

## Supramolecularly Engineered Circular Bivalent Aptamer for Enhanced Functional Protein Delivery

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## S Supporting Information

**ABSTRACT:** Circular bivalent aptamers (cb-apt) comprise an emerging class of chemically engineered aptamers with substantially improved stability and molecular recognition ability. Its therapeutic application, however, is challenged by the lack of functional modules to control the interactions of cb-apt with therapeutics. We present the design of a  $\beta$ -cyclodextrin-modified cb-apt (cb-apt- $\beta$ CD) and its supramolecular interaction with molecular therapeutics via host–guest chemistry for targeted intracellular delivery. The supramolecular ensemble exhibits high serum stability and enhanced intracellular delivery efficiency compared to a monomeric aptamer. The cb-apt- $\beta$ CD ensemble delivers green fluorescent protein into targeted cells with efficiency as high as 80%, or cytotoxic saporin to efficiently inhibit tumor cell growth. The strategy of conjugating  $\beta$ CD to cb-apt, and subsequently modulating the supramolecular chemistry of cb-apt- $\beta$ CD, provides a general platform to expand and diversify the function of aptamers, enabling new biological and therapeutic applications.

Aptamers, also known as the “chemical antibodies”, are oligonucleotides that bind with targeted biomolecules or cells with high affinity and selectivity.<sup>1–11</sup> The excellent molecular recognition property of aptamers has led to their expanding use in biomedical applications,<sup>1,2</sup> for example, as conjugates with cancer chemotherapy drugs.<sup>12–15</sup> The practical application of aptamer-drug conjugates (ApDCs), however, requires tailor-made covalent chemistry to prepare aptamer conjugates.<sup>16,17</sup> These conjugations not only pose a substantial synthetic barrier to aptamer usage, but also may compromise the aptamer's targeting properties, due to the loss of control over its stability and conformational flexibility. Therefore, new

chemistries that modulate aptameric structure with improved stability and recognition capabilities are highly desired.

To meet this need, we recently reported a cyclization strategy to stabilize aptamers by ligating two aptamer-based oligonucleotide sequences.<sup>11</sup> The ligated circular bivalent aptamer (cb-apt) exhibited substantially improved thermal and physical stability in biological media, and showed enhanced target binding affinity, both *in vitro* and *in vivo*. However, the therapeutic potential of cb-apt has not yet been fully studied, mostly due to the lack of functional modules on cb-apt to control its interactions with biomedicines.<sup>18</sup> To improve this, a facile and efficient chemical approach to functionalize cb-apt and control its interactions with molecular therapeutics is required. Recently, use of the supramolecular chemistry of oligonucleotides has proved to be an efficient approach to prepare aptamer conjugates for biomedical applications. For instance, the host–guest interaction of cucurbituril- or cyclodextrin-conjugated DNA has been used to expand aptamer functionality for molecular sensing,<sup>19,20,22</sup> catalysis,<sup>21</sup> and biomedicine.<sup>23,24</sup> Therefore, we hypothesized that installing a supramolecular module onto cb-apt and designing its interaction with therapeutics would lead to a new generation of potentially paradigm-shifting aptamer-based molecular medicine.

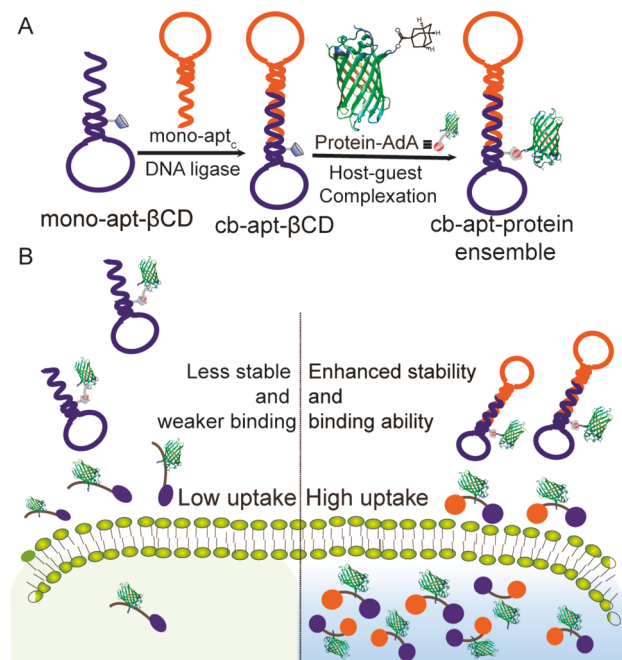
In this paper, we report the design of  $\beta$ -cyclodextrin ( $\beta$ CD)-conjugated cb-apt, and the study of its supramolecular interaction with molecular therapeutics for targeted intracellular delivery. As shown in Scheme 1, we first conjugated a molecular container,  $\beta$ CD, onto a single-stranded sgc8 aptamer that targets protein tyrosine kinase-7 (PTK-7) on the cell surface.<sup>25,26</sup> We then cyclized it with a complementary sequence (monoapt<sub>c</sub>) to form cb-apt- $\beta$ CD. Incorporating  $\beta$ CD with cb-apt not only retained the high serum stability of

