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Self-assembled Multifunctional DNA Nanoflowers for the Circumvention of Multidrug Resistance in Targeted Anticancer Drug Delivery

Lei Mei^{1,†}, Guizhi Zhu^{1,2,†,‡}, Liping Qiu^{1,2}, Cuichen Wu², Huapei Chen¹, Hao Liang¹, Sena Cansiz², Yifan Lv¹, Xiaobing Zhang¹, and Weihong Tan^{1,2}

Xiaobing Zhang: xbzhang@hnu.edu.cn; Weihong Tan: tan@chem.ufl.edu

¹Molecular Sciences and Biomedicine Laboratory, State Key Laboratory for Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, College of Biology, Collaborative Innovation Center for Chemistry and Molecular Medicine, Hunan University, Changsha 410082, China

²Departments of Chemistry, Physiology and Functional Genomics, Center for Research at the Bio/Nano Interface, Shands Cancer Center, UF Genetics Institute and McKnight Brain Institute, University of Florida, Gainesville, FL 32611-7200, USA

Abstract

Cancer chemotherapy has been impeded by side effects and multidrug resistance (MDR) partially caused by drug efflux from cancer cells, which call for targeted drug delivery systems additionally able to circumvent MDR. Here we report multifunctional DNA nanoflowers (NFs) for targeted drug delivery to both chemosensitive and MDR cancer cells and circumvent MDR in both leukemia and breast cancer cell models. NFs are self-assembled via liquid crystallization of DNA generated by Rolling Circle Replication, during which NFs are incorporated with aptamers for specific cancer cell recognition, fluorophores for bioimaging, and Doxorubicin (Dox)-binding DNA for drug delivery. NF sizes are tunable (down to ~200 nm in diameter), and the densely packed drug-binding motifs and porous intrastructures endow NFs with high drug loading capacity (71.4%, wt/wt). The Dox-loaded NFs (NF-Dox) are stable at physiological pH, yet drug release is facilitated in acidic or basic conditions. NFs deliver Dox into target chemosensitive and MDR cancer cells, preventing drug efflux and enhancing drug retention in MDR cells. Consequently, NF-Dox induces potent cytotoxicity in both target chemosensitive cells and MDR cells, but not nontarget cells, thus concurrently circumventing MDR and reducing side effects. Overall, these NFs are promising to circumvent MDR in targeted cancer therapy.

Correspondence to: Xiaobing Zhang, xbzhang@hnu.edu.cn; Weihong Tan, tan@chem.ufl.edu.

[†]These authors contributed equally

[‡]Present address: Laboratory of Molecular Imaging and Nanomedicine, National Institute of Biomedical Imaging and Bioengineering, National Institutes of Health, Bethesda, MD 20892

Electronic Supplementary Material: Supplementary methods and materials, results of predicted secondary structure of DNA templates for NF preparation, NF characterization, agarose gel electrophoresis, SEM images of different NFs and NF-Dox, calibration of Dox fluorescence intensity with Dox concentration at different pH, drug loading and drug release, flow cytometry analysis of P-glycoprotein expression of MDR cells, and MTS assay results of cells treated with NFs only, are available in the online version of this article at http://dx.doi.org/10.1007/s12274-***-****-.

Graphical abstract

Self-assembled by DNA liquid crystallization, multifunctional DNA nanoflowers (NFs) were demonstrated for targeted drug delivery to both chemosensitive and multidrug resistant (MDR) cancer cells and the resultant circumvention of MDR, in both leukemia and breast cancer cell models.

Keywords

aptamer; rolling circle replication; self-assembly; DNA nanotechnology; multidrug resistance; targeted cancer therapy

