Ultrasound in Med. & Biol., Vol. 42, No. 6, pp. 1362–1374, 2016

0301-5629/$ - see front matter

http://dx.doi.org/10.1016/j.ultrasmedbio.2016.01.017

● *Original Contribution*

EFFICIENT SIRNA DELIVERY USING NOVEL CELL-PENETRATING PEPTIDE-SIRNA CONJUGATE-LOADED NANOBUBBLES AND ULTRASOUND

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(*Received* 31 *May* 2015; *revised* 13 *January* 2016; *in final form* 27 *January* 2016)

Abstract—Because of the absence of tolerable and effective carriers for *in vivo* delivery, the applications of small interfering RNA (siRNA) in the clinic for therapeutic purposes have been limited. In this study, development of a novel siRNA delivery system based on ultrasound-sensitive nanobubbles (NBs, nano-sized echogenic liposomes) and cell-permeable peptides (CPPs) is described. A CPP-siRNA conjugate was entrapped in an NB, (CPP-siRNA)-NB, and the penetration of CPP-siRNA was temporally masked; local ultrasound stimulation triggered the release of CPP-siRNA from the NBs and activated its penetration. Subsequent research revealed that the (CPP-siRNA)-NBs had a mean particle size of 201 ± 2.05 nm and a siRNA entrapment efficiency >85%. *In vitro* release results indicated that >90% of the encapsulated CPP-siRNA was released from NBs in the presence of ultrasound, whereas <1.5% (30 min) was released in the absence of ultrasound. Cell experiments indicated higher cellular CPP-siRNA uptake of (CPP-siRNA)-NBs with ultrasound among the various formulations in human breast adenocarcinoma cells (HT-1080). Additionally, after systemic administration in mice, (CPP-siRNA)-NBs accumulated in the tumor, augmented *c-myc* silencing and delayed tumor progression. In conclusion, the application of (CPP-siRNA)-NBs with ultrasound may constitute an approach to selective targeted delivery of siRNA. (E-mail: amms2013@126.com) **© 2016 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Ultrasound, Nanobubbles, Cell-penetrating peptides, siRNA delivery.**

INTRODUCTION

Small interfering RNA (siRNA), suppressing the expression of oncogenes (*e.g.*, *c-myc*) closely related to tumor growth, proliferation, invasion and expansion, has been studied as a potential candidate for cancer treatment for decades. However, the application of siRNA in the clinic would encounter a series of hurdles, such as the rapid degradation by nuclease, renal clearance and poor cellular uptake. Thus, development of a siRNA delivery vehicle that can reinforce specificity for the tumor and has efficient cellular uptake is desirable.

To overcome these challenges, a new approach employing cell-penetrating peptides (CPPs) for payload delivery seems promising. CPPs are positively charged, short peptide sequences that are rich in lysine or arginine (Derossi 1998). These cationic peptides can facilitate the cellular internalization of therapeutic agents, which is attributed to the interaction between the negatively charged plasma membrane and the positively charged CPP (Lindgren and Langel 2011; Zorko and Langel, 2005). Some studies have reported that siRNA/CPP complexes, formed *via* electrostatic interactions between the cationic CPP and anionic siRNA, could facilitate cellular import and elicit RNAi, which results in the silencing of endogenous genes (Simeoni et al. 2003). Researchers reported that entrapment of the gene and CPP into microbubbles is an effective strategy for gene transfection (Ren et al. 2009, 2014). However, the depressed cell penetrating ability of CPPs neutralized by anionic siRNA and the discounted gene silencing efficacy resulting from an unpacked carrier (caused by strongly electrostatic interaction) hinder the potential use of such complexes. An alternative strategy is

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covalent siRNA-CPP conjugates of CPPs and siRNA linked by reducible disulfide bonds. This conjugate could dissociate naked siRNA in the cytoplasm in response to intra-cellular glutathione. However, because CPP is a non-specific functional molecule that can penetrate any cell on encountering it, this drawback was limited the utilization of siRNA-CPP in drug delivery systems (Vivès 2005).

