

Hyaluronic Acid Capped, Irinotecan and Gene Co-Loaded Lipid-Polymer Hybrid Nanocarrier-Based Combination Therapy Platform for Colorectal Cancer

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Background: The current approach for treating colorectal cancer favors the use of drug and gene combination therapy, and targeted nano-systems are gaining considerable attention for minimizing toxicity and improving the efficacy of anticancer treatment. The aim of this study was to develop ligand-modified, irinotecan and gene co-loaded lipid-polymer hybrid nano-carriers for targeted colorectal cancer combination therapy.

Methods: Hyaluronic acid modified, irinotecan and gene co-loaded LPNs (HA-I/D-LPNs) were prepared using a solvent-evaporation method. Their average size, zeta potential, drug and gene loading capacity were characterized. The in vitro and in vivo gene transfection and anti-tumor ability of this nano-system were evaluated on colorectal cancer cells and mice bearing colorectal cancer model.

Results: HA-I/D-LPNs had a size of 182.3 ± 5.1 , over 80% drug encapsulation efficiency and over 90% of gene loading capacity. The peak plasma concentration (C_{max}) and half-life ($T_{1/2}$) achieved from HA-I/D-LPNs were $41.31 \pm 1.58 \mu\text{g/mL}$ and $12.56 \pm 0.67 \text{ h}$. HA-I/D-LPNs achieved the highest tumor growth inhibition efficacy and the most prominent transfection efficiency in vivo.

Conclusion: HA-I/D-LPNs exhibited the most remarkable tumor inhibition efficacy and best gene transfection efficiency in the tumor, which could prove the effects of the drug and gene combination therapy.

Keywords: colorectal cancer, combination therapy, lipid-polymer hybrid nanoparticles, hyaluronic acid, irinotecan

Introduction

Colorectal cancer (CRC) is the world's fourth most deadly cancer which causes the death of 700,000 people every year.¹⁻³ The current approaches to treating CRC favor the use of combination cytotoxic therapy. First-line treatments include the doublet cytotoxic combinations of fluorouracil, leucovorin, irinotecan, leucovorin, and oxaliplatin.^{4,5} Irinotecan, an extract from the Chinese tree *Camptotheca acuminata*, was first approved in the United States in 1996 for the treatment of metastatic CRC refractory to 5-fluorouracil (5-FU).⁶ Clinical trials have shown that irinotecan has a survival advantage in patients with metastatic colorectal cancer, making irinotecan one of the most important drugs in the management of metastatic CRC.⁷ Unfortunately, chemotherapy using irinotecan may be limited by

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multidrug resistance, inability to have a selective distribution, or other adverse effects.⁸

The application of nano-systems offers new prospects for the effective therapy of CRC.⁹ Targeted nano-systems could utilize the surface-modified ligands, which could interact with the expressed molecules on the surface of tumor cells, enabling the effective delivery of antitumor agents.¹⁰ One of the targeted ligands applied is hyaluronic acid (HA), a natural anionic polysaccharide. The high binding affinity of HA to the CD44 receptors overexpressed on the tumor cells surfaces made it a promising moiety for anticancer drug delivery.¹¹ It was reported that the prominent expression of CD44 has been considered as a marker of highly tumorigenic CRC cells and a component of the colorectal cancer stem cell gene signature that predicts disease recurrence in CRC patients. Therefore, CD44 is a potential therapeutic target for the treatment of CRC.¹²

Co-delivery of plasmid DNA and anti-cancer drugs using a single nano-system has emerged as a strategy to combine the advantages of gene therapy and chemotherapy.¹³ Currently used nano-systems for the co-delivery of genes and anticancer drugs include liposomes,¹⁴ lipid nanoparticles,¹⁵ polymeric nanoparticles,¹⁶ inorganic nanoparticles,¹⁷ and so on. Polymeric nanoparticles provide significant stability, high drug loading ability, controlled drug release, and excellent biocompatibility, thus was widely applied as gene and drug delivery system.^{18–20} In our previous study, colorectal cancer was treated with 5-Fluorouracil (5-FU) and enhanced GFP (EGFP) co-delivered polymeric nanoparticles and achieved effective combination results.²¹ Lipid nanoparticles/liposomes are biocompatible and can be used for the specific delivery of gene/drug to tumor tissues and also render them long circulatory lifetime.²² In the research carried out by Han et al, plasmid DNA and doxorubicin were co-delivered by solid lipid nanoparticles for lung cancer therapy.¹⁵

Lipid-polymer hybrid nanoparticles (LPNs) are core-shell nanoparticle structures comprising polymer cores and lipid shells, which combined the advantages of both polymeric nanoparticles and lipid nanoparticles/liposomes, particularly in terms of their physical stability and biocompatibility.²³ In the present study, we would like to construct an HA modified, irinotecan and gene co-loaded LPNs for targeted colorectal cancer combination therapy. The *in vitro* and *in vivo* gene transfection and anti-tumor ability of this nano-system were evaluated.

