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An Aptamer-Nanotrain Assembled from 6-Letter DNA Delivers Doxorubicin Selectively to Liver Cancer Cells

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Abstract

Expanding the number of nucleotides in DNA increases the information density of functional DNA molecules, creating nanoassemblies that cannot be invaded by natural DNA/RNA in complex biological systems. Here, we show how 6-letter GACTZP DNA contributes this property in two parts of a nanoassembly: (1) in an aptamer evolved from a 6-letter DNA library to selectively bind liver cancer cells; and (2) in a 6-letter self-assembling GACTZP nanotrain that carries the drug doxorubicin. The aptamer-nanotrain assembly, charged with doxorubicin, selectively kills liver cancer cells in culture, as the selectivity of the aptamer binding directs doxorubicin into the aptamer-targeted cells. The assembly does not kill untransformed cells that the aptamer does not bind. This architecture, built with an expanded genetic alphabet, is reminiscent of antibodies conjugated to drugs, which presumably act by this mechanism as well, but with the antibody replaced by an aptamer.

Graphical Abstract:

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Experimental Section
Additional details regarding the materials and methods may be found in SI Appendix.

Supporting Information
Supporting Information contains a detailed description of the nanotrain assembly and data from affinity measurement experiments.

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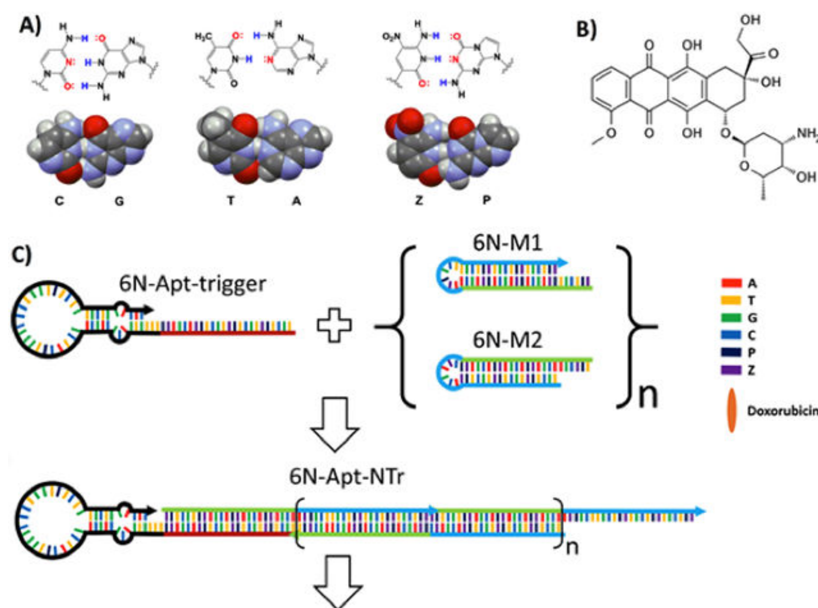

Folded DNA molecules were evolved or designed from a 6-letter alphabet to perform two non-genetic functions: (a) bind selectively to liver cancer cells and (b) bind the toxic drug doxorubicin. Combined into an aptamer-nanotrain conjugate, these selectively kill liver cancer cells. This selective drug delivery is reminiscent of antibody-drug conjugates, but without proteins.

Keywords

6-Nucleotide DNA; nanotrain; aptamer; targeted drug delivery

Introduction

One branch of DNA nanotechnology uses Watson-Crick pairing to assemble DNA and/or RNA (collectively xNA) molecules to give folded structures more complex than linear double helices. These folded xNA molecules often exhibit functional behavior beyond simple information storage, such as signaling dendrimers,[1] beacons,[2] and geometric shapes[3] that are imaged by atomic force microscopy.[4] Suggested applications of DNA as a programmable material include binding molecules ("aptamers"),[5–6] catalytic molecules ("aptazymes"),[7] and drug-carrying assemblies (such as nanotrains).[8–12] Aptamers, for example, have been seen as possible replacements for antibodies.[13]

