ORIGINAL RESEARCH

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The biodistribution and pharmacokinetic evaluation of choline-bound gold nanoparticles in a human prostate tumor xenograft model

Abstract

Purpose: Gold nanoparticles (GNPs) have attracted significant attention in the treatment of cancer due to their potential as novel radiation enhancers, particularly when functionalized with various targeting ligands. The aim of this study was to assess the biodistribution and pharmacokinetic characteristics of a novel choline-bound GNP (choline-GNP) stabilized with polyethelenimine (PEI).

Methods: Choline bound to 27 nm diameter GNPs was characterized using transmission electron microscopy (TEM), X-ray photoelectron spectroscopy (XPS) and Fourier transform infrared spectroscopy (FTIR). Toxicity of choline-GNPs was examined on DU-145 prostate cancer cells using an MTT assay. Using balb/c mice bearing flank DU-145 prostate tumors, choline-GNPs bio-distribution was measured using inductively coupled mass spectroscopy (ICP-MS). Blood, heart, lung, liver, spleen, brain, kidney and tumor gold content were examined at multiple time points over a 24-hour period after tail vein injection.

Results: An MTT assay using DU-145 prostate cancer cells yielded a 95% cell viability 72 hours after choline-GNP administration. The tumor GNP area under the concentration-time curve during the first 4 hours (AUC₀₋₄) was 2.2 µg/ml h, representing 13% of the circulating blood GNP concentration over the same time period. The maximum intra-tumor GNP concentration observed was 1.4% of the injected dose per gram of tumor tissue (%ID/g) one hour post injection.

Conclusions: GNPs functionalized with choline demonstrates a viable future nanoparticle platform with increased intra-tumor uptake as compared to unconjugated GNPs. Decreased intra-hepatic accumulation appears to be the reason for the improved systemic bioavailability. The next logical translational investigation will incorporate external beam radiation with the observed maximum intra-tumor uptake.

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The application of gold nanoparticles (GNPs) to enhance radiation therapy has evolved into a promising frontier in cancer research [1-7]. Roeske *et al.* demonstrated that materials with high atomic numbers, such as gold, when coupled with low energy x-rays, can provide relatively large increases in dose enhancement factors (DEF) [4]. Following the pioneering work by Herold *et al.* with gold microspheres, Hainfeld *et al.* performed pre-clinical *in vivo* experiments to establish proof-of-principle that intravenously-administered GNPs can accumulate within tumors in sufficient enough quantity to impart external beam radiation enhancement [7,8]. Recent *in vivo* experimental work further illustrates that GNPs are capable of providing radiation enhancement in the setting of radiation resistant squamous cell carcinoma cells, yielding enhanced cytotoxicity [2]. Radiation enhancement has been shown to involve mechanisms beyond the predicted local DEF. Roa *et al.* demonstrated that GNPs enhance the effects of radiotherapy on prostate cancer cells through the increased accumulation of cells in the radiation sensitive G2/M phase of the cell cycle [9]. GNPs can be bound to various ligands through a robust amine or thiol covalent bond, conferring unique functional capabilities, in order to improve tumor targeting [9-12].

Choline is a quaternary amine that is ubiquitously distributed in cells, mostly in the form of phospholipids [13]. *In vitro* and *in vivo* magnetic resonance spectroscopy studies have shown an over-expression of choline kinase and a significant increase in choline in prostate cancer cells. Although the liver and kidney are major sites for choline oxidation, the bladder has only negligible activity, which allows for enhanced prostate imaging sensitivity [14-16]. These favorable features have garnered significant clinical interest in the applicability of radiolabelled choline as a potential positron emission tomography (PET) tracer in the imaging of prostate malignancy [17-20].