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### Scheme 1. Equipping Circular Bivalent Aptamers with Supramolecular $\beta$ CD for Enhanced Intracellular Protein Delivery<sup>a</sup>

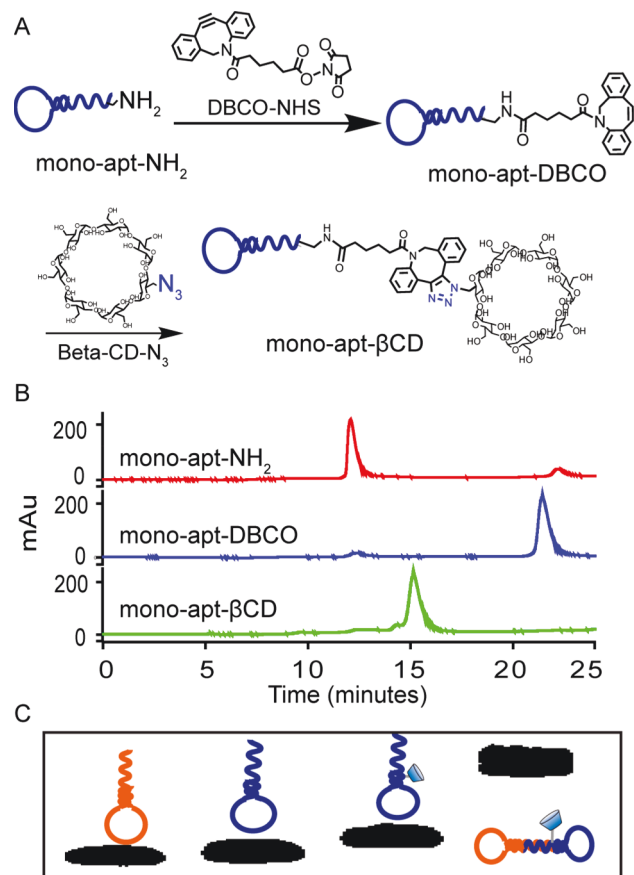


<sup>a</sup>(A) Synthesis route of cb-apt- $\beta$ CD and its supramolecular interaction with adamantane-modified protein. (B) Schematic illustration of cb-apt- $\beta$ CD-mediated enhanced protein delivery.

the circular bivalent aptamer but also enabled encapsulation of a hydrophobic small-molecule, or adamantane-modified protein, into cb-apt, forming a supramolecular ensemble for enhanced intracellular delivery. We demonstrate that cb-apt- $\beta$ CD can efficiently deliver not only small-molecule chemotherapeutics but also a cytotoxic macromolecule (protein) to inhibit tumor cell growth, highlighting the potency of using the supramolecularly engineered cb-apt as a general platform for the delivery of various therapeutics and for new biomedical applications.

To make the cb-aptamer- $\beta$ CD construct, we modified the fifth thymine nucleotide (relative to 3') of an sgc8 aptamer precursor with a primary amine (detailed sequence is provided in Table S1, Supporting Information), which could react with dibenzocyclooctyne-sulfo-*N*-hydroxysuccinimidyl ester (DBCO-NHS) to afford DBCO-conjugated sgc8 aptamer (monoapt-DBCO). As shown in Figure 1A, mixing monoapt-DBCO and 6-monoazido-6-monodeoxy- $\beta$ CD at room temperature yields  $\beta$ CD-conjugated monoapt- $\beta$ CD via click conjugation. After purification using RP-HPLC (Figure 1B), the monoapt- $\beta$ CD was then hybridized with the complementary aptamer component and ligated using T4 DNA ligase to afford circular cb-apt- $\beta$ CD (Scheme 1A). The successful ligation and formation of cb-apt- $\beta$ CD was confirmed by denatured polyacrylamide gel electrophoresis (PAGE). As shown in Figure 1C, the click conjugation of  $\beta$ CD onto monoapt-DBCO slightly retarded its migration, while the cyclization of two aptamers resulted in a significantly higher degree of retardation.

