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Gold Nanotriangles: Scale Up and X-Ray Radiosensitization Effects in Mice

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Abstract

In the past decade, there has been considerable interest in radiosensitization using gold nanoparticles that accumulate specifically in cancerous tissue while sparing normal tissues. Despite this interest, it remains unclear which nanoparticle morphologies, cellular uptake, or cytoplasmic distribution elicit optimal radiosensitization. We introduce gold nanotriangles (AuNTs) as a possible X-ray radiotherapy sensitizer. In this study, we first explored a large-scale synthetic method for the production of high quality monodisperse AuNTs. Second, we conducted *in vitro* and *in vivo* experiments to evaluate the effect of PEGylated AuNTs (pAuNTs) on cellular uptake, cytotoxicity, bio-distribution, and radiosensitization on radiation-resistant human Glioblastoma Multiforme (GBM) cells. Our results suggest that the new scale up synthesis methodology consistently produced high quality AuNTs and pAuNTs which had nonspecific cellular uptake without any obvious cytotoxicity and exhibited excellent radiosensitization.

Graphical abstract

Intravenously administered gold nanotriangles accumulate passively in tumors in mice and potentially sensitize them to radiation therapy.

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Introduction

Gold has historically been a key building block for synthesizing nanomaterials in medicine.^[1–4] A wide variety of different particle morphologies can be obtained using any one of the countless synthetic methods described in the literature. A great deal of work has been focused on anisotropic nanoparticle synthesis because of their tunable, geometry-sensitive optical properties.^[5–8] The most common anisotropic gold nanostructures are gold nanorods (AuNRs) and gold nanoprisms.^[9–11] Both morphologies exhibit tunable optical properties for *in vivo* imaging and are suitable candidates for photothermal therapeutics and photoacoustic contrast agents because of their surface plasmon resonance absorptions at near infrared wavelengths.^[8,11,12] Using another form of radiation, X-rays, gold nanoparticles have been shown to be effective radiation therapy enhancers.^[13,14] The radiation enhancement effect comes from the emission of photo- and Auger electrons when core electrons in the particle's atoms are ejected by X-ray photon bombardment.^[15,16] Because gold nanoparticles have been previously demonstrated as radiation enhancers, it follows that other particle morphologies should as well. We chose to investigate the relatively novel gold nanotriangle (AuNT) for the purposes of our work.

Shape-specific syntheses of gold nanoprisms have been demonstrated using variations on the original pentahedrally-twinned AuNR synthesis described by Murphy and her group.^[17] Addition of a trace amount of iodide and the use of cetyltrimethylammonium chloride (CTAC) dramatically replaces the fraction of AuNRs with AuNTs.^[10,18,19] In 2013, Scarabelli *et al.* described a scalable seed-mediated synthesis of monodisperse, equilateral AuNTs that gives high shape purity *via* depletion sedimentation.^[20] With the advent of new low polydispersity syntheses, the implementation of AuNTs towards *in vivo* applications is possible. Our interest in AuNTs stems from work performed by Niikura *et al.* who showed that gold nanoplates were consumed by cells more readily than spherical particles of the same diameter.^[21] Because of the particle uptake and intrinsic radiosensitization potential of AuNPs, we expected that AuNTs will be effective radiosensitizers. In order to determine the effectiveness of AuNTs on X-ray radiotherapy we performed both *in vitro* and *in vivo* experiments to determine several key factors from fundamental particle optimization to complex *in vivo* experiments: first, we studied the uptake of AuNTs with different molecular weight PEG-SH ligands to determine the optimum length. Second, we determined the cytotoxic dose of PEGylated AuNTs in MCF-7 cells, a commonly used cell line for cancer therapeutic investigations and are models for cytotoxic effects. Third, we performed *in vitro* radiation experiments with radiation-resistant U87MG human glioblastoma multiforme cells to determine if the particles enhanced the efficacy of X-ray radiation and finally we performed *in vivo* experiments in mice to study biodistribution, radiation effects on implanted U87MG tumors and histology.

