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

Yanfang Yang^{a,b,1}, Yang Yang^{c,1}, Xiangyang Xie^d, Xueqing Xu^{a,b}, Xuejun Xia^{a,b}, Hongliang Wang^{a,b}, Lin Li^{a,b}, Wujun Dong^{a,b}, Panpan Ma^{a,b}, Yuling Liu^{a,b*}

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, PR 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, PR China

c. Beijing Institute of Pharmacology and Toxicology, Beijing 100850, PR China

d. Wuhan General Hospital of Guangzhou Military Command, Wuhan 430070, PR China

Short title

Targeted delivery of siRNA

Correspondence to:

Professor Yuling Liu

Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, PR China

Tel: +86 10 89285098

Email: liuyulingbj@163.com

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.

Keywords: Dual stimulus; Heat-activable; intracellular redox environment; Cell-penetrating peptides; Small interfering RNA

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., 2013). 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., 2007), the *in vivo* biological activity of purified

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

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

1 in combination with trigger-responsive nanocarriers could precisely control the release
2 of the cargo, concentrated the cargo at the target sites, and thus will greatly sensitize
3 cancer cells to therapy agents (Koning et al., 2010).

4 Thermo-sensitive liposomes (TSL), whose lipid materials will undergo a
5 gel-to-liquid crystalline phase transition upon heating in a mild-hyperthermia range, will
6 enable a liposome-based drug delivery system to immediately release encapsulated
7 drugs in the heating tissues or organs (Zhang et al., 2011). For applications in oncology,
8 TSL in combination with localized hyperthermia will show more advantages: (i) TSL
9 could maintain the encapsulated cargo stable in the normal body circulation (at 37 °C)
10 and immediately release their contents upon arrival at the heated tumor area
11 (40 °C-42 °C). The release was so fast that it can counteract rapid blood passage time
12 and washout of the encapsulated cargo from a tumor (Tagami et al., 2011); (ii) TSL in
13 combination with hyperthermia is able to increase the amount of drug accumulation in
14 heated tumor versus non-heated controls, leading to an evidently improvement in cancer
15 therapy (Manzoor et al., 2012). Rajan et al (Ranjan et al., 2012) reported that
16 doxorubicin (Dox) encapsulated TSL in combination with hyperthermia showed a 3.5-
17 and 7.6-fold more tumor accumulation than TSL and free Dox, respectively. The
18 research of Manzoor et al (Manzoor et al., 2012) indicated the combination therapy was
19 able to show a 9-fold increase in Dox accumulation over 20 min as compared to control
20 groups of free drug.

21 Therefore, utilizing the combined ascendancies of TSL and hyperthermia, a new
22 strategy by encapsulating siRNA-CPPs into TSL was hypothesized to improve both *in*

1 *in vivo* stability and targeting efficiency of siRNA-CPPs. Once at the area of interest,
2 localized and targeted delivery of siRNA-CPPs could be achieved with minimal
3 influence to other parts of the body under hyperthermia. Then the free siRNA would be
4 transported into cytosol with the help of CPPs and under the redox stimulus of GSH.
5 This strategy may well balance the siRNA stability in the body circulation and its
6 expression efficiency in target action area. As far as we know, no others reported
7 nanosystems so far are flexible enough to response to dual stimulus of hyperthermia and
8 redox effect of GSH, which may increase cancer therapeutic effect of siRNA
9 dramatically. In addition, since active targeting ligands modification on the surface of
10 TSL could enhance the targeting ability of TSL to specific tumors,
11 Asparagines-Glycine-Arginine (NGR) peptide motif would be utilized to elevate the
12 targeting ability of TSL to tumor cells highly expressed CD13 in this study (Yang et al.,
13 2014).

14 Here, utilizing the dual stimulus of hyperthermia and redox effect of GSH, we
15 postulate a new approach to combine NGR, TSL, siRNA-CPPs and external
16 hyperthermia into one protocol and construct a new siRNA delivery system
17 (siRNA-CPPs/NGR-TSL, shown in Fig.1): (i) CPPs (CKRRMKWKK) derived from
18 penetratin are conjugated with siRNA via disulfide bonds to form a conjugate
19 (siRNA-CPPs). Then, the conjugates are entrapped in TSL with NGR moiety
20 modification and **target** to tumors with CD13 over-expressed on the surface; (ii) TSL,
21 used as drug delivery carrier, improves the *in vivo* stability of siRNA-CPPs and cloaks
22 the penetration function of CPPs before reaching the target sites; (iii) CPPs are activated

1 via heat stimulus at the tumor sites. The activated siRNA-CPPs **penetrates into** the cell
2 membrane, **escapes** from the endosomal entrapment and **transports** siRNA into the
3 cytosol efficiently; (iv) the disulfide bonds of the siRNA-CPPs are reduced by GSH in
4 the cytosol and siRNA **will be dissociated** from the carrier easily, resulting in efficient
5 gene knockdown of the tumor. Accordingly, the action mechanisms and gene silencing
6 efficiency of siRNA-CPPs/NGR-TSL in combination with hyperthermia were
7 investigated both *in vitro* and *in vivo*. We show that the siRNA transfection efficiency is
8 a function of the targeted cell association, induction of cellular uptake, endosomal
9 escape and dissociation of free siRNA from the carrier under the redox environment.
10 The successful induction of RNAi requires concurrent siRNA release from the vector
11 and escape from the endosomes. Moreover, the *in vivo* antitumor efficacy of free
12 siRNA-CPPs under hyperthermia and siRNA-CPPs/NGR-TSL without hyperthermia
13 was also evaluated and compared with that of siRNA-CPPs/NGR-TSL under
14 hyperthermia.

15 **2. Materials and methods**

16 *2.1. Materials*

17 Dipalmitoyl phosphatidylcholine (DPPC),
18 Monostearoylphosphatidyl-choline(MSPC),1,2-distearyl-sn-glycero-3-phosphoethanola
19 mine-N-[methoxy(polyethyleneglycol)-2000](DSPE-PEG2000)and1,2-distearoyl-sn-gly
20 cero-3-phosphoethanolamine-N-maleimide(polyethyleneglycol)(DSPE-PEG2000-Mal)
21 were purchased from Avanti Polar Lipids (Alabaster, USA) and used without further
22 purification. Lyophilized c-myc siRNA against c-myc mRNA

1 (5'-AACGUUAGCUUCACCAACAUAU-3') and Negative control siRNA (antisense
2 strand, 5'-ACGUGACACGUUCGGAGAATT-3'), both of which were chemically
3 modified with a thiol group at the 5' end of one RNA strand and a 5' FAM or Cy3
4 modification on the complementary strand, were obtained from GenePharma (Shanghai,
5 China). All primers were synthesized by AuGCT Biotechnology (Beijing, China). NGR
6 motif peptide (CYGGRGNG) and CPPs (CKRRMKWKK) were custom synthesized by
7 Shanghai GL Biochem Co, Ltd, (Shanghai, China). Trypsin was obtained from Solarbio.
8 Modified Eagle's Medium (MEM) and fetal bovine serum (FBS) were obtained from
9 GIBCO, Invitrogen Corp. (Carlsbad, USA). Diamide, propidium iodide (PI), and
10 Hoechst 33258 were purchased from Sigma-Aldrich (St Louis, MO). Penicillin and
11 streptomycin were provided by North China Pharmaceutical Co., Ltd, (Hebei, China).
12 LysoTracker Red, Annexin V-FITC apoptosis detection kit and one step TUNEL
13 apoptosis assay kit was obtained from Beyotime Institute of Biotechnology (Jiangsu,
14 China). TRNzol A+ reagent and Quantscript RT Kit were purchased from Tiangen
15 Biotec Co., Ltd (Beijing, China). Anti-c-myc monoclonal antibody and rabbit
16 anti-β-actin were supplied by Applygen Technologies Inc, (Beijing, China). All other
17 chemicals were analytical or high performance liquid chromatography (HPLC) grade.

18 Female BALB/c nude mice (18-22 g) were purchased from Vital River Laboratories
19 (Beijing, China). All animals were handled according to the code of ethics in research,
20 training and testing of drugs as laid down by the Animal Care and Use Ethics Committee
21 of Chinese Academy of Medical Sciences & Peking Union Medical College.

22 2.2. Synthesis of siRNA-CPPs conjugates

1 **siRNA-CPPs conjugates were** synthesized by coupling CPPs with siRNA via
2 disulfide **bonds** as reported by Muratovska *et al* (Muratovska and Eccles, 2004) with
3 slight modification. Briefly, c-myc siRNA, CPPs and thiol oxidant diamide were
4 dissolved in HEPES buffer (10 mM HEPES, 1 mM ethylenediamine tetraacetic acid
5 (EDTA), pH 8.0) to obtain a final concentration of 17.5 μ M, 20 μ M and 20 μ M,
6 respectively. Then equimolar amounts of siRNA, CPPs and diamide were mixed and
7 incubated for 1 h at 40 °C. **The conjugates prepared were purified by minicolumn**
8 **centrifugation method using dextran gel (Sephadex G-50) column.** At last, the product
9 with a yield of 82.7 % was obtained and verified by MALDI-TOF MS.

