NanoImpact 3–4 (2016) 40–46

Contents lists available at ScienceDirect

NanoImpact

journal homepage: www.journals.elsevier.com/nanoimpact

Research paper

Protein corona influences liver accumulation and hepatotoxicity of gold nanorods

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ARTICLE INFO

Article history:
Received 22 July 2016
Received in revised form 21 September 2016
Accepted 22 September 2016
Available online 24 September 2016

Keywords:
Gold nanorods
Protein corona
In vivo
Liver accumulation
Toxicity

ABSTRACT

This work aimed to examine whether the surface coating with serum proteins could influence the *in vivo* metabolic pattern and toxicity of nanoparticles (NPs). Gold nanorods (AuNRs) were synthesized and employed as a model NPs, and were pre-incubated with mouse serum or mouse serum albumin before intravenous injection. The hepatic uptake and location of AuNRs were examined by ICP-MS and TEM; the hepatotoxicity of AuNRs was evaluated in terms of blood biochemistry, oxidant stress and histopathology. The results showed that AuNRs could be removed from blood circulation by Kupffer cells. Heat stable opsonin proteins played a dominant role in the liver-targeting of AuNRs. Serum albumin corona could confer a certain degree of stealth property to AuNRs, therefore some of them escaped the clearance by Kupffer cells and entered the hepatocytes. It might lead to a long-term retention of AuNRs in the liver. The findings may have important implications for the understanding of the *in vivo* behavior of NPs as well as the design of NPs-based drug delivery system.

1. Introduction

With the development of nanotechnology, more and more engineered nanomaterials have been used in every field of life from the automotive industry to household applications. Among them, gold nanoparticles (NPs) are regarded as one of the most promising candidates for biomedical applications such as targeted labelling, imaging, drug delivery, and photothermal therapy (Dreaden et al., 2012; Zhang, 2015).

Due to the potential biomedical benefits of gold NPs, toxicological study of gold NPs is of great interest from the clinical point of view (Khlebtsov and Dykman, 2011). While gold in its micro- or bulk form are generally thought of as inert, stable, and biocompatible, the health and environmental impact of gold NPs is a far more complex issue. It has been reported that the aspect ratio (*i.e.* the ratio of the length along the long axis to the short axis) can affect the cellular internalization of gold NPs (Qiu et al., 2010). In addition to the aspect ratio, some other factors, including size, geometry, and surface modification, can also affect the toxicity of gold NPs (Bozich et al., 2014; Coradeghini et al., 2013; Li et al., 2014). Compared to the large number of *in vitro* studies dealing with the bio-applications and cytotoxicity of gold NPs, relatively fewer investigations have addressed the biodistribution of gold NPs as well as their toxicity *in vivo*. (Khlebtsov and Dykman, 2011).

Gold nanorods (AuNRs) have unique optical and photothermal proterties, tunable size and shape, as well as the ability to conjugate different drugs/molecules to their surface, therefore have been continuously in the limelight of research (Wang et al., 2011; Zhang et al., 2014). AuNRs are thought to be more toxic than their spherical counterparts (Khlebtsov and Dykman, 2011). Further studies have shown that the toxicity of CTAB-stabilized AuNRs is due to the CTAB ligands, not the core (Boisselier and Astruc, 2009; Qiu et al., 2010; Wang et al., 2013b). Therefore, numerous methods have been developed to either remove, or overcoat, the CTAB bilayer on AuNRs (Alkilany et al., 2012). *In vitro* experiment suggested that the toxicity of CTAB-stabilized AuNRs could be minimized after the formation of serum albumin protein corona (Wang et al., 2013b).