In this report, we detail a parallel synthesis and application of monodisperse AuNTs in radiosensitization experiments both *in vitro* and *in vivo* and their effects on cell proliferation, endocytosis, cytotoxicity, biodistribution, toxicity, and post-irradiation survival in tumor-bearing mice.

Results and Discussion

The production of gold nanoprisms has been demonstrated by nearly all syntheses of AuNRs that result in twinned structures. In seed-mediated syntheses based on the Murphy preparation of AuNRs, a two- or three-step process is used to prepare the particles.^[10,17] Seed particles are added to an intermediate growth solution, which is quickly transferred to a final growth solution. The procedure described by Scarabelli *et al.* demonstrated that the high yield synthesis of AuNTs could be performed using the same method described by Ha *et al.* with CTAC-capped instead of citrate-capped seed particles.^[18,20] The reaction produces a blend of spherical and triangular prism-shaped particles that separate easily in surfactant solutions through depletion.^[22] The proportionately larger, flatter surfaces of AuNTs are more susceptible to surfactant depletion effects than spherical particles and are selectively precipitated with the addition of extra CTAC. Following sedimentation, the contaminated supernatant is decanted and the purified sediment is then resuspended in water resulting in a highly purified solution of AuNTs (Figure 1)

One important limitation to this procedure is the method in which the growth solutions are mixed. Scarabelli *et al.* describes the synthesis up to 250 mL without an increase in polydispersity.^[20] However, the quantity of particles necessary for our experiments required a significant increase in batch size and in order to reduce the effect of increased polydispersity, we chose not to linearly scale the single-batch synthesis. Our solution to this problem was to perform a series of parallel syntheses made of smaller batches prepared in rapid succession from the same initial stock solutions (Figure 1A). The stock solutions were split evenly, and processed simultaneously during the reaction. We chose this route because large volumes of the solutions do not mix as efficiently when hand stirred and achieving high particle monodispersity was hampered as a result. Unlike single crystalline AuNR syntheses, the reaction timescale of AuNT synthesis is much shorter; particle growth begins within seconds of adding the seed solution and the reaction is largely complete within one hour as opposed to the slow growth observed with AuNR syntheses.^[23,24] Because of the speed of the reaction, the solution must be homogeneous within three to five seconds to ensure the seed particles are growing uniformly. The use of a stir bar or mixer can generate unwanted foaming and particle nucleation. Taking these factors into consideration, we opted for a convergent preparation and found that it worked well in our hands. Despite the promise of anisotropic gold nanostructures in medicine, the surfactants (cetyltrimethylammonium bromide, CTAB, or the chloride salt, CTAC) used to synthesize the nanostructures are highly cytotoxic and must be removed from the nanoparticles before they can be used in *in vitro* or *in vivo* studies.^[25,26] The complete removal of surfactant destabilizes the particles and causes irreversible fusion and aggregation. Displacement of the surfactant by a thiol ligand such as methoxy-poly(ethylene glycol) (mPEG-SH) can stabilize and detoxify the particles.^[27,28]

Carried one step further, functional thiols make it possible to functionalize the nanoparticle surface with linkers that can be easily conjugated with biomolecules such as antibodies and peptides.^[29,30]

We functionalized AuNTs with mPEG-SH of various molecular weights using the protocol described by Liopo *et al.*^[31] and inspected the PEGylated AuNTs (pAuNTs) by transmission electron microscopy (TEM) (Figure 1B), UV-vis spectrophotometry (Figure 1C), and zeta potentiometry (Figure 1D). TEM micrographs collected before PEGylation of the purified triangles showed near complete elimination of unwanted spherical or bipyramidal contaminants and a narrow edge length distribution (61.51 ± 2.91 , $n=152$, Figure 1D). Following PEGylation, there is not a significant change in the UV-vis spectra or AuNT edge length (Figure 1C and 1D). The UV-vis absorption spectrum of the pAuNT solution showed typical characteristics of AuNTs, e.g. a single peak (dipole) at 640 nm and a shoulder with a peak at 540 nm (quadrupole) and there were no significant differences in the collected spectra except for a minor 2–3 nm red-shift of the surface plasmon resonance for pAuNTs (Figure 1D).^[19] Zeta potential measurements supported the success of the functionalization (+40 mV vs. –8 mV, Figure 1E).