10 2.3. Preparation of siRNA-CPPs encapsulated liposomes

11 The functional materials of NGR-PEG2000-DSPE used in this work were
12 synthesized via the thiol-ene “click” reaction of the maleimide group with the sulfhydryl
13 group of NGR peptide according to the method reported in our previous study (Yang et
14 al., 2014). NGR-targeted or non-targeted thermal-sensitive liposomes carrying
15 siRNA-CPPs (named as siRNA-CPPs/NGR-TSL or siRNA-CPPs/TSL) were prepared
16 as follows: DPPC: MSPC: (DSPE-PEG2000-NGR or DSPE-PEG2000) in a mass ratio
17 of 87:3:10 were dissolved in chloroform, dried by solvent evaporation, and left
18 overnight in a vacuum desiccator. Then the dried lipid film was hydrated using HEPES
19 buffer (20 mM HEPES, 150 mM NaCl, pH =7.0) containing 10 μ M siRNA-CPPs at
20 50 °C for 30 min. The siRNA-CPPs/liposomes mixture was subjected to ten freeze-thaw
21 cycles in liquid nitrogen and a 50 °C water bath. At last, the liposomal formulation was
22 sonicated to reduce the size of the liposomes. Finally, the liposomes were filtrated by

1 0.22 µm filter for sterilization and stored at 4 °C for further characterization.

2 2.4. Encapsulation efficiency (EE) determination

3 The encapsulation efficiency (EE) of siRNA-CPPs was determined by
4 ultra-filtrating the liposomes containing FAM-siRNA-CPPs using Amicon® Ultra-4
5 centrifugal filter devices (100,000 NMWL, Millipore, Massachusetts), as reported by
6 Gao *et al* (Gao et al., 2012). After thoroughly ultrafiltration with DEPC water,
7 unencapsulated FAM-siRNA-CPPs were collected and quantified using a calibration line
8 obtained with standard FAM-siRNA-CPPs solutions. The fluorescence of
9 FAM-siRNA-CPPs was determined with the spectrofluorometer (Synergy™ 4, Biotek,
10 Wi, USA) using excitation and emission wavelengths of 495 and 525 nm, respectively.
11 The EE was calculated using the formula: $(M_i - M_U)/M_i \times 100\%$. M_U and M_i were defined
12 as the mass of unencapsulated FAM-siRNA-CPPs and initially added
13 FAM-siRNA-CPPs, respectively.

14 2.5. Physicochemical Characterization

15 All size, zeta potential and polydispersity index measurements were made using a
16 Malvern Zeta-sizer Nano ZS90 instrument (Malvern Instruments Ltd., U. K.). The
17 results were determined in three serial measurements and reported as the mean values $\pm$
18 standard deviation. The prepared siRNA-CPPs/NGR-TSL was morphologically
19 characterized by a transmission electron microscope (TEM, HITACHI, H-7650, Japan)
20 and Atomic force microscopy (AFM, NanoWizarc, JPK Ltd., Germany), respectively.
21 The *in vitro* stability of siRNA-CPPs/NGR-TSL in the presence of 10% FBS was
22 evaluated using a Turbiscan Lab® Expert (Formulation, L'Union, France), which is able

1 to determine the small changes of colloidal systems. The liposomes were diluted by cell
2 culture medium (90% MEM) containing 10% FBS and incubated at 37 °C, 5% CO₂
3 incubator simulating the *in vivo* environment. At predesigned time points, 3 mL of the
4 sample was placed into cylindrical glass tubes and submitted to Turbiscan Lab[®] Expert
5 stability analysis. Also, the prepared siRNA-CPPs/NGR-TSL was diluted by different
6 solution (0.01 M PBS, 0.02 M PBS, 0.1 M PBS, 0.1 M PBS+10% FBS, MEM+10%FBS,
7 FBS; pH value of PBS is 7.4) and incubated at 37 °C, 5% CO₂ incubator for 48 h. At
8 different time points (0h, 6h, 12h, 24h and 48h), the size change of the sample was
9 evaluated by the Nano ZS90 instrument. Serum stability of siRNA, siRNA-CPPs and
10 siRNA-CPPs/NGR-TSL was evaluated using agarose gel electrophoresis.

11 *2.6. Storage stability studies of siRNA-CPPs/NGR-TSL*

12 The storage stability study was conducted to monitor physical and chemical
13 stabilities of the liquid form of siRNA-CPPs/NGR-TSL at 4 °C. The stability parameters,
14 such as the mean particle size, PDI, entrapment efficiency and zeta potential were
15 determined as a function of the storage time.

16 *2.7. Thermal stability of FAM-siRNA and heat activation of siRNA-CPPs*

17 The molecular weight change of FAM-siRNA before and after heat treatment
18 (preheated FAM-siRNA at 42 °C for 30 min) was evaluated utilizing Q-TOF (QSTAR
19 Elite, Applied Biosystem/MDS Sciex) equipped with ESI to study the thermal stability
20 of FAM-siRNA.

21 FAM-siRNA-CPPs/TSL and FAM-siRNA-CPPs/NGR-TSL were suspended in
22 phosphate buffered saline (PBS, pH=7.4) and incubated at 37 °C and 42 °C respectively

1 with gentle shaking. In addition, the release of FAM-siRNA-CPPs from NGR-TSL in
2 different medium was also observed by incubating FAM-siRNA-CPPs/NGR-TSL in
3 PBS (pH=7.4), MEM solution containing 10% FBS and PBS (pH=6.5) which is similar
4 to tumor microenvironment at 37 °C and 42 °C, respectively. Release of FAM-siRNA
5 from TSL and NGR-TSL in different medium at different temperature was monitored at
6 several time intervals over 30 min. At each sampling time, the sample was thoroughly
7 ultrafiltrated and the released FAM-siRNA-CPPs was collected and determined using
8 the spectrofluorometer previously described (see section 2.4).

9 *2.8. In vitro evaluation of siRNA-CPPs/NGR-TSL*

10 *2.8.1. Cell culture*

11 Human fibrosarcoma cells (HT-1080 cells) with a high expression of CD13 as
12 confirmed by our previous study (Yang et al., 2014) were purchased from the Cell
13 Resource Centre (IBMS, CAMS/PUMC) and grown in MEM medium supplemented
14 with 10% FBS, 100 IU/mL penicillin, and 100 mg/mL streptomycin. The cells were
15 maintained in a 37 °C humidified incubator with a 5% CO₂ atmosphere.

16 *2.8.2. Competitive binding assay of nanocarriers, cellular uptake and flow cytometric* 17 *analysis*

18 The enhancement in cellular uptake offered by NGR and heat-activable CPPs
19 mediated drug delivery system was confirmed using flow cytometry with HT-1080 cells.
20 HT-1080 cells were seeded into 6-well plates (Corning, NY, USA) with a density of $3 \times$
21 10^5 cells/well. After incubating for 24 h, the medium in each well was replaced with
22 fresh cell medium without FBS containing free FAM-siRNA, free FAM-siRNA-CPPs or

1 an equivalent concentration of FAM-siRNA-CPPs/TSL (unheated),
2 FAM-siRNA-CPPs/NGR-TSL (unheated) and FAM-siRNA-CPPs/NGR-TSL-H
3 (preheated at 42 °C for 30 min). As for the competitive binding assay of
4 siRNA-CPPs/NGR-TSL and siRNA-CPPs/TSL to HT-1080 cells, excess free NGR (1
5 mg/mL) was added to the medium prior to the introduction of the two liposomes. The
6 concentration of FAM-siRNA was 100 nM. Following the treatment for 4 h, the cells
7 were washed twice with cold PBS (0.1 M, pH 7.4), detached with 0.25% trypsin and
8 washed another three times with cold PBS. Finally, the cells were resuspended in 0.2 ml
9 PBS and subjected to flow cytometry (BD FACS Calibur). The autofluorescence of the
10 cells was used as a control. All experiments were performed in triplicate.

11 2.8.3. *In vitro confocal image*

12 Following the culture of HT-1080 cells for 24 hours on a petri dish (3×10^5
13 cells/well), free FAM-siRNA, free FAM-siRNA-CPPs or an equivalent concentration of
14 FAM-siRNA-CPPs/TSL (unheated), FAM-siRNA-CPPs/NGR-TSL (unheated) and
15 FAM-siRNA-CPPs/NGR-TSL-H were added to each dish and incubated at 37 °C for 4
16 hours. The medium was removed and discarded, and then the cells were washed with
17 cold PBS (0.1 M, pH 7.4) for three times, followed by fixing with 4% paraformaldehyde
18 for 20 minutes. Nuclear staining was performed by Hoechst 33258 for 10 min at ambient
19 temperature. The fluorescent images of cells were analyzed using a laser scanning
20 confocal microscope (UltraVIEW Vox, PerkinElmer, USA). FAM-siRNA and Hoechst
21 33258 were excited using 488 nm and 405 nm lasers, respectively.

22 The endosomal release of liposomal FAM-siRNA-CPPs was tracked by confocal

1 image. HT-1080 cells were cocultured with FAM-siRNA-CPPs/NGR-TSL (unheated and
2 preheated) for 4 h. After then, the cells were washed three times with cold PBS and
3 cultured in complete medium for an additional 1 h or 3 h. After incubating for 0.5h, the
4 endosome/lysosome was stained with 2 ml of Lyso-Tracker Red (75 nM). Subsequently,
5 the cells were rinsed with cold PBS and the nuclear staining was treated as described
6 above. The cells were analyzed using confocal image and the LysoTracker Red was
7 excited by a 561 nm laser.

8 *2.8.4. In vitro transfection and analysis of gene expression*

9 HT-1080 cells were seeded into 25 cm² tissue culture flasks at a population of
10 2.0×10^6 cells/flask and incubated in a humidified atmosphere of 5 % CO₂ at 37 °C.
11 Twenty-four hours later, the cells were cultured with siRNA (c-myc siRNA or
12 siN.C.)-loaded samples (unheated or preheated as mentioned above) at the concentration
13 of 250 nM in serum-free medium. After 5 h incubation, the cells were washed with PBS
14 for three times and further incubated in complete medium for an additional 48 h (for
15 mRNA assay) or 72 h (for protein quantification). Subsequently, qRT-PCR (quantitative
16 real-time polymerase chain reaction) and western blot analysis were utilized to evaluate
17 c-myc mRNA and protein, respectively.

