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Cyclic RGD targeting nanoparticles with pH sensitive polymer-drug conjugates for effective treatment of melanoma

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It is high desirable to design and development of multifunctional nanoparticles for improving specificity, tumor accumulation and therapeutic efficacy. A strategy of combining active targeting with tumor microenvironmental response in one polymeric drug delivery system was developed via assembling a c(RGDfK)-linked copolymer and a pH sensitive polymer-epirubicin conjugate in aqueous solution. These hybrid micelles with diameter of less than 100 nm showed pH sensitive drug release behaviours in vitro, which could be used for assisting tumor targeting and drug delivery. Confocal laser scanning microscopy and flow cytometry results indicated that they can enhance the endocytosis by B16F10 cells. Moreover, the hybrid micelles displayed synergistic effect and led to increased cytotoxicity in vitro and enhanced antitumor efficacy in vivo.

1 Introduction

Nanoparticles offer an opportunity to alter the pharmacokinetic profile of drugs, to reduce systemic toxicity, and to improve the therapeutic index through passive and active targeting.[1-4] Passive targeting is a consequence of enhanced permeability and retention (EPR), whereby the leakiness of the tumor vasculature combined with poor lymphatic drainage enables nanoparticles to accumulate within the tumor tissues.[5,6] Although EPR mediates enhanced delivery of nanomedicines to tumors compared with free small-molecule drugs, it is widely believed that active targeting has the potential to increase the content of nanoparticles in tumor sites and enhance the therapeutic efficacy relative to nontargeted nanosystems. The presence of targeting ligands can enhance the cellular uptake of nanoparticles via receptor-mediated endocytosis, although tumor accumulation of nanoparticles is mainly dependent on the physicochemical properties of nanoparticles.[7-10]

The targeted nanoparticles usually are accomplished by coupling low-molecular weight targeting ligands, like folate, peptides and aptamers, onto the starting materials and by self-assembling of these materials.[11,12]Taking advantage of antigens and receptors exclusively (or highly) expressed on tumor cells or tumor vascular endothelial cells the targeted nanocarriers can specially bind to target cells and be quickly internalized. Compared with antibodies and protein targeting ligands, the peptides show some irreplaceable advantages, such as low cost and high conjugation efficiencies and easy purification.[13,14] Although several targeted nanoparticles are now in clinical trials,[15] clinical translation of targeted delivery is still a long way to go.

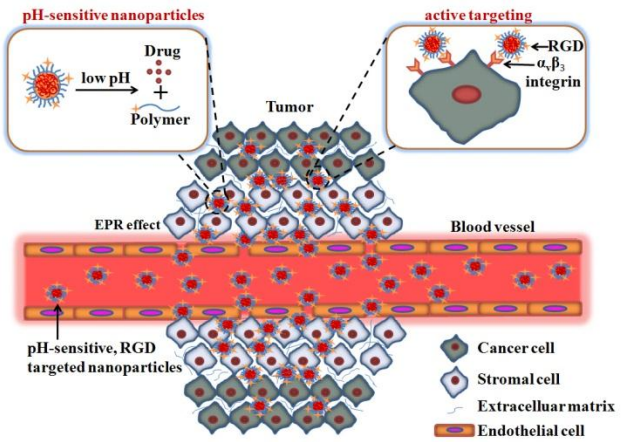
RGD/integrin system has been widely used in anticancer drugsdelivery.[16] Nanocarriers decorated with arginine-glycine-aspartic acid (RGD) peptide can selectively bind to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins which are overexpressed in tumor vascular endothelial cells and some solid tumors (such as glioblastoma, breast cancer, prostate carcinoma).[17,18] Accumulating studies have proven that RGD-targeted systems are capable of improving the efficiency of target delivery in chemotherapeutics treatment.[19-24]

Recently, the tumor microenvironments(various differences compared with normal tissues) have been utilized to design high effective antitumor drugs. Among these differences, the lower pH in tumor site and in tumor cells is frequently considered.[25-27] Compared with pH7.4 of normal tissues, the pH value in tumors usually ranges from 5.7 to 7.4, even much lower pH ranging from 4.5 to 5.5 can be detected in intracellular late endosomes and lysosomes.[28-30] PH-sensitive nanocarriers which are stable under physiological pH can rapidly release the drug when the pH reached trigger point value. Many studies have demonstrated the great potential of pH-sensitive drug nanocarriers in improving the efficiency of cancer treatment.[31-33]

To our knowledge, very few researches combining active targeting and tumor microenvironment stimuli to address the

drug delivery. We speculate that a pH-sensitive, RGD-targeted nanocarrier could greatly improve the drug accumulation and significantly inhibit tumor growth in vivo (Scheme 1). Until now, only one report indicated that the pH-sensitive RGD linked nanoparticles exhibited high efficiency to kill U87 human glioblastoma cancer cells.[34] However, this report only examined the drug release of nanoparticles in U87 cells, and in vivo evaluation was not carried out to examine its antitumor efficacy.

In this study, we developed a pH sensitive and cRGD-linked polymer-epirubicin (EPI) conjugates micelle to delivering EPI to tumor cells. Drug releases in vitro, cell uptake, cell toxicity in vitro and antitumor efficacy in vivo were studied and evaluated in detailed.



Scheme.1 Schematic illustration of tumor penetration and cell uptake of pH-sensitive, c(RGD)-targeted EPI conjugated micelles.

2 Material and Methods

2.1 Materials

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and 9,10-phenan-threnequinone were purchased from Amresco. DAPI (4',6-diamidino-2-phenylindole) was purchased from Shanghai Yuanye Ltd. EPI was purchased from Zhejiang Hisun Pharmaceutical Co. Ltd, cyclic (RGDfK) peptide was customized from Shanghai Chinapeptides Ltd. Ultrapure water was prepared from a Milli-Q system(Millipore, USA). All other chemicals were analytical grade and used directly.