For radiosensitization to be effective, the pAuNTs must undergo endocytosis. We investigated the effect of mPEG-SH molecular weight on particle uptake by functionalizing AuNTs with 2 kDa, 5 kDa, 10 kDa, 20 kDa, and 30 kDa mPEG-SH and incubating them for 24 hours with MCF-7 cells at a concentration of 13.8 ppm (Figure 2A). We observed an inverse relationship between particle uptake and PEG molecular weight except for the 2K MW mPEG-SH sample (Figure 2A). From previous work, we know that 2K MW mPEG-SH functionalized AuNTs are prone to aggregation in solution. Therefore, we suspect the significant difference between the uptake of the 2K and 5K MW samples is due to the pAuNTs aggregating during sample preparation. However, the corresponding LDH assays showed no relationship between particle uptake and PEG molecular weight (Figure S1). For further experiments, we utilized the 5K MW mPEG-SH because it showed the highest particle uptake per cell. We estimate based on the initial number of cells and the mass of gold per cell that approximately 15% of the pAuNTs in the original solution were consumed by the cells during the incubation period. To determine a non-toxic dose, we incubated MCF-7 cells with 13.8–82.8 ppm solutions in 13.8 ppm increments of 5K-pAuNTs and performed LDH assays. We observed a direct correlation between particle concentration and LDH activity, and chose to use 13.8 ppm for further studies (Figure S2). We utilized laser scanning confocal microscopy (LSCM) to image pAuNT uptake by U87MG cells because of its superior resolution and imaging versatility. We detected signals, colored in yellow, from cells incubated with pAuNTs (Figure 2B), indicating pAuNT uptake and aggregation; whereas, no signals were detected from control cells. Because pAuNTs strongly scatter light, we could simultaneously collect fluorescence and reflectance images with the appropriate filter configuration. We detected signals localized near the nucleus that suggested the uptake of the pAuNTs was continuous and localized in the perinuclear region. Despite the clarity enhancement, the reconstructed LSCM image is extremely bright and may not reflect the true *z*-axis positioning of the pAuNTs (Figure 2C). Regardless, LSCM may be a suitable replacement for dark field microscopy in some cases, especially when simultaneous imaging of fluorescent labels and strongly scattering aggregates or large particles is desired. The observed uptake phenomenon is in agreement with other types of PEGylated nanoparticles, as previously reported, for which the expected pathway was the lysosomal internalization in macrophages.^[32,33] Because the pAuNTs are not treated with a target-specific ligand, they

do not have an affinity towards a specific receptor; therefore, pAuNTs are not selective for endocytosis. Furthermore, the intracellular distribution of pAuNTs in this study are consistent with the results from Oh and Park that suggest uptake is by the pinocytotic pathway.^[34]

Next, we performed *in vivo* biodistribution and tumor growth delay studies to determine both if pAuNTs accumulated in a U87MG tumor xenograft and also if the pAuNTs functioned as radiosensitizers *in vivo*. The distribution and concentration of gold in organs (blood, liver, lungs, spleen, brain, heart, kidneys, intestine, muscle, and tumor) at 24 h after intravenous injection in tumor-bearing mice ($n=3/\text{group}$) was measured by inductively coupled plasma-mass spectroscopy (ICP-MS). The results show that the pAuNTs were widely distributed throughout the body with the highest level of accumulation in the spleen (Figure 3). One possible cause of splenic accumulation could be related to uptake by splenic macrophages.