18 For the qRT-PCR assessment, cells were collected, and the total RNA was extracted
19 by using TRNzol A+ reagent. 2 mg of total RNA was subjected to the synthesis of first
20 strand cDNA using a Quantscript RT Kit (first strand cDNA synthesis kit). Then the
21 synthesized cDNA was subjected to qRT-PCR analysis targeting c-myc and
22 glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified as an internal

1 control. The sequence of the c-myc forward primer was
2 5'-GGCTATTCTGCCCATTGTTGGGGAC-3', and the reverse primer was 5'-
3 GGCAGCAGCTCGAATTTCTTC-3'.

4 For western blot analysis, transfected cells were lysed in lysis buffer for 30 min on
5 ice and then the lysates were collected by centrifugation for 10 min (14000 rpm, 4 °C).
6 Subsequently, the protein was separated by 10% sodium dodecyl sulfate-polyacrylamide
7 gel electrophoresis (SDS-PAGE) and transferred to immobilon-P membranes (Millipore,
8 Bedford, MA). The blots were blocked with 5% BSA in tris-buffered saline with
9 Tween-20 (TBST) for 1 h at ambient temperature and incubated overnight at 4 °C in 5%
10 BSA in TBST with anti-c-myc monoclonal antibody (1:1000). Rabbit anti-β-actin
11 (1:1500) was used as the internal control. The bands were visualized by the Molecular
12 Image[®] ChemiDoc TM XRS + imaging system (Bio-Rad).

13 2.8.5. *Cell apoptosis assay*

14 HT-1080 cells were seeded on 25 cm² tissue culture flasks at a density of 2.0×10^6
15 cells per flask in 6 mL of complete MEM medium. After 24 hours, the cells were
16 washed with PBS and incubated with various siRNA (c-myc siRNA or siN.C.)
17 containing samples (unheated or preheated as mentioned above) in serum-free medium
18 at a concentration of 250 nM. After incubating for 6 h, the cells were washed with PBS
19 for three times and incubated in complete medium for an additional 72 h. Finally, the
20 collected cells were stained with the Annexin V-FITC apoptosis detection kit and
21 immediately analyzed by flow cytometry.

22 2.9. *In vivo antitumor evaluation of siRNA-CPPs/NGR-TSL under hyperthermia*

2.9.1. *In vivo* imaging

The antitumor efficacy *in vivo* was evaluated in HT-1080 tumor-bearing female nude mice. Briefly, the mice were subcutaneously injected in the right axilla with 0.2 mL of cell suspension containing 2×10^7 HT-1080 cells. When the tumor volumes reached approximately 200 mm^3 , the mice were administered 0.2 mL of PBS, free Cy3-siRNA, free Cy3-siRNA-CPPs, or different liposomal formulations (TSL or NGR-TSL) containing Cy3-siRNA-CPPs at a dose of 1.2 mg/kg via the tail vein. The mice were heated in a homemade hyperthermia device connected with a thermostatic circulator (HX-1050, Beijing Boyikang Laboratory Apparatus Co., Ltd., China) at 42 °C for 30 min after each tail vein injection. A mice group treated with Cy3-siRNA-CPPs/NGR-TSL and not subjected to hyperthermia (designated as siRNA-CPPs/NGR-TSL-unheated) was used as a control. At different time points (1 h, 6 h, 12 h and 24 h) after injection, the fluorescence signals from the whole body were monitored using an IVIS[®] Lumina II *in vivo* imaging system (Caliper life sciences, USA). After 24 h, the mice were killed, and the excised tumors and major organs, including the liver, heart, stomach, spleen, lung, kidney, and intestine, were also imaged.

2.9.2. *In vivo* tumor growth inhibition study

The antitumor efficacy was evaluated in HT-1080 tumor-bearing female nude mice as described above. Once tumors reached approximately 90 mm^3 , the mice were randomly divided into six groups (n=6) and received free c-myc siRNA, free c-myc siRNA-CPPs, various liposomal formulations (TSL or NGR-TSL) carrying c-myc siRNA-CPPs or NGR-TSL carrying siN.C.-CPPs at a dose of 1.2 mg/kg (0.2 mL) and

1 PBS as control. Different samples were intravenously injected once every other day for a
2 total of 14 d. The mice were heated in a homemade hyperthermia device connected with
3 a thermostatic circulator (HX-1050, Beijing Boyikang Laboratory Apparatus Co., Ltd.,
4 China) at 42 °C for 30 min after each tail vein injection. In addition, a mice group
5 treated with c-myc siRNA-CPPs/NGR-TSL and not subjected to hyperthermia was also
6 used as a control (named as siRNA-CPPs/NGR-TSL-unheated). The body weight and
7 tumor size were measured every day. The tumor volume was calculated using the
8 formula $V=1/2 \times (\text{larger diameter}) \times (\text{smaller diameter})^2$. At the end of the
9 experimental period, the mice were sacrificed, and the tumors were separated for further
10 *in vivo* gene silencing and histological evaluation.

11 2.9.3. *In vivo* gene silencing

12 For the *in vivo* c-myc expression, tumor tissues were excised 48 h after the last
13 administration. The tumor fragments were weighed and processed for total mRNA or
14 protein extraction followed by qRT-PCR and western blot assays, as described in section
15 2.8.4.

16 2.9.4. Immunohistochemical analysis

17 For the immunohistochemical analysis, after sacrificing the mice, the tumor
18 tissues were excised and immediately frozen. The frozen sections were cut into 8-μm
19 slices and fixed with 4% formaldehyde for 30 min at room temperature. After washing
20 with PBS, sections were permeabilized in freshly prepared PBS containing 0.1% Triton
21 X-100 (pH 7.4) for 2 min in an ice bath. Each sample was washed in PBS and treated
22 with 50 μL of labeling reaction mixture (48 μL of FITC-labeled dUTP:

fluorescein-dUTP, 2.0 μ L of TdT enzyme) for 60 min at 37 °C in a dark and humidified atmosphere. The sections were counterstained with propidium iodide (PI) for 45 min at room temperature and visualized using a confocal laser scanning microscope.

2.10. Statistical analysis

All data are shown as means $\pm$ standard deviation (SD) unless particularly outlined. Student's t test or one-way analyses of variance (ANOVA) were performed in statistical evaluation. A P-value less than 0.05 was considered to be significant, and a P-value less than 0.01 was considered as highly significant.

3. Results and discussion

As a successful translation of siRNA delivery nanocarrier for oncotherapy, **it should not only** stably transporting siRNA into the cytoplasm of tumor cells, **but also** dissociate siRNA easily to avoid disturbing the RNAi effect of siRNA. Utilizing the dual stimulus of hyperthermia and the intracellular redox environment, a new delivery system (siRNA-CPPs/NGR-TSL) combining bio-reducible siRNA-CPPs, NGR-modified TSL and hyperthermia was designed for tumor specific siRNA transfection in this work.

3.1. Design and Characterization of siRNA-CPPs encapsulated TSL

Human fibrosarcoma is a malignant tumor, and patients usually die after surgery with a wide range of tumor metastasis. Overexpression and activation of the c-myc gene induces various forms of cancers, including human fibrosarcoma, and the genetic alterations of c-myc are related to ~70,000 US cancer deaths per year (Chen et al., 2014). However, expression of c-myc is also essential for proliferation and regulation in normal mammalian cells. Therefore, a targeted delivery system is required for specific delivery

1 of c-myc siRNA.

2 In this work, c-myc siRNA was chemically modified with a free thiol group at the
3 5' end of one strand so that it could react with a free thiol group from a cysteine amino
4 acid on the CPPs (Fig. 2A). As we know, both thiol group modified CPPs (CPPs-SH)
5 and thiol group modified siRNA (SH-siRNA) molecules are charged (positive and
6 negative, respectively) in HEPES buffer. The same molecule with the same charge will
7 repel each other, and thus has less chance to form the bimolecular structure of
8 CPPs-s-s-CPPs and siRNA-s-s-siRNA. The product yield (82.7 %) of siRNA-CPPs
9 implied that most reactions took place between CPPs-SH and SH-siRNA. Successful
10 synthesis of siRNA-CPPs was confirmed by the molecular weight shifts in the
11 MALDI-TOF MS (m/z , $[M-H]^+$) analysis from 7104.49 to 8197.86. The increased value
12 is the molecular weight of CPPs, as depicted in Figs. 2B and 2C. The reducible disulfide
13 bond of the siRNA-CPPs conjugate benefits the effective dissociation of free siRNA
14 from the carrier in the cancer cell cytosol, thereby minimizing the CPPs' interference
15 with the silencing process and maximizing the antisense effect of siRNA (Lundberg et
16 al., 2007). However, before the successful utilization of the siRNA-CPPs conjugate, the
17 following drawbacks should be addressed: (i) non-specific penetration of siRNA-CPPs
18 into normal cells; (ii) plasma degradation of siRNA-CPPs in the body circulation; and
19 (iii) unwanted reduction of siRNA-CPPs before arrival at the targeted cell. To settle
20 these problems, siRNA-CPPs were encapsulated into the functional nanocarrier-TSL
21 modified with homing moiety NGR on the surface.

