed to attach for 12 h before exposure. Cells were treated in triplicate with the particle suspensions at concentrations of 50, 100, 150 μM for 24 h. Then, the cells were rinsed with ice-cold PBS, trypsinizsed and immediately disrupted by a repeated frozen-thaw process (three times). The cell lysates were centrifuged and frozen at –20 °C for subsequent determination. MDA were measured using the reagent kits purchased from Jiancheng Bioengineering Co. Ltd, Nanjing, China, according to the manufacturer's instructions.

Intracellular NO measurement

RAW 264.7 cells were plated in six-well plates and grown for 72 h. Cell culture medium was removed and fresh medium without phenol red containing GA–DS–AuNPs (100 μM) or danshensu (100 μM) were incubated for 24 h, respectively. After the medium containing

antioxidant was washed off, 4-amino-5-methylamino-2', 7'-difluorescein diacetate (DAF-FM-DA) and lipopolysaccharide (LPS) were added to the cells. Then the plates were incubated at 37 °C for 1 hour. Hereafter, the cells were washed three times using media to remove excess probe. The cover slips and image were amounted using fluorescence microscopy at an excitation/emission maxima of 495/515 nm.

Characterisation

The UV–Vis spectra were recorded on a Hitachi U-3310 spectrophotometer. ESR measurements were made on a Bruker ESP 300 spectrometer. Transmission electron microscope (TEM) observation was carried out on a JEOL-1100. Nitric oxide was detected using Apollo 4000 instrument (WPI Europe). The fluorescence image was obtained by an Olympus FV1000-IX81.

Statistical analysis

Statistical analyses of the values for all experiments were expressed as ± mean standard deviation of three or more independent experiments and *p*-values were calculated using an unpaired Student's t-test ($p < 0.05$ and $p < 0.01$ were defined as statistically significant and statistically very significant, respectively). All statistical analyses were conducted using Origin 8.0 (OriginLab Co, USA).

Results

Preparation and Characterisation of the AuNPs

The gold nanoparticles conjugated with gum arabic and danshensu (GA–DS–AuNPs) were synthesi~~z~~sed according to a similar procedure described in literatures (Nune et al. 2009; Yang et al. 2010; Huang et al. 2010). The UV absorption spectrum of GA–DS–AuNPs showed that the surface plasmon resonance band derived from the GA–DS–AuNPs was at around 530 nm (Figure 2A). The sizes of the GA–DS–AuNPs were found to be in the 60–100 nm range as measured from TEM images (Figure 2C). To check whether danshensu itself could be used to yield highly stable nanoparticles in solution, the gold nanoparticles coated only with danshensu (i.e. DS–AuNPs) were prepared by reducing $HAuCl_4$ in the presence of danshensu. As expected, the DS–AuNP~~s~~ solution was not stable enough for a few hours' storage, which implies that the gum arabic acts synergistically with danshensu to provide a robust coating around the AuNPs and prevent the aggregation. The TEM image, shown in Figure 2B, further demonstrates that the gum arabic-free solution can create various types of 3D shapes, ~~*i.e.*~~ that is triangle-plates, balls or rods.

In vitro stability studies of GA–DS–AuNPs

For the purpose of *in vivo* molecular imaging applications, the nanoparticles should maintain stability over a reasonable time period. Thus, as shown in Figure 3, the stability of GA–DS–AuNPs has been evaluated by monitoring the plasmon wavelength (λ_{max}) and plasmon bandwidth ($\Delta\lambda$) in NaCl (10%), 0.2 M histidine or 0.5% bovine serum albumin (BSA). Meanwhile, the stability of GA–DS–AuNPs was examined at pH 5.5, 7.0 and 8.5 phosphate buffer solutions. The plasmon wavelengths in all above formulations show minimal shifts of approximately 5 nm, which indicates that the GA–DS–AuNPs can keep

intact over half a month, and demonstrates excellent *in vitro* stability in biological fluids at physiological pH.