Entrance of NPs into biological environment endows them with a dynamic protein corona that critically defines the biological identities of NPs (Wang et al., 2013a). The structure and composition of the protein corona depend on the physicochemical properties of the NPs (size, shape, surface functional groups, and surface charges), the nature of the physiological environment, and the duration of exposure (Walkey and Chan, 2012). The influences of the formation of protein corona on the fate and toxicity of NPs have been widely studied *in vitro*. For example, protein corona can regulates cellular recognition and penetration of NPs, and stimulate and/or suppress immune responses (Dobrovolskaia and McNeil, 2007); the formation of protein corona

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http://dx.doi.org/10.1016/j.impact.2016.09.005
2452-0748/

could further influence the intracellular trafficking of NPs (Ge et al., 2015). However, the effect of protein corona on the *in vivo* fate and toxicity of NPs remains largely unknown.

More recently, the relevance of the protein corona in the biological impacts of AuNRs has garnered much attention (Wang et al., 2013b). Therefore, the aim of this work is to examine whether the interactions of AuNRs with serum proteins could influence the metabolic and toxicological pattern of AuNRs. AuNRs with an aspect ratio equal to 4.2 were synthesized and employed as a model NPs to investigate how protein corona affect the *in vivo* fate and toxicity of NPs. The synthesized AuNRs were pre-incubated with mouse serum (MS) or mouse serum albumin (MSA) before intravenous injection. The hepatic uptake and hepatotoxicity of AuNRs under different treatments were studied.

2. Experimental details

2.1. Agents and kits

Sodium borohydride ($NaBH_4$), ascorbic acid (AA), tetrachloroaurate acid ($HAuCl_4$ $4H_2O$), hexadecyl trimethyl ammonium bromide (CTAB), silver nitrate ($AgNO_3$), sodium oleate (NaOL) and mouse serum albumin lyophilized powder (MSA) were obtained from Sigma and were used without any additional purification. Glutathione (GSH), glutathione peroxidase (GSH-PX), malondialdehyde (MDA), catalase (CAT) and total superoxide dismutase (T-SOD) assay kits were provided by Nan Jing Jian Cheng Bioengineering Institute.

2.2. Preparation and modification of AuNRs

AuNRs were prepared according to a typical seed-mediated growth method described previously. (Ye et al., 2013) First, the seed solution was made by mixing 5 mL of $HAuCl_4$ (0.5 mM) with 5 mL of CTAB (0.2 M) solution in a small Erlenmeyer flask. Then, 0.6 mL of fresh ice-cold $NaBH_4$ (0.01 M) was added to the Au(III)-CTAB mixture solution under vigorous stirring (1200 rpm), and the solution color was changed from yellow to brownish yellow. After stirring for another 2 min, the seed solution was aged at room temperature for 0.5 h before use. The growth solution of the AuNRs consisted of a mixture of 1.4 g (0.037 M in the final growth solution) CTAB and 0.2468 g NaOL in 50 mL of warm water (~50 °C) in a 250 mL Erlenmeyer flask. After cooling down the solution to 30 °C, 3.6 mL of 4 mM $AgNO_3$ and 50 mL of 1 mM $HAuCl_4$ were successively added and the mixture was kept undisturbed for 15 min. The solution was then stirred at 700 rpm for 90 min and became colorless. After another 15 min of gentle stirring, 0.25 mL of ascorbic acid (0.064 M) was added and the solution was vigorously stirred for 30 s. At last, 80 μL seed solution was injected into the growth solution. The resultant was stirred for 30 s and left undisturbed at 30 °C overnight for NR growth. The final products were isolated by centrifugation at 10,000*g* for 20 min followed by removal of the supernatant.

The prepared AuNRs solution was diluted to 1.5 mg/mL and divided into 4 aliquots. One of them was incubated with an equal volume of mouse serum for 24 h (MS-AuNR); another one denoted as IMS-AuNR was incubated with heat inactivated mouse serum (56 °C for 30 min, Mankovich et al., 2013) for 24 h; the third aliquot was incubated in mouse serum albumin solution for 24 h (MSA-AuNR) with a final concentration of 0.75 mg/mL AuNRs and 2 mg/mL albumin (about 1/20 of the albumin level in mouse serum); the left one remained unmodified, so designated as AS-AuNR. After the incubation, MS-AuNR, IMS-AuNRs, MSA-AuNRs and AS-AuNRs were collected by centrifugation at ~ 19,000 g for 20 min (4 °C), and then re-suspended in the normal saline; the protein remained in the supernatant was quantified *via* Bradford method as described previously (Hu et al., 2011). The capability of protein loading on AuNRs surface was evaluated by the following: $q = (W_{Total} - W_{Sup})/(W_{AuNRs})$ (W_{Total}, W_{Sup}, W_{AuNRs} represent the total amount of protein, the amount of protein in the suspension, and the amount of AuNRs, respectively).