2.2 Polymer synthesis and characterization

2.2.1 Synthesis of EPI-conjugated polymer

2.2.1.1 Synthesis of mPEG-b-PAGE/MCH

Firstly, block copolymer poly(ethylene oxide)-block-(allylglycidyl ether) (mPEG-b-PAGE) was synthesized according to the literature as shown in Scheme.1. The copolymers used in this paper had a formula of $mPEG_{113}$-$PAGE_8$, where the subscripts are the degree of polymerization of the corresponding repeating units measured by proton nuclear magnetic resonance (^1H NMR). Secondly, in order to introduce hydroxyl groups, 2-mercaptoethanol (MCH) was used to react with the mPEG-b-PAGE by thio-ene reaction. Briefly, the mPEG-b-PAGE (1g, 1.23 mmolallyl) and MCH (0.45 ml, 1.15 mmol) were dissolved in 10 ml THF in a 100 ml round-bottom quartz flask, followed by degassing using N_2 for 30 min to eliminate the dissolved oxygen. Then the mixture was stirred at room temperature under UV light (254 nm, 1.29 mW/cm^2). After 8 h, the light source was turned off and the mixture was concentrated under vacuum. The residue was poured into large amount of cold diethyl ether to cause precipitation to give the final product mPEG-b-PAGE/MCH. (Yield: 90 %).

2.2.1.2 Synthesis of EPI-conjugated polymer via carbamate linkage

The pendant hydroxyl groups of mPEG-b-PAGE/MCH were first activated with 4-nitrophenyl chloroformate (NPC). Briefly, to mPEG-b-PAGE/MCH block copolymer (0.8g) dissolved in methylene chloride (20 ml), NPC (0.198 g) and triethylamine (TEA) (0.3 ml) were added dropwise at 0 ℃ (molar ratio of DHP/NPC/TEA is 1/1/4). The reaction mixture was stirred for 4 h at 0 ℃, and finally the NPC activated polymer mPEG-b-PAGE/NPC was obtained by precipitation in cold diethyl ether, and dried in vacuo.

Then the mPEG-b-PAGE/NPC (0.2 g) dissolved in dimethylformamide (DMF) (10 ml) was reacted with EPI (100 mg) in the presence of TEA (57 μL) for 48 h at room temperature under nitrogen. The product mPEG-b-PAGE-cbm-EPI was purified as polymeric micelles by adding doubly distilled water to the organic phase, followed by dialyzing against water with a cellulose membrane (cutoff Mn=3500) for 3 days. After dialysis, the conjugate polymer was lyophilized and stored. The EPI content was determined by measuring the UV absorbance of a DMSO solution of the conjugate at 485 nm.A calibration curve was constructed using a series of concentrations of free EPI in DMSO.

2.2.1.3 Synthesis of EPI-conjugated polymer via hydrazine linkage

Firstly, hydrazine monohydrate (477 μL) was slowly dropped into the mPEG-b-PAGE/NPC copolymer (0.8 g) dissolved in methylene chloride (10 ml) (molar ratio of activated hydroxyl groups/hydrazine: 1/10). The reaction was carried out for 2 h at room temperature. By precipitation in diethyl ether, the resulting hydrazine conjugated mPEG-b-PAGE copolymer was obtained. Secondly, The hydrazine conjugated mPEG-b-PAGE (0.2g) dissolved in dimethylformamide (10 ml) was further reacted with EPI (100 mg) in the presence of TEA (0.2 ml) for 48 h at room temperature under nitrogen. The EPI conjugate mPEG-b-PAGE-hz-EPI was purified as that prepared in previous section. The drug content was also determined by measuring the UV absorbance at 485 nm.

2.2.2Synthesis of c(RGDfK)-containing copolymer

$HOOC\text{-}PEG_{5K}\text{-}PLA_{3K}$was synthesized in previous work [35]. $HOOC\text{-}PEG_{5K}\text{-}PLA_{3K}$ (200 mg, 25 μmol) was dissolved in 4ml DMSO, then 7.74mg dicyclohexylcarbodiimide (DCC) (37.5 μmol) and 4.32 mg N-hydroxysuccinimide (NHS) (37.5 μmol) were added. The reactions were conducted at room temperature

for 2 hours and then 15mg c(RGDfK) peptide (25 μmol) was added. After dialysis against distilled water for 12 hours (MWCO=3500), the final suspension in dialysis bag containing cRGD-PEG_{5K}-PLA_{3K} was freeze-dried. The c(RGDfK) peptide content of the polymers was determined by measuring the arginine content according to the method of Graf N.[36]

2.3 Preparation of micelles and hybrid micelles

The EPI-containing copolymers mPEG-b-PAGE-cbm-EPI and mPEG-b-PAGE-hz-EPI prepared above were self-assembled into micelles through solvent evaporation method. Briefly, 50 mg EPI-conjugated polymer was dissolved in 2 ml THF, and polymer solution was added dropwise to 10 ml of deionized water. The suspension was stirred until all of THF was evaporated. The micelles obtained were abbreviated as M(cbm) and M(hz), respectively.

Hybrid micelles cRGD-M(cbm) and cRGD-M(hz) were also prepared similarly. For example, to prepare hybrid cRGD-M(cbm), 80 mg mPEG-b-PAGE-cbm-EPI and 20 mg cRGD-PEG_{5K}-PLA_{3K}were dissolved in 2 ml THF. After 2 minutes of sonication, the polymer solution was added dropwise to 10 ml of deionized water. The suspension was stirred vigorously for 10 h until no THF could be detected. The hybrid micelles cRGD-M(hz) composed of 80 wt% of mPEG-b-PAGE-hz-EPI and 20 wt% of cRGD-PEG_{5K}-PLA_{3K}.

2.4 EPI release in vitro

The release profile of EPI from M(cbm) and M(hz) were studied at 37°C in PBS buffers of different pH values (pH5.0, pH 6.5 and pH7.4). Briefly, micelle dispersion containing 2 mg EPI was transferred into dialysis bag (MWCO= 3000) followed by immersing in 20 ml PBS buffer (50 mM, pH5.0, pH 6.5 and pH 7.4) with stirring at 37°C. At certain time points, 1 ml dialysis solution was taken out for UV-Vis measurement (485 nm) and replenished with 1 ml fresh PBS solution.

