A carboxyl-terminated dendrimer enables osteolytic lesion targeting and photothermal ablation of malignant bone tumors

Yang Yan, Xin Gao, Song Zhang, Yitong Wang, Zhengjie Zhou, Jianru Xiao, Qiang Zhang, and Yiyun Cheng

ACS Appl. Mater. Interfaces, Just Accepted Manuscript • DOI: 10.1021/acsami.8b15827 • Publication Date (Web): 07 Dec 2018

Just Accepted

“Just Accepted” manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides “Just Accepted” as a service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. “Just Accepted” manuscripts appear in full in PDF format accompanied by an HTML abstract. “Just Accepted” manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are citable by the Digital Object Identifier (DOI®). “Just Accepted” is an optional service offered to authors. Therefore, the “Just Accepted” Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the “Just Accepted” Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these “Just Accepted” manuscripts.
A Carboxyl-Terminated Dendrimer Enables Osteolytic Lesion Targeting and Photothermal Ablation of Malignant Bone Tumors

Yang Yan†, Xin Gao‡, Song Zhang†, Yitong Wang†, Zhengjie Zhou†, Jianru Xiao‡, Qiang Zhang†,* and Yiyun Cheng†,*

†Shanghai Key Laboratory of Regulatory Biology, School of Life Sciences, East China Normal University, Shanghai, 200241, P. R. China.
‡Department of Orthopaedic Oncology, Changzheng Hospital, the Second Military Medical University, Shanghai, 200003, P. R. China.

KEYWORDS: carboxyl-terminated dendrimer, dendrimer-encapsulated nanoparticles, bone targeting, photothermal therapy, osteolysis

ABSTRACT: Malignant bone tumor accompanied with tumor-associated osteolysis remains a challenging task in clinical practice. Nanomedicines engineered with bone-targeting ligands such as alendronate and pamidronate were developed for targeted delivery of therapeutic agents to bone tumors. However, these targeting strategies usually show relative poor selectivity towards the healthy skeletons and the osteolytic lesions due to the high binding affinity of bisphosphonates with all the bone tissues. Here, we reported a carboxyl-terminated dendrimer as the candidate to preferentially deliver therapeutic nanoparticles to the osteolytic lesions in a malignant bone tumor model. The high density of carboxyl groups on dendrimer surface endow the polymer with natural bone-binding capability. The dendrimer encapsulated with platinum nanoparticle predominantly accumulates at the osteolytic lesions around bone tumors rather than at healthy bone tissues in vivo. The therapeutic experiments reveal that the dendrimer-mediated photothermal therapy efficiently suppresses bone tumors and osteolysis, and the anionic polymer exhibits
minimal cytotoxicity and hematologic toxicity. The results suggest that the carboxyl-terminated dendrimer is a promising candidate for selective delivery of therapeutics to the osteolytic lesions and photothermal treatment of malignant bone tumors.

1. INTRODUCTION

Bone metastatic tumors occur frequently in advanced cancer patients. Malignant bone tumors also lead to osteolytic change in the structural integrity of bone, and induce unbearable bone pain and other skeletal-related events, which additionally cause disability, reduced quality of life and mortality. The conventional surgical resection generally fails to remove the multiple focal lesions of bone tumors, and the chemo- and radio-therapies are always troubled with bone marrow microenvironment-associated therapeutic resistance. All these situations promote the development of new strategies. Photothermal therapy (PTT) is a newly-developed strategy for cancer therapy, and has demonstrated its efficacy in the regression of bone tumors. However, the skeletal tissues lack circulating systems, plus the low blood flow rate in bone (0.05-0.2 mL/min/g), which leads to a poor distribution of the therapeutic nanoparticles in bone tumors.

