PLK1shRNA and doxorubicin co-loaded thermosensitive PLGA-PEG-PLGA hydrogels for osteosarcoma treatment

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A B S T R A C T
Combination cancer therapy has emerged as crucial approach for achieving superior anti-cancer efficacy. In this study, we developed a strategy by localized co-delivery of PLK1shRNA/polylysine-modified polyethylenimine (PEI-Lys) complexes and doxorubicin (DOX) using biodegradable, thermosensitive PLGA-PEG-PLGA hydrogels for treatment of osteosarcoma. When incubated with osteosarcoma Saos-2 and MG-63 cells, the hydrogel containing PLK1shRNA/PEI-Lys and DOX displayed significant synergistic effects in promoting the apoptosis of osteosarcoma cells in vitro. After subcutaneous injection of the hydrogel containing PLK1shRNA/PEI-Lys and DOX beside the tumors of nude mice bearing osteosarcoma Saos-2 xenografts, the hydrogels exhibited superior antitumor efficacy in vivo compared to the hydrogels loaded with PLK1shRNA/PEI-Lys or DOX alone. It is noteworthy that the combination treatment in vivo led to almost complete suppression of tumor growth up to 16 days, significantly enhanced PLK1 silencing, higher apoptosis of tumor masses, as well as increased cell cycle regulation. Additionally, ex vivo histological analysis of major organs of the mice indicated that the localized treatments showed no obvious damage to the organs, suggesting lower systemic toxicity of the treatments. Therefore, the strategy of localized, sustained co-delivery of PLK1shRNA and DOX by using the biodegradable, injectable hydrogel may have potential for efficient clinical treatment of osteosarcoma.

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1. Introduction

Osteosarcoma is the most common primary malignant bone cancer in children and adolescents and shows a peak incidence during the second and third decades of life, representing the second highest cause of cancer-related death in this age group [1]. In children and young adults, osteosarcoma is the eighth most common malignancy of childhood, with approximately 400 new cases of osteosarcoma diagnosed in the United States each year [2]. Osteosarcoma pathologically arises from mesenchymal cells, characterized by spindle cells and aberrant osteoid formation. Typically, the primary sites of osteosarcoma are the distal femur, the proximal tibia, and the distal humerus, with 64% originating from the knee [3].

Up to four decades ago, the overall long-term survival, prior to multi-agents chemotherapy regimens, was close to 10% when the first-line treatment of osteosarcoma was limb amputation, with succumbing less than one year from operation [4]. Currently, the standard treatments of osteosarcoma comprise surgery and chemotherapy regimens (neoadjuvant and adjuvant chemotherapy). As a result, the long-term survival has dramatically increased to 60–70% and the surgical technique and effective multi-agents chemotherapeutic regimes have made limb-salvage a reality [4,5]. However, despite these improvements, there remains more than 30% of patients resistant to chemotherapy regimens, eventually succumbing to metastases, leading to no substantial further improvement [6]. Therefore, many current studies lay emphasis on identifying and characterizing more effective drug delivery systems [7,8] and additional therapeutic targets for osteosarcoma [9,10].

In modern treatment schedules, a combination of different chemotherapeutics drugs are administered either intravenously or orally both before and after surgery [11–13]. However, most chemotherapeutics also carry the risk of both short-term and long-term toxic and adverse effects [14]. Doxorubicin (DOX) is a
broad-spectrum anti-neoplastic drug and shows excellent therapeutic efficacy against osteosarcoma [15,16]. DOX prevents the double helix DNA from being resealed by inhibiting topoisomerase II (Topo II), an enzyme that can relax the supercoils in DNA for transcription, leading to the stop of transcription. Furthermore, DOX intercalates into double helix DNA, resulting in the deregulation of the epigenome [17,18]. Due to its narrow therapeutic index and dose related toxicity, however, DOX causes life-threatening adverse effects, such as cardiotoxicity [19], typhlitis, myelosuppression, nausea, vomiting, alopecia and so on. Thus, it is indeed a matter of great urgency to develop a more effective therapeutic regimen with enhanced antitumor efficacy and minimal adverse effects.

Gene therapy is an emerging targeted therapeutic strategy for the treatment of osteosarcoma. It aims to prevent osteosarcoma proliferation and metastasis by specific targeting the genes closely related with osteosarcoma initiation, progression and prognosis [10]. Polo-like kinase 1 (PLK1) gene is the most extensively studied member of the polo-like kinases, which are a family of high conserved serine/threonine kinases. It is essential in the maintenance of genomic stability, and recognized as a key regulator of mitosis, meiosis and cytokinesis. The activity and concentration of PLK1 is of vital importance for the precise regulation of cell cycle [20,21]. Recently, studies have been performed to uncover the mechanisms of PLK1 regulation, especially its regulation on cycle modulators by interaction with upstream kinases and phosphatases and localization to different specific subcellular structures during cell cycle [22]. It has been found that PLK1 plays an important role in multiple processes including mitotic entry, centrosome maturation, spindle assembly, anaphase promoting complex (APC) and cytokinesis, associated with several modes of regulation [20,23]. Notably, PLK1 is overexpressed in a broad spectrum of human tumors and has prognostic potential in cancer [24–26], including osteosarcoma [27]. Due to its involvement in carcinogenesis, PLK1 reveals a potential as a promising therapeutic target for osteosarcoma therapy [28].