2.5 In vitro cytotoxicity

The cytotoxicity of four kinds of EPI-conjugated micelles was measured via MTT assay with free EPI as a control. Briefly, B16F10 melanoma cells were seeded in 96-well plates at 2×10^3cells/well 12 h prior to incubation with drugs in 100 μL Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), and then incubated at 37°C in 5% CO_2.The medium was refreshed with 200 μl DMEM medium containing different concentrations of EPI or micelles for 48 or 72 h.The concentration of EPI ranged from 0.01 to 10 μg/ml. 20 μl MTT (5 mg/ml) was added to each well and incubation was continued for more 4 h, and then 150 μl DMSO were added to each well to dissolve the blue formazan formed in the live cells. The absorbance was measured on a microplate reader (BioTek, EXL808) at 490 nm. Experiments were repeated three times.

2.6 Cellular uptake study

The cellular uptake of M(cbm), cRGD-M(cbm), M(hz) and cRGD-M(hz) was evaluated by confocal laser scanning microscopy(CLSM) and flow cytometry.

2.6.1 CLSM

B16F10 cells (2×10^5 cells/well) were seeded into a six-well plate. After 12h incubation the medium was replaced with fresh medium (2 ml) containing 20 μg EPI or micelles for 1 or 3 h. For the pre-treatment of chloroquine, 25μg/ml chloroquine in DMEM medium were added to the cells for 1h at 37 °C, and then equal dose of EPI or EPI-loaded micelles were added. The cells were washed three times with PBS, and fixed with 4% (w/v) paraformaldehyde for 10 min at room temperature. The cell nuclei were stained with DAPI (6-diamidino-2-phenylindole) for 15 min. CLSM images were captured via confocal microscope (Carl Zeiss LSM 780) under the same conditions.

2.6.2 Flow cytometry

B16F10 cells were seeded in six-well plates (2×10^5 cells/well) and cultured for 12 h. The medium was replaced with fresh medium (2 ml) containing 20 μg EPI or micelles for 1 or 3 h. The cells were rinsed three times with PBS and processed with 200 μl 0.25% trypsin for 2 min. After adding 1 ml DMEM medium, the cells were centrifuged for 5 min at 1000 rpm. After removing supernatant, the cells were resuspended in 0.5 ml PBS. Flow cytometry analysis was performed by a flow cytometer (Beckman, USA) which collected 1×10^4gated events for each sample.

2.7 Antitumor activity assay

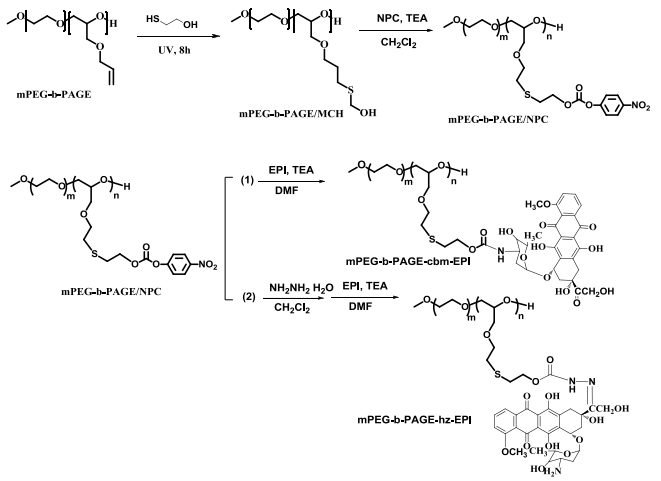
For in vivo anti-tumor activity assay C57BL/6 mice were utilized to implant xenograft B16F10 melanoma. 1 million B16F10 cells were used to produce subcutaneous tumor xenograft. When tumor volume reached 50~100mm^3, xenograft-bearing mice were divided randomly into 6 groups (6 mice per group): saline, free EPI, M(cbm), cRGD-M(cbm), M(hz) and cRGD-M(hz). The drug was administered intravenously for three times and every two days with equal equivalent dose of 10 mg/kg of EPI. The day of first administration was named as day 0. The anti-tumor activities were evaluated by measuring the tumor volume (V) estimated by the following equation: $V = ab^2/2$, where a and b stand for the major and minor axes of the tumor measured via Verniermicrocaliper.

2.8 Statistics

All experiments were performed at least three times and all results are expressed as mean±SD (standard deviation). Students't-test was used to demonstrate statistical significance ($P<0.05$).

3Results

3.1 Synthesis of copolymers



Scheme. 2 Synthesis route of polymer drug mPEG-b-PAGE-cbm-EPI and mPEG-b-PAGE-hz-EPI.

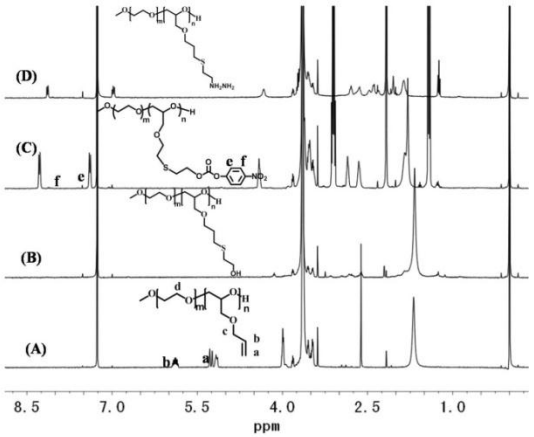
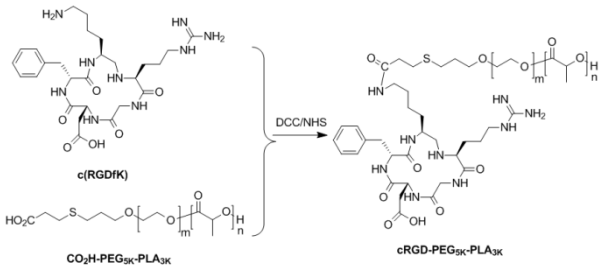


Fig. 1 ^{1}H NMR spectra of mPEG-b-PAGE (A), mPEG-b-PAGE/MCH (B), mPEG-b-PAGE/NPC (C), and mPEG-b-PAGE/NH_2NH_2(D) in $CDCl_3$.