On the other hand, most biomedical applications require that the dilution of gold nanoparticles solutions do not alter its characteristic chemical and photophysical properties. In order to ascertain the effect of dilution on the stability of GA–DS–AuNPs, the plasmon resonance wavelength was monitored after every successive addition of 0.2 mL of ultra-pure water to 1 mL of GA–DS–AuNPs solutions. The absorption intensity at λ_{max} was found to be linearly dependent on the concentration of GA–DS–AuNPs, in accordance with the Beer–Lambert law. It is vital to realize that λ_{max} and $\Delta\lambda$ of the GA–DS–AuNPs did not change with dilutions over a range of 10^{-5}–10^{-6} M, which were typical concentrations encountered when working at a cellular level.

Evaluation of cytotoxicity

In order to reveal the improvement in biocompatibility of GA–DS–AuNPs, *in vitro* cytotoxicity of the nanoparticles was examined by using MTT assay. As shown in Figure 4, cell viability of RAW 264.7 macrophages was determined in terms of the effects of both nanoparticles GA–DS–AuNPs and CT–AuNPs on cell proliferation. Untreated cells as well as cells treated with 60, 120, 180, and 240 μM concentrations of GA–DS–AuNPs for 24 h were subjected to the MTT assay for cell viability determination. In MTT, only cells that are viable after 24–h exposure to the sample are capable of metabolizing a dye (3-(4, 5-dimethylthiazol-2-yl)–2, 5-diphenyltetrazolium bromide) efficiently and produce purple

coloured crystals ~~which~~ that are dissolved in a detergent and analyzed spectrophotometrically. After 24 h of post-treatment, as expected, GA-DS-AuNPs did not show any appreciable cytotoxicity, even at a concentration of 240 μM, which is probably a much higher concentration than that encountered in *in vivo* studies (Khlebstov et al. 2011). On the contrary, as shown in Figure 4, it appears that more cells grew in the time frame after adding GA-DS-AuNPs compared ~~to~~with the control, which might be attributed to the cell proliferation when treated with antioxidants (Itoh et al. 2008; Acosta et al. 2010). In comparison ~~to~~ with MTT assay for GA-DS-AuNPs, when RAW 264.7 cell was pre-incubated with CT-AuNPs, cell viability gradually decreased and a significant negative correlation was found between concentration of CT-AuNPs (μM) and cell viability (%), indicating that CT-AuNPs show dose-dependent cytotoxicity to macrophages and the antioxidant danshensu can alleviate it.

Evaluation of oxidative stress in cell

As an indicator of oxidative stress, ~~malondialdehyde (~~MDA~~)~~, plays a significant role in the pathophysiology of several major cardiovascular and cerebral diseases, such as atherosclerosis, coronary heart disease and atherothrombotic cerebral infarction (Duryee et al. 2010; Cavalca et al. 2001; Alexandrova et al. 2005). Herein, to further examine ~~if~~ whether the danshensu can obviously protect the macrophages from oxidative stress and the associated damage to cellular components, TBARS assay was used to measure cellular MDA concentrations in the presence of gold nanoparticles. As shown in Figure 5, CT-AuNPs evidently elevated the intracellular MDA concentrations in a dose-dependent manner, but the

MDA concentration did not increase significantly when incubated with 0, 50, 100, and 150 μM GA–DS–AuNPs, respectively. In comparison to with CT–AuNPs, GA–DS–AuNPs (i.e. AuNPs is encapped with danshensu) can efficiently prevent oxidative stress that initiated by gold nanoparticles itthemselvesf.

In vitro ROS/RNS scavenging activities of GA–DS–AuNPs

As mentioned above, ONOO-, recognizsed as a key mediator of oxidative stress, wais directly responsible for the tissue damage and dysfunction in various diseases and usually is endogenously formed by a rapid reaction between nitric oxide (NO) and superoxide anion (Midori & Yenari 2004; Liu et al. 2000). Further considering that DS–GA–AuNPs may inhibit oxidative stress by efficiently down-regulating NO, superoxide anion as well as other ROS, *in vitro* ROS/RNS–scavenging activities of GA–DS–AuNPs must be examined. The first experiment was to test whether DS–GA–AuNPs can reduce the releasing of nitric oxide in blood serum, referring to a procedure described in literature (Jia et al. 2009). In this experiment, S-nitrosoglutathione (GSNO) was chosen as a model compound instead of the total endogenous S-nitrosothiols (RSNO) in blood serum. As shown in Figure 6, upon the addition of various concentrations of GSNO into a solution of GA–DS–AuNPs, the NO signal rose much slower than that from CT–AuNPs, indicating that the released NO from the gum arabic/danshensu-capped AuNPs was apparently less than that from the danshensu-free nanoparticles. The decrease in NO signal value from DS–GA–AuNPs is caused by the scavenging effect of danshensu on NO (Kuang et al. 1996).

