Novel Multimeric siRNA-XDNA Nanostructures for Precise Multifunctional Synergistic Therapy

Chyan Ying Ke[1], Soong Ho Um[2,3], Darrell J. Irvine[2,3]
[1]Harvard-MIT Division of Health Sciences and Technology, [2]Department of Material Science and Engineering,
[3]Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge MA 02139

Statement of Purpose: DNA is increasingly being explored as pharmaceutical material as the specific complementary base pairing enables precise design of the DNA architecture and guarantees the reproducibility of the nanostructures. Furthermore, nanoscale DNA delivery vehicles are expected to be biocompatible and capable of accessing disease sites such as tumors or lymphatic vessels due to their small size. Here we describe 'X' DNA nanostructures[1] whose four arms can each attach therapeutic cargo such as RNA via specific nucleic acid hybridization. We propose that these X-DNA nanostructures are ideal delivery vehicles as they are highly modular and can co-deliver multiple attached molecules in a precisely controlled ratio. As a first step toward multifunctional delivery vehicles, here we sought to demonstrate the precise control of siRNA-conjugation on X-DNA and investigate the efficacy of gene silencing of multimeric siRNA-XDNA nanostructures.

Methods: All oligonucleotides were synthesized by Integrated DNA Technologies. Four strands of oligonucleotides, each partially complementary to its adjacent strand, were hybridized to one another at equimolar ratio forming branched X-DNA structure with four-nucleotide overhangs on each arm for siRNA-ligation as previously reported[1]. By varying the number of X-DNA arms with overhangs, we are able to precisely tune the number of duplex siRNA on each X-DNA molecule. Duplex siRNA targeting luciferase with corresponding complementary overhangs were hybridized separately, and ligated to overhangs on each arm of X-DNA by T4 DNA ligases (Promega) (Fig. 1a). The siRNA-XDNA products were then purified by agarose gel extraction. Transfection studies were performed in DC2.4 dendritic cells stably expressing luciferase. Cells were transfected for four hours with siRNA-XDNA structures complexed with Lipofectamine RNAiMAX in Opti-MEM media with GL2+GL3 siRNA (Ambion) as control before fresh media replacement.

Results: The siRNA-XDNA ligation products were visualized by agarose gel electrophoresis. As shown in Fig. 1b, the desired product was the most intense band, along with faint bands corresponding to partially ligated siRNA-XDNA. The major product typically accounted for 35-75% of the total ligation product, based on band intensities on the agarose gel. Highly ligated siRNA-XDNA structures gave lower ligation yield. Following agarose gel extraction, we obtained average yields of 25-30% of initial oligonucleotide input. In Fig. 1b, we show that X-DNA with different number of siRNAs ligated are distinct from one another and can be visualized as a step ladder of increasing molecular weight on agarose gels. Cell viability studies of B16-F10 melanoma cells cultured with increasing concentrations of siRNA-XDNA structures showed minimal cytotoxicity up to at least ~15 mM of the nucleotide nanostructures. Gene silencing

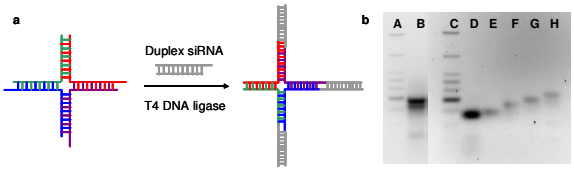

Fig 1. (a) Schematic of siRNA ligation onto an X-DNA molecule with three overhangs, forming (3)siRNA-XDNA. (b) Agarose gel showing DNA ladder (lanes A and C), unpurified (3)siRNA-XDNA (B), X-DNA (D) and purified X-DNA with different number of siRNA arms: 1-armed (E), 2-armed (F), 3-armed (G), 4-armed (H).

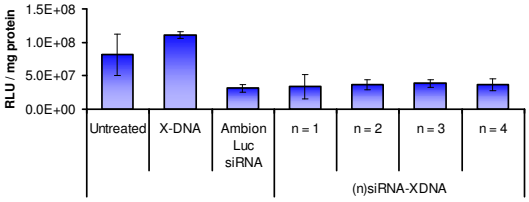

Fig. 2. siRNA-XDNA transfection results with 6.25 mM siRNA, complexed with 0.6 uL RNAiMAX, in Luc$^+$ DC2.4 cells.

studies were carried out in Luc$^+$ DC2.4 cells with untreated and duplex Luc siRNA-treated cells as negative and positive controls respectively, and the concentration of siRNA kept constant at 6.25 mM across all experimental groups (Fig. 2). X-DNA alone without siRNA demonstrated no RNA interference, similar to untreated cells, signifying that the X-DNA core in siRNA-XDNA is inert in gene silencing. Interestingly, X-DNA with up to four siRNAs ligated mediate approximately 75% gene knockdown, comparable to the linear siRNA positive control. The efficiency of gene silencing was independent of the number of siRNA arms on each X-DNA, implying that Dicer processing of the nanostructure-conjugated siRNA was not hindered by steric effects for the multimeric nanostructures. We also observed stable gene silencing beyond five days of transfection and are in the process of validating the kinetics of siRNA-XDNA gene knockdown.

Conclusions: We have shown here a novel multimeric siRNA-XDNA structure that enables precise tuning of the type and number of attachments on each X-DNA core. These siRNA-XDNA structures mediated gene silencing as efficiently as free duplex siRNAs with little or no cytotoxicity, independent of the number of siRNAs ligated. This result is significant particularly for future studies where ligands and siRNAs targeting different genes are ligated to the same X-DNA molecule for synergistic co-delivery into the same cell. Ongoing work demonstrates that the X-DNA can be easily generalized to allow for ligation of other oligonucleotides of interest by designing unique overhangs for each type of oligo. We are currently studying ligand-conjugated siRNA-XDNA to increase endosomal uptake and gene silencing in cells without exogenous transfection reagents for multifunctional synergistic therapy.

References: 1. Um SH, *Nat Mat.* 2006;5:797-801.