In this paper, diblock copolymer PEG-b-PAGE with allyl groups on the right block was designed and synthesized. These allyl groups were converted to hydroxyl groups via thio-lene reaction with 2-mercaptoethanol (MCH) which were used to connect anticancer drug EPI in two different linkages. The synthesis procedure is shown in Scheme 2. First, anionic ring-opening copolymerization of AGE (allylglycidyl ether) in toluene at 45 ^{o}C using mPEG/cesium hydroxide as an initiator afforded functional mPEG-b-PAGE. By adjusting the feed ratio of mPEG and AGE, different block copolymers were successfully prepared and copolymer $mPEG_{113}$-$PAGE_8$ was chosen for subsequent drug conjugation. The anticancer drug EPI was chemically conjugated to the pendant hydroxyl groups via two different covalent bonds, an acid-labile hydrazone linkage (mPEG-b-PAGE-hz-EPI) and a more stable carbamate linkage (mPEG-b-PAGE-cbm-EPI), by reacting with the amino and keto groups in EPI, respectively, as shown in Scheme2.

The chemical structures of the intermediate and final products were characterized by ^{1}H NMR (Fig. 1). As shown in Fig. 1, mPEG-b-PAGE (Fig. 1A) displayed the ^{1}H NMR signals of mPEG (d at 3.65 ppm), and PAGE (a at 5.3 ppm, b at 5.9 ppm and c at 4.0 ppm). The polymerization degree of AGE in the copolymer was calculated from the integral area ratios of $-CH_2CH_2O$ (mPEG at 3.65 ppm) to CH_2=C*H*-CH_2 (b at 5.9 ppm). Then, mPEG-b-PAGE was reacted with excess MCH at room temperature under UV irradiation. After the radical mediated thiol-ene addition reaction between the allyl groups and MCH, the signals of the allyl groups disappeared (Fig. 1B). In order to conjugate with the drug EPI, the obtained pendant hydroxyl groups was further reacted with NPC. The appearance of C_6H_4-NO_2signals at 7.48 ppm and 8.3 ppm indicated the successful activation of OH groups (Fig. 1C). Hydrazine was successfully introduced into the polymer as shown in Fig. 1D and the signals of C_6H_4-NO_2 at 7.48 ppm disappeared. The unconjugated drug was removed by dialysis the production against water. The EPI content was determined to be 31.25 wt% and 30.5 wt% in the polymer conjugates with carbamate linkage and hydrazone linkage, respectively.

cRGD-containing copolymer was prepared by reacting c(RGDfK) with HOOC-PEG_{5K}-$PLA_{3K}$with the help of DCC and NHS as shown in Scheme 3:



Scheme. 3 Preparation of cRGD-PEG_{5K}-PLA_{3K}.

Attachment of c(RGDfK) peptide onto HOOC-PEG_{5K}-PLA_{3K} was confirmed by fluorimetric assay, in which 9,10-phenanthrenequinone was reacted with the arginine residue in c(RGDfK) to generate a specific fluorescent compound. By fluorescence measurement of this compound (λ_{ex}=312 nm, λ_{em}=340~570nm), the molar ratio of cRGD peptide to backbone polymer was determined to be 1:2.17, i.e., averagely 46 % of HOOC-PEG_{5K}-PLA_{3K} was labeled with c(RGDfK).

3.2 Preparation of micelles and hybrid micelles

In this study, the hydrophilic segments of the two block copolymers (mPEG-b-PAGE-cbm-EPI and mPEG-b-PAGE-hz-EPI) were designed with the same length (~5000) and hydrophobic segments were fixed to ~3000. Starting from these two copolymers and cRGD-PEG_{5K}-PLA_{3K}, two homo-micelles (M(cbm), M(hz)) and two hybrid micelles (cRGD-M(cbm), cRGD-M(hz)) were prepared by solvent-evaporation method. The spherical morphology of micelles and hybrid micelles was observed by TEM (Fig. 2), and the particle sizes of the four micelles were determined by DLS (Fig.2, insets) as 18, 50, 23, and 60 nm for M(cbm), M(hz), cRGD-M(cbm) and cRGD-M(hz), respectively. It seems that the spacer between polymer

backbone and EPI influence particle size significantly probably due to the existence of free amino group in mPEG-b-PAGE-hz-EPI. Its charges may result in enhanced hydrophilicity, mutual repellence between molecular chains and enlarged hydrodynamic volume.

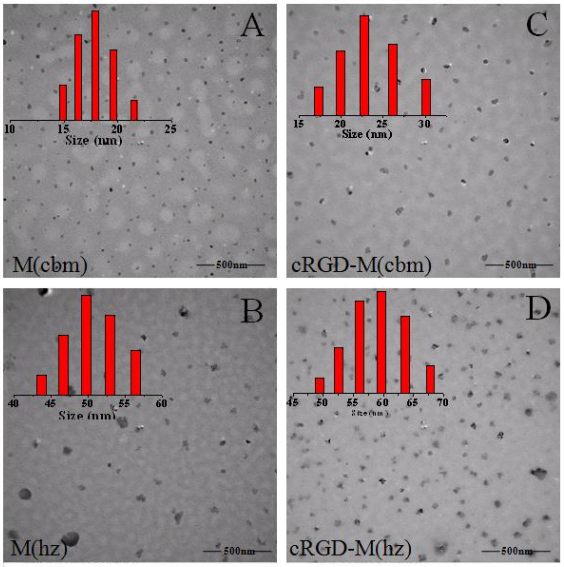


Fig. 2TEM images and DLS profiles of M(cbm) (A), M(hz) (B), cRGD-M(cbm) (C), and cRGD-M(hz) (D).

