Hierarchical Fabrication of DNA Wireframe Nanoarchitectures for Efficient Cancer Imaging and Targeted Therapy

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ABSTRACT: Though small-molecule drugs play a crucial role in cancer treatment, intrinsic issues such as poor solubility and systematic toxicity have considerably mitigated their anticancer functions and caused unwanted side effects. To achieve satisfying therapeutic efficiency, it is essential to develop innovative targeting systems for precise and efficient delivery of anticancer drugs. In this work, a hierarchical self-assembly strategy was applied to fabricate a core−shell nanoarchitecture composed of a DNA octahedral wireframe and chemodrug-functionalized Sgc8c aptamer. The integrated enhanced permeability and retention effect of the DNA nanostructure and active targeting ability of the Sgc8c aptamer allowed the highly selective chemodrug delivery and in vivo efficient imaging and treatment. The advantage of our multifunctional nanostructure was further highlighted by its impressive serum stability, excellent accumulation ability, deep penetration capability, significantly improved therapeutic efficacy, and favorable biosafety. This study showed promising potential of such a core−shell DNA nanoarchitecture in precise drug loading control, drug delivery, and personal medicine.

KEYWORDS: DNA nanostructure, octahedron, hierarchical self-assembly, cancer imaging, targeted cancer therapy

The systematic distribution and nontargeted cytotoxicity of hydrophobic small-molecule chemodrugs cause side effects and the reduction of efficacy, consequently hindering their wider applications in cancer treatment. 1 Thanks to the rapid development of drug carriers including organic polymers, 2 inorganic nanoparticles, 3 and liposomes, 4 numerous targeted chemodrug delivery strategies have been established to overcome these disadvantages. However, these synthetic materials have low biocompatibility, poor degradability, and/or immunogenicity risks. 5 The precise drug loading within a carrier is a key factor affecting the pharmacokinetic properties and delivery efficiency. 6 But for most carriers, especially polymer-based systems, it is difficult to control the overall molecular weight and size, leading to heterogeneous drug-to-carrier ratio and low reproducibility. 7 These intrinsic issues on targeted drug delivery hamper their broad applications in precise medicine and clinic trials. 8

As the size and geometry of DNA nanostructures can be predesigned and easily constructed, they have received extensive applications in biosensing, bioimaging, controlled drug release, and intelligent nanomedicine. 8–11 One- and two-dimensional DNA nanostructures (e.g., DNA nanotubes, DNA nanowires, DNA nanosheets, and DNA nanolattices) have been used to carry anticancer therapeutics based on their structure superiorities including high aspect ratio and facile designability. 12,13 To improve the access chance with target cells, more advanced three-dimensional DNA nanostructures present better tunability and binding ability. DNA origami is perceived as a major breakthrough of the structural DNA nanotechnology as a result of easy control of size, shape, and 3D geometry. 14,15 Many DNA origami structures have been developed for multiple payload loading and stimuli-responsive drug release. 16–18 However, the lability to endogenous nuclease degradation and high cost from hundreds of unemployed staple strands hamper DNA origami from being translated into clinic trials. 19–21 Owing to the excellent programmability, precise addressability, and explicit spatial orientation, DNA polyhedral nanostructures provide a promising platform of molecular medicine as a drug carrier to precisely functionalize drugs at the desired positions. 16,22

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DNA tetrahedra and other polyhedral frameworks are widely reported because of their facile self-assembly, high nuclease-resistant stability, and favorable internalization.\textsuperscript{11,23,24} By taking advantage of these factors, various small-molecule drugs, antisense oligonucleotides, and siRNA were loaded by these polyhedral nanostructures to enhance the cellular uptake, therapeutic efficacy, and biosafety.\textsuperscript{23,25,26} Given limited programmability and insufficient drug-loading capacity of typical DNA tetrahedra assembled by four ssDNA,\textsuperscript{7,11} we herein report our efforts that have been made in the design and application of an innovative nanoarchitecture that consists of a DNA octahedral wireframe and combretastatin A-4 (CA4, a broad-spectrum microtubule inhibitor)-functionalized Sgc8c aptamers (CA4-FS) for precise cancer imaging and efficient targeted therapy.

