Capping and \textit{in vivo} toxicity studies of gold nanoparticles

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Received 28 June 2011
Accepted for publication 23 November 2011
Published 9 February 2012
Online at stacks.iop.org/ANSN/3/015002

Abstract
Water-dispersed colloidal gold nanoparticles (AuNPs) with high concentration were synthesized from metal precursor HAuCl\textsubscript{4}. The bovine serum albumin (BSA) and heterobiofunctionalized thiol polyethylene glycol acid (HS–PEG–COOH) were used as biofunctionalized layers for the synthesized AuNPs. The BSA and HS–PEG–COOH bound to the AuNPs were characterized qualitatively and quantitatively by transmission electron microscope and UV-VS spectrophotometer. The fabricated BSA and HS–PEG–COOH-capped AuNPs were introduced in mouse to study its toxicity and its availability in the liver.

Keywords: gold nanoparticles, bioconjugation, biocompatibility, mouse, toxicity

Classification numbers: 2.04, 4.02

1. Introduction

Gold nanoparticles (NPs) have attracted great scientific and technological interest due to their ease of synthesis, chemical stability and unique optical properties. Gold NPs synthesized in water subsequently linked to biomolecules have many applications in the life sciences, such as drug delivery, gene transfer, bioprobes in cell and tissue analysis and studies of biological processes at the nanoscale [1–5]. In general, gold nanoparticles are recognized as being nontoxic [6–8]. The available literature reports on both \textit{in vitro} and \textit{in vivo} studies, vary widely in their methods and conclusions [9]. Many reports indicate that gold NPs are nontoxic; however, others contradict this finding. But it is sure that the results depend on their size, shape, composition, functionalization and the way of introducing NPs into the organism [10–12].

Polyethylene glycol (PEG) is commonly coated or chemically linked to the particles due to its stealth character with immune system [13]. Moreover, modified thiol–PEG (SH-PEG) is an excellent candidate for stabilizing gold NPs in physiological condition, and enabling long lasting circulation in the blood [7, 14]. However, until now there are not very many reports on \textit{in vitro} and \textit{in vivo} uptake and toxicity of PEG-coated gold NPs. Takahashi \textit{et al} [7] found out that replacing cetyltrimethylammonium bromide (CTAB) by PEG on the surface of nanorods reduced the toxicity of nanorods (6.5 nm × 11 nm) on HeLa cells. Shukla \textit{et al} [15] reported that 3.7 nm PEG-coated gold sphere NPs entered to the HeLa cell nucleus and did not induce toxicity. However, 13 nm SH-PEG capped gold spheres induced inflammation and apoptosis in the mouse tissue. The tissue kinetics, cancer accumulation and biodistribution of PEG-coated gold NPs are size dependent; smaller nanoparticles have longer blood circulation time, greater chance of tissue infiltration and more tumor accumulation [16–18].

Serum albumin is the major protein component of blood plasma but is distributed to the interstitial fluid of body tissues. Serum albumin is capable of binding to a wide variety of drugs, and there is strong interest in this abundant protein because of its effects on drug delivery [19, 20]. Still there is...
no report on capping the gold NPs by bovine serum albumin (BSA) and heterobifunctional thiol polyethylene glycol acid (HS–PEG–COOH), and its in vivo toxicity study.

This work presents the results of bioconjugation of AuNPs and its in vivo toxicity study in mouse. In this study we prepared gold nanoparticles encapsulated with HS–PEG–COOH and BSA, then investigated their in vivo toxicity after intravenous multi-injections into mice.

2. Experimental

2.1. Synthesis

2.1.1. Materials. Tetrachlorouaric acid trihydrate 99.5% (HAuCl₄·3H₂O) and trisodium citrate dehydrate (Na₃C₆H₅O₇·2H₂O), dithiothreitol (DTT) were purchased from Merck. BSA was purchased from Biochem. HS–PEG–COOH with molar weight of 3400 Dalton was purchased from Creative PEGworks. Double distilled water was used throughout the course of this investigation.

2.1.2. Synthesis of AuNPs. Gold colloids were prepared by sodium citrate reduction of HAuCl₄ following our previous work [21]; 90 ml of 3 × 10⁻⁴ M aqueous solution of HAuCl₄ was boiled, at which point 3.6 ml of 6.8 × 10⁻² M sodium citrate was added drop wise with stirring. After approximately 10 min, the solution was cooled to room temperature with continued stirring. The suspension was stored in the refrigerator at 4°C until further use. The average diameter of the citrate capped gold nanoparticles (AuNPs) was determined from a high resolution scanning electron microscope (HSEM, Hitachi-S480) image to be 15.8 ± 3.8 nm.

