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Peptide Decoration of Nanovehicles to Achieve Active Targeting and Pathology-Responsive Cellular Uptake for Bone Metastasis Chemotherapy

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Abstract

To improve bone metastases chemotherapy, a peptide-conjugated diblock copolymer consisting of chimeric peptide, poly(ethylene glycol) and poly(trimethylene carbonate) (Pep-*b*-PEG-*b*-PTMC) is fabricated as a drug carrier capable of bone-seeking targeting as well as pathology-responsive charge reversal to ensure effective cellular uptake at the lesion sites. The chimeric peptide CKGHPGGPQAsp₈ consists of an osteotropic anionic Asp₈, a cathepsin K (CTSK)-cleavable substrate (HPGGPQ) and cationic residue tethered to polymer chain. Pep-*b*-PEG-*b*-PTMC can spontaneously self-assemble into negatively charged nanomicelles (~75 nm). As to the model drug of doxorubicin, Pep-*b*-PEG-*b*-PTMC shows 30.0 ± 1 % and 90.1 ± 2 % for loading content and loading efficiency, respectively. High bone binding capability is demonstrated with that 66 % of Pep-*b*-PEG-*b*-PTMC micelles are able to bind to hydroxyl apatite, whereas less than 15 % is for Pep-free micelles. The nanomicelles exhibit a negative-to-positive charge conversion from -18.5 ± 1.9 mV to 15.2 ± 1.8 mV upon exposure to CTSK, an enzyme overexpressed in bone metastatic microenvironments. Such a pathology-responsive transition would lead to remarkably enhanced cellular uptake of the nanomicelles upon reaching lesion sites, thus improving the drug efficacy as verified by the *in vitro* cytotoxicity assay and the *in vivo* study in myeloma-bearing 5TGM1 mice model.

Keywords

bone metastases; peptide; nanomicelle; active targeting; MTSK-induced charge reversal

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1. Introduction

Most patients with cancer die not because of the tumor in the primary site, but rather because it has spread to other sites of which the skeleton is the most common organ to be affected.^{1, 2} Patients with bone metastases generally cannot be treated curatively, e.g., only 20% of patients with breast cancer are still alive five years after the diagnosis of bone metastases; more than 550,000 people die annually with bone metastases.^{1, 3} Effective chemotherapy of bone metastases has therefore remained an urgent challenge for cancer treatments.⁴

The surface decoration of drug nanovehicles (NVs) with bone-targeting moieties is among the most used strategies to improve the chemotherapy against bone metastases.⁵ Given the remarkable feature of skeletal tissue rich in apatite ($\text{Ca}_{10}(\text{PO}_4)_6$), many anionic agents with high apatite binding affinity including bisphosphonates (BPs) and oligopeptides (Asp₆, Asp₈ and Glu₈), etc., have been purposely incorporated into NV construction for the sake of bone-seeking action.⁵⁻⁷ However, the treatment efficacies mediated by those bone-targeting NVs are still far from satisfactory; some of them were even less efficacious than the unmodified counterparts. The marked deviation from the expectation is thought to associate with the dilemma that the attachment of anionic agent to NVs inevitably discourages cellular translocation due to the charge repulsion by the cell membranes.⁴⁻⁶ In turn the drugs loaded in NVs are difficult to enter cancerous cells to elicit therapeutic action. On the contrary, despite the easy intracellular internalization, the applications of positively charged NVs suffer a lot from rapid *in vivo* clearance and non-specific uptake by healthy tissues resulting from the strong interaction with oppositely charged blood components and the cell membranes. Taking those into account, the solution to bone-targeted chemotherapy may eventually rely on the rational design of anionic bone-seeking NVs simultaneously capable of negative-to-positive charge transition that is preferred to mostly occur in bone metastatic sites.

One notable physicochemical feature of bone metastases is the predominant over-expression of several proteases including matrix metalloproteinase, urokinase plasminogen activator, and cathepsins (cathepsins K, L, and B).⁸ In particular, the development of cancer metastases in bones is found to be highly correlated with the expression level of acidity-activated Cathepsin K enzyme (CTSK). The bone metastatic lesions express even dramatically higher level of CTSK compared to primary tumors and soft tissue metastases.⁹ The strong dependency of bone metastases with CTSK level make CTSK able to serve as a prognostic indicator for the diagnosis of bone metastases and a therapeutic target for disease treatments.¹⁰⁻¹² This pathology-dependent feature of CTSK overexpression evokes our interest to exploit CTSK as the biological stimuli to locally trigger the charge reversal of anionic bone-seeking NVs upon reaching bone metastatic sites.¹³⁻¹⁶