To evaluate whether cb-apt- $\beta$ CD retains the targeting capability of sgc8 aptamer after chemical conjugation and cyclization, we first set out to study the capability of cb-apt-

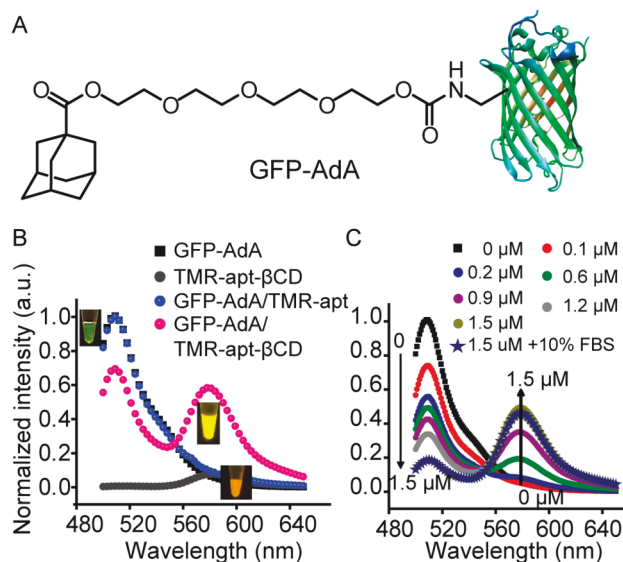


**Figure 1.** (A) Synthesis of  $\beta$ CD-modified monomeric aptamer via DBCO conjugation and copper-free click reaction. (B) RP-HPLC trace of amine, DBCO, and  $\beta$ CD-modified sgc8 aptamer. (C) PAGE analysis of cb-apt- $\beta$ CD.

$\beta$ CD for targeted anticancer drug delivery by making use of the host-guest chemistry of  $\beta$ CD to encapsulate a hydrophobic small-molecule drug. To this end, an anticancer agent that we have previously identified,<sup>13,27</sup> N-heterocyclic carbene (NHC)-gold(I) (AuNHC), was selected and loaded into cb-apt- $\beta$ CD, mainly through the hydrophobic interaction between AuNHC and  $\beta$ CD. AuNHC-encapsulated in cb-apt- $\beta$ CD (cb-apt-AuNHC) was then exposed to CEM cells, a PTK-7-positive cell line, at different concentrations of AuNHC. Cell viability after treatment was measured and compared to that of cells treated with AuNHC encapsulated in monoapt- $\beta$ CD. As shown in Figure S1, monoapt- $\beta$ CD can deliver AuNHC to induce cytotoxicity against CEM cells in Opti-MEM, while showing a significantly decreased efficiency in the presence of serum, suggesting a profound effect from serum degradation of the monomeric aptamer conjugate.<sup>11</sup> Notably, however, cb-apt-AuNHC exhibited a 5-fold increased cytotoxicity against CEM cells compared to that of mon-apt-AuNHC in the presence of serum, indicating the enhanced stability and retention of binding ability of cb-apt- $\beta$ CD in serum for intracellular delivery. Moreover, we observed only minimal cytotoxicity of cb-apt-AuNHC against Ramos cells (Figure S2), a PTK-7 low-expressing cell line, again confirming that the targeting capability of sgc8 is retained in cb-apt- $\beta$ CD, a critical prerequisite for using cb-apt- $\beta$ CD for targeted intracellular delivery.