Polyethylenimine (PEI) has been extensively studied as a vehicle for non-viral gene delivery and therapy. PEI is widely utilized as a polymer-based gene carrier secondary to efficient polyplex (complex of nucleic acid and polymer) transfection, as demonstrated both *in vitro* and *in vivo* [21]. The positively-charged PEI polymer is thought to interact with the anionic cell membrane proteoglycans resulting in improved cellular uptake [22]. PEI retains a substantial buffer capacity over a wide pH range and it has been hypothesized that this simple molecular property is associated with the efficiency by which the complex multistage process of transfection takes place [22]. The "proton sponge" nature of PEI is thought to provide buffering within the endosomes, improving PEI bound GNP stability after cellular internalization [23-27]. Furthermore, the steric hindrance imparted by PEI surface functionalization

imparts added colloidal stability for the gold in solution [27]. For these reasons, its incorporation into a nanoparticle platform may provide enhanced systemic bioavailability and intra-tumor uptake after intravenous administration.

In this study, GNPs, with a diameter of 27 nm, were covalently functionalized with both choline and PEI (Figure 1). In addition to the potential enhancement of GNP cell uptake, PEI provides additional stability of our construct in solution. The objective of this study was to assess the biodistribution pattern of choline-bound GNPs (choline-GNPs) using a human prostate cancer xenograft mouse model.

Materials and Methods

Materials and reagents

All chemicals used in the experiment were obtained from Sigma-Aldrich (WI, USA) or Acros Organics (NJ, USA), unless otherwise stated.

Thio-choline synthesis

The procedure for the preparation of 2-mercaptoethyl-*N,N,N*-trimethylammonium chloride (mta or thiocholine) was adapted from a previously established protocol [28]: 0.5 g (2.52 mmol) of acetylthiocholine chloride was dissolved in 2 ml (1 M) of HCl and refluxed at 110°C under an inert atmosphere for 16 hours. Acetic acid, HCl and deionized water were removed via vacuum distillation leaving behind a white solid. Recrystallization with methanol produced a yield of 83.0%. Thin layer chromatography (2:1 CHCl₃: MeOH) results demonstrated a retention factor = 0.23. ¹H NMR (CD₃OD, 300 MHz): 3.50, 3.11 and 2.91 ppm.

Seed solution preparation

HAuCl₄ (0.5 ml of 1% HAuCl₄ solution) was added to 50 ml deionized water. The solution was heated to reflux and 0.5 ml (0.02 g/ml) of branched PEI (MW: 25000 Da) was added, followed by 1.0 ml of 1% sodium citrate solution. The solution was then boiled for 5 minutes and allowed to cool to room temperature. The resulting colloidal gold solution seen in Figure 2 was used as the seed for the subsequent synthesis of 27.3 nm gold nanoparticles.

Synthesis of PEI stabilized choline bound GNPs

HAuCl₄ (0.5 ml of 1% HAuCl₄ solution) in 50 mL deionized water was added to 2.0 ml of the seed GNP solution produced in the previous step. Sodium citrate (0.15 ml of 1% sodium citrate solution) and 0.5 ml (0.03 M) of hydroquinone were

FIGURE 1. PEI stabilizes choline-GNPs via covalent conjugation.

added and stirred continuously at 750 rpm for 20 min. Thio-choline (0.20 ml; 0.2 M) was added and the resulting solution was stirred continuously for an additional 20 minutes, creating the final PEI stabilized choline bound GNP (choline-GNP) end product. Choline-GNP was stored at 4°C. The mean diameter of synthesized choline-GNPs was achieved by measuring 100 nanoparticles using TEM (JEOL-2100, Japan).

X-ray photoelectron spectroscopy (XPS)

Room-temperature XPS experiments were performed at Alberta Center for Surface and Engineering Science (ACES) using a Kratos Axis spectrometer with monochromatized Al K_{α} ($h\nu = 1486.71$ eV). The spectrometer was calibrated by the binding energy (84.0 eV) of Au $4f_{7/2}$ with reference to Fermi

level. The pressure of analysis chamber during experiments was greater than 5×10^{-10} Torr. A hemispherical electron-energy analyzer working at the pass energy of 20 eV was used to collect core-level spectra while survey spectrum within a range of binding energies from 0 to 1100 eV was collected at analyzer pass energy of 160 eV. Charge effects were corrected by using C 1s peak at 284.8 eV. A Shirley background was applied to subtract the inelastic background of core-level peaks. Non-linear optimization using the Marquardt Algorithm (Casa XPS) was used to determine the peak model parameters such as peak positions, widths and intensities. The model to describe XPS core-level lines for curve fitting was a product of Gaussian and Lorentzian functions.