With the focus of addressing such a problem, the concept of stimulus-responsive nanocarriers was imported to assemble an "off–on" switch, which is based on sensitivity to endogenous triggers (*e.g.*, pH and enzymatic activity) and external triggers (*e.g.*, light and ultrasound) to control the penetrating activity of CPPs. For example, Xiang et al. (2013) developed an "activatable cell-penetrating peptide" system for the targeted delivery of siRNA to prostate-specific antigen–positive prostate tumors. In this system, the activity of CPP was blocked by masking the arginine positive charges with a polyanionic segment (polyglutamate). Between the two peptide segments, there was a prostate-specific antigen cleavable sequence linking them. Thus, the overexpressed matrix metalloproteinases in the vicinity of the tumor cells could initiate the cell penetration of CPPs. In another example, by using different pH levels between tumor tissue and healthy tissue, He's group designed long-circulating pH-detachable polyethylene glycol (PEG)-shielded CPP-liposomes to realize site-specific drug delivery (Zhang et al. 2013a). These liposomes were constructed with PEG chains attached to their surface, and the outside segment of the PEG chain linked to a pH-sensitive bond cloaked the CPP on the surface of the liposome. The slightly acidic tumor environment could trigger removal of the PEG chain, thus activating the cell crossing ability of CPP. However, with respect to the reliance on the endogenous stimulus, the aforementioned triggered release mechanisms may suffer some disadvantages, mainly because of the large inter-individual variability in the expression levels of enzymes or intra-tumoral pH. In addition, as the CPPs are immobilized on the surface of the nanocarriers in these strategies, they may not be well protected from enzymatic degradation *in vivo* before approaching the targeting sites.

To overcome the drawback of endogenous stimulus-triggered CPP, some researchers began to investigate the general triggered release methodology, which was independent of the tumor microenvironment and could realize targeted drug delivery (Yang et al. 2014). In this strategy, the non-specific function of CPPs was sterically shielded during the first phase of drug delivery; after the CPPS reached the target site, their exposure to external triggers would activate their cell penetration and, therefore, enhance the intracellular delivery of their payloads. Among the various external triggers, ultrasound is especially attractive, as it can penetrate deeply into tissues and can be focused on regions of tumor growth to effectively activate sonosensitizers while preserving peripheral healthy tissue. Ultrasound-mediated drug delivery can be amplified by the acoustic disruption of microbubble carriers that undergo cavitation (Escoffre et al. 2013). Recently, microbubbles have been successfully used in preclinical research for drug delivery (de Saint Victor et al. 2014; Tsu-Yin et al. 2013). However, microbubbles (1–10 μm) are incapable of targeting specific tissue *in vivo* because of large particle sizes that limit them from penetrating the vessel wall and entering target tissues (Son et al. 2014). On the other hand, nanocarriers (10–1000 nm) are more suitable for *in vivo* drug delivery to target sites, because they can extravasate from the bloodstream and, because of their smaller size, enter the desired tissues (Cavalli et al. 2013).

To date, the use of nanobubble (NB) systems combined with ultrasound for drug delivery has been studied extensively, and this strategy has been found to have a variety of merits, including: non-invasiveness, local applicability, targeted release, high transfection efficiency and proven tolerability (Kantarci and Cavalli 2012; Kwan et al. 2015; Suzuki et al. 2011). Among the various types of NBs, liposomes, which are temperature sensitive, have attracted increasing attention in drug delivery research for their sonosensitive features (Evjen et al. 2013; Lin et al. 2014; Rizzitelli et al. 2015); thus, they are also called echogenic or sonosensitive liposomes.

Therefore, by using the ascendance of NBs, a new strategy of encapsulating cell-permeable peptides-small interfering RNA (CPP-siRNA) conjugates into NBs to mask the activity of CPPs in circulation was adopted in this work. Here, we planned to verify a new RNA delivery strategy by constructing a nanocarrier, (CPP-siRNA)-NBs, that was sensitive to ultrasound. Figure 1 is a schematic of the design. CPP (CKRRMKWKK), derived from Penetratin, which has increased membrane translocation efficiency (Fischer et al. 2000), is first conjugated to siRNA to form CPP-siRNA *via* a chemical reaction; then, the CPP-siRNA is encapsulated in NBs (also called echogenic liposomes). After systemic administration, (CPP-siRNA)-NBs pass or accumulate in tumor sites through the enhanced permeability and retention (EPR) effect. As the target site is exposed to ultrasound, CPP-siRNA is released from the NBs, which have been burst by ultrasound irradiation. Then, the CPPs deliver the siRNA directly and actively through cellular membranes into the cytoplasm to silence the target gene. In this work, the physicochemical and *in vitro* biological characters of the NBs loaded with CPP-siRNA were investigated, and the *in vivo* tumor therapy efficiency of (CPP-siRNA)-NB was explored.