Superior penetration and retention behavior of 50 nm gold nanoparticles in tumors

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Abstract

Nanoparticles offer potential as drug delivery systems for chemotherapeutics based on certain advantages of molecular drugs. In this study, we report that particle size exerts great influence on the penetration and retention behavior of nanoparticles entering tumors. In comparing gold-coated Au@tiopronin nanoparticles that were prepared with identical coating and surface properties, we found that 50 nanoparticles were more effective in all in vitro, ex vivo and in vivo assays conducted using MCF7 breast cells as a model system. Beyond superior penetration in cultured cell monolayers, 50 nm Au@tiopronin nanoparticles also penetrated more deeply into tumor spheroids ex vivo and accumulated more effectively in tumor xenografts in vivo after a single intravenous dose. In contrast, larger gold-coated nanoparticles were primarily localized in the periphery of the tumor spheroid and around blood vessels, hindering deep penetration into tumors. We found multicellular spheroids to offer a simple ex vivo tumor model to simulate tumor tissue for screening the nanoparticle penetration behavior. Taken together, our findings define an optimal smaller size for nanoparticles that maximizes their effective accumulation in tumor tissue.
Introduction

The ultimate objective of chemotherapy was to specifically kill the cancer cells without adverse effect. In the past few decades, nanoparticle-based drug delivery system has shown unique advantages over small molecules. Some commercially available nanomedicines are widely used in the clinic for cancer therapy including liposomes such as Doxil® (liposome loaded with doxorubicin), nanoparticles such as Abraxane® (albumin nanoparticle loaded with paclitaxel). Due to the prolonged circulation time and the altered biodistribution of the chemotherapeutic agent as well as the enhanced drug solubility of the commercial nanoparticles, the side effect of the drug was significantly reduced, whereas the efficacy of the drug was to some extent restricted because of the limited nanoparticle penetration to the tumor tissues (1). Generally, the anticancer drugs must penetrate into tumor tissues efficiently and reach to all the cancer cells in a sufficient concentration to exert effective therapeutic efficacy (2).

In solid tumors, the microenvironment was complicated, poorly organized vascular architecture, hypoxia, increased interstitial fluid pressure as well as the presence of the extracellular matrix (ECM) (3-5). These characteristics of the tumor microenvironment will significantly limit the delivery of drugs to the cells distant from the blood vessels, and then the efficiency of drug will be compromised. Compared with molecular drugs, the penetration behavior of the nanoparticles in vitro also depend on their properties including size (6), shape (7), surface charge (8), the stage of the cell cycle (9), and so on.
Different from cells in monolayer culture, cells in solid tumor are exposed to a microenvironment mentioned above and to a gradient of drug concentrations due to the distance from the blood vessels. In theory, direct *in vivo* evaluation of the penetration behavior of the nanoparticles is the most ideal way, owing to the pharmacokinetics of nanoparticles will significantly affect their accumulation in tumor tissue (10). Hence, *ex vitro* model system is more simple and practicable to evaluate drug penetration, and multicellular spheroid model is one of the most used methods. Multicellular spheroids are spherical aggregates of tumor cells, which reflect many properties of the microenvironment of solid tumor (2, 11-14). Recently, it has been reported that three-dimensional culture system may better reflect some *in vivo* aspects and can be used to further improve the understanding of the molecular mechanism of Herceptin® action (14). Herein, the breast cancer cell line MCF-7 tumor spheroid was developed, characterized, and used to qualitatively and quantitatively evaluate the penetration and retention behavior of nanoparticles in this study.

The enhanced permeation and retention (EPR) effect contributed to the passive tumor targeting of nanoparticles with the size of sub-100 nm (10, 15). The sizes of Doxil® and Abraxane® mentioned above are about 100 nm and 130 nm, respectively, and it was found that these nanoparticles dominantly located near the blood vessels (1). Hence, size of nanoparticles plays a critical role in their penetration and retention in tumor tissue (6, 10, 16, 17). The 50 nm nanoparticles including gold nanoparticles, silica nanoparticles, and polystyrene nanoparticles were most uptaken by monolayer
cells (18-22). However, the systematic evaluation of nanoparticles’ penetration into the monolayer cells, tumor spheroid, and tumor tissue in vivo was not well-understood until now. Gold nanoparticles have shown unique advantages in nanoparticle-based drug delivery systems due to the significant improvement in the synthesis of gold nanoparticles with easy fabrication, controllable size and shape, good biocompatibility, tunable surface functionalities and so on (7, 8, 10, 19, 20). Therefore, gold nanoparticles with different sizes and similar surface properties were selected in this work. Herein, we hypothesized that smaller nanoparticles have advantages over larger ones in terms of penetration behavior into not only the tumor monolayer cells but also the tumor tissue in vivo (Scheme 1).

