**Branched siRNA nanostructures for enhanced gene silencing**

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**ABSTRACT SUMMARY**

This study presents a well-defined, branched siRNA nanostructure for enhanced gene silencing using cationic carriers, PBAEs. Three single strand (ss) DNA-siRNA chimeras are mixed to form Y-shaped siRNA with three sticky ends, ss G\(_1\)-siRNA, through the self-assembly of nucleic acid base pairs. The hybridization of two kinds of ss G\(_1\)-siRNAs and ss siRNA can generate highly branched siRNA structures with sizes of ~35 nm. The resultant G\(_2\)-siRNA contains nine anti-GFP siRNAs and four DNA junctions, which can increase spatial charge density and structural stability. Upon contact with PBAEs, G\(_2\)-siRNA is effectively collapsed into stable nanoparticles that can facilitate intracellular uptake of siRNAs. The G\(_2\)-siRNA is effectively degraded by endonuclease Dicers, releasing free siRNA with RNAi activity. This study demonstrates that branched siRNA nanostructures can be utilized as an effective condensation with biodegradable PBAEs for enhanced intracellular delivery of siRNA therapeutics.

**INTRODUCTION**

Nucleic acid has been used as a promising tool for constructing predicated nanoscale architectures due to highly specific base paring. Recently, the marked concern of biologically functional nanostructures has led to the development of RNA nanotechnology\(^[10]\). In particular, small double-stranded RNA with 19 ~ 21 bp that known as small interfering RNA (siRNA) exhibited a biological activity of target-specific mRNA degradation via RNA interference in cytoplasm. A variety of self-assembled RNA structures incorporating siRNA fragments such as siRNA oligomers, siRNA hydrogels, and siRNA sponges have exhibited superb cellular uptake and target gene silencing. Although engineered RNA structures have been successfully prepared by chemical or physical assembly, they do not have a single uniform population in terms of size and shape for clinical applications.

Poly(β-amino ester)s (PBAEs) have been extensively studied as efficient cationic polymers for gene delivery in cancer cells and stem cells. In particular, PBAEs are degradable into smaller and nontoxic molecules due to hydrolysis of their backbone esters. The combinatorial library synthesis and high-throughput screening methods have been explored to identify optimal gene carriers with high transfection efficiency and low cytotoxicity. Several PBAEs have demonstrated an effective delivery carrier of plasmid DNAs (pDNA) into cells. They can condense pDNA into stable complexes with ~200 nm in sizes via electrostatic interaction. However, unlike pDNA, siRNAs do not show any significant gene silencing effects when delivered with PBAEs.

This study introduces a three-dimensional branched siRNA nanostructure generated by programmable self-assembly of six ss DNA-siRNA chimeras and ss siRNA. The branched DNA/siRNA structure is easily degraded to release siRNA that activate RNAi pathway. The enhanced spatial charge density and structural flexibility of branched siRNA can effectively interact with PBAEs via electrostatic interaction, producing compact nanoparticles. The branched siRNA are expected to facilitate the intracellular translocation and gene silencing effects.

**EXPERIMENTAL METHODS**

**Preparation of branched siRNA nanostructures (G\(_1\)-siRNA and G\(_2\)-siRNA).** To form G\(_1\)-siRNA, equal moles of three ss DNA-GFP siRNA chimeras, Y\(_1\), Y\(_2\), and Y\(_3\), were hybridized in PBS. The resultant products were assembled with GFP siRNA at a 1:3 molar ratio at room temperatures (RT). G\(_2\)-siRNA was obtained by mixing G\(_{1a}\)-siRNA, G\(_{1b}\)-siRNA, and ss GFP siRNA at a 1:3:6 molar ratio at RT. Hybridization of ss G\(_1\)-siRNA was performed according to the following procedures: (i) At 95 °C for 2 min. (ii) At 65 °C for 2 min. (iii) At 60 °C for 5 min and cooling at 4 °C with a continuous temperature decrease at a rate of 2 °C per 1 min using PCR machine.

**Synthesis of PBAEs.** PBAEs were synthesized by the previous method\(^[2]\). Briefly, C32-based copolymers were prepared by reacting 1,4-butandiol diacrylate (C) and 5-amino-1-pentanol (32) at 90°C for 24 h (1.2:1.0 molar ratio of diacrylate to amine). After cooling, the solution was added with end-capping diamine (10 mmol) in 50 mL THF for 24 h at RT. The resultant products were purified by precipitation in diethyl ether and dried under vacuum. The pellets were dissolved at 100 mg/mL in DMSO. To prepare DD-based terpolymers, dodecylamine (DD), hydrophobic amine, and hydrophilic amine were reacted in DMSO at 90 °C (1.2:0.3:0.7 molar ratio). After 48, the reactions were allowed to cool to RT and added end-capping amine (2 mmol) for 24 h.
siRNA/PBAEs complexes did not show any significant using wit branched siRNA nanostructures with RNAi activity. The cells were mixed with solution consisting of 98% PBS, 2% FBS, and propidium iodide (PI). The resultant cells were transferred to 96-well round-bottom plates. The GFP expression was measured using FACS. PI staining was used to exclude dead cells from the analysis.