By hierarchical self-assembly of six DNA tiles,\textsuperscript{27} we construct a DNA octahedral wireframe to programmatically load and delivery free drug CA4 with this 3D three-layer core–shell nanostructure (Scheme 1a). Based on a solid-phase DNA synthesizer, CA4 is readily functionalized as a drug base and then is combined with other natural bases to modularly synthesize CA4-FS.\textsuperscript{15} In our design of DNA tiles, two single-stranded handles are overhung from each edge of this DNA wireframe to install CA4-FS with a high efficiency. By designing the sequence of extended handles followed by pairing of these handles with our CA4-FS, different numbers of CA4-FS could be precisely loaded on the DNA octahedron to form a core–shell CA-functionalized and aptamer-tethered DNA nanostructure, termed CA4-Oct. Moreover, this hierarchical self-assembly core–shell nanostructure also exhibited several appealing considerations in the design of Scheme 1b: (i) the outer “shell” layer Sgc8c aptamer (targets tumor biomarker PTK7) can play a receptor-binding role. Compared to a monovalent aptamer, these multiple aptamers in 3D space will enhance the binding chance and ability to receptors; (ii) the aptamer-based active target can unite in vivo an enhanced permeability and retention (EPR)-based passive target to enhance the drug delivery efficacy; (iii) the middle layer CA4 is hidden inside a “DNA sphere” to decrease its direct exposure in serum to avoid undesired premature toxicity; (iv) the dense and rigid DNA octahedron core could facilitate the insertion of flexible CA4-FS into a compact solid tumor tissue to enhance diffusion. Therefore, with our rational design, multiple CA4 molecules can be integrated in an aptamer-functionalized DNA octahedral nanocarrier, demonstrating a favorable CA4 delivery for targeted cancer therapy.

RESULTS AND DISCUSSION

Hierarchical Self-Assembly of DNA Octahedral Wireframe. To obtain single-stranded CA4-FS, the CA4 phosphoramidite group was prepared (Figures S1–S5) and then was incorporated into an Sgc8c aptamer via an automated DNA synthesizer (Figure S6, Table S1).\textsuperscript{28,29} Both label-free and Cy5-labeled CA4-FS were verified by mass spectra (Figures S7 and S8). CA4 was attached to this CA4-FS via a cellular phosphoesterase-responsive phosphodiester bond, allowing intracellular phosphoesterase-triggered (not pH-dependent, Figure S9) drug release when CA4-FS internalizes into target cancer cells.\textsuperscript{29,30}

We next constructed a DNA octahedron through a hierarchical self-assembly strategy. By programmable annealing, six DNA tiles of four-way junction were constructed as a vertex of DNA octahedra. Then the DNA tiles linked together by their single-stranded domain, forming a DNA octahedral wireframe (Figure S10, Tables S1 and S2). After we optimized the construction conditions (Figure S11), stepwise assembly of a DNA octahedron from DNA tiles was verified (Figure 1a). Atomic force microscope (AFM) imaging confirmed the morphology of the octahedron (Figure 1a). To demonstrate the tunable drug loading, DNA octahedra with different numbers of handles for hybridizing CA4-FS were constructed (Figure S12).

The addressability and predictability of this DNA octahedron allow us to precisely load different numbers of single-stranded CA4-FS. As a proof-of-concept, we herein constructed half-loading CA4-octahedron (hCA4-Oct) and full-loading CA4-Oct and measured the loading efficiency (Figure 1c, Table S3). To further verify the programmable assembly of hCA4-Oct and CA4-Oct, we next investigated the fluorescence resonance energy transfer (FRET) effect of CA4-Oct by using Cy3-labeled Oct-12H, Cy3-labeled Oct-24H, and Cy5-labeled CA4-FS. As expected, both hCA4-Oct and CA4-Oct showed obvious FRET signals (Figure S13a–c). The hydrodynamic size of the DNA octahedron was 17.0 ± 1.1 nm, and functional Oct-12H and Oct-24H were 24.9 ± 3.0 and 26.5 ± 3.4 nm, respectively. The size of hCA4-Oct was 29.5 ± 3.2 nm, and CA4-Oct was 30.1 ± 3.7 nm (Figure 1d). AFM imaging also confirmed the formation of hCA4-Oct and CA4-Oct (Figure S13d,e). This result revealed the CA4-FS was oriented toward the outside of the DNA octahedron core, forming a three-layer core–shell nanoassembly. The above results demonstrated the precise loading of multiple free drug molecules on a DNA octahedron.
Cellular Internalization and Tumor Spheroid Penetration. To load maximum CA4-FS, a fully loaded CA4-decorated DNA octahedron was chosen for the next studies. First, we focused on the crucial cellular internalization of CA4-Oct using single-stranded CA4-FS, Lib (control of Sgc8c), CA4-Lib (control of CA4-FS), and CA4-lib-Oct (control of CA4-Oct) as controls. Sgc8c aptamer tethered groups showed apparent enhancement of cellular internalization compared to their own control group (Figure 2a,b); especially, CA4-Oct obtained the highest fluorescence signal. Thus, these findings demonstrated the enhanced cellular internalization by modifying aptamers on a DNA nanostructure. The competitive blocking experiment also supported the conclusion (Figure S14). Moreover, the shift of CA4-Oct is greater than CA4-FS. On the basis of the above results, we inferred that the following factors contributed to this enhanced internalization:25,26,31 (i) increase of recognition units (24 CA4-FS vs 1 CA4-FS); (ii) rigidity of the octahedron structure; and (iii) improved stability of CA4-Oct. As for the vital role of the stability factor, we tested the biostability of CA4-Oct and CA4-FS in 10% FBS. After 8 h treatment, CA4-Oct remained intact, but 55% of CA4-FS was degraded (Figure S15). So the time-dependent internalization growth rate of CA4-Oct was faster than CA4-FS (Figure 2b). CA4-Oct and CA4-FS showed the same time-dependent internalization growth rate because they kept the same integrity in FBS-free medium, which further illustrated the vital stability factor decelerated CA4-FS’s time-dependent internalization (Figure S16).

