Hyaluronic acid-modified manganese-chelated dendrimer-entrapped gold nanoparticles for the targeted CT/MR dual-mode imaging of hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) is the most common malignant tumor of the liver. The early and effective diagnosis has always been desired. Herein, we present the preparation and characterization of hyaluronic acid (HA)-modified, multifunctional nanoparticles (NPs) targeting CD44 receptor-expressing cancer cells for computed tomography (CT)/magnetic resonance (MR) dual-mode imaging. We first modified amine-terminated generation 5 poly(amidoamine) dendrimers (G5.NH2) with an Mn chelator, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), fluorescein isothiocyanate (FI), and HA. Then, gold nanoparticles (AuNPs) were entrapped within the above raw product, denoted as G5.NH2-FI-DOTA-HA. The designed multifunctional NPs were formed after further Mn chelation and purification and were denoted as [(Au0)100G5.NH2-FI-DOTA(Mn)-HA]. These NPs were characterized via several different techniques. We found that the [(Au0)100G5.NH2-FI-DOTA(Mn)-HA] NPs exhibited good water dispersibility, stability under different conditions, and cytocompatibility within a given concentration range. Because both AuNPs and Mn were present in the product, [(Au0)100G5.NH2-FI-DOTA(Mn)-HA] displayed a high X-ray attenuation intensity and favorable r1 relaxivity, which are advantageous properties for targeted CT/MR dual-mode imaging. This approach was used to image HCC cells in vitro and orthotopically transplanted HCC tumors in a unique in vivo model through the CD44 receptor-mediated endocytosis pathway. This work introduces a novel strategy for preparing multifunctional NPs via dendrimer nanotechnology.

Hepatocellular carcinoma (HCC) is the most common malignant tumor of the liver and has the sixth highest incidence rate and the second highest mortality rate of malignant tumors worldwide. Nevertheless, as this condition is difficult to diagnose early, it is often treated late, which leads to low survival rates. Rapid developments in molecular imaging technology over the last decade have allowed the collection of highly sensitive and specific information regarding physiological and pathological processes for the accurate diagnosis of disease. Among current clinical imaging techniques, computed tomography (CT) is considered to be one of the most convenient because of its wide availability, speed, efficiency, and inexpensiveness. CT is able to present images with high...
spatial and density resolution, and this technique can provide valuable tomographic information regarding anatomical structures\textsuperscript{12-14} and functional information\textsuperscript{15-17} through the use of high-resolution 3-dimensional (3D) technology\textsuperscript{12,18,19}. Meanwhile, magnetic resonance (MR) imaging is one of the most powerful noninvasive medical imaging techniques with good spatial resolution and high sensitivity\textsuperscript{20,21}, which can provide superior anatomical detail and tomographic information, especially in soft tissues\textsuperscript{22,23}.

However, due to the limitations of individual techniques, only combinations of different techniques can provide more accurate and comprehensive diagnostic data\textsuperscript{24-28}. Therefore, it is essential to develop various contrast agents for dual- or multimode imaging applications\textsuperscript{26-28}. Conventional, small-molecule contrast agents have serious shortcomings, such as a lack of specificity, short half-life, and risk of nephrotoxicity at relatively high concentrations. Fortunately, superior nanoparticles (NPs) have recently been developed, including NPs with the ability to specifically target tumor cells\textsuperscript{9,26-32}.

Previous studies have described the synthesis of different types of CT/MR dual-mode imaging contrast agents\textsuperscript{26-28}. Despite the many advantages of iron as a contrast agent for T\textsubscript{2}-negative MR imaging\textsuperscript{28}, T\textsubscript{1} contrast agents consisting of gadolinium (Gd)/chelator complexes are the commercially available MR contrast agents used in clinical settings\textsuperscript{33,34}. However, Gd ions can become highly toxic if they dissociate from NPs or complexes in vivo, thus resulting in severe renal damage, e.g., nephrogenic systemic fibrosis\textsuperscript{35}. Due to the safe excretion of intracellular manganese (Mn), cytotoxicity issues related to transmetallation and bioretention are considered nominal provided that Mn remains chelated in the bolus/vascular phase\textsuperscript{36}. In addition, as Mn ions are highly magnetized, Mn is a compatible material for MR imaging\textsuperscript{37}. Different molecular probes have incorporated Mn to facilitate the specific delivery of probes to cancer cells and achieve targeted molecular imaging via MR\textsuperscript{38-41}.

