Co-delivery of HIF1α siRNA and gemcitabine via biocompatible lipid-polymer hybrid nanoparticles for effective treatment of pancreatic cancer

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ARTICLE INFO

Article history:
Received 10 September 2014
Accepted 20 December 2014
Available online 15 January 2015

Keywords:
siRNA delivery
Hypoxia-inducible factor 1α
Lipid-polymer hybrid nanoparticles
Combination therapy
Pancreatic cancer
Orthotopic tumor model

ABSTRACT

Hypoxia-inducible factor 1α (HIF1α) has emerged as a promising new target for pancreatic cancer treatment over the past decade. High expression of HIF-1α increases the drug resistance of the current first line chemotherapeutic drug, gemcitabine (Gem). Here we employed biocompatible lipid-polymer hybrid nanoparticles to co-deliver HIF1α siRNA (si-HIF1α) and Gem for pancreatic cancer treatment in subcutaneous and orthotopic tumor models. The cationic ε-polylysine co-polymer (ENPs) can effectively absorb negatively charged si-HIF1α on the surface and encapsulate Gem to the hydrophilic core. Further coating of ENPs with PEGylated lipid bilayer resulted formation of LENPs, with reversed surface charge. The lipid bilayer of LENPs prevented nanoparticle aggregation and si-HIF1α degradation in serum, as well as Gem leakage. Those characteristics endow LENPs encapsulating drug prolonged lifetime in bloodstream and improved drug release via the enhanced tumor vasculature effect in tumor tissues. LENPs can co-deliver Gem and si-HIF1α (LENP-Gem-si-HIF1α) into tumor cells and effectively suppress the HIF1α expression both in vitro and in vivo. LENP-Gem-siHIF1α exhibited significant synergistic antitumor effects. Furthermore, LENP-Gem-si-HIF1α showed excellent capability to inhibit tumor metastasis in orthotopic tumor model.

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1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a hypovascular tumor with a 5-year survival rate of less than 5% [1]. Even if treated with the current first line chemotherapeutic drug, Gemcitabine (Gem), the median overall survival of patients only ranged from 5.0 to 7.2 months [2]. Therefore, new therapy approaches are urgently needed for the vast majority of PDAC patients.

Because of inefficient tumor vascular supply, there are large hypoxic areas in pancreatic cancer tissues [3]. Tumor cells can survive under hypoxic conditions by activating many signaling pathways, in which HIFs (hypoxia-inducible factors) were the most important transcription factors [4]. HIF is a heterodimer that is composed of one of three oxygen-regulated subunits (HIF1α, HIF2α, HIF3α) and a constitutively expressed b-subunit (HIF1β). Compared to the ambiguous role of HIF2 (composed of HIF2α and HIF1β) in tumor progression [5–7], HIF1 (composed of HIF1α and HIF1β) is recognized as a key transcription factor in tumor development [8,9], although there has been reported that HIF1 plays a role in hypoxia-mediated apoptosis [10]. HIF1 can induce a wide range of gene products, which regulate tumor invasion, proliferation, angiogenesis and drug resistance [11–14]. In recent years, targeting HIF1α has become a novel and efficient strategy for cancer therapy [15,16]. Many HIF-1α inhibitors have been identified, in which the vast majority were chemical inhibitors, such as Resveratrol, YC-1, PX-12, SAHA and FK228. However, the relative lack of specificity led to many side effects in clinical trials of HIF-1 chemical inhibitors [17].
RNA interference (RNAi) mediated by small interfering RNA (siRNA) is an effective method to selectively inhibit expression of a target gene [18]. Compared to chemical inhibitors, HIF1α siRNA (si-HIF1α) had stronger suppressive effects [19]. However, naked siRNA has a half lifetime of less than an hour in the bloodstream, and is rapidly degraded by nucleases in plasma or excreted by kidney [20]. Furthermore, naked siRNA hardly penetrate across cell membranes due to their high molecular weight, hydrophilic properties and high density of charge [21]. These issues suggest that the development of safe and effective delivery systems is essential for therapeutics using siRNA in vivo.

Two major classes of biomaterials that have been employed for siRNA delivery are liposomes and polymers [20,22–24]. The liposomes used for siRNA delivery are mainly cationic liposomes. Positively charged lipids can realize effective siRNA loading by electrostatic interaction, but previous studies suggest that cationic lipids are associated with severe toxicity, strong immune or inflammatory responses [22,25]. Polymers used for siRNA delivery include polycations or polycation-containing block copolymers, such as poly(vinyl pyridine), poly(L-lysine), and polyethylenimine. Although many different types of biodegradable polycations have been explored, the carrier-induced toxicity is still a challenge for cationic polymers. Generally, to deliver more siRNA, polycations are designed with high charge densities, which are associated with toxicity. Recently, lipid-polymer hybrid nanoparticles have emerged as effective vehicles for siRNA delivery [26,27]. Lipid-polymer hybrid nanoparticles, with a single layer or bilayer lipid shell around a polymeric core, may combine the advantages of polymers and liposomes. The cationic polymeric core can encapsulate drug and absorb siRNA; meanwhile, lipid shell provide protective effect and excellent biocompatibility and in vivo stability. However, the studies using lipid-polymer hybrid nanoparticles for co-delivery of drug and siRNA were still few [26,27].