Materials and Methods

Materials

pEGFP-N1 was obtained from Solarbio Life Sciences (Beijing, China). Egg yolk lecithin (EYL, PC-98T) was purchased from Kewpie Corporation (Tokyo, Japan). Poly (D,L-lactic-co-glycolic) (PLGA, 50:50, MW 20,000) was purchased from Shandong Institute of Medical Instrument (Shandong, China). HA-PEG-DSPE was provided by Xi'an Ruixi Biological Technology Co., Ltd. (Xi'an, China). Dimethyl sulfoxide (DMSO) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). SW480 cells (ATCC® CCL-228™, human Dukes' type B, colorectal adenocarcinoma), and Human Umbilical Vein Endothelial Cells (HUVEC) were purchased from American Type Culture Collection (ATCC, Manassas, VA). BALB/c nude mice (6–8 weeks old) were purchased from Beijing Vital River Experimental Animal Technical Co., Ltd (Beijing, China). All the animal experiments complied with the ARRIVE guidelines and should be carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments and were approved by the Medical Ethics Committee of Hebei University.

Preparation of Hyaluronic Acid Modified, Irinotecan and Gene Co-Loaded LPNs

Hyaluronic acid modified, irinotecan and gene co-loaded LPNs (HA-I/D-LPNs, Figure 1A) were prepared using a solvent-evaporation method.²³ Irinotecan (50 mg) and PLGA (200 mg) was dissolved in acetone (10 mL) (organic phase). Then, the pEGFP (100 mg), HA-PEG-DSPE (100 mg), and EYL (200 mg) were dispersed in water (90 mL) (aqueous phase). The organic phase was added dropwise into the constantly stirring aqueous phase to form an oil-in-water (o/w) emulsion. Then, the organic solvent was removed by stirring for another 4 h. Non-ligand modified, irinotecan and gene co-loaded LPNs (I/D-LPNs) were prepared using PEG-DSPE instead of HA-PEG-DSPE. Blank LPNs (LPNs) were prepared without adding irinotecan and pEGFP. Free irinotecan and gene solution (Free I/D) were prepared by mixing irinotecan (100 mg) with pEGFP (100 mg).

Characterization of the LPNs

Particle Size, Polydispersity, and Zeta Potential

LPNs were diluted by ultrapure water. Surface morphology of HA-I/D-LPNs was recorded by a transmission electron

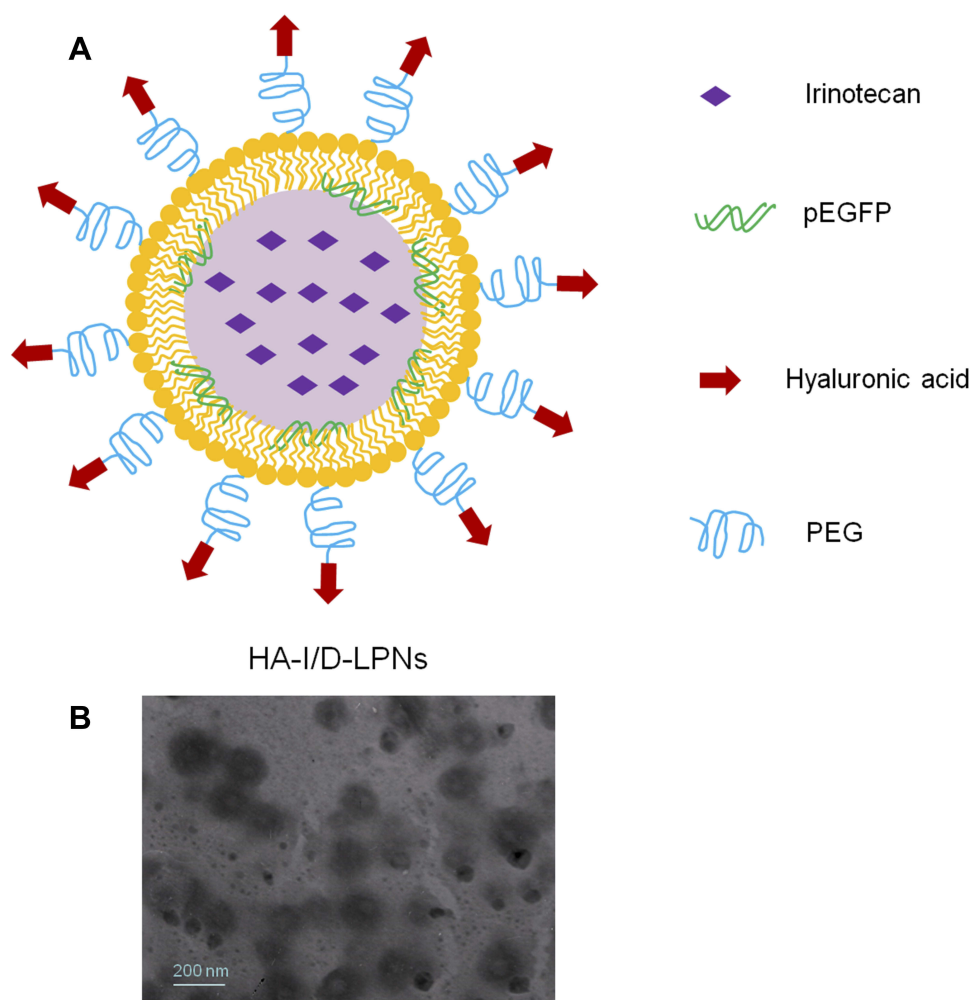


Figure 1 Scheme structure (A) and TEM picture (B) of Hyaluronic acid modified, irinotecan and gene co-loaded LPNs (HA-I/D-LPNs). HA-I/D-LPNs were prepared using a solvent-evaporation method. HA-I/D-LPNs were spherical particles.

microscopy (JEM-1010, JEOL, Japan). The particle size, polydispersity, and zeta potential of the LPNs were determined by dynamic light scattering (DLS) using a NanoZS Zetasizer (Malvern Instruments Ltd., Malvern, UK).²⁴

TEM pictures are Morphologies and size of NI/HA-DDP-PNPs and NI/HA-DDP-LPNs were recorded by a transmission electron microscopy.

