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Automated Modular Synthesis of Aptamer–Drug Conjugates for Targeted Drug Delivery

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Supporting Information

ABSTRACT: Aptamer–drug conjugates (ApDCs) are promising targeted drug delivery systems for reducing toxicity while increasing the efficacy of chemotherapy. However, current ApDC technologies suffer from problems caused by the complicated preparation and low controllability of drug–aptamer conjugation. To solve such problems, we have designed and synthesized a therapeutic module for solid phase synthesis, which is a phosphoramdite containing an anticancer drug moiety and a photocleavable linker. Using this module, we have realized automated and modular synthesis of ApDCs, and multiple drugs were efficiently incorporated into ApDCs at predesigned positions. The ApDCs not only recognize target cancer cells specifically, but also release drugs in a photocontrollable manner. We demonstrated the potential of automated and modular ApDC technology for applications in targeted cancer therapy.

Traditional anticancer chemotherapeutic drugs affect both cancer cells and healthy cells without selectivity, resulting in severe side effects.[1] Targeted drug delivery is promising to reduce drug toxicity in healthy cells and diminish side effects, thereby improving therapeutic efficacy.[2] Active targeted therapy exploits the specific recognition ability of targeting elements (e.g., antibodies and aptamers) to disease tissue. For instance, antibody–drug conjugates (ADCs) combine less selective cytotoxic drugs with antibodies, so that ADCs can selectively target and kill disease cells, including cancer cells, with reduced side effects.[3] Several ADCs have received marketing approval by the FDA, and more ADCs are in clinical trials.[3b,d,4] This demonstrates the feasibility of targeted therapy by conjugating less selective drugs with molecular targeting elements that can selectively recognize target disease sites.

In addition to antibodies, aptamers comprise another class of molecular targeting elements for selective disease tissue recognition in targeted therapy. Aptamers are single-stranded oligonucleotides that can specifically recognize their targets, including live cancer cells, and bind to the targets with high affinity.[5] DNA aptamers targeting cancer cells can be generated by cell-SELEX (systematic evolution of ligands by exponential enrichment) technology developed by our group.[6] Similar to antibodies, some aptamers themselves can be used as macromolecular drugs.[7] Compared with antibodies, aptamers are more stable as dry powders or in solution, less toxic, easier to modify for diverse purposes, and more economical to produce. Hence, aptamers have been exploited as exceptional targeting elements for cancer cells and have been widely studied in targeted cancer therapy.[8]

Aptamer–drug conjugates (ApDCs) have been developed to exploit aptamer-based targeted drug delivery.[8a,e,f] ApDCs/ADCs are similar to molecular trains (MT) in structure, and drugs are manually loaded into the "vehicles" (aptamers/antibodies) and selectively transmitted to target cells. Compared to ADCs, ApDCs present unique advantages, including vehicle economy and procedural simplicity, because aptamers are relatively small in size and can be chemically synthesized. For aptamer–drug coupling, current ApDC technologies largely rely on the noncovalent association of drug and specific DNA sequences,[8g] or complicated and less efficient organic synthesis.[8a] These technologies suffer from complicated preparation, low controllability of site-specific drug conjugation on vehicles, low synthesis yield in some cases, low drug loading capacity and the accompanying high cost and low spatiotemporal controllability in drug release. For instance, only a limited number of drug copies can be conjugated onto one aptamer strand in ApDCs, resulting in low drug loading capacity. This is also the case for antibody vehicles, which can deliver only a very few drug molecules in each ADC. Moreover, the preparation of these ApDCs or ADCs is typically complicated: aptamer and antibody "vehicles" are prepared first, followed by the chemical reactions to conjugate drugs and the vehicle manually.[3,8,9]

Solid-phase synthesis technology represents a paradigm of automated and highly controllable molecular synthesis.[10] This technology is able to generate DNA from individual phosphoramidite building blocks (A, T, C, and G) with high efficiency, providing a platform for automated and sequence-predesigned DNA synthesis. Inspired by this technology, we are interested in the development of therapeutic modules that can be integrated into ApDCs, as well as the downstream

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automated and modular ApDC preparation by solid-phase synthesis technology. Here we report the design and synthesis of the next-generation of ApDCs for targeted drug delivery, by first developing a drug-incorporated module and then using the solid-phase synthesis technology to automatically integrate aptamers and drug modules. As shown in Figure 1A, the