Herein, we employed biocompatible lipid-polymer hybrid nanoparticles to co-deliver si-HIF1α and Gem for pancreatic cancer treatment in subcutaneous and orthotopic tumor models (Scheme 1A). All of component materials have been approved by the US Food and Drug Administration (FDA) for clinical uses or as food additives. The designed nanoparticles were composed with a cationic co-polymer core and a PEGylated lipid bilayer shell. Gem can be effectively encapsulated into the hydrophilic core of the cationic co-polymer; meanwhile, negatively charged si-HIF1α can be absorbed on the surface of the cationic co-polymer. The PEGylated lipid bilayer shell was designed for the following reasons: (a) Lipid bilayer shell encapsulated siRNA inside the nanoparticles, which can protect siRNA from serum nucleases and the immune system recognition; (b) The shell can decrease leakage of Gem and the replacement of siRNA by other negative charged substance in the bloodstream; (c) Unfavorable aggregation due to positive surface charge can be addressed by the negative surface charge of the lipid shell [20,28]; (d) The PEG on the lipid shell provides “stealth” properties such as prevention of serum protein absorption [29].
Those characteristics can endow the nanoparticles with better stability and prolonged lifetime in the bloodstream, and improve drug release via the enhanced tumor vasculature effect in tumor tissues [30] (Scheme 1).

2. Materials and methods

2.1. Materials and reagents

Gem, cholesterol, and FITC-phallolidin were purchased from Sigma–Aldrich (St. Louis, MO, USA). Monomethoxy poly(ethylene glycol) (mPEG)-poly (lactate-co-glycolic acid) (PLGA, molar ratio of D, L-lactic to glycolic acid, 75: 25), was purchased from Jinan Daigang Biotechnology Co. Ltd. polyethylene (EPL) was purchased from Zhengzhou Bainafao Bioengineering Co. Ltd. L-carnosine from J & K. Cell Counting Kit-8 (CCK8) was purchased from Dojindo Molecular Technologies (Tokyo, Japan). Dulbecco’s modiﬁed eagle’s medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Lyso-Tracker Red, 4,6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen. All chemicals used were of analytical reagent quality.

2.2. Preparations of nanoparticles

Co-polymer nanoparticles were prepared by the double emulsion (W/O/W) method with minor modiﬁcations [24,31]. Briefly, 20 mg of mPEG-PLGA was dissolved in 1 mL of methylene chloride with 0.2 mL water (containing Gem if required), and then transferred 1 to a centrifuge tube. The mixture was emulsified by sonication for 5 min. The emulsion was added to 2 mL of 2% polyvinyl alcohol (PVA) with EPL, and emulsified for a second time by sonication for 5 min. The emulsion was then slowly dropped into 10 mL of 0.6% PVA and stirred for 10 min at room temperature. The solvent was be removed by vacuum evaporation. The co-polymer nanoparticles were collected by centrifugation at 13,000 rpm for 10 min at room temperature and dispersed in distilled water again. Phospholipids and cholesterol mixture [lecithin, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(methoxy(polyethylene glycol)-2000] (DSPE-PEG-2000), and cholesterol in a 60: 12: 15 mass ratio] were dissolved in dichloromethane, and then a lipid film was formed in a round bottom flask under reduced pressure using a vacuum rotary evaporator, followed by the addition of the polymer emulsion. The lipid film will coat on the EPL-modified co-polymer nanoparticles (ENPs) during sonication for 10 min. The resulting lipid-polymer hybrid nanoparticles (LENPs) were collected by centrifugation at 13,000 rpm for 10 min at room temperature and washed twice with distilled water.

2.3. Morphological characterization

For the morphology characterization, nanoparticles were negatively stained with 2% uranyl acetate solution, deposited on a carbon-coated copper grid, and examined with a transmission electron microscope (TEM, JEM-2000C, Jeol Ltd., Japan).

2.4. Size distribution and zeta potential measurements

The nanoparticle size (diameter, nm), size distribution and surface charge (zeta potential, mV) were determined using a ZetaSizer Nano series Nano-ZS (Malvern Instruments Ltd., Malvern, UK) equipped with a He–Ne Laser beam at a wavelength of 633 nm and a fixed scattering angle of 90°. Determinations were performed at 25 °C for samples appropriately diluted in distilled water.

2.5. High performance liquid chromatography (HPLC) analysis

A waters HPLC system, comprised of an auto sampler (model 717 plus), binary pump (model 1525), UV photodiode array detector (model 996) was used. Samples were applied to a symmetry C18 reverse phase column (20 mL, 4.6 × 150 mm) and eluted using an acetonitrile, water, trifluoroacetic acid gradient. The starting mobile phase was acetonitrile 80%, water 20%, trifluoroacetic acid (0.1% v/v), with a linear progression linearly to 80% acetonitrile over 15 min. The mobile phase composition was then kept constant at 5% acetonitrile for 1–3 min, followed by a further linear change up to 50% acetonitrile. The flow rate was 1.0 mL/min and the eluent was monitored at 270 nm for Gem, respectively. All separations were performed at room temperature.