2.1.3. Preparation of BSA-coated AuNPs. An amount of BSA was added into AuNPs solution, synthesized by the method presented above, while stirring at room temperature till optically clear. The minimum amount of protein BSA required to stabilize AuNPs was determined by employing the surface plasmon resonance absorption assay. In this assay, serial dilutions of the BSA-AuNPs (Au@BSA) at pH = 4 and pH = 6.5 (pH of primary gold solution) were prepared varying the quantities of BSA from 0 to 140 µg in 1 ml of AuNPs solution (µg ml⁻¹) followed by UV-Vis spectrophotometric analysis. The minimum amount of protein BSA necessary to cap AuNPs was deduced graphically from the concentration at which the absorbance at pH = 4 becomes nearly constant. The AuNPs and BSA-AuNPs solutions were characterized using transmission electron microscope (TEM, JEM 1011) and UV-Vis spectrophotometer (JASCO-V570-UV-VIS).

2.1.4. Preparation of PEG-coated AuNPs. HS–PEG–COOH was diluted in citrate solution while stirring at room temperature. An amount of DTT was added in the solution with molar ratio DTT/HS–PEG–COOH about 1/1. Here the DTT produces the free sulphydryl bonds. The solution DTT–HS–PEG–COOH then was added into primary AuNPs solution while stirring at room temperature for about 15 min. The minimum amount of HS–PEG–COOH required to stabilize AuNPs was determined by the method similar to the BSA case. Two serial dilutions of the HS–PEG–COOH–AuNPs (Au@PEG) at pH = 4 and pH = 6.5 were prepared, varying the quantities of HS–PEG–COOH from 0 to 150 µg in 1 ml of AuNPs solution (µg ml⁻¹), and followed by UV-VIS spectrophotometric analysis. The minimum amount of HS–PEG–COOH necessary to coat AuNPs was deduced graphically from the concentration with which the absorbance at pH = 4 becomes nearly constant. All of these solutions were measured by UV-Vis spectrophotometry. The size and shape of AuNPs were characterized by a transmission electron microscope (TEM).

2.2. Toxicological studies

2.2.1. Animals. Healthy 8–10 week old male Swiss Albino mice with a body of weight of 18 ± 1.5 g were used for the experiments. The mice had free access to food and water, and were maintained on a 14/10 h light/dark cycle. Thirty-three mice were randomly divided into eleven groups: one control group and ten experimental groups with different doses of AuNPs.

2.2.2. Experiment design. Mice received tail vein injections of approximately 200 µl of Au@BSA or Au@PEG solution at total doses of 1.16, 2.32, 3.52, 4.68 and 5.84 mg kg⁻¹ in a period of eight days. The last injection was done at the ninth day for all groups. Control group was treated with phosphate buffered saline (PBS) solution. The bodyweights of animals were recorded and their behavior was carefully observed daily during the course of the experiment. Twenty-four hours after the last injection (day 10), several mices from the group which received the highest doses of Au@PEG or Au@BSA were sacrificed, the kidney and liver organs were collected, stained en bloc with uranyl acetate and embedded in epoxy resin. Ultra-thin (50 nm) sections were cut, stained with uranyl acetate and lead citrate, and examined with a TEM (JEM 1011, JEOL).

3. Results and discussion

3.1. Synthesis

3.1.1. BSA-coated AuNPs. Figure 1 (black line) shows the UV-Vis absorption spectra of AuNPs at pH = 7.4. A strong plasmon absorption peak at 519 nm attributed to AuNP collective electron oscillations or localized surface plasma resonance (SPR) is presented and is a feature of 16 nm AuNPs. The red line in Figure 1 shows the UV-Vis absorption spectra of BSA protein stabilized AuNPs. It is clear that the plasmon absorption peak of AuNPs shifts from 519 to 526 nm after the modification by BSA. The resonance wavelength and bandwidth of AuNPs are dependent on the particle size and shape, the refractive index of the surrounding medium, and the temperature. This shift after conjugation of AuNPs with BSA is attributed to the changes of the dielectric nature surrounding the AuNPs due to BSA presence (n = 1.9). The inset in figure 1 shows the TEM image of the BSA-stabilized AuNPs. Compared to citrate-capped AuNPs, a shadow around the AuNPs is clearly observed in the BSA-stabilized nanoparticles and represents the BSA protein.
Figure 1. UV-VIS spectra of colloidal AuNPs (black line) and Au@BSA NPs (red line). Inset: TEM image of Au@BSA NPs, scale bar is 50 nm.

Figure 2. (a) Absorption spectra and (b) absorbance of Au@BSA-NPs versus BSA concentration at pH = 4.

Figure 3. (a) UV-Vis spectra of colloidal AuNPs (black line) and Au@PEG NPs (red line). (b) Absorbance of Au@PEG-NPs versus PEG concentration at pH = 4. Inset: TEM image of Au@PEG-NPs, scale bar is 20 nm.

layer on the surface of gold NPs. The thickness of shadow surrounding the gold nanoparticles is about 4 nm and is quite close in size to that of the BSA which has a molecular weight of 69 kDa with dimensions $4 \times 4 \times 14$ nm [20].