RESULTS AND DISCUSSION

The preparation procedures of branched siRNA nanostructures with RNAi activity are schematically illustrated in Figure 1. The novel strategy of the self-assembly of single-strand (ss) DNA-GFP siRNA chimeras and ss GFP siRNA can generate programmable branched siRNA nanostructures. Equal molar ratios of three ss DNA-GFP siRNA chimeras were hybridized to produce Y-shaped siRNA with the three sticky ends, ss G1-siRNA, through complementary base pairing of DNA sequences. The ss G1-siRNA had a three-way DNA junction in a core and three single strands bearing GFP siRNA in arms. The melting temperature of DNA junction was approximately 55 °C, which can enhance the structural stability of Y-conformation at RT. The ss G2-siRNA was prepared by annealing Y1, Y2, and Y3, which had an identical GFP siRNA sequences in three arms, while ss G1b-siRNA composed of Y4, Y5, and Y6 contained two different GFP siRNA sequences in arms. One arm of G1b-siRNA can only hybridized with G1b-siRNA arms, which can precisely control the sizes and structures. G2-siRNA was obtained by self-assembling ss G1-siRNA, ss G1b-siRNA, and ss GFP siRNA at a 1:3:2 molar stoichiometry. During the hybridization, ss G1-siRNA served as an initiator core, ss G1b-siRNA extended the generation, and ss GFP siRNA performed an overhang terminator. The G2-siRNA was composed of two kinds of GFP siRNA and DNA, which served as RNAi initiators and branching points, respectively.

To determine gene silencing, KB-GFP cells were seeded at 2.0 × 10^{4} cells/well in 96-well plates. The cells were treated with various siRNA/PBAEs complexes that prepared at a weight ratio (PBAEs/siRNA) of 40 with 35 nM siRNA in 10% serum medium for 4 h. The medium was replaced with fresh medium. After 48 h, the cells were washed with PBS and detached using 0.25% trypsin-EDTA. The expression was measured using FACS. To determine gene silencing, KB-GFP cells were treated with various siRNA/PBAEs complexes prepared at a weight ratio (PBAEs/siRNA) of 40 with 35 nM siRNA in 10% serum medium for 4 h. The medium was replaced with fresh medium. After 48 h, the cells were washed with PBS and detached using 0.25% trypsin-EDTA. The medium was then used to exclude dead cells from the analysis.

We also examined the siRNA condensation, target mRNA degradation, cytotoxicity, and morphology of branched siRNA and branched siRNA/PBAEs complexes. The gene silencing in KB-GFP cells was assessed using FACS analysis (Figure 2). As expected, naked siRNA/PBAEs complexes did not show any significant GFP suppression. However, G1- and G2-siRNA efficiently inhibited the GFP expression by DD-based terpolymers, while C32-based copolymers were not effective at the same conditions. The levels of the GFP expression in cells treated with G1-siRNA/PBAEs complexes decreased to 45.3 ± 1.8% by DD24-C12-122, 59.1 ± 2.0% by DD60-C24-122, and 44.0 ± 0.9% by DD90-C24-122, and those of G2-siRNA/PBAEs complexes were 46.0 ± 2.5% by DD24-C24-122, 44.9 ± 2.4% by DD60-C24-122, and 43.3 ± 1.9% by DD90-C24-122. Notably, G2-siRNA showed higher gene silencing efficiency than G1-siRNA. It was suggested that G2-siRNA had higher spatial charge density and structural flexibility than G1-siRNA, which can effectively interact with PBAEs to form more stable nanoparticles that facilitated cellular uptake.

![Figure 1](image1.png)

**Figure 1.** Schematic illustration for the preparation of branched siRNA nanostructures with RNAi activity.

**Figure 2.** GFP suppression of KB-GFP cells after transfection with various siRNA/PBAEs complexes.

We also examined the siRNA condensation, target mRNA degradation, cytotoxicity, and morphology of branched siRNA and branched siRNA/PBAEs complexes.

CONCLUSION

This study demonstrated a novel strategy to prepare three-dimensional branched siRNA nanostructures with RNAi through the programmable self-assembly of six ss DNA-siRNA chimeras and ss siRNA. Branched siRNA with increased charge density and structural flexibility was effectively condensed to form stable nanoparticles with PBAEs carriers. Branched siRNA/PBAEs complexes showed much higher cellular uptake and enhanced gene silencing in KB-GFP cells than naked siRNA/PBAE complexes. Thus, engineered siRNA nanostructures can be potentially utilized as a new platform and strategy for polymer-based siRNA delivery systems.

REFERENCES