To the end, we herein designed a bone-seeking, CTSK-responsive charge-reversal NV system for efficient chemotherapy of bone metastases. This delivery system was constructed on the core-shell nanomicelles self-assembled from a triblock copolymer (Pep-*b*-PEG-*b*-PTMC) composed of the peptide segment with the sequence of CKGHPGGPQAsp₈, poly(ethylene glycol) and poly(trimethylene carbonate). The chimeric peptide segment

consisted of three main functional modules: the anionic Asp₈ is responsible for the direction and accumulation of NVs into the skeletal tissues; the HPGGPQ serves as the CTSK-cleavable substrate;⁸ and the remaining cationic residue is chemically conjugated to PEG shell of the micelles. In this way, the combination of the targeting function of Asp₈ and the stealth effect of PEG shell would help the drug-loaded Pep-*b*-PEG-*b*-PTMC nanomicelles prolong the circulation time and minimize undesirable cellular uptake to healthy cells. Of special note is that the bone metastatic microenvironment featured with CTSK overexpression would locally stimulate the enzymatic cleavage of HPGGPQ moieties, leaving cationic peptide residue on shell periphery. This pathology-responsive charge reversal transition is expected to largely contribute to the selectively efficient internalization of nanomicelles into the neighboring cancerous cells (Scheme 1), thus improving the therapy efficacy. In principle, this strategy of bone-seeking, pathology-responsive charge-reversal NVs may be also applicable to other malignant diseases of skeletal tissue if the development of those diseases involves overexpression of certain enzymes. To verify our hypothesis, an anticancer drug of DOX was used as the drug model and the *in vitro* and *in vivo* properties of the Pep-*b*-PEG-*b*-PTMC nanomicelles were comparatively explored in the CTSK-rich and CTSK-absent conditions.

2. Experimental section

2.1. Materials

Monomethyl terminated polyethylene glycol monoamine (MeO-PEG₁₁₃-OH, Mw = 5000 Da) was purchased from Sigma-Aldrich. It was dried by azeodistillation of anhydrous benzene prior to use. Heterofunctional PEG with monomaleimide and monohydroxyl terminus (Mal-PEG₁₁₀-OH, Mw = 5000 Da) was purchased from Laysan Bio (Arab, AL) and dried under high vacuum. Trimethylene carbonate (TMC) was prepared as previously described.¹⁷ It was recrystallized from THF/ether mixture, and further purified via sublimation prior to use. 1,8-Diazabicyclo [5.4.0] undec-7-ene (DBU) was distilled from CaH₂ under dry N₂ prior to use. N-(3,5-Trifluoromethyl)phenyl-N-cyclohexylthiourea (TU) catalyst was prepared as described elsewhere.¹⁸ Stannous octoate (Sn(Oct)₂, Sigma) was purified according to a method described in the literature.¹⁹ Anhydrous solvents were purchased from Sigma-Aldrich and stored over molecular sieves (4 Å). Doxorubicin was purchased from Selleck Inc. (Houston, TX). Cy5.5 was purchased from Lumiprobe (Russia). Cathepsin K was purchased from EMD biosciences. Hydroxyapatite (HA) was purchased from Bio-Rad (Bio-Gel HTP, DNA grade; Hercules, CA). 1-Hydroxybenzotriazol (HOBt), O-benzotriazole-N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU), Rink amide resin, NovaSyn TGT alcohol resin and all of the amino acids for solid phase peptide synthesis were purchased from Novabiochem (CA). All other chemical reagents were available commercially and used as received unless otherwise noted.

2.2. Peptide synthesis

ISOLUTE column reservoirs (Charlottesville, VA) were used for the solid phase synthesis. The functional peptide with the sequence of CKGHPGGPQAsp₈ was synthesized by standard solid phase peptide synthesis (SPPS) procedure by using Rink amide resin solid support and HBTU/HOBt as coupling reagents. Coupling efficiencies were monitored by the

Kaiser ninhydrin test. The peptide was cleaved from the resin by treatment with TFA/EDT/H₂O/TIBS (94/2.5/2.5/1) mixture as scavengers. The crude was precipitated with cold ethyl ether and purified by preparative RP-HPLC. The purity of peptide was checked by analytical RP-HPLC and the molecular weight was confirmed by MALDI-TOF mass spectrometry, MS (*m/z*, [*M*]⁺): 1800.68, (calculated for C₆₈H₉₇N₂₁O₃₅S); 1800.54 (found).

2.3. Synthesis of MPEG-*b*-PTMC di-block copolymer (MPEG-*b*-PTMC)

HO-PEG₁₁₃-OMe (5 g, 1 mmol), TMC (5.1 g, 50 mmol) and TU ((380.6 mg, 2.5 mmol, 5 mol% of TMC monomer) were dissolved in 25 mL dichloromethane (DCM), then DBU (380.6 mg, 2.5 mmol, 5 mol% of TMC monomer) was added. The reaction was performed under N₂ atmosphere for 24 hours. The reaction mixture was concentrated under a reduced pressure and the product was obtained by precipitation in ether. The residue was re-dissolved in a minimal amount of DCM and then poured into cold ether to precipitate the product, total yield: 8.6 g (85.2%).

2.4. Synthesis of maleimide-terminated PEG-*b*-PTMC polymer (Mal-PEG-*b*-PTMC)

Mal-PEG₁₁₀-*b*-PTMC₅₂ was synthesized by ring-opening polymerization using Mal-PEG₁₁₀-OH (0.5 g) as the initiator, Sn(Oct)₂ as the catalyst and anhydrous toluene as the solvent. The reaction was performed at 100 °C under N₂ atmosphere for 16 hours. The reaction mixture was concentrated under a reduced pressure and the product was obtained by precipitation in ether. The residue was re-dissolved in a minimal amount of THF and then poured into cold ether to precipitate the product. Yield: 0.72 g (71.3 %).