Strategy of targeting nanoparticles to bone is employed for favorable drug delivery to bone tumors. Bisphosphonates and peptides are the two widely exploited bone-targeting moieties. Although they have demonstrated their efficacy in selective drug delivery to bone, there are still disadvantages limit their applications. Bisphosphonate-conjugated molecules and polymeric nanoparticles represent highly increased deposition in bone tissues. However, their strong bone-binding affinity results in nonspecific deposition over entire bone tissues, which somehow reduces the accumulation of therapeutic agents in bone tumors, and increases the risk of adverse effects in healthy bone tissues. Peptides consisting of aspartic acid (Asp) or glutamic acid (Glu) repeating sequences, such as Asp octapeptides, are able to selectively recognize the resorption sites in bone tissues due to their relatively weak bone binding affinity compared with
bisphosphonates\textsuperscript{33, 34} but the instability and unexpected immune response of peptides hinder their utilizations. Therefore, the development of novel bone-targeting moieties with high affinity towards osteolytic lesions is urgently required.

Several studies reveal that the affinity of Asp- or Glu-rich peptide to bone strongly depend on the number of Asp or Glu residues, but have no relationship with their species (Asp or Glu) or their optical characters\textsuperscript{35-37} which indicates that carboxyl groups in Asp and Glu play a critical role in the bone-binding action. Moreover, it is found that single Asp-modified polymeric nanoparticles can also interact with skeletal tissues effectively\textsuperscript{38}. In this case, the multivalency effect of Asp residues (high density of Asp on particle surface) might offset the bone-binding affinity of peptide sequences. The hypothesis is confirmed by another investigation, in which the bone-binding affinity is proportional to the number of bisphosphates conjugated on the surface of polymeric nanoparticles\textsuperscript{39}. All these inspire that carboxyl-terminated dendrimers might be a promising candidate for selective delivery of therapeutic agents to bone tumors. The plentiful carboxyl terminals on the surface of dendrimer might endow the nanoparticles intrinsic bone-binding capability. Moreover, the dendrimers also have well-defined size and shape\textsuperscript{40} and their hollow interior can be utilized to load photothermal nanoparticles and other therapeutic agents\textsuperscript{41-43}. In this study, we synthesized carboxyl-terminated dendrimer-encapsulated platinum (Pt) nanoparticles (DEPt-COOH) for targeted PTT of malignant bone tumors (Scheme 1). To well learn the function of carboxyl groups on bone targeting, amine-terminated dendrimer-encapsulated Pt nanoparticles (DEPt-NH\textsubscript{2}) and acetylated DEPt-NH\textsubscript{2} (DEPt-AC) were synthesized as control materials. The bone-binding affinity of the three materials was assessed on hydroxyapatite or bone \textit{in vitro} and \textit{in vivo}. Finally, malignant bone tumors treated by DEPt-associated PTT was conducted in an orthotropic bone tumor model.
Scheme 1. Illustration depicts targeted delivery of DEPt-COOH to the osteolytic lesions in bone tumors and DEPt-COOH-associated treatment of malignant bone tumors by PTT.

2. MATERIALS AND METHODS

2.1. Materials. The generation 5 amine-terminated (G5-NH₂) polyamidoamine (PAMAM) dendrimer and the generation 4.5 carboxyl-terminated (G4.5-COOH) PAMAM dendrimer were obtained from Dendritech Inc. (Midland, MI, USA). Acetic anhydride and triethylamine were bought from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Potassium hexachloroplatinate (K₂PtCl₆) and sodium borohydride (NaBH₄) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid, methanol and sodium hydroxide were bought from Shanghai Titan Scientific Co., Ltd (Shanghai, China). All the reagents were used as received without further purification.

2.2. Synthesis of acetylated G5-NH₂ (G5-AC) PAMAM dendrimer. G5-NH₂ PAMAM dendrimer (100 mg, 3.47 μmol) was dissolved in 2 mL methanol. Acetic anhydride (34.5 μL, 0.364 mmol) and triethylamine (63.5 μL, 0.456 mmol) were then added. After 48 h reaction, the reaction solution was dialyzed with deionized (DI) water by using a dialysis bag with molecular weight cut off (MWCO) of 3500 Da (Biosharp, USA).
The purified samples were freeze-dried and the acetylation degree was confirmed by $^1$H NMR.

