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Hyperthermia-triggered drug delivery from iRGD-modified temperature-sensitive liposomes enhances the anti-tumor efficacy using high intensity focused ultrasound

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ABSTRACT

An important limitation to successful cancer treatment with chemotherapeutics is the inability to achieve therapeutically effective drug concentrations while avoiding healthy tissue damage. In this work, a new tumor-targeting peptide iRGD (CCRGDKGPDC) was used to modify drug-loaded low temperature-sensitive liposomes (iRGD-LTSL-DOX) to explore the anti-tumor effects in combination with high intensity focused ultrasound (HIFU) *in vitro* and *in vivo*. iRGD-LTSL-DOX can specifically target to αvβ3-positive cells and locally release the encapsulated doxorubicin (DOX) in a hyperthermia-triggered manner. *In vivo* results showed that DOX from iRGD-LTSL-DOX was intravascularly released and rapidly penetrated into tumor interstitial space after HIFU-triggered heat treatment, thereby overcoming the limited tumor penetration of anticancer drugs. Significantly stronger anti-tumor efficacy further supported the effective combination of iRGD-LTSL-DOX with HIFU-induced hyperthermia. Our study provided a novel tumor-targeting LTSL-DOX and demonstrated its usefulness in HIFU-induced hyperthermia-triggered drug delivery.

1. Introduction

The effectiveness of cancer chemotherapy in solid tumors greatly depends on sufficient delivery of therapeutic agents into tumor cells. Nanoparticles such as liposomes have been developed to increase drug accumulation at the tumor site by taking advantage of the enhanced permeability and retention (EPR) effect due to the leaky tumor vasculature [1–3]. In a manner of passive targeting of tumors, the EPR effect has been exploited for various nanosomal formulations and macromolecular drug conjugates in a plethora of preclinical studies [4–6]. And yet, this approach has its limitations in the clinical practice. Commonly, human tumors grow slower and have less pronounced EPR effect than these aggressive tumors used in preclinical models [7]. Moreover, drug encapsulation in nanocarriers such as PLGA or liposomes may reduce drug uptake in healthy tissue, but simultaneously hampers the bioavailability to tumor cells. This is the case for long circulating cisplatin-loaded liposomes which failed in a phase III clinical trial due to the lack of drug efficacy [8].

An alternative strategy that may overcome the former limitation is to design and fabricate the tumor-targeted nanovehicles. By modifying these nanovehicles with tumor-homing peptides or antibodies, site-directed drug delivery to tumor tissues can be realized, resulting in the increased drug concentrations in the tumor while decreasing off-site toxicity [9,10]. To date, targeted drug delivery systems have attracted more and more attention and attempted with various degrees of success in cancer therapy [11,12]. Recently, Ruoslahti et al. identified a novel tumor-homing peptide (iRGD, CRGDK/EGPD/EC) which has not only tumor-targeting functions but also highly efficient tumor-penetrating capability [13]. iRGD peptide, like conventional RGD peptides, can home to tumors by binding to αv integrins selectively overexpressed on the tumor angiogenic endothelial cells as well as tumor cells (*e.g.*, the 4T1 cell line). Evidences have demonstrated that iRGD can increase drug tissue permeability in a tumor-specific and neuropilin-1 dependent manner even if iRGD was chemically conjugated to nano-drugs [14,15]. All of these findings indicated that iRGD could function as a promising tumor-homing peptide to construct tumor-targeted drug delivery systems.

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Effective and controllable drug release from nano-carriers is another challenge for successful tumor chemotherapy. Due to the different materials properties, including biocompatibility, biodegradability and physiological stability [16–18], the therapeutic advantage of traditional nano-drugs have been limited despite of their high accumulation in the tumor tissue [19,20]. Low temperature-sensitive liposomes (LTSL) were developed as a nanovesicle for temperature-triggered local drug release at the tumor site by local hyperthermia (HT) [21–24]. By using of LTSL, the drug is encapsulated and remains in the aqueous lumen of the LTSL at body temperature, but releases the drug from the TSL at the melting phase transition temperature (Tm) of the lipid bilayer in the range of 40–45 °C [21]. Temperature-triggered drug delivery *via* LTSL has been tested in a plethora of preclinical studies and has reached the clinical trial phase. ThermoDox® (doxorubicin-loaded low temperature-sensitive liposomes, LTSL-DOX) has reached phase III clinical trials for liver cancer and phase II trials in breast cancer recurrence at the chest wall [25]. Recent findings suggest hyperthermia (HT) not only may promote drug release from the LTSL into the tumor vasculature, but also can increase free drug in the interstitial space [22,26].

Local heating has been established with various approaches, including radiofrequency, light, water bath and ultrasound [27]. Of all, high intensity focused ultrasound (HIFU) is a promising technology for the non-invasive, local heating treatment of deep-seated tissue by focusing multiple ultrasound waves. By using of pulsed HIFU in combination with relatively short duty cycles, temporal average ultrasound intensities will be decreased, resulting in the generation of mild hyperthermia or non-lethal temperature elevations in local tumors. Besides of heat generation which may favor drug release from LTSL, the complex bio-effects from ultrasound, such as acoustic cavitation and radiation forces [28], provide pulsed HIFU with extra advantages to improve drug delivery [29,30].

