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Dual stimulus of hyperthermia and intracellular redox environment triggered release of siRNA for tumor-specific therapy

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ABSTRACT

Small interfering RNA (siRNA) offers a new and potential therapeutic strategy for tackling many diseases at the molecular level. Recently, cell-penetrating peptides (CPPs) conjugated with siRNA *via* disulfide-bonds (designated as siRNA-CPPs) were reported to form glutathione-sensitive carriers. However, non-cell specificity, CPPs degradation and the unwanted reduction of siRNA-CPPs before reaching the targeted tissue *in vivo* hampered the development of siRNA-CPPs. Herein, utilizing the dual stimulus of hyperthermia and the intracellular redox environment, we devised a thermosensitive liposome (TSL) containing an Asparagine-Glycine-Arginine (NGR) peptide and reducible siRNA-CPPs for tumor-specific siRNA transfection (siRNA-CPPs/NGR-TSL), in which siRNA-CPPs were "caged" in NGR-TSL to overcome their limitations *in vivo*. The functional nanocarrier possessed a small particle size of approximately 90 nm, a high drug encapsulation efficiency of approximately 86% and good serum stability. Both free siRNA-CPPs and siRNA-CPPs/NGR-TSL (preheated) silenced c-myc in human fibrosarcoma (HT-1080) cells *in vitro*. However, in an HT-1080 xenograft murine model, siRNA-CPPs/NGR-TSL with hyperthermia displayed superior *in vivo* antitumor efficacy (about 3-fold) and gene silencing efficiency (about 2-fold) compared with free siRNA-CPPs under hyperthermia. This study demonstrates that the constructed vesicle in combination with hyperthermia could greatly improve the *in vivo* stability of siRNA-CPPs and synergistically enhance its cancer therapy efficiency.

1. Introduction

RNA interference (RNAi), mediated by small interfering RNA (siRNA), has emerged as a well-established strategy to specifically target pathogenic proteins on the post-transcriptional level of malignant tumors both *in vitro* and *in vivo* (De Backer et al., 2015). The highly specific nature and clear-cut design principle demonstrate the potential of siRNA as anticancer drugs in the future (Yu et al., 2014). siRNA molecules must be delivered intracellularly to trigger RNAi, but their large, anionic and hydrophilic structure prevent them from diffusing across cell membranes to reach their site of action (Lorenzer et al., 2015). Therefore, a specialized delivery vehicle is required to localize siRNA to the cytoplasm of tumor cells.

Cell-penetrating peptides (CPPs), with potent cell-penetrating ability, show potential for ameliorating the delivery of siRNA and represent a promising class of siRNA carriers (Yang et al., 2013a,b). In general, CPPs mediate siRNA penetrating into cell *via* electrostatic interaction or covalent bonds. Among them, disulfide bond linked siRNA-CPPs conjugates are noteworthy because they show reversible stability upon cleavage (reduction) in the cell interior in response to the increased glutathione (GSH) concentration, which is 100–1000 times higher than that in the cell exterior (Saito et al., 2013). Reversible stability is an important feature for nucleic acid delivery vehicles because the release of siRNA into cytoplasm is required to initiate the RNAi process (Davidson et al., 2004). However, due to the non-specificity and enzymatic degradation of CPPs *in vivo* as well as the unwanted reduction of siRNA-CPPs before arrival at the target cell (Lundberg et al.,

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2007), the *in vivo* biological activity of purified siRNA-CPPs conjugates were unsatisfactory (Turner et al., 2007; Moschos et al., 2007). Therefore, a highly effective siRNA delivery system should result from exploring a carrier for selectively shielding/deshielding siRNA-CPPs function and protecting siRNA-CPPs from plasma degradation, thus maximizing the siRNA transfecting effect of siRNA-CPPs. However, to the best of our knowledge, there is no report about developing a functional nanocarrier to take full advantage of siRNA-CPPs.

It is well known that one of the most important issues for gene delivery is how to stably deliver siRNA to the target sites. Cationic nanocarrier, which can easily complex siRNA *via* electrostatic interaction, are widely used in siRNA delivery. However, since the stiff structure and low spatial charge density of siRNA made it not easily condensed by cationic vesicles, most of those reported carriers are not stable enough *in vivo* (Lee et al., 2013). On the other hand, if the siRNA complexed carrier is too stable, siRNA will be not expressed in the target site (Geoghegan et al., 2012). Therefore, how to well balance the *in vivo* stability as well as expression efficiency at the action site of siRNA remains a troublesome problem to be resolved in siRNA delivery. To address these challenges, the approach reported in this paper is to combine the above mentioned bio-reducible siRNA-CPPs with trigger-responsive liposomes under stimulus. Currently, an effective therapeutic approach against cancer also requires the combination of several modalities such as chemotherapy, gene therapy, radiation, internal/external stimulus and so on (Frazier and Ghandehari, 2015). External stimulus-hyperthermia, which could increase the permeability of tumor vasculature and cell membranes and lower hydrostatic pressure, is considered as the "fourth leg" of cancer treatment. Hyperthermia in combination with trigger-responsive nanocarriers could precisely control the release of the cargo, concentrated the cargo at the target sites, and thus will greatly sensitize cancer cells to therapy agents (Koning et al., 2010).