2.3. Synthesis of DEPts. DEPts including DEPt-COOH, DEPt-NH$_2$ and DEPt-AC were prepared according to the well-developed methods. In a typical synthesis of DEPt-COOH, 0.550 mL G4.5-COOH aqueous solution (0.346 mM, pH = 2) was added with 0.476 mL K$_2$PtCl$_6$ (20 mM) under magnetic stirring. The reaction was conducted for 12 h followed by changing the pH of solution to 9. After that, 0.476 mL NaBH$_4$ (200 mM) was added, and the reaction was conducted for 2 h. Finally, the reaction solution was dialyzed against phosphate buffered saline (PBS) by using a dialysis bag (MWCO = 3500 Da) at pH = 9. The purified DEPt-COOH were collected, and the inductively-coupled plasma mass spectrometry (ICP-MS) was employed to measure the concentration of Pt element. The pH of the above reaction solution was changed by adding hydrochloric acid (100 mM) or sodium hydroxide (100 mM). For the synthesis of DEPt-NH$_2$ and DEPt-AC, the same methods was used.

2.4. Characterization. Transmission electron microscopy (TEM) images were obtained on a HT7700 TEM instrument (100 kV, Hitachi, Japan). The high-resolution TEM (HRTEM) images were recorded on a JEM-2100 TEM instrument (200 kV, JEOL, Japan). The hydrodynamic sizes of nanoparticles were determined by using a Zetasizer Nano ZS90 (Malvern Instruments, UK). The quantitative analysis of Pt element in samples was conducted with MC-ICP-MS (Neptune, Thermo, USA). The ultraviolet-visible (UV-Vis) absorption spectra of nanoparticles were recorded on an Agilent Cary 60 UV-Vis spectrophotometer (Technologies, USA).

2.5. Photothermal effect of DEPts. DEPt aqueous solution (300 µM, 1 mL) was held in a cuvette (cross sectional dimension: 1 × 1 cm), and was exposed to a near-infrared (NIR) laser (4.65 W·cm$^{-2}$, 808 nm, CNI laser, China) for 5 min. An infrared camera (Magnity Electronics, China) was employed to record the temperature changes.

2.6. Assay to determine bone-binding affinity of DEPts. The affinities of DEPts to
bone were assessed. The hydroxyapatite tablets with high crystallinity were obtained as a gift from Prof. Kaili Lin at Shanghai Jiaotong University. Briefly, the hydroxyapatite tablets were incubated with 2 mL DEPt-NH₂, DEPt-AC and DEPt-COOH (100 μM) for 24 h, respectively. After that, the hydroxyapatite tablets were washed and then air dried. The hydroxyapatite tablets were irradiated by NIR light. Their temperatures and thermographic images were collected. Finally, the quantitative analysis of Pt element in the hydroxyapatite tablets was conducted with ICP-MS.

The binding ability of DEPtS to bone fragments was further assessed according to the same method for hydroxyapatite tablets.

2.7. Cell culture. NIH3T3 cells and MDA-MB-231 cells stably expressing luciferase (MDA-MB-231-Luc cells) from ATCC were cultured in MEM (Gibco) and DMEM (Invitrogen), respectively. The cell culture contained 10% FBS, 100 μg/mL penicillin and 100 μg/mL streptomycin, and the cells were cultured at 37 °C under 5% CO₂.

2.8. Cytotoxicity assay of DEPtS. NIH3T3 or MDA-MB-231-Luc cells were cultured in a 96-well plate (10⁴ cells/well) overnight, and then were incubated with DEPtS at different concentrations in a range of 0-300 μM for 24 h. A standard 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was conducted to determine the cytotoxicity.

2.9. In vitro Killing cancer cells by DEPt-associated PTT. MDA-MB-231-Luc cells in a 96-well plate (10⁴ cells/well) were incubated with the culture media suspended with three DEPtS (100 μM) for 24 h. The cells were then treated with NIR irradiation at 3.6 W cm⁻² for 5 min. After another 2 h incubation, MTT assay was employed to measure the cell viabilities.