We observed a substantial amount of gold accumulated in the tumor compared to the muscle, brain, heart, and blood. The high concentration of gold detected in tumors compared to blood 24 h post-injection suggests that the tumor uptake of pAuNTs has not been overestimated due to residual blood associated with tumor at autopsy because the concentration of gold in blood is well below the concentration of gold in other organs. Increased accumulation in the tumor may be related to the enhanced permeability and retention (EPR) effect.^[37] Reduced macrophage clearance of neutral mPEG-SH decorated AuNTs is in general agreement with published studies using other PEGylated nanoparticles.^[32,33,37,38] *In vivo* circulation of small molecule drugs or nanoparticles can be extended by the incorporation of long PEG chains.^[35] It may be possible to enhance circulation time, cellular uptake, and consequently radiosensitization effectiveness by incorporating longer PEG chains. However, it should be noted that increasing the PEG chain length beyond a certain threshold may make it difficult for the particles to clear the organism and could result in unwanted accumulation.^[36] pAuNTs may have a future role as a radiotherapeutic agent because of the prolonged circulation time and rapid accumulation in tumors (24 h post-injection, Figure 3B). Before performing *in vivo* radiosensitization experiments, we tested the *in vitro* radiosensitization with pAuNTs in cultured human glioblastoma multiforme (GBM) cells. The goal of this test was to determine if pAuNTs acted as radiosensitizers intracellularly, or extracellularly. Two treatment scenarios were tested. In the first, cells were treated with pAuNTs for 24 h, washed, and then subjected to irradiation 250 kVp X-rays (radiation therapy, RT). Here, only internalized nanoparticles could contribute to radiosensitization. In the second scenario, cells incubated with pAuNTs for 24 h received RT with washing. The first scenario potentially represents tumors with AuNTs internalized by tumor cells whereas the second accounts for uninternalized AuNTs embedded in the tumor microenvironment or vasculature without tumor cell internalization. These washing conditions represent the two extremes in an *in vivo* situation where all nanoparticles accumulating in a tumor are either in tumor cells or outside tumor cells and the concentrations used here are comparable to what was observed *in vivo* (13.8ppm *in vitro* vs. 5ppm *in vivo*). Both washing conditions were significantly more effective at reducing the surviving cell population compared to RT alone at 4 Gy ($p<0.01$ for both washed and unwashed groups) and 6 Gy ($p<0.01$ for the washed group and $p<0.001$ for unwashed

group) (Figure S3). These *in vitro* results suggest that pAuNTs are effective radiosensitizers. Furthermore, the effect of pAuNT-induced radiosensitization on cancer cells may be achieved both by internalized and uninternalized particles.

The *in vivo* study was performed in two parts. First, we performed a tumor volume study on a group of mice ($n=3$) to establish the *in vivo* activity of pAuNTs. Our results demonstrate that pAuNTs alone do not cause tumor growth inhibition (Figure 3C). Second, we compared tumor volumes in mice administered vehicle alone (PBS) without radiation treatment (RT, control), RT alone without pAuNTs, and pAuNTs with RT ($n=5$ for control, $n=7$ for RT and $n=8$ for RT + pAuNTs, Figure 3D). The prolongation in time to tripling of tumor volume was ~3 days with RT alone and ~8 days with RT + pAuNTs, suggesting a dose enhancement factor of 2.67. This benefit was maintained up to 32 days (Figure S4).

Furthermore, the average volume of tumors in the experimental group (RT + pAuNTs) was significantly smaller than that of mice in the RT alone group ($p=0.004$, Figure 3E). This decrease in tumor volume should correlate with the efficacy of pAuNTs as potential radiosensitizers in a therapeutic setting. Notably, this translated to a trend towards improved survival of RT + pAuNTs treated mice as well ($p=0.08$, Figure 3F).

Nanoparticle-induced *in vitro* and *in vivo* toxicity are important factors for the development of nanoparticle-mediated therapy. Chemical and physical parameters such as nanoparticle morphology, surface functionality and charge, composition, colloidal stability, metabolic byproducts, and clinical factors such as dosage and treatment regimen have a profound effect upon acute toxicity. Keeping these parameters in mind, we assessed the effects of a single dose of pAuNTs at a concentration of 2.7 mg/kg. Blood collected 18 days following intravenous administration of particles was analyzed by classical biochemical and hematological toxicity panels (Figure 4 and Table S1). There were no significant biochemical and hematological differences between untreated control mice and mice treated with pAuNTs alone. We did observe a minimal and clinically insignificant elevations of lymphocyte count and aspartate aminotransferase (AST) count in treated mice (Figure 4 and Table S1).