However, the limited number (just four) of building blocks in standard xNA limits its functional versatility. The low information density, in turn, can causes ambiguous folding, even in natural xNA.[14] This ambiguity also limits the functional performance of designed DNA, including catalytic DNA evolved in a laboratory.[15]

Further, complex biological systems also contain many xNA molecules that are also built from G, A, C, and T/U). This means that in human blood and other medically relevant environments, the A:T and G:C pairing that gives programmed DNA molecules their

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functional folds can be invaded by background xNA, destroying both the programmed fold and ist function[16].

Recently, we showed that as many as 8 nucleotides from an Artificially Expanded Genetic Information System (AEGIS) can be added to the nucleic acid "alphabet" to give a "hachimoji" DNA and RNA (from Japanese, hachi = eight and moji = letter).[17] The additional 4 nucleotides form two pairs (the **P**:**Z** and **B**:**S** pairs) with the same size and shape as the natural A:T and G:C pairs. Subsets of hachimoji DNA have been built into signaling beacons that avoid invasion by natural DNA,[18] translation systems to encode additional amino acids,[19] diagnostics products,[20] and laboratory *in vitro* evolution (LIVE) platforms[21] that create aptamers with additional nucleotides that bind to cells[22] and proteins.[23]

Here, we use components of one of the additional pairs (the **P**:**Z** pair, Fig. 1A) to serve *two different* functional roles in one nanoassembly. In one role, the **P**:**Z** pair is exploited to assemble a "nanotrain" that carries the drug doxorubicin (Dox, Fig. 1B). Nanotrains are long, linear duplexes built from two components that individually form stable hairpins with loops; single-stranded overhangs are designed as toeholds at their ends (Fig. 1C, 1D and Fig. S1). When a triggering DNA sequence is added that can hybridize to the toehold of one component, strand exchange opens the hairpin and forces its complementary strand to become single-stranded. The resulting single strand has a region that, in turn, hybridizes to the toehold of the second component and invades its hairpin. The nanoassembly that results is an alternating concatamer of both components, a series of "boxcars" of repeating elements (Fig 1C). In nanotrain assembly, **P** and **Z** increase the information density of the sequences, and also ensure that the base-paired regions cannot be invaded by any standard nucleic acids that might be present in any sample.

A second role for **P** and **Z** is performed in an aptamer that had been selected by 6-letter (GACT**ZP**) laboratory *in vitro* evolution (LIVE) experiments to bind to liver cancer HepG2 cells.[24] Immortalized non-cancerous liver cells were used in the counter-selection to remove aptamers bound non-cancer related targets. The structure of one of several aptamers that emerged (the LZH5 aptamer) was optimized. Here, the modified version (LZH5B) was modeled to have a long binding loop with a stem containing a bulge (Fig. S2). A trigger sequence was then added to the 5'-end of the LZH5B stem to allow the aptamer itself to initiate formation of the nanotrain.

We hypothesized that doxorubicin, intercalated into the assembled nanotrain, could be guided to an unknown feature on the liver cancer cell (but not on the normal cell) by the tethered aptamer. The feature-aptamer-nanotrain-drug complex would then be internalized into a lysosome, where it would be digested to release doxorubicin. By this mechanism, we hoped to achieve specific cytotoxicity of doxorubicin against the target liver cancer cell.

Further, both the nanotrain and aptamer contain components that do not bind to G, A, C, or T/U. Thus, neither structure could be disrupted via the invasion of any natural DNA or RNA. Thus, the extra **Z** and **P** building blocks would prevent the release of doxorubicin prematurely to solution, and thus prevent non-targeted toxicity.