The hydroxyl radical-scavenging capacity of GA–DS–AuNPs has been further determined by ESR spin–trapping method. The hydroxyl radical was generated by UV/H_2O_2 system (Olive et al. 2000) and then was simultaneously trapped by 5-tert-butoxycarbonyl-5-methyl-1-~~P~~pyrroline *N*-oxide (BMPO). As shown in Figure 7, the amount of BMPO-OH adducts was decreased with an increase in the concentrations of GA–DS–AuNPs (IC_{50} ~ 750 μM). Similarly, the superoxide anion radical-scavenging capacity of GA–DS–AuNPs has also been examined by ESR–spin–trapping technique, in which IC_{50} value was determined as 192 μM (data not shown). The superoxide anion radical was generated using light-PSII (plant photosystem II) ~~system~~ (Song et al. 2006).

Intracellular inhibition of NO release

Although the gum arabic/danshensu-capped AuNPs effectively inhibited the release of NO caused by the catalysis of AuNPs to GSNO, however, we do n’ot even know ~~if~~ whether the nanoparticles can intracellularly inhibit the release of NO~~,~~ and meanwhile prevent the damage caused by oxidative stress. Thus, it was necessary to perform a fluorescent imaging to directly visuali~~z~~se NO production. As shown in Figure 8, the intracellular nitric oxide in LPS-stimulated RAW 264.7 cells was measured by monitoring changes in the fluorescence of 5 μM 4-amino-5-methylamino-2’, 7’–difluorescein diacetate (DAF-FM-DA). The imaging results clearly indicated that the intracellular nitric oxide release was inhibited upon the addition of the GA–DS–AuNPs or danshensu to the RAW 264.7 cells and the nanoparticles were much more effective than danshensu itself did. The reason why GA–DS–AuNPs exhibit stronger antioxidant activity than danshensu does is that the gum arabic

danshensu-capped AuNPs may more efficiently penetrate the cell wall and deliver the antioxidants into cells and therefore exert better protective effect. It could be therefore inferred that ~~the~~ GA-DS-AuNPs might provide an ideal platform for the treatment of the oxidative stress-related diseases due to its high antioxidant~~s~~ activity potential.

Discussions

Previous study demonstrated that AuNPs could highly upregulate the expression of protein NDUFS1, ~~that~~ which is the core and largest subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase in complex I (Li et al. 2011). Complex I not only functions in the transfer of electrons from NADH to the respiratory chain, but also is the major source of superoxide anion and ROS in human fibroblasts (Iuso et al. 2006). As a result, the upregulation of NDUFS1 is probably responsible for the production of ROS in AuNP~~s~~-treated cells.

On the other hand, it is generally accepted that oxidative stress represents an imbalance between the production and manifestation of reactive oxygen species and, therefore, is often associated with increased production of oxidi~~z~~sing species or a significant decrease in the capability of antioxidant defen~~c~~ses (Halliwell et al. 1999). ~~Malonyldialdehyde (~~MDA~~)~~, a biomarker to measure the level of oxidative stress, is a three-carbon dialdehyde ~~which~~ that is widely produced in mammalian organisms as an end product of polyunsaturated lipid peroxidation. It is lipid peroxidation in which oxidi~~z~~sing free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage *via* a free radical chain reaction. In the

present study, the MTT assay results demonstrate a dose-dependent cytotoxicity in RAW 264.7 cell lines after exposure to the danshensu-free nanoparticles, ~~i.e.~~that is CT–AuNPs. Meanwhile, TBARS values increased with increasing the concentration of CT–AuNPs, indicating that the danshensu-free nanoparticles initiate oxidative stress and ROS production. However, in contrast to CT–AuNPs, the danshensu-capped AuNPs, ~~i.e.~~that is GA–DS–AuNPs, pre-treated RAW 264.7 macrophage cells show lower cytotoxicity and lower MDA levels. Based on MTT and TBARS observations together with earlier concept on oxidative stress-induced cell death (Lin et al. 2006), we further suppose that protective effect of danshensu on cell viability of RAW 264.7 macrophages is attenuated *via* its antioxidative activity.