There were no significant histopathological differences between the untreated control mice and mice treated with pAuNTs alone (Figure 5), except for some foreign body-type granulomas and mononuclear infiltrates in the liver (Figure 5D), and inflammatory infiltrates consisting of both mononuclear cells and granulomas in the spleen (Figure 5F). The mononuclear inflammatory infiltrates in the spleen were more variable in both size and shape in comparison to those seen in the liver. The foreign body granulomas in the reticuloendothelial organs (liver and spleen) likely represent opsonized AuNTs that are recognized by membrane-bound scavenger receptors localized on tissue-resident macrophages such as Kupffer cells and splenic macrophages. Nonetheless, biochemical parameters were not influenced by this reticuloendothelial sequestration and elimination of pAuNTs. These results are in agreement with the results of Cho *et al.* showing that PEGylated particles exhibited low toxicity and were not concentrated in the kidneys and liver.^[39] There were no appreciable histological abnormalities in the heart, kidney and lung (Figure 5B, 5H, 5J).

Conclusions

In conclusion, we have demonstrated that the large-scale synthesis of AuNTs is possible using a convergent batchwise seed-mediated method. The purified triangles showed near complete elimination of unwanted spherical or bipyramidal contaminants and a narrow edge length distribution. We claim that this robust synthetic method can produce large quantities of high quality AuNTs in our hands. This synthetic methodology has the potential to enable the use of AuNTs, and potentially other nanostructures, in large, multistage biomedical studies where a consistent effector is crucial for accurate results. *In vitro* and *in vivo* test results for pAuNT cellular uptake, cytotoxicity, biodistribution and radiosensitization effect on radiation-resistant human glioblastoma multiforme (GBM) cells suggest that pAuNTs have no obvious cytotoxicity and exhibit excellent radiosensitization. Accordingly, with the advent of new synthetic techniques, we envision clinical translation potential of AuNTs as radiosensitizers is realistic and feasible.

Experimental

Materials and Methods

Hydrogen tetrachloroaurate trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, 49%, metals basis), CTAC (25% w/w in water), sodium borohydride (NaBH_4 , >99%, ReagentPlus), sodium iodide (NaI, 99.5%, ACS Reagent), and ascorbic acid (99%, ACS Reagent) were purchased from Sigma-Aldrich. Methoxy-poly(ethylene glycol) thiol (2K, 5K, 10K, 20K, 30K MW) were purchased from Laysan Bio. MCF-7 (ATCC® HTB-22™) and U87MG (ATCC® HTB-14™) cell lines were obtained from ATCC. All reagents were used without further purification. *Aqua regia* solutions were prepared with trace metals grade HNO_3 and HCl (3:1). All glassware was cleaned with *aqua regia* prior to use and all solutions were prepared in Milli-Q water.

Preparation of Gold Nanotriangles

AuNTs were prepared using an adaptation of the procedure described by Scarabelli *et al.*^[20]

Seed Solution—First, gold seed particles were synthesized by adding 43 μL of 0.0289 M $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ to 4.7 mL of 0.1 M CTAC in an 8-dram vial. A stir bar was placed in the vial and the solution was stirred at 1200 RPM. 300 μL of 0.01 M NaBH_4 was rapidly added in one injection by pipet. The color of the solution turned brown and was left stirring for 5 min before being left to age for 30 min. After aging, 600 μL of the seed solution was diluted in 6 mL of 0.1 M CTAC in a separate vial. **Growth Solutions.** Two growth solution stocks were prepared, **G1**, and **G2**. **G1** was prepared in a 50 mL Erlenmeyer flask and contained 0.879 mL of 25% CTAC and 37.5 mL of water. 276.8 μL of 0.0289 M $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ was added to **G1** followed by 60 μL of 0.01 M NaI to form a yellow-colored solution. **G2** was prepared in a 2 L Erlenmeyer flask and contained 65.76 mL of 25% CTAC and 894.4 mL of water. 20.76 mL of 0.0289 M $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ was added followed by 7.2 mL of 0.01 M NaI and gently mixed. **G1** was then carefully split into four 9.6 mL volumes in 6-dram vials and 80 μL of 0.01 M AA was added to each vial and gently mixed. Likewise, **G2** was split into four 240 mL portions in 500 mL Erlenmeyer flasks and 2.4 mL of 0.01 M AA was added to