Having demonstrated the capability of cb-apt- $\beta$ CD to encapsulate hydrophobic AuNHC for targeted delivery based

on the host–guest chemistry of cb-apt- $\beta$ CD, we next studied whether cb-apt- $\beta$ CD could be engineered to deliver protein-based therapeutics, which are generally cell-impermeable biomacromolecules. Many proteins are attractive “biologics” for disease treatment, but the lack of approaches for efficient and effective intracellular delivery restricts their therapeutic potential.<sup>28–31</sup> Addressing this in the present study, we chemically modified protein with adamantane (AdA), a hydrophobic moiety that forms a tight host–guest inclusion complex with  $\beta$ -CD.<sup>21,23</sup> As a proof of concept, we first chose green fluorescent protein (GFP) as a model protein. The chemical conjugation of GFP with AdA ligand (Figure 2A; see

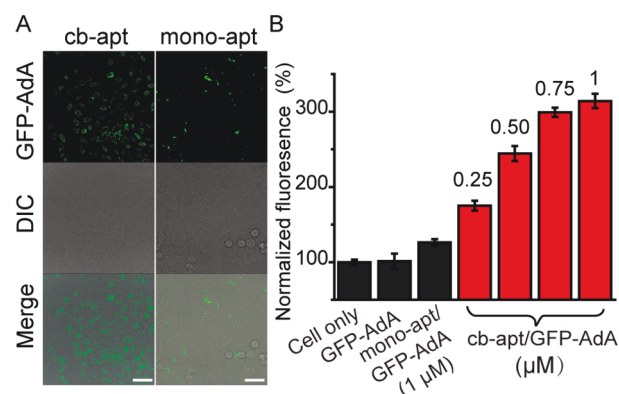


**Figure 2.** (A) Chemical structure of GFP-AdA. (B) Fluorescence spectra of GFP-AdA alone (1  $\mu$ M), GFP-AdA (1  $\mu$ M) in the presence of TMR-apt (2  $\mu$ M) or TMR-cb-apt- $\beta$ CD (0.5  $\mu$ M) in PBS. All spectra were measured with excitation at 470 nm. The inset photographs show the fluorescence of images of GFP-AdA and TMR-apt- $\beta$ CD alone, or their mixture under ultraviolet light. (C) Fluorescence titration of GFP-AdA with TMR-apt- $\beta$ CD from 0 to 1.5  $\mu$ M with and without serum.

experimental details in SI) resulted in GFP-AdA with, on average, one AdA group conjugated onto GFP. GFP-AdA was characterized using matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectroscopy, as shown in Figure S3. To study and confirm the efficient host–guest inclusion between apt- $\beta$ CD and GFP-AdA, we fluorescently labeled apt- $\beta$ CD with TMR (TMR-apt- $\beta$ CD) to titrate the fluorescence of GFP-AdA. The efficient FRET between TMR and GFP provides direct evidence for the inclusion complex of apt- $\beta$ CD and GFP-AdA. As shown in Figure 2B, mixing GFP-AdA with TMR-apt- $\beta$ CD at a 2:1 molar ratio resulted in ample fluorescence quenching of GFP-AdA and enhanced TMR emission, indicating the occurrence of efficient FRET between GFP-AdA and TMR-apt- $\beta$ CD and, hence, the formation of the inclusion complex. Importantly, when TMR-apt, an aptamer without  $\beta$ -CD conjugation, was mixed with GFP-AdA, no occurrence of FRET was observed (Figure 2B), again highlighting the necessity of the AdA- $\beta$ CD interaction to form the supramolecular aptamer and protein inclusion ensemble. A systematic titration of TMR-apt- $\beta$ CD with GFP-AdA showed a concentration-dependent FRET ratio (Figure

2C). Moreover, the supramolecular GFP-AdA and TMR-apt- $\beta$ CD complex were highly stable in the presence of serum, as evidenced by the minimal FRET change of the GFP-AdA and TMR-cb-apt- $\beta$ CD in the presence of serum (Figure 2C). This stability is essential for intracellular protein delivery.

The efficient and stable supramolecular interaction between cb-apt- $\beta$ CD and GFP-AdA prompted us to study whether the complex could transport proteins into cells via aptamer-mediated uptake of cb-apt- $\beta$ CD complex. Our preliminary study of the cytotoxicity of the cb-apt- $\beta$ CD/GFP-AdA complex (Figure S4) ensured the high biocompatibility of cb-apt- $\beta$ -CD for intracellular delivery. The cellular delivery of GFP to human cervical carcinoma HeLa cells, which also overexpress tyrosine kinase-7 on their cell surfaces, was studied by treating cells with cb-apt- $\beta$ CD/GFP-AdA, or monoapt- $\beta$ CD/GFP-AdA, as a negative control. Confocal laser scanning microscopy (CLSM) and flow cytometry were used to quantify and compare GFP delivery efficiency. As shown in Figure 3, and