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Results

We first asked whether GACT**ZP** repeats placed in a nanotrain could bind doxorubicin. Here, a series of GACT**ZP** oligonucleotides were designed from a report that a single unit of duplex GCA/CGT or CGA/GCT can intercalate one molecule of doxorubicin.[22] The designed sequences were self-complementary, forming 8 consecutive GCA/CGT, GC**Z**/CG**P**, or **PZ**A/**ZP**T paired units. These were connected by TTT linkers (Fig. S3A). The three sequences (DOXCAR1, DOXCAR2 and DOXCAR3) were synthesized by solid-phase synthesis from the corresponding phosphoramidites (firebirdbio.com) and annealed (snap-cooling) to form self-complementary duplexes. The concentration-dependence of doxorubicin binding was studied by mixing different concentrations (0–400 nM) of DOXCARs with doxorubicin (1 μM, 2 h, 5 mM $MgCl_2$, PBS, r.t.) and examining the quenching of doxorubicin fluorescence upon intercalation.

Binding of doxorubicin to **PZ**-containing DNA was confirmed by the loss of its fluorescence. This loss, studied at various concentrations of different DOXCARs, suggested that replacement of GC/CG by **PZ**/**ZP**, or A/T by **Z**/**P**, neither impacted the intercalation of doxorubicin nor the quenching of its fluorescence (Fig. S3B). Fitting the binding curve showed that fluorescence decreases as the concentration of DOXCARs increases, with apparent disassociation constants (K_d) of ~20 nM (Fig. S3C); these were similar for all DOXCARs.

We then designed the nanotrain and an interacting modified aptamer, starting with aptamer LZH5 reported previously as a product of GACT**ZP** LIVE targeting liver cancer HepG2 cells. In that LIVE, an immortalized untransformed non-cancerous cell line (Hu1545V) was used in a counter selection. Aptamer LZH5 was chosen from about a dozen other aptamers because of its superior affinity (41 nM) and its ready internalization into HepG2 cells.

The structure of LZH5 was then truncated to give LZH5B, which had shorter sequence and improved affinity (12 nM) (Fig. S2). Internalization of LZH5B was confirmed by a binding assay and confocal microscopy (Fig. S4).

For the nanotrain itself, two hairpin monomers (6N-M1, 6N-M2, Table S1) were designed from a 6-nucleotide GACT**ZP** DNA so that the energy stored in the loops would be protected by the corresponding stems. A DNA trigger probe was appended to the 5'-end of LZH5B to initiate the assembly of the 6-letter DNA nanotrain.

Introduction of the LZH5B-trigger to a mixture of 6N-M1 and 6N-M2 initiated the polymerization of these building blocks through mutual hybridization, and was expected to result in the self-assembly of the DNA nanotrains tethered to the LZH5B aptamer (LZH5B-NTrs, Fig. 1). The molar ratio of the LZH5B-trigger to monomers was optimized.

Doxorubicin was then loaded by incubating the assembly with doxorubicin (50:1 doxorubicin:assembly, PBS, 5 mM Mg^{2+}, r.t., 2 h) followed by washing. Initiation by the aptamer of a nanotrain formation was confirmed by AFM (Fig. 2B). The nanotrains have a worm-like shapes up to 100's of nanometers. Images of the GACT**ZP** nanotrains were similar to those of nanotrains built from standard nucleotides.[8]

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We next evaluated the stability of 6N-LZH5B-NTr-Dox complexes through a drug diffusion experiment using a dialysis units. Results showed negligible independent diffusion of the drug once it was bound to the 6N-LZH5B-NTrs. This result stands in contrast to fast diffusion of the drug presented as free in solution (PBS, with 5 mM Mg^{2+}). This indicated that the stability of 6N-LZH5B-NTr-Dox com-plexes is adequate to prevent cargo leakage (Fig. 3A). Interestingly, 6N-LZH5B-NTr-Dox showed better stability than an LZH5B-initiated nanotrain built from standard 4-letter DNA carrying doxorubicin (LZH5B-NTr-Dox, PBS with 10% fetal bovine serum) (Fig. 3B). This may arise from the non-invadability of duplexes containing **P**:**Z** pairs.