each flask and gently mixed to produce colorless solutions. Then, 300 μ L of the diluted seed solution was added to each of the 6 dram vials containing the **G1** growth solutions followed by rapidly hand-mixing the vials for approximately 2 s. The resulting solution in each of the four vials was then added to one of the four corresponding flasks containing the **G2** growth solutions. The combined solutions transitioned from red, to violet, and finally to dark blue over the course of 5 min after which, the solutions were left to rest at room temperature for 2 h.

Purification—To remove spherical particles and impurities, 30.24 mL of 25% CTAC was added to each flask and gently mixed. The solutions were left to rest at room temperature for 16 h and the supernatant in each flask was carefully decanted. Each flask was rinsed with 10 mL of 0.1M CTAC and the rinse solution was discarded. Then, 25 mL of Milli-Q H₂O was added to each flask followed by vigorously mixing by hand to resuspend the purified AuNTs. After resuspension, the AuNTs were concentrated by centrifugation at 4200 RCF for 10 min.

Synthesis of PEGylated AuNTs

pAuNTs were synthesized using the protocol described by Liopo et al.^[31] Briefly, 8 mL of CTAC-AuNTs concentrated to an optical density of 2.0 were added to five 15 mL centrifuge tubes and 1 mL of 0.002 M K₂CO₃ and 1 mL of 0.5 mM mPEG-SH of 2K, 5K, 10K, 20K, or 30K MW were sequentially added to each tube. The pAuNTs were left on a rocking table for 24 h, centrifuged at 4200 RCF to remove residual mPEG-SH, and finally resuspended in water. UV-vis absorbance spectra (Cary 5000 UV-vis-NIR Spectrometer), DLS (Dynamic Light Scattering; Zetasizer, Malvern), and Transmission Electron Microscopy (TEM; JEOL JEM-1230 HC-TEM operating at 80 KeV accelerating voltage) were used to characterize the pAuNTs.

Cell Culture

MCF-7 human breast cancer cells were cultured in Eagle's modified essential media (EMEM, Lonza) supplemented with 1% (v/v) penicillin-streptomycin (Lonza) and 10% FBS (Fetal Bovine Serum, heat inactivated, Seradigm). Cells were cultured at 37°C in 4.5% CO₂ prior to subculture. U87MG cells were cultured similarly in Dulbecco's MEM media supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C in 5% CO₂.

Cytotoxicity of pAuNTs

MCF-7 cells were seeded in a 96-well plate to a density of 1×10^4 /well and incubated for 18 hrs for the cells to adhere. Afterwards, the media was removed and replaced with 100 μ L of 13.8 ppm mPEG-SH-AuNTs of varying mPEG-SH molecular weights (2 kDa-30 kDa) dispersed in complete media and incubated for 24 hrs. After 24 hrs, 25 μ L of media was removed and transferred to a clean 96-well plate to conduct an LDH release assay. After 4 h of incubation, absorbance of the plate was read at 562 nm using a colorimetric plate reader (Biotek). LDH activity was determined as a relative percent of the control samples that were not exposed to the pAuNTs.

Cellular Uptake and Imaging

To characterize the cellular uptake of AuNTs, dark field microscopy (DFM), confocal microscopy, and transmission electron microscopy (TEM) imaging was performed. Twenty-four hours before the experiment, 0.5×10^5 cells were seeded into the wells of Lab-Tek 8-well chambered coverglass (Nalge Nunc) (for DFM and confocal imaging) or 24-well plate (for inductively coupled plasma – mass spectrometry, ICP-MS). pAuNTs diluted in DMEM (2.76–24.8 ppm) were incubated with the cells for an additional 24 hrs at 37°C in a humidified incubator under 5% CO₂ atmosphere. Cells were then washed with PBS and fixed with 3.7% paraformaldehyde. For optical imaging, nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole, 2 µg/ml) in phosphate buffered saline (PBS).

