Colloids and Surfaces B: Biointerfaces 146 (2016) 607–615

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Thermal and magnetic dual-responsive liposomes with a cell-penetrating peptide-siRNA conjugate for enhanced and targeted cancer therapy

Yanfang Yang[a,b,c,1], Xiangyang Xie[d,1], Xueqing Xu[a,b], Xuejun Xia[a,b], Hongliang Wang[a,b], Lin Li[a,b], Wujun Dong[a,b], Panpan Ma[a,b], Yang Yang[c,*], Yuling Liu[a,b,**], Xingguo Mei[c]

[a] State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China
[b] Beijing Key Laboratory of Drug Delivery Technology and Novel Formulation, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, China
[c] State Key Laboratory of Toxicology and Medical Countermeasure, Department of Pharmaceutics, Beijing Institute of Pharmacology and Toxicology, Beijing 100850, China
[d] Department of Pharmacy, Wuhan General Hospital of Guangzhou Command, Wuhan 430070, China

ARTICLE INFO

Article history:
Received 2 February 2016
Received in revised form 29 June 2016
Accepted 1 July 2016
Available online 2 July 2016

Keywords:
Thermosensitive release
Magnetic guidance
Cell-penetrating peptides
siRNA delivery
Liposomes

ABSTRACT

Due to the absence of effective *in vivo* delivery systems, the employment of small interfering RNA (siRNA) in the clinic has been hindered. Here, we describe a novel siRNA targeting system that combines features of biological (cell-permeable peptides, CPPs) and physical (magnetic) siRNA targeting for use in magnetic hyperthermia-triggered release. A siRNA-CPPs conjugate (siRNA-CPPs) was loaded into thermal and magnetic dual-responsive liposomes (TML) (siRNA-CPPs/TML), and *in vitro* siRNA-CPPs thermosensitive release activity, targeted cellular uptake, gene silencing efficiency, *in vivo* targeted delivery and *in vivo* antitumor activity were determined. The results demonstrated that siRNA-CPPs/TML exhibited good physicochemical properties, effective cellular uptake, endosomal escape and a significant gene silencing efficiency in MCF-7 cells *in vitro*. Additionally, in the *in vivo* study, siRNA-CPPs/TML under an alternating current (AC) magnetic field displayed a superior *in vivo* targeted delivery efficacy, antitumor efficacy and gene silencing efficiency in a MCF-7 xenograft murine model. In conclusion, the application of siRNA-CPPs/TML under an AC magnetic field represents a new strategy for the selective and efficient delivery of siRNA.

1. Introduction

The delivery of small interfering RNA (siRNA) to block an oncogene is a new strategy aimed to increase therapeutic armament. However, the application of siRNA in the clinic have encountered several hurdles, such as rapid degradation by nucleases in the serum, fast elimination, poor cellular uptake, endosomal trapping, inefficient translocation to the cytoplasm, and a lack of targeting ability [1]. To effectively transfer siRNAs to the tumor cells, various nanocarriers have been developed by many groups. Among nanocarriers, activated tumor-targeted cationic liposomes have drawn significant attention for the delivery of siRNA to targeted sites. However, such active targeting is sometimes characterized by limitations, such as a low degree or low heterogeneity of receptor expression among different tumor cells and differences in ligands-receptor affinities and receptor-mediated internalization rates [2,3]. Thus, a delivery platform with increased specificity for tumors is highly desirable.