Drug Entrapment Efficiency and Gene Loading Capacity

The irinotecan entrapment efficiency (EE) was determined by high-performance liquid chromatography method.²⁵ Irinotecan was separated by a C18 column (200×4.6 mm, 5 μm). The mobile phase consisted of acetonitrile and water (40/60, v/v) with a flow rate of 1.0 mL/min. The fluorescence detection was set at 370 nm. Gene loading capacity (GL) of LPNs was determined by the PicoGreen-fluorometry method.²⁶ pEGFP was isolated from LPNs by centrifugation

(15,000 rpm, 30 min). The concentration of pEGFP was assessed by a fluorescence spectrophotometer at 480 nm.

Serum Stability

LPNs were added into the phosphate-buffered saline (PBS, pH 7.4) containing 10% FBS (v/v) at 37°C under gentle stirring to exam the stability in serum.²⁷ LPNs formulas were incubated with 10% FBS (v/v) solution for 1, 2, 4, 8, 24, 48, or 72 h. At predetermined time points, 1 mL of each sample was taken out and the particle size and polydispersity were measured.

In vitro Drug and Gene Release

In vitro release of irinotecan and pEGFP from LPNs were performed in PBS (pH 7.4) by suspending samples in different Eppendorf® tubes and vortexed.²⁸ The tubes were placed in a shaking water bath (100 rpm, 37°C). At predetermined time points, the LPNs suspensions were

centrifuged (15,000 rpm, 30 min) and the amount of irinotecan and pEGFP released was analyzed by methods assay mentioned in “Drug entrapment efficiency and gene loading capacity” section.

Cell Culture

All cell lines were cultured in DMEM containing 5% fetal bovine serum and incubated at 37°C with 5% CO₂ and 90–100% relative humidity. When the cells had reached 80–90% fusion, they were sub-cultured.

Cellular Uptake

Cellular uptake characteristics of LPNs were analyzed by flow cytometry.²⁹ HA-I/D-LPNs and I/D-LPNs (200 mg/mL) were added at concentrations of into SW480 cells equilibrated with Hank's buffered salt solution (HBSS) at 37°C. After incubation for 24 h, the cells were washed once with 1 mL of PBS; detached with trypsin/EDTA, centrifuged at 1500 rpm, 4°C for 5 min; resuspended in 300 µL of PBS and directly introduced to a flow cytometer.

In vitro Cytotoxicity

In vitro cytotoxicity of LPNs was estimated by MTT assay.²⁹ Briefly, SW480 cells and HUVEC were seeded in 96-well plates (2,000 cells/well) and incubated for 24 h. Drug and gene loaded LPNs and Free I/D at various concentrations were added and incubated for 72 h. MTT (5 mg/mL) was then added and incubated for another 4 h. The formazan crystals were dissolved in DMSO (100 µL) and the absorbance of the formed dye was measured at 570 nm using a microplate reader. Cell viability (%) was calculated according to the equation: (Absorbance of test cells)/(Absorbance of control) × 100.

In vivo Pharmacokinetics and Anticancer Activity

Colorectal cancer-bearing mice were produced by injecting SW480 cells (10⁶ in 100 µL 0.9% saline) to the dorsal side of the mice. When tumors reached 4–5 mm in diameter, mice were randomly divided into three groups. HA-I/D-LPNs (10 mg irinotecan per kg mice), I/D-LPNs

(10 mg irinotecan per kg mice), and Free I/D (10 mg irinotecan per kg mice) were administered to the mice via the tail vein.³⁰ Blood samples which were taken from the retro-orbital plexus at predetermined time points, centrifuged (4,000 rpm, 15 min) and plasma was collected and stored at –20°C until further analyzed by methods assay mentioned in “Drug entrapment efficiency and gene loading capacity” section.

For the anticancer activity evaluation, colorectal cancer-bearing mice were randomly divided into five groups. HA-I/D-LPNs (5 mg irinotecan, 10 mg pEGFP per kg mice), I/D-LPNs (5 mg irinotecan, 10 mg pEGFP per kg mice), LPNs, Free I/D (10 mg irinotecan, 10 mg pEGFP per kg mice), and 0.9% saline were administered to the mice via the tail vein every 3 days. The tumors were measured every 3 days with calipers during the period of study and were calculated according to the equation: (longest diameter × shortest diameter²)/2. Mice were weighed at the time of treatment and the body weight of mice was monitored as an index of systemic toxicity.

In vivo Transfection Efficiency

Colorectal cancer-bearing mice were administered with the same five samples as mentioned in the anticancer activity evaluation section. The mice were sacrificed at 24 or 72 h after administration and the tumor tissue samples were taken out, homogenized, washed three times with PBS.³¹ The cells were finally obtained after centrifugation (4°C, 1000 rpm, 5 min) and were seeded into 24-well plates in 1 mL of DMEM with 10% FBS. The fluorescent cells were observed using an inversion fluorescence microscope. Then, the cells were quantified using flow cytometry.

Statistical Analysis

Experiments were performed at least three times and expressed as mean ± standard deviation (mean ± SD). The statistical analysis was performed using a post hoc test following ANOVA. * P < 0.05 was considered as statistical significance and ** P < 0.01 as extreme statistical significance.