22 Using TEM and AFM, we observed that siRNA-CPPs/NGR-TSL have a regular

1 circular shape and diameters ranging from 80 to 100 nm, in accordance with that
2 determined by the Nano ZS90 instrument, as shown in Figs. 3A, 3B, and 3C. The 24 h *in*
3 *vitro* stability of siRNA-CPPs/NGR-TSL in the medium containing FBS was evaluated
4 by Turbiscan Lab[®] Expert. A salient feature of this method is that it can detect the early
5 small changes in transmission or backscattering profiles before the appearance of a
6 macroscopic scale physical modification of colloidal emulsion, thus shorten the lapse of
7 time necessary for the identification of instability phenomena (Celia et al., 2009).
8 Variations greater than 10% either as a positive or negative value in the graphical scale
9 of backscattering are the representative of an instable formulation (Marianecci et al.,
10 2010). The result shows the changes in transmission or backscattering profiles of the
11 sample were less than 5% (Fig. 3D), indicating there was no apparent aggregation or
12 sedimentation during the culture. The storage stability in Table 1 shows that
13 siRNA-CPPs/NGR-TSL was physically and chemically stable at 4 °C for up to 60 days
14 and no significant changes in mean particle size, PDI, drug entrapment encapsulation
15 and zeta potential were observed during the course of stability study. In addition, as
16 shown in Table 2, the size changes of siRNA-CPPs/NGR-TSL in PBS with different
17 ionic strength (0.01 M, 0.02 M and 0.1 M) and different FBS concentration (0, 10% and
18 100%) observed by the Nano ZS90 instrument also indicate the constructed system is
19 stable in solutions and conditions simulating the *in vivo* environment during 48 h. As
20 shown in Figure 3E, pure siRNA and siRNA-CPPs were fully degraded after 8 h and 12
21 h, respectively. In contrast, siRNA in siRNA-CPPs/NGR-TSL were not fully degraded
22 even after 24 h, indicating that siRNA-CPPs could be well protected from serum

degradation by encapsulating into NGR-TSL. All of these results are favorable for the further *in vivo* application of the system (Yang et al., 2014).

The mean diameter, encapsulation efficiency and zeta potential of the liposomes are shown in Table 3. The results show that all the liposomes had a mean particle size of approximately 90 nm and an encapsulation efficiency of approximately 86%, which facilitates the vesicles to be transported in the vasculature of the tumor tissue, permits greater accumulation of the liposomes in the tumor tissue by the enhanced permeability retention (EPR) effect and evades elimination by the kidney (Yang et al., 2013). The zeta potentials of siRNA-CPPs, siRNA-CPPs/TSL, siRNA-CPPs/NGR-TSL and siRNA-CPPs/NGR-TSL-H (preheated at 42 °C for 30 min) were 11.25 ± 1.05 mV, -22.30 ± 0.56 mV, -18.98 ± 0.62 mV and 10.98 ± 1.21 mV, respectively. Compared with unheated siRNA-CPPs/NGR-TSL, the increased zeta potential of siRNA-CPPs/NGR-TSL-H confirmed our hypothesis that the functions of CPPs could be shielded by encapsulation into TSL and activated via heat-stimulus.

3.2. Thermal stability of FAM-siRNA and heat-triggered activation of siRNA-CPPs

In this work, since the cumulative release of siRNA, cellular internalization as well as endosomal escape of siRNA were all detected via fluorescence labelled FAM-siRNA, the stability of FAM-siRNA before and after heat treatment (preheated FAM-siRNA at 42 °C for 30 min) is important. The molecular weight change of FAM-siRNA before and after heat treatment was evaluated to study the thermal stability of FAM-siRNA. The data shown in Figs. 4A and 4B suggest that there is no change of the molecular weight of FAM-siRNA before (the molecular weight is 7102.97 and

1 7104.98 calculated from 1016.2848 and 1185.4956 in Fig.4A) and after heat treatment
2 (the molecular weight is 7102.94 and 7104.96 calculated from 1016.1369 and 1185.4893
3 in Fig.4B), indicating that the heat treatment in this work did not lead to the
4 decomposition of FAM-siRNA.

5 The release of siRNA-CPPs from TSL and NGR-TSL in different medium at 37 °C
6 and 42 °C is depicted in Fig. 4 (C, D). The data indicate that the release of siRNA-CPPs
7 from TSL and NGR-TSL in three medium was all time- and temperature-dependent. The
8 release data were all very low at 37 °C, and the optimal activation condition for
9 siRNA-CPPs was at 42 °C for 30 min. These results were correlated with DPPC, the
10 major component of TSL, which has a phase transition temperature at 42 °C, and could
11 cause permeability and drug release from TSL at 42 °C (AL-Jamal et al., 2012). The
12 results also suggest that TSL modified with or without NGR did not influence the release
13 of siRNA-CPPs obviously (Fig.4C). There is no siRNA-CPPs leakage from NGR-TSL
14 in three medium of PBS (pH=7.4), MEM solution containing 10% FBS and PBS
15 (pH=6.5) which is similar to tumor microenvironment at 37 °C (shown in Fig.4D),
16 suggesting the function of siRNA-CPPs could be well masked in body circulation and
17 the tumor microenvironment. The release of siRNA-CPPs in three medium at 42 °C
18 verified that CPPs could be activated via heat stimulus. Although the release of
19 siRNA-CPPs from MEM solution containing 10% FBS showed a little higher than that
20 from PBS (pH7.4 and pH 6.5), the difference is not significant.

21 3.3. *In vitro* evaluation of siRNA-CPPs/NGR-TSL

22 HT-1080 cells were used to evaluate the *in vitro* antitumor efficiency of the

1 constructed siRNA delivery vesicles. In this part, free siRNA, siRNA-CPPs,
2 siRNA/CPPs, siRNA-CPPs/TSL and siRNA-CPPs/NGR-TSL mentioned both in the
3 figures and article mean these formulations were co-cultured with the cells directly
4 without preheating. Meanwhile, siRNA-CPPs/NGR-TSL-H and
5 siN.C.-CPPs/NGR-TSL-H mean siRNA-CPPs/NGR-TSL and siN.C.-CPPs/NGR-TSL
6 were preheated at 42 °C for 30 min before co-culturing with the cells.

7 *3.3.1. Competitive binding assay of nanocarriers, heat-triggered cellular uptake and* 8 *internalization of nanocarriers*

9 The competitive binding of siRNA-CPPs/NGR-TSL and siRNA-CPPs/TSL to
10 HT-1080 cells was evaluated firstly. As shown in Fig. 5A, the cellular uptake of
11 siRNA-CPPs/NGR-TSL in the presence of excess free NGR (1 mg/mL) in HT-1080
12 cells was suppressed significantly, becoming almost equivalent to that of
13 siRNA-CPPs/TSL. However, transfection efficiency of siRNA-CPPs/TSL was not
14 influenced by free NGR.

15 The *in vitro* internalization efficiency of various formulations was evaluated by both
16 flow cytometry and confocal laser scanning microscopy (CLSM). Considerable
17 differences in the distribution and extent of cell association were detected in various
18 formulations. As shown in Figs. 5B and 5C, siRNA-CPPs/NGR-TSL-H exhibited the
19 highest cellular uptake efficiency and the greatest cellular distribution, benefiting from
20 the synergism between NGR receptor-mediated endocytosis and the efficient
21 internalization of heat-activated CPPs. Without heat activation, the uptake of
22 fluorescently-labeled siRNA was much greater in HT-1080 cells treated with

1 siRNA-CPPs/NGR-TSL than cells treated with siRNA-CPPs/TSL. This indicates that
2 the NGR ligand increased the delivery efficiency of the nanoparticles for the
3 CD13-expressing HT-1080 cells. Due to the cell-penetrating ability of CPPs, free
4 siRNA-CPPs displayed a stronger distribution and higher cellular uptake than
5 siRNA-CPPs/NGR-TSL. Conversely, the large M_w (~13 kDa), hydrophilic nature and
6 negative zeta potential may prevent entrance of siRNA into cells by passive diffusion
7 mechanisms (Zheng et al., 2015), and no translocation of free siRNA into the HT-1080
8 cells was detected. Overall, the data from the flow cytometric and confocal laser
9 scanning microscopy are consistent with each other.

10 3.3.2. Endosomal escaping of siRNA

11 After internalization by cells via receptor-mediated endocytosis and heat activated
12 CPPs, whether siRNA-CPPs/NGR-TSL-H can overcome the intracellular endolysosomal
13 delivery barriers and efficiently deliver siRNA into the cytoplasm is crucial for its efficient
14 gene silencing activity (Hou et al., 2013). FAM-siRNA formulated in liposomes and
15 endosomes/lysosomes stained with LysoTracker (red) at different time points were tracked
16 by CLSM. As shown in Fig. 6A, for FAM-siRNA-CPPs/NGR-TSL-H, endosomal escape is
17 time-dependent. Within 0.5 h of transfection, the yellow fluorescence in the picture
18 suggests the colocalization of the green fluorescence FAM-siRNA and the LysoTracker
19 Red labeled endosomes/lysosomes. After transfecting for 2.5 h, the FAM-siRNA escaped
20 from the lysosomes and was released into the cytoplasm. And most of the green
21 fluorescence was observed in the cytosol and was not colocalized with the LysoTracker
22 (red), as shown in Fig. 6B. In addition, the yellow fluorescence observed in Fig. 6C

1 indicated that most of the unheated FAM-siRNA-CPPs/NGR-TSL was entrapped by
2 endosomes/lysosomes and could not escape the entrapment of endosomes/lysosomes even
3 after transfecting for 2.5 h.

