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Synthesis of Gold Nanoparticles by Electro-reduction Method and Their Application as an Electro-hyperthermia System

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We report the successful preparation of gold nanoparticles (Au NPs) using a novel electroreduction process, which is simple, fast, and environmentally friendly (toxic chemicals such as strong reducing agents are not required). Our process allows for the mass production of Au NPs and adequate particle size control. The Au NPs prepared show high biocompatibility and are non-toxic to healthy human cells. By applying radio-frequency (RF) ablation, we monitored the electro-hyperthermia effect of the Au NPs at different RFs. The Au NPs exhibit a fast increase in temperature to 55 °C within 5 min during the application of an RF of 13 MHz. This temperature rise is sufficient to promote apoptosis through thermal stress. Our work suggests that the selective Au NP-mediated electro-hyperthermia therapy for tumor cells under an RF of 13 MHz has great potential as a clinical treatment for specific tumor ablation.

Key Words : Gold nanoparticles, Radiofrequency ablation, Electro-hyperthermia, Heat shock, Biocompatible nanomaterials

Introduction

Radiofrequency (RF) ablation is currently the most popular and widely used thermal therapy for the treatment of various solid tumors.[1,2] In this treatment, a needle-type electrode is inserted directly into the targeted tumor to deliver an RF (375–500 kHz) alternating current. This process creates a localized area of heating through electric vibration of metal ions in the tumor cells. To induce cellular death *via* coagulation necrosis, the ideal temperature to be achieved during RF ablation ranges from 50 to 60 °C.[3]

However, RF ablation is a relatively non-specific treatment. Although the RF electrode is positioned into the target tumor, the range of heating cannot be controlled, and thermal damage occurs in both tumor cells and healthy cells surrounding the electrode. Therefore, due to insufficient ablation, tumor recurrence often occurs, whereas excessive ablation may result in collateral damage to normal tissues and the adjacent organs.[4] Recently, several research groups have endeavored to overcome these problems by using a combination of RF ablation and nanoparticles (NPs). Ideally the NPs should be responsive to specific RFs, which do not induce electric vibration of metal ions present in healthy cells, and naturally possess biocompatibility. Cardinal, J. *et al*. reported that Au NP colloids generate heat in excess of 50 °C when subjected to RF radiation, which suggests their potential use in RF ablation to induce selective tumor cell death.[5] However, toxic chemicals such as strong reducing agents are often used in the synthesis of Au NPs and are often difficult to remove; this is a potential drawback in the use of Au NPs.[6]

We now report the preparation of Au NPs using an electro-reduction method without toxic chemicals (such as strong reducing agents). Furthermore, this environment-friendly method allows for control of the Au NP size and potential one-pot scale-up by increasing Au NP concentrations up to ~10 nM. Existing methods of Au NP preparation use seed nanoparticles, with a conducted seed-growth tactic reaction after Au ion addition, to increase the Au NP size under dilute conditions (few nM).[7]

We tested our Au NPs in a mediated electric-hyperthermia system using high RF (13 MHz) in a hepatocellular carcinoma cell line (HepG2). Specific response of the Au NPs resulted in an increase in temperature sufficient to induce thermal cell death *in vitro*.

Experimental

Materials. $HAuCl_4 \cdot 3H_2O$, sodium citrate tribasic dehydrate, and other chemicals were purchased from Sigma-Aldrich and used without purification. Distilled water (< 18 MΩ) was obtained by using an ELGA purification system. The *in vitro* toxicology kit for MTT assay was obtained from Sigma-Aldrich. An RF system {RF generator (HM8131, HAMEG instruments, Germany) and an RF power amplifier (350L, ACQUITEK, France)} were used for Au NP preparation and electro-hyperthermia experiments.

Characterization. The Au NPs prepared were analyzed for shape and size distribution by transmission electron microscopy (TEM, JEOL, JEM-2100) and dynamic light

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scattering studies (DLS, Malvern, Zetasizer Nano ZSP). The absorption spectra of the Au NPs were obtained by UV-Vis spectroscopy (Scinco, S-3100). The Au NPs prepared were tested for cell toxicity and biocompatibility using a conventional MTT assay kit.

Synthesis of Au NPs using Electro-Reduction Method. A 0.23 mM aqueous solution of Au^{3+} (50 mL) was poured into a 100 mL beaker and magnetically stirred. A 38.8 mM solution of sodium citrate tribasic dihydrate (1 mL) was added, and the resulting solution stirred for 5 min at 4 °C in an ice–water bath. An AC electric field (50 W, 13 MHz) was applied to the reaction mixture for 5 min (See Figure 1 in Supporting Information). The reaction mixture gradually turned purple in color, indicating the formation of Au NPs. The Au NP size was controlled by adjusting the molar ratio of Au^{3+} and citrate to 3, 10, and 15.

