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Streptavidin-mirror DNA tetrahedron hybrid as a platform for intracellular and tumor delivery of enzymes

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ABSTRACT

Despite the extremely high substrate specificity and catalytically amplified activity of enzymes, the lack of efficient cellular internalization limits their application as therapeutics. To overcome this limitation and to harness enzymes as practical biologics for targeting intracellular functions, we developed the streptavidin-mirror DNA tetrahedron hybrid as a platform for intracellular delivery of various enzymes. The hybrid consists of streptavidin, which provides a stoichiometrically controlled loading site for the enzyme cargo and an L-DNA (mirror DNA) tetrahedron, which provides the intracellular delivery potential. Due to the cell-penetrating ability of the mirror DNA tetrahedron of this hybrid, enzymes loaded on streptavidin can be efficiently delivered into the cells, intracellularly expressing their activity. In addition, we demonstrate tumor delivery of enzymes in an animal model by utilizing the potential of the hybrid to accumulate in tumors. Strikingly, the hybrid is able to transfer the apoptotic enzyme specifically into tumor cells, leading to strong suppression of tumor growth without causing significant damage to other tissues. These results suggest that the hybrid may allow anti-proliferative enzymes and proteins to be utilized as anticancer drugs.

1. Introduction

Protein enzymes are essential natural molecules that catalyze various biochemical reactions. They are involved in the biosynthesis and regulation of most of the biological molecules required for intracellular functions and homeostasis. As enzymes have the high substrate specificity and can amplify cellular signals by their catalytic reactions, the application of enzymes for therapeutic purposes is an attractive approach for disease treatment [1–4]. However, all of the therapeutic enzymes that are currently in use function extracellularly because protein enzymes are unable to enter cells [5–12]. Since potential therapeutic targets are much more abundant inside of cells, small molecule drugs have been traditionally used to achieve stoichiometric efficacy. Improving the cellular internalization of enzymes would allow the development of game-changing therapeutics and biotechnologies to modify intracellular functions and activities. In an effort to develop carriers for intracellular delivery of enzymes, cationic lipid-based carriers conventionally used for gene delivery have been adopted on a limited basis for delivering enzymes containing protein domains with multiple negatively charged residues or complexed with polyanionic oligonucleotides [13,14]. Conjugation of enzymes with protein transduction domains (PTDs) has similarly improved the intracellular uptake of enzymes [15–18]. Cellular uptake of enzymes can also be facilitated by nanoparticulate carriers, such as polymeric lipid nanoconstructs [19], inorganic nanoparticles [20,21], and protein nanoparticles [22,23]. Although these previous developments represent exciting opportunities for enzyme delivery, they are limited to *in vitro* intracellular delivery and topical delivery of protein enzymes, leaving *in vivo*

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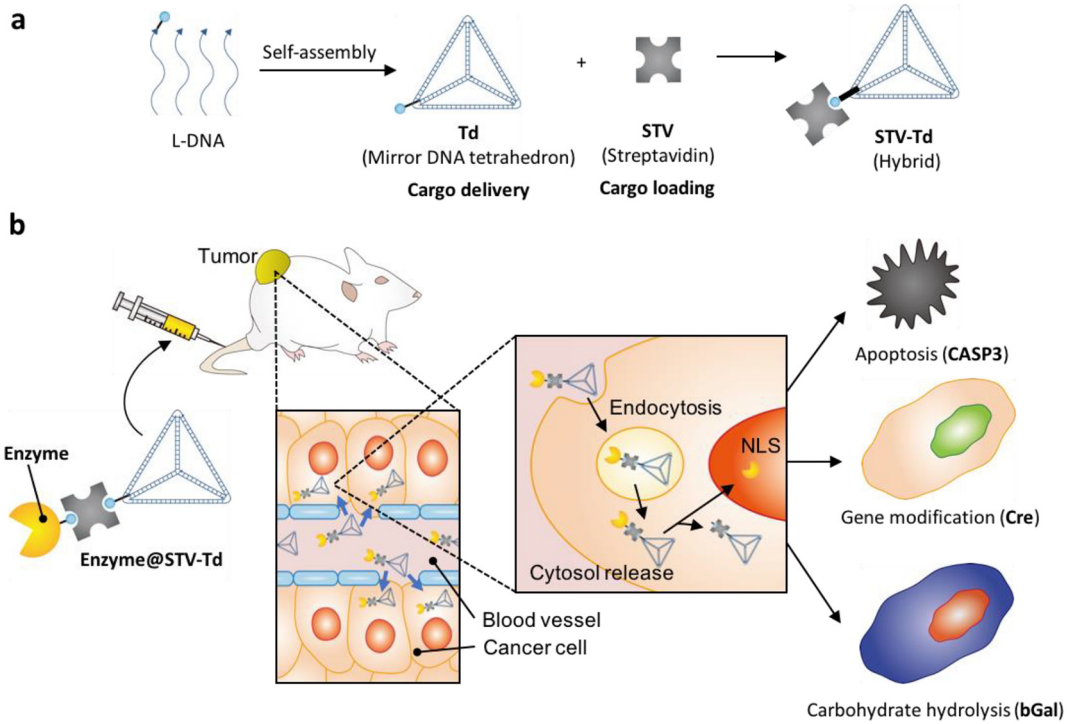