2.10. In vivo biodistribution of DEPtS. BALB/c nude mice (4 weeks old, ~20 g) were obtained from SLAC Laboratory Animal Co. Ltd. (Shanghai, China). The in vivo experiments were approved by the ethics committee of East China Normal University, and the experiments were conducted according to the National Institutes of Health guidelines.
for care and use of laboratory animals. The orthotropic bone tumor-bearing model was established by injecting MDA-MB-231-Luc cells into the medulla of mice tibias. Two weeks later, the mice with tumor luminescence were selected and divided into 6 groups, and each group had 3 mice. DEPt-NH$_2$, DEPt-AC and DEPt-COOH (3.9 mg/kg, Pt mass, two groups for each DEPt) were then administrated to the mice via intravenous injection. After that, three groups of mice with different injections were executed at 12 h, and the remaining groups of mice sacrificed at 24 h. The main organs and tissues of mice were harvested and measured for Pt content by ICP-MS.

2.11. In vivo treatment of malignant bone tumors by PTT. The bone tumor-bearing mice were classified in five groups (5 mice/group). Four groups of mice were intravenously administrated with PBS (0.16 mL), DEPt-NH$_2$ (3.9 mg/kg, Pt mass, dissolved in 0.16 mL PBS), DEPt-AC (3.9 mg/kg, Pt mass, 0.16 mL PBS) and DEPt-COOH (3.9 mg/kg Pt mass, 0.16 mL PBS), respectively. The tumors were then exposed to NIR irradiation (3.6 W cm$^2$, 10 min) 12 and 24 h after each injection. Mice in the fifth group were just injected with DEPt-COOH (3.9 mg/kg, Pt mass, 0.16 mL PBS). The treatments were performed three times with a time interval of three days. The evolution of the temperatures at tumor locations and the thermographic images of mice were also recorded while NIR treatment. An IVIS Lumina II in vivo imaging system (Caliper Life Sciences, USA) was employed to record the luminescence images of mice.

2.12. Assay to determine tumor apoptosis. The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining assay was employed for determining apoptosis. Briefly, the tumor tissues were fixed in paraffin, and then were incised into slices after 48 h incubation in 4% formalin solution. After that, the tumor slices were stained with an in situ cell-death detection kit from Roche (Mannheim, Germany). Finally, apoptosis cells were observed under a fluorescence microscope.

2.13. Histopathological examination. After treatment, the main organs and tissues of mice in PBS+NIR and DEPt-COOH+NIR including heart, liver, spleen, kidneys, and lungs
were collected. They were fixed with formalin and cut into slices, and then were stained with hematoxylin and eosin. The samples were examined under an optical microscope.

2.14. Tumor-bearing tibia reconstruction by micro-computed tomography (micro-CT). The mice were executed, and the tumor-bearing legs were excised. The legs were then scanned by a Skyscan 1077 micro-CT scanner (Antwerp, Belgium). After that, the three dimensional (3D) features of the tumor bearing-tibias were reconstructed by using the CTVox program.

2.15. Hemolysis assay. DEPt-COOH were incubated with red blood cells (RBC, 2% in PBS, from nude mice) suspension at different concentrations at 37 °C. RBC were also treated with PBS and Triton X-100 (0.5%) as controls. After incubation for 1 h, the supernatants were collected via centrifugation (2000 rpm, 5 min), and their absorbance at 540 nm was measured. The hemolysis ratio (%) was calculated as follows: $\frac{(A_{\text{sample}} - A_{\text{negative control}})}{(A_{\text{positive control}} - A_{\text{negative control}})} \times 100 \%$, where A was defined as the absorbance at 540 nm.

2.16. Blood test assay. To assess the influence of DEPt-COOH on hematology, two groups of mice (3 mice/group) were intravenously administrated with PBS (0.16 mL) and DEPt-COOH (3.9 mg/kg, Pt mass, 0.16 mL PBS), respectively. 24 h later, the blood was collected from sacrificed mice for hematology analysis by Adicon clinical laboratory (Shanghai, China).