2.6. Gem encapsulation efﬁciency calculation

The Gem encapsulation efﬁciency of the LENPs was calculated by the following equation. Encapsulation Efﬁciency – (A − B)/A × 100%, where A is the initial amount of drug put in the system, B represents the amount of drug obtained in the supernatant of the after centrifugation determined by HPLC.

2.7. Cell culture

Panc-1 human PDAC cells and B-16 human melanoma cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. Cells were grown at 37 °C in a humidified atmosphere of 95% air and 5% CO2 using DMEM with 10% FBS.

2.8. siRNA duplexes and transient transfection

Three siRNAs against HIF1α were designed and synthesized from Ribobio (Guangzhou, China), and the most effective one was selected for further study (Fig. 5i, si-HIF1α#2). For transfection, cells were plated at a density of 5 × 10⁵ cells/well in 6-well plates. When the cells were 80% confluent, 50 nm siRNA were transfected into cells using lipofectamine-2000 (Invitrogen) for 48 h.

2.9. Stability measurement in serum

The in vitro stabilities of DNA and Gem encapsulated ENPs (ENP-Gem-DNA) and DNA and Gem encapsulated LENPs (LENP-Gem-DNA) were investigated by dispersing the nanoparticles in 10% FBS and examining the changes of size by dynamic light scattering (DLS).

2.10. Cytotoxicity studies in vitro

For the in vitro cell viability assay, panc-1 cells were seeded into 96-well plates at a density of 5000 cells per well. Twelve hours after seeding, the cells were treated with various drug formulations for 48 h. The proportion of viable cells was evaluated using a CCK-8 assay according to the manufacturer’s instructions (Dojindo, Japan).

2.11. Western blot

Adherent cells were collected and washed twice with ice-cold phosphate buffer solution (PBS). Total cell protein was extracted, and the concentrations were quantiﬁed by the bicinchoninic acid protein assay kit (23225; Thermo). Cell lysates were resolved with SDS-PAGE electrophoresis and then transferred to PVDF membranes. These membranes were blocked and incubated with primary antibodies at 4 °C overnight. Subsequently, the membranes were washed and treated with appropriate secondary antibodies for 1 h. The immunoreactivity was visualized by enhanced chemiluminescence reagents. β-actin was used as an internal control.

2.12. Immunohistochemistry

Immunohistochemistry for HIF-1α of PDAC patient tissues was performed according to instructions, using a DAB substrate kit (Maxin, Fuzhou, China). The results were scored by two examiners who were blinded to clinicopathologic data. Intensity of staining was scored as (0 = negative; 1 = low; 2 = medium; 3 = high). Extent of staining was scored as 0 = 0% stained; 1 = 1–25% stained; 2 = 26–50% stained; 3 = 51–100% stained. Five random ﬁelds were observed under a light microscope. The ﬁnal score was determined by multiplying the scores of intensity with the extent of staining, ranging 0–9. Final scores of less than 1 were considered as negative staining (−), 1–2 as low staining (+), 3–4 as medium staining (+++) and 6–9 as high staining (+++++). Then the correlation between level of HIF-1α expression and outcome of patients was analyzed using Kaplan–Meier curves.

2.13. Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from cells using TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions. A reverse-transcription polymerase chain reaction (RT-PCR) system (Promega) was used. Then, 1 mg sample of the cDNA was quantiﬁed by real-time PCR using primer pairs with SYBR Green PCR Master mix (Promega). Each sample was done in triplicate. β-actin was used as loading control. The vascular endothelial growth factor (VEGF), downstream gene of HIF1, was used as a positive control.


Panc-1 cells were seeded onto a 35 mm borosilicate chambered cover glasses (Nunc, USA), at a density of 2 × 10⁵ cells/well. Cells were then treated by rhodamine B (Rhod) and carboxylfluorescein labeled siRNA (FAM-siRNA) encapsulated LENPs (LENP-Rhod-FAM-siRNA) for different time. The cells were washed three times with PBS and were observed with an LSM 710 confocal microscope (Carl Zeiss, USA) at 60× magniﬁcation. The excitation wavelengths of FAM-siRNA, and Rhod were 490 and 540 nm, respectively, and the emission ﬁlters were 525 and 588 nm. If cells were treated by FAM-siRNA encapsulated LENPs (LENP-FAM-siRNA), lysosomes were stained by LysoTracker Red according to the manufacturer’s instructions before imaging.

2.15. Knockdown effect in vitro

Panc-1 cells were seeded onto 6-well plates at a density of 1 × 10⁶ cells per well. Cells were treated with saline, LENPs, free si-HIF1α, si-HIF1α encapsulated ENPs (ENP-si-HIF1α) and si-HIF1α encapsulated ENPs (LENP-si-HIF1α) containing medium for 6 h. Then cells grew in fresh FBS containing medium for another 48 h, and were analyzed for HIF1α gene and protein using RT-PCR and western blot. Knockdown action was chosen for visual observation of knockdown effect, and B-16 cells were chosen because of their obvious actin cytoskeleton. Cells were treated with saline, LENPs, free siRNA against actin (si-Actin), si-Actin encapsulated ENPs (ENP-si-Actin) and si-Actin encapsulated LENPs (LENP-si-Actin) containing medium for 6 h. Then cells grew in fresh FBS containing medium for another 48 h. Before imaging
using confocal microscope, actin cytoskeleton was stained with FITC-phalloidin (Sigma) according to the manufacturer’s instructions.