2.5. Synthesis of peptide-*b*-PEG-*b*-PTMC copolymer (Peptide-*b*-PEG-*b*-PTMC)

Peptide (CKGHPPGGPQAsp₈, 50 mg, 27.8 μmol) was dissolved in PBS buffer (pH = 7.2) and treated with tris(2-carboxyethyl)phosphine (TCEP, 40 μmol) for 2 hours under N₂ atmosphere. After that, 213 mg of Mal-PEG-*b*-PTMC in 5 mL of dimethylacetamide (DMAc) was added. The reaction was performed at room temperature overnight. The reaction mixture was then subject to dialysis against water (MWCO = 6~8 KD, Spectrumbiolabs) at 4 °C for 24 hours. The solution was further purified by MicrosepTM Centrifugal Devices with OmegaTM Membrane (MWCO = 10 K, Pall Life Sciences). The final product was recovered and dried by lyophilization. Yield: 151 mg (57.5 %).

2.6. NMR characterization

MERCURY 400 MHz spectrometer was used for ¹H NMR spectra to determine the structure and composition of the block copolymers. Deuterium oxide (D₂O), deuterated chloroform (CDCl₃) and dimethyl sulfoxide (DMSO-D₆) were used as the solvent as noted for NMR measurements.

2.7. Determination of critical micelle concentration (CMC)

Critical micelle concentrations of the copolymers were estimated by a fluorescence spectroscopic method using pyrene as the fluorescence probe.²⁰ A predetermined amount of pyrene solution in acetone was added into a series of volumetric flasks, and the acetone was then evaporated completely. A series of copolymer solutions at different concentrations

ranging from 1.0×10^{-5} to 1.0 mg/mL were added to the bottles, whereas the concentration of pyrene in each flask was fixed at a constant value (6.0×10^{-7} mol/L). The excitation spectra were recorded at 20 °C on a Shimadzu RF-5301PC spectrofluorophotometer with the detection wavelength at 390 nm and a slit width of 3 nm. The intensity ratio of the bands at 340 and 338 nm (I_{340}/I_{338}) as a function of the logarithm of the concentration of copolymers was plotted and the CMC value was taken as the intersection of the tangents to the horizontal line of intensity ratio with relatively constant values and the diagonal line with rapid increased intensity ratio.

2.8. Preparation of self-assembled nanoparticles

Preparation of Blank Nanoparticles—Blank micelles were prepared by a dialysis method. Briefly, block copolymer (10 mg) was dissolved in 1 mL of DMSO and stirred for 10 min at room temperature. Then, 5 mL of double distilled water was added dropwise into the polymer solution under stirring. After 30 min, the solution was transferred into dialysis membrane tubing (MWCO = 6–8 KD, Spectrumlabs) and dialyzed for 24 h against distilled water to remove the organic solvent. The solution was recovered and lyophilized to afford blank nanoparticles.

Preparation of Fluorescence-labeled Nanoparticles—In order to track nanoparticles, fluorescence-labeled nanoparticles were prepared by using Cy5.5 as a fluorescent tracer. Cy5.5-labeled nanoparticles were similarly prepared following the above-described dialysis method.

Preparation of DOX-loaded NVs—Unless otherwise noted, all procedures for preparation and handling of DOX-loaded NVs were performed in the dark. DOX-loaded micelles were prepared similarly to the blank micelles. Briefly, 10 mg of each polymer was dissolved in 1 mL of DMSO, followed by adding a predetermined amount of DOX·HCl and two molar equivalents of triethylamine (TEA) and stirred at room temperature for 1 h. Then, 5 mL of double distilled water was added dropwise. After being stirred for an additional 1 h, the solution was dialyzed (MWCO = 6–8 KD, Spectrumlabs) against water for 24 h. Drug-loaded NVs were dried via lyophilization.

2.9. Determination of drug loading capacity

Drug loading content was determined spectrophotometrically by measuring the absorption of the lyophilized sample which was dissolved in DMSO at a wavelength of 482 nm. The absorbance of DOX in nanoparticles was measured to determine drug content in the solution using an established calibration curve with known concentration of free DOX. The percentages of DLC (drug loading content) and DLE (drug loading efficiency) were calculated according to the following equations:

$$DLC (\%) = \frac{\text{amount of DOX in micelle}}{\text{amount of DOX-loaded micelles}} \times 100 \%$$

$$DLE (\%) = \frac{\text{amount of DOX in micelle}}{\text{amount of DOX used for nanomicelle preparation}} \times 100 \%$$

2.10. Drug release profile

Drug release experiments were performed in a buffer with a fixed ionic strength at 150 mM by using saline. Appropriate pH was maintained with phosphate buffer (10 mM, pH 5.5 or 7.4) solutions. DOX-loaded micelle solution was diluted to 1 mg mL⁻¹, and transferred into a dialysis membrane tubing (Spectra/Por, Float-A-Lyzer G2, MWCO = 8 ~ 10 KDa). The tubing was immersed in 100 mL of the release medium and kept in a horizontal laboratory shaker maintaining a constant temperature (37°C) and stirring (100 rpm). Samples were periodically removed and replaced by the same volume of fresh medium. The amount of released DOX was analyzed with a spectrophotometer at 482 nm. The drug release studies were performed in triplicate for each of the samples.