Experiments then tested the ability of the nanoassembly built from this expanded genetic alphabet to selectively kill cells that the aptamer recognizes. This design requires that (i) doxorubicin be effectively sequestered by its intercalation into the nanotrain, and therefore not “free” to be toxic (as free doxorubicin is), but (ii) the doxorubicin bound to an aptamer-nanotrain assembly be internalized into a cell after the aptamer binds to the cell surface, where (iii) doxorubicin is released into the cell in a toxic form after the nucleotides that sequester it are digested. Thus, while free doxorubicin is expected to be toxic to any cells, [25] doxorubicin bound to this aptamer-nanotrain complexed should not kill any cells except those that are bound by the LZH5B aptamer.

To confirm this, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was used to assess the survival of cells after they were exposed to free doxorubicin compared to the aptamer-nanotrain-Dox nanostructure. In this assay, both HepG2 cells, which bind the aptamer, and control Hu1545 cells, which do not, were treated with free Dox and LZH5B-NTr-Dox complex.

Free doxorubicin showed the standard dose-dependent cytotoxicity in both HepG2 cells and Hu1545 cells. [26] In contrast, doxorubicin bound in LZH5B-NTrs for transport to target HepG2 cells (Fig. 4) displayed a dose-dependent cytotoxicity similar to that of free doxorubicin, but only against HepG2 cells. Essentially, no toxicity was seen with non-transformed liver cells that had been used as the counter-selection in the GACT**ZP** LIVE experiment that generated the LZH5B aptamer. This showed a robust cytotoxic efficacy of LZH5B-NTr-Dox against target cells and excellent selective cytotoxicity of this molecular drug transported by aptNTrs. The lack of cytotoxicity of LZH5B-NTrs in Hu1545V cells indicates the biocompatibility of these transporters under our experimental conditions (Fig. 4).

Discussion

The results combine four recent themes in applied biomolecular sciences:

- An emerging class of medicines that use protein derivatization chemistry to attach drugs to antibodies to target to specific cells compounds that are too toxic to administer without targeting (e.g. trastuzumab emtansine and brentuximab vedotin).[27]

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- The desire to replace antibodies by aptamers, reflecting a concern that antibodies are "a major driver of what has been deemed a reproducibility crisis" in medicine.[28]
- Using DNA molecules emerging from expanded genetic alphabets to improve the performance of classical aptamers made from only the four standard nucleotides.
- The programmability of DNA to form nanostructures.

In this work, a toxic drug (doxorubicin) was appended to an aptamer that has been improved by expanding the genetic alphabet. However, the attachment exploited the ability of expanded DNA to self-assemble; no conjugation chemistry was required. The self-assembling nanostructure likewise benefits from the expanded DNA alphabet because it cannot be invaded by standard DNA or RNA, both abundant in a biological background. Thus, GACT**ZP** nanostructures can assemble in stable form in complex biological systems without invasion by natural DNA or RNA.

Perhaps surprising is the observation that replacing standard nucleotide pairs by **P**:**Z** pairs did not alter, and possibly improved, the affinity of the DNA for doxorubicin. Available structures of DNA containing **P**:**Z** pairs show that they distort very little the standard duplex structure. Indeed, the A:T pair, joined by only two hydrogen bonds, provides a greater distortion.