To prove our hypothesis, we systematically evaluated whether or not the smaller gold nanoparticles were beneficial for penetration into cancer cells in monolayer cells in vitro, tumor spheroids ex vivo, and tumor tissues in vivo. Firstly, we prepared the gold nanoparticles with the size of 50 nm and 100 nm with identical surface coating of tiopronin (Au@tiopronin NPs). As we all know tiopronin is a thiol drug with good biocompatibility used to control the rate of cysteine precipitation and excretion in the disease of cystinuria (23). In addition, tiopronin is used as a stabilizing agent for metal nanoparticles, as its thiol groups can bind on the surface of nanoparticles to prevent coagulation (24). More importantly, the carboxyl groups of tiopronin can be modified with molecules, peptides, or drugs. In our previous work, Au@tiopronin NPs were functionalized with the targeted and therapeutic peptide showed enhanced cancer therapy (25). Then the MCF-7 tumor spheroid as an ex vivo model was developed and
characterized particularly to study the gold nanoparticles’ penetration and retention behavior. Furthermore, the pharmacokinetics and biodistribution of the nanoparticles in vivo were performed in the tumor-bearing mice.

Materials and methods

Chemicals. Gold chloride trihydrate (≥99.9%, HAuCl₄•3H₂O) and N-(2-Mercaptopropionyl) glycine (Tiopronin, C₅H₉NO₃S) were purchased from Sigma-Aldrich (St Louis, USA). L (+)-Ascorbic acid (99.0%) was supplied by Acros (USA). Sodium citrate (≥99.5%, C₆H₅Na₃O₇•2H₂O) was obtained from Solarbio (Beijing, China). Nitric acid and hydrogen peroxide of MOS grade were bought from Beijing Chemical Reagents Institute (China). Stock standard solution of Au (1000 μg/mL) was obtained from the National Analysis Center for Iron and Steel, Beijing, China. All glass wares used for the preparation and storage of gold nanoparticles were cleaned with aqua regia (HCl: HNO₃=3:1, v:v). All chemicals were used without further purification and Milli-Q water was used throughout this study.

Synthesis of 15 nm Au@citrate seed nanoparticles. 15 nm Au@citrate NPs were prepared by the standard citrate reduction method as reported (26). Briefly, 0.5 mL of HAuCl₄•3H₂O solution (1%, w/v) in 50 mL of Milli-Q water was heated to boiling and then 1.5 mL of sodium citrate solution (1%, w/v) was added to the boiling solution quickly with vigorous stirring. After the color change finished in 5 min, the mixture solution was kept boiling for another 15-30 min and then was allowed to cool down to room temperature with stirring. Finally, the 15 nm Au@citrate NPs were obtained, and the nanoparticles were kept as seed solution to synthesize the larger
gold nanoparticles with the size of 50 nm and 100 nm.

**Synthesis of 50 nm and 100 nm Au@citrate nanoparticles.** 50 nm and 100 nm Au@citrate NPs were synthesized using the seed growth method (27). Generally, 2.25 mL of 15 nm Au@citrate nanoparticle seed solution was mixed with 2.44 mL of HAuCl₄•3H₂O solution (10 mM) and then the mixture was diluted to 150 mL with Milli-Q water. Then, 100 mL of ascorbic acid solution (0.4 mM) was added to the above stirring solution at a rate of 10 mL/min with a peristaltic pump. Finally, the 50 nm Au@citrate NPs in purple red were obtained. For the preparation of 100 nm Au@citrate NPs, the method was the same as that of 50 nm Au@citrate NPs, only the volume of seed solution and HAuCl₄•3H₂O solution (10 mM) were 0.24 mL and 2.49 mL, respectively.

