Glutathione-mediated drug release from Tiopronin-conjugated gold nanoparticles for acute liver injury therapy

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\textbf{A B S T R A C T}

Tiopronin-conjugated gold nanoparticles (TPN@GNPs), with glutathione (GSH)-responsive drug release property, were developed for acute liver injury therapy. The TPN@GNPs were prepared using a one-pot synthesis method and characterized by UV–vis and transmission electronic microscopy methods. The TPN@GNPs displayed typical surface plasmon resonance of nanogold with a narrow size distribution (ca. 2 nm). The in vitro drug release profiles of the conjugates indicated that TPN@GNPs were able to release TPN in a sustained fashion for 4 h at a simulated intracellular level of GSH. pH values or ionic strengths of the release media had no obvious influence on TPN release from the surface of nanoparticles. The pharmacokinetic studies in rats showed that the TPN@GNPs had longer MRT (7.71 h) than TPN (3.96 h), indicating sustained release pattern of TPN@GNPs in vivo. The sustained release of TPN at the relative high GSH concentration could ameliorate the instability of TPN and enable the drug release in the target cells. Although the IC\textsubscript{50} value of TPN@GNPs with TPN/AuCl\textsubscript{4} of 3:1 (mol/mol) showed slight increase in comparison with that of the free TPN in HepG2 cells (1.26 ± 1.07 vs. 1.73 ± 1.16 mg/mL), the TPN@GNPs displayed better effects over TPN in the treatment of acute liver injury in vivo. In a liver injury mice model induced by CCl\textsubscript{4}, the histological analysis showed both the TPN@GNPs and free TPN group could repair the liver injury. In addition, the biochemical parameters showed TPN@GNPs could reduce the aminotransferase to a lower level compared with TPN, which might be due to the sustained drug release and passive liver targeting properties of TPN@GNPs. It demonstrated that gold nanoparticle-based drug delivery system allowed smart functions and superior properties by taking advantages of the unique small size effects and surface chemical properties.

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1. Introduction

Gold nanoparticles (GNPs) have attracted tremendous attentions in the field of drug delivery due to shape- and size-controllable preparation methods (Schmid, 1992), high drug loading capacity (Templeton et al., 1999a,b; Gibson et al., 2007), and the ability to utilize the enhanced permeability and retention (EPR) effect to passively target drugs to tumor cells or inflammatory cells via the leaky vasculature (Ghosh et al., 2008). Moreover, GNPs-based drug delivery system (DDS) also possesses the following unique and attractive advantages for drug delivery applications such as the easy surface modification through the place-exchange reaction and thiol chemistry (Templeton et al., 1999a), excellent light scattering signal for image-guided drug delivery (Boisselier and Astruc, 2009), toxicity-free and inert gold core for safe cell and small animal studies (Klebtssov and Dykman, 2011; Tsoi et al., 2005; Bhattacharya and Mukherjee, 2008; Connor et al., 2005), and light-mediated hyperthermia combined with chemotherapy for the cancer treatment. Up to now, various therapeutics (e.g. small molecules, biomacromolecules, and diagnostic probes) have been loaded on the surface of gold nanoparticles via the physical adsorption, electrostatic interaction, specific recognition, as well as S–Au covalent bonds (Ghosh et al., 2008; Boisselier and Astruc, 2009; Duncan et al., 2010).

Most recently, triggered-release drug delivery systems based on gold nanoparticles have been developed, in which drug release can be triggered via a variety of stimulations such as low pH in tumor tissue (Wang et al., 2011), photo- and thermal-stimulations (Vivero-Escoto et al., 2009), and intracellular high level of glutathione (GSH) (Hong et al., 2006; Navath et al., 2008; Kim et al., 2012). Among them, due to the wide application of thiol chemistry in GNPs-based drug delivery system, GSH-sensitive drug release attracts more attentions (Hostettler et al., 1998) and offers
2.1. Behavior