The versatility of the GACT**ZP** system is especially illustrated by its dual use in these nanostructures. First, it was used to create the aptamer that binds specifically to liver cancer cells. Especially for cell-targeted LIVE, the literature suggests that the GACT**ZP**-expanded alphabet generates cell-specific aptamers with higher affinity and does so faster than standard GACT LIVE. By exemplifying new ways of approaching current problems in applied nanotechnology, further examination of such systems is warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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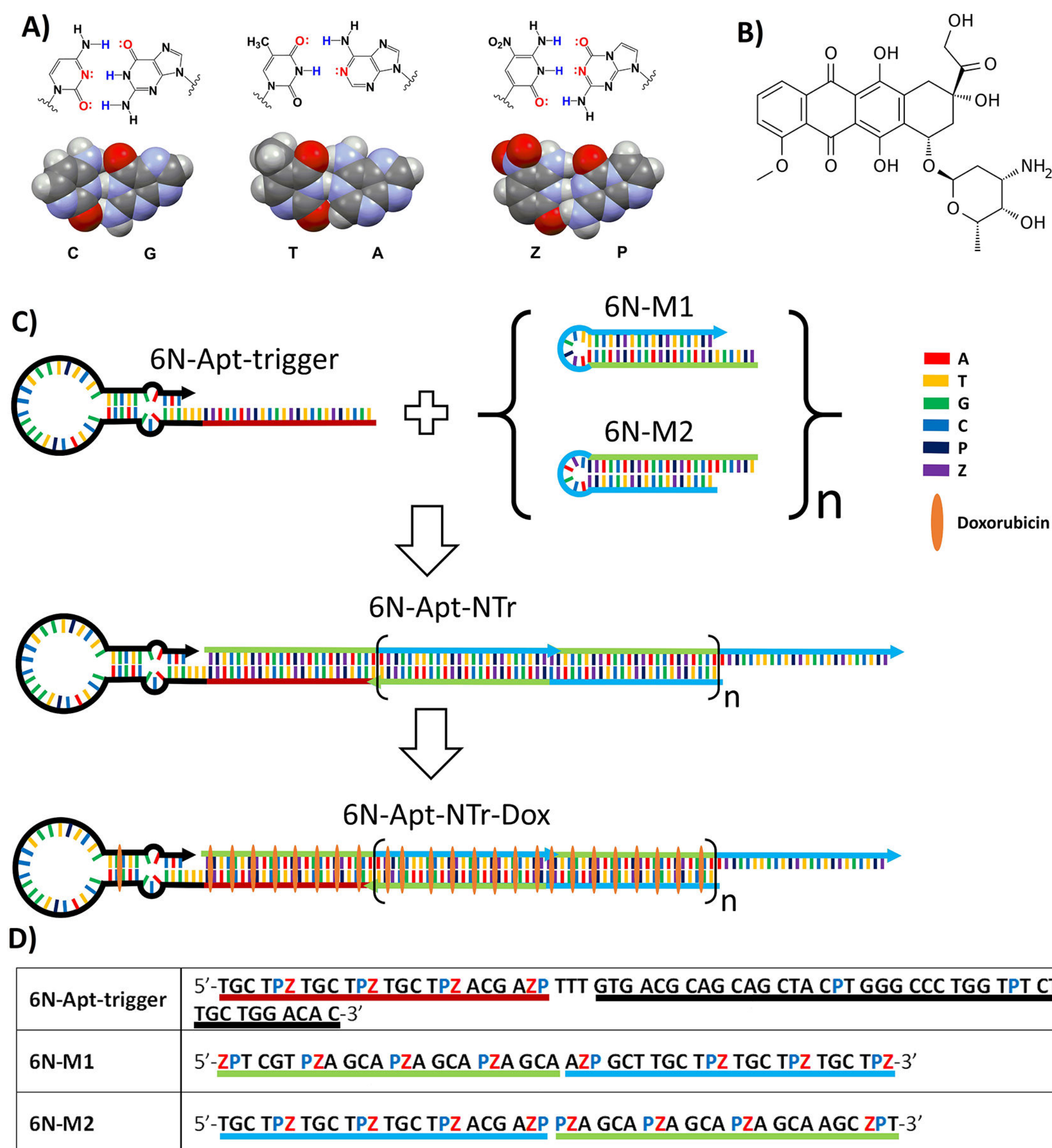

Figure 1.