Meanwhile, several reports have described the application of NPs in CT/MR dual-mode imaging to evaluate subcutaneous transplanted tumors in vivo\textsuperscript{26-28}. The orthotopic tumor transplantation model is known to represent physiological and pathological processes better than the subcutaneous tumor transplantation model. Hence, we aimed to synthesize a novel NP-based CT/T\textsubscript{1} MR imaging contrast agent for in vivo use in the orthotopic HCC model.

In some cancers, CD44 is a major cell surface marker of progression and metastasis\textsuperscript{42}. Certain established noninvasive cell lines and normal cell types do not express CD44 receptor\textsuperscript{43,44}. Metastatic cancer growth is closely related to interactions between the tumor stroma and the microenvironment involving CD44\textsuperscript{43,44}. Moreover, hyaluronic acid (HA) is a molecule that can specifically bind to CD44 receptors in a highly efficient manner\textsuperscript{45}. This important characteristic of HA, as well as its biocompatibility, biodegradability, and easily modified structure, makes HA-based materials extremely attractive for applications such as the tumor-targeted delivery of imaging agents and the diagnosis and treatment of cancer\textsuperscript{46-50}.

In this study, 2,2′,2″-(10-(2-(2,5-dioxopyrrolidin-1-yloxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl) triacetic acid (DOTA-NHS) and fluorescein isothiocyanate (FI) were conjugated to the surface of G5.NH\textsubscript{2} dendrimers, and targeting HA ligands were also coupled onto the dendrimer surface via 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) coupling chemistry. Then, the product (i.e., G5.NH\textsubscript{2}-FI-DOTA-HA) was used as a template to entrap AuNPs and chelate Mn. HA-targeted Mn-loaded dendrimer-entrapped AuNPs (i.e., {(Au\textsuperscript{0})\textsubscript{100}G5.NH\textsubscript{2}-FI-DOTA(Mn)-HA}) were formed (Fig. 1) without acetylation of the remaining terminal amines of dendrimers. The developed {(Au\textsuperscript{0})\textsubscript{100}G5.NH\textsubscript{2}-FI-DOTA(Mn)-HA} NPs were characterized via \textsuperscript{1}H nuclear magnetic resonance (NMR) spectroscopy, dynamic light scattering (DLS), transmission electron microscopy (TEM), and inductively coupled plasma-atomic emission spectroscopy (ICP-AES). The cytocompatibility of the NPs was evaluated using a cytotoxicity assay and cell morphology observations. Furthermore, the formed {(Au\textsuperscript{0})\textsubscript{100}G5.NH\textsubscript{2}-FI-DOTA(Mn)-HA} NPs were applied in the CT/MR dual-mode imaging of HCCLM3 cells in vitro and orthotopically transplanted HCC tumors in vivo.
**Experimental Materials.** Ethylenediamine core amine-terminated G5.NH₂ poly(amidoamine) dendrimers with a polydispersity index (PDI) less than 1.08 were purchased from Dendritech (Midland, MI). DOTA-NHS was purchased from Chematech (Dijon, France). HA with molecular weight of 6 kDa, sodium hydroxide, EDC, and N-hydroxysuccinimide (NHS) were purchased from J&K Chemical Ltd. (Shanghai, China). HCCLM3 cells (an HCC cell line) were obtained from the Liver Cancer Institute, Zhongshan Hospital, Fudan University (Shanghai, China). Fetal bovine serum (FBS), streptomycin, penicillin, and Dulbecco's modified Eagle medium (DMEM) were obtained from Hangzhou Jinuo Biomedical Technology (Hangzhou, China). Triethylamine, acetic anhydride, HauCl₄·4H₂O, and all other chemicals and solvents were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). The water used in all experiments was purified by a Milli-Q Plus 185 water purification system (Millipore, Bedford, MA) with a resistivity greater than 18 MΩ cm. Regenerated cellulose dialysis membranes (MWCO = 14,000 or 1,000) were acquired from Thermo Fisher Scientific (Pittsburgh, PA).