In vitro Radiosensitization

U87MG cells were seeded in 60 mm petri dishes and incubated for two days until they reached 70% confluency. Then, petri dishes were randomly assigned to RT only group, pAuNT + RT (washed) group, or pAuNT + RT (unwashed) group. Groups receiving pAuNT treatment received 13.8 ppm pAuNT diluted in media while the RT only group received media alone. After 24 hours of incubation, both groups of petri dishes were washed twice with PBS then given fresh media before radiation. For these groups, only internalized nanoparticles could contribute to radiosensitization. Cells in the pAuNT + RT (unwashed) group did not receive fresh media and were exposed to RT while still incubating with 13.8 ppm pAuNT. As such, both internalized and uninternalized pAuNTs could contribute to radiosensitization. In this way, the pAuNT + RT (unwashed) group served as a model for uninternalized nanoparticles caught in the tumor microenvironment or tumor vasculature which may also contribute to tumor radiosensitization in the *in vivo* setting. RT was conducted using the XRAD 320 orthovoltage irradiator. Immediately following radiation, cells were trypsinized, neutralized, counted, and reseeded in 6-well plates. 6-well plates were incubated with fresh media for two weeks before being stained with 0.5% crystal violet diluted in ethanol. Plates were photographed at high resolution using an Oxford optotronic gel counter and counted manually by the same evaluator. Surviving fraction was calculated as the number of colonies divided by the product of the number of cells seeded and the plating efficiency.

In vivo Radio-sensitization and Mouse Survival Studies

All mice (male (Swiss nu/nu)) were purchased, maintained, and handled using protocols approved by Institutional Animal Care and Use Committee (IACUC) at M.D. Anderson Cancer Center (MDACC). The U87MG tumor models were generated by subcutaneous injection of 2×10^6 cells suspended in 50 µL of PBS into the right thigh of mice aged 4 to 6 wks. When tumors grew to 8 mm in diameter, mice were randomly divided into three groups; (1) control (vehicle treatment without particles or radiation), (2) a single radiation therapy (RT) session using 250 kVp X-rays, and (3) pAuNTs with RT. A separate set of mice was used to investigate tumor growth inhibition with or without administering pAuNTs. The pAuNTs were injected via the tail vein at a dose of 2.7 mg/kg suspended in 200 µL of PBS while mice were anesthetized with isoflurane. Tumor growth was measured every two or three days using digital calipers, and tumor volume was calculated according to

the formula: $v = \pi ab^2/6$ where v = tumor volume (mm^3), a = long axis (mm) and b = short axis (mm). The mean tumor volume for each group was plotted over time until all mice reached the mandated institutional sacrifice limit when tumors grew more than 20 mm in diameter and were sacrificed. Survival data were plotted using Kaplan-Meier techniques with survival time being calculated from the date of pAuNTs injection to the date of sacrifice for each mouse.