4 The ability of FAM-siRNA-CPPs/NGR-TSL-H to escape the endosomal
5 entrapment was attributed to its cell internalization form, which was in the form of
6 siRNA-CPPs. The delivery carrier-CPPs (CKRRMKWKK) used in this work was
7 derived from penetratin. Molecules covalently attached to penetratin can be efficiently
8 delivered into the cytosol (Varkouhi et al., 2011). Our results shown in Fig. 6B also
9 confirmed this report. Although the escape mechanism of penetratin is not clear in the
10 current reports, in this study, the mechanism is likely related with the CPPs used, which
11 are rich in lysine and arginine amino acid residues. Several studies have demonstrated
12 that lysine-rich peptides could mimic the endosomal disruptive properties of viral
13 particles, penetrate cells and facilitate the delivery of nucleic acids (Morris et al., 1999).
14 In addition, arginine moieties with cell-penetrating ability also enhance siRNA transport
15 into the cytosol (Zhao et al., 2012). However, the cell internalization form of
16 FAM-siRNA-CPPs/NGR-TSL was liposomes, and most of which will end up inside the
17 cell within endosomes (Yudina et al., 2011).

18 3.3.3. *c-myc* gene silencing efficiency *in vitro* and cell apoptosis assay

19 The *in vitro* gene silencing activity of *c-myc* siRNA against the human *c-myc* gene
20 delivered by the nanocarrier was further determined by qRT-PCR and western blot
21 analysis. As shown in Figs. 7A and 7B, the *c-myc* mRNA and protein expression of
22 HT-1080 cells treated with siRNA-CPPs/NGR-TSL-H were significantly inhibited ($P <$

0.01). In addition, HT-1080 cells treated with unheated siRNA-CPPs/NGR-TSL showed similar c-myc mRNA and protein expression as the unheated siRNA-CPPs/TSL group, both of which displayed only slight downregulation of c-myc. Compared with siRNA-CPPs, free c-myc siRNA did not show greater silencing activity. siN.C.-CPPs/NGR-TSL-H did not show any gene silencing activity on c-myc. To clarify the gene silencing activity of siRNA-CPPs assisted by GSH in the cytosol, a comparison study of the gene silencing activity was performed between the siRNA-CPPs conjugate and the siRNA/CPPs complex (siRNA and CPPs complexed via electrostatic interaction, zeta potential: 26.32 ± 2.01 mv). From the results (Figs. 7A and 7B), we can conclude that the siRNA-CPPs conjugate enables an advantageous improvement in the endogenous gene-silencing efficiency when compared to the simple electrostatic complex of siRNA/CPPs counterparts.

In the treated cells, apoptosis induced by various formulations carrying c-myc siRNA and siN.C. was also evaluated by flow cytometry. As shown in Fig. 7C, cells exposed to c-myc siRNA-loaded formulations showed significant apoptosis, whereas only a slight effect was observed in the control, free c-myc siRNA and siN.C.-CPPs/NGR-TSL-H groups, indicating that apoptosis mainly originated from the downregulated expression of c-myc in the HT-1080 cells. Cell apoptosis was in the order of siRNA-CPPs/NGR-TSL-H (56.41%) > siRNA-CPPs (49.34%) > siRNA/CPPs (39.78%) > siRNA-CPPs/NGR-TSL (19.57%) $\approx$ siRNA-CPPs/TSL (19.23%) > free siRNA (3.81%) > control (2.28%) > siN.C.-CPPs/NGR-TSL-H (1.47%), which was consistent with the c-myc protein expression results described above.

1 Because cytoplasm is the right place for the RNAi of siRNA, an ideal siRNA
2 transfection vector should fulfill the following: (i) transport siRNA into the target tumor
3 cell to avoid the potential of an ‘off-target’ effect (Oh and Park, 2009); (ii) penetrate into
4 the cell membrane and escape from the endosomes to deliver siRNA into the cytoplasm
5 (Zheng et al., 2015); and (iii) effectively dissociate free siRNA from the carrier in the
6 cytoplasm to reduce the interference with the processing and antisense effect of siRNA
7 (Zheng et al., 2015). In this study, the designed vector satisfied the above three keys. The
8 strongest gene knockdown and cell apoptosis induced by siRNA-CPPs/NGR-TSL-H can
9 be attributed to: (i) NGR ligands increased the cell binding ability and cellular uptake of
10 the vesicles by receptor-mediated internalization; (ii) heat-activated siRNA-CPPs could
11 penetrate the cell membrane and escape from the entrapment of endosomes to deliver
12 siRNA into the cytoplasm; and (iii) the covalent disulfide bond of siRNA-CPPs could be
13 reduced by GSH in the cytoplasm, thus releasing bioactive siRNA to cause
14 sequence-specific mRNA degradation in HT-1080 cells. Although unheated
15 siRNA-CPPs/NGR-TSL showed higher levels of cellular uptake than unheated
16 siRNA-CPPs/TSL, their efficiency in mediating c-myc gene silencing was almost
17 identical. One possible explanation for the lack of gene silencing mediated by
18 unheated siRNA-CPPs/NGR-TSL and unheated siRNA-CPPs/TSL is that they were
19 internalized by cells in the form of the liposome, which could not escape the endosomal
20 compartment upon cellular internalization to complete the siRNA delivery process (as
21 shown in Fig. 6C). As mentioned above, the superior efficacy of siRNA-CPPs to
22 siRNA/CPPs in silencing c-myc *in vitro* was in virtue of its effective dissociation of

1 siRNA from CPPs upon redox stimulus by GSH. In other words, the siRNA/CPPs
2 complex could not deliver free siRNA effectively, and the complexed CPPs interfered
3 with the RNAi efficacy of siRNA. The results complied with the report that peptides
4 binding too strongly to siRNA prevent successful RNAi (Frazier and Ghandehari, 2015).
5 Because the anionic charge of backbone phosphates (~ 40 /molecule) makes it impossible
6 for siRNA to interact with anionic phospholipid cell membranes (Ruvinov et al., 2015),
7 free siRNA showed weak gene silencing activity and cell apoptosis. The negligible gene
8 knockdown efficacy of siN.C.-CPPs /NGR-TSL-H *in vitro* suggested that the c-myc
9 siRNA used in this work is gene-specific. The results of this study verified that the
10 constructed siRNA-CPPs/NGR-TSL could saliently facilitate the transport of c-myc
11 siRNA under the dual stimulus of hyperthermia and the intracellular redox environment.

12 3.4. *In vivo antitumor evaluation of siRNA-CPPs/NGR-TSL under hyperthermia*

13 In this part, HT-1080 xenografted nude mice were used to evaluate the *in vivo*
14 antitumor efficiency of the constructed siRNA transporting vesicles. Herein, free siRNA,
15 siRNA-CPPs, siRNA-CPPs/TSL, siRNA-CPPs/NGR-TSL and siN.C.-CPPs/NGR-TSL
16 mentioned both in the figures and text mean the mice groups treated by these
17 formulations were all subjected to hyperthermia after tail vein injection. On the other
18 hand, siRNA-CPPs/NGR-TSL-unheated mentioned means the mice group administered
19 by this formulation was not combined with hyperthermia.

20 3.4.1. *In vivo distribution of c-myc siRNA carried formulations*

21 After observing the improved biological performance of c-myc siRNA nanocarriers
22 *in vitro*, we sought to clarify the potential for these systems to traffic to solid tumors and

1 induce knockdown following systemic administration. *In vivo* fluorescence imaging
2 provides a convenient and rapid assessment of real-time biological processes.

3 Here, a tumor xenograft model was generated in female nude mice by subcutaneous
4 injection with HT-1080 cells, and the model was used to study the *in vivo* distribution of
5 Cy3-labeled siRNA in different carriers with hyperthermia. The *in vivo* imaging system
6 was used to visualize the fluorescence intensity of Cy3-siRNA-encapsulated vesicles at
7 different time points after intravenous injection. The whole-animal imaging (Fig. 8A)
8 indicated that in mice treated with PBS, no fluorescence signals were detected during the
9 experimental period. The fluorescence intensity of free siRNA rapidly decreased after
10 administration, and there was no or less accumulation in the tumor. The reason for this
11 phenomenon is related with the inherent problems of siRNA, such as its instant
12 degradation by RNase (a type of nucleases) and rapid renal excretion after intravenous
13 injection. Under hyperthermia, siRNA-CPPs/NGR-TSL-treated mice exhibited the most
14 intense tumor distribution during the whole period. This phenomenon suggested the
15 introduction of NGR-induced tumor-targeting, hyperthermia as well as activated CPPs,
16 enhanced its accumulation in tumors. Compared with siRNA-CPPs/NGR-TSL, although
17 siRNA-CPPs/TSL lost the superiority of the NGR moiety, mice treated by this
18 formulation also displayed intense fluorescence accumulation in the tumors. This could
19 be attributed to the hyperthermia in the tumor sites, which could benefit the targeting
20 accumulation to some extent (Viglianti, 2009). In contrast, siRNA-CPPs-treated mice
21 showed discrete fluorescence distribution in many organs, including the tumor, which is
22 attributed to the non-specific cell penetration of CPPs. Significantly reduced

1 fluorescence intensity after 12 h occurred in the siRNA-CPPs group verified that
2 siRNA-CPPs was not stable *in vivo*. The results also indicate that mice injected with
3 siRNA-CPPs/NGR-TSL-unheated showed less fluorescence intensity in the tumor
4 because the cell-penetrating ability of CPPs was not activated.

