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Aptamer-Targeted DNA Nanostructures for Therapeutic Delivery

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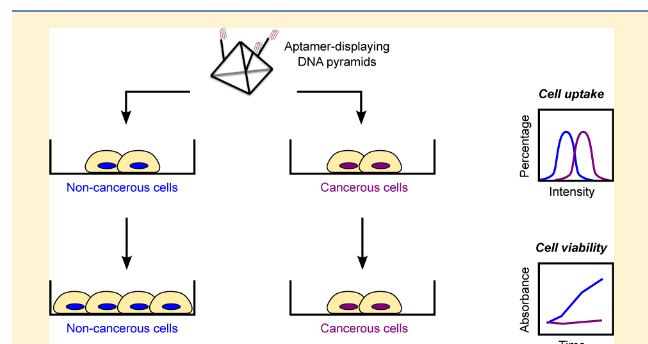
Supporting Information



ABSTRACT: DNA-based nanostructures have been widely used in various applications due to their structural diversity, programmability, and uniform structures. Their intrinsic biocompatibility and biodegradability further motivates the investigation of DNA-based nanostructures as delivery vehicles. Incorporating AS1411 aptamers into DNA pyramids leads to enhanced intracellular uptake and selectively inhibits the growth of cancer cells, achieved without the use of transfection reagents. Furthermore, aptamer-displaying pyramids are found to be substantially more resistant to nuclease degradation than single-stranded aptamers. These findings, along with their modularity, reinforce the potential of DNA-based nanostructures for therapeutic applications.

KEYWORDS: *self-assembly, nanomedicine, cancer therapy, transfection*

Three-dimensional DNA-based nanostructures can be self-assembled in solution with precise architectures and high efficiency.[1−3] These characteristics motivate the design of DNA nanostructures as drug and gene delivery vehicles, given that nanoparticle size and architecture are known to play significant roles in therapeutic bioavailability.[4−6] Due to their modular nature, DNA nanostructures are able to simultaneously carry multiple therapeutic cargoes. In particular nucleic acids such as antisense, aptamers, and siRNA are readily carried without need of chemical modification.[7] The flexibility in controlling the spatial organization of ligands and cargoes makes DNA nanostructures attractive as compared to conventional delivery vehicles such as liposomes or polyplexes. In addition, due to their intrinsic biocompatibility and biodegradability, DNA nanostructures tend to elicit minimal immune response and avoid chronic accumulation, corresponding to lower toxicity in vivo.[8]

Our group has previously shown that DNA nanostructures can be used to deliver antisense DNA to cancer cell lines.[9] These first-generation carriers unfortunately lacked target specificity and required transfection reagents for their intracellular uptake. To address these limitations, the Anderson group covalently incorporated folic acid, a small molecule targeting ligand, to DNA nanostructures and demonstrated delivery of antiluciferase siRNA to tumors in a xenograft mouse model.[8] However, they did not investigate whether such DNA nanostructures can deliver therapeutic molecules or exhibit subsequent bioactivity. Here we demonstrate that DNA nanostructures can (1) be self-assembled into precise architectures with controllable cargo location, (2) preferentially localize to target cells by use of DNA aptamers, (3) enable intracellular uptake without the need for transfection reagents, (4) deliver therapeutic cargoes and selectively exhibit bioactivity in target cells, and (5) display increased resistance to nuclease degradation.

Four oligonucleotides are stoichiometrically combined to self-assemble into a pyramid cage nanostructure with four triangular faces and six double-stranded edges (Figure 1A). This type of structure has now been extensively studied and characterized.[1,9−15] All edges are 20 base pairs of approximately 7 nm in length. DNA nanostructures generally allow therapeutic molecules to be encapsulated within their interior space, intercalated along their double-helical edges, or incorporated as a part of the structure itself. Inspired by the strategy of Lee et al.,[8] we place multiple overhangs into the nanostructures, providing sites for targeting ligands. This overhang strategy allows for physical extension of ligands away from the carrier vehicle and facilitates their interaction with receptors. Specifically, we use the overhangs to display targeting ligands that are DNA aptamers (Figure 1A). We have chosen the AS1411 aptamer due to its prior use as a cancer-targeting ligand.[16,17] The receptor for the AS1411 aptamer is thought to be nucleolin, a glycoprotein upregulated on a plasma membrane of several cancer cells.[18−20] In addition, AS1411 by itself has been shown to inhibit growth activity in cancer cells.[21,22] The formation of aptamer-displaying DNA pyramids is confirmed by native polyacrylamide gel electrophoresis (PAGE) (Figure 1B). As strands are added from lane 1 to 4, the mobility shifts of distinct bands indicate the formation of uniform larger structures. As the number of aptamer strands per nanostructure is increased, lanes 4 to 7, the mobility decreases further, indicating the successful incorporation of aptamers.