2.16. Circulation time and targeting in vivo

For evaluating of circulation time, female BALB/c mice were injected with free Cy3 labeled siRNA (Cy3-siRNA), Cy3-siRNA encapsulated ENPs (ENP-Cy3-siRNA) and Cy3-siRNA encapsulated LENPs (LENP-Cy3-siRNA) through tail vein. At different time, blood was drawn from tail vein, and then imaged using a Maestro™ in vivo spectrum imaging system (CRi, Woburn, MA, USA) at an excitation: emission of 548: 562 nm. To study the targeting ability of nanoparticles in vivo, BALB/c nude mice with pancreatic cancer xenografts were injected with saline, free RhoB, RhoB encapsulated ENPs (ENP-RhoB) and RhoB encapsulated LENPs (LENP-RhoB) through tail vein. After 24 h, the ex vivo fluorescent images of organs and tumors were obtained using a Maestro™ in vivo spectrum imaging system (CRi, Woburn, MA, USA) at an excitation: emission of 540: 588 nm.

2.17. Antitumor effects in vivo

Subcutaneous pancreatic cancer xenografts were formed in female BALB/C nude mice by injecting $5 \times 10^6$ panc-1 cells (4 mice/group). When the tumor volume reached 21–23 mm$^3$, different drug formulations (4 mg/kg Gem, 1.33 mg/kg siRNA) were injected through the tail vein. Tumor size was measured, and tumor volumes were calculated using the formula $V = \frac{1}{2}ab^2$, where $a$ is the long axis and $b$ is the short axis. Mice with tumor implants were euthanized after 15 day, and the tumor xenografts were excised and weighed. The tumor xenografts were sectioned, and HIF1α was stained by immunohistochemistry. The positive cancer cells were with a brown staining.

For orthotopic pancreatic cancer models, luciferase expressing panc-1 cells (panc-1-luc) were injected into pancreatic tail through operation. Treatments began 20 days after injection (n = 3 per group). Bioluminescent imaging of the mice was performed at day 1 and 10. Different drug formulations (4 mg/kg Gem, 1.33 µg/kg siRNA) were injected at day 1, 4 and 7. Mice with tumor implants were euthanized after 10 d, and the tumor xenografts were excised and weighed. For bioluminescent imaging, D-luciferin potassium salt (15 mg/mL, 10 µL/g/mouse) was injected into abdominal cavity. After 10 min, mice were imaged using IVIS Spectrum.

2.18. ELISA assays for acute immune response-related cytokines

Female BALB/c mice were injected with saline, free siRNA, siRNA encapsulated ENPs (ENP-siRNA) and siRNA encapsulated LENPs (LENP-siRNA) through tail vein (4 µg/kg siRNA). After 1 h and 3 h, IFN-α and IL-6 were measured by enzyme-linked immunosorbent assay (ELISA, Solarbio S&T Co., Beijing, China).

2.19. Statistical analysis

Student’s t test for unpaired data was used to compare mean values. The log-rank test was used to obtain a $p$ value for the significance of Kaplan–Meier curves’ divergence. Analyses were performed using the SPSS17.0 statistical analysis software.

3. Results and discussion

3.1. Kaplan–Meier survival analysis of HIF1α expression in PDAC patients and effects of HIF1α knockdown on cell proliferation and cytotoxicity of Gem

In a cohort of 81 PDAC patients treated at the Tianjin Cancer Hospital in China, patients with medium or high (++) or (+++) HIF1α protein expression had significantly worse overall survival than those with negative or low (− or +) HIF1α expression (Fig. 1A). In cell study (Fig. 1B), HIF1α knockdown inhibits proliferation of pancreatic cancer cells. Pancreatic cancer therapy with si-HIF1α in vivo has been reported previously [32]. In addition, inhibiting HIF-1α in early-stage tumors was found to be more efficacious than
that in more established ones [32]. These results suggested that HIF1α indeed is a promising therapeutic target in pancreatic cancer.

Gem can arrest tumor growth via inhibiting replication and inactivating the ribonucleotide reductase. After HIF1α knockdown, cytotoxicity of Gem significantly enhanced (Fig. 1C). If Gem and si-HIF1α could be co-delivered by nanoparticles into cells, potential synergistic antitumor effects are expected.