3.3 Release of EPI in vitro

The release of EPI from M(cbm) and M(hz) was determined in PBS buffer (pH5.0, pH 6.5 and pH7.4) respectively. The EPI dissociated from the backbone polymer was determined by UV spectrometer at 485 nm. As shown in Fig. 3, a continuous EPI release in three PBS solutions (pH5.0, pH 6.5 and pH7.4) was observed from M(hz), especially at pH5.0 and pH 6.5; while only 10% EPI release was detected from M(cbm) in 120 hours at pH5.0, pH 6.5 and pH7.4. Two release profiles of M(cbm) at pH 6.5 and 7.4 were almost overlapping, indicating the nature of stable connection between EPI and polymeric carrier. In contrast, M(hz) showed a increasing release with decreasing the pH value, for example, cumulative released EPI was 39, 33 and 27 at pH5.0, 6.5 and 7.4 for 72 h, respectively, suggesting a pH-sensitivity characteristics of M(hz). This is ascribed to both the pH-sensitivity of hydrazine linkage and the existence of free amino groups. It is well known that hydrazine is apt to hydrolysis.[27] Enhanced hydrophilicity and extended molecular chains caused by the amino groups are also responsible for the hydrolysis of hydrazine linkage.

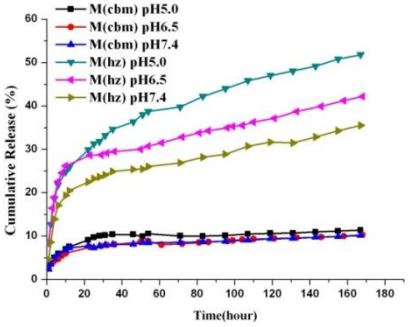


Fig. 3 In vitro cumulative release profiles of EPI from M(cbm) and M(hz) at different pHs (pH 5.0, 6.5 and 7.4) at 37℃.

3.4 In vitro cytotoxicity

In order to examine the cytotoxicity of free EPI, M(cbm), cRGD-M(cbm), M(hz) and cRGD-M(hz) to B16F10 cells, MTT analyses were performed at different concentrations (0.01~10μg/ml). The survival rates of B16F10 cells after incubation with free EPI or micelles for 48, 72 h were examined. As shown in Fig. 4, free EPI showed the highest cytotoxicity in all cases. This is ascribed to the super capability of EPI to be internalized by cells and to diffuse rapidly in cytoplasm. Among the four micelles, the cell viability showed following order at both 48 and 72 h and at all drug concentrations examined: M(cbm)>cRGD-M(cbm)>M(hz) >cRGD-M(hz), indicating the reversed order of cytotoxicity. This order is understandable if the pH-sensitivity of hydrazine linkage and the contribution of cRGD receptors are considered. The CDI value at the concentration of 0.5, 1, 5 μg/ml were 0.96, 0.88 and 0.87 respectively, indicating a synergy effect of pH-sensitive property and RGD modification in cytotoxicity in vitro.

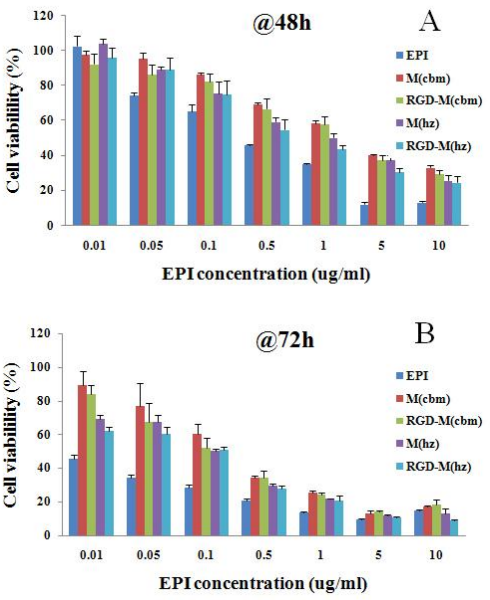


Fig. 4 MTT analysis. Free EPI,M(cbm), cRGD-M(cbm), M(hz) and cRGD-M(hz) were incubated with B16F10 cells for 48 (A) and 72 (B) h for various time intervals. All the results are repeated three times, and presented as mean±SD.

3.5 In vitro cell uptake

The uptake of four micelles was detected by confocal laser scanning microscopy (CLSM) and flow cytometry by virtue of the inherent fluorescence of EPI itself. B16F10 melanoma cells were chosen as test cells because they can express $\alpha_v\beta_3$ integrins at a high level.[16] As shown in Fig. 5A and 5B, the fluorescence of EPI comes from the whole cytoplasm and sometime later even from the nucleus region, indicating that all micelles can be internalized by B16F10 cells, the release of EPI occurred in cytoplasm, and the released EPI can enter the nuclei readily. Among the four micelles, the fluorescence intensity seems to follow the order of M(cbm)<cRGD-M(cbm)<M(hz)<cRGD-M(hz). Moreover, the CLSM studies of cells with the pre-treatment of chloroquine were carried out. As shown in Figure 5c, the fluorescence of four micelle groups mainly distributed in the cytoplasm, with nearly no distribution in cell nucleus. This result is ascribed to pre-treatment of chloroquine, which inhibit lysosomal degradation and result in locating in cytoplasm of drug-loaded micelles. However, the location of free EPI in cell was unaffected, because free EPI was taken in by diffusion, not by endocytosis. Uptake of micelles by cancer cells was also studied quantitatively by flow cytometry, the results (Fig. 6A and 6B) showed that all the targeted micelles show high uptake than that of non-targeted counterparts, and pH-sensitive micelles (RGD targeted and non-targeted) show much more endocytosis than that of insensitive micelles, whether one or three hour; and cRGD-M(hz) shows the strongest fluorescence intensity than other three micelles after 3 h of incubation, indicating the effects of pH-sensitivity and cRGD attachment on the micelle internalization and subsequent EPI release.