**Synthesis of ([Au₀]₁₀₀G₅.NH₂-FI-DOTA(Mn)-HA) NPs.** In all, 35 molar equivalents of DOTA-NHS (26.25 mg, 35 μmol) dissolved in DMSO (10 mL) were added dropwise into a DMSO solution of G₅.NH₂ dendrimers (26 mg, 1 μmol, 10 mL), forming the raw G₅.NH₂-DOTA product after vigorous magnetic stirring for 1 d. Then, 20 molar equivalents of HA (120.49 mg, 20 μmol) preactivated by NHS (11.91 mg, 100 μmol, in 1 mL of DMSO) and EDC (19.11 mg, 100 μmol, in 1 mL of DMSO) were added dropwise into the aforementioned G₅.NH₂-DOTA solution to form the raw G₅.NH₂-DOTA-HA product after vigorous magnetic stirring for 3 d. After 1 mL of DMSO was then conjugated to G₅.NH₂-DOTA-HA to form the raw G₅.NH₂-FI-DOTA-HA product after vigorous magnetic stirring for 1 d.

The procedure used to synthesize ([Au₀]₁₀₀G₅.NH₂-FI-DOTA(Mn)-HA) was similar to what has been previously reported, with slight modifications. In brief, 100 molar equivalents of HauCl₄·4H₂O in an aqueous solution (30 mg/mL, 2.78 mL) were added into the G₅.NH₂-FI-DOTA-HA aqueous solution mentioned above under vigorous stirring for 30 min. An ice-cold aqueous NaBH₄ solution (18.92 mg, 0.5 mmol, in 1 mL of H₂O) with a 5-fold molar excess of Au salt was added into the aforementioned solution under vigorous stirring for 2 h. Then, 70 molar equivalents of MnSO₄·4H₂O in an aqueous solution (13.52 mg, in 1 mL of H₂O) were added dropwise into the aforementioned G₅ dendrimer solution under vigorous stirring for 24 h to synthesize the targeted ([Au₀]₁₀₀G₅.NH₂-FI-DOTA(Mn)-HA) NPs. Extensive dialysis was applied to remove the excess reactants and by-products in the reaction mixture, and subsequent lyophilization was used to obtain the final product.

The small positive surface charge of the ([Au₀]₁₀₀G₅.NH₂-FI-DOTA(Mn)-HA) NPs allowed the commonly used acetylation step to be omitted. In the control group, the CD44 receptors were blocked with free HA at a concentration of 25 mM for half an hour before the targeted NPs were injected via the tail vein.

**Characterization techniques.** DLS and zeta potential measurements were conducted using a Malvern Zetasizer Nano ZS model ZEN3600 (Worcestershire, U.K.) with a standard 633-nm laser. Prior to taking the measurements, the samples were dissolved in water (0.1 mg/mL). We used TEM (JEOL 2010F, Tokyo, Japan) with an accelerating voltage of 200 kV to characterize the morphology of the ([Au₀]₁₀₀G₅.NH₂-FI-DOTA(Mn)-HA) NPs. TEM samples were prepared by depositing a dilute NP suspension (5 μL) onto carbon-coated copper grids and allowing it to air-dry before observation. The TEM image selections were random, and the average size and size distribution of more than 300 NPs were recorded using ImageJ software. ICP-AES (Leeman Prodigy, USA) was employed to analyze the composition of Au and Mn within the multifunctional NPs. A GE LightSpeed VCT imaging system (GE Medical Systems) was used for CT scanning at 80 mA, 100 kV, and a slice thickness of 0.625 mm. Solutions of ([Au₀]₁₀₀G₅.NH₂-FI-DOTA(Mn)-HA) NPs (0.2 mL) with different concentrations of Au were prepared in 2.0-mL Eppendorf tubes, and the CT value (Hounsfield units, HU) of each sample was measured. The concentration of Au in each sample ranged from 0.01 to 0.12 mM. T₁ relaxometry of {(Au₀)₁₀₀G₅.NH₂-FI-DOTA(Mn)-HA} NPs (1 mL) with different concentrations of Mn were prepared in 2-mL Eppendorf tubes by diluting the NP suspensions with water containing an Mn concentration ranging from 0–100 μM. The following system parameters were used: repetition time (TR) = 500 ms; echo time (TE) = 25 ms; slice thickness = 2 mm; matrix = 256 × 256; field of view (FOV) = 120 mm; and excitation number = 1. T₁ relaxivity (r₁) was determined by linearly fitting 1/T₁ (s⁻¹) as a function of the Mn concentration (mM).