Fourier transform infrared spectroscopy (FTIR)

Transmission spectra for the gold nanoparticles and its choline-GNP complex were obtained by forming transparent KBr pellets containing the choline-GNPs centrifuged in the FTIR spectrometer (Nicolet 8700, Nicolet-Thermo). The KBr mixtures were placed on a vacuum line overnight before pellet formation, and then the pellets were again placed on a vacuum line before use. The transmission spectra were obtained, after purging in dry air, and were background corrected using a reference “blank” KBr pellet. FTIR spectra were recorded in the range of $400\text{--}4000\text{ cm}^{-1}$ at a resolution of 2 cm^{-1} .

DU-145 prostate cancer cell culture

Human prostate carcinoma cell line DU-145 was used in all the experiments (American Type Culture Collection, VA, USA). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 20 mM D-glucose, 100 UI/ml penicillin G and 100 $\mu\text{g}/\text{ml}$ streptomycin in a humidified incubator with 5% CO_2 in the air at 37°C .

In vitro MTT assay

DU-145 prostate cancer cells were plated in 96 well plates at a density of 2×10^3 cells in 200 μL of medium. The 96 well plates were then incubated for 24 hours to allow adherence of the cells prior to the administration of various samples for cell viability testing. The culture medium was then replaced with 200 μL of solution containing a mixture of fresh medium and a choline-gold nanoparticle solution prepared in deionized water. The effects of 0.32 μM native PEI, 2.0 mM choline chloride and choline-GNPs were examined representing a PEI containing GNP concentration greater than a previously published concentration using GNPs bound with PEI [26]. Control wells containing the same cell density received only 200 μL of fresh

FIGURE 2. Transmission electron microscopy image of PEI stabilized gold seed solution used for subsequent fabrication of 27 nm choline-GNPs (mean diameter = 7.3 nm, polydispersity index = 0.138).

culture medium. The 96 well plates were then incubated for 72 hours. The cells remained in the wells for this period without exchanging the media. All experiments were performed in triplicate. MTT (20 μL of 5 mg/mL of MTT solution) was added to each well and then incubated for an additional 4 hours. The media was removed and 150 μL of DMSO was added to dissolve the formazan crystals resulting from the reduction of the tetrazolium salt by metabolically active cells according to manufacturer-recommended protocol (Promega, WI, USA). The absorbance of dissolved formazan was measured at a wavelength of 540 nm using a Thermo Scientific (ON, Canada) microplate reader. Percent viability was normalized to untreated controls.

DU-145 prostate cancer mouse model

Five-week old balb/c nude mice were implanted subcutaneously in the flank with 2×10^6 human DU-145 prostate cancer cells suspended in 0.1 ml of PBS. The tumor was allowed to grow for two to three weeks until the tumor volume reached 300 mm^3 . Tumor volumes were calculated as $V = 1/2xy^2$, where x and y are the longest and shortest diameters respectively [29].

Pharmacokinetics, Biodistribution of Choline-GNPs

All the mice experiments performed were approved by our institutional Animal Ethics Review Board. Biodistribution experiments were carried out once a tumor volume of 300 mm^3 had been reached. Mice ($n=15$ total) were anaesthetised with

FIGURE 3. Transmission electron microscopy images of synthesized unfunctionalized GNPs (mean diameter = 27.3 nm, polydispersity index = 0.132).

isoflurane and 7.2 mg/kg of choline-GNPs were injected via the tail vein. Mice ($n=3$ per time point) were euthanized at 15 min, 1 h, 2 h, 4 h and 24 h after intravenous injection. Blood was sampled via cardiac puncture and placed into a heparinized glass tube. Tissues (tumor, heart, liver, lung, spleen, kidneys and brain) were harvested. The tissues were briefly washed with normal saline. The isolated tissues and blood samples were then weighed and subsequently stored at temperatures of -40°C and $+4^{\circ}\text{C}$, respectively, until processing for ICP-MS analysis.