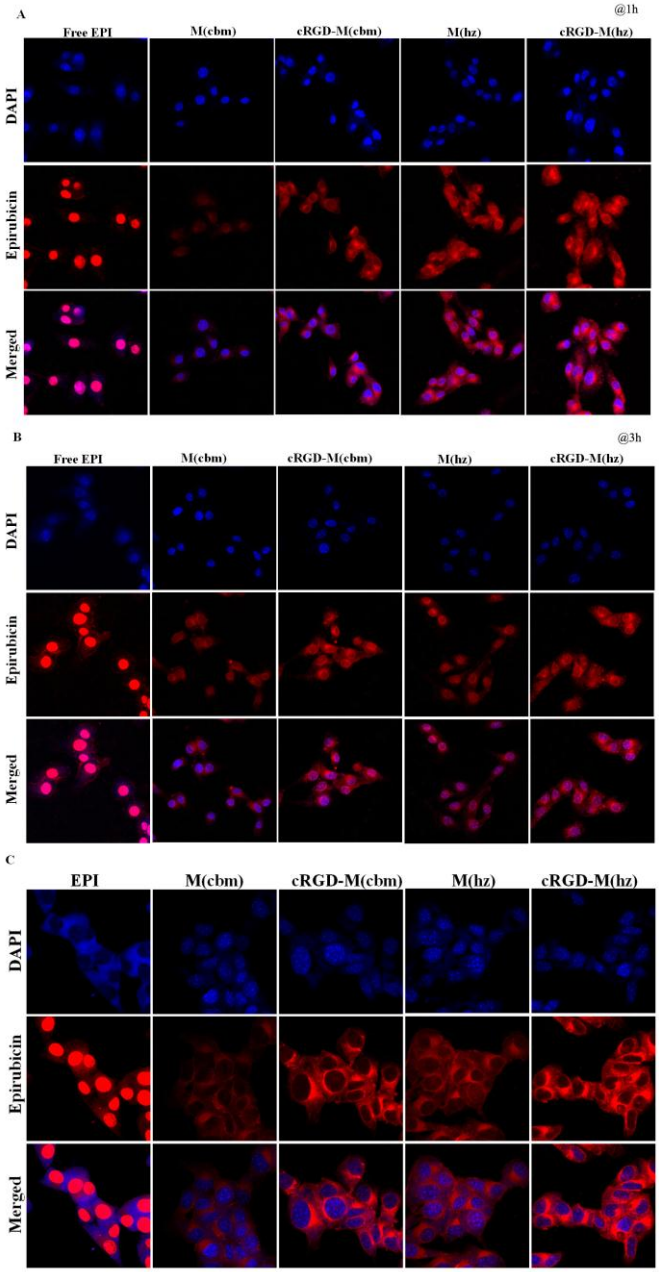


Fig. 5 Cellular uptake studies of M(cbm), cRGD-M(cbm), M(hz) and cRGD-M(hz) evaluated by B16F10 cells via CLSM. The cells were incubated with free EPI or respective micelle for 1 (A) and 3 h (B) at 37 ℃, and the cells were pre-treated using chloroquine for 1 h at 37 ℃ (C).

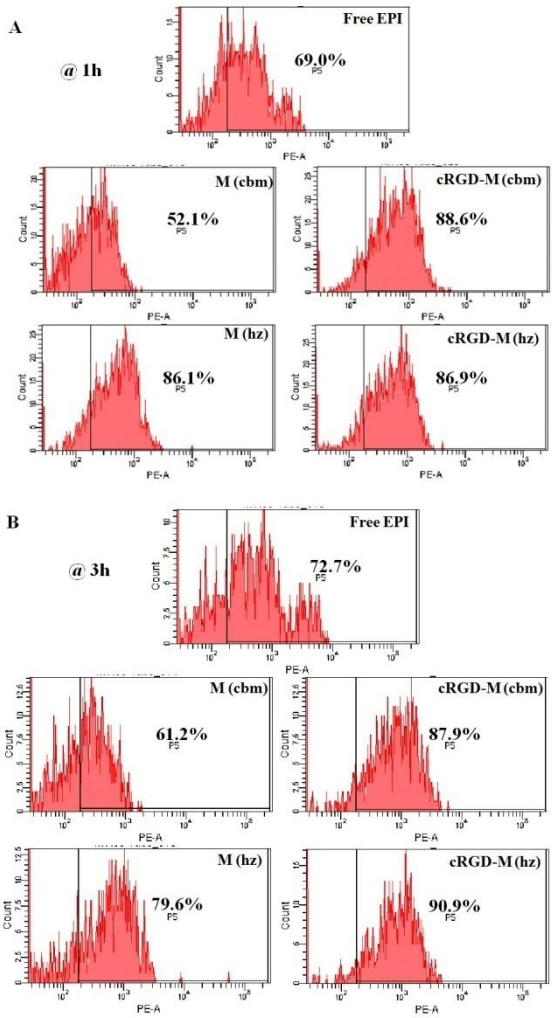


Fig. 6 Cellular uptake studies of M(cbm), cRGD-M(cbm), M(hz) and cRGD-M(hz) evaluated for 1 (A) and 3 h (B) at 37 °C by flow cytometry.

3.6 In vivo anti-tumor efficacy

To examine whether pH-sensitivity and c(RGDfK) decoration could play roles in inhibiting tumor growth in vivo or not, the antitumor effects of four micelles were examined with mouse B16F10 melanoma xenograft in C57BL/6 mice. When tumor volume reached 50~100mm^3, mice were divided into six groups randomly. Equivalent dose of EPI (10 mg/kg) was injected via tail vein on day 0, 2, 4, respectively. The tumor volume and body weight were measured for 15 days. As shown in Fig. 7A, all micelles showed better anti-tumor efficacy than free EPI; cRGD-M(cbm), M(hz), cRGD-M(hz) showed more significant tumor growth inhibition than M(cbm); and cRGD-M(hz) was the best in terms of tumor growth inhibition. Changes in body weight were used to analyze the systemic toxicity. As shown in Fig. 7B, free EPI showed less weight gain than four EPI-loaded micelles in 15 days due to the high systemic toxicity of it while all the micelles led to modest weight increase corresponding to their reduced systemic toxicity.