**Cytotoxicity assay and cell morphology observations.** An MTT assay was applied to measure the in vitro cytotoxicity of the ([Au₀]₁₀₀G₅.NH₂-FI-DOTA(Mn)-HA) NPs in a routine culture environment with 10% FBS (heat-inactivated, 1% penicillin-streptomycin) and 5% CO₂ at 37°C. HCCLM3 cells suspended in medium were seeded in a 96-well plate at a density of 1 × 10⁴ cells/well with 200 μL per well and were incubated overnight. NPs with an Mn concentration ranging from 0–100 μg/mL were added into each well, and the cells were incubated for an additional 24 h. The mixture was then carefully removed, and the cells were washed twice with phosphate-buffered saline (PBS). Then, 20 μL of MTT solution (5 mg/mL in PBS) was added into each well, and the cells were cultured for another 4 h at 37°C and 5% CO₂. To dissolve the insoluble formazan crystals, the medium was carefully discarded and replaced with 200 μL of DMSO. Finally, the absorbance of each sample was measured at 570 nm using a Thermo Fisher Scientific Multiskan MK3 ELISA reader (Thermo Fisher Scientific, Hudson, NH).

The morphology of the HCCLM3 cells was observed to evaluate the cytotoxicity of the ([Au₀]₁₀₀G₅.NH₂-FI-DOTA(Mn)-HA) NPs after the cells were treated with the NPs at Mn concentrations of 0, 10, 20, 50, 75, and 100 μg/mL for 24 h. A Leica DM IL LED inverted phase-contrast microscope was employed to observe the cell morphology of each sample at a magnification of 200×.
Table 1. The zeta potential and hydrodynamic size of the \( \text{(Au}\)\textsubscript{100}G5.NH\textsubscript{2}-FI-DOTA(Mn)-HA) NPs.

| Sample         | G5.NH\textsubscript{2}-FI-DOTA-HA | \( \text{(Au}\)\textsubscript{100}G5.NH\textsubscript{2}-FI-DOTA(Mn)) | \( \text{(Au}\)\textsubscript{100}G5.NH\textsubscript{2}-FI-DOTA(Mn)-HA) |
|----------------|----------------------------------|---------------------------------------------------------------|---------------------------------------------------------------|
| Zeta potential (mV) | +6.4 ± 0.6                       | +24.7 ± 0.3                                                 | +6.1 ± 0.7                                                 |
| Hydrodynamic size (nm) | 189.2 (PDI = 0.219)              | 196.5 (PDI = 0.221)                                         | 245.3 (PDI = 0.293)                                         |
| Medium         | Water                            | PBS                                                          | PBS                                                          |
|                | 187.5 (PDI = 0.231)              | 190.1 (PDI = 0.245)                                         | 238.7 (PDI = 0.315)                                         |

**In vitro cellular uptake assay.** The cellular uptake of the \( \text{(Au}\)\textsubscript{100}G5.NH\textsubscript{2}-FI-DOTA(Mn)-HA) NPs in vitro was assessed by flow cytometry. HCCLM3 cells, which highly express CD44, were used for this experiment. Cells were seeded into each well of a 12-well plate at a density of \( 2 \times 10^5 \) cells/well and incubated with 2 mL of DMEM overnight. NPs with Mn concentrations of 10, 25, 75, and 100 \( \mu \text{g/mL} \) were added into the wells, and the cells were incubated for an additional 4 h. Cells treated with PBS and cells treated with HA followed by NPs were used as controls.