**Synthesis of 50 nm and 100 nm Au@tiopronin nanoparticles.** In our study, a surface molecule exchange reaction was adopted to obtain 50 nm and 100 nm Au@tiopronin NPs using the corresponding Au@citrate NPs. Briefly, the reaction was performed under stirring at 40°C for more than 48 h by mixing a certain volume of as-prepared Au@citrate NPs with an aqueous solution containing a large excess of tiopronin. When the reaction finished, both Au@citrate and Au@tiopronin NPs were centrifuged three times at 10000 rpm for 30 min to remove any residual salt, unbounded tiopronin and citrate.

In order to characterize all the gold nanoparticles mentioned above including 15 nm Au@citrate NPs, 50 nm and 100 nm Au@citrate NPs, and 50 nm and 100 nm Au@tiopronin NPs, some instruments including Tecnai G² 20 S-TWIN Transmission
Electron Microscope (TEM, Philips, Netherlands) with 200 kV acceleration voltage, Nano ZS Zetasizer (Malvern, England), X-ray photoelectron spectroscopy (XPS, ESCALAB250Xi X-ray photoelectron spectrometer, Thermo Fisher Scientific, USA), Lambda 950 UV/vis/NIR spectrophotometer (Perkin-Elmer, USA), X-ray diffraction (XRD, X'Pert PRO MPD X-ray diffractometer, PANalytical B.V., Netherlands) are used in this work.

**Uptake of gold nanoparticles by monolayer MCF-7 cells.** Human breast cancer cell line MCF-7 was maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% fetal bovine serum in a humidified atmosphere containing 5% CO₂ at 37 °C. MCF-7 cells were cultured in 6-well plates at about 60% confluence. And then medium was removed and 1 mL fresh medium containing 50 nm or 100 nm Au@tiopronin NPs at a dose of 1 nM was added to the wells. After 24 h incubation, cells were washed gently with 150 mM phosphate-buffered saline (PBS, pH7.4), digested with 0.25% trypsin-EDTA, centrifuged at 1000 rpm for 3 min, then collected and counted by Vi-CELL® (Beckman Coulter).

**MCF-7 multicellular spheroid culture.** MCF-7 spheroid was produced by the liquid overlay method as previously described (28). Briefly, cells were detached from monolayer and single cell suspension was transferred into the flat-bottomed 96-well plate pre-coated with 1% agarose (w/v) at 200 µL per well containing 600 cells. Afterwards, the cells were incubated for about 7 days in the condition as monolayer cells described above. In addition, the culture medium was partially (100 µL) replaced by fresh medium every other day.
Penetration behavior of gold nanoparticles into spheroids. For the 7-day-old spheroid at the seeding concentration of 600 cells per well, 100 μl medium was replaced by medium containing 50 nm or 100 nm Au@tiopronin NPs at a concentration of 2 nM, therefore the final treated concentration is 1 nM. After 24 h incubation, the spheroids were taken out using pipette, and gently washed with PBS for the following analysis. The distribution of gold nanoparticles in the spheroid was qualitatively observed using microscopy under bright field and dark field. The ultra-localization of gold nanoparticles in the outer and inner cells of the spheroid was evaluated by Bio-TEM (JEM-1400, JEOL, Japan). In addition, the Au content in every spheroid was quantified by ICP-MS (ELAN DRC-e Inductively Coupled Plasma Mass Spectrometer Perkin-Elmer, USA).

Pharmacokinetics and biodistribution of gold nanoparticles in tumor-bearing mice. Female balb/c nude mice (18-20 g) were purchased from Beijing Vital River Laboratories. All care and handling of animals were performed with the approval of the Animal Ethics Committee of the Medical School, Peking University. To establish the tumor-bearing mice, the mice were subcutaneously inoculated with 4×10^6 cells into the right flank of the mice. When the average volume of the tumor reached about 100 mm^3, the mice were randomly divided into groups for the study of pharmacokinetics and distribution of Au@tiopronin NPs.

For pharmacokinetics study, the tumor-bearing mice were intravenously injected with the 50 nm and 100 nm Au@tiopronin NPs at a dose of 5 mg Au/kg. About 30-100 μl blood were taken from the tail vein with a quantitative capillary at 10 min,
1 h, 3 h, 8 h, and 24 h after the administration. In addition, the nanoparticles were injected at the lower part of one tail vein, and the blood sample was taken at the other side of the tail vein, thereby protecting the sample from pollution. The blood volume was calculated as 0.0778 mL/g body weight, and the blood samples were used to measure Au content by ICP-MS. For the tissue distribution evaluation, tissues including tumor, heart, liver, spleen, lung, and kidney were removed at 24 h after injections, and part of collected tissues were used for ICP-MS measurement, Bio-TEM observation, and histological evaluation, respectively.