***In vivo* Toxicity**

An *in vivo* toxicity study was performed on an independent set of six mice ($n=3$ control mice treated with vehicle and $n=3$ for experimental mice treated with pAuNTs). Control and experimental mice were injected as previously described with the experimental mice being injected with pAuNTs at a dose of 2.7 mg/kg suspended in 200 μL of PBS. Mice were sacrificed at 18 d post-injection and the blood, heart, liver, spleen, lung, and kidney were collected for hematology, biochemistry, and toxicological analysis. A panel of 16 routine biochemical analyses (albumin (ALB), alkaline phosphatase (ALP), alanine transferase (ALT), Asparagine transferase (AST), blood urea nitrogen (BUN), calcium (CA), chloride (Cl), creatinine (Cr), globulin (GLOB), potassium (K), lactate dehydrogenase (LDH), sodium (Na), phosphorous (PHOS), random and a panel of 8 standard routine hematological parameters (white blood cell (WBC) count, red blood cell (RBC) count, hemoglobin (HGb), red blood cell specific volume (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelet (PLT) were assessed using a biochemical analyzer and a hematological autoanalyzer respectively).^[39] Blood was drawn from mice via cardiac puncture and collected in appropriate vacuum tubes (potassium EDTA collection tubes for hematology analysis and lithium heparin collection tubes for serum biochemistry analysis). For histopathology analysis, the organs were collected, fixed in 10% buffered formalin, stored at 4°C overnight, and then embedded in paraffin blocks. 4 μm thick tissue sections were prepared and subsequent slides were stained with hematoxylin and eosin (H&E), photographed using an optical microscope (DM750, Leica, Germany) at 400X, and examined by a pathologist.

Biodistribution

U87MG tumor-bearing mice were anesthetized using isoflurane and pAuNTs (57 $\mu\text{g}/\text{mouse}$) were injected as previously described. After 24 h, the mice were euthanized and the blood, spleen, liver, lung, kidney, intestine, muscle, brain, heart, and tumor were collected. All organs were precisely harvested, weighed, lyophilized, digested using *aqua regia*, and quantified using ICP-MS. Standards were prepared and measured along with the samples to calculate amount of gold (ppb/gram) in dry weight tissues.

Statistical Analysis

The results of all experiments were presented as the mean values $\pm$ standard deviation (SD). All cell viability data analyses were performed by using the Student's t-test for differences among the groups. Values of $p < 0.05$ were considered as statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Synthesis and characterization of AuNTs. (A) Schematic of split large-scale synthesis. (B) Transmission electron micrograph of CTAC-capped AuNTs showing highly purified AuNTs. Scale bar is 100 nm. (C) UV-vis spectrum of CTAC-capped AuNTs and 5K MW poly(ethylene glycol) thiol-functionalized (mPEG-SH) AuNTs (pAuNTs). (D) Histogram of CTAC-AuNT and pAuNT particle distribution, edge lengths are in 5 nm bins. (E) Zeta potential of CTAC-AuNTs and pAuNTs

Figure 2. pAuNT uptake by adherent cells. (A) Effect of PEG MW on pAuNT uptake by MCF-7 cells dosed at 13.8 ppm. (B) Laser scanning confocal micrograph of pAuNTs consumed by U87MG cells. pAuNTs are shown in yellow. Nuclear stain (DAPI) is shown in blue. (C) Three-dimensional reconstruction of pAuNTs internalized by U87MG cells. Small aggregates can be seen within the volume of the nucleus. Scale bars are 20 μm in length.

Figure 3.

In vivo biodistribution and radiosensitization. (A) Biodistribution studies showed that a substantial portion of the intravenously administered gold was isolated from the spleen after 24 h. Other organs showed significantly lower concentrations with the liver and intestines having the next highest concentrations. (B) Tumor tissue showed a significantly larger concentration of gold when compared to the brain, heart, and blood, suggesting that particle accumulation in the blood was not a significant source of error. (C) Tumor growth inhibition of subcutaneous U87MG xenografts by intravenously administered pAuNTs compared to vehicle alone (control). (D) Tumor growth delay following treatment of subcutaneous U87MG xenografts with radiation alone (RT), pAuNTs + RT, and vehicle alone (control). (E) Change in tumor volume with each of the above treatments. (F) Actuarial survival of mice treated with vehicle alone, RT alone, and pAuNTs + RT.

Figure 4.

Biochemical (A) and hematological (B) toxicity panel results shown with averages and 1σ -deviations for mice receiving no treatment (control) and mice receiving a 2.7 mg/kg dose of AuNTs after 18 days.

Figure 5. Hematoxylin and eosin-stained histological samples from mice exposed to PBS (Left Column) and pAuNTs (Right Column). (A, B) Heart; (C, D) Liver; (E, F) Spleen; (G, H) Lung; (I, J) Kidney, respectively.