2.3. Characterization

The size and morphology of AuNRs were observed by transmission electron microscopy (TEM) and scanning electron microscopy (SEM). Their hydrodynamic distribution and surface charge were determined using dynamic light scattering (DLS). Optical extinction spectra were recorded using a UV–Vis spectrophotometer.

2.4. Animal experiment and assays

120 male 6–8 weeks old mice (CD-1, 25–30 g) were purchased from Beijing Vitalriver Experiment Animal Corporation. The animal experiments were carried out in compliance with the ethics guideline for the care and use of animals in research (formulated by Perking University Health Science Center). After adaptation, mice were separated randomly into 20 groups, by 5 treatments (control, AS-AuNRs, MS-AuNRs, IMS-AuNRs, MSA-AuNRs) and 4 time points (1 day, 3 days, 7 days, 28 days), with 6 mice per group. Mice were injected intravenously with 200 μL of AuNRs (a single dose of 5 mg/kg) or 0.9% saline solution, respectively. At 1, 3, 7, and 28 day(s) post-injection, mice were anesthetized and sacrificed to collect the liver. The liver was immediately cut into small pieces. One of them was fixed with 10% formalin. Another one was fixed with PBS containing 2.5% glutaraldehyde and postfixed with OsO4; after dehydration, ultrathin sections of samples embedded in Epon resin were prepared for TEM measurements at 80 kV. The left tissue samples were quick-frozen in liquid nitrogen and then stored at − 80 °C.

2.5. The accumulation of AuNRs in the liver

To quantify the accumulation of AuNRs in mice liver, tissues were weighed, digested, and measured by ICP-MS. Briefly, HNO_3/H_2O_2 (v/v = 4:1) mixture of a total volume of 5 mL was added to a conical flask to predigest samples (with wet-weights of ~0.2 g) overnight, then the samples were transferred to a heating plate. The remaining solution (about 0.5–1 mL) was cooled and diluted to 5 mL with mixed acid solution containing 2% HCl and 1% HNO_3. Bismuth (20 ppb) in a mixed acid solution was used as an internal standard to compensate for signal drifting and matrix suppression. The AuNRs distribution in the liver at 3 days post-injection was studied by TEM observation.

2.6. Bio-assay for hepatotoxicity

The potential hepatotoxicity of AuNRs was evaluate by monitoring the representative serum biochemical indicators relevant to liver functions, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyltransferase (GGT), alkaline phosphatase (ALP), total bilirubin (TBil), and total bile acid (TBA). These serum parameters were measured with standard spectrophotometric methods on a Hitachi 7170A clinical automatic chemistry analyzer (Yang et al., 2012). The antioxidant defenses in the liver were studied in terms of SOD and catalase activities, and level of GSH, GSH-Px and MDA using each kit provided by Nanjing Jiancheng Bioengineering Institute, Nanjing, China (Meng et al., 2014). The histopathological tests were performed using standard laboratory procedures. Briefly, the tissues were embedded in paraffin blocks, then sectioned into 5 μm slice and mounted onto glass slides. After hematoxylineosin staining, the sections were observed and the photos were taken using optical microscope. The pathological sections were evaluated by a pathologist blinded to the investigator's measurement.

2.7. Statistics analysis

Results were expressed as mean ± standard deviation (S. D.). Between-group differences of the gold contents in the liver were analyzed by the two-way ANOVA followed by post-hoc LSD-t multiple