Gold content determination

Whole blood was collected at 15 min, 1 h, 2 h, 4 h and 24 h after injection of PEI stabilized choline-GNPs. Gold concentrations in the harvested tumor, blood and organ specimens (heart, liver, spleen, kidneys, brain and lung) were quantified by ICP-MS. Briefly, whole blood, tumor and organ specimens ($n = 3$ each) were dried at room temperature and weighed. Tissues were digested using freshly prepared aqua regia solution (nitric acid and hydrochloric acid with a 1:3 ratio, respectively), which is capable of dissolving GNPs. The gold content of each sample was then determined by ICP-MS. GNP biodistribution data is expressed as a percentage of injected dose per gram of tissue/blood (%ID/g).

Statistical analysis

All data are represented as means, while uncertainties are standard deviations of the mean, unless otherwise stated. The variance associated with the area under the GNP concentration-

time curve (AUC) was calculated using Bailer's method. Statistical differences between groups were determined using a two-tailed, unpaired Student's *t*-test with a $p < 0.05$ considered to be significant.

Results

Nanoparticle characterization

GNPs with a diameter of 27.3 nm (Figures 3A & 3B) were functionalized with choline and PEI for experimental evaluation. The chemical and electronic structure of choline-functionalized gold nanoparticles were further analyzed by XPS. XPS results confirmed that the choline-GNP pellet did, in fact, contain nitrogen, sulfur and carbon elements in addition to gold (Figure 4A). In the core-level spectra of the Au 4f region, shown in Figure 4B, there exists the presence of a shoulder on Au 4f_{5/2} and Au 4f_{7/2}, confirming that S was bound with gold. Both XPS survey and deconvolution peaks of core-level of gold 4f confirmed that GNP and choline were bound together through a thiol bond [29,30].

Figure 5 shows the FTIR spectrum of the thiol monolayer between 400 and 4000 cm^{-1} . The formation of a covalent gold-sulfur bond is confirmed by the presence of the large band in the range of 500 to 750 cm^{-1} . This large band is assigned to stretch the mode of C-S groups. Another band appears at 1700 cm^{-1} , corresponding to a C=O stretch vibration mode. The peak observed at 1310 cm^{-1} corresponds to the C-H scissors vibration mode. A small main band at 2900-2950 cm^{-1} is clearly observed in the high frequency region. This is assigned

FIGURE 4. (A) XPS spectra of the choline-GNP nanoparticle pellet. (B) XPS spectrum shows the Au4f peaks of the choline-GNP conjugate.

FIGURE 5. FTIR spectra of choline-GNPs dispersed in a KBr pellet.

to the vibration mode of alkyl thiol chains. Bands at 1125-1335 cm⁻¹ are assigned to the N-C stretch [31]. These results confirm the presence of covalent thiol and amine bonds found in our PEI stabilized choline-GNPs.

In Vitro Cytotoxicity

An MTT assay was performed in order to evaluate the possible cytotoxic effects of choline-GNPs. After 72 hours, minimal cytotoxicity was observed (Figure 6). DU-145 cell viability

FIGURE 6. MTT results demonstrating mean percent normalized DU-145 cell viability 72 hours after exposure (±SD). 0.32 μM native PEI, 2.0 mM choline chloride and choline-GNPs were examined.

after exposure to choline-GNPs, choline alone and PEI alone did not vary significantly from control (95.39 %, 94.41% and 89.78 %, respectively, $p > 0.05$).