3.2. Preparation and characterization of nanoparticles

LENPs were fabricated reproducibly by combining a double emulsion method for polymer nanocore and ultrasound assisted self-assembly of a lipid film for lipid shell [31,33]. The internal polymer components were constructed with amphiphilic copolymer mPEG-PLGA and EPL. PLGA and mPEG are two well-characterized and biodegradable materials which have been approved for clinical use by FDA [34]. EPL is a homopolymer of 25–35 L-lysine residues produced by Streptomyces Albulus. This natural compound has been used as a food additive approved by the FDA [35]. Here the positively charged polymer nanocore based on mPEG-PLGA and EPL through a double emulsion method (Scheme 1A). The hydrophilic drug gemcitabine can be loaded into the hydrophilic core of the polymer nanoparticles in the first emulsification, which resulted in a water-in-oil (W/O) emulsion. Then the cationic EPL was added, followed by the second emulsification, which generated a water-in-oil-in-water (W/O/W) emulsion. Then we added the emulsion into surfactants to form ENPs. The anion siRNA or DNA can be bound at the surface of the cationic ENPs through electrostatic interactions. A lipid mixture formed lipid film, was then used to coat ENPs by self-assembly under ultrasound to form LENPs. The ENPs were encapsulated in the PEGylated lipid bilayer shell (Scheme 1A).

We first examined the surface charge density of ENPs at different weight ratios of EPL: mPEG-PLGA to assess the EPL binding ability at the co-polymer nanoparticle surface. The results showed that EPL did effectively bind at the surface of the nanoparticles as the zeta potential of the ENPs changed radically, from −16.8–22.7 mV, when EPL increased in mass ratio (Fig. 2A). The nucleic acid-binding capacity of ENPs was evaluated by using those nanoparticles to absorb DNA. When DNA was mixed with ENPs in different proportions, the zeta potential of ENPs changed from 22.7 to −36.0 mV with increased mass ratio of DNA: mPEG-PLGA (Fig. 2B). These results can be observed in an electrophoretic mobility shift assay more intuitively. DNA was neutralized in agarose gel after mixed with ENPs at various DNA: mPEG-PLGA ratios. When the DNA: mPEG-PLGA ratios were decreased to 1:16, the DNA was fully neutralized as no further mobilization of DNA band was observed (Fig. 2C). These results demonstrated that ENPs has a large capacity to absorb negatively charged nucleic acids.

Then we examined whether a PEGylated lipid bilayer film could assemble effectively on the surface of ENPs. As shown in Fig. 2D, when the weight ratios of lipid: mPEG-PLGA increased from 0.15/1

![Fig. 2. Surface charge changes of the nanoparticles at the different stages of preparations. (A) Zeta potential changes of ENPs after modification with different amounts of EPL. PNP: co-polymer nanoparticles without EPL. (B) Zeta potential changes of ENPs loaded with different amounts of DNA. The anion DNA was bound at the surface of the cation ENPs through electrostatic interactions. (C) Electrophoretic mobility of DNA when they were mixed with ENPs in different proportions. (D) Changes in the zeta potentials of LENPs after coating with lipid bilayer shell at different mass ratios (lipid/mPEG-PLA).](image-url)
to 1.05/1, zeta potential changed gradually from 22.7 to −36.0 mV. When the lipid/PEG-PLGA weight ratios exceeded 1.05/1, the zeta potential remained at a relative stable value (33–36 mV). These results demonstrated that PEGylated lipid bilayer did effectively coat on the surface of all of ENPs.

The morphology and structure of Gem encapsulated ENPs (ENP-Gem), ENP-Gem-DNA and LENP-Gem-DNA were characterized by TEM respectively. All the hybrid nanoparticles were dispersed, with a well-defined spherical core-shell structure (Fig. 3A–C). The sizes of ENP-Gem and ENP-Gem-DNA were about 50 nm. The LENP-Gem-DNA was a little larger with diameter of about 60 nm. The average hydrodynamic diameters according to DLS measurement results of ENP-Gem, ENP-Gem-DNA, and LENP-Gem-DNA were 122.4, 122.9, and 141.8 nm, respectively (Fig. 3A–C). The zeta potential of ENP-Gem was 21.9 mV due to the EPL on the surface (Fig. 3A). DNA (equivalent to therapeutic dose of siRNA) adsorptions changed the zeta potential to 9.03 mV from 21.9 mV (Fig. 3B). Zeta potential of LENP-Gem-DNA changed to −34 mV due to the lipid bilayer shell coating (Fig. 3C). The nanoparticle size was important character for long circulation lifetime in vivo, because it is imperative to be large enough (>20 nm) to avoid renal clearance. Long lifetime in bloodstream can guarantee effective accumulation in solid tumor sites because of enhanced tumor vasculature effect. Surface charges were also important for stability of nanoparticles in plasma. Nanoparticles with high positive surface charge more likely aggregated and absorb serum proteins (such as opsonin).

### 3.3. Stability examination in serum and in vitro drug release

The in vitro stabilities of ENP-Gem-DNA and LENP-Gem-DNA were investigated by dispersing the nanoparticles in 10% FBS and examining the changes of size by DLS over time (Fig. 4A and B). There was no significant change in the size distribution of LENP-Gem-DNA before 48 h incubation, but ENP-Gem-DNA treated in the same way were of a much larger size and broader size distribution. As mentioned previously, the negative surface charges made LENPs hard to aggregate and absorb serum protein. In addition, the “stealth” properties provided by PEG in the lipid shell also played a role in high stability of LENPs in serum.