In general, nucleic acids do not efficiently cross cell membranes due to their negative charge. Formulation with

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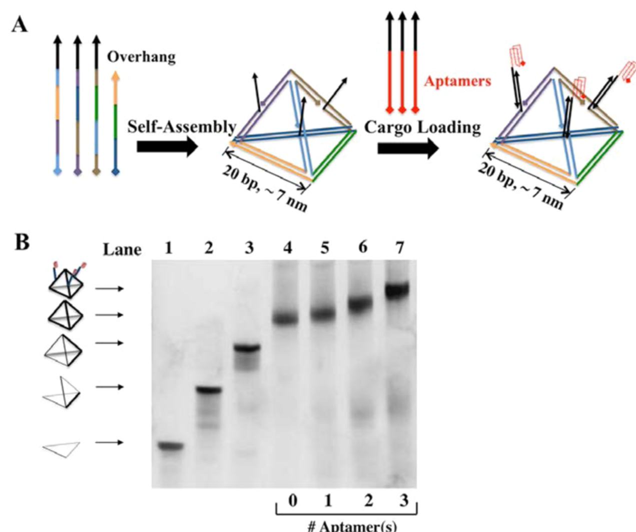

Figure 1. Assembly and characterization of pyramidal DNA nanostructures. (A) Stoichiometric quantities of four oligonucleotides are combined and thermally annealed. Three edges of DNA pyramid (which share the same vertex) display overhangs, which allows hybridization of DNA aptamers to the nanostructures. (B) Native polyacrylamide gel electrophoresis verifies the formation of DNA pyramids. Lane 1: strand 1. Lane 2: strands 1 + 2. Lane 3: strands 1−3. Lane 4: strands 1−4. Lanes 4−7 are DNA pyramids decorated with a progressively increasing number of aptamers.

transfection reagents such as cationic lipids and polymers are commonly used to enhance intracellular uptake of nucleic acids, although these reagents can induce cytotoxicity.[23] Several works have recently demonstrated that assembling nucleic acids into compact three-dimensional objects enhances their intracellular uptake without the need for transfection reagents.[24,25] Motivated by these past works, we investigated the uptake and efficacy of pyramidal DNA nanostructures bearing multiple copies of a DNA aptamer in a human cervical cancer cell line (HeLa), also without the use of transfection reagents.

Pyramids are labeled with a Cy5 or TAMRA fluorophore so as to monitor cellular internalization. Using flow cytometry, we find that uptake of DNA pyramids in HeLa cells is an energy-dependent process (Figure 2). Bare pyramids (i.e., no aptamer) entered HeLa cells at 37 °C without the aid of transfection agents, while their internalization was reduced to control levels at 4 °C. Incorporating AS1411 aptamers as targeting ligands significantly increased pyramid uptake by HeLa cells. This result suggested that the improved uptake of AS1411-pyramids is likely mediated by the specific interaction between AS1411 and the nucleolin receptor[20] since increased uptake was not observed with nontargeting pyramids displaying control aptamers. The flow cytometry results of Figure 2 are confirmed by similar uptake trends determined by fluorescence microscopy (Figure S1 in the Supporting Information).

With the precise control enabled by DNA nanostructures, the presentation of the displayed aptamers can be easily adjusted and is found to strongly influence the uptake of DNA pyramids. Our current design allows from zero to three aptamers to be attached per DNA pyramid via overhangs, simply by using combinations of strands bearing or lacking the overhang sequence (Table S1 in the Supporting Information). Figure 3A shows the normalized uptake level of AS1411-pyramids relative to bare pyramids in HeLa cells. As the pyramid concentration is increased, the uptake of AS1411-