Table I Characterization of LPNs (Mean ± SD, n=6)

Formulations	Particle Size (nm)	Polydispersity	Zeta Potential (mV)	EE (%)	GL (%)
HA-I/D-LPNs	182.3 ± 5.1	0.17 ± 0.02	–21.3 ± 2.2	81.5 ± 3.5	90.3 ± 2.6
I/D-LPNs	151.2 ± 4.2	0.15 ± 0.02	–33.4 ± 2.9	83.2 ± 2.7	91.5 ± 2.1
LPNs	133.7 ± 3.3	0.12 ± 0.01	–39.1 ± 2.6	/	/

Results and Discussion

Characterization of LPNs

HA modified nanoparticles have been widely used for the delivery of drugs and genes for tumor targeting.^{32–34} They were expected to accumulate in cancer tissues as a combined function of the magnetic targeting-enhanced EPR effect and HA-mediated active targeting after intravenous injection.³⁵

Particle sizes smaller than 200 nm are conducive to drug accumulation at the tumor site based on the EPR effect, thereby reducing the drug dose and minimizing toxicity.³⁶

The size of HA-I/D-LPNs was 182.3 ± 5.1 (Table 1), which was smaller than 200 nm and larger than that of I/D-LPNs and LPNs. Zeta potential of nanoparticles higher than 20 mV was reported to make the nanoparticles repel each other,

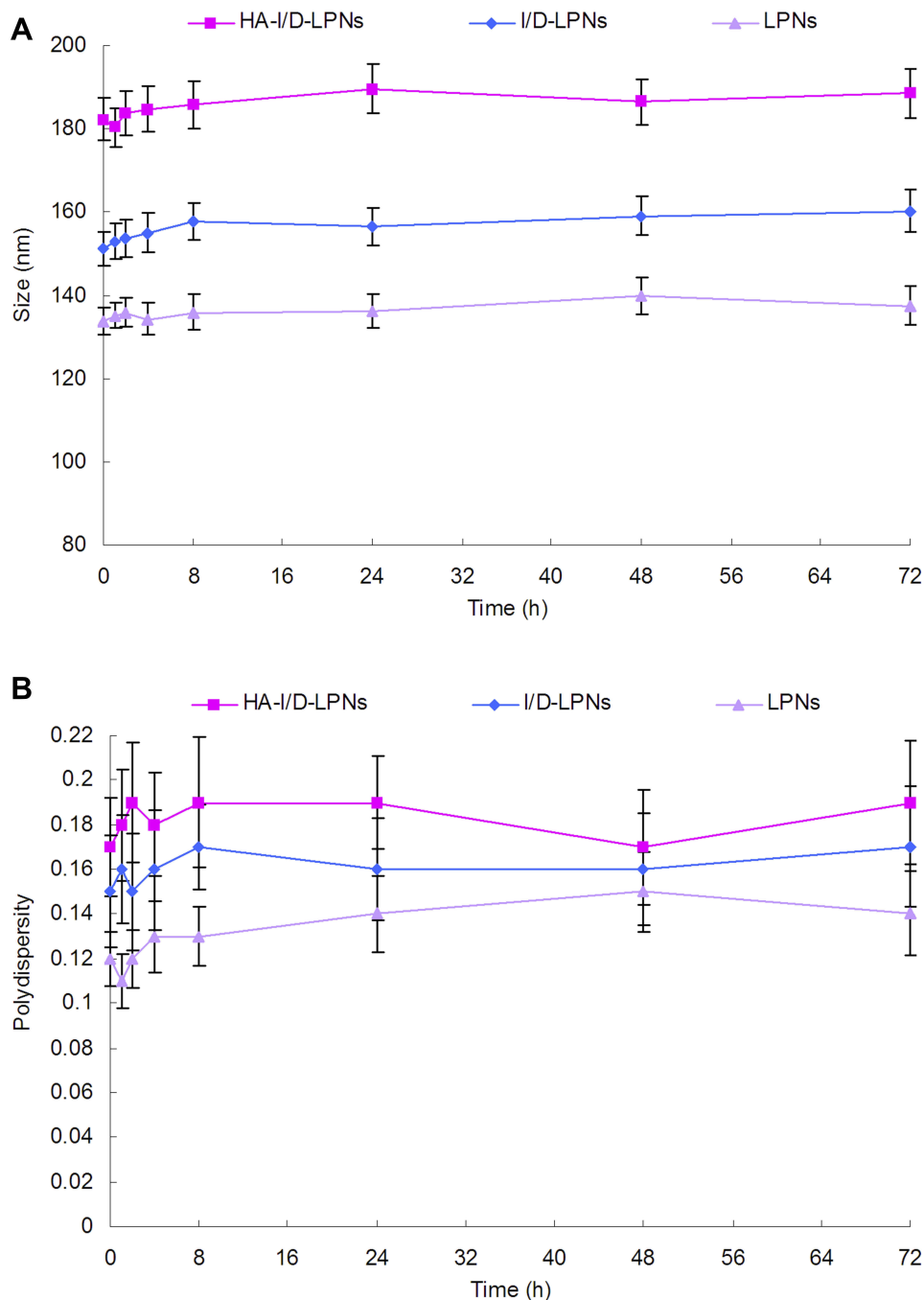


Figure 2 Serum stability of LPNs evaluated by the changes in the particle size (**A**) and polydispersity (**B**) after mixing with serum media. Data are presented as mean $\pm$ SD, $n=6$. The adsorption of proteins on the nanoparticles could cause aggregation, thus leading to increase in particle size. The size and polydispersity showed no obvious change during the tested time, indicating the stability of the LPNs in serum.