Key components of AEGIS aptamer-nanotrain assembly. (A) Structures of three nucleobase pairs (G:C, A:T, and P:Z) that support nanoassembly in GACTZP 6-nucleotide DNA (PDB ID: 4RHD) (B) Doxorubicin. (C) Self-assembling nanostructure triggered by an aptamer (6N-Apt-trigger) that hybridizes to the toehold of 6N-M1, invades its hairpin to generate a single strand that can invade the hairpin of 6N-M2. This yields a 6-nucleotide DNA nanotrain (6N-Apt-NTrs) formed from short 6-nucleotide DNA fragments (6N-M1 and 6N-M2) tethered to a 6-nucleotide aptamer specific for liver cancer cells. The nanotrain may be loaded with the doxorubicin drug. (D) Sequences of 6-nucleotide DNA fragments. Underlined region highlighted with red color shows the sequence of the trigger; Black color underlined region shows the sequence of the aptamer; Green and blue color underlined regions in 6N-M1 and 6N-M2 show the complementary regions when forming nanotrains.

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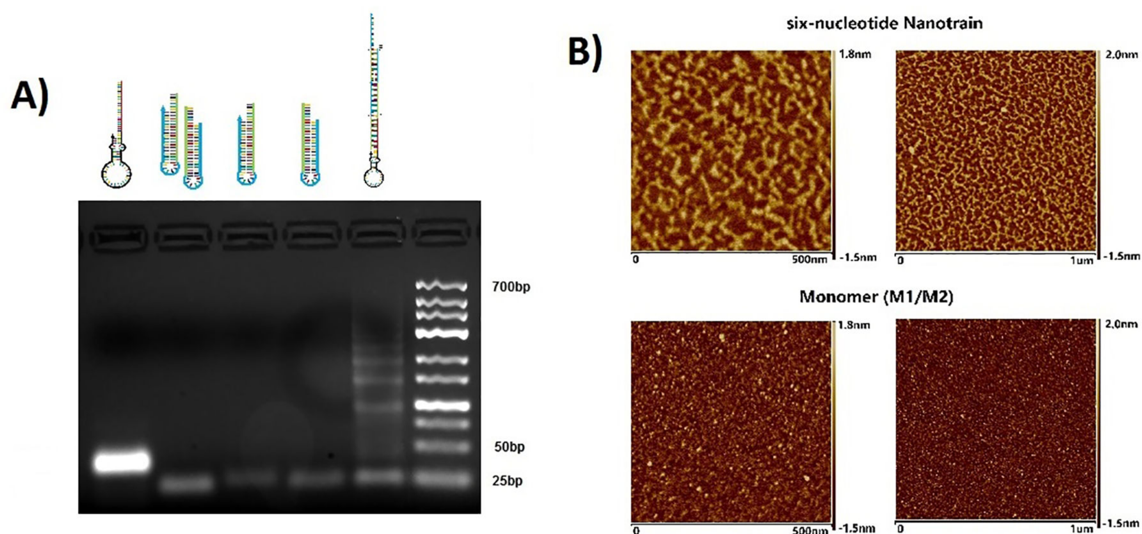

Figure 2.

Characterization of nanotrain assembly. (A) Agarose gel image shows migration of (left to right) 6N-Apt-trigger, mixture of 6N-M1 and 6N-M2, 6N-M1, 6N-M2, 6N-Apt-NTrs, DNA ladders. (B) Atomic force microscopy images show the formation of 6-letter nanotrains (top two images) compared to the 6-letter monomers (bottom two images).