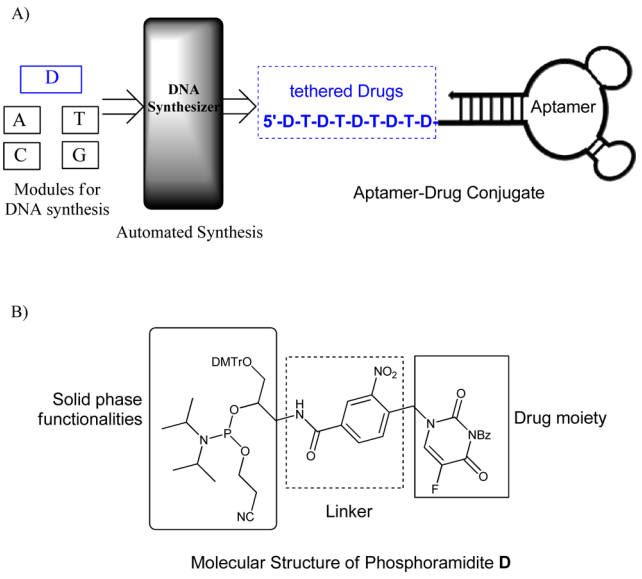


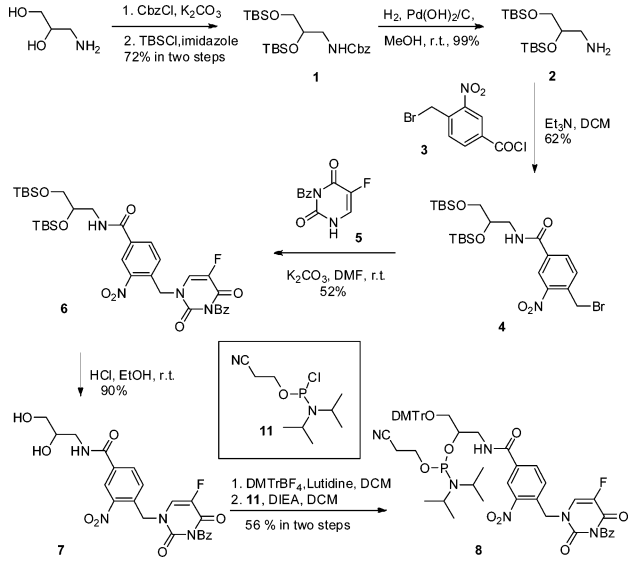
Figure 1. (A) Automated and modular synthesis of ApDCs from phosphoramidites A, T, C, G, and **D**. (B) Structural features of phosphoramidite **D**.

aptamer moiety on one end serves as the locomotive of the MT, and the tandem drug modules serve as the "boxcars". Each boxcar contains a drug (**D**) molecule, which was first integrated into phosphoramidite as a therapeutic module phosphoramidite **D** (Figure 1B). In addition, we incorporated spatiotemporal controllability of drug release into the therapeutic module through a photocleavable chemical linkage of drugs and vehicles. The ApDCs can be automatically prepared from modules **D**, A, T, C, and G on an automated DNA synthesizer in a tailor-designed manner (Figure 1A). In this way, multiple drug moieties can be conjugated onto one aptamer at predesigned positions and drug loading capacity. The automated conjugation of aptamers with drugs is operationally simple and highly efficient. It also improves vehicle economy and achieves spatiotemporally controllable drug release.

To realize the automated and modular synthesis of ApDCs, the key is the design and synthesis of the therapeutic module: phosphoramidite **D**. As shown in Figure 1B, phosphoramidite **D** is composed of three parts: solid phase synthesis functionalities, drug moiety, and a linker between. For synthesis simplicity, we chose glycol as the backbone instead of ribose.[11] Various drug molecules can be covalently incorporated into the phosphoramidite as prodrugs, but subtle protecting strategy will be required for those with nucleophilic functionalities such as hydroxyl and amino groups. We chose Fluorouracil (5-FU), a simple and widely used anticancer drug for treatment of many types of cancers, including colorectal cancer and pancreatic cancer,[12] as a model in this study to test our idea. Between the backbone and drug, a functional linker is necessary for efficient and controllable drug release (as the results of ApDC **sgc8-5FU** demonstrated). In this design a nitrobenzene derivative was selected as the photocleavable linker (PC-linker) between drug and the backbone of MT to achieve photocontrollable drug release.[13]

The synthesis of phosphoramidite **D** is started with 3-amino-1,2-propanediol, which was selectively protected with Cbz and TBS groups providing compound **1** in 72% yield in two steps (Scheme 1). Removal of N-Cbzgroup followed by coupling of