To attain this goal, a number of novel nanocarriers have been developed that are triggered by endogenous (*i.e.,* enzyme, metal ions and pH) or external stimuli (*i.e.,* magnetic, hyperthermia and light) [4–7]. The most precise control, however, is achieved when external triggers are employed because these can be applied to define localized areas to induce drug release. Over the past few decades, hyperthermia has been administered as an adjuvant therapy to improve therapeutic effects when combined with

* Corresponding author.
** Corresponding author at: State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China.
E-mail address: amms2013@126.com (Y. Yang).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.colsurfb.2016.07.002
0927-7765/

radiation or chemotherapy in the clinic. Therefore, among the various external triggers, thermal triggering is particularly attractive. Thermosensitive liposomes (TSL), whose lipid materials undergo a gel-to-liquid crystalline phase transition upon heating over a mild-hyperthermia range, could be used to develop a liposome-based drug delivery system to immediately release encapsulated payloads in heated tissues or organs [8]. Although TSL could improve the targeting delivery efficiency to some extent, their mono-targeting mechanism makes them inefficient in terms of designing an effective targeted delivery system for both biodistribution and systemic administration. Recently, ThermoDOX® (Celsion Corp) announced that their clinical study did not meet the primary endpoint of the phase III study (www.celsion.com). One of the reasons was that the drug concentration at the target site may not have been high enough to produce the therapeutic effect necessary for clinical effectiveness. To overcome these challenges, establishing a suitable protocol based on a dual-responsive strategy is desirable, which may result in successful targeted cancer therapy. Magnetic nanoparticles are known for their immense potential in the field of drug delivery [9]. Magnetic liposomes (magnetic nanoparticles encapsulated in liposomes), which can be accumulated in a target tumor by applying a magnetic field, have been used as drug carriers [10]. Magnetic nanoparticles have also been used successfully in magnetic hyperthermia cancer therapy [11]. In such therapies, Fe_3O_4 nanoparticles are injected directly into the tumor and exposed to an alternating current (AC) magnetic field. Inspired by these results, we constructed thermal and magnetic dual-responsive liposomes (TML) composed of magnetic fluid Fe_3O_4 and thermosensitive lipids in this study. The TML were able to combine both magnetic drug targeting and temperature sensitive payload release, such that the triggered release of payloads can be realized when the environmental temperature exceeds the phase-transition temperature of the liposomal components.

An ideal siRNA delivery system should not only selectively accumulate siRNA at the tumor after systemic administration but also deliver siRNA to tumor cells with a high efficacy [12]. To address this need, new approaches employing cell-penetrating peptides (CPPs) for macromolecular payload delivery are promising. CPPs hold an extraordinary potential to achieve intracellular access. In general, CPPs mediate siRNA penetration into cells via electrostatic interactions or covalent bonds. Disulfide bond-linked siRNA-CPPs conjugates are noteworthy because they exhibit reversible stability upon cleavage (reduction) in the cell interior in response to increased glutathione (GSH) concentrations, which are 100–1000 times higher compared to the cell exterior [13]. However, due to the non-specificity and enzymatic degradation of CPPs *in vivo* and the unwanted reduction in siRNA-CPP before arrival at the target cell [14], the *in vivo* biological activity of purified siRNA-CPP conjugates is unsatisfactory [15]. Therefore, a highly effective siRNA delivery system should incorporate a nanocarrier for selectively shielding/deshielding the function of siRNA-CPPs and protecting siRNA-CPPs from plasma degradation, thus maximizing the siRNA transfection effect of siRNA-CPPs.