Electro-Hyperthermia Effect of Au NPs. A 10 nM solution of the pre-prepared 10-nm Au NPs (50 mL) was poured into a 100 mL beaker. Two pad-type electric probes were fitted to both sides of the beaker wall, and the solution maintained at 20 °C. RFs of 0.48, 1, 5, and 13 MHz were applied to the reaction mixture, and the reaction temperature monitored directly using a digital thermometer.

Results and Discussion

Aqueous-dispersible Au NPs were successfully prepared without the need of toxic reducing agents such as $NaBH_4$ by using an electro-reduction method with an AC field (50 W, 13 MHz) [See Figure 1(a) in Supporting Information]. The Au^{3+} ions were agglomerated to nanosized particles after reducing to Au^0. To maintain the particle size and dispersion in the hydrophilic solution, sodium citrate was added to the solution to act as a surface stabilizer at a molar ratio 15 times that of the concentration of Au^{3+}.

The prepared Au NPs showed a regular size distribution of ~10 nm with a highly crystalline structure (Figure 1). Moreover, the Au NP size could be increased to ~100 nm with a relatively monodisperse distribution by decreasing the citrate concentration, i.e., 1:3 molar ratio of Au^{3+} to citrate. The Au NPs prepared using this method was analyzed for shape and size distribution using TEM and DLS studies. The larger Au NPs (~100 nm) exhibited a red shift with a maximum absorption peak at 515–550 nm in the UV-Vis spectrum. The increasing Au NP size is a result of the decrease in the concentration of the surface stabilizer, and therefore, the Au NP size is dependent on the concentration of citrate. This result is different to those observed with existing methods of Au NP preparation *i.e.*, the seed growing method. In general, with Au colloidal solutions, low concentrations of Au particles (0.1–2 nM) are produced by normal chemical reduction method in the presence of a strong reducing agent (such as $NaBH_4$). Our electro-reduction method affords a higher concentration of Au NPs (~10 nM) when high concentrations of Au^{3+} are added to the reaction solution. Mass production of Au NPs is possible, as characterized by UV-Vis absorption spectra (See Figure 1(b) in Supporting Information).

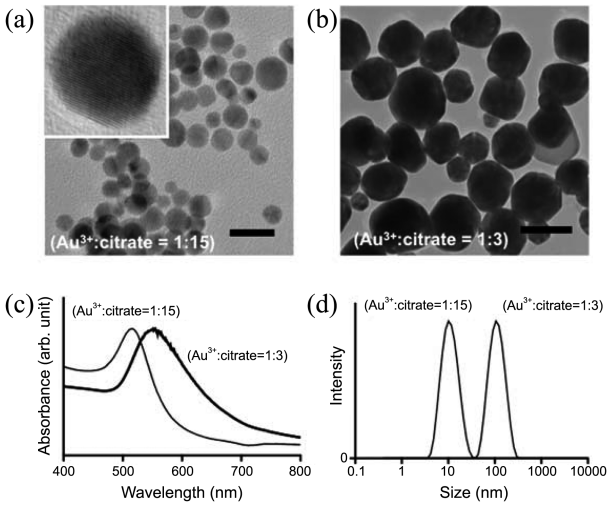

Figure 1. (a) and (b) TEM images for Au NPs prepared with an average size of 10 nm and 100 nm (in A and B, the scale bar is 20 and 100 nm, respectively). The inset in A indicates the high crystallinity of Au NPs, similar to a zinc blend structure. (c) UV-Vis spectra of Au NPs. (d) Size distribution of Au NPs by DLS study.

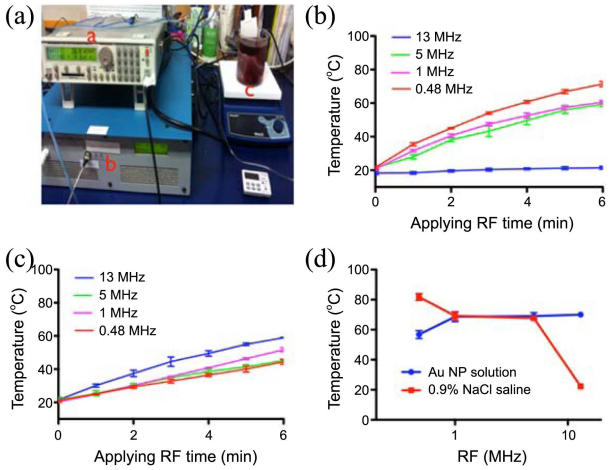

Figure 2. (a) The electro-hyperthermia system (where a = RF generator, b = RF power amplifier, and c = pad-type electrodes). (b) and (c) Heating temperatures of a 0.9% saline solute ion (b) ion and Au NP solution (c) at different RFs as a function of time. (d) The maximum heating temperature observed with the applied RFs.