Scheme 1. Synthetic Route of Phosphoramidite 8 as a Module for DNA Synthesis



amine **2** with benzoyl chloride **3** gave nitrobenzyl bromide **4**. The nucleophilic substitution of **4** with 3-*N*-benzoyl 5-FU (**5**) provided compound **6** in 52% yield. The solution of compound **6** in MeCN/H_2O was submitted to UV light (365 nm) irradiation, and the results demonstrated efficient photocontrollable release of the drug moiety (see Supporting Information (SI)). Deprotection of *O*-TBS groups by hydrochloride solution in ethanol gave diol **7** in 90% yield, which was converted into phosphoramidite **8** in two steps following standard protocols.[14]

Using the modular phosphoramidite **8**, any number of drug moieties can be automatically conjugated with an aptamer in a chained mode at predesigned sites on a DNA synthesizer. Aptamer sgc8 was chosen as a model for the automated and modular synthesis of ApDCs with a high capacity of drugs at predetermined positions. Sgc8 can bind to target protein PTK7, which is overexpressed on target HCT116 colon cancer cells, but not on nontarget Ramos lymphoma cells.[6a,15] To test the efficacy of the MTs for targeted drug delivery using phosphoramidite **8**, we synthesized two ApDCs, **MT-I** (sgc8-(PC-5FU)) and **MT-II** (sgc8-$(PC\text{-}5FU)_5$) with one and five 5FU units, respectively, at the 5′-end of the aptamer (Table S1 (SI)). We also synthesized an ApDC **sgc8-5FU** incorporated with 5FU from commercially available phosphoramidite, in which no PC-linker was integrated. A random-sequence DNA-drug conjugate (**LIB**, Table S1 (SI)) was also prepared as a control.

We then evaluated the specific recognition ability of MTs to target cancer cells. To do so, biotinylated MTs were used in combination with a streptavidin-PE-Cy5.5 (Phycoerythrin) conjugate dye for a cell binding assay. In this assay, flow cytometry was used to monitor the fluorescence intensities of cells, with aptamer sgc8 as a positive control and random

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sequences (**LIB**) as a negative control. As shown in Figure 2A, compared to the negative control, the fluorescence enhancement of cells incubated with MTs indicates these MTs maintained their specific binding ability to target HCT116 cells. The fluorescence intensities of cells bound with either MTs or **sgc8-5FU** were weaker than those bound with sgc8, presumably because the 5FU moiety influences aptamer–target interaction. In contrast to specific binding to target cells, neither **LIB** nor MTs bound to Ramos cells (Figure 2A), indicating the selectivity of MTs for cancer cell recognition. The maintenance of selective recognition ability of MTs to target cancer cells provides the basis for downstream application of targeted drug delivery.

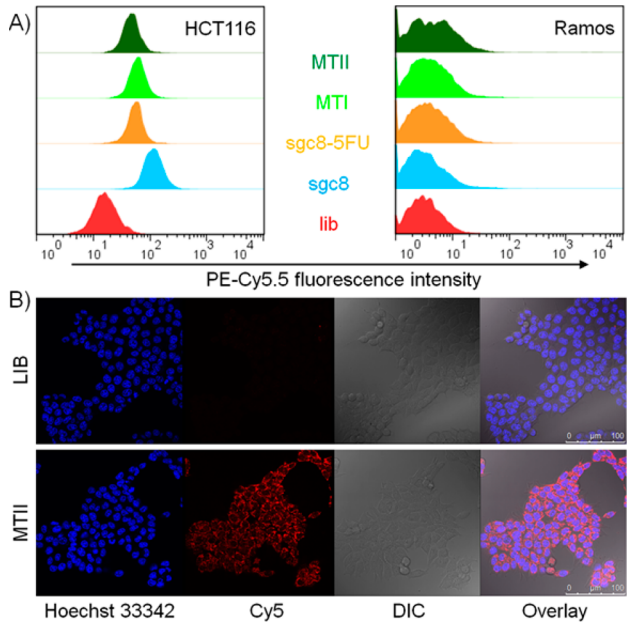

Figure 2. (A) Flow cytometric results indicating the selective recognition abilities of MTs and **sgc8-5FU** to target HCT116 cells, but not nontarget Ramos cells. Sgc8 was used as a positive control, and **LIB** as a negative control. All probes were labeled with biotin at the 3′ ends. (B) Confocal miscroscopy images demonstrating the specific binding and internalization of **MT-II** into target HCT116 cells, as shown by strong fluorescence intensity of the membrane and cytoplasm in cells treated with **MT-II**, in contrast to negligible fluorescence intensity of cells treated with **LIB**. Both probes were labeled with Cy5 at the 3′ ends. Cells were stained with Hoechst 33342 for nucleus identification.