RNA interference provides an excellent strategy for functional gene silencing in tumor cells [29,30], which enables us to achieve targeted genetic loss-of-function in tumor [31]. However, considerable barriers need to be overcome for efficient gene therapy, including biological safety, efficient gene delivery, sustained and steady gene expression. To improve the efficiency of gene delivery, various controlled delivery systems have been developed for systemic delivery of genes, such as liposomes, microspheres, nanoparticles, polymeric micelles and conjugates [32]. In our recent study, an efficient cytofectin, polylysine-modified polyethylenimine (denoted as PEI-Lys), was developed for enhanced gene transfection [33].

In addition, localized and sustained drug delivery systems based on injectable hydrogels have attracted increasing attention due to their superior advantages including easy formulation, target injection, biodegradability, less systemic toxicity, as well as localized and sustained drug/gene release profiles. Thermosensitive hydrogels based on poly(DL-lactide-co-glycolide)-poly(ethylene glycol)-poly(DL-lactide-co-glycolide) triblock copolymers (PLGA-PEG-PLGA) have been shown great potential for clinical applications, due to their excellent biocompatibility, tunable gelation behavior and biodegradability [34,35]. Sustained release behaviors of several protein drugs and an hydrophobic anti-cancer drug, paclitaxel, from the injectable PLGA-PEG-PLGA hydrogels have been investigated [36,37]. Furthermore, the PLGA-PEG-PLGA hydrogels containing DNA/cationic polymer complexes have been shown to improve the gene transfection efficiency [38,39]. Additionally, a similar thermosensitive hydrogel based on a PEG-PLGA-PEG triblock copolymer also promoted the prolonged gene expression and enhanced diabetic wound healing [40,41]. Nevertheless, to-date, reports on biodegradable, injectable hydrogels for localized co-delivery of anti-cancer drugs and genes for combination cancer therapy are still limited.

In the present work, we developed a strategy by localized, sustained co-delivery of PLK1shRNA/PEI-Lys complexes and DOX using the biodegradable PLGA-PEG-PLGA hydrogel for treatment of osteosarcoma in vitro and in vivo (Scheme 1). To study the synergistic anti-tumor efficacy of the hydrogels containing PLK1shRNA/PEI-Lys complexes and DOX in vitro, the influence of the hydrogels incubated with osteosarcoma Saos-2 and MG-63 cells on the tumor cell cytotoxicity, PLK1 gene silencing, apoptosis of osteosarcoma cells, and cell cycle arrest was investigated. Furthermore, the in vivo synergistic anti-tumor efficiency of the hydrogel treatment was studied by subcutaneous injection of the hydrogel loaded with PLK1shRNA/PEI-Lys complexes and DOX beside the tumors in nude mice bearing human osteosarcoma Saos-2 xenografts. The treatment effects on tumor growth suppression, PLK1 silencing, tumor apoptosis, and tumor cell cycle regulation were evaluated in detail.

Scheme 1. Proposed schematic illustration for the synergistic effect of PLK1shRNA/PEI-Lys and DOX co-loaded hydrogel on the tumor in nude mice.
2. Materials and methods

2.1. Materials

Poly(ethylene glycol) (PEG, Mn = 1000) and stannous octoate (Sn(OtC2)2, 95%) were purchased from Sigma–Aldrich (USA). Lactate (LA) and glycolide (GA) were obtained from Purac (Netherlands). 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma (USA). The PLK1shRNA expression vector was constructed and purchased from GenePharma (China). Trizol reagent was purchased from Invitrogen (USA). DOX hydrochloride (DOX·HCl) was obtained from Zhejiang Hisun Pharmaceutical Co., Ltd. China. Polyethylene-modified polystyrene (denoted as PEI-lys, Mn = 41.0 KDa with PEI wt% of 60.9%) was synthesized according to our previous method[33] and used as a cationic cytofectin. Saos-2, MG-63 and 2929 cells were obtained from the American Type Culture Collection (ATCC).

2.2. Synthesis and characterization of PLGA-PEG-PLGA copolymers

The PLGA-PEG-PLGA triblock copolymer was synthesized via ring-opening copolymerization of LA and GA by using PEG (Mn = 1000) and Sn(OtC2)2 as the macoinitiator and catalyst, respectively. The copolymer was obtained by precipitation into diethyl ether, followed by filtration. The resulting product was further purified by dialysis and lyophilization.

1H NMR spectrum was performed on a 300 MHz Bruker spectrometer (DMX300) with CDCl3 as solvent to confirm the chemical structure and composition. Molecular weights (MW) and the polydispersities (PDI) of the copolymer were monitored by gel permeation chromatography (GPC, Waters) with tetrahydrofuran as eluent at a flow-rate of 1.0 mL/min at 35 °C. The MW was calibrated by monodispersed poly-styrene standard. Based on the 1H NMR result, the molecular weight of the triblock copolymer is 3500 with a WA/GA molar ratio of 1.8:1, which are coincident with the theoretic values (Table S1).