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2.2. Synthesis of GNPs and TPN@GNPs

All the glassware used in our experiments were treated with aqua regia and cleaned with purified water. Citrate-protected GNPs were prepared by the method describes elsewhere (Daniel and Austron, 2004). Briefly, 150.1 mg sodium citrate and 1.845 mL (5 × 10^{-5} mol/L) HAuCl₄ aqueous solution were added into 25 mL of purified water in a baker with magnetic stirring in an ice bath. 3 mg sodium borohydride was added into 25 mL of purified water in another baker with magnetic stirring in an ice bath. After 20 min, the later mixture in the baker was poured into the former baker slowly, the color of the cocktail turned to wine red. After another 20 min, the citrate-protected GNPs were collected for use.

The one-pot synthesis method was employed to prepare TPN@GNPs (Templeton et al., 1999b) with some modification. Briefly, TPN and HAuCl₄·3H₂O with the molar ratio of 1:1, 3:1, and 5:1 were dissolved in a certain amount of methanol/acetic acid mixture solvent (6:1, v/v) to keep the concentration of TPN to be 11.2 mg/mL. Sodium borohydride was added in the mixture and stirred vigorously for 15 min at 44 °C. The obtained dark brown suspension was stirred for 30 min after its cooling to room temperature. The solvent was removed under vacuum at 35 °C, and the crude TPN@GNPs were obtained. To purify the conjugates, the crude TPN@GNPs were dissolved in water and dialyzed (MWCO = 10,000 Da) against deionized water for 24 h. The purified solution was lyophilized and kept at 4 °C for further use.

2.3. In vitro drug release studies

0.1 mL of TPN@GNPs aqueous solution (0.13 g/mL) was placed in a dialysis tube (MWCO = 10,000 Da), in which 0.9 mL of release media (PBS buffers with or without 10 mM GSH) was added. After sealed tightly at each end with cable ties, the dialysis tubes were immersed into 10 mL of release media in a beaker, stirring at 37 ± 0.5 °C. The release media used were 50 mM PBS (pH 5.5 and pH 7.4) with different the ionic strengths (ranging from zero to 1.0 M). At predetermined time points, 200 μL of samples were withdrawn and replenished with 200 μL of fresh release media. All the samples were performed in triplicates.

A reversed-phase HPLC with UV detection was selected to analyze TPN. The HPLC system was Shimadzu LC-20A series (Shimadzu Corporation, Japan), consisting of a quaternary pump, a vacuum degasser, an auto-sampler, a thermostated column compartment, and an analytical column (Phenomenex, ODS 250 mm × 4.6 mm, ID 5 μm; Hanbon Sci. & Tech Co., Ltd., China). According to the UV–vis absorption spectra of TPN, UV detection wavelength was set at 210 nm. The column temperature was maintained at 30 °C with isocratic elution at a flow rate of 1.0 mL/min and the injection volume was 20 μL. The mobile phase consisted of acetonitrile and aqueous KH₂PO₄ (10 mM, adjusted to pH 3.2 with phosphoric acid) (5:95, v/v).

2.4. Cell culture and viability tests

HepG2 cell lines were grown in the Dulbecco’s modified Eagle medium (DMEM, Thermo Fisher Scientific Inc., China) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin at 37 °C with 5% CO₂. To determine the IC₅₀ values of TPN and TPN@GNPs, the HepG2 cells were plated into 96-well
plates and incubated overnight. After 24 h, the medium was replaced with fresh DMEM containing different concentrations of TPN@GNPs or free TPN and further incubated for 24 h. Then, PBS and MTT solution were added, incubating at 37°C for 4 h. And DMSO was added to dissolve the MTT formazan and the absorbance was measured on a microplate reader (Multiskan MK3, Thermo Fisher Scientific Inc., USA) at 570 nm and corrected at 630 nm.