Result and discussion

Characterization of 50 nm and 100 nm Au@citrate nanoparticles. In order to obtain the 50 nm and 100 nm Au@citrate NPs, 15 nm Au@citrate NPs were firstly prepared as seeds. As shown in Supplementary Fig. S1A and B, the prepared 15 nm Au@citrate NPs showed uniform spherical morphology with the size of 14.8±0.6 nm and zeta-potential of -23.8 mV. In addition, the 15 nm Au@citrate NPs was red in color with the maximum adsorption at 519 nm as shown in the visible absorption spectrum (Supplementary Fig. S1C). Subsequently, both the 50 nm and 100 nm Au@citrate NPs were prepared using the 15 nm Au@citrate NPs as seeds. The results in Fig. 1 revealed that the size of the Au@citrate NPs were 49.8±6.6 nm and 101.1±13.8 nm and the zeta potential of the 50 nm and 100 nm Au@citrate NPs were -11.3 mV and -5.8 mV, respectively.

Characterization of 50 nm and 100 nm Au@tiopronin nanoparticles. The 50 nm and 100 nm gold nanoparticles coated with tiopronin were successfully obtained
in this study. Because of the higher binding affinity of thiol groups to gold in comparison with to the electrostatic interaction between gold nanoparticles and citrate, the 50 nm and 100 nm Au@tiopronin NPs were prepared by the surface exchange of tiopronin with corresponding Au@citrate NPs. In Fig. 2A, the size of Au@tiopronin NPs were 50.7±6.7 nm and 102.6±11.2 nm, which indicated that the size of the Au@tiopronin NPs were hardly changed after the surface exchange of tiopronin with corresponding Au@citrate NPs. Compared to the corresponding Au@citrate NPs in Fig. 2B, the zeta-potential of 50 nm and 100 nm Au@tiopronin NPs decreased from -11.3 mV to -38.8 mV and -5.81 mV to -40.0 mV, respectively. Zeta-potential of gold NPs mainly depends on the surface modification of the nanoparticles. For Au@citrate NPs, it was stabilized with citrate through electrostatic interaction between gold NPs and citrate. For Au@tiopronin NPs, it was stabilized with tiopronin through the formation of Au-S bond. There were free negatively charged carboxy group of tiopronin exposed on the outside surface of Au@tiopronin NPs. Hence, the zeta-potential of Au@tiopronin NPs was lower than that of Au@citrate NPs.

XPS was used to investigate the different element components on the surface of gold nanoparticles. Fig. 1C and Fig. 2C showed the atomic % of C, N, S and Au on the surface of the Au@citrate and Au@tiopronin NPs with the size of 50 nm and 100 nm, which indicated that the molar ratio of S to Au of the 50 nm, 100 nm Au@citrate NPs were both 0.02, whereas the ratio increased to 0.19 and 0.29 in the 50 nm and 100 nm Au@tiopronin NPs, respectively, demonstrating the thiol groups have been
bonded to the surface of the gold nanoparticles. The similar molar ratio of N to Au was also observed at the same time.

Optical absorption spectra of the gold nanoparticles are related to the shape, size, and dispersion of the gold nanoparticles. When the size of Au@citrate NPs were 49.8 and 101 nm, their visible absorption peaks observed were situated at 534 nm and 599 nm, respectively (Fig. 2D). In general, with increasing size of gold nanoparticles, the corresponding surface plasmon resonance peaks would shift toward longer wavelength (29). The Fig. 2D showed that after the surface exchange of tiopronin with corresponding Au@citrate NPs, the surface plasmon resonance peaks of the Au@tiopronin NPs essentially remained, which was consistent with the results shown in Fig. 1A and Fig. 2A. Meanwhile, 50 nm and 100 nm gold nanoparticles dispersed in pure water were purple red and yellow slurry in color. In a word, there was no significant difference in visible spectrum between Au@citrate and the corresponding Au@tiopronin NPs.