thereby avoiding particle aggregation and keeping the long-term stability of nanoparticles.³⁷ The zeta potentials of LPNs were between -21.3 ± 2.2 and -39.1 ± 2.6 mV, which was high enough to keep a stable system. Good drug encapsulation efficacy and gene loading capacity are required for the construction of a successful nanoparticle system.³⁸ Over 90% of GL and EE above 80% proved good loading ability of LPNs. HA-I/D-LPNs were spherical particles (Figure 1B). Serum stability was tested to simulate the in vivo hemocompatibility of LPNs.³⁹ The adsorption of proteins on the nanoparticles could cause aggregation, thus leading to increase in particle size. The size and polydispersity showed no obvious change during the tested time, indicating the stability of the LPNs in serum (Figure 2).

In vitro Drug and Gene Release

PEG is the most important prolonged circulation modification moiety, which provides a very attractive combination

of properties such as excellent solubility in aqueous solutions, high flexibility of its polymer chain, very low toxicity, immunogenicity, and PEG can be used as a linker for covalent attachment of active targeting moieties.⁴⁰ In this study, HA was conjugated to the end of the PEG chain and used for the modification of LPNs. Figure 3 revealed that HA modified LPNs showed slower drug/gene release than that of unmodified LPNs. We also found that the DNA release was faster than drug release in the same kind of LPNs, this may be explained by the loading of gene was at the outer layer of the LPNs.²¹

In vitro Cellular Uptake and Cytotoxicity

Figure 4A illustrated that the cellular uptake of HA-I/D-LPNs was higher than I/D-LPNs ($P < 0.05$), which may be the evidence that HA-I/D-LPNs have CD44-targeting effect.⁴¹ In vitro cytotoxicity results showed that HA-I/D-LPNs exhibited remarkable better cell inhibition

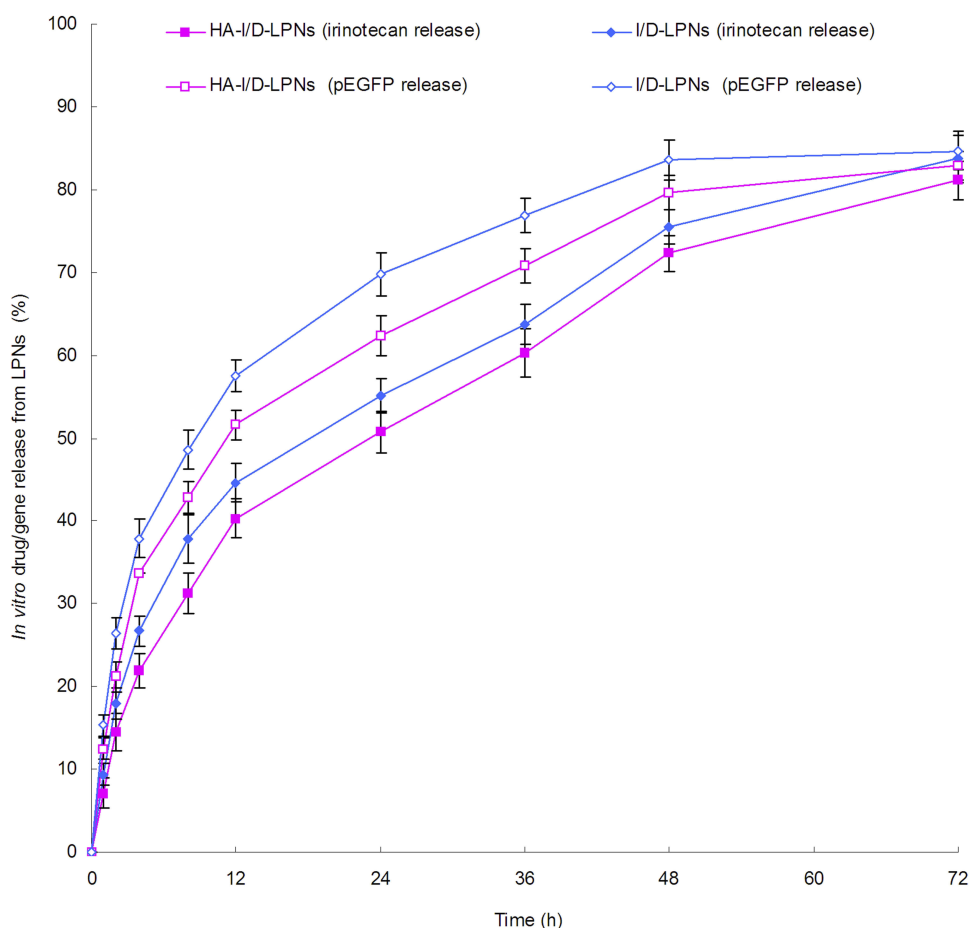


Figure 3 Cumulative release of irinotecan and pEGFP from HA-I/D-LPNs and I/D-LPNs. Data are presented as mean $\pm$ SD, $n=6$. HA modified LPNs showed slower drug/gene release than that of unmodified LPNs. Faster DNA release was found than drug release in the same kind of LPNs, which may be explained by the loading of gene was at the outer layer of the LPNs.

