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Enhanced antitumor efficacy by cyclic RGDyK-conjugated and paclitaxel-loaded pH-responsive polymeric micelles

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ABSTRACT

Cyclic RGDyK (cRGDyK)-conjugated pH-sensitive polymeric micelles were fabricated for targeted delivery of paclitaxel to prostate cancer cells based on pH-sensitive copolymer poly(2-ethyl-2-oxazoline)-poly(D,L-lactide) (PEOz-PLA) and cRGDyK-PEOz-PLA to enhance antitumor efficacy. The prepared micelles with an average diameter of about 28 nm exhibited rapid release behavior at endo/lysosome pH, effectively enhanced the cytotoxicity of paclitaxel to PC-3 cells by increasing the cellular uptake, which was correlated with integrin $\alpha_v\beta_3$ expression in tumor cells. The active targeting activity of the micelles was further confirmed by *in vivo* real time near-infrared fluorescence imaging in PC-3 tumor-bearing nude mice. Moreover, the active targeting and pH-sensitivity endowed cRGDyK-conjugated micelles with a higher antitumor effect in PC-3 xenograft-bearing nude mice compared with unmodified micelles and Taxol with negligible systemic toxicity. Therefore, these results suggested that cRGDyK-conjugated pH-sensitive polymeric micelles may be a promising delivery system for efficient delivery of anticancer drugs to treat integrin $\alpha_v\beta_3$-rich prostate cancers.

1. Introduction

Currently, androgen ablation therapy remains the first choice of prostate cancer treatment, however, the cells gradually acquire hormone-resistance as the disease progresses [1]. After endocrine therapy, chemotherapy with drugs such as paclitaxel and docetaxel is adopted as the primary clinical treatment regimen [2]. However, the benefit of chemotherapy is limited due to its serious systemic toxicity. To overcome this inconvenience, many new and innovative strategies to entrap antitumor drugs in different types of nanocarriers have been developed including liposomes, microemulsion, nanoparticles, and polymer-drug conjugates. Among them, polymeric micelles present great potential to improve water solubility of anticancer drugs, prolong blood circulation time, and enhance their accumulation at tumor sites by the enhanced permeability and retention (EPR) effect and therefore have attracted considerable attention [3–5]. However, their efficiency of passive targeting to tumor by EPR effect is limited. Consequently, recognition and uptake of micelle delivery system by tumor cells remain a considerable challenge [2,6–8], which highlights the urgent need for more effective design strategies.

The use of various targeting ligands on the surface of nanocarriers, being recognized by their specific receptors/antigens on tumor cell surface, has been demonstrated to promote cellular uptake [9]. Cyclic Arg-Gly-Asp-Tyr-Lys (cRGDyK) has high affinity to integrin $\alpha_v\beta_3$, a tumor angiogenesis biomarker overexpressed in tumor neovasculature and most tumor cells [10,11]. It was reported that cRGDyK conjugated onto the nanocarriers facilitated their uptake by tumor cells via integrin-mediated endocytosis, thereby enhancing the cytotoxicity of antitumor drug-loaded nanocarriers against tumor cells [11–14]. Herein, we focused on cRGDyK as a candidate decoration.

Another major concern is that slow release of anticancer drug from nanocarriers in tumor cells may result in a low level of intracellular free drug concentration and thereby induce limited antitumor effect [3,15]. Even worse, maintaining a low level of free drug concentration in tumor cells for a long time may cause occurrence of drug resistance. Consequently, to ensure the delivery of anticancer drug to tumor site with sufficient drug concentration, nanocarriers, such as polymeric micelles, can be used for increased drug stability in circulation and rapid drug release in the tumor. The phenomenon of rapid release might be achieved by targeted polymeric micelles with a triggered release mechanism that responds to the pH or enzymes inside the cells [16,17]. Further, ligand-modified nanocarriers are generally internalized into tumor cells via an endocytic pathway with an experience of a pH gradient

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from 5.5–6.5 in endosomes to 4.5–5.0 in lysosomes in their intracellular trafficking pathway, thereby leading to an inferior antitumor efficacy due to the degradation of the cargos by the lysosome enzymes [18]. Therefore, it is very important to facilitate drug escape from endo/lysosome vesicles [19,20], and pH-responsive polymeric micelles may be suitable for intracellular drug delivery. As known, pH-responsive hydrophobic polyacids or polybases usually aggregate to form an inner core of polymeric micelles that convey pH-sensitivity to drug release. For example, the protonation of poly(L-histidine) frequently constituting the hydrophobic core of mixed PEG-poly(L-histidine)/PEG-poly(D,L-lactic acid) micelles results in destabilization of micelle cores and expedient drug release [20]. However, polymeric micelles with pH-responsive outer shell have rarely been reported.

Here, we aimed to overcome the current limitations mentioned above. cRGDyK-conjugated pH-sensitive polymeric micelles were designed based on pH-sensitive diblock copolymer poly(2-ethyl-2-oxazoline)-poly(D,L-lactide) (PEOz-PLA) and cRGDyK-PEOz-PLA for integrating the merits of ligand-modified polymeric micelles for enhanced accumulation at tumor site and increased uptake by tumor cells, and pH-sensitive polymeric micelles for rapid intracellular drug release and endo/lysosome escape. We hypothesized that the designed micelles would be endowed with tumor cell-targeting ability and pH-response to intracellular compartments and thereby an effective delivery system for anticancer drugs to treat prostate cancers.