**In vivo CT/MR imaging.** All animal experiments were approved by the ethical committee of Zhongshan Hospital. A subcutaneous HCCLM3 tumor transplantation model was established in 6-week-old BALB/c nu/nu male mice (SLAC, Shanghai, China) via the subcutaneous injection of HCCLM3 cells (\( 5 \times 10^6 \) cells in 150 \( \mu \text{L} \) of PBS). When the maximum diameter of the subcutaneous tumors reached 8 mm, the tumor-bearing mice were euthanized. Then, each subcutaneous tumor was removed surgically, and the non-necrotic tumor tissue was cut into small pieces (1 mm\(^3\)) for further orthotopic implantation into the livers of a different set of 6-week-old male mice, thereby establishing the orthotopic HCCLM3 tumor model.

During the operation, the mice were anesthetized by an intraperitoneal injection of pentobarbital sodium (40 mg/kg). Three weeks after the orthotopic tumor implantation, the mice underwent CT/MR scanning. Then, a solution of \( \text{(Au}\)\textsubscript{100}G5.NH\textsubscript{2}-FI-DOTA(Mn)-HA) NPs (0.3 mL, \[\text{Au}] = 120 \text{mM}) in PBS was administered to the mice via a tail vein injection. CT scans were performed at 0 min, 30 min, 1 h, and 2 h post-injection using a GE LightSpeed VCT system. A GE Advantage Workstation 4.5 (AW4.5) was used for image reconstruction. MR scanning was performed at 0 min, 30 min, 1 h, and 2 h post-injection using a 3.0-T Magnetom Verio MR system with a wrist receiver coil and the following parameters: TR/TE = 582/24 ms; FOV = 120 mm; FOV phase = 100%; matrix = 416 × 416; and slice thickness = 1.5 mm. Three mice were included in each group.

**In vivo biodistribution of \( \text{(Au}\)\textsubscript{100}G5.NH\textsubscript{2}-FI-DOTA(Mn)-HA) NPs.** The tumor-bearing mice were used to study the in vivo biodistribution of the \( \text{(Au}\)\textsubscript{100}G5.NH\textsubscript{2}-FI-DOTA(Mn)-HA) NPs. The mice were first anesthetized by an intraperitoneal injection of pentobarbital sodium (40 mg/kg). Then, \( \text{(Au}\)\textsubscript{100}G5.NH\textsubscript{2}-FI-DOTA(Mn)-HA) NPs (0.3 mL in PBS solution, \[\text{Au}] = 120 \text{mM}) were injected via the tail vein; the mice were euthanized at 24 h post-injection. The major organs, i.e., liver, spleen, kidneys, lungs, and heart, were surgically removed along with the tumor and weighed. The tissues were cut into small pieces (1-2 mm\(^3\)) for aqua regia digestion (nitric acid:hydrochloric acid = 1:3 (v/v)). The Au content was measured by ICP-AES.

**Statistical analysis.** The experimental data were analyzed by a single-factor analysis (one-way ANOVA). The results were considered significant at \( P < 0.05 \); (*), (**) and (***)) were used to indicate \( P < 0.05 \), \( P < 0.01 \), and \( P < 0.001 \), respectively.

**Results and Discussion**

**Synthesis and characterization of \( \text{(Au}\)\textsubscript{100}G5.NH\textsubscript{2}-FI-DOTA(Mn)-HA) NPs.** In this study, \( \text{(Au}\)\textsubscript{100}G5.NH\textsubscript{2}-FI-DOTA(Mn)-HA) NPs were formed using an NaBH\textsubscript{4} reduction route, as previously reported\textsuperscript{26,27}. Both AuNPs and Mn were integrated into one multifunctional NP platform for CT/MR dual-mode imaging applications. First, DOTA-NHS was covalently linked to the surface of G5.NH\textsubscript{2} dendrimers. Then, HA and FI were coupled to the dendrimers separately. AuNPs were then entrapped by G5.NH\textsubscript{2}-FI-DOTA(Mn)-HA NPs via the same method to calculate that approximately 13.6 HA and 3.7 FI moieties were linked to each G5 dendrimer (Figure S1b,c). The zeta potential and hydrodynamic size of the \( \text{(Au}\)\textsubscript{100}G5.NH\textsubscript{2}-FI-DOTA(Mn)-HA) NPs were recorded (Table 1). The final product clearly exhibited a slight positive charge (+6.1 mV) due to the HA coupled to the dendrimer terminal amines and the omission of acetylating the remaining dendrimer terminal amines\textsuperscript{26,27}. The small amount of positive charge on the NP surface may have been caused by some of the G5 amines being used to stabilize the AuNPs. DLS was used to assess the hydrodynamic size of the developed NPs dissolved in water (Table 1). The \( \text{(Au}\)\textsubscript{100}G5.NH\textsubscript{2}-FI-DOTA(Mn)-HA) NPs clearly displayed a hydrodynamic size of 245.3 nm. The NPs exhibited both an acceptable PDI and excellent colloidal stability.