Choline-GNP Pharmacokinetics

GNP concentration, as determined by ICP-MS analysis, was used to assess the pharmacokinetic profile of PEI stabilized choline-GNP. The blood GNP concentration was examined over the initial 24 hours after intravenous administration. Tu-

FIGURE 7. Twenty-four hour DU-145 tumor and blood choline-GNP concentration after tail vein injection in DU-145 prostate tumor-bearing mice ($\pm$ SD). N=3 for each time point. Injected dose = 7.2 mg/kg based on Au weight.

TABLE 1. Choline-GNP Pharmacokinetics at 4 and 24 hours

	Mean AUC ₀₋₄ (μ g/ml $\cdot$ hr)	SD	Mean AUC ₀₋₂₄ (μ g/ml $\cdot$ hr)	SD
Tumor	2.162	0.59	* ₋	* ₋
Blood	16.905	2.12	34.292	2.01

Mean area under the GNP concentration-time curve (AUC) for both blood and flank DU-145 prostate tumor over the initial 4 and 24 hours after IV injection

*Value below detection limit of ICP-MS

FIGURE 8. Twenty-four hour biodistribution of choline-GNPs after tail vein injection in DU-145 prostate tumor bearing balb/c mice ($\pm$ SD). N=3 for each time point. Injected dose = 7.2mg/kg based on Au weight.

mor concentrations were measured between 15 minutes to 4 hours after injection (24 hour concentration below reliable ICP-MS detection). Both tumor and blood pharmacokinetic profiles are shown in Figure 7. GNP concentration in blood peaked at 15 min, and rapidly declined until 4 hours post-injection, after which a steadier rate of blood clearance was observed over the subsequent 20 hours. The GNP blood area under the concentration-time curve between 0-4 hours (AUC_{0-4}) and 0-24 hours (AUC_{0-24}), and the GNP tumor AUC_{0-4} data is summarized in Table 1.

Choline-GNP Biodistribution

15 min post-injection

Figure 8 displays the time dependent biodistribution of GNPs in blood, organs (lung, heart, liver, spleen, kidney, brain) and DU-145 tumor at various time points after a single IV injection of choline-GNPs. Fifteen min after IV administration, blood demonstrated the highest GNP concentration (8.40 ± 2.57 %ID/g) followed by the spleen (5.86 ± 2.4 %ID/g), lung (5.37 ± 0.49 %ID/g), heart (3.50 ± 1.79 %ID/g), liver (1.33 ± 0.20 %ID/g), kidney (1.22 ± 0.13 %ID/g), tumor (0.38 ± 0.20 %ID/g) and brain (0.21 ± 0.04 %ID/g).

1 hour post-injection

The spleen exhibited the highest GNP uptake (14.05 ± 6.00 %ID/g), with blood concentration dropping (5.67 ± 2.34 %ID/g) 1 hour after IV administration. Lung (3.42 ± 1.03 %ID/g), heart (2.67 ± 1.35 %ID/g), kidney (0.82 ± 0.16 %ID/g), and brain (0.13 ± 0.07 %ID/g) GNP uptake all decreased slightly. Hepatic uptake (1.89 ± 0.87 %ID/g) continued to increase slowly. Tumor GNP concentration peaked at this time point (1.35 ± 1.06 %ID/g).

2 hours post-injection

The spleen continued to exhibit the highest uptake of GNPs (13.27 ± 8.33 %ID/g) with blood GNP concentration dropping from the previous hour (3.68 ± 0.48 %ID/g). Tumor GNP concentration dropped significantly (0.37 ± 0.27 %ID/g) from the previous hour whereas continued hepatic uptake (3.27 ± 0.90 %ID/g) was observed.

4 hours post-injection

The spleen still displayed the highest GNP uptake (12.57 ± 7.47 %ID/g). Lung GNP concentration doubled as compared to the previous 2 hours (9.01 ± 1.4 %ID/g). Tumor ($0.20 \pm$

0.05 %ID/g), blood (1.17 ± 0.48 %ID/g) and hepatic (1.83 ± 0.28 %ID/g) GNP concentrations continued to decline.