2.3. Loading of DOX and PLK1shRNA/PEI-Lys in PLGA-PEG-PLGA hydrogel

The complex of polylysine-modified polystyrene (denoted as PEI-lys) and PLK1shRNA was prepared in distilled water by mixing plasmid DNA and PEI-Lys with gentle vortexing, and then the complex was incubated at room temperature for 30 min. The required amount of PLGA-PEG-PLGA copolymer and DOX·HCl were carefully weighed and mixed in a vial. After that, the appropriate amount of DOX and copolymer was well suspended. The PLK1shRNA/PEI-Lys and DOX co-loaded hydrogel was formed after incubation of the mixed solution at 37 °C until DOX and copolymer were well suspended. The PLK1shRNA/PEI-Lys and DOX co-loaded hydrogel was formed as no gel phase transition behaviors of the PLGA-PEG-PLGA triblock copolymers against different cell lines (Saos-2, MG-63, and 2929 cells) were investigated. Each data point was the average of 3-4 replicates and the error bars represent the standard deviation of the mean. The cytotoxicities of PLGA-PEG-PLGA triblock copolymers against different cell lines (Saos-2, MG-63, and 2929 cells) were investigated by the MTT assay. The cytotoxicities of PLGA-PEG-PLGA triblock copolymers against different cell lines (Saos-2, MG-63, and 2929 cells) were investigated by MTT assay. Generally, the cells were seeded in 96-well plates at 10,000 cell per well in 200 μL of complete Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum, supplemented with 50 U/mL penicillin, and 50 U/mL streptomycin, and incubated at 37 °C in 5% CO2 atmosphere for 24 h. After removing culture medium, copolymer solutions in complete DMEM at different concentrations were added. The cells were subjected to MTT assay after being incubated for another 24 h. The absorbency of the solution was measured on a Bio-Rad 680 microplate reader at 492 nm. Cell viability (%) was calculated based on the following equation:

\[
\text{Cell viability}(\%) = \left(\frac{A_{\text{sample}}}{A_{\text{control}}}\right) \times 100\%
\]

2.4. Phase diagram

The sol–gel phase transition behaviors of the PLGA-PEG-PLGA triblock copolymer aqueous solutions were determined by a vial inverting approach with a temperature increment of 1 °C per step. The sol–gel transition temperatures were recorded as no flow was observed within 30 s after inverting the vials. The sol–gel phase transitions of the PLGA-PEG-PLGA aqueous solutions containing DOX and plasmid DNA/PEI-lys were also investigated. Each data point was the average of three measurements.

2.5. In vitro gel degradation

The PLGA-PEG-PLGA aqueous solutions (20 wt%, 0.5 mL) were transferred into the vials with an inner diameter of 16 mm and incubated at 37 °C for 10 min. After the hydrogels were formed, 3 mL of phosphate buffer saline (PBS, pH 7.4) was added to the top of the gels and the samples were further incubated at 37 °C with continuous shaking at 50 rpm. At predetermined time intervals, the buffer was removed, and the remaining gels were accurately weighed.

2.6. In vivo gel degradation and biocompatibility

Wistar rats were used for the in vivo gel degradation and biocompatibility tests. In brief, 0.5 mL of PLGA-PEG-PLGA aqueous solution (20 wt%) was subcutaneously injected into the dorsal areas of rats by a syringe with a 21-gauge needle. At given time intervals, the rats were sacrificed and the gels in the subcutaneous layer were photographed. The skin tissues surrounding the gels were surgically removed and histologically stained by hematoxylin–eosin (H&E) for examination of inflammatory response to the materials in rats.

2.7. In vitro cell viability

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) proliferation kit (Sigma). The relative cytotoxicities of PLGA-PEG-PLGA triblock copolymers against different cell lines (Saos-2, MG-63, and 2929 cells) were investigated by the MTT assay. Generally, the cells were seeded in 96-well plates at 10,000 cell per well in 200 μL of complete Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum, supplemented with 50 U/mL penicillin, and 50 U/mL streptomycin, and incubated at 37 °C in 5% CO2 atmosphere for 24 h. After removing culture medium, copolymer solutions in complete DMEM at different concentrations were added. The cells were subjected to MTT assay after being incubated for another 24 h. The absorbency of the solution was measured on a Bio-Rad 680 microplate reader at 492 nm. Cell viability (%) was calculated based on the following equation:

\[
\text{Cell viability}(\%) = \left(\frac{A_{\text{sample}}}{A_{\text{control}}}\right) \times 100\%
\]

2.8. Cell cycle analysis

The cell cycle of osteosarcoma Saos-2 cell line exposure to hydrogels containing DOX and PLK1shRNA/PEI-lys was evaluated by Flowcetm-Activated Cell Sorter (FACS) analysis. Cells were seeded in 6-well plates at 3.0 × 105 cells per well in 2.0 mL of complete DMEM and cultured for 24 h. After that, the culture media were removed and the hydrogel precursor solutions (10 μL) were added to the wells. After the hydrogels formed at 37 °C, the 24-well plates were replenished with 1 mL of DMEM. The hydrogel formulations included PLGA-PEG-PLGA hydrogel only (control), scrambled shRNA/PEI-Lys loaded hydrogel (negative control), DOX-loaded hydrogel, PLK1shRNA/PEI-Lys loaded hydrogel and hydrogel containing both DOX and PLK1shRNA/PEI-lys. The DOX concentrations in the hydrogels were 0.1 mg/mL, 0.2 mg/mL and 1 mg/mL, respectively, and the concentration of PLK1shRNA/PEI-lys in the hydrogels was 0.2 mg/mL with the N/P ratio of 2.5:1. After incubation for another 24 h, 48 h and 72 h, the cells were then subjected to MTT assay and the cell viability was evaluated.

