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Bioreducible branched poly(modified nona-arginine) cell-penetrating peptide as a novel gene delivery platform

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ABSTRACT

Cell-penetrating peptides (CPPs) have been widely used to deliver nucleic acid molecules. Generally, CPPs consisting of short amino acid sequences have a linear structure, resulting in a weak complexation and low transfection efficacy. To overcome these drawbacks, a novel type of CPP is required to enhance the delivery efficacy while maintaining its safe use at the same time.

Herein, we report that a bioreducible branched poly-CPP structure capable of responding to reducing conditions attained both outstanding delivery effectiveness and selective gene release in carcinoma cells. Branched structures provide unusually strong electrostatic attraction between DNA and siRNA molecules, thereby improving the transfection capability through a tightly condensed form. We designed a modified type of nona-arginine (mR9) and synthesized a branched-mR9 (B-mR9) using disulfide bonds. A novel B-mR9/pDNA polyplex exhibited redox-cleavability and high transfection efficacy compared to conventional CPPs, with higher cell viability as well. B-mR9/VEGF siRNA polyplex exhibited significant serum stability and high gene-silencing effects *in vitro*. Furthermore, the B-mR9 polyplex showed outstanding tumor accumulation and inhibition ability *in vivo*. The results suggest that the bioreducible branched poly CPP has great potential as a gene delivery platform.

1. Introduction

Cell-penetrating peptides (CPPs) composed of 5 to 30 amino acids have been used intensively in biological systems. Generally, CPPs containing lysine or arginine residues are positively charged at physiological pH levels to interact effectively with negatively charged cell plasma membranes [1]. Positively charged CPPs are capable of facilitating penetration into cells using three types of penetrating mechanisms: the formation of a pore through a membrane, the destruction of the membrane morphology, and endocytosis [2,3]. Owing to these unique penetrating characteristics, CPPs have been investigated in various biomedical fields. In gene delivery systems, CPPs acting as a condensing material are complexed with guest molecules such as plasmid DNA (pDNA) and siRNA using electrostatic interactions or covalent bonds. The complexes can then be delivered to the desired sites. pVEC, TAT, Transportan, Penetratin and Polyarginine have been widely used as cell-penetrating agents in the biomedical fields. Among the various CPPs, polyarginines composed of simple and short sequences are readily applicable as gene delivery carriers and cell permeable enhancers [4–7].

However, in gene delivery systems, CPPs by themselves cannot easily be used as an encapsulating agent due to their unstable linear structures, low molecular weights, and relatively low electron charge density compared to nucleic acids such as DNA and siRNA. To overcome the weak condensation of both CPP and nucleic acid, CPPs are chemically modified with reactive groups at the both ends so as to connect to other segments and thus increase the molecular weight of the resulting polycations. Therefore, poly CPPs can effectively condense very large nucleic acid molecules due to their enhanced electron charge capacity. Furthermore, for controlled release at targeted sites, the CPP segments are covalently bonded by several linkers cleavable by pH, proteases, and redox-potential [8–11].

A disulfide bond is a prevalent bioreducible linker at tumor sites due to even higher intracellular reduction conditions than other normal sites [12–14]. In previous reports, the chain length of conventional CPPs modified with thiol groups at both ends of the chain was significantly incremental through the formation of disulfide bridges [10,11,15]. Won et al. achieved unusual transfection efficacy with elongated polycations through the disulfide bonding of R9 [16,17]. Elongated polycations simultaneously provide improved gene transfection efficacy and controlled release characteristics against low-molecular-weight CPPs. According to previous reports, conventional branched polycations showed more successful cytosolic

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gene delivery than linear conformations [18–21]. To construct branched structures through disulfide bridges, cysteine, a natural amino acid, is a feasible solution because disulfide bonds can easily form between each CPP segment. Furthermore, a disulfide linkage, which is vulnerable to bio-reducing agents, attains selective release at desirable sites because Glutathione (GSH), a bio-reducing agent, is over-secreted in cytoplasm. Therefore, it may be effective for gene delivery systems simultaneously to introduce both branched conformations and disulfide bonds in polycations.

Herein, this study reports a novel bioreducible branched poly(nona-arginine) CPP (B-mR9) with outstanding gene delivery effectiveness. A modified type of R9 (mR9) was designed with cysteine residues at both ends and in the middle of mR9 (Cys-R9-Cys-R9-Cys), resulting in a novel branched shape through disulfide bonding. The driving force of its formation was the three reactive positions in mR9, as shown in Fig. 1. The additional thiol group was capable of capturing another mR9 segment. This branched poly CPP could more strongly interact with nucleic acid molecules as compared to the linear type. These polyplexes could be internalized through endocytosis or direct penetration, and the packed gene molecules likely escape from the carriers through deconstruction of disulfide bonds. The released pDNA or siRNA can then efficiently transfect cells, or suppress gene expression. Therefore, this novel bioreducible B-mR9 is a promising tool for use as a gene delivery platform.