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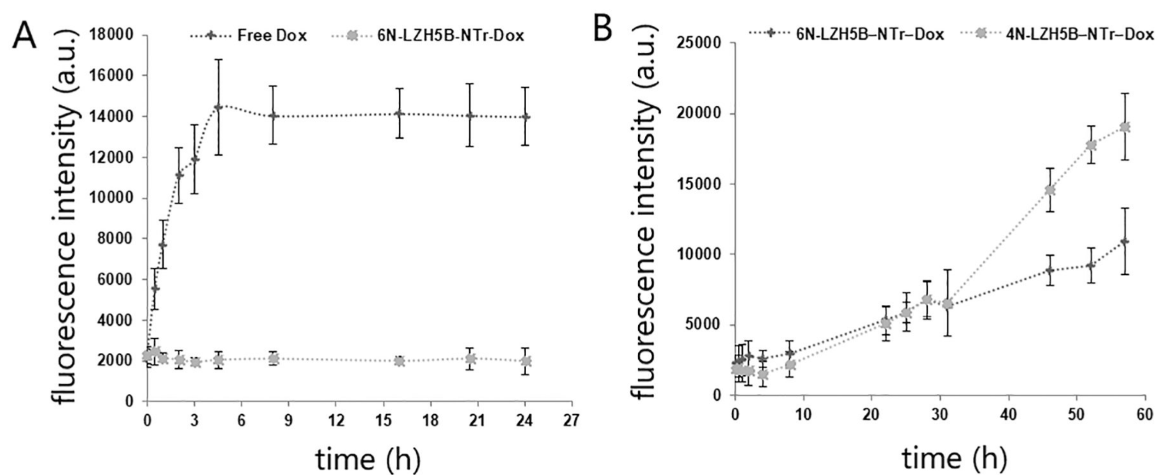

Figure 3.

Loading and releasing of doxorubicin. Fluorescence of dialyzed doxorubicin was detected to represent the concentration of diffused doxorubicin. (A) Drug diffusion of free doxorubicin and doxorubicin carried by 6-nucleotide nanotrain in PBS with 5 mM Mg^{2+}. (B) Drug diffusion of doxorubicin carried by 6-nucleotide nanotrain and 4-nucleotide nanotrain in PBS with 10% fetal bovine serum.

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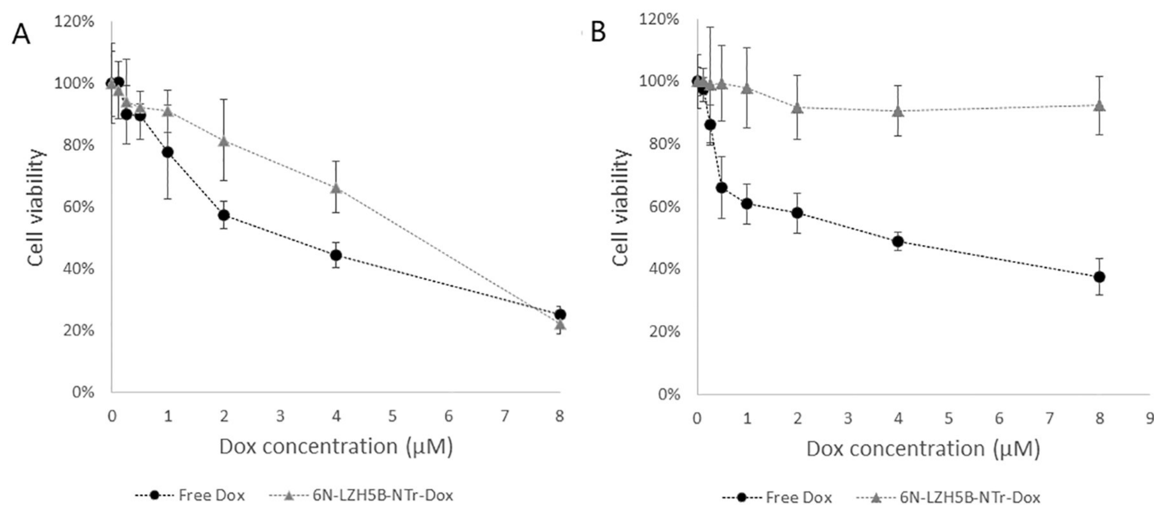

Figure 4.

Selective cytotoxicity of molecular drugs (Dox) transported by aptNTrs. (A and B) MTS assay results showing that Dox transported by LZH5B-NTrs (LZH5B-NTr-Dox) selectively induced potent cytotoxicity and inhibited cell proliferation in target HepG2 cells (A), but not in non-target Hu1545 cells (B), in contrast to nonselective cytotoxicity induced by free Dox in both target and non-target cells.