2.17. Excretion analysis. Two groups of mice (3 mice/group) were intravenously administrated with PBS (0.16 mL) and DEPt-COOH (3.9 mg/kg, Pt mass, 0.16 mL PBS), respectively. The urines and feces of the mice were collected at 12 and 24 h after injection. After that, the urines and feces were digested by aqua regia. Pt contents in the urines and feces were determined by ICP-MS.

2.18 Data analysis. Statistical significance was analyzed by student's t-test ($^N_S p > 0.05$, $^* p < 0.05$, $^{**} p < 0.01$ and $^{***} p < 0.001$).
3. RESULTS AND DISCUSSION

DEPt-COOH, DEPt-NH$_2$ and DEPt-AC were synthesized by using PAMAM dendrimer scaffolds with different surface functionalities. The TEM images showed that Pt nanoparticles in the three DEPts were ultrasmall and highly dispersed (Figure 1a-c). The HRTEM images revealed that they had a lattice fringe of 0.22 nm (Figure 1a-c), which was corresponding to the (111) plane of Pt.$^{44}$ The three DEPts also had narrow size distributions, and their average diameter sizes (measured from the TEM images) were 1.48 ± 0.24 nm for DEPt-NH$_2$, 1.46 ± 0.26 nm for DEPt-AC, and 1.51 ± 0.28 nm for DEPt-COOH, respectively (Figure 1d-f). The hydrodynamic sizes of the three DEPts were almost the same (Figure 1g). The zeta potential of three DEPts were different due to their distinct surface functionalities (Figure S1, 42.5 ± 1.5 mV for DEPt-NH$_2$, 12.9 ± 1.4 mV for DEPt-AC, and -38.3 ± 2.8 mV for DEPt-COOH,). The UV-Vis spectra showed the three DEPts had the same absorption in the NIR region (Figure 1h), which indicated that they might have the similar photothermal effects. The suspensions of the three DEPts were further irradiated by a NIR laser, and their temperature evolved in the same tendency (Figure 1i), indicating that they had the same photothermal conversion efficiency.
Figure 1. (a-c) TEM images of DEPt-NH$_2$, DEPt-AC and DEPt-COOH. Insets are HRTEM images of a single particle. (d-f) The size distribution of DEPts. (g) The hydrodynamic sizes of DEPts in DI water. (h) The UV-Vis spectra of DEPts. (i) Temperature changes of DEPts suspensions (300 μM, 1 mL) while NIR irradiation (808 nm, 4.65 W·cm$^{-2}$) for 5 min.

The bone-binding capabilities of the three DEPts were evaluated in vitro. The crystallinities of hydroxyapatite in the skeletal tissues are varied. The newly-generated hydroxyapatite at the bone-formation sites has a low crystallinity, while the hydroxyapatite exposed at bone-resorption locations is highly crystallized. In this case, a home-made hydroxyapatite tablet with high crystallinity was employed to mimic the bone-resorption
lesions and assess the bone-binding capability. As shown in Figure 2a, the hydroxyapatite tablet turned from white into black color after incubation with DEPt-COOH, while the tablets incubated with DEPt-NH$_2$ or DEPt-AC became light grey color. The result indicated that there were more DEPt-COOH adsorbed on hydroxyapatite tablet than DEPt-NH$_2$ or DEPt-AC. The tablets were further analyzed by ICP-MS, and the Pt content on each tablet was quantified. The data revealed that Pt content in DEPt-COOH-adsorbed tablet was three times more than those in DEPt-NH$_2$- and DEPt-AC-adsorbed ones (Figure 2a), which confirmed that DEPt-COOH had the highest affinity to bone. The tablets were additionally treated with NIR irradiation, and their temperature changes were recorded. As shown in Figure 2b, the temperature of DEPt-COOH treated tablet rapidly increased to ~70 °C, while those of DEPt-NH$_2$- and DEPt-AC-adsorbed tablets only increased to 36 and 45 °C, respectively. To further assess their bone-binding capability, the three DEPts were incubated with bone fragments of mice tibias. The ICP-MS analysis showed that Pt content in DEPt-COOH-adsorbed bone fragments was 1086 ng/g, while those in DEPt-NH$_2$- and DEPt-AC-adsorbed ones were only 202 and 192 ng/g, respectively (Figure 2c). The data demonstrated that DEPt-COOH had the highest affinity to the real bone tissues compared with the two control DEPts. The bone-binding affinity of DEPt-COOH should attributed to the multivalence effect of carboxyl groups on the dendrimer surface, which was consistent with the previous report.$^{33}$
Figure 2. (a-c) The bone-binding capability of different DEPts. (a) The affinity of DEPts to hydroxyapatite tablets. Inset are the photographs of hydroxyapatite tablets incubated with different DEPts. (b) Temperature changes of different DEPt-adsorbed hydroxyapatite tablets upon NIR irradiation at 3.60 W cm\(^{-2}\). Thermographic images of the hydroxyapatite tablets were showed in the insets. (c) The affinity of DEPts to bone fragments. (d, e) Cytotoxicity of different DEPts on NIH3T3 (d) and MDA-MB-231-Luc cells (e), respectively. (f) The in vitro killing MDA-MB-231-Luc cells by PTT. The cells incubated with different DEPts (100 \(\mu\)M) were exposed to NIR light at 3.6 W cm\(^{-2}\) for 5 min.