The morphology and size of the \( \text{(Au}\)\textsubscript{100}G5.NH\textsubscript{2}-FI-DOTA(Mn)-HA) NPs were observed by TEM (Fig. 2). The formed NPs were nearly spherical in shape (Fig. 2a) and had a mean diameter of 2.1 nm with a relatively uniform size distribution (Fig. 2b). The size measured by DLS was larger than that measured by TEM, likely because DLS characterizes the size of \( \text{(Au}\)\textsubscript{100}G5.NH\textsubscript{2}-FI-DOTA(Mn)-HA) NP clusters in an aqueous solution, whereas TEM reveals the size of individual AuNPs\textsuperscript{26-28}.
Stability. The stability of these NPs is essential for their biomedical application. To evaluate the stability of the prepared \([\text{Au}^0]_{100}\text{G5.NH}_2\text{-FI-DOTA(Mn)-HA}\) NPs, the NPs were dissolved in different solutions (i.e., water, PBS, or cell culture medium). After the NPs were stored for one month at room temperature, no precipitate was observed (Figure S2). Meanwhile, as described in the literature, we also measured the hydrodynamic size of the NPs after 7 days of storage at room temperature. The hydrodynamic diameter of the NPs was 266.5 nm, which was not significantly different from the value observed before storage.

T1 relaxometry. The paramagnetic Mn complexes render the NPs suitable for MR imaging. A 0.5-T Mini MR system (Niumeg, Shanghai, China) was used to calculate the longitudinal relaxation time (T1) of the \([\text{Au}^0]_{100}\text{G5.NH}_2\text{-FI-DOTA(Mn)-HA}\) NPs by measuring the MR signal intensity of aqueous NP solutions with different Mn concentrations (i.e., water, PBS, or cell culture medium). After the NPs were stored for one month at room temperature, no precipitate was observed (Figure S2). Meanwhile, as described in the literature, we also measured the hydrodynamic size of the NPs after 7 days of storage at room temperature. The hydrodynamic diameter of the NPs was 266.5 nm, which was not significantly different from the value observed before storage.
X-ray attenuation measurements. The \( ([\text{Au}^0]_{100}\text{G5.NH}_2-\text{FI-DOTA(Mn)-HA}) \) NPs could be used for CT imaging because of presence of the AuNPs. As previously reported by Cao et al.\textsuperscript{52}, Au is superior to iodine in terms of X-ray attenuation (e.g., Omnipaque) because Au has a higher atomic number. Therefore, AuNPs have been widely applied in CT contrast agents. The results clearly show that the CT value increases as the Au concentration increases (Fig. 3c,d). By linearly fitting the attenuation intensity versus the Au concentration, a dose-dependent relation was obtained.

In vitro cytotoxicity. Prior to biomedical application, the cytocompatibility of the developed \( ([\text{Au}^0]_{100}\text{G5.NH}_2-\text{FI-DOTA(Mn)-HA}) \) NPs was evaluated using an MTT colorimetric assay (Fig. 4). After 24 h of incubation with HCCLM3 cells, no significant cytotoxicity from the \( ([\text{Au}^0]_{100}\text{G5.NH}_2-\text{FI-DOTA(Mn)-HA}) \) NPs was observed at any of the tested Mn concentrations. Compared to the PBS control, no significant difference (\( P > 0.05 \)) in HCCLM3 cell viability was observed even at the highest Mn concentration (100 \( \mu \text{g/mL} \)). Cell viability remained at more than 80%. Clearly, the produced NPs are cytocompatible in the given Mn concentration range.

