Biomaterials 104 (2016) 192–200

Contents lists available at ScienceDirect

Biomaterials

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

A redox-sensitive, oligopeptide-guided, self-assembling, and efficiency-enhanced (ROSE) system for functional delivery of microRNA therapeutics for treatment of hepatocellular carcinoma

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ARTICLE INFO

Article history:
Received 17 May 2016
Received in revised form
11 July 2016
Accepted 13 July 2016
Available online 16 July 2016

Keywords:
miRNA therapy
Delivery system
Hepatocellular carcinoma
Multifunctional delivery

ABSTRACT

Lack of efficient adjuvant therapy contributes to a high incidence of recurrence and metastasis of hepatocellular carcinoma (HCC). A novel therapeutic is required for adjuvant treatment of HCC. We developed a polymer-based nanosystem (ROSE) for functional gene therapy by synthesizing a supramolecular complex self-assembled from polycations and functional adamantyl modules. The ROSE system condensing tumor suppressor microRNA-34a (miR-34a) therapeutics becomes ROSE/miR-34a nanoparticles that could facilitate gene transfection in HCC cells with satisfied stability and efficiency, possibly due to proton sponge effect by polycations, PEGlyation protection, and controlled release by breakdown of disulfide bonds. Meanwhile, modification with a targeting oligopeptide SP94 in ROSE/miR-34a enables approximately higher affinity for LM3 HCC cells than hepatocytes in vitro and greater HCC specificity in vivo. Furthermore, ROSE/miR-34a nanoparticles significantly inhibits HCC cell proliferation and in vivo tumor growth, representing a notable effect improvement over conventional gene delivery strategies. ROSE/miR-34a, featuring redox-responsiveness, oligopeptide-guided specificity, self-assembly, and enhanced transfection, is therefore a potential therapeutic agent in future adjuvant therapy for HCC treatment.

1. Introduction

Lack of efficient systemic therapy contributes to a high risk of recurrence and metastasis of hepatocellular carcinoma (HCC), even after potentially curative locoreginal or surgical treatment [1]. Current adjuvant approaches, such as widely-used sorafenib and combination chemotherapy, have limited effect on advanced HCC in addition to conventional management strategies [2–4]. A novel therapeutic is required for systemic treatment and adjuvant prevention against HCC. Along with the RNAi technology broadly applied to cancer treatment that disables specific pieces of mRNA to silence oncological targets [5,6], a different type of small RNA, microRNA (miRNA), which directly coordinates post-transcriptional activities in cells rather than regulates protein-synthesizing mRNA, has showed promising therapeutic effect on several malignancies [6,7]. Dysfunction of key miRNA regulatory network in HCC significantly correlates with tumor pathological behaviors, like rapid development and metastasis [7]. miRNA-34a (miR-34a), activated by p53, negatively regulates carcinogenesis, tumor growth, and metastasis [8]. Specifically in HCC pathological environment, miR-34a activation is blocked, resulting in loss of miR-34a expression and subsequent cancer development [9]. The reinstatement of oncosuppressor miRNAs, for example miR-34a replacement therapy [10], might be a potential option for systemic therapy against HCC.

However, excessive accumulation of exogenous miRNA therapeutics travelled to the non-malignant tissues would interfere with normal cell activities, causing off-target side effect [5,6]. Given that naked miRNA would not survive in complicated in vivo environment before arriving at tumor site by long-time circulation, a

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http://dx.doi.org/10.1016/j.biomaterials.2016.07.016
0142-9612/

miRNA delivery system must be introduced to achieve satisfying outcomes. Importantly, the delivery system should fulfill the minimum requirements including (1) vector-based protection that reduces miRNA loss and improves pharmacodynamics in circulation and endocytosis, (2) functional delivery, featuring tumor targeting ability, stimuli-responsiveness, size control, or biodegradability, that eventually increases intratumoral miRNA expression while producing little adverse impact on normal cells [11].

In our previous approach, we developed a delivery system based on polyethylenimine-crosslinked β-cyclodextrins (PEI-βCD) complex, which showed superior transfection efficiency of carrying nucleotides with negative charges, such as plasmid, siRNA, and miRNA [12]. We then used a hydroxypropyl-rich cyclodextrin to replace the β-cyclodextrin component to attain better solubility and biocompatibility [13]. Notably, cyclodextrin-based polymeric system could be modified by self-assembly of adamantyl functional modules, to obtain nanosystems supporting functional delivery, benefiting from host-guest interaction of cyclodextrins and adamantyl moieties [14,15]. Therefore, we are able to develop a supramolecular nanosystem that meets the requirements for miRNA delivery, optimized by assembled modules. Herein, we report a novel delivery system possessing combination attributes of redox-responsiveness, oligopeptide-guided targeting, self-assembling synthesis, and high transfection efficiency (ROSE), for miRNA replacement therapy.

2. Materials and methods

A full description of the methods is provided in the Supplementary Data.