In another hand, XRD patterns of all the gold nanoparticles were able to be indexed to the cubic form of Au with PDF number of 04-0784 (cubic, a=b=c=0.2884 nm, d (111) = 0.2355 nm). As shown in Fig. 2E, there were five primary diffraction peaks with 38.187, 44.385, 64.576, 77.567 and 81.722, respectively, they could be correspondingly indexed to the crystal planes of (111), (200), (220), (311) and (222) of Au. This result demonstrated that the prepared material was gold definitely. Further observations showed that the peak shape was wider than that of the corresponding bulk material, which is one of the characteristics of nanocrystal materials, and the
particle is smaller, the corresponding XRD peak is wider (30). What’s more, the calculation based on the (111) peak line-width at half-maximum intensity roughly showed that the average size of the nanoparticles were about 50 and 100 nm.

All together, the above data demonstrated that we successfully prepared 50 nm and 100 nm Au@tiopronin NPs, which are well-dispersed in water and other relevant biological media including 0.9% saline, PBS, 5% glucose, cell-culture medium (Supplementary Fig. S2), spherical-shaped and stable for more than one month at 4°C. More importantly, the only difference among these nanoparticles used in this study is the size, which is required for the following studies.

_Uptake of gold nanoparticles by monolayer cells_. In this study, the concentration of gold nanoparticles was calculated from the concentration of Au by ICP-MS measurements for all the experiments according to the equation reported (19). First of all, the cell viability was investigated by the CCK-8 assay, and the data showed that exposure to the Au@tiopronin NPs for 24 h was not toxic to the MCF-7 cells in Supplementary Fig. S3, suggesting the good biocompatibility of the nanoparticles, and then MCF-7 cells were treated with the 50 nm and 100 nm Au@tiopronin NPs at a concentration of 1 nM. From the images taken under bright field in Fig. 3A, the uptake of 50 nm and 100 nm gold nanoparticles were obviously observed. However, after the quantitative analysis by ICP-MS, the profile of the number of gold nanoparticles in each cell versus size of gold nanoparticles indicated that cellular uptake of gold nanoparticles was in a size-dependent manner at a concentration of 1 nM nanoparticles with 24 h treatment in Fig. 3B, that is, more
uptake (more than 5 times) was observed for the 50 nm nanoparticle compared with that of 100 nm nanoparticle. And this result was in agreement with the reported maximum uptake by cells occurred for the 50 nm citrate stabilized gold nanoparticles (19). It was reported that only monomeric “glycovirus” (~50 nm) substantially entered cells via receptor-mediated endocytosis more efficiently than smaller or larger nanoparticles (31). Bio-TEM was performed to obtain the intracellular distribution of the gold nanoparticles after treatment, and the images in Fig. 3C showed that both 50 nm and 100 nm gold nanoparticles were localized in the cytoplasm in an aggregated state after treatment for 24 h, and the 50 nm gold nanoparticles in the cell observed were much more than 100 nm nanoparticles, which is in accordance with the ICP-MS results.

**Characterization of MCF-7 tumor spheroids.** Multicellular tumor spheroids as one of three-dimensional (3D) culture systems have been proved to have unique advantages over two-dimensional (2D) culture systems for cancer research (2, 12-14). Therefore, the spheroid of breast cancer cell (MCF-7) was developed as ex vivo tumor model in this study. To provide direct evidence of the morphology for the 3D culture, ESEM was performed to demonstrate that MCF-7 cells formed tightly packed and rounded spheroids as shown in Fig. 4A. In the cellular spheroid, the cells are spindle-shaped and the interactions between cells are very tight, which is similar to the in vivo tumor tissue, while different from the monolayer cells.

The cell condition in the spheroid was evaluated using qualitative HE staining and quantitative flow cytometry. And the results in Fig. 4B and C indicated that cells
in the 7-day-cultured spheroid were able to be divided into two groups. One group was metabolically active cells with larger size, stained with calcein AM by live/dead cell vitality assay kit, and mainly localized in the outer of spheroids in HE staining. Another group was apoptotic or necrotic cells with smaller size, stained with EthD-1, and distributed in the inner of spheroids. Furthermore, the cell condition in the 14-day-cultured spheroid was similar to that of the 7-day-old spheroid, only much more apoptotic and necrotic cells were observed with increasing the culture time from 7 days to 14 days.