2.5. Pharmacokinetics study in rats

2.5.1. Study design
Male rats weighed 200–250 g were divided into two groups (n = 4) and administered with TPN solution and TPN@GNPs conjugates (18 mg/kg) via tail vein injection, respectively. 0.5 mL of blood samples were withdrawn from retro orbital sinus at 0.83, 0.17, 0.33, 0.5, 1, 2, 3, 4, 6, 8, 10, and 12 h after injection. The blood samples were collected in heparinized tubes and centrifuged at 3000 rpm for 5 min to obtain plasma samples and stored at −20°C until assay.

2.5.2. Sample preparation
For a 100 μL aliquot of each plasma sample, 20 μL of methanol, 10 μL of internal standard solution (25 μg/mL Fudosteine in methanol), 10 μL of L-Cysteine (20 μg/mL), and 10 μL 1,4-Dithiothreitol (3 mg/mL) were added and vortex mixed for 30 s. After incubated in a water bath at 30°C for 10 min, the samples were deproteinized by 200 μL of methanol, vortex mixed for 3 min and centrifuged at 15,000 rpm for 10 min. 20 μL of the supernatant of each sample was injected for LC–MS/MS analysis.

2.5.3. LC–MS/MS analysis
The chromatographic separation was performed on a RP-HPLC column (ZORBAX Eclipse Plus C18, 2.1 mm × 150 mm, 3.5 μm, Agilent Technologies, USA). The column temperature was maintained at 30°C with an isocratic elution at a flow rate of 0.3 mL/min and the injection volume was 20 μL. The mobile phase consisted of methanol and 0.1% formic acid (10/90, v/v).

The HPLC (Agilent 1290 Infinity series, USA) was connected to the mass spectrometry (MS/MS, Agilent 6460 triple quadrupole, USA) equipped with an electrospray interface (ESI) operating in a negative-ion mode. The assay was carried out using multiple-reaction monitoring (MRM) with the transition from m/z 162.0→74.0 for TPN and m/z 178.0→91.0 for the IS. The source parameters were as follows: gas temperature, 350°C; gas flow, 7 L/min; nebulizer, 30 psi; capillary voltage, 3.5 kV.

The plasma pharmacokinetic parameters were estimated by Kinetica (4.4 version, Thermo Electron Corporation) software for both TPN and TPN@GNPs injections.

2.6. Efficacy of TPN@GNPs against acute liver injured mice

2.6.1. Model construction of acute liver injury in mice
Healthy male ICR mice (body weight 18–22 g) used in the experiment were purchased from Zhejiang Experimental Animal Center (Certificate number: SCXK (Zhe) 20080033). The mice were randomly divided into five groups (ten mice per group) and administered with saline, saline, TPN solution, TPN@GNPs, and free GNPs with the same TPN dosage (26 mg/kg) via tail vein injection for seven consecutive days. After 2 h of the last administration, except the first group (as the control), the mice were treated with 0.1% CCl4 peanut oil (v/v) via intraperitoneal administration (10 mL/kg of body weight) to induce acute liver injury in mice. 16 h after the administration of CCl4, the blood samples were collected and the livers were excised, washed with saline and weighed.

2.6.2. Biochemical parameters of hepatic function
The relative liver weight percentage was calculated by liver weight dividing the body weight of a mouse. The amino transferase activities, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in the serum were measured using diagnostic kits (Nanjing Jiancheng Institute of Biotechnology). Origin soft ware (Version 8.6) was selected to address the data.

2.6.3. Histological analysis
The excised livers were weighed and was fixed with 4% paraformaldehyde. The samples were processed and sectioned, and the thin tissue sections were stained with hematoxylin and eosin (H and E) and Masson trichrome for histological observation (Olympus TH4-200, Olympus Optical Co., Ltd., Japan).

2.7. Statistics
All the results are presented as mean ± SD. The data were analyzed by one-way analysis of variance (ANOVA) with the appropriate Bonferroni correction to determine the significant differences for multiple comparisons. Significance was assumed at P < 0.05.