The protective effect of lipid shell on preventing the nucleic acid from desorption was also detected. We incubated the ENP-Gem-DNA or LENP-Gem-DNA in the serum, and the released DNA in the supernatant was examined by electrophoretic mobility at different time points, respectively. According to the results in Fig. 4C, the replacement of nucleic acids absorbed on the surface of ENPs by other negative charged substances present in the serum was suppressed by the lipid bilayer. Disassembly of DNA on ENPs gradually increased over time, while little DNA were released from LENP-Gem-DNA to the supernatant (Fig. 4C). Considering the free siRNA will be cleared up quickly in vivo, the LPNPs display great potential as siRNA delivery platform.

We also confirmed the successful encapsulation of Gem (absorption peak at 270 nm, Fig. S2A) in LENPs by HPLC spectra.
As shown in Fig. S2C, the highest encapsulation efficiency was about 42%. The release profiles of Gem encapsulated ENPs (ENP-Gem) and Gem encapsulated LENPs (LENP-Gem) in vitro were obtained at pH 7.4 and 4.4 in room temperature (Fig. S2D). Rapid Gem release of ENP-Gem was observed at pH 7.4 and 4.4. Nevertheless, LENP-Gem lost only a small proportion (less than 10%) of their cargoes over 4 days at both pH 7.4 and 4.4. The lipid bilayer shell can prevent drug leakage from the co-polymer core as a diffusion barrier by preventing drug molecules diffusing out freely and reducing the water penetration rate into the polymeric core. Those will reduce the degradation rate of the particles and afford the particles a prolonged circulation time in vivo. The slow kinetics of drug release feature can significantly reduce the unfavorable drug leakage in the blood circulation. When the drug-loaded-LENPs accumulated in tumor sites through enhanced penetration effect, the continuous release in the tumor tissue will dramatically enhance the antitumor efficacy.

3.4. Cellular uptake of nanoparticles and siRNA escape from lysosome

Two prerequisites for efficient siRNA-mediated gene silencing effect are high siRNA uptake levels and successful release of siRNA to cytoplasm. Free siRNA cannot be taken up by cells, because their high molecular weight and hydrophobicity not to diffuse across cell membranes. FAM-siRNA (green fluorescence) and RhoB (red fluorescence, instead of Gem) were used to study cellular uptake of LENP-RhoB-FAM-siRNA. When human pancreatic cancer panc-1
Fig. 5. Cellular uptake, intracellular distribution and gene silencing of siRNA of ENPs and LENPs. (A) Cellular uptake of LENPs. Confocal microscopic images of panc-1 cells treated with LENP-RhoB-FAM-siRNA at different time points. Cell nucleuses (blue) were stained by DAPI. (B) Intracellular distribution of LENPs. Confocal microscopic images of panc-1 cells treated with LENP-FAM-siRNA for different time intervals. The lysosomes were stained with LysoTracker Red. (C) Gene silencing ability of LENPs-si-Actin. Cells were treated with LENPs, free si-Actin, ENP-si-Actin and LENP-si-Actin in serum-free media for 6 h. After 48 h, cells actin cytoskeletons were stained with phalloidin-FITC, and cells were observed under a confocal microscopy. (D) Gene silencing ability of LENP-si-HIF1α. Cells were treated with free si-HIF1α, ENP-si-HIF1α, LENP-si-ctrl and LENP-si-HIF1α in serum-free media for 6 h. After 48 h, the mRNA levels of HIF1α and VEGF were measured by RT-PCR. The expression of HIF1α protein was analyzed by western blot. *p < 0.05 vs. si-HIF1α group; #p < 0.05 vs. ENP-si-HIF1α group. si-ctrl, negative control siRNA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
cells were incubated with LENP-RhoB-FAM-siRNA, obvious green and red fluorescence in the same cells rapidly appeared and this observation indicated that the LENPs facilitate the siRNA internalization while delivering drug into cells (Fig. 5A).

However, siRNA must be released into the cytoplasm to exert their interference function. As illustrated in Fig. 5B, after 0.5 h incubating with LENP-FAM-siRNA, overlay of green (FAM-siRNA) and red (LysoTracker Red stained lysosome) fluorescence demonstrated that the LENP-FAM-siRNA located in lysosomes. After 2 h incubation, most FAM-siRNA were released from lysosomes to cytoplasm, as shown by the separation of green and red fluorescence. As previously mentioned, naked siRNA hardly penetrates across biomembranes due to their high molecular weight, hydrophilic properties and high density of charge. Therefore, we hypothesize that siRNA escapes from the lysosomes in the form of encapsulation in LENPs and then the siRNA is released into the cytoplasm.

3.5. Knockdown efficiency of siRNA loaded LENPs

To evaluate the knockdown efficiency of siRNA loaded LENPs, we first chose actin as target gene in B-16 cells. Actin cytoskeletons were stained with phalloidin-FITC (Fig. 5C). Exposure with empty LENPs and free si-Actin did not reduce the amount of cytoskeletons. However, treatment with ENP-si-Actin and LENP-si-Actin led to significant decrease of cytoskeletons. Next, the knockdown efficiency of LENP-si-HIF1α was examined in panc-1 cells. The expression of HIF1α mRNA and protein were measured by RT-PCR and western blot as shown in Fig. 5D. Compared with cells in control groups, about 76% and 91% down regulation of HIF1α mRNA expression were observed in ENP-si-HIF1α and LENP-si-HIF1α groups, respectively. Meanwhile, the expression of VEGF (a downstream gene of HIF1), the positive control gene, was decreased by 36% and 70% in ENP-si-HIF1α and LENP-si-HIF1α groups, respectively. According to the mRNA results, the expression level of HIF1α protein treated with LENP-si-HIF1α was significantly lower than ENP-si-HIF1α groups.