From above, it demonstrated that the multicellular tumor spheroid used in this study was actually similar to part of the tumor tissue in vivo. Cells in the outer region of the spheroid were corresponding to the tumor tissue beside the blood vessel, and cells were proliferating with enough oxygen and nutrients. However, cells in the inner region were quiescent, similar to the tissue far away from the blood vessel due to decreased oxygen, nutrients, and pH (11, 13). Therefore, the tumor spheroid was a suitable model for evaluating the penetration behavior of gold nanoparticles.

**Penetration behavior of gold nanoparticles into tumor spheroids.** The penetration behavior of nanoparticles depends on their properties. For example, positive particles were taken up to a greater extent by proliferating cells, whereas negative particles were able to delivery drugs deep into tissues due to their quick diffuse ability (8). In order to enhance the nanoparticle delivery into the spheroid, RGD peptide-conjugated dendrimers were developed and significantly enhanced the entry of dendrimer/siRNA nanoparticles into U87 malignant glioma spheroid to
improve siRNA delivery, which presumably caused by the decreased integrin-binding affinity in the tumor model (32, 33). In this work, 50 nm and 100 nm Au@tiopronin NPs with the same surface properties were prepared, and this ensured the possibility of the size effect on the penetration behavior in tumor spheroid.

From images in Fig. 5A, the black spheroids after treatment with 50 nm and 100 nm gold nanoparticles at 1 nM for 3 h and 24 h were observed, which is caused by the gold nanoparticles surrounding the spheroids compared to the spheroid without treatment. In order to confirm the exact location site of the gold nanoparticles in the spheroid, HE staining and the corresponding dark field microscopy observation were carried out. As shown in Fig. 5B, gold nanoparticles including 50 nm and 100 nm were primarily localized at the periphery of the spheroid at 3 h after treatment, and the penetration of 50 nm nanoparticles increased with prolonging incubation time from 3 h to 24 h. However, 100 nm gold nanoparticles were hindered outside of the tumor spheroid.

In addition, the quantitative analysis of ICP-MS data demonstrated the number of gold nanoparticles in each spheroid in Fig. 5C. After 24 h treatment, more penetration (more than 4 times) in the spheroids was observed at the nanoparticle size of 50 nm compared to that at the size of 100 nm. And the content of gold nanoparticles in spheroid increased while prolonging the incubation time from 3 h to 24 h for 50 nm gold nanoparticles, but not for 100 nm nanoparticles. This result is consistent with the report that penetration of the nanoparticles into the spheroid core was limited to particles smaller than 100 nm (34). As mentioned above, ECM presents a transport
barrier that restricts nanoparticle penetration, and collagenase-coated 100 nm carboxylated polystyrene nanoparticles showed a 4-fold increase in the number of particles delivered to the spheroid core compared with the untreated nanoparticles due to the site-specific degradation of ECM proteins (34). Bio-TEM was used to evaluate the distribution of these nanoparticles in the spheroid in terms of micro-structure, the images in Fig. 5D showed that the location site of 50 nm and 100 nm gold nanoparticles was only the several layers of cells outside of the spheroid, and they were found only in the cytoplasm of each cell.

**Pharmacokinetics and biodistribution of gold nanoparticles in tumor-bearing mice.** Many kinds of nanoparticles for drug delivery system are around 100 nm in diameter due to the EPR effect by the leaky vasculature and poor lymphatic drainage associated with solid tumors (15, 35). The penetration of the nanoparticles from the blood into the tumor tissues primarily depend on the diffusion and convection through endothelial fenestræ (36, 37). And the high cell density, presence of ECM and interactions with cells also decreases the diffusion of nanoparticles into the tumor compartment. In order to improve the accumulation of the nanoparticles in the tumor, active targeting can be used to increase cellular internalization and reduce the clearance of the nanoparticles from the tumor interstitium (38).

To evaluate the accumulation behavior of the 50 nm and 100 nm gold nanoparticles in the tumor tissue *in vivo*, the tumor-bearing mice was developed. At first, the pharmacokinetics behavior of the nanoparticles were investigated after a single intravenous injection at a dose of 5 mg Au/kg, and the data indicated that all
The nanoparticles were eliminated rapidly from blood (Fig. 6A). The concentration of Au in blood decreased to 11.0 μg/ml at 10 min, 1.9 μg/ml at 8 h and 0.2 μg/ml at 24 h for 50 nm nanoparticles. To some extent, 100 nm nanoparticles cleared more quickly from the blood than 50 nm nanoparticles. The pharmacokinetics of the nanoparticles can be influenced by many factors mainly including the size and surface properties of the nanoparticles which affect the extent of opsonization and clearance. In addition, presence of a non-ionic, steric stabilizing hydrophilic polymer like polyethelene glycol (PEG) has been shown to prolong the circulation time of the nanoparticles by avoiding opsonization and subsequent clearance by the mononuclear phagocytic system (MPS) (38). Gold nanoparticles used in this study were coated with tiopronin, they were opsonized easily by the proteins and then cleared from blood.