Fig. 1. (a) Preparation of protein-mirror DNA tetrahedron hybrid. The mirror DNA tetrahedron (Td) was assembled with four L-DNA strands. One of the strands was biotinylated and conjugated to streptavidin (STV) in a 1:1 ratio to yield the hybrid. (b) Various biotinylated enzymes can be loaded onto the STV subunit of the hybrid and systemically delivered to tumor cells through the cell-penetrating and tumor-localizing properties of Td. The activity of the delivered enzymes (CASP3, Cre, and bGal) will exert corresponding functions (apoptosis, gene modification, and carbohydrate hydrolysis) in tumor cells. An enzyme bearing a nuclear localization signal (NLS) can be further localized to the nucleus.

systemic delivery of the enzymes to tumors as a formidable challenge.

A wireframe DNA tetrahedron is a self-assembled DNA nanoconstruct with a tetrahedral shape [24,25]. The structures of DNA tetrahedrons are determined by the precise organization of complementary base-pairs. Since DNA tetrahedrons are made of a highly biocompatible material and are known to have cell-penetrating properties [26], they have recently been employed for intracellular delivery of various bioactive molecules [27,28]. DNA tetrahedrons assembled with enantiomeric mirror DNA (L-DNA) instead of natural DNA (D-DNA), particularly show an enhanced cellular uptake efficiency and intracellular stability compared with natural DNA tetrahedrons [29]. Utilizing these novel aspects of the mirror DNA tetrahedron (Td), we developed the streptavidin-Td (STV-Td) hybrid as a platform for intracellular delivery of a diverse range of enzymes (Fig. 1a). STV in the hybrid provides a loading site for biotinylated enzymes while Td can deliver the loaded enzymes into cells. Unlike other described nanocarrier systems, the hybrid is expected to be able to carry virtually any type of enzymes, whether extracted from cells or expressed *in vitro*, with a precise loading ratio (Fig. 1b). In this study, we prepare this STV-Td hybrid and analyze the mechanism and efficiency of its cellular uptake. Then, we investigate the power of the hybrid to serve as a platform for intracellular and tumor delivery of various enzymes. Further, we use the hybrid for *in vivo* tumor-targeted delivery of an apoptotic enzyme, demonstrating its potential in the development of cancer therapeutics.

2. Materials and methods

2.1. Preparation of the STV-Td hybrid

Biotinylated Td was prepared by following previously reported procedures [29]. Briefly, the mixture of four strands (S1, biotin-labeled S2, S3 and S4, 250 nM of each strand, shown in Table S1) were annealed in TM buffer (10 mM Tris-HCl, 5 mM $MgCl_2$, pH 8) by heating to 95 °C and cooling slowly to 4 °C. STV-Td was prepared by incubating a 1:1 mixture of biotinylated Td and STV at room temperature for 1 h.

2.2. Preparation of the enzyme-loaded hybrid

For biotinylation of enzymes, CASP3 (1 mg, bacterially expressed, Supplemental Information) was reacted with a 10-fold excess of NHS-PEG_{12}-Biotin linker (EZ –Link™ NHS-PEG_{12}-Biotin, Thermo Fisher Scientific, USA) for 30 min at room temperature. For biotinylation of Cre (1 mg, Excellgen, USA), a disulfide-containing agent (EZ –Link™ Sulfo-NHS-SS-Biotin, Thermo Fisher Scientific, USA) was used. Biotinylated bGal was purchased from Sigma-Aldrich (USA). After the reaction, the unreacted biotinylation reagent was removed by Zeba micro spin desalting column (MWCO = 7 K, Thermo Fisher Scientific, USA). CASP3@STV-Td, Cre@STV-Td, and bGal@STV-Td were prepared by incubation of the STV-Td hybrid with biotinylated CASP3, biotinylated Cre, or biotinylated bGal at 1:1 M ratio in TM buffer. The enzyme-loaded hybrids were used without further purification.

2.3. Intracellular delivery of enzymes

To examine the cellular uptake of CASP3@STV-Td, Cre@STV-Td, and bGal@STV-Td, HeLa cells (for CASP3 and bGal delivery) and Nanog fibroblast cells (for Cre delivery) were seeded (5×10^4 cell/well) onto 24-well plate. After 24 h, the cells were washed twice with PBS, and then incubated with the enzyme-loaded hybrid (50 nM for CASP3 and bGAL delivery, 100 nM for Cre delivery) in serum free DMEM at 37 °C in a 5% CO_2 incubator. After 6 h, the cells were washed twice with PBS, trypsinized, washed with cold PBS twice, and then resuspended in ice cold PBS (500 μL). The internalized enzymes were further analyzed by flow cytometry, activity assays, and Western blotting (see Supplementary material).

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