2.1. Preparation and general characterization of the ROSE system

PEI-hβCD, Ad-SS-PEG, and Ad-PEG-SP94 were synthesized as described in Supplementary Data section. ROSE^{SP94+} [PEI-hβCD@(Ad-SS-PEG/Ad-PEG-SP94)] was self-assembled by mixing PEI-hβCD with Ad-SS-PEG and Ad-PEG-SP94 in water, followed by mixture stirring and lyophilization to dryness. Similarly, ROSE^{SP94-} (PEI-hβCD@Ad-SS-PEG) was synthesized from mixing PEI-hβCD and Ad-SS-PEG in water. ROSE solution was added dropwise to nucleotide solution in equivalent volume according to the predetermined nitrogen to phosphorus (N/P) molar ratios to form the ROSE system. The ROSE complexes were characterized by ^{1}H NMR and NOESY spectroscopy using a Varian 400 MHz NMR spectrometer (Palo Alto, CA). The ROSE/nucleotide system was tested for electrophoretic mobility by gel electrophoresis experiment, and was then visualized using a Hitachi HT-7700 transmission electron microscope (TEM; Tokyo, Japan). The hydrodynamic size and the surface charge of the ROSE/nucleotide complexes were measured on a Zetasizer Nano ZS (Malvern, Worcestershire, UK).

2.2. Redox-responsiveness test

To evaluate the reduction sensitivity of the ROSE/nucleotide complex (ROSE^{SS+}/miR-34a), the solutions were incubated at 37 °C with constant shaking, with or without the presence of 5 mM of dithiothreitol (DTT). The ROSE without disulfide linkage (ROSE^{SS-}, PEI-hβCD@Ad-PEG) complexed with nucleotide was used as control. The particle size change was monitored using a Zetasizer Nano ZS (Malvern) for 4 h with DTT co-incubation.

2.3. Verification of tumor specificity and targeting ability

Tumor specificity was evaluated from affinity of ROSE-cell interaction. In brief, ROSE^{SP94+} complexed with fluorescein-5,6-isothiocyanate (FITC)-labeled negative control siRNA (nc-siRNA) were co-incubated with LM3 HCC cells or HL-7702 hepatocytes for 1 h. After removal of unbound complexes, mean fluorescence intensity was measured by a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ), and was then plotted versus ROSE concentration to generate a saturation binding curve to calculate dissociation constant (K_d). For in vivo targeting study, ROSE^{SP94+} carrying Cy5-labeled nc-siRNA (ROSE^{SP94+}/Cy5-siRNA), with PBS, naked Cy5-siRNA, or ROSE^{SP94-}/Cy5-siRNA as control, was administrated to the tumor-bearing mice via tail-vein injection, and was visually tracked by a FX Pro In-Vivo Imaging System (Kodak, Rochester, NY). Following the in vivo fluorescence tracking, the mice were sacrificed, and their tumors, along with heart, liver, spleen, lung, and kidney, were directly measured for fluorescence visualization.

2.4. Investigation of cellular uptake and transfection efficiency

LM3 cells were treated with nanocarriers (PEI-hβCD, ROSE^{SP94-}, or ROSE^{SP94+}) complexed with FITC-labeled nc-siRNA for 4 h. Fluorescence distribution was plotted. Mean fluorescence intensity of the samples was then measured by a FACSCalibur flow cytometer (Becton Dickinson) to determine cellular uptake efficiency. For visualization, the cellular membrane was stained with Alexa Fluor® 647 conjugate, with the nuclei stained with DAPI. The cells were then imaged under a Radiance 2100 confocal laser scanning microscope (Bio-Rad, Hercules, CA). For transfection study, LM3 cells were treated with nanocarriers (PEI-hβCD, ROSE^{SP94-}, or ROSE^{SP94+}) complexed with pRL-CMV for 24 h. The luciferase assays were performed to determine the normalized relative light units per milligram of cell protein lysate against that after PEI-hβCD treatment.

2.5. In vitro therapeutic effect study

LM3 cells were treated with PBS, naked miR-34a, or ROSE/miR-34a for evaluation of in vitro therapeutic effect. In vitro proliferation after specific treatments was evaluated using a cell counting kit-8 assay (CCK8; Dojindo, Kumamoto, Japan) and a 5-ethynyl-2′-deoxyuridine assay (EdU; RiboBio, Guangzhou, China). Relative cell viability was calculated as a percentage of untreated controls. The half-maximal inhibitory concentration (IC_{50}) was determined by non-linear fitting. EdU^{+} ratios were calculated to further evaluate the proliferation activity. Invasion activity was examined by the ability to pass through a gel matrix (Matrigel; Becton Dickinson) using the Transwell chambers (Corning, New York, NY). The invaded cells stained with crystal violet were counted, and the results were expressed in a normalized value to the untreated control. Migration ability was evaluated using wound healing assays, and the wound area was assessed by using Image-Pro Plus 6.0 (Media Cybernetics, Acton, MA), normalized to the wound area at the 0 h time point. The E-cadherin, vimentin, Snail1, and GAPDH expression after indicated treatments was evaluated by Western blotting assays.

2.6. In vivo therapeutic effect study

Tumor-bearing mice were treated with PBS, naked miR-34a, ROSE^{SP94-}/miR-34a, or ROSE^{SP94+}/miR-34a for evaluation of in vivo therapeutic effect. The treatment was performed every 3 days for 6 times via tail-vein injection, and tumor growth was monitored by calipers every 3 days. All mice were sacrificed 21 days after first injection, followed by ^{18}F-FDG microPET evaluation with a MicroPET Rodent R4 scanner (Concorde Microsystems, Knoxville, TN), and their tumors were dissected, weighed, and then imaged.