2.11. Dynamic light scattering (DLS)

The hydrodynamic size of the micellar nanoparticles was analyzed using a Brookhaven Instruments BI-200SM system equipped with a 5 mW helium neon laser with a wavelength output of 633 nm. The effective diameter and population distribution were computed from the diffusion coefficient. Measurements were made at 25 °C at an angle of 90°, and each sample was analyzed in triplicate. The micelle solution was filtered using a 0.45 µm membrane filter prior to measurement for all of the experiments.

2.12. Binding affinity of nanoparticles to hydroxyapatite (HA)

The binding affinity of nanoparticles to hydroxyapatite was assessed with free Cy5.5, Cy5.5-loaded nanoparticles or Cy5.5-peptide conjugate.²¹ Cy5.5-peptide conjugate was obtained by direct conjugation of Mal-Cy5.5 (Lumiprobe, Russia) with peptide via thiol-maleimide reaction. The samples were dissolved in phosphate buffered saline (pH 7.4) and then subjected to incubation with 1 mg of HA powder (HA, Bio-Gel HTP, DNA grade; BIO-RAD, Hercules, CA) in an eppendorf tube at RT. At predetermined time intervals, the incubation was stopped by centrifugation. The UV absorbance of the supernatant was monitored at a wavelength of 660 nm. Each sample was measured in duplicate.

2.13. Cell culture

Myeloma cells (5TGM1 and ARP-1) and breast cancer cells (MCF-7) were cultured in RPMI1640 containing 10% heat-inactivated fetal calf serum (FCS), 4 mM L-glutamine (Gibco), penicillin (100 U/mL), and streptomycin (100 µg/mL) at 37°C in humidified atmosphere containing 5% CO₂.

2.14. Cell-proliferation assay

The cells were seeded in 96-well plates at 2,000 cells per well in 100 µL of complete medium, and incubated at 37 °C in a 5% CO₂ humidified atmosphere, followed by adding DOX-loaded nanoparticles or blank micelles (25 µL) at pre-determined concentrations. Following 48-hour incubation, 12.5 µL of PrestoBlue™ Cell Viability Reagent (Invitrogen)

was added into cultured cells. After incubation for an additional 3 h, the absorbance of the solution was measured at 570 and 600 nm using a microplate reader (Molecular Device). The cell number was calculated according to the manufacture's instruction, and the cell viability was normalized to that of cells without drug treatment.

2.15. Confocal laser scanning microscopy (CLSM)

Confocal laser scanning microscopy was carried out using an Olympus FV-1000 laser scanning microscope operated with FLUOVIEW software (Olympus, Tokyo, Japan). Images were produced by using the lasers sequentially with a 20 × objective lens.

2.16. Biological evaluation of CTSK-pretreated nanoparticles

Cathepsin K (EMD Biosciences) was pre-incubated at 37°C for 5 min to activate the enzyme in the active site, followed by addition of the nanoparticles. The nanoparticles were incubated with cathepsin K (150 nM) at 37°C in acetate buffer (0.1 M, pH 5.5). The incubation mixture contained 0.15 µM of cathepsin K, 2.5 mM cysteine, 5 mM EDTA. After CTSK pretreatment for 30 minutes, nanoparticles were recovered via ultrafiltration (MWCO=100K), and then subjected to zeta potential measurement (Malvern Zetasizer 3000), cell incubation for CLSM observation (Olympus FV-1000) or cytotoxicity evaluations. Nanoparticles pretreated in the cleavage buffer but in the absence of CTSK were used as the control.

2.17. In vivo test of nanotherapeutics

The preliminary test of Dox-loaded nanotherapeutics was carried out in a 5TGM1 myeloma cell-induced bone metastatic mouse model. Experiments were approved by the Institutional Animal Care and Use Committee of the University of Utah. To establish the mice with bone manifestations of metastatic cancers, 1×10^6 5TGM1 cells (in 100 µL PBS) were injected into each mouse through the tail vein and the tumors were allowed to grow for a week. The bone-targeted, CTSK-cleavable nanotherapeutics, as well as the corresponding non-targeted nanotherapeutics (neutral NVs), or free DOX were investigated. The doses of doxorubicin were kept equivalent in different groups. Another group with PBS was used as a negative control. Three C57BL/KaLwRij mice with 5TGM1 inoculations were used for each group. At the time of the experiments, the animal was injected with different therapeutic formulations or controls at the dose of 0.75 mg/Kg twice a week. The tumor burden in the mice was monitored by weekly ELISA measurements of monoclonal immunoglobulin (Ig) in mouse sera. Survival was evaluated from the day of tumor inoculation until death.

2.18. Statistical analysis

All measurements were conducted in duplicate or triplicate and expressed as mean ± standard deviation where indicated. Differences between the mean values were analyzed by two-sided Student's t test or one-way ANOVA. Kaplan–meier curve and log rank test were used for mouse survival. P values of <0.05 are considered statistically significant.

