Biomaterials 53 (2015) 330–340

Contents lists available at ScienceDirect

Biomaterials

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

Single-component self-assembled RNAi nanoparticles functionalized with tumor-targeting iNGR delivering abundant siRNA for efficient glioma therapy

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ARTICLE INFO

Article history:
Received 10 February 2015
Received in revised form
16 February 2015
Accepted 19 February 2015
Available online

Keywords:
Self-assembled nanoparticle
RNAi
Tumor-homing and penetrating
iNGR
Glutathione triggered
Glioma therapy

ABSTRACT

Existing limitations of common RNA interference (RNAi) oncotherapy severely compromised their therapeutic effects. In this study, a novel glioma-targeting RNAi system was developed. Single-component RNAi nanospheres were tactfully self-assembled *in vitro*, combining the carrier and cargo as a whole. An artificially synthesized polycation (pOEI) with redox-sensitive disulfides in structure condensed the RNAi nanospheres into more compacted nanoparticles. Then a novelly designed tumor-homing and penetrating cyclopeptide iNGR was further modified on the surface. iNGR modified RNAi nanoparticles demonstrated significantly enhanced accumulation in glioma site, remaining stable in circulation until the release of naked RNAi nanospheres were triggered off by the paranormal concentration of glutathione within glioma cells. Naked RNAi nanospheres were digested into abudant siRNA afterwards. Remarkable luciferase gene down-regulations have confirmed their outstanding RNAi effects. With specific design of sequences, the iNGR modified RNAi nanoparticles were supposed to be of great potential in safe and efficient glioma therapy in future.

1. Introduction

Currently, RNAi therapy, in the form of nanoparticles encapsulating siRNA with lipids or polymers, has been extensively applied for curing various kinds of cancers [1–3]. However, as is well-known, the instability of siRNA during the delivery in blood, inefficient drug loading capability, relatively high toxicity caused by excess positive charge, and biodegredation inadequacy of carriers severely compromise the expected therapeutic effects of siRNA nanoparticles [4,5]. To overcome the above limitations and achieve an efficient therapy for cancers, a novel single-component RNAi system has been developed [6]. Plenty of RNA self-assembled into one nanosphere (~500nm in diameter) with the carrier and cargo as a whole, carrying thousands of siRNA sequences. These RNAi nanospheres were of good stability during circulation until entered tumor cells, where were diced by the inherent intracellular enzymes and implemented RNAi effect. The onefold endogenous ingredient effectively guaranteed the safety and biodegradability *in vivo*. Moreover, for different kinds of tumors, siRNA sequences of these RNAi nanospheres could be specially designed for extensive application in cancer therapy.

In order to achieve an efficient delivery in blood and an increased accumulation at tumor site, the novel sponge-like RNAi nanospheres (~500 nm in diameter) had to be further optimized into a smaller and compacted scale (~100 nm in diameter) with cationic polymers. However, common polycations available for gene condensation were all of a large molecular weight with strong electropositivity, which had difficulties in biodegradation and controlled release of interference RNA, rendering obvious toxicity and inefficient therapy *in vivo* [7–10]. In this way, a novel polymer was delicately designed and synthesized, aiming to keep a good balance among the condensation ability, internal toxicity and controlled release of therapeutic materials.

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http://dx.doi.org/10.1016/j.biomaterials.2015.02.084
0142-9612/

Branched oligoethylenimine 800 Da (OEI800) with abundant primary amine groups were polymerized by DTSSP (3,3-Dithio-bis-(sulfosuccinimidyl)propionate) linkers into a high-molecular-weight cationic polymer (pOEI). pOEI was of sufficient molecular weight and electropositivity, displaying outstanding ability to condense the sponge-like RNAi nanospheres into an optimized scale for remarkable EPR effect [11–13]. DTSSP has been reported as a novel linker, which endowed an easy polymerization for OEI oligomers and provided abundant redox-sensitive disulfide bonds for pOEI [14]. Thanks to the disulfide bonds in DTSSP molecules, pOEI also revealed an excellent redox sensitivity to the high concentration of GSH in tumor cytes. When entering tumor cells, the disintegration of pOEI was easily triggered off through the reduction of disulfide bonds [15]. pOEI disintegrated into fractional sections with much lower electropositivity, released the naked RNAi nanospheres and exposed them to enzyme systems *in vivo*. Fractional OEI then got biodegraded, and the naked RNAi nanospheres were rapidly digested into large amount of siRNA, taking effect of RNAi afterwards.