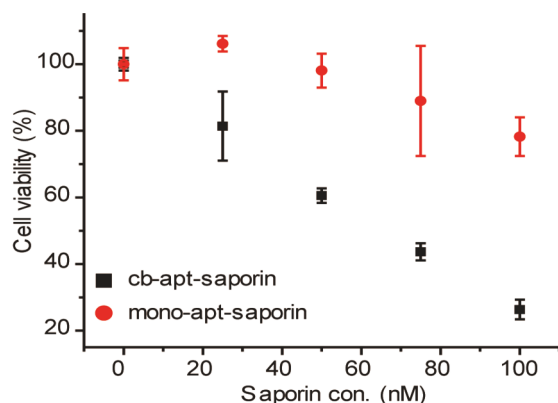


**Figure 3.** Cb-apt- $\beta$ CD-mediated enhanced GFP delivery into HeLa cells. (A) CLSM images of HeLa cells treated with 600 nM GFP-AdA complexed with cb-apt- $\beta$ CD (left panel) or monoapt- $\beta$ CD (right panel). Scale bar: 40  $\mu$ m. (B) Flow cytometry analysis of HeLa cells treated with PBS (cell only), GFP-AdA (1  $\mu$ M), monoapt/GFP-AdA (1  $\mu$ M), and the indicated concentration of cb-apt/GFP-AdA. The fluorescence intensity was normalized to cells with PBS treatment. Error bars represent the standard deviations of three parallel measurements.

Figure S5, GFP-AdA alone could not enter cells, and monoapt- $\beta$ -CD only facilitated the delivery of GFP-AdA with very low efficiency in the presence of serum. In contrast, the cb-apt- $\beta$ CD/GFP-AdA complex delivered GFP into cells with efficiency as high as 80% in cell culture medium (Figure S5), showing dependence on protein concentration (Figure 3B). The inefficient cellular uptake and delivery of GFP-AdA by monoapt- $\beta$ CD probably resulted from the lower stability of the monomeric aptamer in the presence of serum, leading to decreased binding to the cell surface receptor for intracellular delivery.<sup>11,15</sup>

Encouraged by the efficient delivery of GFP using cb-apt- $\beta$ CD, we finally investigated its potency for functional protein delivery for therapeutic interventions. To this end, saporin, a cytotoxic protein that inhibits protein synthesis by disrupting ribosomes, was selected, because its potent toxicity has been harnessed for protein-based cancer therapy.<sup>32,33</sup> We similarly conjugated saporin with an AdA handle (Figure S6) and loaded it into  $\beta$ -CD-conjugated aptamer before exposure to HeLa cells. As shown in Figure 4, treatment of HeLa cells with 100 nM cb-apt- $\beta$ CD and saporin-AdA complex (cb-apt-saporin) reduced





**Figure 4.** Cell viability of HeLa cells treated with different apt- $\beta$ CD-saporin complexes. HeLa cells treated with cb-apt-saporin or monoapt-saporin in 10% FBS containing medium at indicated saporin concentrations for 36 h, followed by determining cell viability by MTS assay. The error bars represent the standard deviations of three parallel measurements.

cell viability to 20%, whereas no obvious cytotoxicity was observed for the cells treated with the same concentration of free saporin or saporin-AdA alone, indicating that cb-apt- $\beta$ CD effectively enhanced the intracellular delivery of saporin (Figure 4 and Figure S7). Significantly, cb-apt- $\beta$ CD delivered saporin to inhibit cell proliferation with much higher efficiency than that of monoapt-saporin at any protein concentration level, again confirming a superior protein delivery efficiency using cb-apt- $\beta$ CD in biological settings.

In conclusion, we report herein a supramolecular approach to modulate the interaction of circular bivalent aptamer with molecular therapeutics for enhanced intracellular protein delivery. By controlling the host-guest chemistry of cb-apt- $\beta$ CD, we are able to efficiently deliver a small-molecule or cytotoxic protein for targeted cancer therapy. The supramolecularly engineered circular bivalent aptamer ensemble provides a general platform for creating a functional and stable synthetic aptamer construct to solve biological and physiological challenges associated with the clinical translation of aptamer-based molecular medicine.

## ■ ASSOCIATED CONTENT

### ■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.8b03442.

Experimental details (PDF)

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### Notes

The authors declare no competing financial interest.

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