2.9. In vivo antitumor tests

The in vivo antitumor efficacy of DOX and PLK1shRNA co-loaded hydrogels was evaluated using 5-week-old male BALB/c nu/nu nude mice. The mice were handled...
under protocols approved by the School of Life Sciences Animal Care and Use Committee of Jilin University. Human osteosarcoma Saos-2 cell line was inoculated into the nude mice. The mice (19–20 g) were injected subcutaneously at the armpit of right anterior limb with 0.3 mL of cell suspension containing 5.0 × 10⁶ Saos-2 cells in PBS. Treatments were started after 1 week as the volume of the tumors reached to ~50 mm³. The mice were weighed and randomly divided into 5 groups (6 mice per group). The mice were then treated by single injection of 0.1 mL of 20 wt% various hydrogel formulations beside the tumors, including PLGA-PEG-PLGA hydrogel only (Gel), scrambled shRNA/PEI-Lys loaded hydrogel as a negative control (concentration of scrambled shRNA = 0.5 mg/mL, Gel + shNS), DOX-load hydrogel (5.0 mg DOX per kg mice weight, Gel + DOX), PLK1shRNA/PEI-Lys loaded hydrogel (concentration of PLK1shRNA = 0.5 mg/mL, Gel + PLK1shRNA/PEI-Lys), as well as DOX and PLK1shRNA/PEI-Lys co-loaded hydrogel (Gel + PLK1shRNA/PEI-Lys + DOX).

The treatment efficacy and safety evaluation were assessed by measuring the tumor volume and body weight at intervals of 2 days for up to 16 days, respectively. Tumor volume was calculated by the equation:

\[ V = L \times W^2 / 2, \]

where \( L \) (mm) and \( W \) (mm) are the largest and smallest diameters of tumor [44]. After the treatments, the animals were sacrificed and the tumor mass and the organs were harvested for further analysis.

2.10. Apoptosis detections in vitro and in vivo

The apoptosis of osteosarcoma cells in vitro induced by the treatments of hydrogels containing PLK1shRNA/PEI-Lys and DOX was evaluated by flow cytometry. The expression of apoptosis genes of osteosarcoma cells in vitro and tumor mass ex vivo was tested by quantitative real-time PCR, respectively. For flow cytometry analysis, the cells were collected after each treatment, and centrifuged at 1500 rpm for 5 min. Then the cells were resuspended in binding buffer and stained with Annexin V-APC for 15 min at room temperature. The 7-AAD was added and incubated with the cells for 15 min before being transferred to flow cytometric analysis within 30 min.

For quantitative real-time PCR tests, the total RNA of osteosarcoma cells incubated with hydrogels in vitro or in tumor masses dissected from the mice was isolated using trizol reagent (Invitrogen) according to the protocol. The tumor masses were grinded in liquid nitrogen before addition of trizol reagent. The RNA was extracted and converted to a complimentary DNA (cDNA) by reverse transcription kit (Takara, Japan). Quantitative real-time PCR was performed on an Mxpro3005 system (Stratagene, USA) by using SYBR Premix Ex Taq™ kit (Takara, Japan). Specific primers were designed for the apoptosis genes Bcl-2, BAX, caspase-3, caspase-9 and the housekeeping gene β-actin, which are listed in Table 1. To analysis the result, gene expression values were relative to housekeeping gene β-actin and subsequently normalized against the respective genes of control group to obtain gene expression fold values.

2.11. Mechanism of PLK1 gene on cell cycle

The expressions of cyclin dependent proteins in osteosarcoma cells in vitro or ex vivo tumor masses after treatments were detected in order to study the mechanism of PLK1 on cell cycle regulation by quantitative real-time PCR. The procedure was similar to Section 2.10. Here, Specific primers were designed for the PLK1, CDK1, cyclin B1 and cdc25c and the housekeeping gene β-actin, which are listed in Table 1. Then the data were normalized against the respective genes of control group to obtain gene expression fold values.

2.12. Histology and TUNEL assay

At the end of the in vivo antitumor test, the mice were sacrificed and hearts, lungs, livers, spleens, kidneys and tumors were collected and fixed in 4% (w/v) paraformaldehyde. Tissue sections were stained with hematoxylin/eosin (H&E) for histological analysis to evaluate the organ damage and the tumor pathology analysis.

In addition, terminal nucleotidyltransferase-mediated nick end labeling (TUNEL) assay was carried out according to the manufacturer’s protocol (Roche, Basel, Switzerland). The nicked DNA ends of tissue sections were labeled by the reaction mixture. The apoptosis of cells was observed by fluorescence microscopy.

2.13. Statistical analysis

The statistical difference of the control and experimental groups was analyzed using the paired Student’s t-test. A statistically significant difference was reported if \( p < 0.05 \). All experiments were performed at least three times, and the data were presented as mean ± standard deviation.

### 3. Results and discussion

3.1. Synthesis and characterization of PLGA-PEG-PLGA triblock copolymer

The PLGA-PEG-PLGA triblock copolymer was synthesized via ring-opening polymerization of lactide (LA) and glycolide...
(GA) by using PEG (Mn = 1000) as a macroinitiator in the presence of Sn(Oct)$_2$ [36,37,45]. The chemical structure and molecular weight of the copolymer were confirmed by $^1$H NMR spectrum and GPC. Based on the $^1$H NMR spectrum (Fig. S1 in Supporting Information), the molecular weight of the triblock copolymer was 3500 with the LA/GA molar ratio of 1.8:1, which agreed well with the theoretical values (Table S1 in Supporting Information).

3.2. Sol–gel transition of PLGA-PEG-PLGA aqueous solutions

PLGA-PEG-PLGA triblock copolymers tend to self-assemble into micelles with hydrophobic PLGA cores and hydrophilic PEG shells in aqueous solution at lower temperatures [45]. The micelle formation and aggregation behavior are closely associated with the molecular structure and the hydrophobic-hydrophilic balance of PLGA-PEG-PLGA triblock copolymers. With increasing the temperature, the aggregation number of the micelles increases abruptly and inter-micellar aggregation forms, due to the increase in the hydrophobic interactions of PLGA blocks and partial dehydration of PEG shell. When the polymer concentration is high enough, a sol-to-gel phase transition occurs. However, further increase in temperature to higher region results in serious dehydration of the PEG shell and marked shrinkage of the micelles, causing the precipitation of the copolymer from the solution and a gel-to-sol phase transition [46].