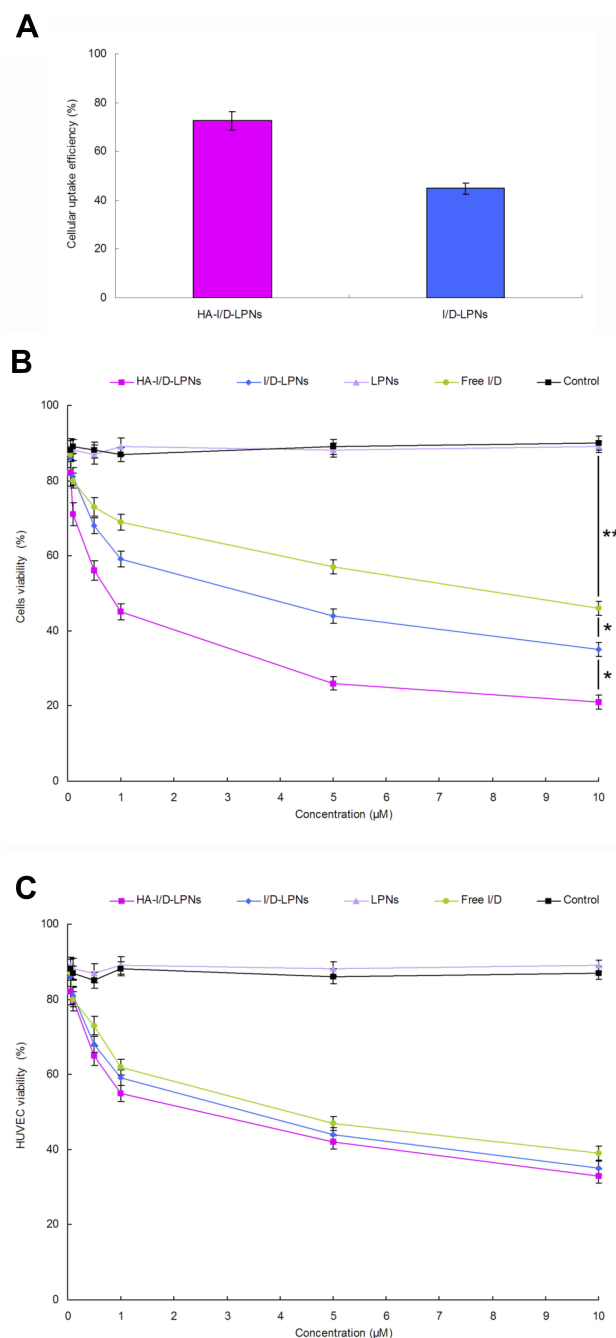


Figure 4 In vitro cellular uptake efficiency of HA-I/D-LPNs and I/D-LPNs (A). In vitro cytotoxicity of LPNs evaluated on SW480 cells (B) and HUVEC (C) measured by MTT assay. Data are presented as mean $\pm$ SD, n=6. *P < 0.05; **P < 0.01. The cellular uptake of HA-I/D-LPNs was higher than I/D-LPNs, which may be the evidence that HA-I/D-LPNs have CD44-targeting effect. In vitro cytotoxicity results showed that HA-I/D-LPNs exhibited remarkable better cell inhibition efficiency than I/D-LPNs on SW480 cells. However, on HUVEC, cytotoxicity of HA-I/D-LPNs and I/D-LPNs exhibited no significant difference.

efficiency than I/D-LPNs on SW480 cells (Figure 4B, P < 0.05). However, on HUVEC, cytotoxicity of HA-I/D-LPNs and I/D-LPNs exhibited no significant difference (Figure 4C). This may be due to the HA in the surface of LPNs that could bind to CD44 receptors overexpressed