In this study, utilizing dual stimuli of hyperthermia and a magnetic field, we propose a new approach that combines magnetic fluid Fe_3O_4, thermosensitive lipids and CPPs into one construct to form thermal and magnetic dual-responsive liposomes (TML) for siRNA delivery (siRNA-CPPs/TML). CPPs (CKRRMKWKK) derived from penetratin, which has increased membrane translocation efficiency [15], were first conjugated with siRNA (siRNA-CPPs) via a chemical reaction (see Section S.1. Synthesis of siRNA-CPPs conjugates). Then, the siRNA-CPPs were encapsulated with magnetic fluid Fe_3O_4 into thermosensitive liposomes. Magnetic fluid Fe_3O_4 was used as the core and functioned as a magnetic target and heating source of the TML. A schematic of the siRNA-CPPs/TML procedure is shown in Fig. 1. siRNA-CPPs/TML will accumulate in tumor sites under the magnetic force generated by a horseshoe magnet (NdFeB permanent magnet, static magnetic force), then replace the horseshoe magnet by a copper coil with alternating current (AC) to produce an alternating magnetic field, which can induce the Fe_3O_4 nanoparticles to produce heat that can trigger the drug release. Consequently, the siRNA-CPPs penetrated the tumor cell membrane, escaped from the endosomal entrapment and efficiently transported siRNA to the cytosol. Finally, the disulfide bonds of the siRNA-CPPs were reduced by GSH in the cytosol and easily dissociated siRNA from the conjugates, resulting in efficient gene knockdown of the tumor. In this work, the physicochemical and *in vitro* biological characters of the siRNA-CPPs/TML were investigated, and the *in vivo* efficiency of the tumor therapy induced by the nanocarriers was also explored.

2. Materials and methods

2.1. Materials and cell lines

The CPPs (CKRRMKWKK) were custom-synthesized by Shanghai GL Biochem Co, Ltd. (Shanghai, China). 1,2-dipalmitoyl-snglycero-3-phosphatidylcholine (DPPC), monostearoylphosphatidyl-choline (MSPC), and 1,2-distearyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethyleneglycol)-2000] (DSPE-PEG2000) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Magnetic Fe_3O_4 was supplied by Qingdao Xinghan Nanotechnology Co. Ltd (Shandong, China). Horseshoe magnet (NdFeB permanent magnet) was supplied from Ningbo Beilun Meank Magnetics Co., Ltd (Zhejiang, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from GIBCO, Invitrogen Corp. (Carlsbad, USA). Lyophilized c-Myc siRNA against c-Myc mRNA (5′-AACGUUAGCUUCACCAACAUU-3′) and negative control siRNA (antisense strand, 5′-ACGUGACACGUUCGGAGAATT-3′), both of which were chemically modified with a thiol group at the 5′ end of one RNA strand and with a 5′ FAM or Cy3 on the complementary strand, were obtained from GenePharma (Shanghai, China). LysoTracker Red and Annexin V-FITC apoptosis detection kits were obtained from the Beyotime Institute of Biotechnology (Jiangsu, China). TRNzol A+ reagent and Quantscript RT Kit were purchased from Tiangen Biotec Co., Ltd (Beijing, China). Anti-c-myc monoclonal antibody and rabbit anti-β-actin were supplied by Applygen Technologies, Inc. (Beijing, China). All chemicals were of reagent grade and were obtained from Sigma-Aldrich, unless otherwise stated.

Human breast adenocarcinoma cells (MCF-7 cells) purchased from the Cell Resource Centre of IBMS (Beijing, China) were maintained in culture medium consisting of DMEM supplemented with 10% FBS, 100 IU/mL penicillin, and 100 mg/mL streptomycin. The cells were maintained in a 37 °C humidified incubator in a 5% CO_2 atmosphere.

2.2. Preparation of siRNA-CPPs-encapsulated liposomes

This dual-responsive siRNA delivery system was based on thermosensitive liposomes (TSL). The TSL carrying siRNA-CPPs (siRNA-CPPs/TSL) were prepared, as described by our previous report [16], with minor modifications. Briefly, DPPC:MSPC:DSPE-PEG_{2000} at a mass ratio of 87:3:10 were dissolved in chloroform, dried via solvent evaporation and left overnight in a vacuum desiccator. Then, the dried lipid film was hydrated using HEPES buffer (20 mM HEPES, 150 mM NaCl, pH = 7.0) containing 10 μM siRNA-CPPs at 50 °C for 30 min. The siRNA-CPPs/liposome mixture was subjected to ten freeze-thaw cycles in liquid nitrogen and a 50 °C water bath. Finally, the liposomal formulation was sonicated and filtrated through 100 nm membranes five times to reduce the size