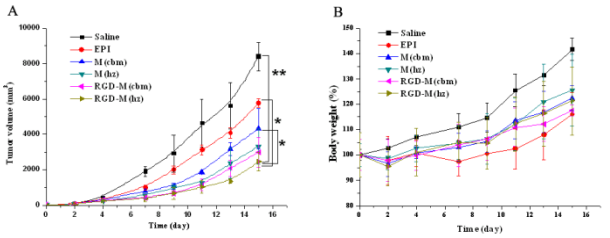


Fig. 7 In vivo anti-tumor efficacy (A) and body weight (B) of free EPI, M(cbm), cRGD-M(cbm), M(hz) and cRGD-M(hz).

4 Discussion

Growing evidences have shown the important role of pH-sensitive characteristics in improving drug target delivery.[37-41] Taking advantage of cellular pH gradient in tumors, pH-sensitive polymers can release its drugs in tumor interstitial space or in tumor cells more effectively. Due to overexpression of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins on tumor cells but being nearly undetectable in normal tissues, RGD peptides have been widely used in polymers, liposomes, micelles, viruses and gene delivery vehicles to selectively transport to tumor tissues.[18-24] Via ligand-receptor interaction, RGD-targeted conjugates and their receptors are quickly invaginated to form endosomes to achieve rapid endocytosis; with the maturation of endosomes, the decreased pH values could activate the lysosomes. The drugs are released from the conjugate to the cytoplasm and finally transport to their target organelle.

In this study, we develop pH-sensitive, c(RGDfK)-linked, EPI-conjugated micelles to mediate EPI target delivery to B16F10 melanoma in vivo and in vitro. The CLSM showed the cRGD-targeted micelles and pH-sensitive micelles were internalized more significantly by B16F10 cells than untargeted and pH-insensitive micelles. After 3h of incubation, intracellular and even perinuclear localization of the cRGD-M(cbm), M(hz), cRGD-M(hz) were observed. As expected, cRGD-M(hz) showed strongest fluorescence intensity in cell uptake experiments compared with other micelles. As reported previously, pH-sensitive nanocarriers can rapidly release their drugs from backbone in the acidic environments (such as endosomes and lysosomes).[38] Our CLSM and flow cytometry results suggest the great advantage of cRGD-M(hz) in target delivery of EPI to melanoma cells.

Consistent with the cell uptake experiments in vitro, a significant antitumor effect was observed from B16F10 melanoma xenograft injected with cRGD-M(hz). This result suggests that not only in vitro, but also in vivo pH-sensitive property and cRGD decoration promote the EPI accumulation in melanoma xenograft. Concerning cRGD-M(hz), the synergistic effect of fast EPI release in intercellular and intracellular regions and selective tumor targeting contributed to significantly improve the therapeutic efficacy of EPI in vivo. These results indicated that both common characteristics and personality of tumor could be considered for more effective cancer therapy.

5 Conclusion

A pH-sensitive, cRGD-linked, EPI-conjugated micelle system was successfully developed to enhance the delivery of EPI to mouse melanoma. The enhanced anti-tumor therapy of this system can be attributed to pH-sensitive characteristics, fast cellular uptake mediated by RGD/integrins, and fast drug release from acidic organelles endowed by pH-sensitive polymer-drug linkage. PH-responsive characteristics and c(RGDfK) modification showed a synergistic effect in drug delivery from tumor vessels to their target organelle. This work highlights the potential of combining active targeting and tumor microenvironment stimuli as a method for developing highly efficient nanomedicine for cancer treatment.