3. Results and discussion

3.1. Synthesis and characterization of TPN@GNPs
The TPN@GNPs was prepared by the one-step reduction method of HAuCl4 by sodium borohydride in the presence of stabilizer of TPN (Templeton et al., 1999b). The as-prepared TPN@GNPs (with TPN/AuCl4− reaction molar ratio of 1:1, 3:1, and 5:1) were characterized with UV-Vis absorption spectroscopy. As shown in Fig. 2, the surface plasmon resonance (SPR) band of all TPN@GNPs samples
was displayed at 560 nm, which was a little red-shifted comparing with that of gold nanoparticles protected by the citrate salt (not shown here). This red-shift was due to the successful formation of S–Au bonds on the gold surface. It was found that with the increase of molar ratio of TPN/AuCl₄⁻, the UV–vis absorption of TPN@GNPs increased and the sample with molar ration of 5:1 showed the highest SPR band at 560 nm. However, we found that the TPN@GNPs (5:1) sample in the solution was quite unstable and some black precipitation can be found when its freeze-dried sample was resuspended in the deionized water. While TPN@GNPs samples with other TPN/AuCl₄⁻ reaction molar ratios were clear solutions when resuspended in the deionized water, and the TPN@GNPs solution kept unchanged for a long time such as several months. For the comprehensive consideration of high stability and TPN loading in TPN@GNPs samples, TPN@GNPs with TPN/AuCl₄⁻ reaction molar ratio of 3:1 was chosen for the further studies.

TEM assay has been carried out to characterize the size and shape of the TPN@GNPs. As shown in Fig. 3, TPN@GNPs showed spherical structure with a narrow distribution (the diameter of ca. 2 nm), which was consistent with the UV–vis results demonstrated in Fig. 2. From the TEM image, it can be found that all particles displayed a spherical shape and were well-dispersed.

To evaluate the number of TPN molecules modified on the surface of gold nanoparticles (Hostetter et al., 1998; Roger, 1995), TGA has been performed and the result was shown in Fig. 4. The organic ligands on the GNP surface decompose to volatile disulfides at a gradually rising temperature. While for gold it possesses a high melting point that is robust enough to undergo the high temperatures applied in the TGA process and leave as the elemental gold residues after thermal analysis process. It can be seen in Fig. 4 that he weight fraction of TPN molecules passivated on the surface of TPN@GNPs is about 24% and therefore the number of TPN molecules modified on the surface of gold nanoparticles can be accurately calculated to be 463 (Daniel and Austruc, 2004). This result was consistent with that of the TPN assay via HPLC, conducted by dialysis in a high concentration (50 mM) of GSH in 10 mL of water.

3.2. In vitro release study of TPN from TPN@GNPs

In vitro release testing of TPN from the TPN@GNPs was operated with or without GSH (10 mM) in the release media. Since the content of TPN was not detectable in the release media even after 6 h in the absence of GSH (not shown here), Fig. 5 shows the drug release results in the presence of 10 mM GSH. Firstly, pH influence was evaluated, and the release behavior of TPN in the release media of PBS at pH 5.5 and pH 7.4 was shown in Fig. 5A. Although it was found that different pH values of release media slightly affected the accumulative release of TPN, the TPN release rate was comparatively faster in a higher pH 7.4 vs. pH 5.5. The mechanism of drug release from the TPN@GNPs was related to thiol group exchange reaction, in which GSH functioned as a reducing agent to exchange TPN molecules from the surface of TPN@GNPs. The reduction activity of GSH was relevant to the number of its thiol groups. Since the pKa value of GSH was about 8.8 and its thiolate form had much higher reduction activity than that of its thiol form, relative higher pH value of release media would lead to rapid ligand exchange rate, and subsequently the release rate of TPN from TPN@GNPs surface (Winterbourn and Metodiewa, 1999). Therefore, it could be concluded that GSH was a sensitive trigger for drug release from the
TPN@GNPs conjugate, especially in the interior of the cell and at the physiological pH, and the release profiles displayed a sustained release pattern within 4 h.