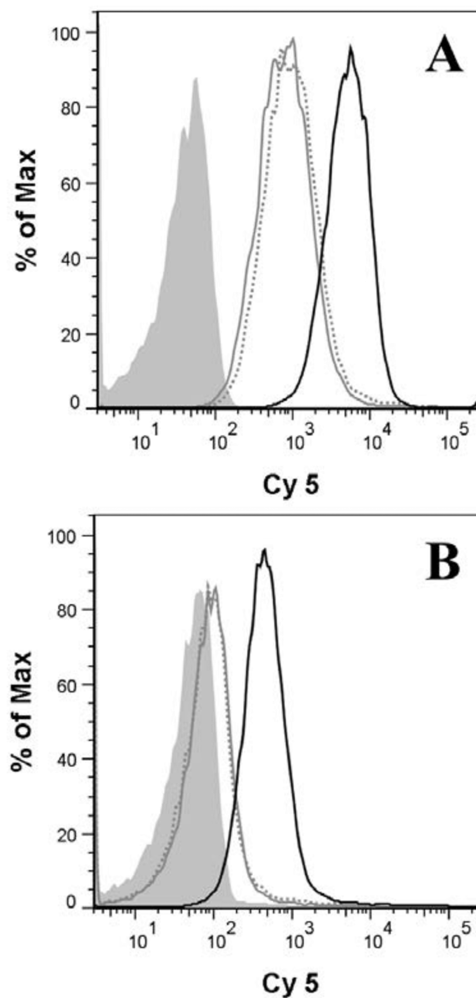

Figure 2. Intracellular uptake of Cy5-labeled DNA nanostructures at 250 nM by HeLa cells. Flow cytometry histograms of bare pyramids (dotted gray line), pyramids bearing three nontargeting aptamers (control, solid gray line), and pyramids bearing three AS1411 aptamers (targeting, solid black line) at (A) 37 °C and at (B) 4 °C. Filled gray histograms represent no treatment.

pyramids is also increased, whereas no effect was observed for nontargeting pyramids (Figure S2 in the Supporting Information). To examine the role of aptamer valency on cellular uptake, the aptamer concentration was held constant (by varying the scaffold concentration). We find that DNA pyramids bearing at least two AS1411 aptamers exhibited improved uptake (Figure 3B). This result indicates that AS1411 aptamer multivalency promotes internalization, presumably through engagement with multiple receptors in close proximity. The presence of serum was found to increase all uptake levels including that of bare and nontargeted samples (Figure S3 in the Supporting Information). However, aptamer-targeted samples always had the greatest uptake. We also note that the orientation of aptamers on the DNA pyramid has an effect on uptake (Figure S4 in the Supporting Information).

In addition to its targeting ability, the AS1411 aptamer has been investigated in several clinical trials as a potential cancer treatment either by itself or in combination with other drugs.[19,26,27] Soundararajan et al. demonstrated that the AS1411−nucleolin interaction destabilizes BCL-2 mRNA to cause cell cycle arrest.[21] Similarly, our results show that

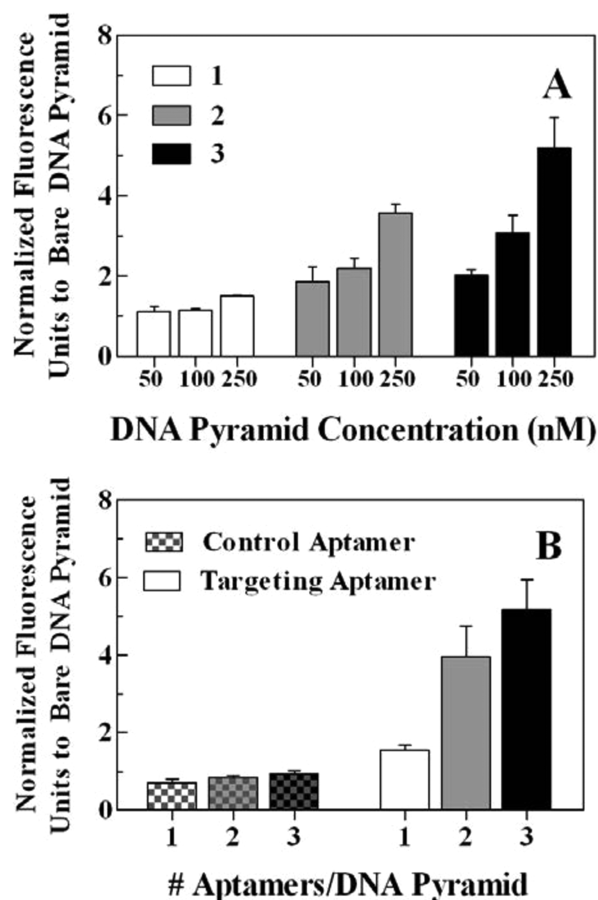

Figure 3. Effect of concentration and aptamer valency on the intracellular uptake of DNA nanostructures by HeLa cells. (A) Uptake level of DNA pyramid with one, two, and three AS1411 aptamers at different concentrations, normalized to uptake by bare DNA pyramids. (B) Normalized uptake of DNA pyramids displaying control (nontargeting) or AS1411 (targeting) aptamers with different valency at a fixed aptamer concentration (250 nM).