The sol–gel transition phase diagrams of the PLGA-PEG-PLGA aqueous solutions with or without drugs/genes were determined by a vial inverting method, as shown in Fig. S2 in Supporting Information. For the PLGA-PEG-PLGA aqueous solution without drug, the sol-to-gel transition temperature was reduced slightly from 29 to 24 °C, as the polymer concentration increased from 15 wt% to 25 wt%. It was found that the sol–gel transition temperature increased 1–4 °C after addition of DOX-HCl and shRNA/PEI-Lys (Fig. S2B). It may be attributed to the effects of hydrophilic DOX-HCl and shRNA/PEI-Lys, leading to the increase in the overall hydrophilicity of the systems [46].

Fig. 2. Viabilities of Saos-2 (A) and MG-63 (B) cells after incubation with 5 μL of hydrogel only (Gel), scrambled shRNA/PEI-Lys loaded hydrogel (Gel + shNS/PEI-Lys, negative control), PLK1shRNA/PEI-Lys loaded hydrogel (Gel + PLK1shRNA/PEI-Lys), DOX loaded hydrogel (Gel + DOX), or PLK1shRNA/PEI-Lys and DOX co-loaded hydrogel (Gel + PLK1shRNA/PEI-Lys + DOX). The DOX concentrations in the Gel + DOX or Gel + PLK1shRNA/PEI-Lys + DOX group were 0.1 mg/mL, 0.2 mg/mL, and 1 mg/mL, respectively. The PLK1shRNA/PEI-Lys concentration was 0.2 mg/mL in hydrogels with the N/P ratio of 2.5/1. ($n = 3$) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Fig. 3. Effects of the various hydrogel formulations on silencing of PLK1 expression in Saos-2 (A) and MG-63 (B) cells in vitro determined by quantitative real-time PCR. The DOX concentration was 1 mg/mL and the PLK1shRNA/PEI-Lys concentration was 0.2 mg/mL in hydrogels with the N/P ratio of 2.5/1. Data were normalized to β-actin ($n = 3$). (**$p < 0.01$, ***$p < 0.001$).
3.3. Degradation and biocompatibility of PLGA-PEG-PLGA hydrogel

Desired degradation behavior and excellent biocompatibility of hydrogels are important for biomedical applications. The degradation behaviors of various thermosensitive PEG/polyester hydrogels have been investigated. Qiao et al. studied the degradation of PLGA-PEG-PLGA hydrogels with various LA/GA ratios, and it was found that a higher LA/GA led to a slower degradation rate as a result of the stronger hydrophobic interaction among the micelles [47]. Kim et al. studied the degradation and biocompatibility of thermosensitive methoxy-poly(ethylene glycol)-poly(ε-caprolactone-co-d,l-lactide) (MPEG-PCLA) diblock copolymer hydrogel [48]. Ding et al. investigated the degradation and biocompatibility of the hydrogels based on the mixture of two types of PLGA-PEG-PLGA copolymers [49]. In the present study, the degradation and biocompatibility of the 20 wt% PLGA-PEG-PLGA hydrogels were investigated in vitro and in vivo, as shown in Fig. 1. When incubated in PBS, about 70% of the PLGA-PEG-PLGA hydrogels degraded in 40 days (Fig. 1A). The in vitro biodegradation and biocompatibility of the PLGA-PEG-PLGA hydrogels were investigated by subcutaneous injection of the copolymer solution (20 wt%) into rats. About 10 min after injection, the gels were formed in situ subcutaneously. It was found that the gels degraded gradually in the subcutaneous layer in 4 weeks and completely disappeared after 5 weeks (Fig. 1B). The hydrogels displayed faster degradation rate in vivo than in vitro, which may be attributed to the following reasons [49–51]. First, the cells and enzymes in the subcutaneous layer accelerated the degradation of the hydrogels. Another reason may lie in the degradation method by using the small vials in the in vitro test. Only the surface of the hydrogels exposed to PBS suffered the gel erosion. Moreover, the accumulation of degradation intermediates in the subcutaneous layer may produce a slightly acid environment [41], which also accelerate the biodegradation [52].

The in vitro biocompatibility of the PLGA-PEG-PLGA triblock copolymer was evaluated by the MTT assay against osteosarcoma (Saos-2, MG-63) cell lines and mouse fibroblast (L929) cell line, and the in vivo biocompatibility of the PLGA-PEG-PLGA hydrogel was tested by histological analysis of the tissues surrounding the hydrogels in the subcutaneous layer of rats. As shown in Fig. S3 in Supporting Information, the Saos-2, MG-63 and L929 cells exposure to the copolymer solutions remained over 90% viable at all concentrations up to 4 g/L, indicating no obvious cytotoxicity of the
Furthermore, the in vivo biocompatibility was evaluated by the H&E staining of the tissues surrounding the hydrogels at given time intervals after subcutaneous injection of the hydrogels into rats, as shown in Fig. 1C. No necrosis, edema, hemorrhaging and hyperemia were observed in all tissue sections, except for a mild acute inflammation with enhanced neutrophils in the tissues at 7 day post-injection. However, the inflammation was reduced markedly and gradually turned into a weak chronic inflammation, as the hydrogels degraded gradually. After the hydrogels disappeared, the surrounding tissue turned into normal, suggesting an acceptable biocompatibility of the hydrogels in vivo [50].