Figure 1. Hierarchical self-assembly of a DNA octahedron and a CA4-loaded octahedron. (a) Agarose gel electrophoresis analysis of stepwise assembly of a DNA octahedron. M: 1 kb DNA marker; lanes 1–6: six different vertexes based on DNA tiles; lanes 7–10: conjugates of different numbers of vertexes; lane 11: DNA octahedron. (b) AFM imaging of a DNA octahedron. (c) Agarose gel electrophoresis analysis of stepwise assembly of HCA4-Oct and CA4-Oct. L: 100 bp DNA ladder, lane 1: Sgc8c, lane 2: CA4-FS, lane 3: DNA octahedron, lane 4: octahedron with 12 DNA handles (Oct-12H), lane 5: hCA4-Oct, lane 6: octahedron with 24 DNA handles (Oct-24H), lane 7: CA4-Oct. (d) DLS measurements of a DNA octahedron, functional DNA octahedron, and CA4-loaded octahedron.

Figure 2. Cellular internalization and tumor spheroid penetration of a CA4-loaded DNA octahedron. (a) Representative Cy5 intensity histogram of different Cy5-labeled DNA incubated with HCT116 cells for 8 h. (b) Normalized Cy5 mean fluorescence intensity of different Cy5-labeled DNA incubated with HCT116 cells for 2, 4, or 8 h. (c) Representative Cy5 intensity histogram of CA4-Oct and CA4-FS incubated with HCT116 cells or NCM460 cells for 8 h. (d) Cellular selective coefficient of the Cy5 intensity ratio of HCT116 to NCM460 incubated with CA4-Oct or CA4-FS for 8 h. (e) Confocal images were captured at intervals of 10 μm from the top to the middle section of tumor spheroids. Red channel: Cy5. Scale bars: 50 μm. (f) Interactive 3D surface plot of max intensity projection of the Cy5 channel in (e). All data are mean ± SD, n = 3. Statistical significance: ***p < 0.001, **p < 0.01, *p < 0.05.
To investigate the cellular selectivity, PTK7-negative NCM460 cells (normal colorectal cell line) were also tested. Compared to NCM460 cells, CA4-Oct and CA4-FS showed more uptake into HCT116 cells (Figure 2c). It is noteworthy that CA4-Oct performed much greater cellular internalizing selectivity of CA4 delivery between HCT116 cells and NCM460 cells compared to single-stranded CA4-FS (Figure 2d). The visualized confocal imaging also confirmed the above findings (Figure S17). These results demonstrate CA4-Oct performed more cellular internalization and selectivity over single-stranded CA4-FS.

To study the delivery depth of CA4 in solid tumor tissue, an HCT116 3D multicellular tumor spheroid was prepared. Compared with the single-stranded CA4-FS, CA4-Oct showed higher intensity in the center of the tumor spheroid (Figure 2e). Moreover, CA4-FS rested on the surface of the tumor spheroid, while CA4-Oct distributed on both the surface and in the center of every depth section. Besides, based on the quantitative result, CA4-Lib-Oct, lacking an aptamer shell, lost more penetration depth and cellular uptake compared to CA4-FS, lacking an octahedron core, if we took CA4-Oct as the standard (Figure 2f). These findings supported the hypothesis that the aptamer shell played a dominant role, owing to its recognition and binding role, and that the DNA octahedron core enhanced the insertion of CA4-FS into tumor tissues.

**Targeted Cytotoxicity and Stability.** After identifying the cellular internalization and penetrating ability of CA4-Oct, we determined cytotoxicity against its target cancer cells. To study the influence of the targeted delivery mediated by aptamer, we chose a short-term incubation of 8 h and an additional incubation of 40 h, in turn, to separate out the anticancer function from nonspecific cell adsorption and nucleolysis. We found that the control groups including Sgc8c, Lib, CA4-Lib, DNA octahedron, and CA4-Lib-Oct showed no or negligible cytotoxicity against HCT116 cells (Figure 3a, Figure S18). As expected, CA4-Oct showed higher inhibitory cytotoxicity than CA4-FS against HCT116 cells (Table S4). The contrastive flow cytometry assay further validated the inhibitory cytotoxicity was caused by apoptosis (61.8% vs 46%, Figure S19). To further highlight the cell-specific cytotoxicity of CA4-Oct, we also compared the cytotoxicity against HCT116 and NCM460 cells. As indicated in Figure 3a, all groups except for CA4 exhibited no or negligible cytotoxicity toward NCM460 cells after 48 h of incubation (washing after an 8 h pretreatment, Figure S20). Based on these results, CA4-Oct shows the selectivity to target cancer cells by the enclosure of CA4-FS, thus presenting selective cytotoxicity.