2. Materials and methods

2.1. Materials

Paclitaxel (PTX) was purchased from Guilin Huiang Biopharmaceutical Co. Ltd. (Guilin, China). cRGDyK was supplied by Shanghai C-Strong Co., Ltd. (Shanghai, China). D,L-lactide purchased from Daigang Biological Technology Co. Ltd. (Jinan, China) was purified by recrystallization from ethyl acetate. Ethyl 3-bromopropionate and stannous octoate were products of Aladdin reagent company (Shanghai, China). 2-ethyl-2-oxazoline supplied by Sigma–Aldrich (St Louis, MO, USA) was dried by vacuum distillation over calcium hydride. mPEG5000-PLA5000 was synthesized by our laboratory as reported previously [21]. N-hydroxysuccinimide (NHS) and N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC·HCl) were obtained from J&K scientific Ltd. (Beijing, China). Sulforhodamine B sodium salt (SRB) and coumarin-6 (donated as C6) were purchased from Sigma–Aldrich (St Louis, MO, USA). Bis Benzimide Hoechst 33258 was supplied by Biodee Biotechnology Co. Ltd. (Beijing, China). LysoTracker® Red was purchased from Life Technologies (Gaithersburg, MD, USA). DiR was obtained from Biotium, Inc. (Hayward, CA, USA). DiO and DiI were purchased from J&K Chemical Ltd. (Shanghai, China).

2.2. Synthesis and characterization of HOOC-PEOz-PLA

HOOC-PEOz-PLA was synthesized through a two-step reaction according to our published report [22].

2.3. Synthesis and characterization of cRGDyK-PEOz-PLA

cRGDyK was linked to the terminal of HOOC-PEOz-PLA using an EDC/NHS technique [23,24]. Typically, to a suspension of HOOC-PEOz-PLA in deionized water, NHS and EDC·HCl were added at a molar ratio of 1:2:2, and then pH was adjusted to 5.0–6.0 with HCl solution under moderate stirring in ice bath. After 15 min of reaction, 1 equiv of cRGDyK was added and pH value was adjusted to 8.0–8.5. After incubation for 24 h at room temperature, the resultant mixture was dialyzed with a dialysis bag (MWCO 3500, Millipore, USA) against deionized water for 24 h to remove the residual EDC, NHS and cRGDyK, and then lyophilized.

2.4. Determination of critical micelle concentration of HOOC-PEOz-PLA

The critical micelle concentration (CMC) of the copolymer was determined by fluorescence spectroscopy using pyrene as a hydrophobic probe as previously reported [25].

2.5. Preparation of drug-loaded polymeric micelles

PTX-loaded polymeric micelles (denoted as PTX/PM) were prepared by film-hydration method as previously reported [22]. The ratio of HOOC-PEOz-PLA to paclitaxel was 10:1 (w/w).

cRGDyK-conjugated and PTX-loaded polymeric micelles (denoted as PTX/PM-R) were prepared as the same to PTX/PM except that HOOC-PEOz-PLA was replaced by cRGDyK-PEOz-PLA and HOOC-PEOz-PLA (1:1, w/w).

C6-, DiR-, DiI/DiO-loaded polymeric micelles were prepared as the same to PTX/PM except that the C6/polymer ratio was 1:1000 (w/w), the DiO/polymer and DiI/polymer ratio was 1:500 (w/w), and the DiR/polymer ratio was 1:250 (w/w), respectively.

2.6. Physicochemical characterization of polymeric micelles

Size and size distribution, and Zeta potential were determined by dynamic light scattering (DLS) on a Zetasizer Nano ZS (Malvern, UK) at 25 °C. The loading content (LC) and encapsulation efficiency (EE) of polymeric micelles were determined as previously described [26]. Transmission electron microscopy (TEM) images were observed on a JEM-1230 transmission electron microscope (JEOL, Japan) with an accelerating voltage of 100 kV.

The *in vitro* release of PTX from various PTX-loaded micelles was evaluated using a dialysis-bag diffusion method as previously described [26]. The release medium was selected to be PBS (pH 5.0, 7.4) with 0.5% Tween 80.

2.7. Cell culture

PC-3 cells were obtained from Cell Culture Center of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College. Cells were cultured in RPMI 1640 medium (MAC Gene Technology) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% Penicillin–Streptomycin in 5% CO_2 humidified atmosphere at 37 °C.

2.8. Stability of polymeric micelles after contacting with cells

The stability of the micelles when contacting with PC-3 cells was evaluated through the leakage of core-loaded molecules from micelles by using the Förster resonance energy transfer (FRET) method [27]. In brief, PC-3 cells were seeded on a glass bottom culture dish and cultured at 37 °C under 5% CO_2 for adherence. After the cells were subcultivated at 80–90% confluence, the culture media were removed, and the cells were washed thrice with serum-free medium at 37 °C. Subsequently, the medium containing FRET micelles (final concentration of both DiI and DiO was 4 μg/mL) was added. After 1 h of incubation, the cells were washed thrice with cooled PBS and fixed with 4% paraformaldehyde at 37 °C for 20 min followed by washing thrice with PBS. FRET images were obtained with a confocal laser scanning microscope (CLSM, TCS SP5, Leica, Germany). The excitation and emission wavelengths for DiO were 484 nm and 500–530 nm, respectively, and 549 nm and 555–655 nm for DiI, respectively. For determination

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