In terms of the accumulation of gold nanoparticles in tumor, more amount of Au was found after 24 h administration for 50 nm NPs (0.42 μg/g), the less for 100 nm NPs of 0.14 μg/g (Fig. 6B). For other tissues, 50 nm and 100 nm Au nanoparticles accumulated dominantly in liver and spleen with a total percentage of more than 80% of the injected dose. Furthermore, only little amount of nanoparticles distributed in the tissues of lung and heart (Supplementary Table S1). After the quantitative analysis of Au content in the tissue, HE staining and corresponding dark field analysis were also performed to observe the site where gold nanoparticles were distributed. As displayed in Fig. 6C and D, 50 nm gold nanoparticles showed a little more amount than 100 nm gold nanoparticles in tumor tissue, and a non-homogeneous distribution of the gold nanoparticles was observed. It is speculated that the nanoparticles mainly distributed
beside the vessel, especially for the larger 100 nm gold nanoparticles. For liver and spleen, the distribution of gold nanoparticle is more obvious. However, no obvious gold nanoparticles were observed in other tissues including kidney, lung, and heart (Supplementary Fig. S4).

Recently, researchers have gradually realized that the penetration of the nanoparticles into tumor is definitely important for the delivery system and it should not be neglected. In order to improve the nanoparticle delivery into tumors, functional nanoparticles, in which the size of the nanoparticles shrank from 100 nm to 10 nm triggered by the matrix metalloproteinases (MMP) overexpressed in the tumor microenvironment, exhibited both the long circulation half-life necessary for the EPR effect and the deep tumor penetration for the delivery into the tumor tissues in vivo (17). In addition, a novel approach of the non-covalent drug delivery by PEGylated Au NPs demonstrated surprisingly efficient drug release and deep penetration into the center of tumor tissues (39). Taken together, the size of the nanomedicines critically affects the penetration and efficacy of the drugs in the tumors.

Conclusion

In summary, our work provides new insights into the size effect of gold nanoparticles of 50 nm and 100 nm on the localization and penetration behavior via 2D-culture in vitro, 3D-culture of multicellular tumor spheroid ex vivo, and xenograft tumor model in vivo. Although the tumor microenvironment significantly limited the deep penetration of nanoparticles into tumor tissue, the smaller gold nanoparticles (50 nm) demonstrated more advantages over the larger nanoparticles (100 nm) in the
uptake and permeability in tumor tissues. Researchers have placed great importance on the prolonged circulation and altered biodistribution of the nanoparticle-based drug delivery systems, however, the tumor uptake and tissue penetration of the nanoparticles is another key issue for exerting effective cancer therapy. More importantly, this work reveals that smaller nanoparticles are more effective than larger particles at delivering drugs to tumor cells, with implications for many kinds of cancer nanotherapy studies.

**Supplementary data** Methods including Bio-TEM observation of spheroids and tumors, ICP-MS, HE staining, Flow cytometry and ESEM observation of MCF-7 spheroids and monolayer cells. Characterization of 15 nm Au@citrate nanoparticles. Characterization of 50 nm and 100 nm Au@tiopronin NPs in relevant biological media. Cytotoxicity of MCF-7 monolayer cells after treated with 50 nm and 100 nm Au@tiopronin nanoparticles for 24 h. Images of tissues after HE staining, and the corresponding tissue’s images taken under dark field. Average Au concentration in different organs (μg Au/g tissue) after 24 h administration of 50 nm and 100 nm Au@tiopronin nanoparticles.

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Figure Legends

**Scheme 1.** Schematic illustration of the localization and penetration behavior of 50 nm and 100 nm Au@tiopronin nanoparticles in monolayer cancer cells *in vitro*, multicellular tumor spheroid *ex vivo*, and xenograft tumor model *in vivo*.