We next increased the incubation time to 48 h to evaluate the possibility of off-target risk caused by potential instability-mediated degradation of DNA sequences. CA4-Oct and CA4-FS showed cytotoxicity comparable to that of CA4 against HCT116 cells (Figure 3a). However, the CA4-Lib group also showed low viability against HCT116 cells, indicating indiscriminate cytotoxicity in a manner like that of CA4-FS at 20 nM (Figure S21). CA4-Lib-Oct showed weaker cytotoxicity in comparison to CA4-Lib, CA4-FS, CA4-Oct, and CA4 (Figure S20). On the basis of these findings and previous cytotoxicity results under short-term treatment, we inferred that long-term treatment of CA4-FS and CA4-Lib in culture medium containing FBS caused their fast degradation (Figure S15), causing the leakage of free drug (off-target effect) and further inducing more toxicity than that noted for the short-time treatment. To further confirm this inference, we also examined the cytotoxicity of CA4-Oct and CA4-FS against the NCM460 cell line. We found that CA4-FS showed higher toxicity against NCM460 cells than CA4-Oct at concentrations of 10 and 20 nM (Figure 3a, Figure S22). On the basis of these findings, we suggest that the considerably improved nuclease resistance ability of CA4-Oct could significantly mitigate the...
off-target effect of monovalent CA4-FS, resulting in a much better safety profile.

To further systematically reveal the targeted cytotoxicity of CA4-Oct, a cell coculture system was constructed to simulate the realistic impact of drugs on the in vivo multicellular coexistent environment. Herein, CA4-Oct or CA4-FS was used to treat the coculture system of GFP-transfected HCT116 (GFP-positive) and NCM460 (GFP-negative) cell mixtures (1:1, Q3/Q4, Figure 3b), respectively. After 48 h of incubation without any treatment, the ratio between live cancer cells and live normal cells (RCN) rose to about 2:1 because of the malignant proliferation of cancer cells (Figure 3c). For short-term treatment (washing after 8 h), RCN in the CA4-Oct group became 1:4, and the RCN in the CA4-FS group was only 1:2 (Figure 3d,e). Besides the inhibitory proliferation of cancer cells (Figure 3c). For short-term treatment (washing after 8 h), RCN in the CA4-Oct group became 1:4, and the RCN in the CA4-FS group was only 1:2 (Figure 3d,e). For short-term treatment (no washing), CA4-FS killed 52% of the NCM460 cells, although it induced apoptosis of 71% of HCT116 cells (Figure 3g). However, CA4-Oct also killed 71% of HCT116 cells, but only caused apoptosis of 28% of NCM460 cells (Figure 3f). As a result, the RCNs for CA4-FS and CA4-Oct are about 1:3 and 1:5, respectively. Apparently, the off-target side effects of CA4-FS are much more serious than those of CA4-Oct because of the difference of stability in extracellular serum. Meanwhile, Sgc8c-pretreated block experiments demonstrated the higher receptor-mediated internalization could account for the cytotoxicity of CA4-Oct against target cells (Figure S23). These results revealed that the DNA octahedron enhanced the targeted cytotoxicity to cancer cells and reduced instability-mediated off-target risk to normal cells.

**Imaging Analysis in Vivo.** Encouraged by favorable in vitro performances, we implemented in vivo assessments. First, Cy5-labeled CA4-Oct, Cy5-labeled CA4-FS, and free Cy5 were injected intravenously to SD rats to evaluate their pharmacokinetics by tracking the fluorescent signal of orbital venous blood, respectively. Compared with the residue of free Cy5 at 2 min, the equivalent fluorescence was found at 20 min of CA4-FS and 120 min of CA4-Oct (Figure S24a). This result supported that no serious off-label phenomena of Cy5 occurred in in vivo experiments. The Cy5 signal of CA4-FS was fully eliminated at 60 min, while about 60% of the residue of CA4-Oct was still retained (Figure S24b). To further clarify the pharmacokinetics of CA4-Oct and CA4-FS, the CA4 concentration (equal to the Cy5 concentration) was determined through an external standard quantitative method of Cy5 intensity (Figures S25 and S26). CA4-Oct has about a 5-fold half-life compared with CA4-FS (Table S5). In addition, the mean residence time (MRT) of the CA4-Oct group in rat blood was about 4 times longer than that of the CA4-FS group. More importantly, the total CA4 concentration of the CA4-Oct group was 2601 μg/L·min, or about 33 times greater than that of the CA4-FS group. All these results indicated that the CA4-Oct carrying CA4 showed much higher blood concentration and circulation time than single-stranded CA4-FS.
To further investigate the potential of CA4-Oct for in vivo targeted anticancer therapy, we next constructed the HCT116 tumor-bearing xenografted mouse model to investigate the biodistribution of CA4-Oct via optical imaging. Cy5-labeled CA4-Oct, Cy5-labeled CA4-FS, and free Cy5 were injected intravenously to HCT116 tumor-bearing mice, respectively.