An *in vitro* application of our electro-hyperthermia system using 10-nm Au NPs prepared in this study involved monitoring the heating effect at differing RFs (50 W; Figure 2). A 0.9% NaCl saline solution was used as a control, which simulates the normal cellular environment of the human body (Figure 2(b)), The highest temperature was observed under an RF of 0.48 MHz, the most commonly utilized frequency in conventional RF ablation. The heat generation at a relatively low RF (0.48, 1, and 5 MHz) was attributed to electro-vibration of metal ions in the saline solution. Interestingly, the saline solution was not altered temperature increasing under the applying of 13 MHz RF. A 10 nM dispersion of Au NPs in triply distilled water was used as

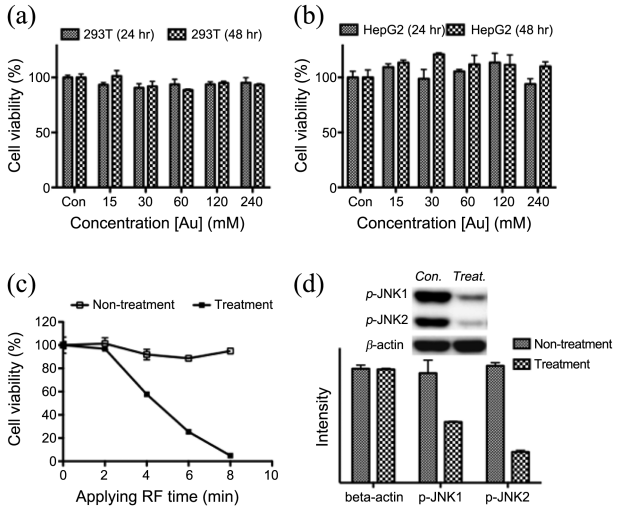

Figure 3. (a) and (b) Cell cytotoxicity of Au NPs, as determined by the cell viability of 293T (fibroblast cell line) and HepG2 (liver cancer cell line) using a conventional MTT assay kit, as a function of incubation time and concentration. (c) Cell viability of HepG2 after treating Au NPs with RF and without RF, as a function of time. (d) Quantitative analysis of *p*-JNK 1 and 2 proteins to evaluate cell death with and without the application of RF. Note: b-actin was used as a loading control and as a reference protein.

positive control. The temperature increased gradually with time under all the RFs applied. The temperature increase was in proportion with the RF applied, with the maximum temperature obtained when using an RF of 13 MHz (Figure 2(c)). Therefore, the application of a low RF generates heat in both the saline and Au NP solutions. However, at a higher RF (13 MHz), electro-hyperthermia by electro-vibration of the Au NPs is observed, but not for the saline solution (Figure 2(d)). These results suggest the possibility for the clinical application of selective Au NP-mediated electro-hyperthermia to tumor cells at an RF of 13 MHz. The increase in temperature to 55 °C within 5 min is sufficient to cause destabilizing thermal stress on the membrane of the tumor cells, leading to necrosis and/or apoptosis.[8,9]

To determine the biocompatibility of the Au NPs prepared in our work, 293T normal cells and HepG2 liver cancer cells were treated with the Au NPs at different concentrations and incubation times (Figure 3). The as-prepared Au NPs (without purification) did not cause any acute toxicity and showed a high cell viability of > 95%, by conventional cell viability assay procedures. In the presence of the Au NPs, rapid death of the HepG2 liver cancer cells *in vitro* was observed after the application of 13 MHz RF for 8 min. The non-treated cells used as the negative control showed no change in cell viability. To elucidate the cellular pathway, the expression of apoptosis marker phosphorylate c-Jun N-terminal kinases (*p*-JNK), which responds to various stress stimuli (*e.g.*, heat shock), was determined by the Western blot technique.[10] The results revealed a remarkable downregulation of *p*-JNK in the Au NP-treated HepG2 cells in comparison with non-treated cells used in the negative control. This suggests that electro-hyperthermia observed when using RF in the presence of the Au NPs results in degradation of the *p*-JNK protein and induces apoptosis.

Conclusion

We developed a simple and fast method to Au NPs solution by electroreduction in the presence of a citrate acting as a surface stabilizer. The process is environmentally friendly, as the use of toxic chemicals (such as strong reducing agents) is not required. The size of the Au NPs formed (from 10 to 100 nm) can be controlled by adjusting the molar ratio of Au^{3+} and citrate. In addition, our process can be carried out at higher concentrations (mM) without any detrimental effect on the Au NP size. The Au NPs prepared using this method was characterized for particle morphology and size distribution by conventional analysis using TEM, DLS, and UV-Vis spectrometry. An *in vitro* application of our electro-hyperthermia system involved monitoring the heating effect of the Au NP solutions at various RFs with time. The Au NP solution reached a maximum temperature of 55 °C within 5 min at the optimized RF of 13 MHz. In addition, the maximum temperature was sufficient to promote apoptosis through thermal stress. Significantly, the application of a high RF field (13 MHz) led to selective Au NP-mediated electro-hyperthermia, which has great potential as a clinical treatment for specific tumor ablation.

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