5 The fluorescent signals of Cy3-siRNA in deep organs could not be judged from
6 the whole-animal imaging because of the optical impedance by soft tissues.
7 Consequently, isolated organs and tumor tissues were further observed by sacrificing the
8 mice 24 h after administration (Fig. 8B). As observed from the images, under
9 hyperthermia, tumors from the siRNA-CPPs/NGR-TSL-treated groups displayed the
10 strongest fluorescence signals, whereas less or no fluorescence was observed in other
11 isolated organs of this group. The results implied that siRNA-CPPs/NGR-TSL combined
12 with hyperthermia efficiently targeted solid tumors and decreased non-specific
13 accumulation in normal organs. In addition to tumors, siRNA-CPPs/TSL-treated groups
14 under hyperthermia also exhibited bright fluorescence signals in the liver, indicating that
15 this formulation might undergo non-specific RES (reticular endothelial system) uptake
16 *in vivo*. Free siRNA-treated mice groups revealed less fluorescence in isolated tumors
17 but bright fluorescence intensity in the liver and kidney, indicating that the *in vivo* fate
18 of siRNA involved renal excretion as well as non-specific RES uptake. The free
19 siRNA-CPPs-treated groups showed fluorescence in all isolated organs, which was
20 stronger in the liver and kidney, resulting from the non-specificity of CPPs, RES uptake
21 and renal excretion *in vivo*. For the siRNA-CPPs/NGR-TSL-unheated group,
22 fluorescence was mainly distributed in the liver, tumor and kidney, demonstrating that

1 the vesicles could target tumors to some extent via NGR mediation. The vesicles could
2 also be subject to RES uptake and renal excretion. However, the related mechanisms of
3 the strong liver uptake of NGR-mediated vesicles *in vivo* warrant further study.

4 These *in vivo* imaging data indicate that siRNA-CPPs/NGR-TSL combined with
5 hyperthermia could maintain siRNA-CPPs stable in the body circulation and specifically
6 transport siRNA into HT-1080 cells compared to free siRNA-CPPs with hyperthermia
7 and siRNA-CPPs/NGR-TSL-unheated.

8 3.4.2. *In vivo* antitumor efficacy

9 Inspired by the satisfactory tumor targeting results, we further explored the *in vivo*
10 anticancer activity of each sample carrying siRNA in HT-1080 xenografted nude mice.
11 As expected, the results shown in Fig. 9A indicate that siRNA-CPPs/NGR-TSL under
12 hyperthermia possessed the best antitumor efficiency ($147.51 \pm 29.79 \text{ mm}^3$), which is
13 3.05- and 3.14-fold greater compared to free siRNA-CPPs under hyperthermia
14 ($451.23 \pm 70.01 \text{ mm}^3$) and siRNA-CPPs/NGR-TSL-unheated ($463.97 \pm 78.12 \text{ mm}^3$)
15 respectively. The less tumor growth inhibition of free siRNA-CPPs was due to the
16 degradation and reduction of siRNA-CPPs in the body circulation. Although
17 siRNA-CPPs/NGR-TSL-unheated can be internalized by tumor cells via NGR-mediated
18 active targeting and internalization, the internalized vesicles are trapped in the
19 endosomes (verified in the endosomal escape section) and can't deliver siRNA
20 efficiently into its action sites. It was reported that mild hyperthermia augments the
21 accumulation of liposomes at tumor sites, and the effect was the doubling of
22 accumulation for every increase in degree from 39 °C to 42 °C (Viglianti, 2009; Kong et

1 al., 2001). Our results of *in vivo* biodistribution (shown in Fig. 8) also verified that
2 hyperthermia could augment the accumulation of liposomes at tumor sites, and thus
3 siRNA-CPPs/TSL without NGR moieties under hyperthermia also displayed stronger
4 tumor growth inhibition ($250.63 \pm 32.01 \text{ mm}^3$). Due to the inherent shortfalls of free
5 siRNA mentioned above, mice in the free siRNA group showed no significant tumor
6 growth inhibition ($495.67 \pm 78.67 \text{ mm}^3$) compared with the control groups (PBS
7 $498.79 \pm 77.31 \text{ mm}^3$ and siN.C.-CPPs/NGR-TSL $506.15 \pm 77.16 \text{ mm}^3$).

8 The clinically therapeutic benefits of cancer patients are mainly determined by the
9 enhancement of life quality and increased survival time (Friedman et al., 2009). To
10 further estimate the antitumor efficacy, the body weight of the tumor-bearing mice was
11 assessed. As shown in Fig. 9B, except for the mice group treated with free siRNA-CPPs,
12 all mice treated in this work showed no pronounced body weight loss, indicating the
13 body safety of our formulations. The significant weight loss of the siRNA-CPPs-treated
14 mice groups is correlated with the nonspecific targeting of CPPs, which leads to damage
15 of normal tissues.

16 To confirm the gene silencing activity of siRNA-CPPs/NGR-TSL under mild
17 hyperthermia more clearly *in vivo*, the c-myc expression at the mRNA and protein level
18 in tumors was assayed by qRT-PCR and western blot, respectively, after sacrificing
19 three mice in each group at the end of the experiment. Consistent with the
20 aforementioned results, under hyperthermia, siRNA-CPPs/NGR-TSL induced the
21 highest gene silencing effect of $37.25 \pm 6.24\%$, which is 2.36- and 2.30-fold greater than
22 free siRNA-CPPs under hyperthermia ($87.74 \pm 7.05\%$) and

1 siRNA-CPPs/NGR-TSL-unheated ($85.53 \pm 5.98\%$) respectively (shown in Fig. 9C). As
2 for free siRNA and siN.C-CPPs/ NGR-TSL, the expression of c-myc mRNA and c-myc
3 protein showed no obvious alterations compared to the PBS-treated group. Compared
4 with siRNA-CPPs/NGR-TSL, siRNA-CPPs/TSL under hyperthermia displayed a lower
5 gene silencing effect but showed a higher downregulating effect compared to other
6 formulations. Overall, from these *in vivo* gene transfection results, it can be concluded
7 that there is a direct causality between delayed tumor progression and the silencing of
8 the c-myc gene.

9 Tumor cell apoptosis is usually assayed by analyzing DNA strand breaks using the
10 terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL)
11 assay (Yang et al., 2012). As shown in Fig. 10, there are no detectable TUNEL-positive
12 tumor cells (green) in either the PBS or free siRNA-treated groups. However, compared
13 with both the free siRNA-CPPs and no hyperthermia groups, under hyperthermia,
14 siRNA-CPPs containing TSL and NGR-TSL exhibited pronounced cell apoptosis. The
15 apoptotic analysis expressed a similar trend with the antitumor efficacy *in vivo*.

16 Generally speaking, the *in vitro* and *in vivo* results verified our hypothesis that
17 siRNA-CPPs/NGR-TSL in combination with hyperthermia explored in this study is an
18 ideal platform for tumor-specific delivery of siRNA. The superior antitumor activity of
19 siRNA-CPPs/NGR-TSL with hyperthermia is due to the following combined effects: (i)
20 NGR targeting to the CD13-over-expressed HT-1080 cells; (ii) siRNA-CPPs protection
21 by TSL in body circulation and function activation by thermal-stimulus at the targeting
22 sites; (iii) successful endosomal escape with the help of the CPPs used in this study; and

(iv) efficient dissociation of free siRNA from CPPs in the cytosol with GSH stimulus, which improved siRNA knockdown efficiency by reducing the interference of carriers. More importantly, our results confirmed that although free siRNA-CPPs displayed efficient gene silencing effect *in vitro*, this formulation showed less bioactivity *in vivo*, which may be attributed to the degradation of CPPs and reduction of siRNA-CPPs in body circulation. The results in this work demonstrate that comparing with free siRNA-CPPs under hyperthermia and siRNA-CPPs/NGR-TSL without hyperthermia, siRNA-CPPs/NGR-TSL in combination with hyperthermia developed could maintain siRNA-CPPs stable in the body circulation, and thus improve its cancer therapeutic effect remarkably.

4. Conclusions

A method to efficiently transport siRNA into the cytosol of target tumor cells remains to be developed for siRNA-carrying vesicles. In this study, under the dual stimulus of hyperthermia and the redox effect of GSH in the cytosol, a functional delivery system (siRNA-CPPs/NGR-TSL) combining the triple effects of NGR, TSL and bio-reducible siRNA-CPPs was constructed. The research data demonstrated that the vesicle possessed appropriate physicochemical properties and could successfully deliver c-myc siRNA into the cytosol of HT-1080 tumor cells. The vesicle with heat stimulus exhibited higher cellular uptake, ideal endosomal escape, excellent inhibition of c-myc expression and strong HT-1080 cell apoptosis compared with other control formulations. Using an HT-1080 xenograft murine model, *in vivo* studies showed that siRNA-CPPs/NGR-TSL combined with hyperthermia results in elevated tumor

1 accumulation, augmented c-myc silencing, delayed tumor progression, good body safety
2 and reinforced tumor cell apoptosis. The results of this study indicated the tremendous
3 potential of these dual-triggered vesicles for the efficient delivery of siRNA for
4 oncotherapy.

5 **Conflict of interest disclosure**

6 The authors declare no competing financial interests.

7 **Acknowledgments**

8 This work was supported by the National Science Foundation of China (No.
9 81402874 & No. 81202466). The authors also acknowledge the financial support from
10 PUMC Youth Fund and the Fundamental Research Funds for the Central Universities
11 (No. 3332015139).