Figure 5. In vivo therapeutic assessments of HCT116 tumor-bearing xenografted mice by intravenous administration of PBS, CA4-Oct, CA4-FS, and CA4. (a) Tumor growth curves of HCT116 tumor-bearing xenografted mice. Arrows denote the administration. (b) Tumor weight statistics after dissecting euthanized mice. (c) Photos of tumor tissues after dissecting euthanized mice. (d) Relative body weight curves of HCT116 tumor-bearing xenografted mice. Arrows denote the administration. (e) Statistical result of main visceral weight from each group. (f) Representative TUNEL-staining immunofluorescence images of tumor sections and quantitative statistical results. Blue, DAPI. Green, TUNEL. (g) Representative Ki67-staining immunohistochemistry images of tumor sections and quantitative statistical results. Brown dots denote Ki67 signal. (h) H&E staining of liver sections. Arrows denote lymphocytic infiltration. (i) H&E staining of kidney sections. Arrows denote cellular exfoliation and expansion. Scale bars: 100 μm. All data are mean ± SD, n = 4 mice in (a), (b), (d), (e), n = 8 fields in (f), (g). Statistical significance: ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05; ns, no significant difference.
The CA4-FS group showed a much brighter Cy5 signal at the tumor site in 4 and 8 h but showed a very weak fluorescence signal at 12 h postinjection (Figure 4a). To our delight, the fluorescence signal of the CA4-Oct case was still bright at 1 day postinjection. Meanwhile, mice were sacrificed for ex vivo analysis to evaluate the biodistribution. At 12 h after intravenous administration, Cy5 and CA4-FS are mainly distributed in the kidneys, while CA4-Oct displayed vigorous fluorescence signal in the tumor site for more than 24 h (Figure 4b). From the semiquantitative results of Cy5-labeled fluorescence from 1 to 12 h, the CA4-Oct group indicated the slowest elimination rate, with about 2.5-fold decline of Cy5 intensity, while the CA4-FS and Cy5 groups showed a 5-fold and 7-fold decline, respectively (Figure S27a). As a result, CA4-Oct still indicated higher selectivity than CA4-FS and Cy5 intensity between the tumor and the normal tissue, liver (Figure S27b). This selectivity became increasingly obvious as time went by because CA4-Oct could rest on the tumor tissue for a longer time. These results clarified that DNA octahedra could enhance the enrichment concentration and retention time of the CA4 drug compared to CA4-FS.

To further determine whether CA4-Oct has superior accumulation capacity and penetration ability in solid tumor tissue, tumor tissues of the CA4-Oct group and CA4-FS group were collected to implement section analysis. The CA4-Oct group showed a brighter Cy5 signal than the CA4-FS group at all the experimental time points (Figure 4c,d), indicating that CA4-Oct possessed better accumulation ability to deliver much more CA4 drug to tumor tissues. Based on the colocalization results between CA4-FS and FITC-labeled CD31, a biomarker of the blood vessel, most CA4-FS distributed inside or around tumor vessels (Figure 4d). In contrast, most CA4-Oct crossed tumor vessels and reached the tumor parenchyma (Figure 4c), which would be beneficial for deep drug delivery. Multivalent, compact, and rigid CA4-Oct indeed enhanced the penetration ability of single-stranded CA4-FS and its therapeutic potential in solid tumor tissue.

**Targeted Cancer Therapy in Vivo.** After the imaging characterizations, we conducted antitumor experiments in HCT116 tumor-bearing xenografted mice followed by determining no hemolysis phenomenon (Figure S28). After five times intravenous administration (day 18 after tumor inoculation), the tumor growth inhibitory rate of CA4-Oct, CA4-FS, and CA4 groups reached 86%, 65%, and 46%, respectively (Figure 5a). However, once the administration was stopped, the tumor inhibitory rate of CA4-Oct still remained about 85%, while that of the CA4-FS and CA4 groups further decreased to 54% and 30%, respectively (day 32). This result could be attributed to the deeper penetration and longer time retention ability of CA4-Oct. Moreover, both the ex vivo tumor weight and tumor images demonstrated that CA4-Oct was the best choice as a targeted agent (Figure 5b,c).