**Figure 1.** Characterization of 50 nm and 100 nm Au@citrate nanoparticles. (A) TEM images and the corresponding size histograms of as-synthesized 50 nm and 100 nm gold nanoparticles coated with citrate. (B) Zeta-potential of the above nanoparticles. (C) XPS graph of the Au@citrate nanoparticles for 50 nm and 100 nm, which showed the atomic % of C, N, S and Au in the nanoparticles.

**Figure 2.** Characterization of 50 nm and 100 nm Au@tiopronin nanoparticles. (A) TEM images and the corresponding size histograms of as-synthesized 50 nm and 100 nm gold nanoparticles coated with tiopronin. (B) Zeta-potential of the 50 nm and 100 nm Au@tiopronin nanoparticles. (C) XPS graph of the Au@tiopronin nanoparticles for 50 nm and 100 nm. (D) Visible spectrum of the Au@citrate and Au@tiopronin nanoparticles with the size of 50 nm and 100 nm at the wavelength between 400 nm and 800 nm, and the corresponding photographs of nanoparticles dispersed in pure water. (E) XRD patterns of as-synthesized Au@citrate and Au@tiopronin nanoparticles.

**Figure 3.** Cellular uptake of the 50 nm and 100 nm Au@tiopronin nanoparticles after incubation for 24 h at a concentration of 1 nM gold nanoparticles in the MCF-7 cells. (A) Images under bright field after treatment with the gold nanoparticles at a dose of 1
nM nanoparticle. (B) Quantitative gold nanoparticles uptaken by each cell measured by ICP-MS. (C) Representative TEM micrographs of the cellular uptake of the 50 nm and 100 nm Au@tiopronin nanoparticles.

**Figure 4.** Characterization of MCF-7 multicellular tumor spheroid. (A) Morphology of both the monolayer cells and the 7-day-old spheroids at the seeding concentration of 600 cells per well observed by ESEM. (B) Images of the spheroids cultured for 7 days and 14 days at the seeding concentration of 400 or 1600 cells per well after HE staining. (C) Cell conditions including live or dead cells in the spheroid with growth time of 7 days and 14 days at the seeding concentration of 800 cells each well analyzed by flow cytometry.

**Figure 5.** Penetration behavior of the 50 nm and 100 nm Au@tiopronin nanoparticles into the MCF-7 tumor spheroid at a concentration of 1 nM for 3 h and 24 h. (A) Images under bright field after treatment with the gold nanoparticles. (B) Images after HE staining and under dark field after culture with the nanoparticles. (C) ICP-MS analysis of the number of Au@tiopronin nanoparticles in each treated spheroid. (D) Representative TEM images including the external and internal region of the treated spheroid for 24 h.

**Figure 6.** Blood elimination profiles and biodistribution of the 50 nm and 100 nm Au@tiopronin nanoparticles at a dose of 5 mg Au/kg after intravenous administration. (A) Blood elimination profiles of Au following a single intravenous injection of 50 nm and 100 nm Au@tiopronin nanoparticles at a dose of 5 mg Au/kg in the
tumor-bearing mice. Data represent mean±S.D. (n=3) (B) Au content in the tissues including tumor, heart, liver, spleen, lung, and kidney at 24 h after i.v. injections of gold nanoparticles. Data represent mean±S.D. (n=3) (C) Images of tumor, liver, and spleen after HE staining and the corresponding images observed under dark field. (D) Bio-TEM images of the tumor tissue taken 24 h after administration of the Au@tiopronin nanoparticles.
Figure 1

- 50 nm Au@tiopronin NPs
- 100 nm Au@tiopronin NPs
Figure 3

50 nm Au@tiopronin NPs

100 nm Au@tiopronin NPs

A: TEM

B: Zeta-potential

C: XPS

D: UV-vis

E: XRD

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Figure 6

A

control  50 nm Au@tiopronin NPs  100 nm Au@tiopronin NPs

3 h

24 h

B

3 h

24 h

C

Number of gold nanoparticles per spheroid (10^6)

3 h

24 h

50 nm NPs  100 nm NPs

D

50 nm Au@tiopronin NPs

100 nm Au@tiopronin NPs

100 nm Au@tiopronin NPs
Superior penetration and retention behavior of 50 nm gold nanoparticles in tumors

Huo Shuaidong, Huili Ma, Keyang Huang, et al.

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