12 **References**

- 13 AL-Jamal, W.T., AL-Ahmady, Z.S., Kostarelos, K., 2012. Pharmacokinetics & tissue
14 distribution of temperature-sensitive liposomal doxorubicin in tumor-bearing mice
15 triggered with mild hyperthermia. *Biomaterials* 33, 4608-4617.
- 16 Chen, Y., Wu, J.J., Huang, L., 2010. Nanoparticles targeted with NGR motif deliver
17 c-myc siRNA and doxorubicin for anticancer therapy. *Mol. Ther.* 18, 828-834.
- 18 Celia, C., Trapasso, E., Cosco, D., Paolino, D., Fresta, M., 2009. Turbiscan Lab[®] Expert
19 analysis of the stability of ethosomes[®] and ultradeformable liposomes containing a
20 bilayer fluidizing agent. *Colloids Surf B. Biointerfaces* 72, 155-160.
- 21 De Backer, L., Braeckmans, K., Stuart, M.C.A., Demeester, J., De Smedt, S.C.,
22 Raemdonck, K., 2015. Bio-inspired pulmonary surfactant-modified nanogels: A

1 promising siRNA delivery system. *J. Control. Release* 206, 177-186.

2 Davidson, T.J., Harel, S., Arboleda, V.A., Prunell, G.F., Shelanski, M.L., Greene, L.A., et

3 al., 2004. Highly efficient small interfering RNA delivery to primary mammalian

4 neurons induces MicroRNA-like effects before mRNA degradation. *J. Neurosci* 24,

5 10040-10046.

6 Frazier, N., Ghandehari, H., 2015. Hyperthermia Approaches for Enhanced Delivery of

7 Nanomedicines to Solid Tumors. *Biotechnol. Bioeng.* 112, 1967-1983.

8 Friedman, H.S., Prados, M.D., Wen, P.Y., Mikkelsen, T., Schiff, D., Abrey, L.E., et al.,

9 2009. Bevacizumab Alone and in Combination with Irinotecan in Recurrent

10 Glioblastoma. *J. Clin. Oncol.* 27, 4733-4740.

11 Geoghegan, J.C., Gilmore, B.L., Davidson, B.L., 2012. Gene Silencing Mediated by

12 siRNA-Binding Fusion Proteins Is Attenuated by Double-Stranded RNA-Binding

13 Domain Structure. *Mol. Ther.; Nucleic Acids* 1, e53.

14 Gao, J., Yu, Y., Zhang, Y., Song, J., Chen, H., Li, W., et al., 2012. EGFR-specific

15 PEGylated immunoliposomes for active siRNA delivery in hepatocellular

16 carcinoma, *Biomaterials* 33, 270-282.

17 Hou, K.K., Pan, H., Ratner, L., Schlesinger, P.H., Wickline, S.A., 2013. Mechanisms of

18 nanoparticle-mediated siRNA transfection by melittin-derived peptides. *ACS Nano*

19 7, 8605-8615.

20 Koning, G.A., Eggermont, A.M.M., Lindner, L.H., ten Hagen, T.L.M., 2010.

21 Hyperthermia and Thermosensitive Liposomes for Improved Delivery of

22 Chemotherapeutic Drugs to Solid Tumors. *Pharm Res.* 27, 1750-1754.

1 Kong, G., Braun, R.D., Dewhirst, M.W., 2001. Characterization of the effect of
2 hyperthermia on nanoparticle extravasation from tumor vasculature. *Cancer Res.* 61,
3 3027-3032.

4 Lorenzer, C., Dirin, M., Winkler, A.M., Baumann, V., Winkler, J., 2015. Going beyond
5 the liver: Progress and challenges of targeted delivery of siRNA therapeutics. *J.*
6 *Control. Release* 203, 1-15.

7 Lundberg, P., WI-Andaloussi, S., Sütli, T., Johansson, H., Langel, U., 2007. Delivery of
8 short interfering RNA using endosomolytic cell-penetrating peptides. *FASEB J.* 21,
9 2664-2671.

10 Lee, S.J., Son, S., Yhee, J.Y., Choi, K., Kwon, I.C., Kim, S.H., et al., 2013. Structural
11 modification of siRNA for efficient gene silencing. *Biotechnol. Adv.* 31, 491-503.

12 Moschos, S.A., Jones, S.W., Perry, M.M., Williams, A.E., Erjefalt, J.S., Turner, J.J., et al.,
13 2007. Lung delivery studies using siRNA conjugated to TAT (48-60) and penetratin
14 reveal peptide induced reduction in gene expression and induction of innate
15 immunity. *Bioconjugate Chem.* 18, 1450-1459.

16 Manzoor, A.A., Lindner, L.H., Landon, C.D., Park, J.Y., Simnick, A.J., Dreher, M.R., et
17 al., 2012. Overcoming limitations in nanoparticle drug delivery: triggered,
18 intravascular release to improve drug penetration into tumors. *Cancer Res.* 72,
19 5566-5575.

20 Muratovska, A., Eccles, M.R., 2004. Conjugate for efficient delivery of short interfering
21 RNA (siRNA) into mammalian cells. *FEBS Lett.* 558, 63-68.

22 Marianecci, C., Paolino, D., Celia, C., Fresta, M., Carafa, M., et al., 2010. Non-ionic

1 surfactant vesicles in pulmonary glucocorticoid delivery: characterization and
2 interaction with human lung fibroblasts. *J. Control. Release* 147, 127-135.

3 Morris, M.C., Chaloin, L., Méry, J., Heitz, F., Divita, G., 1999. A novel potent strategy
4 for gene delivery using a single peptide vector as a carrier. *Nucleic Acids Res.* 27,
5 3510-3517.

6 Oh, Y.K., Park, T.G., 2009. siRNA delivery systems for cancer treatment, *Adv. Drug*
7 *Delivery Rev.* 61, 850-862.

8 Ranjan, A., Jacobs, G.C., Woods, D.L., Negussie, A.H., Partanen, A., Yarmolenko, P.S.,
9 et al., 2012. Image-guided drug delivery with magnetic resonance guided high
10 intensity focused ultrasound and temperature sensitive liposomes in a rabbit Vx2
11 tumor model. *J. Control. Release* 158, 487-494.

12 Ruvinov, E., Kryukov, O., Forti, E., Korin, E., Goldstein, M., Cohen, S., 2015.
13 Calcium-siRNA nanocomplexes: What reversibility is all about. *J. Control. Release*
14 203,150-160.

15 Saito, G., Swanson, J.A., Lee, K.D., 2013. Drug delivery strategy utilizing conjugation
16 via reversible disulfide linkages: role and site of cellular reducing activities. *Adv.*
17 *Drug Delivery Rev.* 55, 199-215.

18 Turner, J.J., Jones, S., Fabani, M.M., Ivanova, G., Arzumanov, A.A., Gait, M.J., 2007.
19 RNA targeting with peptide conjugates of oligonucleotides, siRNA and PNA. *Blood*
20 *Cells, Mol., Dis.* 38, 1-7.

21 Tagami, T., Foltz, W.D., Ernsting, M.J., Lee, C.M., Tannock, I.F., Maya, J.P., et al.,
22 2011. MRI monitoring of intratumoral drug delivery and prediction of the

1 therapeutic effect with a multifunctional thermosensitive liposome. *Biomaterials* 32,
2 6570-6578.

3 Varkouhi, A.K., Scholte, M., Storm, G., Haisma, H.J., 2011. Endosomal escape pathways
4 for delivery of biological. *J. Control. Release* 151, 220-228.

5 Viglianti, B.L., 2009. Target molecular therapies: methods to enhance and monitor
6 tumor drug delivery. *Abdom Imaging*. 34, 686-695.

7 Yu, Q.Q., Liu, Y.N., Cao, C.W., Le, F.L., Qin, X.Y., Sun, D.D., et al., 2014. The use of
8 pH-sensitive functional selenium nanoparticles shows enhanced in vivo
9 VEGF-siRNA silencing and fluorescence imaging. *Nanoscale* 6, 9279-9292.

10 Yang, F.F., Li, Y.F., Hang, W., Chen, W., Jin, M.J., Gao, Z.G., 2013. Cell-penetrating
11 peptide TAT modified chitosan for siRNA delivery. *J. Control. Release* 172,
12 E100-E101.

13 Yang, Y.F., Yang, Y., Xie, X.Y., Cai, X.S., Zhang, H., Gong, W., et al., 2014. PEGylated
14 liposomes with NGR ligand and heat-activable cell-penetrating
15 peptide-doxorubicin conjugate for tumor-specific therapy. *Biomaterials* 35,
16 4368-4381.

17 Yang, Y.F., Xie, X.Y., Yang, Y., Zhang, H., Mei, X.G., 2013. A review on the
18 influences of size and surface charge of liposome on its targeted drug delivery in
19 vivo. *Acta Pharm. Sin.* 48, 1644-1650.

20 Yudina, A., de Smet, M., Lepetit-Coiffé, M., Langereis, S., Van Ruijssevelt, L., Smirnov,
21 P., et al., 2011. Ultrasound-mediated intracellular drug delivery using microbubbles
22 and temperature-sensitive liposomes. *J. Control. Release* 155, 442-448.

- 1 Yang, X.Z., Dou, S., Wang, Y.C., Long, H.Y., Xiong, M.H., Mao, C.Q., et al., 2012.
2 Single-step assembly of cationic lipid-polymer hybrid nanoparticles for systemic
3 delivery of siRNA. ACS Nano 6, 4955-4965.
- 4 Zhang, X., Luckham, P.F., Hughes, A.D., Thom, S., Xu, X.Y., 2011. Development of
5 lysolipid based thermosensitive liposomes for delivery of high molecular weight
6 proteins. Int J Pharm. 421, 291-292.
- 7 Zheng, N., Song, Z.Y., Zhang, R.J., Zhang, R.Y., Yao, C., Uckun, F.M., et al., 2015.
8 Redox-responsive, reversibly-crosslinked thiolated cationic helical polypeptides for
9 efficient siRNA encapsulation and delivery. J. Control. Release 205, 231-239.
- 10 Zhao, Z.X., Gao, S.Y., Wang, J.C., Chen, C.J., Zhao, E.Y., Hou, W.J., et al., 2012.
11 Self-assembly nanomicelles based on cationic mPEG-PLA-b-Polyarginine (R15)
12 triblock copolymer for siRNA delivery. Biomaterials 33, 6793-6807.