To further evaluate NP cytotoxicity, after the HCCLM3 cells were treated with \( ([\text{Au}^0]_{100}\text{G5.NH}_2-\text{FI-DOTA(Mn)-HA}) \) NPs at Mn concentrations of 10, 20, 50, 75, and 100 \( \mu \text{g/mL} \) for 24 h, the morphology of the cells was then observed by phase-contrast microscopy (Figure S3). The results showed that the morphology of the HCCLM3 cells treated with the \( ([\text{Au}^0]_{100}\text{G5.NH}_2-\text{FI-DOTA(Mn)-HA}) \) NPs at Mn concentrations of 10–100 \( \mu \text{g/mL} \) (Figure S3b–f) was similar to that of the cells in the control group (treated with PBS) (Figure S3a). The MTT
data are supported by the cell morphology results, thereby validating the cytocompatibility of the \[[\text{Au}^0]_{100}\text{G5.NH}_2\text{-FI-DOTA(Mn)-HA}\] NPs and their suitability for in vivo CT/MR imaging applications.

**Flow cytometry assay.** The NPs were modified by FI molecules and could be analyzed by flow cytometry through the binding of the \[[\text{Au}^0]_{100}\text{G5.NH}_2\text{-FI-DOTA(Mn)-HA}\] NPs to the target cells. Cells with blocked CD44 receptors were used as a control. At different Mn concentrations, the fluorescence intensity of HCCLM3 cells with unblocked CD44 receptors was much stronger than that of cells with blocked CD44 receptors (Figures S4 and 5). The enhanced cellular uptake of the \[[\text{Au}^0]_{100}\text{G5.NH}_2\text{-FI-DOTA(Mn)-HA}\] NPs should be related to the modified HA molecules that can specifically target HCCLM3 cells via the CD44 receptor-mediated pathway.

**In vivo targeted CT/MR imaging of orthotopic HCC tumors.** The \[[\text{Au}^0]_{100}\text{G5.NH}_2\text{-FI-DOTA(Mn)-HA}\] NPs were next employed to verify the feasibility of targeted CT/MR imaging of tumors in vivo (Figs 6 and 7). CD44 receptor blocking was used for the control. Figures 6 and 7 show the contrast enhancement in the CT and T1-weighted MR images of tumors at different time points after the tail vein injection of the \[[\text{Au}^0]_{100}\text{G5.NH}_2\text{-FI-DOTA(Mn)-HA}\] and free HA + \[[\text{Au}^0]_{100}\text{G5.NH}_2\text{-FI-DOTA(Mn)-HA}\] NPs. The CT and MR signal intensity values of the tumors appeared to reach their maxima at 0.5h post-injection. In the experimental group, this should be attributable to the fact that the NPs accumulate in the tumor region via the enhanced permeability

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**Figure 6.** In vivo CT images of orthotopic liver tumors at different times after a 0.3-mL intravenous injection of a \[[\text{Au}^0]_{100}\text{G5.NH}_2\text{-FI-DOTA(Mn)-HA}\] NP solution (0.3 mL in PBS, \([\text{Au}] = 120 \text{ mM}\)).