As shown in Fig. 5B, ionic strength did not show significant influence on the release rate of TPN from the TPN@GNPs. Within the ionic strength studied, TPN release rate slightly decreased with the increasing ionic strength. The process of drug release from the conjugate was synchronized with the process of GSH adsorbing on GNPs (Lim et al., 2008).

3.3. Cell viability

The cytotoxicity of TPN@GNPs is a vital parameter to apply the gold conjugate in vivo, so the MTT assay was carried out in both the free TPN and the TPN@GNPs containing the same amount of TPN. In view of the chromatic interference of the conjugate, the cell viability in previous reports were determined by the method of direct counting the cell numbers (Cai et al., 2011) or using the Cell Counting Kit-8 (CCK-8 assay) (Huang et al., 2012) to avoid the disturbance. Due to 3-(4,5)-dimethylthiazolium(3,5-diphenyltetrazoliumromide (MTT) has no absorption at 630 nm, MTT assay performed in this work was adopted to determine the cell viability by calculating the absorption differences at 570 nm and 630 nm. As shown in Fig. 6, the IC50 values of the TPN@GNPs and TPN on the HepG2 cell line were 1.26 ± 1.07 and 1.73 ± 1.16 mg/mL, respectively, indicating that the TPN@GNPs was relatively safe.

3.4. Pharmacokinetics study in rats

The mean plasma concentration–time curves of free TPN and TPN@GNPs were shown in Fig. 7 and main pharmacokinetic parameters were summarized in Table 1. The mean retention time (MRT) of TPN@GNPs was significantly longer than that of free TPN (7.714 h vs. 3.962 h), indicating that TPN release from TPN@GNPs in vivo was in a sustained manner. The in vivo result was consistent with the in vitro drug release profiles discussed earlier in this paper. In addition, the TPN from TPN@GNPs was eliminated considerably faster than free TPN.

Table 1

<table>
<thead>
<tr>
<th>Parameters</th>
<th>TPN</th>
<th>TPN@GNPs</th>
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</thead>
<tbody>
<tr>
<td>MRT (h)</td>
<td>3.962 ± 1.182</td>
<td>7.714 ± 2.142</td>
</tr>
<tr>
<td>AUC0-12 h (µg/mL)</td>
<td>103.985 ± 26.819</td>
<td>111.232 ± 13.323</td>
</tr>
<tr>
<td>t1/2d (h)</td>
<td>2.746 ± 1.739</td>
<td>9.796 ± 2.844</td>
</tr>
<tr>
<td>Vd (mL)</td>
<td>0.196 ± 0.127</td>
<td>0.330 ± 0.217</td>
</tr>
<tr>
<td>Cl (mL/h/µg)</td>
<td>0.0406 ± 0.0172</td>
<td>0.0407 ± 0.0206</td>
</tr>
</tbody>
</table>

MRT: mean retention time; AUC0-12h: area under curve from 0 h to 12 h; t1/2d: elimination half-life; Vd: apparent distribution volume; Cl: clearance rate.

* P < 0.05 compared with TPN@GNPs.
slowly in vivo with a larger $t_{1/2b}$ of 9.796 h. It could be deduced from the prolonged MRT and $t_{1/2b}$ that TPN@GNPs could ameliorate the instability and rapid elimination of TPN in vivo. Other parameters such as $V_d$, Cl, and AUC$_{0-\infty}$ showed no significance between TPN and its GNP conjugates.