As the three DEPts were intravenously administrated, their colloid stability in physiological conditions was evaluated. The hydrodynamic sizes of the three DEPts in PBS containing 50% FBS were measured at different times. The data revealed that their sizes had no distinguished changes along with time (Figure S2), which suggested that they were all very stable in the physiological solution. The cytotoxicity of the three DEPts were also determined on NIH3T3 and MDA-MB-231-Luc cells. As shown in Figure 2d and e, DEPt-COOH and DEPt-AC had negligible cytotoxicity in 0-300 \(\mu\)M concentration range, while DEPt-NH\(_2\) were a little toxic due to the excess positive charges on dendrimer surface. The MDA-MB-231-Luc cells incubated with different DEPts were additionally irradiated by a
NIR laser, and the cell viabilities were all reduced by over 80% (Figure 2f), indicating PTT mediated by the three DEPts killed cancer cells efficiently.

**Figure 3.** Biodistribution of different DEPts. Pt content at 12 (a) and 24 (b) h after injection was determined in different organs and tissues.

Before the therapeutic treatment, the biodistribution and the bone-targeting capabilities of the three DEPts were evaluated *in vivo*. The bone tumors were composed of soft tumor tissues and osteolytic bone tissues. To determine the bone-targeting capability, the tumor tissues were isolated from the tibias and the cracked bone fragments, and the Pt contents in tumor and bone tissues were analyzed individually. Biodistributions of the materials at 12 and 24 h post-injection were determined. The three DEPts were mainly captured by the reticuloendothelial system in the liver and spleen. In other organs and tissues like blood, heart, lungs and kidneys, they had relatively low accumulations (Figure 3a and b). As the three DEPts had the similar hydrodynamic sizes, their biodistributions in the main organ and tissues should majorly influenced by their different zeta potentials. The literatures suggested nanoparticles with positive or high negative charge were more efficiently captured by liver and spleen.\(^{46,47}\) In this case, DEPt-NH\(_2\) and DEPt-AC had positive charges and DEPt-COOH had relatively high negative charge. Therefore, they were all highly taken by liver and spleen. In the tumor tissues, the accumulations of three DEPts were a little different but all at very low concentration levels (Figure 3a and b), which was due to that
the three nanoparticles had no specific targeting moieties to the tumor tissues. In comparison, the distributions of the three DEPts were significantly different in the bone tissues (Figure 3a and b). In each mouse, the healthy tibia and the tumor-bearing tibia were both analyzed for Pt content. It was exciting to observe that DEPt-COOH showed high selectivity towards tumor-bearing tibias and healthy tibias. The amount of DEPt-COOH accumulated at the tumor-bearing tibias was about ten-fold higher than that at the healthy ones. Considering that the osteolytic lesion was only a small part of the tibias, the selectivity of DEPt-COOH on binding with osteolytic bones and healthy ones should be much larger than ten-fold. Besides, DEPt-NH$_2$ and DEPt-AC showed poor selectivity towards osteolytic bones and healthy ones (Figure 3a and b). The data suggested that DEPt-COOH had not only the bone-targeting capability but also the ability to recognize the osteolytic bone lesions in vivo. The result was consistent with the previous report for carboxyl-rich peptides, which also favorably recognized the bone-resorption sites. The carboxyl rich polymers had relative weak bone-binding affinity compared with bisphosphonates. Therefore, they were sensitive to the crystallization of hydroxyapatite and favorably recognized the highly-crystallized hydroxyapatite in the mature osteolytic bone lesions.
Figure 4. Malignant bone tumors treated by PTT. (a) Luminescence images of mice taken before and after treatment. (b and c) Thermographic images (b) and the temperature changes at tumor site (c) recorded while the first NIR irradiation. (d) The relative tumor luminescence intensities after treatment. (e) The circumference changes of tumor-bearing legs. (f) The time-elapsed evolution of the body weight of mice. (g) Apoptotic tumor cells (red) detected by a TUNEL assay.