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Notes and references

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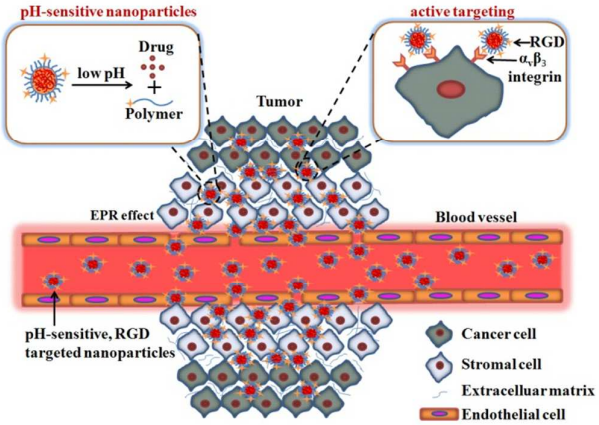
1. Z. Cheng, A. Al Zaki, J. Z. Hui, V. R. Muzykantov and A. Tsourkas, *Science*, 2012, **338**, 903-910.
2. F. Alexis, E. M. Pridgen, R. Langer and O. C. Farokhzad, *Drug Delivery*, Springer, 2010, 55-86.
3. P. Couvreur, *Adv. Drug. Deliver. Rev.*, 2013, **65**, 21-23.
4. K. Kataoka, A. Harada and Y. Nagasaki, *Adv. Drug. Deliver. Rev.*,2001, 47, 113-131.
5. E. K.-H. Chow and D. Ho, *Sci. Transl. Med.*, 2013, 216, 216rv4.
6. H. Maeda, *Adv. Enzyme Regul.*, 2001, **41**, 189-207.
7. J. Shi, A. R. Votruba, O. C. Farokhzad and R. Langer, *Nano Lett.* 2010, **10**, 3223-3230.
8. D. W. Bartlett, H. Su, I. J. Hildebrandt, W. A. Weber and M. E. Davis, *Proc. Nat. Acad. Sci. U.S.A.*, 2007, **104**, 15549-15554.
9. K. F. Pirollo and E. H. Chang, *Trends Biotechnol.*, 2008, **26**, 552-558.
10. J. Yue, S. Liu, Z. Xie, Y. Xing and X. Jing, *J. Mater. Chem. B.*, 2013, **1**, 4273-4280.
11. R. Wang, X. Hu, S. Wu, H. Xiao, H. Cai, Z. Xie, Y. Huang and X. Jing, *Mol.Pharmaceut.*, 2012, **9**, 3200-3208.
12. H. Chen, B. Li, J. Qiu, J. Li, J. Jin, S. Dai, Y. Ma and Y. Gu, *Nanoscale*, 2013, **5**, 12409-12424.
13. R. Wang, X. Hu, J. Yue, W. Zhang, L. Cai, Z. Xie, Y. Huang and X. Jing, *J. Mater. Chem. B.*, 2013, **1**, 293-301.
14. J. Yue, S. Liu, R. Wang, X. Hu, Z. Xie, Y. Huang and X. Jing, *Mol.Pharmaceut.*, 2012, **9**, 1919-1931.
15. M. E. Davis and D. M. Shin,*Nat. Rev. Drug.Discov.*, 2008, **7**, 771-782.
16. J. S. 2003-7-25Desgrosellier and D. A. Cheresh, *Nat.Rev. Cancer.*, 2010, **10**, 9-22.
17. K. Temming, R. M. Schiffelers, G. Molema and R. J. Kok, *Drug.Resist.Update.*, 2005, **8**, 381-402.
18. N. Yonenaga, E. Kenjo, T. Asai, A. Tsuruta, K. Shimizu, T. Dewa, M. Nango and N. Oku, *J. Control. Release.*, 2012, **160**, 177-181.
19. J. Yang, Y. Hou, G. Ji, Z. Song, Y. Liu, G. Dai, Y. Zhang and J. Chen, *Eur. J. Pharm. Sci.*, 2014, **52**, 180-190.
20. Z. Zhen, W. Tang, H. Chen, X. Lin, T. Todd, G. Wang, T. Cowger, X. Chen and J. Xie, *ACS nano*, 2013, **7**, 4830-4837.
21. Y. Miura, T. Takenaka, K. Toh, S. Wu, H. Nishihara, M. R. Kano, Y. Ino, T. Nomoto, Y. Matsumoto and H. Koyama, *ACS nano*, 2013, **7**, 8583-8592.
22. W. Song, Z. Tang, D. Zhang, Y. Zhang, H. Yu, M. Li, S. Lv, H. Sun, M. Deng and X. Chen, *Biomaterials*, 2014, 35, 3005 – 3014.
23. F. Selvestrel, F. Moret, D. Segat, J. H. Woodhams, G. Fracasso, I. M. R. Echevarria, L. Bau, F. Rastrelli, C. Compagnin, E. Reddi, C. Fedeli, E. Papini, R. Tavano, A. Mackenzie, M. Bovis, E. Yaghini, A. J. MacRobert, S. Zanini, A. Boscaini, M. Colombatti and F. Mancin, *Nanoscale*, 2013, **5**, 6106-6116.
24. H. Tian, L. Lin, J. Chen, X. Chen, T. G. Park and A. Maruyama, *J. Control. Release.*, 2011, **155**, 47-53.
25. F. Danhier, O. Feron and V. Préat, *J. Control. Release.*, 2010, **148**, 135-146.
26. D. Schmaljohann, *Adv. Drug. Deliver. Rev.*, 2006, **58**, 1655-1670.
27. J. Liu, Y. Huang, A. Kumar, A. Tan, S. Jin, A. Mozhi and X.-J. Liang, *Biotechnol.Adv.* 2013.
28. K. Engin, D. Leeper, J. Cater, A. Thistlethwaite, L. Tupchong and J. McFarlane, *Int.J.Hyperther.*, 1995, **11**, 211-216.
29. M. Stubbs, P. M. McSheehy, J. R. Griffiths and C. L. Bashford, *Mol. med.*, 2000, **6**, 15-19.
30. E. S. Lee, K. T. Oh, D. Kim, Y. S. Youn and Y. H. Bae, *J. Control. Release.*, 2007, **123**, 19-26.
31. W. She, N. Li, K. Luo, C. Guo, G. Wang, Y. Geng and Z. Gu, *Biomaterials*, 2013, **34**, 2252-2264.
32. H. Wang, F. Xu, D. Li, X. Liu, Q. Jin and J. Ji, *Polym. Chem.*, 2013, **4**, 2004-2010.
33. Y. Liu, L. Feng, T. Liu, L. Zhang, Y. Yao, D. Yu, L. Wang and N. Zhang, *Nanoscale*, 2014, **6**, 3231-3242.
34. Z.-Y. Qiao, S.-L. Qiao, G. Fan, Y.-S. Fan, Y. Chen and H. Wang, *Polym. Chem.*, 2014, **5**, 844-853.
35. J. Yue, X. Li, G. Mo, R. Wang, Y. Huang and X. Jing, *Macromolecules*, 2010, **43**, 9645-9654.
36. N. Graf, D. R. Bielenberg, N. Kolishetti, C. Muus, J. Banyard, O. C. Farokhzad and S. J. Lippard, *ACS Nano*, 2012, **6**, 4530-4539.
37. N. Bhattarai, J. Gunn and M. Zhang, *Adv. Drug. Deliver. Rev.*, 2010, **62**, 83-99.
38. W. Chen, F. Meng, R. Cheng and Z. Zhong, *J. Control. Release.*, 2010, **142**, 40-46.
39. X. D. Guo, L. J. Zhang, Y. Chen and Y. Qian, *AIChE J.*, 2010, **56**, 1922-1931.
40. A. Makhlof, Y. Tozuka and H. Takeuchi, *Eur. J. Pharm.Biopharm.*, 2009, **72**, 1-8.
41. M. Shen, Y. Huang, L. Han, J. Qin, X. Fang, J. Wang and V. C. Yang, *J. Control. Release.*, 2012, **161**, 884-892.



Cyclic RGD targeting polymeric nanomedicines prepared from pH sensitive polymer-drug conjugates for effective treatment of melanoma.