24 hours post-injection

24 hours after choline-GNP administration, an increase in splenic (38.19 ± 17.47 %ID/g), pulmonary (12.37 ± 5.95 %ID/g) and hepatic (2.29 ± 0.90 %ID/g) GNP uptake continued to be seen. Blood (0.57 ± 0.29 %ID/g) GNP concentration declined to 6.8% of the peak %ID/g observed. Tumor GNP concentration fell below reliable ICP-MS detection levels.

Discussion

The blood choline-GNP AUC_{0-4} and AUC_{0-24} were 16.91 ± 2.12 $\mu\text{g}/\text{ml}\cdot\text{hr}$ and 34.29 ± 2.01 $\mu\text{g}/\text{ml}\cdot\text{hr}$, respectively, and the tumor choline-GNP AUC_{0-4} was 2.16 ± 0.59 $\mu\text{g}/\text{ml}\cdot\text{hr}$. This demonstrated that 13% of the choline-GNP AUC_{0-4} in circulation were localized to the subcutaneously located DU-145 tumor over the initial 4 hours after intravenous administration.

Blood GNP concentration is an important driving force affecting tumor localization *in vivo*. The GNP blood concentration peaked at 8.4 %ID/g, 15 minutes after IV administration. Interestingly, even 24 hours after choline-GNP administration, 6.8% of the peak blood GNP concentration still remained in the systemic circulation. There is great interest in increasing intra-tumor GNP concentration, which, in the literature, has been demonstrated to be very low. Zhang *et al.* demonstrated a peak intra-tumor GNP concentration of 0.8 %ID/g [33]. Our functionalized GNP concentration within the DU-145 prostate tumor peaked 1 hour (1.4 %ID/g) after injection then decreased in a time-dependent manner by 4 h (0.2 %ID/g) after injection. Therefore, one hour after IV injection would be the ideal time to administer external beam radiation to thoroughly take advantage of the radiation enhancing ability of GNPs.

Choline-GNP demonstrated rapid circulation clearance, with the majority being taken up via the various reticuloendothelial systems of the spleen, lung and liver for subsequent clearance or degradation. The majority of GNP uptake at 24 hours was shown to be splenic (38.19 %ID/g) and pulmonary (12.37 %ID/g), where GNPs were likely sequestered through the action of macrophages and lymphocytes [34-37]. Interestingly, the liver internalized only a small percentage of GNPs, with maximum levels at 24 hours (2.30 %ID/g). Similarly, brain GNP concentration remained very low over the 24 hours (max = 0.21 %ID/g).

GNPs in the literature have demonstrated significant hepatic and splenic GNP accumulation, with their combined uptake representing more than 80% of the injected dose [38-40]. In comparison, our PEI stabilized choline-GNP demonstrated significantly lower combined splenic and hepatic GNP uptake (max combined was 40 %ID/g). This significant decrease in the accumulation of choline-GNPs would likely aid in the reduction of potential chronic toxicity.

We have demonstrated that choline-GNPs represent viable nanoparticle platforms for *in vivo* applications. The incorporation of PEI onto a GNP surface results in a positively charged nanoparticle [26]. Nanoparticles with a net positive charge have been purported to have improved cancer cell uptake [22,26]. Furthermore, the steric hindrance imparted by PEI improves the circulation stability.

Conclusions

GNPs have shown potential for the enhancement of radiotherapy in the treatment of cancer both *in vivo* and *in vitro*. In order to enhance the tumor localization of GNPs, a GNP was designed that is covalently conjugated to both choline and PEI, retaining the biological function of choline and thereby enhancing the uptake of GNPs by a DU-145 prostate tumor *in vivo*. In this report, important *in vivo* pharmacokinetic and biodistribution data of PEI stabilized choline-GNPs has been provided. Choline-GNPs display significantly reduced hepatic accumulation as compared with literature-cited unmodified GNPs. Further investigation is required to evaluate the potential clinical application of this novel GNP platform.

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