Finally, the therapeutic efficacies of DEPt-mediated photothermal treatment of malignant bone tumors were evaluated. Four groups of tumor-bearing mice were intravenously administrated with PBS, DEPt-NH$_2$, DEPt-AC and DEPt-COOH and treated with NIR irradiation. The fifth group was injected with DEPt-COOH without NIR irradiation. All the mice had the similar luminescence intensities at the tumor sites before the different treatments (Figure 4a). The thermographic images revealed that the tumor-site temperatures were obviously higher in DEPt-COOH+NIR group than in the other irradiation groups (Figure 4b). The temperature curves showed that the tumor-site temperatures rapidly increased to 44 °C in DEPt-COOH+NIR group, while those in DEPt-NH$_2$+NIR and DEPt-AC+NIR groups only increased to ~40 °C (Figure 4c and S3). The
therapeutic temperature was controlled below 45 °C by tuning the laser power in our experiment, which was aimed to avoid the hyperthermia damage of healthy tissues. After three round injections and NIR irradiations, the tumor-site luminescence intensities had no distinguishing changes in DEPt-COOH+NIR group before and after treatment (Figure 4a). However, the tumor-site luminescence intensities in the other four groups were significantly increased (Figure 4a), and they were all increased by over 15 times (Figure 4d). The circumferences of tumor-bearing legs in DEPt-COOH+NIR group increased slowly compared with those of the tumor-bearing legs in the other groups (Figure 4e). After treatments, the tumors were isolated and weighed. The image revealed that the tumors were smaller in DEPt-COOH+NIR group compared with the ones in the other groups (Figure S4a). The tumor weight in DEPt-COOH+NIR group was only 0.2 g, and those in the other groups were all larger than 0.7 g (Figure S4b). The isolated tumor tissues were further stained for apoptosis analysis. As shown in Figure 4g, the tumor cells in DEPt-COOH+NIR group represented a high apoptosis level, while those in the control groups were minimal apoptotic. The body weights of mice evolved similarly in different groups during the treatment (Figure 4f).
Figure 5. Evaluation of osteolysis. (a) The structural features of the tibias in bone tumors post-treatment. (b-e) The changes of bone volume (b), bone surface (c), trabecular number (d), and trabecular separation (e) after treatment.

The inhibition of osteolysis could significantly reduce bone pain and pathological bone fractures and enhance the quality of life. After treatment, the structural integrity of the tumor-bearing tibias and their 3D architecture parameters were evaluated by using 3D micro-CT. As shown in Figure 5, the tibias in the DEPt-COOH+NIR group well maintained their original morphologies, while osteolytic bone destruction was observed at proximal tibias in all the control groups. The 3D profiles and the transverse sections of tumor-bearing tibias were also reconstructed (Figure 6). The data confirmed that the tumor-bearing tibias were minimally damaged in DEPt-COOH+NIR group, while those in the other groups were severely destroyed. The structure parameters such as bone volume, bone surface, trabecular number, and trabecular separation were also analyzed. The values of bone volume, bone surface and trabecular number of the tumor-bearing tibias in DEPt-COOH+NIR group were higher in comparison with those in the other groups, and that of trabecular separation was lower in the DEPt-COOH+NIR group (Figure 5b-e). These data suggested that the osteolysis was efficiently suppressed in DEPt-COOH+NIR group.