To achieve efficient anticancer drug delivery, drug carriers should be internalized into cancer cells. It has been reported that many aptamers are capable of internalization into cancer cells through various pathways. In particular, sgc8 was reported to be internalized into target CEM T-cell leukemia cells.[16] We then studied whether MTs could be specifically internalized into target cancer cells. In this study, **MT-II** and **LIB** were labeled with fluorophore Cy5 and incubated respectively with HCT116 cells in a cell culture incubator for 3 h. Cells were stained with Hoechst 33342 to localize the nucleus and washed prior to confocal microscopy observation. Results in Figure 2B showed that strong fluorescence was identified on the cell membrane or in the cytoplasm of HCT116 cells treated with **MT-II**, indicating that **MT-II** not only bound to target HCT116 cells, but was also internalized into cancer cells. In contrast, cells treated with **LIB** displayed negligible fluorescence intensity, demonstrating the specificity of **MT-II** recognition and internalization into target cancer cells. The ability of MTs to be internalized into target cancer cells provides the basis of efficient targeted drug delivery.

Once MTs were validated for selective cancer cell recognition and internalization, they were subsequently evaluated for targeted therapy. The cytotoxicity was evaluated using an MTS assay. The cytotoxic efficacy of 5FU released from DNA–drug conjugates was first demonstrated using a short oligonucleotide incorporated with phosphoramidite **8** ($T(PC\text{-}5FU)_3T$), which was either irradiated with UV to release 5FU (precleaved) before treatment of cells, or irradiated by UV after incubation with cells (Figure S1 (SI)). It is worth noting that UV irradiation was optimized and did not cause dramatic cytotoxicity under our experimental conditions, removing the concerns of side effects caused by UV overexposure. The selective in vitro cytotoxicity induced by **MT-II** was then studied. Free 5FU, **LIB**, and **sgc8-5FU** (prepared from commercially available 5FU phosphoramidite, without PC linkers between 5FU and backbones) were used as controls. Specifically, target HCT116 cells were treated with free 5FU, **LIB**, **sgc8-5FU**, and **MT-II**, respectively, at a series of different 5FU equivalent concentrations. The resultant cells were incubated for 2 h to allow specific binding and internalization of drug or drug carriers into cells, prior to washing and UV exposure for 1 h. The capability of cell proliferation was evaluated using an MTS assay. While neither **LIB** nor **sgc8-5FU** induced appreciable cytotoxicity, both free 5FU and **MT-II** showed dose-dependent cytotoxicity in target HCT-116 cells (Figure 3). This demonstrated the robust

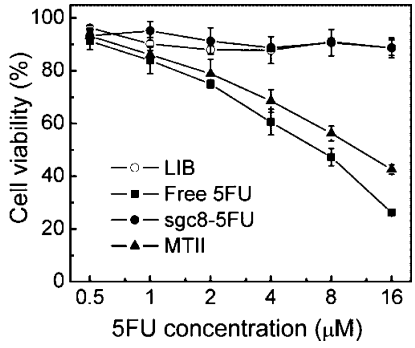

Figure 3. MTS results showing the specific and dose-dependent cytotoxicity induced in target HCT116 cells by 5FU delivered via MTs. Free 5FU, **LIB**, and **sgc8-5FU** were used as controls.

cytotoxic efficacy of 5FU delivered by **MT-II** into target cells and photocontrollably released from these molecular trains at target cancer cells, as well as the specificity of cytotoxicity mediated by the aptamer moiety in the MT system.

In conclusion, we have developed MTs as an automated and modular ApDC technology. Using this technology, anticancer drugs can be synthesized into ApDCs at predesigned positions and are capable of photocontrollable release in targeted cancer therapy. This technology takes advantage of the well-established solid-phase synthesis platform, in which a novel therapeutic module, a phosphoramidite incorporated with an anticancer drug, can be incorporated into ApDCs in a tailored manner. 5FU was used as a model drug in this study to demonstrate the principle. It is worth noting that this phosphoramidite was further functionalized with the ability of photocontrollable drug release. Our flow cytometry study

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demonstrated the specific binding ability of the resultant ApDCs, and confocal microscopy results further showed the ability of ApDCs to be internalized into target cancer cells for efficient intracellular drug delivery. Subsequently, in vitro cytotoxicity assay proved the specific cytotoxicity in target cancer cells. Overall, the MTs we developed here demonstrate the potential of modular ApDC technology for applications in targeted cancer therapy.

■ ASSOCIATED CONTENT

Supporting Information

Procedures and additional data. 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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