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An effective tumor-targeting strategy utilizing hypoxia-sensitive siRNA delivery system for improved anti-tumor outcome

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ABSTRACT

Hypoxia is a feature of most solid tumors, targeting hypoxia is considered as the best validated yet not extensively exploited strategy in cancer therapy. Here, we reported a novel tumor-targeting strategy using a hypoxia-sensitive siRNA delivery system. In the study, 2-nitroimidazole (NI), a hydrophobic component that can be converted to hydrophilic 2-aminoimidazole (AI) through bioreduction under hypoxic conditions, was conjugated to the alkylated polyethyleneimine ($bPEI_{1.8k}$-C_6) to form amphiphilic $bPEI_{1.8k}$-C_6-NI polycations. $bPEI_{1.8k}$-C_6-NI could self-assemble into micelle-like aggregations in aqueous, which contributed to the improved stability of the $bPEI_{1.8k}$-C_6-NI/siRNA polyplexes, resulted in increased cellular uptake. After being transported into the hypoxic tumor cells, the selective nitro-to-amino reduction would cause structural change and elicit a relatively loose structure to facilitate the siRNA dissociation in the cytoplasm, for enhanced gene silencing efficiency ultimately. Therefore, the conflict between the extracellular stability and the intracellular siRNA release ability of the polyplexes was solved by introducing the hypoxia-responsive unit. Consequently, the survivin-targeted siRNA loaded polyplexes shown remarkable anti-tumor effect not only in hypoxic cells, but also in tumor spheroids and tumor-bearing mice, indicating that the hypoxia-sensitive siRNA delivery system had great potential for tumor-targeted therapy.

Statement of Significance

Hypoxia is one of the most remarkable features of most solid tumors, and targeting hypoxia is considered as the best validated strategy in cancer therapy. However, in the past decades, there were few reports about using this strategy in the drug delivery system, especially in siRNA delivery system. Therefore, we constructed a hypoxia-sensitive siRNA delivery system utilizing a hypoxia-responsive unit, 2-nitroimidazole, by which the unavoidable conflict between improved extracellular stability and promoted intracellular siRNA release in the same delivery system could be effectively solved, resulting in enhanced siRNA silencing efficiency in tumor cells. To our knowledge, the described work is the first demonstration of a siRNA delivery system using a hypoxia trigger for regulation of siRNA release, which represents a new strategy for tumor-targeted therapy, and it is expected that this meaningful strategy must be widely applied in the future.

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1. Introduction

Most solid tumors contain a tumor-specific microenvironment that is characterized by low pO_2, also known as hypoxia. Owing to the rapid proliferation of cancer cells, the tumor quickly exhausts the nutrient and oxygen supplied from the vasculature, and becomes hypoxic [1,2]. Up to now, numerous researches have found that hypoxia is a negative prognostic factor owing to its multiple contributions to tumor chemoresistance, radioresistance, invasiveness, metastasis, etc [3]. However, taking account of the significant difference in oxygen tension between tumor and normal tissues, the existence of hypoxia also provides an opportunity for tumor-targeting therapy, mainly including bioreductive prodrugs and inhibitors of molecular targets upon which hypoxic cell survival depends [4,5].

Recently, a new approach for hypoxia-targeting was realized using stimuli-responsive nanocarriers modified with a hypoxia-

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sensitive moiety, 2-nitroimidazole. In fact, nitroimidazole derivatives have been widely utilized as hypoxia imaging agents and bioreductive prodrugs since they undergo selective bioreduction in hypoxic cells to form reactive products that irreversibly bind to cell components [6–9]. Interestingly, 2-nitroimidazole (NI), which is poorly water-soluble, can be converted to hydrophilic 2-aminoimidazole (AI) via a series of bioreductions under hypoxic conditions. Taking advantages of this unique property, Thambi et al. [10] constructed a hypoxia-responsive nanoparticle consisted of carboxymethyl dextran and hydrophobically modified 2-nitroimidazole derivative for tumor-targeted doxorubicin delivery. Yu et al. [11] produced a hypoxia-sensitive insulin delivery system using 2-nitroimidazole conjugated hyaluronic acid to mimic the function of pancreatic cells, and this system had great potential to improve the life quality of diabeties.

On the other hand, the specificity and potency of small interfering RNA (siRNA) regulation of gene expression holds great promise for cancer therapy. Even so, the efficiency of siRNA is significantly compromised by their poor stability and insufficient cellular transport [12,13]. To overcome these deficiencies, many kinds of synthetic or natural polycations have been widely used in the siRNA delivery system for enhanced gene transfection efficiency. Of the most extensively studied polymers for siRNA delivery, polyethyleneimine (PEI) with low molecular weight is of considerable interest, because it shows relatively low toxicity compared to that with high molecular weight, yet accompanied by decreased efficacy [14]. Hence, enormous efforts have been made to improve the transfection efficiency without toxicity increasing. Previous studies showed that alkylation of low molecular weight PEI helped to improve the stability and enhance the cellular uptake efficiency of the polycation/siRNA polyplexes, whereas such stable structures would hinder the siRNA dissociation in the cytoplasm, thus desirable silencing efficacy still couldn't be achieved [15,16]. Therefore, the stability and the release behavior of the siRNA delivery system should deserve equal attention.