3.4. In vitro synergistic transfection efficiency and cytotoxicity of the PLK1shRNA/PEI-Lys and DOX co-loaded PLGA-PEG-PLGA hydrogels

To investigate the transfection efficiency of polylysine-modified polyethylenimine (denoted as PEI-Lys) developed by our group [33], the luciferase assay (Promega) was carried out on Saos-2 and MG-63 osteosarcoma cell lines with lipofectamine 2000 and PEI25K as positive controls. The transfection efficiency was measured by the luciferase intensity of the reporter gene expression. As shown in Fig. S4 in Supporting Information. The DNA/PEI-Lys complexes displayed almost 3 order of magnitude higher transfection efficiency compared to naked pGL3, and higher than the commercially available transfection agents, lipofectamine 2000 and PEI25K. Additionally, the luciferase intensity was highest when the N/P was 2.5/1, indicating that the N/P ratio of 2.5 showed the highest transfection efficiency and therefore this N/P ratio was used in the following tests.

The in vitro cytotoxicities of the hydrogels containing PLK1shRNA/PEI-Lys and DOX against osteosarcoma Saos-2 and MG-63 cells were evaluated by MTT assay. The cells were treated with hydrogel only (Gel), hydrogel containing scrambled shRNA/PEI-Lys (Gel + shNS/PEI-Lys, negative control), hydrogel containing PLK1shRNA/PEI-Lys (Gel + PLK1shRNA/PEI-Lys), DOX-loaded hydrogel (Gel + DOX), and hydrogel containing PLK1shRNA/PEI-Lys and DOX (Gel + PLK1shRNA/PEI-Lys + DOX), respectively. The cell viabilities were measured at 24 h, 48 h and 72 h. As shown in Fig. 2, the hydrogels containing either PLK1shRNA/PEI-Lys or DOX exhibited significantly higher cytotoxicities against both Saos-2 and MG-63 cells.

Fig. 6. In vivo antitumor effects of various treatments on BALB/c nude mice bearing human osteosarcoma Saos-2 xenografts. The groups were divided into Gel, Gel + shNS/PEI-Lys, Gel + DOX, Gel + PLK1shRNA/PEI-Lys and Gel + PLK1shRNA/PEI-Lys + DOX. (A) Tumor growth inhibition, (B) The weights of tumors obtained after the mice were sacrificed, (C) The change in body weight of mice during treatments. The DOX concentration was 5.0 mg DOX per kg mice weight and PLK1shRNA/PEI-Lys concentration in hydrogels was 0.5 mg/mL with N/P ratio of 2.5/1. Data were presented as mean ± standard deviation (n = 6). (*p < 0.05, ***p < 0.001).

Fig. 7. Ex vivo analysis of on silencing of PLK1 expression in the tumor masses obtained from the mice after various treatments by quantitative real-time PCR. Data were normalized to β-actin (n = 3). (**p < 0.001).
MG-63 osteosarcoma cells compared to hydrogels only or hydrogels containing scrambled shRNA/PEI-Lys (denoted as shNS/PEI-Lys). This suggested that silencing of PLK1 by PLK1shRNA showed efficient proliferation inhibition of Saos-2 and MG-63 cells [53]. Moreover, the hydrogels loaded with both PLK1shRNA/PEI-Lys and DOX exhibited the markedly enhanced cytotoxicities than those containing either PLK1shRNA/PEI-Lys or DOX, indicating a synergistic antitumor efficacy of PLK1shRNA and DOX in vitro.

3.5. Effects of hydrogels containing PLK1shRNA/PEI-Lys and DOX on the expression of PLK1 in vitro

The gene silencing efficiency of the cells treated with different hydrogel formulations was evaluated by quantitative real-time PCR to measure the expression of osteosarcoma cells. As shown in Fig. 3, the expression of PLK1 was significantly down-regulated in both Saos-2 and MG-63 cells after the cells were treated with the
hydrogels containing PLK1shRNA/PEI-Lys or DOX. Moreover, it was found that the silencing efficiency of PLK1shRNA/PEI-Lys and DOX co-loaded hydrogel was significantly higher than that of the hydrogel containing PLK1shRNA/PEI-Lys or DOX alone. This confirmed that DOX and PLK1shRNA/PEI-Lys presented synergistic effects in silencing the expression of PLK1, which was in coincidence with the cell cytotoxicity test.

3.6. Effects of hydrogels containing PLK1shRNA/PEI-Lys and DOX on cell apoptosis in vitro

The in vitro apoptosis of osteosarcoma Saos-2 and MG-63 cells was detected by Annexin V-APC/7-AAD assay and the apoptosis gene expression was measured by quantitative real-time PCR. For the Annexin V test, the osteosarcoma cells were double stained for viability (7-AAD) and apoptosis (Annexin V-APC). Annexin V is an intracellular protein that specifically binds to phosphatidylserine which can transfer from the intracellular membrane to external leaflet during early apoptosis [54]. As shown in Fig. 4, treatment with hydrogels containing both DOX and PLK1shRNA/PEI-Lys induced much higher apoptosis compared with that containing DOX or PLK1shRNA/PEI-Lys alone, even though either of them also induced apoptosis of the osteosarcoma cells.