Figure 6. The 3D profiles (a) and transverse sections (b) of tumor-bearing tibias after treatment.

Since DEPt-COOH had demonstrated their efficacy in bone-targeted PTT of bone
tumors, their potential hematologic toxicity and excretion profile were further evaluated. To test the hemolytic activity, DEPt-COOH were incubated with RBC in PBS suspension. As shown in Figure 7a, DEPt-COOH had no observable hemolysis in the concentration range of 0-150 μM. Furthermore, DEPt-COOH were intravenously injected in healthy mice, and the blood was collected 24 h later for hematology analysis. The hemoglobin (HGB), platelets (PLT), RBC and white blood cells (WBC) had no different changes compared with those of mice injected with PBS (Figure 7b). In addition, there were no detectable pathological changes in the main organs of mice in DEPt-COOH+NIR group compared to the mice treated with PBS (Figure 7d). All these data suggested that DEPt-COOH had an excellent hemocompatibility. Finally, the excretion profile of DEPt-COOH was studied. The previous studies suggested that the low generation dendrimers were quickly filtered out of kidney after intravenous injection, while the high generation ones were mainly accumulated in liver. In our case, the urines and feces of mice were collected and analyzed for Pt content. The data revealed that DEPt-COOH were mainly excreted via the liver and biliary system, and a small part of them were filtered out of the body via kidneys (Figure 7c).
Figure 7. Hematologic toxicity of DEPt-COOH. (a) Hemolytic activity of DEPt-COOH at different concentrations. PBS and Triton X-100 (0.5%) were used as the negative and positive controls, respectively. (b) The hematological analysis of mice blood after injection with PBS and DEPt-COOH. (c) Excretion profiles of DEPt-COOH in health mice at 12 and 24 h. (d) Histological examination of the main organs of mice in PBS+NIR and DEPt-COOH+NIR groups. The insert bar was 200 μm.

4. CONCLUSIONS

In summary, the carboxyl-terminated dendrimer was developed as a novel vehicle to favorably deliver Pt nanoparticles to the osteolytic bone lesions for PTT of bone tumors. The plentiful carboxyl groups on the dendrimer surface endowed DEPt-COOH the intrinsic bone-binding affinity. DEPt-COOH represented high affinity to hydroxyapatite and bone fragments \textit{in vitro}. The biodistribution revealed that DEPt-COOH were able to selectively recognize the osteolytic bone lesions in bone tumors, resulting in a higher accumulation in the tumor-bearing tibias than in the healthy ones. The therapeutic experiments demonstrated that DEPt-COOH-mediated PTT efficiently regressed the bone tumor growth and tumor-associated osteolysis.
ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS publications website at DOI:
Zeta potential of three DEPts, evolution of the hydrodynamic sizes of three DEPts, temperature changes in tumor while NIR irradiation, isolated bone tumors and their average weights

AUTHOR INFORMATION

Corresponding Author
*Email: qzhang@bio.ecnu.edu.cn,
*Email: yycheng@mail.ustc.edu.cn.

Author Contributions
Y. Yan and X. Gao contributed equally on this manuscript.

Notes
The authors declare no competing financial interest.

ACKNOWLEDGEMENTS
We would like to thank financial supports from the National Key Research and Development Program of China (2016YFC0902100), the National Natural Science Foundation of China (21725402 and 81671822), and the SMSTC (17XD1401600) on this work.

REFERENCES


(47) Xiao, K.; Li, Y.; Luo, J.; Lee, J. S.; Xiao, W.; Gonik, A. M.; Agarwal, R.; Lam, K. S. The Effect of Surface Charge on In Vivo Biodistribution of PEGoligocholic Acid Based