3.6. Cytotoxicity of different drug formulations in vitro

Since LENPs can co-deliver Gem and si-HIF1α into panc-1 cells and significantly silence HIF1α gene in vitro, next we investigated synergistic cytotoxicity of Gem and si-HIF1α encapsulated LENPs. Panc-1 cells were treated with different drug formulations for 48 h followed by quantification of cell viability (Fig. 6). Free Gem-si-HIF1α and free Gem had similar inhibitory rates of tumor cell growth, because free si-HIF1α could not be taken up by cells. The advantages of LENP-Gem and ENP-Gem-si-HIF1α (Gem and si-HIF1α encapsulated ENPs) were not obvious compared with free Gem. However, the LENP-Gem-si-HIF1α (Gem and si-HIF1α encapsulated LENPs) group showed marked improvement in inhibition of tumor cells relative to other treatment groups.

3.7. Prolonged circulation time in vivo

In vivo stability of nanoparticles is an important parameter for their further development. BALB/c mice were injected intravenously with free Cy3-siRNA, ENP-Cy3-siRNA or LENP-Cy3-siRNA, respectively. Blood was collected from tail veins for fluorescent imaging at different time points (Fig. 7A and Fig. S3). The fluorescence intensities were measured. The fluorescence intensity was normalized in each group to 0 h intensities considered as 100%. Free Cy3-siRNA had a half lifetime less than 1 h. ENP-Cy3-siRNA was somewhat better with a half lifetime of about 2 h. However the LENP-Cy3-siRNA exhibited a half lifetime more than 3 h. As mentioned previously, the PEcylated lipid bilayer shell protects siRNA from serum nucleases and immune recognition, and decreases the replacement of siRNA by other negative charged substance in the serum. All these qualities contributed to long circulation time of LENP-Cy3-siRNA in vivo.

3.8. In vivo tumor targeting

To demonstrate the targeting ability in vivo, panc-1 cells were grown as subcutaneous xenografts in BALB/c nude mice. Mice were divided into 4 groups and received tail vein injections: saline, Free RhoB, ENP-RhoB and LENP-RhoB. After 24 h, mice were sacrificed and liver, heart, lung, spleen, kidney and tumor were collected for ex vivo imaging (Fig. 7B). No obvious fluorescence signals in organs and tumor of free RhoB group were detected. The fluorescence signals were clearly visible in organs and tumors of the mice injected with ENP-RhoB, but the tumor targeting ability was not significant. In LENP-RhoB group, the fluorescence signals were much stronger, and most signals concentrated at tumor tissues, exhibiting excellent tumor targeting ability. Although size of ENPs was similar with LENPs in solution, ENPs had larger size and broader size distribution in serum because of aggregation and adsorption with other proteins induced by its positive charges. However, due to their appropriate size and long lifetime in the bloodstream, LENPs can accumulate in tumor tissues probably because of the enhanced tumor vasculature access.

3.9. Inhibition of tumor growth in subcutaneous and orthotopic tumor models

Next the inhibition of tumor growth in vivo was investigated. We took BALB/c nude mice with panc-1 subcutaneous xenografts and randomly divided them into eight groups: saline, LENPs, free Gem, free Gem-si-HIF1α, LENP-Gem, LENP-si-HIF1α, LENP-Gem-si-HIF1α and ENP-Gem-si-HIF1α. When the tumor volume reached a predetermined size (21~23 mm³, approximately 20 days after the tumor cell inoculation), we began the treatments according to the schedule shown in Fig. 7C. Tumors in the saline group grew rapidly, and empty LENPs had no effect on tumor growth. The growth of tumors in LENP-Gem-si-HIF1α group was significantly slower than LENP-Gem and LENP-si-HIF1α groups, demonstrating the synergistic antitumor effects of the Gem and si-HIF1α. Especially,
because of better stability and tumor uptake, LENP-Gem-si-HIF1α exhibited significant advantage in its antitumor effects, compared with ENP-Gem-si-HIF1α. After sacrificing the mice, tumors were collected and weighed. Consistently with the growth curves, the mean weight of the tumors in the LENP-Gem-si-HIF1α group was the lowest among all groups (Fig. 7D and Fig. S4).