At pH = 4, the AuNPs are not stable due to charge neutralization following the aggregation of gold particles [19], their absorption spectra are large and low in intensity compared with those at pH = 7.4 (figure 2(a)). As shown, the added protein BSA forms new capping by replacing the citrate ions on gold surface. If the added amount of BSA protein is not enough to replace all the citrate capping molecules, the residue citrate ions continue to react with hydrons in solution to form citrics, leaving gold to tend to aggregate following the dependence of its absorption spectra on added BSA amounts (figure 2(a)). When the BSA molecules are enough to cap all the surface of gold to form stable protein–gold conjugate, its absorption spectra become constant. The minimum amount of protein BSA necessary to prevent aggregation of AuNPs was deduced graphically from the concentration at which the absorbance at pH = 4 becomes nearly constant. This value must be determined for every gold solution. In this case, the minimum BSA amount necessary for capping is about 100 $\mu$g ml$^{-1}$ (figure 2(b)).

3.1.2. HS–PEG–COOH-coated AuNPs. Figure 3(a) (black line) shows the UV-Vis absorption spectra of AuNPs at
Figure 4. Weight development of certain mice groups (circled indicate the days injected). Data are presented as mean ± SD.

Figure 5. Thin-section TEM images of mouse liver after 10-day intravenous injections of BSA- and PEG-coated AuNPs. The micrographs show entrapped BSA-coated AuNPs (a, b) and PEG-coated AuNPs (c, d), and their clustering and localization in intracellular organelles, such as lysosomes in liver Kupffer cells at scope magnifications of 8000 × (a, c), and 30 000 × (b, d).

pH = 6.5. The absorption peak at 519 nm is AuNP localized surface plasma resonance (SPR) of 16 nm AuNPs. The red line in figure 3(a) shows the UV-Vis absorption spectra of HS–PEG–COOH stabilized AuNPs. The plasmon absorption peak of AuNPs shifts from 519 to 522.5 nm after the modification by PEG. This shift shows the changes of the dielectric nature surrounding the AuNPs due to the association of HS–PEG–COOH molecules with gold nanoparticles to form a stable covalent bond Au–S [18]. The inset in figure 3 shows the TEM image of the PEG-stabilized AuNPs. Similarly to the BSA case, the minimum amount of HS–PEG–COOH necessary to coat all the surface of AuNPs was deduced graphically from the concentration at which the absorbance at pH = 4 becomes nearly constant and it is about 50 µg ml⁻¹ for the 16 nm gold solution of OD ~ 3 (figure 3(b)).

3.2. AuNPs’ toxicity

We tested whether AuNPs treatment induces any sub-acute toxicity in mice during the course of the study or not.
We observed no mortality or any gross behavioral changes in mice receiving AuNPs at the doses studied. The mice in all groups were healthy and exhibited no abnormality in physiology. Based on weight data recorded, we reported that all groups have a similar rate of weight growth. There is no statistical significance in the difference between C5A and C5B (that received the highest doses of Au@PEG and Au@BSA, respectively), or the control group (figure 4). The results indicate that there was no affect of either Au@BSA or Au@PEG on the normal growth of the animals.

Figure 5 shows that both Au@BSA and Au@PEG NPs were accumulated in the liver until the end of the study (10 days). Also, TEM images showed that AuNPs were trapped in liver Kupffer cells. However, no AuNPs were found by TEM in other cell types or kidney organs (data not shown). TEM images also showed that numerous liver lysosomes contained the AuNPs but no uptake was observed in the nuclei, mitochondria, or the Golgi complex structures. No changes in the cell morphology were observed for Au@BSA entrapped organelles (figures 5(a) and (b)). But there were little changes in cells of Au@PEG entrapped tissue; the broken (degraded) mitochondria were detected from their TEM images (figures 5(c) and (d)). The TEM images show also that both the Au@BSA and Au@PEG are stabilized in liver organs, and no aggregation appeared after injection. There are no signs of AuNPs detected on TEM images of kidney ultrastructures. Other studies must be done for clearing up the uptake and toxicity of BSA and heterobifunctional HS–PEG–COOH-capped AuNPs, in vivo as well as in vitro.

4. Conclusion

We reported here a simple method to cap AuNPs by BSA and heterobifunctional HS–PEG–COOH necessary to coat gold colloidal NPs. The gold NPs did not produce any mortality or any gross behavioral changes in mice at the doses studied, all experimented animals were healthy and had normal weight development. Neither did the accumulation of BSA-capped gold NPs in liver organs after intravenous multi-injection produce any changes in cell morphology, except for one instance of broken (degraded) mitochondria detected in the liver of mice injected by Au@PEG at 5.84 mg kg\(^{-1}\)\(\cdot\)dose. Overall, these results demonstrated that AuNPs are effectively stabilized by the coat under physiological conditions, proving their biocompatibility characteristics. Those are the first results of in vivo toxicity studies of BSA- and SH–PEG–COOH-capped nanogolds. Other studies must be done for the use of these NPs as biomarker.

Acknowledgment

This work was supported by National Project no 4/2/742/2009/HD-DTDL.

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