To assess therapeutic safety, we first monitored the body weight variation of these mice. The CA4 treatment produced an obvious body weight loss (about 7%) owing to the side effect of systemic distribution (Figure 5d). The CA4-FS group also lost 6% ponderal growth compared with the CA4-Oct group. Then, to evaluate the possible damage of these drugs to healthy tissue, the main viscera were collected and weighed on the 32nd day. The administration of CA4 and CA4-FS both showed more serious abnormal weight gain of the liver compared with the PBS group and CA4-Oct group (Figure 5e). The damage to this detoxification organ may be attributed to off-target toxicity of CA4-FS and nonselective toxicity of CA4. To further elucidate CA4-Oct therapeutic efficacy and safety, we collected tumor tissues, main viscera, and blood to carry out a more detailed study. CA4-Oct indicated about 65% apoptosis with TUNEL-positive cells (TUNEL⁺), while CA4-FS and CA4 just showed about 35% and 20% killing ability (Figure 5f). Proliferation mark Ki67 signal indicated that CA4-Oct performed the most inhibition against tumor proliferation, compared to the CA4-FS group and CA4 group (Figure 5g).

We next evaluated therapeutic safety by analyzing collected main viscera and blood. To reasonably evaluate possible side effects and other significant differences, we also collected normal organs and blood of normal mice (no tumor inoculation) under the same condition. First, we investigated possible hepatic toxicity because CA4 could induce liver pain. Compared to the normal group of no tumor inoculation, the PBS group indicated slight lymphocytic infiltration in the liver, but the administration of CA4-Oct alleviated that tendency (Figure 5h). Moreover, neither the PBS nor CA4-Oct indicated obvious dead liver cells. By contrast, the CA4-FS group indicated local necrosis of liver cells and hyperplasia of HSCs (hepatic stellate cells). Local lymphocytic infiltration was observed around the central veins of mouse liver (Figure 5h). More seriously, large patchy necrosis of liver cells and more obvious hyperplasia of HSCs were observed after the administration of CA4. Many infiltrative areas of neutrophils and lymphocytes could be found in the CA4 group, and the abnormal increase of alanine transaminase and aspartate transaminase also confirmed this tendency (Figure S29a,b). These results revealed different levels of CA4-FS and CA4 hepatic toxicity, while CA4-Oct showed a certain potential safety profile.

Then we evaluated the histopathology of H&E-stained sections from the kidneys of each group. Compared with the normal group, no significant difference was noted between the PBS group and CA4-Oct (Figure 5i). However, after the administration of CA4-FS, focal exfoliation of the brush border of renal tubules and slight cellular swelling could be seen. We even found obvious elevation of serum creatinine and interstitial edema with inflammatory cellular infiltration besides the obvious cellular exfoliation and swelling in the free CA4 group (Figure 5i, Figure S29c). These results demonstrated the aptamer modification alleviated the renal damage of CA4 and the DNA octahedron protected CA4-FS from overflowing off-target CA4 to kidney tissue.

We continued to check the other biochemical and hematological data. Compared to the normal group and PBS groups, the CA4-Oct group showed no significant increase of creatinine phosphokinase (CK), while the CA4-FS group and CA4 group showed different levels of increased CK (Figure S29d). This result indicated that CA4-FS and CA4 caused different degrees of cardiac damage. It was also reported that free CA4 could induce thrombocytopenia. The administration of CA4 indeed caused obvious reduction of platelets, but the CA4-FS and CA4-Oct groups had no damage (Figure S29e). Another discrepant index is the number of white blood cells (WBCs). Surprisingly, the CA4-Oct group had no obvious elevation of WBCs, compared with the normal group, while the PBS, CA4-FS, and CA4 groups showed an obvious increase of WBCs (Figure S29f). These findings further illustrated that the administration of PBS vehicle, CA4-FS, and CA4 could cause potential inflammation and myelosuppression. Only the
anticancer scheme of CA4-Oct could obtain the most efficient therapy with enough biological safety. Unfortunately, we did not find other superiorities of CA4-Oct from the assessments of H&E sections of heart, liver, and spleen (Figure S30). Additionally, all groups except for CA4 did not induce apparent elevation of immune response indicators (Figure S31).