Although the temperature-triggered local drug delivery using drug-loaded LTSL is well-established, many relative factors including the targeting capability of LTSL and heating modality should be really considered as a synergistic system to achieve sufficient drug levels in the tumor, while reducing off-site drug uptake. In this study, we designed the iRGD-modified LTSL-DOX and explored its feasibility in hyperthermia-triggered drug delivery to enhance the anti-tumor efficacy using pulsed HIFU (Scheme 1). Using doxorubicin (DOX) as the model drug, the targeting capability, hyperthermia-triggered drug release efficiencies, and anti-tumor efficacy of the iRGD-LTSL-DOX were systematically evaluated *in vitro* and *in vivo*.

2. Materials and methods

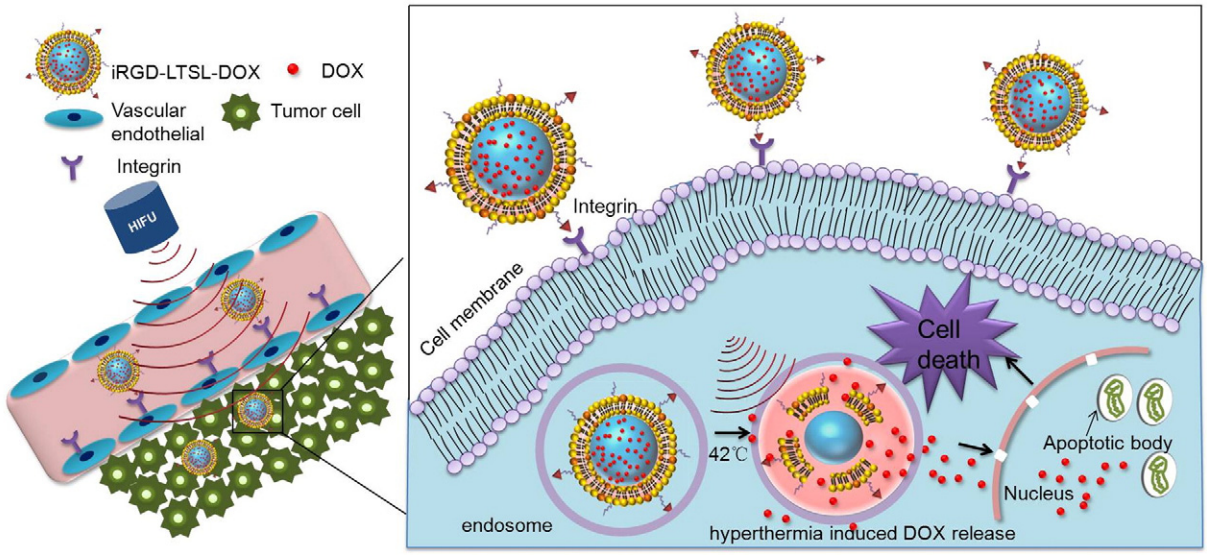
2.1. Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (MPPC), cholesterol, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-maleimide (polyethylene glycol)2000 (DSPE-PEG2000-maleimide), and 1,2 distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-Methoxy polyethyleneglycol-2000 (DSPE-PEG2000) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Doxorubicin hydrochloride (DOX, >98%) and 4 lyethylenino-2-phenylindole (DAPI) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and Endothelial Cell Medium (ECM) were purchased from HyClone Inc. (Logan, UT, USA) and ScienCell (Carlsbad, USA). Fetal bovine serum (FBS) was obtained from GIBCO (Grand Island, NY, USA). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Tokyo, Japan), cyclic iRGD c(CRGDKGPDC)-SH (>90%) and FAM-c(CRGDKGPDC)-SH (>90%) were customized by GL Biochem Ltd. (Shanghai, China). Annexin V-FITC apoptosis kit was purchased from Biovision (Milpitas, California). *In Situ* Cell Death Detection Kit, POD (TUNEL) was purchased from Roche (Indianapolis, USA).

2.2. Preparation and characterization of liposomes

iRGD-DSPE-PEG2000 or FAM iRGD-DSPE-PEG2000 were prepared by coupling FAM-c(CRGDKGPDC)-SH or c(CRGDKGPDC)-SH to DSPE-PEG2000-maleimide at a 1:1 M ratio by making use of a free cysteine sulfhydryl group on the peptide. The reaction was performed in aqueous solution at room temperature for 4 h (Fig. 1A). Finally, the reaction mixture was dialyzed extensively against water to remove all impurities and lyophilized. The formation of iRGD-DSPE-PEG2000 was confirmed by MALDI-TOF Mass Spectrometry. As shown in SP Fig. 1a and b, there was a fragment shift between DSPE-PEG2000-maleimide (≈3000 Da) and synthetic product (≈3975 Da). The experimental molecular weight (MW) of iRGD-DSPE-PEG2000 was determined by MALDI-TOF MS at 3975 Da in accordance with the theoretical MW, confirming that iRGD peptide was successfully conjugated with DSPE-PEG2000-maleimide.

Three different liposomal formulations were prepared by lipid film hydration and remote loading method [31]. DPPC:MPPC:DSPE-PEG2000 at 86:10:4 M ratio for low temperature-sensitive liposomes (LTSL-DOX), DPPC:MPPC:DSPE-PEG2000:DSPE-PEG2000-iRGD at



Scheme 1. Schematic design of DOX-loaded iRGD-LTSL combined with HIFU for targeting and hyperthermia-triggered drug delivery.