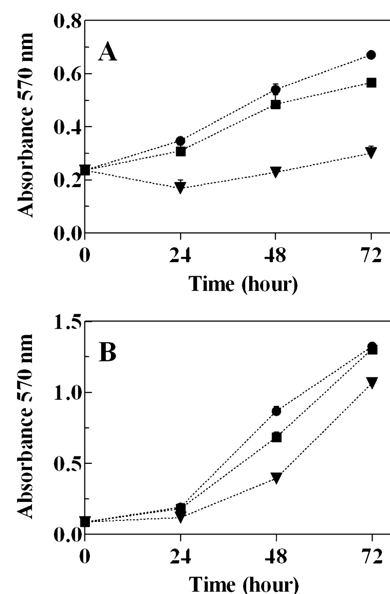

Figure 4. Cell proliferation following treatment with DNA pyramids, as determined by MTT assay. (A) HeLa cells and (B) NIH3T3 cells treated with saline (circles), 250 nM bare DNA pyramids (squares), and 250 nM DNA pyramids bearing three AS1411 aptamers (triangles). Note the different vertical scales for panels A and B. Error bars are smaller than the symbols.

AS1411-pyramids can inhibit HeLa cell growth within 24 h (Figure 4A). The viability of cells treated with AS1411-pyramids is significantly lower compared to saline treatment and cells treated with bare pyramids (Figure 4A). The selectivity of AS1411-pyramids is demonstrated by no adverse effect on the growth of a noncancerous cell line (Figure 4B), despite intracellular uptake (Figures S1 and S5 in the Supporting Information). These findings indeed agree well with previous reports on the uptake mechanism of AS1411 aptamers.[17,20] In cancerous cells, AS1411 enters primarily through a macropinocytosis pathway and can escape endolysosomal degradation. On the other hand, in noncancerous cells, AS1411 is routed into the endolysosomal pathway and is subsequently degraded. Based on the results of Figures 3 and 4, AS1411-pyramids might presumably show this same selectivity in internalization routes, thereby exhibiting antiproliferative activity in cancerous cells but no adverse effects in noncancerous cells. Toward gaining further mechanistic insight, we are currently investigating uptake pathways of these pyramids and intracellular levels of BCL-2 protein.

In addition to selectivity for the target, another requirement for any drug carrier is its stability in the physiological environment. We examined the stability of aptamer-displaying pyramids, bare pyramids, and aptamers alone in the presence of fetal bovine serum (FBS). FBS is a commonly used blood surrogate and is a source of both endo- and exonucleases, among other proteins. Since AS1411-pyramids are self-assembled from oligonucleotides with three different lengths (84, 62, and 44 nucleotides, see Table S1 in the Supporting Information), three distinct bands corresponding to these DNA strands are observed on denaturing PAGE (Figure 5A). The degradation of oligonucleotides generates shorter fragments which can overlap with lower bands and complicate interpretation. Therefore, we analyze the topmost band as a representative of the degradation profile of aptamer-displaying pyramids. Overall, there is no significant difference in degradation rates between aptamer-displaying pyramids and bare pyramids, while aptamers alone were degraded at a substantially faster rate (Figure 5A–C). By fitting data to the first-order decay kinetics, the decay time constants for pyramids were found to be approximately three times greater than for the AS1411 aptamer (Figure 5F). These results can be understood in the context of work by Seferos et al., who demonstrated that the dense negative charge of DNA nano-objects results in a locally high salt concentration, thereby inhibiting nuclease activity.[28]