3.5. In vivo assessment of TPN@GNPs on acute liver injured mice induced by CCl$_4$

Based on the above studies, TPN@GNP conjugate proved to be able to release the free TPN in a sustained way under a GSH stimulated condition. To better understand the practical superiority of TPN@GNP conjugate comparing to the free TPN, it is necessary to evaluate the treatment efficacy of hepatitis for TPN@GNP formulation in vivo. In the animal experiment, acute liver injury model in mice was constructed by administering the animals intraperitoneally with carbon tetrachloride (CCl$_4$). The hepatic markers of acute liver injury were determined, including the relative liver weight (LW) percentage, AST, and ALT activity levels shown in

![Graph showing effects of TPN and TPN@GNPs on relative LW, AST, and ALT activity.](image)

**P < 0.01 represented the significance of inter groups.**

Fig. 8. Effects of the TPN and TPN@GNPs on the relative LW percentage, AST and ALT activity. Each of the data was presented as mean ± SD, and **P < 0.01 represented the significance of inter groups.

![Histological analysis of liver slices](image)

Fig. 9. Histological analysis of liver slices for the five groups of mice after treatment. The images on the left column (A–E) are H and E staining, and on the right are Masson’s trichrome staining (F–J). All the images were captured by the Olympus microscope at a 400× magnification of the original images. The arrows represented the abnormal state of the liver, especially the inflammation.
Fig. 8, respectively. The relative LW is calculated by the following formula:

\[
\text{Relative LW} = \frac{\text{liver weight (mg) / body weight (g)}}{100}
\]

Compared to the control group, the CCl4 group resulted in significant increasing of the relative LW as well as the AST, and ALT activity, which implied that the acute live injury model in mice was successfully established. There was no significant difference between the citrate-protected GNPs and CCl4 group in LW, AST, and ALT activities, which means that the GNPs themselves did not enhance or attenuate the efficacy of liver injury. In the CCl4 induced groups, both TPN and TPN@GNPs groups could significantly reduce the elevated relative LW and AST activity caused by CCl4 to the normal level. Although there was no significance between two groups, the TPN@GNPs group showed lower AST activity level in comparison with that of TNP group, which indicated better recovery of liver function using TPN@GNPs. In addition, compared with TPN group, the effect of TPN@GNPs on reducing ALT activity was of significant difference (P < 0.01, Fig. 8), which could be due to the TPN accumulation in liver via the passive targeting of gold nanoparticles and GSH-mediated TPN release after the conjugates were uptake by liver cells.

The images of liver sections stained with H and E or Masson's trichrome were presented in Fig. 9. In this study, the liver injury was not serious according to the experimental design, so the Masson's trichrome staining (Fig. 9F–J) showed no fibrosis in all the experimental groups. However, in the H and E stained tissues, compared with the saline group, the CCl4 (Fig. 9B) and GNP (Fig. 9C) group appeared obvious inflammation and abnormal structure of liver cells (marked by arrows). While the free TPN (Fig. 9D) and its GNP conjugates (Fig. 9E) could restore the impaired liver cells to normal state, without inflammation and structure abnormalities. The histological analysis of five groups was in accordance with the results of the biochemical parameters of hepatic function.

4. Conclusion

In summary, TPN-conjugated gold nanoparticles were prepared by using a one-pot synthesis method, and characterized by UV–vis, TEM, and TGA measurements. The in vitro studies suggest the conjugate was GSH-sensitive and free TPN could release from the conjugate completely in a sustained manner at the presence of intracellular level of GSH. The pharmacokinetics study showed TPN@GNPs could improve the pharmacoeutics of free TPN and sustain the drug release in vivo, in consistency with the results of drug release in vitro. Although, compared with that of free TPN, the cell cytotoxicity of TPN@GNPs on the HepG2 cell line was slightly increased, the sustained drug release behavior and passive liver targeting property of TPN@GNP conjugates render them a better therapeutic efficiency on acute liver injury therapy than that of the conventional injection. The TPN@GNPs could substantially reduce the rising aminotranferase ALT caused by CCl4 to the normal level. Compared to the conventional preparation, the TPN@GNPs possessed greater advantages in delivering drug to targeted cells and preventing liver from acute injury. From this study, there is a huge potential of applying the drug-loaded GNPs to clinical use and this novel drug delivery system will be notably promising in the upcoming future.

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