The result was further confirmed by the expression of apoptosis genes in the osteosarcoma cells by quantitative real-time PCR, as shown in Fig. S5 in Supporting Information. Silencing of PLK1 can induce the cell apoptosis through a series of apoptosis and anti-apoptosis genes, including the Bcl-2 family and caspase family. The Bcl-2 family is considered as key regulators to the mitochondrial pathway of apoptosis by the permeabilization of the mitochondrial outer membrane. The deregulation of Bcl-2 and up-regulation of BAX play a major role in tumor formation and the tumor response to anticancer therapy [55]. Caspases are a family of cysteine proteases, which are responsible for osteosarcoma initiation and progression, especially caspase-3 and caspase-9. It has been proved that the down-regulation of caspase-3 promotes the tumorigenesis, and caspase-9 is in low expression in cancer [56]. Caspase-3 can propagate and augment the apoptosis pathways through the positive feedback that leads to the burst of caspase-9 and further propagating the apoptosis [57,58]. Furthermore, Bcl-2 is considered as an anti-apoptosis gene, which causes a chain of events that block activation of certain procaspases [59].

Therefore, the down-regulation of Bcl-2 promotes the apoptosis of the osteosarcoma cells [60]. On the other hand, BAX, caspase-3 and caspase-9 are apoptosis genes in the apoptosis pathway, and the up-regulation of these genes can cause higher apoptosis rate [61]. In our study, as shown in Fig. S5 in Supporting Information, the combination of PLK1shRNA/PEI-Lys and DOX increased the expression of caspase-3, caspase-9 and BAX significantly, whereas the expression of anti-apoptosis gene Bcl-2 was reduced obviously in both Saos-2 and MG-63 cells. Thus, this further confirmed that the osteosarcoma cells treated with PLK1shRNA/PEI-Lys and DOX co-loaded hydrogels showed the highest apoptosis.

3.7. Effects of hydrogels containing PLK1shRNA/PEI-Lys and DOX on the cell cycle in vitro

PLK1, a serine/threonine kinase, plays a crucial role in mitosis and in the maintenance of genomic stability, and contributes to multiple processes including mitotic entry, centrosome maturation, spindle assembly and cytokinesis, associated with several modes of regulation [20]. However, the mechanisms of PLK1 regulation remained unclearly. PLK1 plays a key role in the G2/M transition, including initiation of mitosis by activating the cyclin-dependent-kinase 1 (CDK1) and phosphorylation of cyclin B1 and cdc25c to promote the cell cycle [22]. It has been shown that PLK1 inhibition leads to the G2/M arrest, apoptosis and multiple mitotic errors [62], implying a promising gene therapy target for a broad range of human tumors, including osteosarcoma [24].

As shown in Fig. 5, treatment with PLK1shRNA/PEI-Lys loaded hydrogel induced marked G2/M arrest compared to treatment with hydrogel only, shNS/PEI-Lys loaded hydrogel or DOX-loaded hydrogel. Notably, even though treatment with hydrogel containing DOX alone showed no obvious G2/M arrest, DOX and...
PLK1shRNA/PEI-Lys co-loaded hydrogel led to the highest G2/M arrest. This suggested that PLK1shRNA and DOX showed synergistic effects on the cell cycle arrest, contributing to the apoptosis of the osteosarcoma cells.

Additionally, in order to study the mechanism of PLK1 on cell cycle regulation, some cyclin dependent proteins were detected, which are known as evolutionarily conserved regulators of the cell cycle. As shown in Fig. S6 in Supporting Information, silencing of PLK1 by treatment with PLK1shRNA/PEI-Lys and DOX co-loaded hydrogel resulted in significant increase in the expressions of CDK1, cdc25c and cyclin B1, indicating synergistic effects on the cell cycle regulation in vitro.

3.8. In vivo synergistic antitumor efficiency of PLK1shRNA/PEI-Lys and DOX co-loaded hydrogels

The in vivo antitumor efficiency of PLGA-PEG-PLGA hydrogels containing PLK1shRNA/PEI-Lys and DOX was investigated on a human osteosarcoma xenograft model by inoculation of human osteosarcoma Saos-2 cells into the armpit of right anterior limb of nude mice. When the volume of the tumors reached to ~50 mm³, the mice were treated by single injection of the hydrogels containing PLK1shRNA/PEI-Lys and DOX beside the tumors. As shown in Fig. 6, the tumor volumes of the control groups treated with hydrogels alone or shNS/PEI-Lys loaded hydrogels increased rapidly. In contrast, treatment with hydrogels loaded with DOX or PLK1shRNA/PEI-Lys alone displayed markedly higher suppression efficacy on tumor growth compared to the control groups up to 16 days after treatment, suggesting PLK1 is no doubt an effective gene target for the treatment of osteosarcoma [28]. Notably, treatment with hydrogels containing both PLK1shRNA/PEI-Lys and DOX exhibited the highest anti-tumor efficacy and the tumor growth was almost completely suppressed up to 16 days. At the end of the test, the osteosarcoma tumor masses were dissected from the mice and weighted. The results were the in consistent with the tumors volumes (Fig. 6B).

Besides, the body weight of rats was measured for the safety evaluation during the whole experimental period (Fig. 6C). No obvious body weight loss was observed in all treated groups, implying no obvious systemic toxicity by the treatments with localized injection of hydrogels containing DOX and genes.

The silencing efficiency of PLK1 in tumor mass was evaluated by quantitative real-time PCR. As shown in Fig. 7, the expressions of PLK1 were significantly lower in the tumors treated with hydrogels containing PLK1shRNA/PEI-Lys or DOX compared to that of the control groups. Moreover, PLK1shRNA/PEI-Lys and DOX co-loaded hydrogels showed the highest silencing efficiency of PLK1 in tumors. This confirmed the synergistic effects of PLK1shRNA and DOX in silencing the expression of PLK1 in vivo.