**Figure 7.** In vivo MR images of orthotopic liver tumors at different times after an intravenous injection of 0.3 mL of a \[[\text{Au}^0]_{100}\text{G5.NH}_2\text{-FI-DOTA(Mn)-HA}\] NP (300 μg Mn) solution in PBS.
and retention (EPR) effect as well as active targeting. However, in the control group, this effect is likely only attributable to NP accumulation via the EPR effect because of the blocked CD44 receptors. Based on the quantitative changes in the CT and MR signal intensity values over time after the actively targeted NPs were injected, the tumor CT density and MR signal intensity values are highest at 0.5 h post-injection and partly recover at 1 h post-injection. The CT and MR signal intensity values of the tumors treated with the NPs in the experimental group were obviously higher than those of the free HA in the control group at the same time point ($P < 0.001$). At 1 h post-injection with free HA + NPs, the NPs began to be metabolized and leave the tumor site, which led to the recovery of the CT value and MR signal, precisely the opposite of the significantly higher CT density and MR signal intensity values maintained after the NP injection without free HA. The CT/MR imaging results of the orthotopic tumors showed that the synthesized $([Au^3]_{100}G5.NH_2$-FI-DOTA(Mn)-HA) NPs can be used as a nanoprobe for effective, targeted CT/MR dual-mode imaging $in vivo$. Our results suggest that $([Au^3]_{100}G5.NH_2$-FI-DOTA(Mn)-HA) NPs have the ability to target cancer cells through an HA-mediated targeting pathway because the targeting ability was weakened when CD44 receptors were blocked. Meanwhile, we also measured the CT and MR signal intensity values of normal livers, and no significant differences were found between the two groups at the different time points (Fig. 8). These results also clearly showed that the CT density and MR signal intensity values were greater in the tumor tissue than in the normal liver tissue. The CT/MR images of orthotopic HCC $in vivo$ could be used to easily distinguish tumor tissue from normal tissue.
**In vivo biodistribution.** It is crucial to know the biodistribution of the synthesized \(\{(\text{Au}^0)_{100}\text{G5.NH}_2\text{-FI-DOTA(Mn)-HA}\}\) NPs prior to their application in advanced *in vivo* biomedical imaging. The *in vivo* biodistribution of the \(\{(\text{Au}^0)_{100}\text{G5.NH}_2\text{-FI-DOTA(Mn)-HA}\}\) NPs in major organs, such as the liver, spleen, kidneys, lungs, and heart, as well as in the tumor, was analyzed by ICP-MS at 24 h post-injection (Fig. 8). After the injection of both NP samples, the Au concentration in the measured organs was obviously higher in the treatment group than in the blank control group. The main uptake of Au occurred in the liver, spleen and lungs in the treatment group, while relatively little uptake occurred in the other tissues (i.e., heart, kidneys, and tumor). The biodistribution data of the Au indicated that the particles could be delivered to the tumor tissue after escaping the reticuloendothelial system. The targeting property of these \(\{(\text{Au}^0)_{100}\text{G5.NH}_2\text{-FI-DOTA(Mn)-HA}\}\) NPs was clearly demonstrated, as the Au concentration in the tumor region of the mice treated with the targeted \(\{(\text{Au}^0)_{100}\text{G5.NH}_2\text{-FI-DOTA(Mn)-HA}\}\) NPs was double that in mice treated with the non-targeted NPs. HA plays an important role in this process by enabling the highly efficient delivery of NPs to the tumor region *in vivo* for targeted CT/MR tumor imaging.

**Conclusions**

In summary, \(\{(\text{Au}^0)_{100}\text{G5.NH}_2\text{-FI-DOTA(Mn)-HA}\}\) NPs with X-ray attenuation favorable for CT imaging and *r_1* relaxivity suitable for *T_1*-weighted MR imaging were developed and applied in an orthotopic HCC tumor model. Dendrimers were used as a template to entrap AuNPs within and chelate Mn ions onto the template surface. EDC coupling chemistry was used to couple HA molecules onto the G5 dendrimer surfaces, thereby endowing the NPs with the ability to actively target CD44 receptor-expressing cancer cells. The favorable characteristics of these multifunctional NPs, including water solubility, colloidal stability, and biocompatibility, make them extremely attractive for potential use in the CT/MR imaging of tumors *in vivo* via an active, HA-mediated targeting pathway. With the application of this strategy, various dual- or multimode imaging contrast agents may be designed for the accurate diagnosis of various types of malignant tumors.

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Author Contributions

X.W. and X.S. contributed to the design of the experiment, analysis of the data and writing the manuscript. R.W. and Y.L. performed the experiments and data analysis, and wrote the draft of the manuscript. S.Y., J. Lin, D.G., X.W. and X.S. contributed to the design of the experiment, analysis of the data and writing the manuscript. R.W. performed the experiments and data analysis, and wrote the draft of the manuscript. S.Y., J. Lin, D.G., X.W. and X.S. contributed to the design of the experiment, analysis of the data and writing the manuscript. R.W. would like to thank the National Natural Science Foundation of China (21273032 and 81371623), the Sino-German Center for Research Promotion (GZ899), and the Program for Professor of Special Appointment (Eastern Scholar) at Shanghai Institutions of Higher Learning. Y. Luo would like to thank the Innovation Funds of Donghua University Doctorate Dissertation of Excellence (15D310509).

Additional Information

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