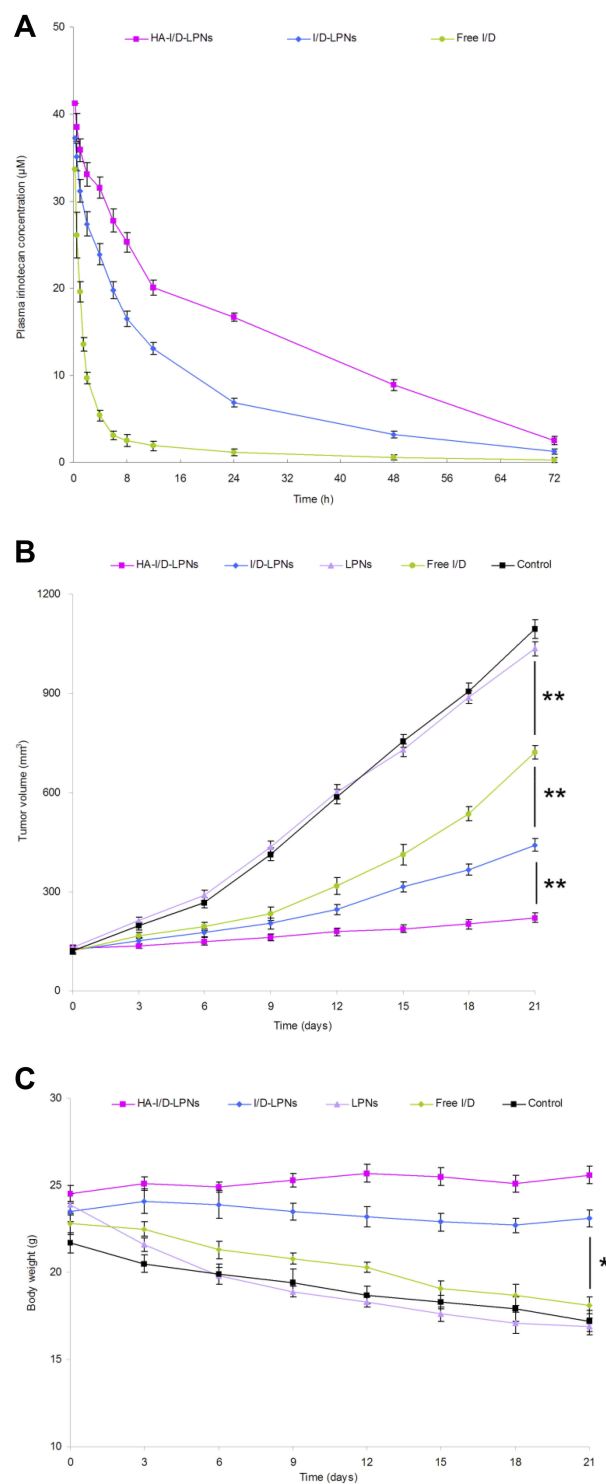


Figure 5 In vivo plasma drug concentration profiles (A), in vivo antitumor efficiency evaluated by tumor volume (B) and body weight (C). Data are presented as mean $\pm$ SD, n=8. *P < 0.05; **P < 0.01. The peak plasma concentration (C_{max}) achieved from HA-I/D-LPNs ($41.31 \pm 1.58 \mu$ g/mL) was significantly higher than that from free I/D ($33.72 \pm 1.85 \mu$ g/mL, P < 0.05). The half-life ($T_{1/2}$) of irinotecan in HA-I/D-LPNs, I/D-LPNs, and free I/D was 12.56 ± 0.67 , 8.78 ± 0.49 , and 6.35 ± 0.32 h. Area Under Curve (AUC) of HA-I/D-LPNs was 1.8-fold greater than that of I/D-LPNs and 7.9-fold longer than free I/D. It was observed that the tumor volumes of HA-I/D-LPNs group were smaller than those of I/D-LPNs group. The mice treated with HA-I/D-LPNs and I/D-LPNs depicted no obvious body weight changes from the day of the administration of different formulations to the end of the experiment.

Table 2 Plasma Pharmacokinetic Parameters (Mean $\pm$ SD, n=8)

Parameters	HA-I/D-LPNs	I/D-LPNs	Free I/D
C_{max} (μ g/mL)	41.31 $\pm$ 1.58*	37.34 $\pm$ 2.07	33.72 $\pm$ 1.85
$T_{1/2}$ (h)	12.56 $\pm$ 0.67**	8.78 $\pm$ 0.49*	6.35 $\pm$ 0.32
AUC _{0-∞} (mg/L·h)	1001.04 $\pm$ 49.56**	545.28 $\pm$ 13.75*	126.23 $\pm$ 5.41
MRT (h)	12.67 $\pm$ 0.52**	8.59 $\pm$ 0.61*	6.47 $\pm$ 0.43

Notes: *P < 0.05 compared with Free I/D, **P < 0.01 compared with Free I/D.

on the tumor cell surface thus bring about higher cytotoxicity.⁴² Higher cell inhibition ability observed by I/D-LPNs than free I/D (P < 0.05) indicated that the LPNs might have the enhanced ability to adhere to the cell membrane due to the similar nature of the lipids and the cell membrane.⁴³ Blank LPNs did not show any cytotoxicity compared with control, indicating the safety and biocompatibility of the materials used for the preparation of the LPNs.⁴⁴ The biocompatibility of LPNs is consistent with previous findings on LPNs and proved that this nano-system can be safely used as a drug/gene delivery vehicle.⁴⁵

In vivo Pharmacokinetics and Anticancer Activity

The plasma drug concentration versus time profiles and the pharmacokinetic parameters were summarized (Figure 5A, Table 2). The peak plasma concentration (C_{max}) achieved from HA-I/D-LPNs ($41.31 \pm 1.58 \mu\text{g/mL}$) was significantly higher than that from free I/D ($33.72 \pm 1.85 \mu\text{g/mL}$, P < 0.05). The half-life ($T_{1/2}$) of irinotecan in HA-I/D-LPNs, I/D-LPNs, and free I/D was 12.56 ± 0.67 , 8.78 ± 0.49 , and 6.35 ± 0.32 h. Area Under Curve (AUC) of HA-I/D-LPNs was 1.8-fold greater than that of I/D-LPNs and 7.9-fold longer than free I/D. It was reported that nanoparticles can be excreted by the kidney or stealthy enough to evade the macrophage phagocytic system (MPS), formerly the reticuloendothelial system (RES).⁴⁶ It was found that the structure of ligand-modified nanoparticles was propitious to reduce the capture of MPS, and benefit to selectively accumulate at the tumor site after intravenous injection via active tumor targeting cooperated with the