3. Results and Discussion

A peptide-conjugated diblock copolymer consisting of peptide, poly(ethylene glycol) and poly(trimethylene carbonate) (Pep-*b*-PEG-*b*-PTMC) has firstly been synthesized. PEG was used as the hydrophilic block because of its established role to help NVs escape from protein opsonization and macrophage uptake in the reticuloendothelial system. The rationale behind the choice of PTMC as the hydrophobic block was that PTMC can degrade with relatively slower rate and have weak inflammatory effect of degradation compounds, which is a marked advantage over polyester and particularly attractive to sustained drug delivery application.²²

The synthesis of peptide-conjugated diblock copolymer of Pep-*b*-PEG-*b*-PTMC was based on the efficient click reaction between the cysteine terminated Pep peptide and maleimidevinyl terminated Mal-PEG-*b*-PTMC diblock copolymer, as illustrated in Fig. 1. Via the well-established organocatalytic (*i.e.* TU) ring-opening polymerization approach, non-functional MPEG-*b*-PTMC copolymer could be readily prepared with a defined molecular weight and narrow molecular distribution when using MPEG-OH (Mw=5000) as the macroinitiator.²³ This strategy was problematic, however, when it came to the synthesis of maleimide-terminated copolymer of Mal-PEG-*b*-PTMC starting from Mal-PEG-OH. ¹H NMR analysis revealed that the maleimidevinyl protons at $\sigma = 6.74$ ppm in the product disappeared. Apparently, the maleimide group might be very labile to super basic condition, and failed to remain intact during the polymerization process. Alternatively, the Mal-PEG₁₁₀-*b*-PTMC₅₂, in which 52 referred to the repeating units of TMC, was successfully synthesized in a mild condition under Sn(Oct)₂ catalysis (Fig. 1A). ¹H NMR analysis demonstrated that the maleimidevinyl protons at $\sigma = 6.74$ ppm in the product remained available after polymerization (Fig. 2). The copolymer composition and number-averaged molecular weight of Mal-PEG₁₁₀-*b*-PTMC₅₂ was determined from the integral ratio of PTMC protons at $\sigma = 4.21$ ppm versus that of the PEG proton at $\sigma = 3.64$ ppm. For comparison purpose, the peptide-free methoxy-terminated MPEG₁₁₃-*b*-PTMC₅₀ was also prepared.

Next, we synthesized the functional peptide with the specially designed sequence of CKGHPPGGPQAsp₈ based on the solid phase peptide synthesis (SPPS) technique (Fig. 1B). Purity (98%) and molecular weight (calculated: 1800.64, found: 1800.54) of the target peptide were confirmed by high-performance liquid chromatography (HPLC) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, respectively (Fig. S1). Via click chemistry, the chemical conjugation of CKGHPPGGPQAsp₈ to Mal-PEG₁₁₀-PTMC₅₂ afforded the target copolymer PEP-*b*-PEG₁₁₀-*b*-PTMC₅₂ (Fig. 1C), which was purified by dialysis method (MWCO = 10 K) followed by ultrafiltration treatment. Successful conjugation was verified by ¹H NMR, which clearly showed the characteristic resonances of the peptide protons at $\sigma = 3.1\sim 3.6$ ppm and complete disappearance of the maleimidevinyl signal at $\sigma = 6.74$ ppm (Fig. 2).

As expected, Pep-*b*-PEG-*b*-PTMC could readily self-assemble into the micelles in aqueous solution like many other amphiphilic copolymers,²⁴ which was confirmed by fluorescence assay using pyrene as the probe. According to the excitation spectra of pyrene at different

polymer concentrations, critical micelle concentration (CMC) of *Pep-b-PEG₁₁₀-b-PTMC₅₂* was determined to be approximately 8.02×10^{-3} mg/mL (Fig. 3C). The mean hydrodynamic diameter of the polymeric micelles was measured by dynamic light scattering (DLS) to be 75 ± 10 nm, showing unimodal size distribution with PDI ~ 0.1 .

In principle, the hydrophobic PTMC segments should localize in the inner core of nanomicelles while peptide and PEG blocks form a corona-type shell due to their highly hydrophilic nature. This core-shell structure was verified by the marked difference between its ^1H NMR spectra in DMSO (Fig. 3A) and D₂O (Fig. 3B). All the characteristic signals could be detectable in DMSO-*d*₆. Unlike it, the proton signals of PTMC block were significantly suppressed and nearly disappeared in aqueous D₂O but those from hydrophilic blocks remained prominent. Taken together, those characterizations demonstrated that *Pep-b-PEG₁₁₀-b-PTMC₅₂* were able to readily form the NVs with the core-shell architecture in the aqueous medium.

The strong binding ability of *Pep-b-PEG₁₁₀-b-PTMC₅₂* micelles to hydroxyapatite (HA), commonly used as a model mineral to mimic bone tissue mineral, was examined to preliminarily evaluate the contribution of peptide functionality to the bone-seeking efficacy.^[5-7] A near infra-red fluorescence (NIRF) Cy5.5 probe has been one of the frequently used fluorescence indicators due to its deeper tissue penetration capacity.²⁵ Cy5.5 was encapsulated into the micellar cores by the dialysis method thus to quantitatively determine the binding affinities of the nanomicelles with and without peptide functionality, respectively.