The stability of AuNPs is another important factor that should be seriously considered and evaluated, before making their application in practice. In our case, the *in vitro* stability experiments show that GA–DS–AuNPs can keep stable over two weeks at room temperature not only in the presence of NaCl, histidine~~,~~ or bovine serum albumin, but also in phosphate buffer solutions with different pH values (pH 5.5, 7.0, 8.5). It was further found that the absorption intensity was linearly dependent on the concentration of GA–DS–AuNPs in accordance with the Beer–Lambert law, suggesting that the successive dilution of GA–DS–AuNPs would not alter its characteristic chemical and photophysical properties. All the above results suggest that GA–DS–AuNPs is stable enough for the biomedical and diagnostic applications.

Conclusions

A simple and versatile green process for the preparation of antioxidant-functionalized gold nanoparticles from the danshensu and gum arabic has been described. The prepared gold nanoparticles not only exhibited remarkable physical and chemical stability, but also did not demonstrate any appreciable cytotoxicity. Further studies revealed that high cell viability from GA–DS–AuNPs (over 100%) may be derived from its high ROS/RNS-scavenging capacity in both intracellular and chemical level. The antioxidant behaviour also implies that the gum arabic danshensu-capped AuNPs probably lowers the potential oxidative stress caused by gold nanoparticles itself. Thus, the "green" gold nanoparticles provide a good opportunity for their applications in nanobiology both for versatile diagnostic tool and for targeted drug delivery.

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Declaration of interest

The authors report no conflicts of interest. The[L5] authors alone are responsible for the content and writing of the paper. None of the authors has a financial conflict of interest related to this research.

Figure 1. Preparations of gold nanoparticles with danshensu and gum Arabic.

Figure 2. The UV–Vis (A) and TEM spectra of DS–AuNPs (B) and GA–DS–AuNPs (C).

Figure 3. UV–Vis spectra showing the *in vitro* stability of GA–DS–AuNPs in aqueous solutions after one day (A) and 15 days (B).

Figure 4. Cell viability was measured by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay after the treatment of RAW 264.7 cells with GA–DS–AuNPs and CT–AuNPs at indicated concentrations.
Figure 5. Intracellular malondialdehyde (MDA) concentrations in the RAW 264.7 cells exposed to nanoparticles for 24 h. Cells were treated with 50, 100, 150 μM of CT–AuNPs (70 nm) and GA–DS–AuNPs for 24 h. Results are expressed by mean ± SEM, $n = 3$. $^{\#}p < 0.05$ versus control.
Figure 6. The amount of NO release due to different concentration of GSNO added to the different gold nanoparticles solutions (CT–AuNPs, GA–DS–AuNPs).
Figure 7. Hydroxyl radical-inhibiting activities of different concentration of GA–DS–AuNPs. Insert: ESR spectrum was obtained from the trapped BMPO-OH adducts by illuminating H_2O_2 system. ESR parameters setting: microwave frequency, 9.5 GHz; modulation amplitude, 0.05–0.15 mT; modulation frequency, 100 kHz; microwave power, 12.9 mW; conversion time, 82 ms; time constant, 164 ms; and receiver gain, 10^4–10^6. UV photolysis was performed using a 200W high-pressure mercury lamp.
Figure 8. Intracellular production of nitric oxide in RAW 264.7 cells. Cells were incubated with danshensu (100 μM) and GA–DS–AuNPs (100 μM) for 24 h. After cells loaded 4-amino-5-methylamino-2', 7'-difluorescein diacetate (DAF-FM DA) for 1 h, the LPS-induced NO burst was determined using a microplate reader. Results are expressed by mean ± SEM, $n = 3$. # $p < 0.05$ versus control.

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