Thermo-sensitive liposomes (TSL), whose lipid materials will undergo a gel-to-liquid crystalline phase transition upon heating in a mild-hyperthermia range, will enable a liposome-based drug delivery system to immediately release encapsulated drugs in the heating tissues or organs (Zhang et al., 2011). For applications in oncology, TSL in combination with localized hyperthermia will show more advantages: (i) TSL could maintain the encapsulated cargo stable in the normal body circulation (at 37 °C) and immediately release their contents upon arrival at the heated tumor area (40–42 °C). The release was so fast that it can counteract rapid blood passage time and washout of the encapsulated cargo from a tumor (Tagami et al., 2011); (ii) TSL in combination with hyperthermia is able to increase the amount of drug accumulation in heated tumor *versus* non-heated controls, leading to an evidently improvement in cancer therapy (Manzoor et al., 2012). Ranjan et al. (2012) reported that doxorubicin (Dox) encapsulated TSL in combination with hyperthermia showed a 3.5- and 7.6-fold more tumor accumulation than TSL and free Dox, respectively. The research of Manzoor et al. (Manzoor et al., 2012) indicated the combination therapy was able to show a 9-fold increase in Dox accumulation over 20 min as compared to control groups of free drug.

Therefore, utilizing the combined ascendancies of TSL and hyperthermia, a new strategy by encapsulating siRNA-CPPs into TSL was hypothesized to improve both *in vivo* stability and targeting efficiency of siRNA-CPPs. Once at the area of interest, localized and targeted delivery of siRNA-CPPs could be achieved with minimal influence to other parts of the body under hyperthermia. Then the free siRNA would be transported into cytosol with the help of CPPs and under the redox stimulus of GSH. This strategy may well balance the siRNA stability in the body circulation and its expression efficiency in target action area. As far as we know, no others reported nanosystems so far are flexible enough to response to dual stimulus of hyperthermia and redox

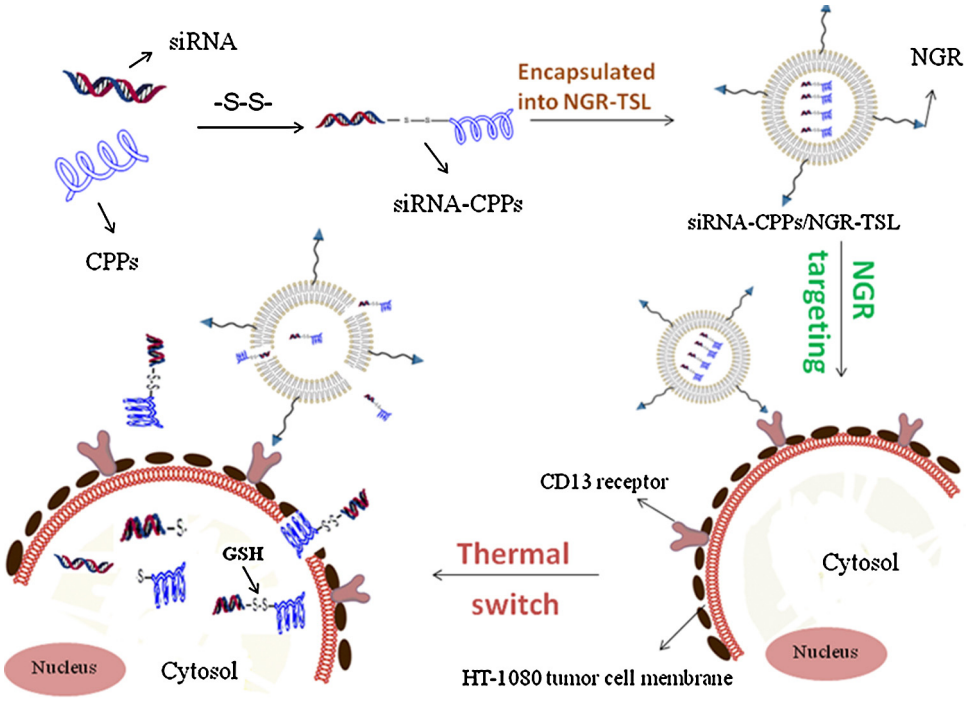

Fig. 1. Schematic illustration of siRNA-CPPs/NGR-TSL for specific siRNA delivery to tumor cells under dual stimulus of hyperthermia and intracellular redox environment. NGR-modified liposomes are retained in the tumor due to the active targeting effect by the NGR ligand. In the body circulation, as siRNA-CPPs were encapsulated in NGR-TSL, they can not penetrate into the normal cell membrane and be degraded by enzyme in the blood. However, upon heat-stimulus at the tumor site, siRNA-CPPs were released, the interaction of CPPs with the cell membrane is restored and mediated siRNA rapidly enter into the cells membrane and escape from the endosomal entrapment into the cytosol. At last, free siRNA was released from the CPPs *via* disulfide-bond broken under the stimulus of GSH in the cytosol and silenced c-myc gene over expressed in HT-1080 cells.