In summary, we have demonstrated attractive characteristics of DNA nanostructures for use as alternative therapeutic vehicles. These DNA-based vehicles possess a simple fabrication process while achieving controllable and uniform structures. In addition to their enhanced stability over the aptamers alone, DNA nanostructures are efficiently internalized by cells without the use of transfection reagents and selectively deliver bioactive payloads to their targets. The advantage of such nanostructures over single-stranded aptamers becomes especially clear in light of the multivalent effects on uptake.[29] Importantly, the modular nature of DNA nanostructures enables multiple therapeutic molecules to be simultaneously incorporated and delivered. Although a single cargo type was demonstrated in this study, DNA nanostructures are applicable

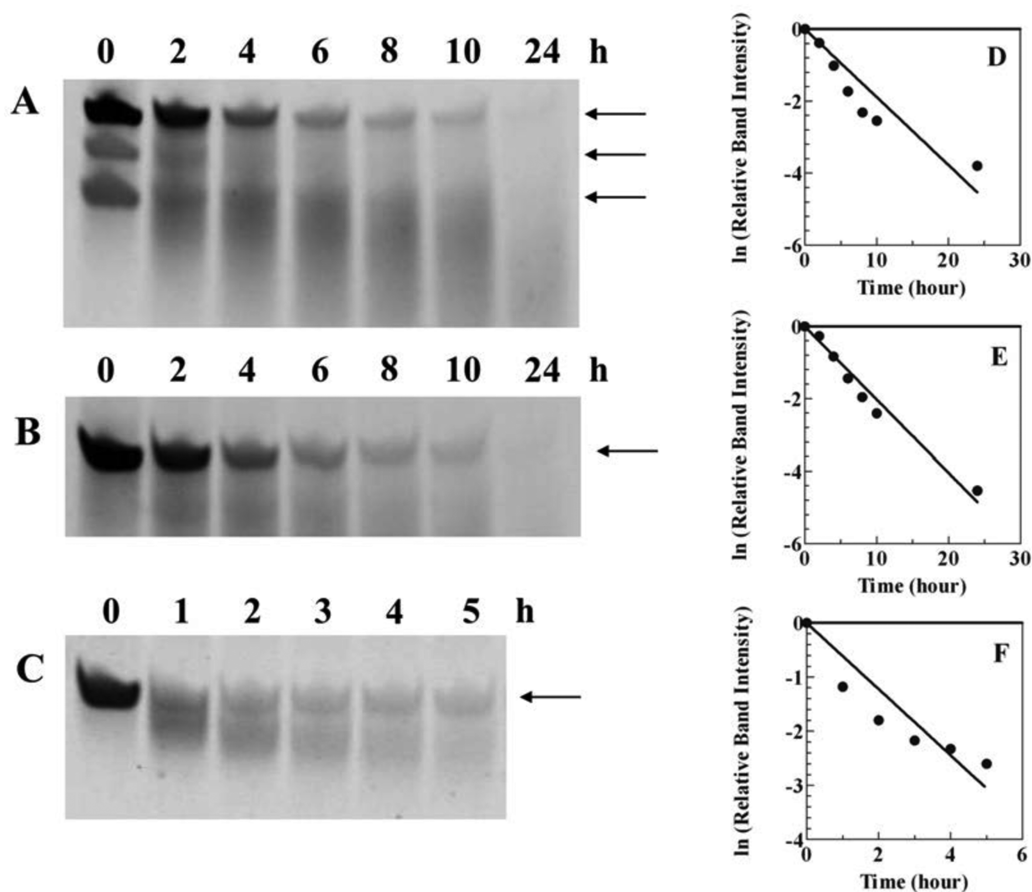

Figure 5. Stability in 10% fetal bovine serum as determined by denaturing PAGE. Individual lanes are marked with incubation time in hours, with arrows denoting uncut DNA. (A) DNA pyramids bearing three AS1411 aptamers. (B) Bare DNA pyramids. (C) AS1411 aptamers. (D−F) Corresponding band intensities of panels A−C are fit by first-order decay kinetics. Note that panel D corresponds to the topmost band in panel A.

for use in combination therapy, which has been proven to be more effective for cancer treatment.[30,31] We are currently developing DNA-based vehicles for delivering multiple bioactive molecules that inhibit the BCL-2 family, aiming to achieve synergistic therapeutic benefits.

■ ASSOCIATED CONTENT

Supporting Information

Details of DNA sequences and experimental procedures, fluorescence images of cell uptake, effect of concentration and aptamer valency for control pyramids, effect of serum on uptake, and effect of aptamer strand orientation on uptake. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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