After incubation for 30 min, approximately 55% of the *Pep-b-PEG-b-PTMC* micelles from the solution were rapidly bound to HA. Further incubation for 24 hours led to a moderate increase of HA-binding up to about 66% (Fig. 4). In marked contrast, less than 15% of *MPEG-b-PTMC* micelles or free Cy5.5 were bound to HA across the entire incubation process, indicating minimal nonspecific binding to HA by the *MPEG-b-PTMC* micelles absent of peptide functionality. The low-molecular-weight peptide-Cy5.5 conjugate exhibited a much stronger affinity to HA with complete binding after incubation for 30 min. Those results correlated well with the reported findings that modification with osteotropic Asp8 would dramatically facilitate the specific binding towards HA-rich bone tissue.²⁶⁻²⁸ Though the outer PEG shell with steric hindrance may somewhat restraint the complete binding of *Pep-b-PEG-b-PTMC* micelles onto HA, they still displayed dramatically enhanced HA affinities over *Pep-free MPEG-b-PTMC* micelles after all.

Typically, the decoration of bone-targeting anionic Asp8 to NVs would disfavor the cellular uptake process due to the charge repulsion by cell membrane, thus impairing the chemotherapy efficacy.²⁹ Within the structure of *Pep-b-PEG-b-PTMC*, a CTSK cleavable substrate (HPGGPQ)^{25, 30} was specially incorporated to link Asp8 and the rest of block copolymer. By virtue of such a CTSK-cleavable spacer, it was speculated that the bone metastatic microenvironment featured with CTSK overexpression would induce the enzymatic chain cleavage and the following removal of Asp8 moieties from the shell periphery of the nanomicelles. Of the particular interest, this CTSK-induced transition may reverse the surface charge and thus enhance the cellular uptake of the nanomicelles, which

was attributed to the remaining cationic peptide residues after Asp₈ removal. To verify this speculation, the zeta potentials of polymeric micelles were measured in the presence and absence of CTSK, respectively. Following 30-min treatment with CTSK, the zeta potential of Pep-*b*-PEG-*b*-PTMC micelles had an instantly marked promotion from -18.5 ± 1.9 mV to 15.2 ± 1.8 mV (Fig. 5). Nevertheless, the control experiment without CTSK treatment afforded nearly no variation of zeta potential. Apparently, it is just CTSK exposure that allowed the charge reversal of the Pep-*b*-PEG-*b*-PTMC micelles.

For comparison, the CTSK-responsiveness of zeta potential was also tested for MPEG-*b*-PTMC micelles with the identical CTSK treatment. No apparent variation was found upon CTSK exposure and the micellar zeta potential stayed almost constant within a narrow range from -8.6 ± 2.1 mV to -8.4 ± 0.7 mV. Those results pointed out that the introduction of CKGHPPGGPQAsp₈ functionality indeed played a dominant role in the CTSK-induced negative-to-positive charge conversion of Pep-*b*-PEG-*b*-PTMC micelles.

To evidence the contribution of CTSK-responsive charge reversal to the intracellular internalization, confocal laser scanning microscopy (CLMS) was used to investigate the cellular uptake of polymeric micelles under the conditions with or without CTSK treatment. The nanomicelles assembled from Pep-*b*-PEG-*b*-PTMC were loaded with Cy5.5 fluorescence indicator, followed by incubating with MCF-7 cells in the presence or absence of CTSK. Following 30-min incubation, the MCF-7 cells were subjected to CLMS observation. As shown in Fig. 6, very weak red fluorescence can be detectable in the cells co-incubated with Pep-*b*-PEG-*b*-PTMC micelles in the medium free of CTSK, indicating that few fluorescence-labeled micelles were taken up by MCF-7 cells under this condition. The poor cellular uptake of untreated micelles was reasonably expected, because it was difficult for the negatively charged NVs to be intracellularly internalized owing to the charge repulsion effect. However, upon CTSK exposure, significant fluorescence enhancement was observed in the CLSM images. This was ascribed to cationic nature of the micelles resulting from CTSK-induced charge-reversal, which was known to favor the approach of NVs to the negatively charged cell membranes and thus facilitate their cellular uptake. For the control of MPEG-*b*-PTMC micelles, it is not strange that minimal difference was observed between the CLSM images obtained before and after CTSK treatment (data not shown)

The CTSK-dependent cellular uptake of Pep-*b*-PEG-*b*-PTMC nanomicelles seemed very attractive in terms of the chemotherapy potential for bone metastases. It can be imaged that those nanomicelles would be hardly taken up by the cells in the normal tissues for the lack of CTSK expression; once the nanoparticles was guided and accumulated in CTSK-rich environments (i.e., CTSK over-expressed bone metastases lesions), however, the transition to cationic NVs would considerably facilitate the entry into the neighboring tumor cell and thus allow the effective treatment with lower systematical biotoxicity.