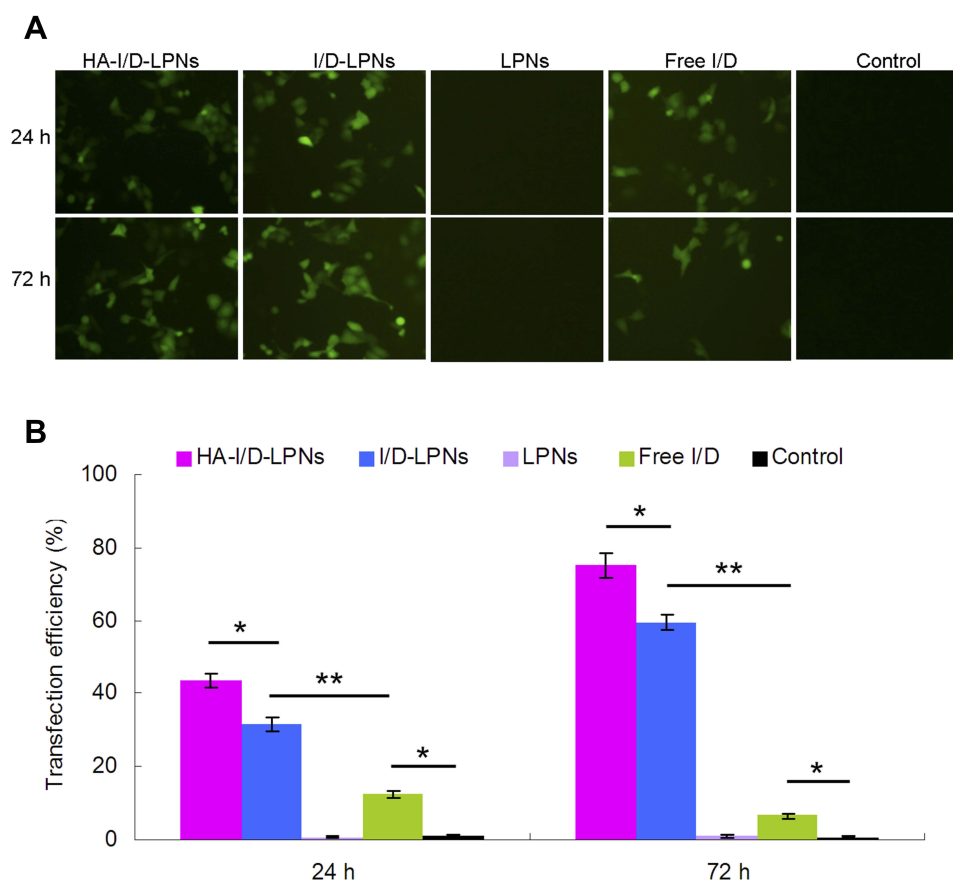


Figure 6 In vivo gene transfection efficiency of LPNs evaluated by fluorescent images (A) and flow cytometry (B). Data are presented as mean $\pm$ SD, n=8. *P < 0.05; **P < 0.01. For gene loaded LPNs groups, better transfection efficiencies were achieved at 72 h than 24 h. This could be explained by the sustained release of the LPNs. On the contrary, free I/D showed weaker fluorescence at 72 h than 24 h.

enhanced permeability and retention (EPR) effect.⁴⁷ The in vivo antitumor effects of LPNs were assessed using colorectal cancer-bearing mice. It was observed that the tumor volumes of HA-I/D-LPNs group were smaller than those of I/D-LPNs group (Figure 5B, $P < 0.01$). This may be explained by the HA CD44-receptor-mediated tumor targeting, indicating that HA-I/D-LPNs can inhibit the tumor growth most significantly, which is consistent with the results of the cytotoxicity assay. No antitumor effect was observed in the control group and blank LPNs group, while free I/D showed effectively inhibit tumor growth than the control ($P < 0.01$). I/D-LPNs illustrated better antitumor ability than that of free I/D ($P < 0.01$) even at a lower dose (10 mg/kg irinotecan for free I/D and 5 mg/kg irinotecan for I/D-LPNs), indicating the remarkable efficiency of the LPNs. The mice treated with HA-I/D-LPNs and I/D-LPNs depicted no obvious body weight changes from the day of the administration of different formulations to the end of the experiment (Figure 5C). However, free I/D caused reduction of body weight along with time. This weight loss induced by free I/D was higher than that of LPNs groups.

In vivo Transfection Efficiency

HA-I/D-LPNs showed the most prominent fluorescence during the in vivo transfection experiment (Figure 6A). For gene loaded LPNs groups, better transfection efficiencies were achieved at 72 h than 24 h (Figure 6B). This could be explained by the sustained release of the LPNs.⁴⁸ On the contrary, free I/D showed weaker fluorescence at 72 h than 24 h. At 72 h post administration, HA-I/D-LPNs showed $75.3 \pm 3.3\%$ fluorescence positivity, which is higher than I/D-LPNs ($59.4 \pm 2.2\%$, $P < 0.05$) and free I/D ($6.5 \pm 0.6\%$, $P < 0.01$). Co-delivery of drug and DNA into the same tumor site is a key for achieving synergistic effect in the combined drug and gene therapy of cancer.¹⁶ In the present study, HA-I/D-LPNs exhibited the most remarkable tumor inhibition efficacy and best gene transfection efficiency in the tumor, which could prove the effects of the drug and gene combination therapy.

Conclusion

In summary, an HA modified, irinotecan and gene co-loaded LPNs were prepared for targeted colorectal cancer combination therapy. HA-I/D-LPNs achieved the highest tumor growth inhibition efficacy and the most prominent transfection efficiency in vivo, which could prove the drug and gene combination therapy effects of the system.

Disclosure

The authors report no conflict of interest in this paper.

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