At the end of the test, the mice were sacrificed and the tumor masses were dissected into sections for further pathology analysis by H&E and TUNEL staining. As shown in Fig. 8, a large number of diving cells with increased size of nuclei and different shapes were observed in the tumor mass treated with hydrogel only or hydrogel containing shNS/PEI-Lys. In contrast, karyolysis, pyknosis and karyorrhexis were observed clearly in the tumors treated with the hydrogel containing PLK1shRNA/PEI-Lys and DOX, indicating obvious tumor necrosis. Furthermore, the tumor sections were TUNEL stained and the group treated with PLK1shRNA/PEI-Lys and DOX co-loaded hydrogel exhibited the highest fluorescence, implying the highest apoptosis ratio of tumor cells.

Fig. 11. Histological H&E staining analyses of the organs of mice after different treatments: Gel, Gel + shNS/PEI-Lys, Gel + DOX, Gel + PLK1shRNA/PEI-Lys and Gel + PLK1shRNA/ PEI-Lys + DOX.
Furthermore, the apoptosis related genes, Bcl-2, BAX and caspase-3, caspase-9 of the osteosarcoma tumor masses of different groups were investigated by quantitative real-time PCR to show the mechanisms of apoptosis pathway. As shown in Fig. 9, compared to the control groups treated with hydrogel only or shNS/PEI-Lys containing hydrogel, the expression of anti-apoptosis gene Bcl-2 was sharply decreased, while the apoptotic genes BAX, caspase-3 and caspase-9 increased significantly in the groups treated with hydrogels containing PLK1shRNA/PEI-Lys or DOX. Additionally, obvious synergistic effects of PLK1shRNA and DOX on the Bcl-2, BAX, caspase-3 and caspase-9 expressions were observed. As a conclusion, localized co-delivery of PLK1shRNA/PEI-Lys and DOX induced the synergistic apoptosis of osteosarcoma tumors through a variety of apoptosis genes, including down-regulation of anti-apoptosis gene Bcl-2 and up-regulation of apoptosis genes BAX, caspase-3 and caspase-9.

To further clarify the mechanism of the PLK1 gene on cell cycle in vivo, PLK1shRNA was used to silence the expression of the PLK1 in the Saos-2 xenografts models. The cyclin dependent proteins of the tumor masses after various treatments were evaluated by the quantitative real-time PCR, including CDK1, cyclin B1 and cdc25c. As shown in Fig. 10, silencing of PLK1 by treatment using PLK1shRNA/PEI-Lys loaded hydrogel induced significant increase in the expressions of CDK1, cdc25c and cyclin B1. Furthermore, treatment with PLK1shRNA/PEI-Lys and DOX co-loaded hydrogel led to enhanced expressions of CDK1, cyclin B1 and cdc25c compared to treatment with the hydrogel containing PLK1shRNA/PEI-Lys alone, suggesting a synergistic effect of PLK1shRNA and DOX on the cell cycle regulation.

From the above results, it was confirmed that DOX is an efficient chemotherapeutics to human osteosarcoma and PLK1 represents an effective target for the treatment of osteosarcoma in vivo [12]. In previous studies, PLK1 has been used as a target for cancer treatment. It was found that PLK1mRNA was over-expressed in pediatric rhabdomyosarcomas through siRNA library screen [63]. PLK1 silencing was found to be effective in the in vivo antitumor treatment [64]. For instance, PLK1shRNA was found to be effective in suppressing the HeLa xenografts models [65] as well as xenograft osteosarcoma model [27]. In the present study, a significant synergistic antitumor efficiency in vivo was achieved by a strategy through localized injection of the biodegradable hydrogels incorporated with PLK1shRNA/PEI-Lys and DOX. The synergistic anti-tumor effects were approved in detail by tumor growth suppression, silencing of PLK1 gene, promoting tumor apoptosis, and influence on cell cycle regulation.

3.9. Safety evaluation of the treatments with hydrogels containing PLK1shRNA/PEI-Lys and DOX

The systemic toxicities of treatments with the hydrogels incorporating PLK1shRNA/PEI-Lys and DOX were evaluated by H&E staining of the tissue sections, including heart, liver, spleen, lung and kidney. At the end of the animal test, the mice were sacrificed and the organs were sectioned and H&E stained for morphological analysis. Since DOX and PLK1shRNA/PEI-Lys were released locally through localized injection of the biodegradable hydrogels incorporated with PLK1shRNA/PEI-Lys and DOX co-loaded hydrogel beside the tumors of nude mice bearing osteosarcoma Saos-2 xenografts, the hydrogel exhibited synergistic anti-tumor efficacy in vivo. It was found that the combination treatment in vivo resulted in almost complete suppression of tumor growth up to 16 days, enhanced PLK1 silencing, increased apoptosis of tumor masses, higher expression of apoptosis-related genes and cell cycle regulation. Moreover, ex vivo histological analysis of major organs of the treated mice showed no obvious damage of the organs, indicating lower systemic toxicity of the localized treatments. Thus, the combination treatment of osteosarcoma by localized, sustained co-delivery of PLK1shRNA and DOX using the non-cytotoxic and biodegradable PLGA-PEG-PLGA hydrogel can be a promising strategy for efficient clinical treatment of osteosarcoma. Further studies in orthotopic models of metastatic disease are needed.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.biomaterials.2014.06.045.

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