To further explore the potential of Pep-*b*-PEG-*b*-PTMC as a drug carrier, an anticancer drug of DOX was used as a model drug to investigate the drug loading and release properties. Poorly water-soluble DOX was encapsulated into the micelles of Pep-*b*-PEG-*b*-PTMC or MPEG-*b*-PTMC by the dialysis method. Typical DOX-loading capacity for mPEG-*b*-PTMC

micelles were determined as $7.5 \pm 0.5\%$ and $22.7 \pm 1.5\%$ for DLC (drug loading content) and DLE (drug loading efficiency), respectively. Dramatic increases of DLC ($30.0 \pm 1\%$) and DLE ($90.1 \pm 2\%$) were observed when Pep-*b*-PEG-*b*-PTMC was used as the drug carrier. Such a high loading efficiency may be a result of the charged nature of Pep-*b*-PEG-*b*-PTMC, for which the outer PEG chains extend more freely and more aggregation numbers per micelle are required to ensure the hydrophilic-hydrophobic balance in the aqueous medium. Thus the DOX drug molecules enter the micelle interior more readily and the larger core is more capable of containing more drug cargos. This is in consistence with the finding reported in many literatures that charged NVs generally possess high DOX-loading capacity.^{13, 31-33} The representative example is that the highest drug loading (47% w/w) of DOX-loaded micelles reported to date was achieved when anionic polymeric glutamic acid (PGA) was used as a building block within PTMC-*b*-PGA.³¹ The *in vitro* drug release study was performed under a simulated physiological condition (pH 7.4). Fig. 7A compares the drug release profiles between free DOX and the DOX loaded in Pep-*b*-PEG-*b*-PTMC micelles. The results suggested that DOX-loaded micelles exhibited a moderately rapid release in the first stage (around 10%) followed by a sustained release period. The sustained DOX release could be attributed to the hydrophobic interaction of drug molecules with the hydrophobic core of the polymeric micelles.

The *in vitro* cytotoxicity profiles of free DOX or DOX-loaded polymeric micelles were evaluated in the cultured cells including myeloma (the most frequent cancer type for bone metastases) suspension cell lines (5TGM1 and ARP-1) and a breast cancer (the second most frequent cancer type for bone metastases) adherent cell line (MCF-7) by a PrestoBlue™ (Invitrogen) assay. The PrestoBlue assay worked very well to quantitatively measure cell proliferation and to establish the relative viability of both suspension and adherent cells. As shown in Fig. 7C, free DOX exhibited a relatively higher toxic effect than that of both DOX-loaded Pep-free and Pep-functionalized polymeric micelles after 4-h culture. The lowered potency of DOX after encapsulation into the micelles was probably due to the sustained release pattern of DOX from the micelles and delayed drug efficacy in the cultured cells. Overall, DOX-loaded Pep-PEG-*b*-PTMC nanomicelles showed lower cytotoxicity than the Pep-free MPEG-*b*-PTMC micelles. Since the CTSK activity have strong association with the interactions between tumor cells and the bone microenvironment, to give more exact information about the influence of CTSK-responsive charge-reversal on the biological properties of Pep-*b*-PEG-*b*-PTMC micelles, the 5TGM1 cytotoxicity of the DOX-loaded micelles were comparatively explored after a short co-incubation period of 30 min under the conditions with or without CTSK treatment. At an equivalent DOX dose (1 $\mu\text{g/mL}$), drug-loaded Pep-*b*-PEG-*b*-PTMC micelles showed lower capability to induce the cell death than the Pep-free MPEG-*b*-PTMC control. CTSK treatment resulted in significant improvement of drug efficacy in the case of Pep-*b*-PEG-*b*-PTMC micelles (Fig. 7B), which was even comparable to the acute toxicity of free DOX. Noticeably, the CTSK-responsive improvement of toxic effect was not observed for free DOX or DOX-loaded MPEG-*b*-PTMC micelles. This marked contrast manifested the close tie between the CTSK responsiveness and the Pep functionality in the micelles, which contributed much to the enhanced drug efficacy. In combination with the result of intracellular internalization obtained from confocal images, it is naturally inferred that the anion nature associated with

the targeting Asp8 moieties would disfavor the intracellular internalization of the Pep-*b*-PEG-*b*-PTMC micelles and therefore impair the drug efficacy, whereas CTSK-responsive charge reversal may more or less overcome this biological barrier.

Despite the advance of drug delivery nanosystems for bone metastases, to our best knowledge, nearly no *in vivo* efficacy evaluation in animal studies have yet been reported. This could be in part ascribed to the limited availability of suitable detection means for the disease progression of bone metastases. Herein, a preliminary *in vivo* study was conducted to test the efficacy of DOX-loaded Pep-*b*-PEG-*b*-PTMC nanomicelles in a 5TGM1-induced myeloma-bearing mice model. Multiple myeloma is the most frequently cancer-induced osteolytic disease,^{1, 2} which intends to invade skeletal tissues in a similar mechanism as cancer cells that interact with the bone marrow microenvironment.³⁴ Likewise, the interactions between myeloma cells and osteoclasts yield substantial cathepsin K up-regulation, resulting in osteolytic lesions.³⁵ Noticeably, the real-time tumor burden in 5TGM1 mice can be easily monitored by measuring the 5TGM1 idiotype (IgG2b) in mouse sera.³⁶ The higher IgG2b level in sera, the bigger tumor in the body. This is a distinct advantage since the disease progression of other types of cancers can only be identified with advanced imaging modalities (μ CT, x-ray etc.).³⁷ Myeloma-bearing 5TGM1 mice were established through inoculation of 5TGM1 cells (1×10^6 cells in 100 μ L PBS) via tail vein injection, and the tumors were allowed to develop and grow for one week before initiating study treatments. The mice were injected twice weekly with different therapeutic formulations at equivalent DOX dose (0.75 mg/Kg) or blank PBS, and the IgG2b level in sera was monitored by ELISA measurement.