CONCLUSION
To address the problems of small-molecule drugs for cancer treatment, we designed and engineered an innovative DNA wireframe nanoarchitecture that was composed of a DNA octahedron and CA4-FS. In this work, the solid-phase synthesis technique was utilized to prepare CA4-FS, and DNA hierarchical self-assembly technology was applied to construct the DNA octahedron with number-controllable outward handles. With a programmatically loading process, multiple CA4-FS could be accurately attached onto the DNA octahedron to form a 3D nick-hidden core-shell nanoarchitecture. Compared with a flexible aptamer carrier with exposed 5′-terminal and 3′-terminal, the DNA octahedral nanocarrier had a much higher nuclease-resistant stability with a compact, rigid, and mainly nick-hidden DNA nanostructure. This DNA nanostructure displayed a precise and high-capacity drug loading control property because of functionalization of the DNA handle. Meanwhile, the DNA wireframe nanocarrier reshaped the properties of single-stranded CA4-FS and showed a much better biostability, higher cellular internalization, excellent imaging characteristic, and deeper tumor penetration to gain more efficient cancer imaging and targeted therapy. Taken together, based on the hierarchical self-assembly, this DNA octahedron with drug positioning function provided an excellent imaging characteristic, and deeper tumor penetration to gain more efficient cancer imaging and targeted therapy. Precise medicine.

EXPERIMENTAL METHODS
Construction of DNA Nanoassemblies. All DNA nanostructures were constructed in 1x TE buffer (20 mM Tris base, 1 mM EDTA) containing 10 mM MgCl₂ by programmable annealing (Table S2). For construction of the drug-loaded octahedron assembly, corresponding numbers of CA4-FS or CA4-Lib were loaded to the octahedral nanostructures (with corresponding numbers of handles) by strand hybridization for 2 h at room temperature (shaking) to obtain CA4-Oct, hCA4-Oct, or CA4-Lib-Oct. For AFM and DLS measurements, primary DNA nanostructures were purified with 50 kD cutoff Amicon filters. The DNA nanostructures were centrifuged at 10000g for 6 min two times to remove unhybridized ssDNA. The purified DNA was recovered by spinning at 5000g for 3 min.

Agarose Gel Electrophoresis. To study the DNA nanostructures, 2% agarose gel electrophoresis was carried out in 1x TBE (89 mM Tris base-borate, 2 mM EDTA) containing 10 mM MgCl₂ buffer at 60 V, 4 °C, 2 h. In the stability analysis, 100 nM octahedron, 100 nM CA4-Oct, 2.4 μM Sgc8c, and 2.4 μM CA4-FS were run in 2% agarose gel electrophoresis in 1x TBE/10 mM MgCl₂ buffer at 110 V, 4 °C, 10 min. Then the gel was stained by EB or Super GelRed (US Everbright Inc.) for imaging using a ChemiDoc imaging and analysis system (Sage Creation, Beijing, China).

AFM Measurement. AFM images were captured on a Multimode VIII system under peak force tapping mode (Bruker). Purified DNA structures (5 μL) were deposited and left on the surface of a freshly cleaved mica chip for approximately 2 min to allow for absorption. Then 40 μL of 1x TE (10 mM MgCl₂ and 1 mM of NiCl₂) was added onto the mica surface. Commercial silicon nitride cantilevers with integrated sharpened AFM tips (Bruker, SNL-10) were used.

DLS Measurement. A 100 μL amount of 100 nM octahedra and purified DNA structures were used to determine the hydrodynamic diameter with a Zetasizer Nano-ZS (Malvern Instruments).

Cell Culture and Animal Models. All cell lines were purchased from ATCC. HCT116 cells, GFP-transfected HCT116 cells, and NCM460 cells were cultured with RPMI-1640 medium, including 10% FBS supplemented with 10% (v/v) fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 mg/mL streptomycin. Female SD (Sprague–Dawley) rats (about 100 g) and female Balb/c nude mice (7–8 weeks) were obtained from Hunan SJA Laboratory Animal Co. Ltd. and used under protocols approved by Hunan University Laboratory Animal Center. SD rats were used to carry out the pharmacokinetics assay. To obtain a subcutaneous colorectal tumor model, around 8 × 10⁶ HCT116 cells in 100 μL of DPBS were subcutaneously injected in the right underarm of each Balb/c nude mouse.

Flow Cytometry Assay. All flow cytometry measurements were done with DxP Athena (US Cytek Biosciences Inc.). For the quantitative internalization assay, a total of 2 × 10⁶ cells (HCT116 or NCM460 cells) were seeded in a 24-well plate and incubated overnight for adherence before treatment. After removing the culture medium, cells were incubated with different Cy5-labeled (equivalent to 200 nM Cy5) DNA for 2, 4, and 8 h incubation in complete medium or FBS-free medium. After that, cells were washed twice with DPBS and then detached with 50 μL of trypsin. Then the trypsin was quenched by 200 μL of complete RPMI-1640 medium. Finally, cells were resuspended in 400 μL of DPBS twice before testing with a flow cytometer. For competitive internalization assays, HCT116 cells were preincubated with 200 nM label-free Sgc8c aptamer or Lib ssDNA for 1 h (no washing) and then incubated with Cy5-labeled CA4-Oct for 2 h. Then the cells were washed before testing with a flow cytometer.