14 **Legends**

15 **Fig.1.** Schematic illustration of siRNA-CPPs/NGR-TSL for specific siRNA delivery to
16 tumor cells under dual stimulus of hyperthermia and intracellular redox environment.
17 NGR-modified liposomes are retained in the tumor due to the active targeting effect by
18 the NGR ligand. In the body circulation, as siRNA-CPPs were encapsulated in
19 NGR-TSL, they can't penetrate into the normal cell membrane and be degraded by
20 enzyme in the blood. However, upon heat-stimulus at the tumor site, siRNA-CPPs were
21 released, the interaction of CPPs with the cell membrane is restored and mediated
22 siRNA rapidly enter into the cells membrane and escape from the endosomal entrapment

1 into the cytosol. At last, free siRNA was released from the CPPs via disulfide-bond
2 broken under the stimulus of GSH in the cytosol and silenced c-myc gene over
3 expressed in HT-1080 cells.

4 **Fig. 2.** (A) Schemes on the synthesis of the siRNA-CPPs conjugate. (B) MALDI-TOF
5 mass spectra of siRNA. (C) MALDI-TOF mass spectra of the synthesized siRNA-CPPs
6 conjugate.

7 **Fig. 3.** Design and characterization of the constructed siRNA-CPPs/NGR-TSL: (A)
8 Transmission electron micrographs. (B) Atomic force microscopic images (three
9 dimension image). (C) Particle size distribution. (D) Transmission and backscattering
10 profiles of liposomes using Turbiscan Lab® Expert, data are reported as a function of
11 time (0-24 h) and sample height (from 28 to 50 mm). (E) siRNA serum stability assay.

12 **Fig. 4.** Thermal stability of FAM-siRNA before and after heat treatment (preheated at
13 42 °C for 30 min) (A, B) and Temperature-triggered release behaviors of siRNA-CPPs
14 at 37 °C and 42 °C, respectively, (n = 3) (C, D). (A) Molecular weight of FAM-siRNA
15 determined by Q-TOF equipped with ESI before heat treatment; (B) Molecular weight
16 of FAM-siRNA determined by Q-TOF equipped with ESI after heat treatment; (C) From
17 the fabricated TSL and NGR-TSL in PBS (pH7.4); (D) From NGR-TSL in different
18 medium of PBS (pH7.4), MEM solution containing 10% FBS and PBS (pH=6.5) which
19 is similar to tumor microenvironment.

20 **Fig. 5.** Cellular uptake and internalization of nanocarriers. (A) Competitive binding
21 assay of siRNA-CPPs/NGR-TSL and siRNA-CPPs/TSL into HT-1080 cells with or
22 without 1 mg/mL free NGR. (B) FACS analysis of the cellular uptake after incubation

1 with various formulations. (C) Cellular localization of siRNA containing samples in
2 HT-1080 cells by confocal microscopy. Hoechst 33258 for nuclei staining (blue) and
3 FAM-siRNA fluorescence (green) were recorded. The data are presented as the means $\pm$
4 SD (n = 3). ** indicates $P < 0.01$.

5 **Fig. 6.** Intracellular trafficking of siRNA encapsulated in siRNA-CPPs/NGR-TSL with
6 or without heat stimulus. (A) 0.5 h after the duration of siRNA-CPPs/NGR-TSL-H
7 (preheated ones) administration. (B) 2.5 h after the duration of
8 siRNA-CPPs/NGR-TSL-H (preheated ones) administration. (C) 2.5 h after the duration
9 of siRNA-CPPs/NGR-TSL (unheated ones) administration. Hoechst 33258 for nuclei
10 staining (blue), FAM-siRNA fluorescence (green) and LysoTracker Red for
11 endosomes/lysosomes (red) were recorded.

12 **Fig. 7.** In vitro evaluation of various siRNA containing samples. (A) The level of c-myc
13 mRNA determined by qRT-PCR. (B) c-myc protein expression determined by western
14 blot analysis. (C) Cell apoptosis following exposure to different formulations. HT-1080
15 cells were individually treated with various formulations carrying c-myc siRNA or siN.C.
16 (100 nM) at 37 °C for 5 h, followed by 48 h (A) or 72 h (B, C) of regular culture.
17 Early apoptotic cells are shown in the lower right quadrant, and late apoptotic cells are
18 shown in the upper right quadrant. The data are presented as the means $\pm$ SD (n = 3). * P
19 < 0.05 ; ** $P < 0.01$.

20 **Fig. 8.** Biodistribution of Cy3-siRNA contained in various samples in mice bearing
21 HT-1080 tumor xenografts. (A) Whole body imaging at different time points after
22 systemic administration. (B) Fluorescence detection of isolated main tissues and organs

1 from mice at the end point of observation. The control was PBS.

2 **Fig. 9.** Anticancer efficacy in the HT-1080 xenografts in female nude mice after the
3 treatment with varying formulations. (A) Tumor volume (mm³) and (B) body weight
4 changes in HT-1080 tumor-bearing mice after treatment with PBS and various samples
5 carrying c-myc siRNA or siN.C. Arrows represent drug administration. Data are
6 presented as means $\pm$ SD (n=6). *P < 0.05 for siRNA-CPPs/NGR-TSL group versus
7 siRNA-CPPs/TSL. **P < 0.01 for PBS, free siRNA, free siRNA-CPPs and
8 siRNA-CPPs/NGR-TSL–unheated groups versus both siRNA-CPPs/NGR-TSL and
9 siRNA-CPPs/TSL groups. (C) Expression of c-myc mRNA and (D) c-myc protein in
10 tumors was detected 24 h after the last administration. *P < 0.05, **P < 0.01 (n = 3). The
11 exposure was performed at a 1.2 mg/kg siRNA equivalent dose once every other day for
12 a total of 14 d.

13 **Fig. 10.** TUNEL detection of apoptotic cells in tumor tissues following treatment with
14 different formulations. The tumor tissues were collected 24 h after the final
15 administration. Green: apoptotic cells marked by FITC; Red: PI for staining cell nucleus.

Table 1 Storage stability of siRNA-CPPs/NGR-TSL at 4 °C

Time (days)	Diameter (nm)	PDI	Encapsulation efficiency (%)	Zeta potential (mv)
0	90.23 ± 0.00	0.018 ±0.007	84.13 ± 2.03	-18.98±0.62
1	89.95 ± 0.03	0.016 ±0.009	84.09 ± 2.98	-18.78±0.73
3	90.15 ± 0.06	0.015 ±0.007	83.03 ± 2.79	-17.99±0.89
5	89.98 ± 0.01	0.017 ±0.008	84.21 ± 1.99	-17.98±0.88
7	89.92 ± 0.03	0.013 ±0.009	84.31 ± 1.56	-18.39±0.74
10	90.18 ± 0.02	0.016 ±0.006	82.99± 2.53	-18.68±0.56
15	90.25 ± 0.01	0.018 ±0.003	83.67 ± 2.01	-17.98±0.97
30	90.05 ± 0.05	0.019 ±0.002	84.01 ± 2.68	-18.76±0.52
45	89.99 ± 0.04	0.017 ±0.007	83.97 ± 1.67	-18.19±0.54
60	90.07 ± 0.05	0.016 ±0.008	84.07 ± 2.14	-18.76±0.71

Table 2 size changes of siRNA-CPPs/NGR-TSL in different solution with different time

Solution /Time/nm	0h	6 h	12 h	24 h	48 h
0.01M PBS	90.02	90.02	89.98	90.01	90.03
0.02M PBS	89.98	89.99	89.97	90.01	90.01
0.1M PBS	90.23	90.21	90.23	90.22	90.19
0.1MPBS+10%FBS	90.32	90.29	90.25	90.31	90.33
MEM+10%FBS	91.01	90.96	90.93	91.02	91.03
FBS	91.21	91.21	91.19	91.22	91.22

The pH value of PBS used in this table is 7.4, which modeled the in vivo pH value.

Table 3 Characteristics of siRNA contained samples

samples	Diameter (nm)	PDI	Encapsulation efficiency (%)	Zeta potential (mv)
siRNA-CPPs	—	—	—	11.25±1.05
siRNA-CPPs/TSL	88.49 ± 0.41	0.021 ±0.005	85.07 ± 1.91	-22.30±0.56
siRNA-CPPs/NGR-TSL	90.23 ± 0.00	0.018 ±0.007	84.13 ± 2.03	-18.98±0.62
siRNA-CPPs/NGR-TSL-H	—	—	—	10.98±1.21

The data are expressed as the mean ± SD value for at least three different preparations

Figure 1

Figure 1

Figure 2

(B)

(C)

Figure2

Figure 3

Figure 3

Figure 4

(A)

(B)

(C)

(D)

Figure 4

Figure 5

(A)

(B)

(C)

Figure 5

Figure 6

Figure 6

Figure 7

(A)

(B)

(C)

Figure 7

Figure 8

Figure 8

Figure 9

(A)

(C)

(B)

(D)

Figure 9

Figure 10

Figure 10

Graphical Abstracts

Scheme 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't 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.