Previously, intratumorally injected RNAi nanoparticles showed effective gene down-regulation in ovarian tumor subcutaneous xenograft model nude mice [6]. Taking advantage of the excellent gene silencing efficiency, this kind of RNAi nanoparticles were further developed to be first applied in glioma model through intravenous injection. Systemic administration has been proved to be an effective way for oncotherapy, avoiding unnecessary invasions [16]. However, the nonspecific distribution of therapeutic agents *in vivo* would severely limit the final curative effects [17]. Therefore, a tumor-targeting modification for condensed RNAi nanoparticles is urgently needed. Common tumor-targeting strategies have been developed based on the overexpression of various unique cell membrane proteins on neovasculars, like comprising certain integrins, growth factor receptors, extracellular proteases, extracellular matrix proteins and so on [18–20]. However, the disability of functional molecules to mediate an extravasation from vessels and penetration into tumor tissues compromised their final tumor-targeting efficacy.

To meet the both demands, a novelly designed CendR tumor-penetrating cyclopeptide iNGR (CRNGRGPDC) was exploited. iNGR has an unique structure, including three indispensable features: a vascular homing motif, an R/KXXR/K tissue penetration motif (CendR motif), and a protease recognition site [21]. These three modules cooperate to ensure a multistep, highly specific, tumor-homing and penetration process.

Endothelial CD13 is widely overexpressed on the surface of glioma neovascular endothelial cells [22]. The annular iNGR in blood specifically and rapidly bound to CD13, guaranteeing the potent tumor-vessel-targeting capacity [23]. Afterwards, proteolytic process took place on the surface of tumor vessels, exposing the internal R/KXXR/K motif at the C-terminus of this truncated peptide [24]. In consequence, the resulting linear iNGRt got prepared for the following CendR pathway tumor-penetrating process, mediated by neuropilin-1 (NRP-1) receptors, which is extensively overexpressed on the surface of glioma tumor cells [25]. So far, iNGR has revealed an even better homing and penetration property to glioma, compared with the familiar widespread used iRGD peptide [26]. Therefore, we speculated its high potential in glioma-targeting when being modified on RNAi nanoparticles.

In this paper, a novel kind of redox-sensitive and self-assembled RNAi nanoparticles, decorated with the tumor-homing and penetrating cyclopeptide iNGR through the connection of PEG, was developed and applied in glioma model mice, intending for a safe and efficient tumor-targeting RNAi glioma therapy.

2. Materials and methods

2.1. Materials

The linear single-stranded DNA (ATAGTGAGTCGTATTAACGTACCAACAAC TTACGC TGAGTACTTCGATTACTTGAATCGAAGTACTCAGCGTAAGTTTAGAGGCATATCCCT) and T7 promoter (TAATACGACTCACTATAGGGAT) were synthesized by Genscript Biotechnology Company (Nanjing, China). T4 DNA Ligase were purchased from Promega Corporation (Madison WI, USA). The AmpliScribeTM T7 High Yield Transcription Kit was obtained from Epicenter Technologies (Madison WI, USA). Recombinant Human Dicer Enzyme Kit was purchased from the Genlantis (San Diego, USA). Cyclopeptide iNGR (CRNGRGPDC, cyclized between the cysteines) and chain polypeptide iNGRt (CRNGR) was synthesized by Chinese Peptide Company (Hangzhou, China). OEI (Polyethylenimine, ethylenediamine branched. Mw ~800) was obtained from Sigma–Aldrich (St. Louis, USA). 3, 3′-Dithiobis (sulfo-succinimidylpropionate) (DTSSP) was purchased from PIERCE (Thermo, USA). Thiol PEG NHS Ester (HS-PEG3500-NHS, Mw = 3500) was synthesized by Jenkem Technology (Beijing, China). Sulfo-LC-SMPT linker was purchased from Pierce (Rockford, USA). Anti-Neuropilin 1 antibody and Anti-CD34 antibody were purchased from Abcam (Cambridge, UK), while CD13 antibody was obtained from Santa Cruz Biotechnology (Texas, USA). SYBR II RNA Dye, BODIPY® FL NHS Ester (succinimidyl ester) and DAPI Nucleic Acid Stain (4, 6-Diamidino-2-phenylindole) were all purchased from Life Technologies Corporation (Carlsbad, USA). D-luciferin was purchased from Pierce (Rockford, USA).