As shown in Fig. 8A, the myeloma-bearing 5TGM1 mice group treated with DOX-loaded Pep-*b*-PEG-*b*-PTMC micelles showed prolonged survival rate in comparison with the control groups with the administration of DOX-loaded MPEG-*b*-PTMC micelles, free DOX or blank PBS. Since the tumor burden was positively correlated with IgG2b level, the data in Fig. 8B distinctly indicated that Pep-*b*-PEG-*b*-PTMC/DOX nanotherapeutic was meanwhile more effective to slow down tumor growth than the other formulations with the equivalent DOX dose. Together with the *in vitro* results, the observed improvement of both *in vivo* systematic biosafety and drug efficacy manifested the superiority of our pathology-specific design by combining both the advantages of efficient DOX delivery to bone metastatic tissues and CTSK-responsive cellular uptake of DOX within lesion sites.

4. Conclusions

Cancer induced bone metastases often lead to substantial morbidity and mortality, which remain a significant therapeutic challenge for cancer treatment. In attempts to address this challenge, a peptide decoration strategy was proposed to render the NVs both the bone-seeking function and the charge-reversal capability in response to the pathological microenvironment. The nanomicelles self-assembled from Pep-*b*-PEG-*b*-PTMC polymer showed strong apatite binding affinity and readily underwent the negative-to-positive charge reversal upon exposure to CTSK, an overexpressed enzyme in bone metastatic microenvironments. This unique characteristic led to dramatically enhanced cellular uptake within bone metastatic sites in a pathology-responsive manner, thus improving the drug

efficacy of loaded DOX *in vitro* and *in vivo*. Although our attempt presented herein for the enhanced bone metastasis chemotherapy is still in an initial stage, the obtained *in vivo* and *in vitro* results are fairly encouraging. More attractively, the success of this strategy may open a new avenue to the chemotherapy for the malignant diseases in bone tissue including but not limited to bone metastases, provided that the development of therapy for those diseases involves overexpression of certain enzymes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Synthetic procedure and chemical structure of Mal-PEG₁₁₀-*b*-PTMC₅₂ (**A**), the Pep peptide (**B**) and Pep-*b*-PEG₁₁₀-*b*-PTMC₅₂ (**C**).

Fig. 2.
 ^1H NMR spectra of MPEG₁₁₃-*b*-PTMC₅₀ (CDCl₃), Mal-PEG₁₁₀-*b*-PTMC₅₂ (CDCl₃) and
Pep-*b*-PEG₁₁₀-*b*-PTMC₅₂ (DMSO-*d*₆).

Fig. 3. ^1H NMR spectra of $\text{Pep-}b\text{-PEG}_{110}\text{-}b\text{-PTMC}_{52}$ in DMSO-d_6 (A) and D_2O (B); CMC measurement by plotting of the I_{340}/I_{338} ratio against logarithm of the polymer concentration (C).

Fig. 4.
Binding capacity of various Cy5.5 formulations to the bone mineral HA.

Fig. 5. Change in the zeta potential of Pep-*b*-PEG-*b*-PTMC micelles upon co-incubation with 150 nM CTSK for 30 minutes.

Fig. 6. CLSM images of MCF-7 cells incubated Pep-*b*-PEG-*b*-PTMC micelles with or without CTSK treatment.

Fig. 7.

(A) Cumulative DOX release from the polymeric micelles at pH=7.4; (B) *In vitro* inhibition to 5TGM1, ARP-1 and MCF-7 cells after 48h incubation with free DOX or DOX-loaded NVs at various doses; (C) Cell viability of 5TGM1 cells after incubation with free DOX, DOX-loaded MPEG-*b*-PTMC nanomicelles, and DOX-loaded Pep-*b*-PEG-*b*-PTMC nanomicelles at the same DOX concentration of 1 µg/mL⁻¹. Formulations were pre-treated with (blue) or without (red) 150 nM CTSK for 30 minutes. Data are presented as the average ± standard deviation (n=3).

Fig. 8.

(A) Efficacy of free DOX or DOX-loaded nanomicelles in bone metastatic 5TGM1 mice bearing myeloma; (B) Kaplan-meyer survival curves of mice following tumor injection and treatments. Relative tumour burden in mice was determined by IgG 2b level in sera.

Scheme 1.

Schematic illustration of nanotherapeutic formation and its mechanism of action *in vivo* for bone metastasis treatment.