For the apoptosis assay of HCT116 cells, 10⁵ HCT116 cells were seeded in a six-well plate and incubated overnight for adherence before the experiment. After removing the culture medium, cells were treated with CA4-bearing CA4-Oct or CA4-FS (equivalent to 20 nM CA4) for 4 h, respectively. Cells were washed and then incubated for another 40 h. Next cells were collected and stained with 5 μL of annexin-V FITC and 5 μL of PI in 195 μL of binding buffer for 15 min, following the manufacturer’s protocol (Beijing Solarbio Science & Technology Co., Ltd.). Finally, the samples were tested by flow cytometer.

For apoptosis assay of the cell coculture system, a total of 10⁵ cell mixtures (GFP-transfected HCT116:NCM460 = 1:1) were seeded in a 12-well plate and incubated for adherence before the experiment. Then, one well of cells, as the 0 h control group, was collected and then stained with 1 μM PI for 10 min prior to being tested by a flow cytometer. The second well was considered as the 48 h control group (cultured for 48 h without drug treatment). The other wells were treated with CA4-bearing CA4-Oct or CA4-FS (equivalent to 20 nM CA4, washing after 8 h of incubation or 48 h of incubation without washing), respectively. After that, cells were collected and then stained with 1 μM PI for 10 min. Then the samples were tested with a flow cytometer using previous experimental conditions. For the Sgc8c-pretreated competitive assay, the two cell mixtures were preincubated with 200 nM label-free Sgc8c for 1 h (no washing) and then incubated with CA4-Oct or CA4-FS (equivalent to 20 nM CA4) for 8 h at 37 °C prior to be washed. After another 40 h of incubation, the cells were stained with 1 μM PI for 10 min prior to being tested by a flow cytometer.

Three-Dimensional Multicellular Tumor Spheroids. Tumor spheroids were cultured by the liquid overlay method. Soluble 1.5% agarose gel with serum-free 1640 medium was placed at the bottom of a 96-well plate (NEST Biotechnology). The collected HCT116 cells were resuspended by 2.5% Matrigel with complete 1640 medium after air drying. Next, each well was seeded with 1000 cells and then centrifuged at 1000 rpm for 10 min at 4 °C. Cells were cultured for about 7 days, and half the culture medium was changed every 2 days. Then spheroids were incubated with 5 μg/mL Hoechst33342 overnight and Cy5-labeled CA4-Oct, CA4-FS, CA4-Lib-Oct, and CA4-Lib (equivalent to 200 nM Cy5 or CA4), respectively, for 8 h at
37 °C. After that, spheroids were washed three times with DPBS and then pipetted to a conical dish. Finally, spheroids were subjected to imaging by confocal microscopy using the FV1000-X81 confocal microscope (Olympus, 10× OBJ). The 3D tumor spheroids were imaged with a zeta scan for 3D reconstruction.

**In Vivo Imaging and ex Vivo Biodistribution.** When the tumor size of the Balb/c nude mice reached ~300 mm³, Cy5-labeled CA4-Oct, Cy5-labeled CA4-FS, or free Cy5 (equivalent dose of Cy5, 10 nmol in 100 μL of DPBS) was intravenously injected into the tumor-bearing mice, respectively. Images were obtained using an IVIS Lumina XR before injection and at 1, 4, 8, 12, and 24 h postinjection. After 1, 12, and 24 h postinjection, the mice were sacrificed. Next, we dissected the mice and imaged the tumor tissue and main viscera (heart, liver, spleen, lung, kidney) ex vivo to analyze the biodistribution of CA4-Oct and CA4-FS based on Cy5 intensity.

**Antitumor Evaluation in Vivo.** At day 8 postinoculation, when the tumor volume of the Balb/c nude mice had reached 150–200 mm³ (volume = 0.5 × length × width³), PBS, CA4-Oct, CA4-FS, and CA4 (the equivalent CA4 dose of 4 mg/kg) were i.v.-injected to HCT116 tumor-bearing mice once every 2 days for five times in total. CA4 was dissolved by PBS containing 5% DMSO and 20% PEG, and other materials were dissolved by PBS only. Then the administration was stopped, and tumor size and body weight were monitored continuously. At day 32, the mice were sacrificed. Blood, tumor tissues, and main organs were collected for further analysis.

**ASSOCIATED CONTENT**

- Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsnano.0c07495. Experimental details, DNA sequences, and characteristics in vitro and in vivo (PDF)

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Y.K., R.P., and D.W. designed the project. D.W. and R.P. prepared the molecular platforms and corresponding characteristics. D.W. tested all assessments in vitro. Y.P. tested all assessments in vivo. Z.D. and Y.S. participated in the cell experiments. F.X., P.W., and L.L. participated in structure characteristics. D.W. and Y.P. analyzed all data. Y.K. interpreted the data. D.W. and R.P. cowrote the manuscript. X.W., Y.K., and W.T. guided the project’s orientation and finalized the manuscript.

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