2.2. Cell lines

U87 cells and Human Umbilical Vein Endothelial Cells (HUVECs) were both obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured at 37 °C in a humidified 5% CO_2 atmosphere with Dulbecco's modification of Eagle's medium (DMEM), supplemented with fetal bovine serum (FBS) (10%), streptomycin (100 mg/mL) and penicillin (100 units/mL). All the related cell cultural media and reagents were purchased from Life Technologies Corporation (Carlsbad, USA).

2.3. Animals

Balb-c nude mice (male, 18–20 g) were supplied by the Experimental Animals Department of Fudan University. All animal experiments were carried out in accordance with guidelines evaluated and approved by Fudan University Institutional Animal Care and Use Committee (IACUC).

2.4. Production of naked RNAi nanospheres

1 μM of single-stranded DNA was hybridized with 1 μM of T7 promoter by heating at 95 °C for 2 min and slowly cooling to 25 °C during 1 h, yielding 0.5 μM circular DNA template. The nick in the circular DNA was chemically closed by T4 DNA ligase, following the commercial protocol. Ligased circular DNA templates (0.5 μM) together with four kinds of 7.5 mM ribonucleotide triphosphate (ATP, UTP, CTP and GTP) were incubated with T7 RNA polymerase (2 μl) at 37 °C for 18 h in the reaction buffer (including 10 mM DTT, 2 μl RNase Inhibitor and 2 μl Enzyme Solution), at a total reaction volume of 20 μl. The transcription reaction was stopped by adding 1 MBU of RNase-Free DNase I to the standard 20 μl reaction system and incubating for 15 min at 37 °C. The corresponding RNAi nanospheres were extracted with TE-saturated phenol/chloroform, followed by chloroform. Afterwards, RNAi nanospheres were precipitated by 5 M ammonium acetate and washed with 70% ethanol. For the purpose of imaging, RNAi nanospheres were further washed with RNase-Free water for several times. They were finally redissolved in RNase-Free water with addition of RNase Inhibitor, and sonicated for 10 min to break any possible connection between each two nanospheres for further usage. For fluorescently-labeled RNAi nanospheres, 10 × SYBR II RNA Dye was added for incubation.

2.5. Synthesis and characterization of the polycation materials

Equimolar amounts of OEI800 and DTSSP were dissolved in pure water and stirred continuously for 48 h at 35 °C. The reaction system was then dialyzed against water using cellulose dialysis bags (molecular weight cutoff 7 kDa) for 24 h and lyophilized. Pure pOEI was further dissolved in D_2O and analyzed by 1H NMR spectroscopy. Equimolar amounts of pOEI and HS-PEG-NHS were dissolved in PBS 8.0 and reacted at room temperature for 2 h. The reaction system was then purified and lyophilized. Pure HS-PEG-pOEI was further dissolved in D_2O and analyzed by ^1H NMR spectroscopy. 10 mM Sulfo-LC-SMPT in pure water was slowly added to iNGR solution (1 mM, PBS 7.2) and reacted at room temperature for 2 h. The reaction system was then purified and lyophilized. Pure Sulfo-LC-peptide was further dissolved in DMSO-D_6 and analyzed by ^1H NMR spectroscopy. One molar of Sulfo-LC-peptide was reacted with 5 M of HS-PEG-pOEI in pure water at room temperature for 4 h. The reaction system was then purified and lyophilized. Pure pOEI-PEG-LC-peptide was further dissolved in D_2O and analyzed by ^1H NMR spectroscopy. The pOEI-PEG-LC-peptide was also dissolved in H_2O for water phase GPC.