In this study, we have successfully modified branched polyethyleneimine (M_W = 1.8 kDa, $bPEI_{1.8k}$) with alkylated NI (C_6-NI), and the synthesized $bPEI_{1.8k}$-C_6-NI could effectively condense siRNA to form a hypoxia-responsive polycation/siRNA polyplexes. It was found that, $bPEI_{1.8k}$-C_6-NI could self-assemble into micelle-like aggregations in physiological conditions, for improved stability and enhanced cellular uptake efficiency of the polyplexes. After being transported into the hypoxic tumor cells, the selective nitro-to-amino reduction of NI would elicit a relatively loose structure to facilitate the siRNA dissociation in the cytoplasm. That's to say, the conflict between the extracellular stability and the intracellular release ability of the polycation/siRNA polyplexes was resolved utilizing the specific hypoxia environment of solid tumors. As a result, this novel system shown remarkable gene silencing efficacy not only in hypoxic cells, but also in tumor spheroids and tumor-bearing mice.

2. Materials and methods

2.1. Materials

Branched polyethyleneimine (M_W = 1.8 kDa, $bPEI_{1.8k}$) was purchased from Alfa Aesar (Ward Hill, MA, USA). 2-nitroimidazole (NI), methyl 6-bromohexanoate and sodium dithionite were purchased from J&K Chemical Ltd. (Beijing, China). 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), triethylamine and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma–Aldrich Inc. (Shanghai, China). RNase A and RNase Inhibitor were purchased from Solarbio Ltd. (Beijing, China). Trypsin and RPMI-1640 media were obtained from Thermo Fisher Scientific Co., Ltd. (Beijing, China). Luciferase assay kit was from Promega Corporation (Madison, WI, USA). Cell counting kit-8 (CCK-8) was obtained from Dojindo Laboratories (Kumamoto, Japan). siRNA, targeting luciferase (siLuc): 5′-CUUACGCUGAGUACUUCGATT-3′ (sense), targeting survivin (siSur): 5′-GCAUUCGUCCGGUUGCGCUTT-3′ (sense), and negative control siRNA (siNC): 5′-UUCUCCGAACGUGUCACGUTT-3′ (sense) were synthesized by GenePharma Co., Ltd. (Shanghai, China). DNA primers, including firefly luciferase (forward 5′-TGACAAGGATGGATGGCTACA-3′, reverse 5′-ACGGCGGCGGGAAGTT-3′), β-actin (forward 5′-GAGGGAAATCGTGCGTGAC-3′, reverse 5′-CCAAGAAGGAAGGCTGGAAAA-3′) were synthesized by Taihe Biotechnology Co., Ltd. (Beijing, China). Famale BALB/c mice were obtained from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All other reagents were of analytical grade and used without further purification.

2.2. Synthesis and characterization of $bPEI_{1.8k}$-C_6-NI

The synthetic procedures were performed as previous reports [10,17] and summarized in Scheme S1. To obtain 6-(2-nitro-1-imidazolyl) hexanoic acid (NI-$(CH_2)_5$-COOH), a stirred suspension of 2-nitroimidazole (1 equiv.), methyl 6-bromobutanoate (1.2 equiv.), and K_2CO_3 (1.1 equiv.) in DMF was heated at 110 °C for 2 h. After that, solvent was removed under reduced pressure, and the residue was partitioned between EtOAc and water. The organic layer was worked up, and hydrolyzed by concentrated HCl at 20 °C for 16 h. Afterwards, the hydrolysate was extracted by EtOAc and recrystallized in water to yield NI-$(CH_2)_5$-COOH as light yellow crystal.

Next, NI-$(CH_2)_5$-COOH was conjugated to $bPEI_{1.8k}$ through amide formation in the presence of EDC and NHS. Briefly, NI-$(CH_2)_5$-COOH (227 mg, 1 mmol) was dissolved in DMSO, then EDC (383.4 mg, 2 mmol) and NHS (230 mg, 2 mmol) were added and stirred at room temperature for 2 h. $bPEI_{1.8k}$ (180–900 mg, 0.1–0.5 mmol) solution in DMSO mixed with 200 μL of triethylamine was added to the reaction mixture drop by drop and stirred for another 24 h. The crude product was following purified by dialysis (MWCO = 1 kDa) against distilled water for 48 h, and the final product was recovered by freeze-drying. The structures of NI-$(CH_2)_5$-COOH and $bPEI_{1.8k}$-C_6-NI were characterized by ^{1}H NMR spectroscopy (Varian Mercury-600 MHz spectrometer, Varian Medical Systems, Inc., Palo Aito, CA, USA) using D_2O as solvent, and further confirmed by FTIR (Nicolet 5700, Thermo Inc., USA).

2.3. Hypoxia-sensitivity of $bPEI_{1.8k}$-C_6-NI

The sensitivity of NI modified PEI derivatives to hypoxic reductive environment was assessed by measuring the change in the absorption peaks after incubation with sodium dithionite. Generally, sodium dithionite (0.1 mg/mL) was added to an aqueous solution of $bPEI_{1.8k}$-C_6-NI at a concentration of 1 mg/mL, and the solution was stirred at room temperature for 2 h. The UV absorptions of $bPEI_{1.8k}$-C_6-NI solutions before and after the reduction were monitored using a UV–vis spectrophotometer (Beijing Purkinje General Instrument Co. Ltd., Beijing, China). The parameters of spectrum scanning were as follows: wavelength range, 200–400 nm; wavelength interval, 1 nm. Additionally, the reductive solution of $bPEI_{1.8k}$-C_6-NI was following dialyzed (MWCO = 1 kDa) against distilled water and lyophilized to give $bPEI_{1.8k}$-C_6-AI, for further investigations. The structure of $bPEI_{1.8k}$-C_6-AI was confirmed by ^{1}H NMR spectroscopy in D_2O as well.

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