To demonstrate the role of HIF1α knockdown in the interference of tumor growth, we sectioned tumor xenografts and analyzed the levels of HIF1α protein. As shown in Fig. 7E, relative to strong expressions of HIF1α in saline group, the expressions of HIF1α were dramatically suppressed by LENP-si-HIF1α and LENP-Gem-si-HIF1α treatments. However, the free si-HIF1α did not decrease the expression of HIF1α in tumor tissues. In other groups, the expressions of HIF1α were similar with that in saline group. These results show that siRNA can effectively diminish the HIF1α gene expression in vivo only when it is delivered effectively by LENPs.
To further explore the antitumor effects of LENP-Gem-si-HIF1α in vivo, we injected luciferase expressing panc-1 cells (panc-1-luc) into the tail of pancreas to simulate clinical anatomy and physiology of pancreatic tumors. About 20 days after injection, bioluminescent imaging of the mice was performed. Mice were divided into 4 groups: saline, free Gem-si-HIF1α, ENP-Gem-si-HIF1α and LENP-Gem-si-HIF1α. Various drug formulations were injected through tail vein at day 1, 4 and 7. Before sacrifice at day 10, bioluminescent imaging of the mice was performed again to depict the extent of tumor burden (Fig. 8A). Major organs were removed as a whole to restore situation in the abdominal cavity (Fig. 8B). Compared to saline group, free Gem-si-HIF1α inhibited tumor growth to a limited extent. Tumors in ENP-Gem-si-HIF1α group were smaller than those in the free Gem-si-HIF1α group. More importantly, the enhancement of antitumor effects in LENP-Gem-si-HIF1α group was more significant than ENP-Gem-si-HIF1α. Tumor tissues were then collected and weighed (Fig. 8C and D). The mean tumor mass of LENP-Gem-si-HIF1α group was the lowest among all groups. In addition, there were many liver metastasis and peritoneal metastasis in saline and free Gem-si-HIF1α groups (Fig. 8E). ENP-Gem-si-HIF1α dramatically reduced the liver metastasis. No metastasis was detected in the animals injected with LENP-Gem-si-HIF1α. These data in orthotopic pancreatic cancer models demonstrate that LENPs possess the potential as nanoparticles of siRNA and chemotherapy drugs for future clinical application.

3.10. Reduced immune response in vivo

Innate immune activation by siRNA represents a significant undesirable side effect in vivo. Some delivery systems aim to protect siRNA from immune recognition by encapsulating it inside nanoparticles. After tail vein injection with saline, free siRNA, ENP-siRNA and LENP-siRNA, we examined two acute immune response-related factors (IFN-α and IL-6) by ELISA. As shown in Fig. 9, free siRNA activated the innate immune system to induce high levels of IFN-α and IL-6 release. Although ENP-siRNA reduced the activation,

![Fig. 8. The antitumor effects in orthotopic tumor model. Luciferase-expressed panc-1 cells (panc-1-luc) were implanted into the tail of pancreas of BALB/c nude mice. After 2 weeks, mice were imaged for luciferase activity and divided into 4 groups: saline, free Gem-si-HIF1α, ENP-Gem-si-HIF1α and LENP-Gem-si-HIF1α. Mice were injected with various treatments through tail vein at day 1, 4, 7, and imaged before sacrificing at day 10. (A) Whole body bioluminescent images of mice at 1 and 10 day of treatment. (B) Images of tumors in situ. (C) Tumor image ex vivo. (D) Tumor weights measurement. (E) Liver and intraperitoneal metastasis. *p < 0.05 vs. saline group; #p < 0.05 vs. free Gem-si-HIF1α group; &p < 0.05 vs. ENP-Gem-si-HIF1α group.](#)
LENP-siRNA decreased the IFN-α and IL-6 close to normal levels, with a more profound effect.

4. Conclusion

In summary, in the current study, biocompatible lipid-polymer hybrid nanoparticles encapsulating si-HIF1α and Gem were fabricated for pancreatic cancer treatment and investigated for their synergistic antitumor effects in subcutaneous and orthotopic tumor models. All materials used in the current study were approved by FDA for clinical usage. LENPs are composed with a cationic EPL-modified co-polymer core, ENPs, and a PEGylated lipid bilayer shell. ENPs effectively absorb negatively charged si-HIF1α in addition to high loading contents of Gem. The lipid bilayer shell significantly decrease the Gem leakage and the replacement of si-HIF1α by other negatively charge substances in the serum. LENPs showed better stability and longer circulating time in the bloodstream than ENPs. These characteristics endow LENPs improved drug targeting properties via the enhanced tumor vasculature access effects in tumor tissues. LENPs can co-deliver Gem and si-HIF1α into panc-1 cells and effectively suppress the HIF1α expression. Consequently, LENP-Gem-siHIF1α exhibited significantly better synergistic anti-tumor effects in vivo than ENP-Gem-siHIF1α. More importantly, LENP-Gem-siHIF1α also showed excellent capability to inhibit tumor metastasis in an orthotopic tumor model. Furthermore, innate immune activation by siRNA was suppressed effectively by the LENPs encapsulation. The LENPs can be used for a wide spectrum of tumors to co-deliver siRNA and chemotherapy drugs (hydrophobic or hydrophilic or both). The current work lays the foundation for a combination therapy strategy with siRNA and chemotherapy drugs using lipid-polymer hybrid nanocarriers.

Acknowledgments

This work was supported by MoST 973 (2012CB934004); National Distinguished Young Scientists program (31825010); National Natural Science Foundation of China (81302082, 81272685, 31301151, 31470957, 31471340, 81402264, 81401957 and 81172355); Key Program of Natural Science Foundation of Tianjin (11JCZDJC18400, 11YCYBYC37400); Major Anticancer Technologies R&D Program of Tianjin (12ZCDYSY16700).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2014.12.028.