2. Materials and methods

2.1. Materials

The R9 (RRRRRRRRR, 1424 Da) and modified R9 (CRRRRRRRRRCRRRRRRRRRRC, 3138 Da) peptides used in this study were purchased from Peptron (Daejeon, Korea). Dulbecco's modified Eagle's medium (DMEM), Fetal Bovine Serum (FBS), Antibiotic Antimycotic Solution (AAS), Dimethyl sulfoxide (DMSO), Phosphate-buffered saline (PBS), Agarose, (4.5-dimethylthiazol-2-yl)-2.5-dipenyltetrazolium bromide (MTT), Tris Acetate-EDTA (TAE) buffer, *L*-Glutathione (GSH), DL-Dithiothreitol (DTT), Paraformaldehyde (PFA), DL-buthionine-[S,R]-sulfoximine (BSO), DPX mountant, heparin sodium, mouse serum, and branched polyethyleneimine (MW 25000, PEI 25 k) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Plasmid green fluorescent protein DNA (pGFP, 5715 bp) was provided by KRIBB (Daejeon, Korea). Reduced serum media (Opti-MEM) was obtained from Invitrogen, Co (Carlsbad, *CA*, USA). The 6× loading star was purchased from Dynebio (Seongnam, Korea). siRNA was purchased from Bioneer (Daejeon, Korea) (siVEGF: 5′-GGAGUACCCUGAUGAG-AUCdTdT-3′, 5′-GAUCUCAUCAGGGUACUCCdTdT-3′; sc-VEGF: 5′-UUCUCCGA ACGU-GUCACGUdTdT-3′, 5′-ACGUGACACGUUCGGAGAAdTdT-3′). HEK 293, Hela, SKOV3, and NCI-H460 cells were cultured in DMEM, 10% FBS, and 1% AAS (100 IU/mL penicillin, 100 μg streptomycin) at 37 °C with 5% CO_2.

2.2. Synthesis of cleavable branched-modified R9 (B-mR9)

Thirty mM of the mR9 peptide was dissolved in PBS (pH 7.4) containing 30% DMSO and was then gently stirred overnight. When the mR9 solution had formed a gel, the reaction was terminated by adding 15 mL of a 5 mM HEPES buffer. Dialysis was performed to remove the DMSO and the low-molecular-weight mR9s (MWCO: 10,000). Purified peptides were then obtained through lyophilization. B-mR9 was diluted in distilled water to concentrations of 1, 0.5, 0.25, 0.125, and 0.0625 mg/mL, after which the absolute molecular weight

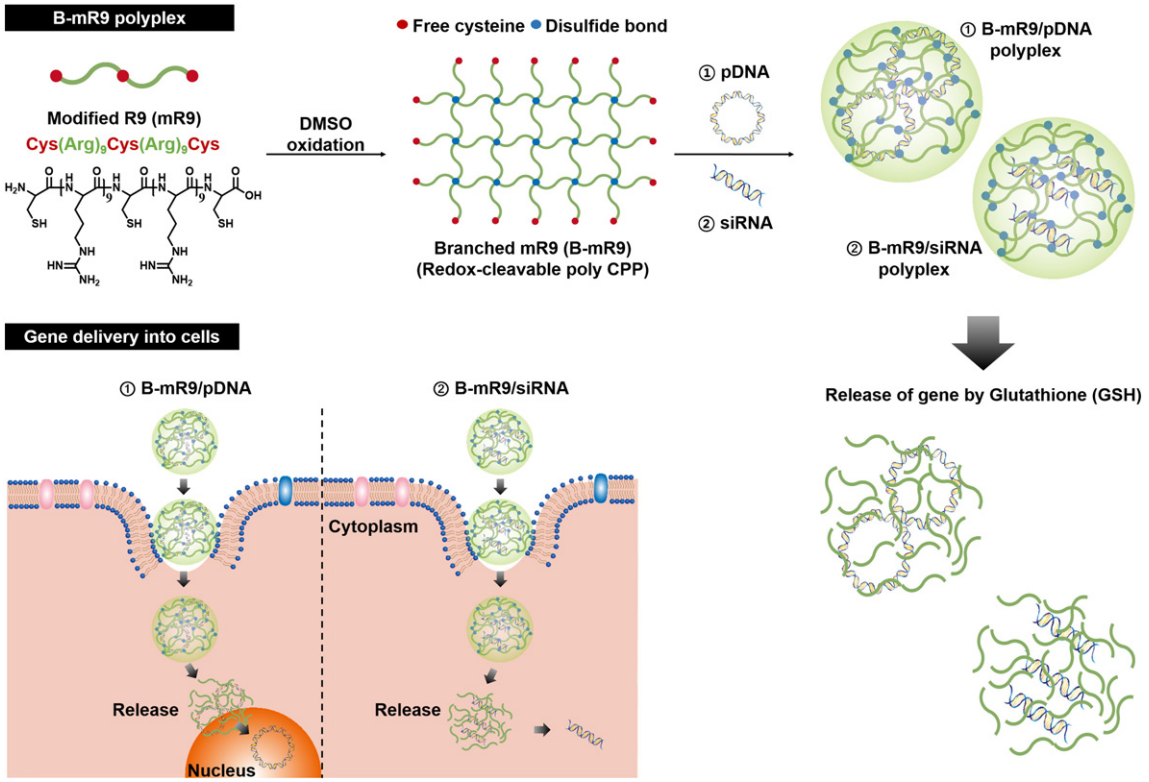

Fig. 1. Schematic illustration of the synthesis of the branched-modified R9 (B-mR9) cell-penetrating peptide (CPP) and construction of pDNA and siRNA polyplexes. Positively charged B-mR9 can be constructed with negatively charged genes through electrostatic interactions. B-mR9 polyplexes were delivered into cells by means of the permeability of the CPP. The branched structures of B-mR9 were then cleaved by the reductive conditions of the intracellular matrix. Finally, pDNA or siRNA was released into the nucleus or cytoplasm, respectively.