1. Introduction

Chemotherapy is one of the principal modalities of cancer therapy. However, conventional chemotherapy has often been impeded by side effects resulting from nonspecific drug delivery [1] and multidrug resistance (MDR) [2]. Nonspecific drug delivery is, of course, inefficient, but also causes toxicity in healthy tissues when drugs are systemically administered, resulting in suboptimal antitumor efficacy and side effects [1]. For example, Dox, an anthracycline drug widely used in cancer chemotherapy [3], can cause cardiomyopathy which leads to lethal congestive heart failure [4]. Suboptimal cancer suppression and cancer recurrence can also be attributed to MDR that often results from drug efflux from cancer cells [5, 6]. Drug efflux is driven by adenosine triphosphate-binding cassette (ABC) transporter proteins, such as P-glycoprotein (P-gp or ABCB1) and ABCG2, which are overexpressed in many types of cancer cells. They are transmembrane proteins with distinctive nucleotide-binding domains (NBDs) which hydrolyze ATP to generate energy for active transport of various substrates across the membrane [7]. Hundreds of compounds as small as 330 Daltons and up to 4000 Daltons can be substrates of ABC transporters, making cancer chemotherapy rather challenging. Other factors that contribute to MDR include acquiring MDR during therapy, enzymatic drug inactivation [2, 6], cancer heterogeneity and cancer stem cells [8]. The pharmacokinetic effect of MDR limits effective drug delivery into subcellular organelles, which many drugs need to reach for optimal therapy. While it is desirable to enhance therapeutic efficacy by escalating drug dose, such

escalation will, ironically, produce an array of side effects. Thus, achieving optimal chemotherapy under these conditions presents a daunting challenge.

As an alternative, targeted drug delivery seeks to deliver drugs only to cancer cells [9]. One mode, active targeting, utilizes molecular recognition ligands, ranging from antibodies [10] and aptamers [11] to growth factors and vitamins [12], to specifically bind to overexpressed cognate receptors on target cancer cells. Screened through Systematic Evolution of Ligands by EXponential enrichment (SELEX) [13, 14], DNA aptamers are single-stranded DNA that can specifically bind to cognate targets. Recently, our group developed cell-SELEX to select aptamers for living cells of various cancers [11, 15–17]. These aptamers possess many remarkable features, such as facile screening against various targets, strong binding affinity, receptor-mediated cell internalization, as well as low cytotoxicity and low immunogenicity [18, 19], making aptamers excellent targeting ligands for targeted drug delivery [19].

Another mode, passive targeting, utilizes drug nanocarriers to exploit the tumor enhanced permeation and retention (EPR) effect resulting from leaky blood vasculature and poor lymphatic drainage [20], which allows nanocarriers to easily penetrate tumor vasculature and have prolonged tumor retention time, thus enabling specific tissue targeting and improved drug delivery. Nanocarriers are usually featured with high drug loading capacity, protection of loaded drugs, and ease of biofunctionalization [9]. Remarkably, many nanocarriers have been reported to be able to circumvent MDR. This distinction arises from their high drug payload capacity and protection of loaded drugs against cell efflux or enzymatic inactivation. The use of nanocarriers for targeted drug delivery has thus provided a new means of circumventing MDR, in addition to other MDR-inhibiting modalities, such as inhibitors of ABC transporters and polymers [2, 5, 9, 21–31].

DNA nanotechnology has allowed the development of various DNA nanocarriers for drug delivery [22, 32–37], owing to such unique features of DNA as sequence programmability, automated synthesis and modification, and intrinsic functionalities (*e.g.*, aptamers for recognition and DNA antisenses for therapy [29]). Previously, we reported a noncanonical approach to the self-assembly of DNA nanoflowers (NFs), which are, in fact, DNA nanogels by their nature of hydrogel. In contrast to conventional assembly of DNA nanostructures by hybridization [22, 32–36], NFs were self-assembled by liquid crystallization of highly concentrated DNA generated from rolling circle replication (RCR), an enzymatic reaction for efficient generation of repetitive ssDNA using a designer circular DNA as template [37]. By customized design of RCR templates, NFs were incorporated with many unique properties, including simple DNA design and preparation, size tunability, and resistance to enzymatic degradation and denaturation. NFs can be assembled from any DNA templates suitable for RCR, allowing for customized incorporation of DNA functionalities, including DNA motifs for drug loading and aptamers for specific recognition and internalization into target cancer cells.

Taking advantage of these features, here we report the development of DNA NFs for targeted drug delivery to both chemosensitive and chemoresistant cancer cells and the resultant circumvention of MDR in both leukemia and breast cancer cell models. During NF assembly, NFs were concurrently integrated with aptamers for specific cancer cell recognition and internalization, fluorophores for bioimaging, and drug-binding DNA motifs

for drug delivery. The sizes of these NFs can be tuned down to about 200 nm in diameter. The hierarchical internal porous structures and densely packed drug-binding DNA motifs in NFs endow them with drug loading capacity as high as 71.4% (wt/wt). The Dox-loaded NFs (NF-Dox) were stable at physiological pH (7.4), yet they gradually released drugs in either acidic or basic environment. Medicated by aptamers, NF-Dox delivered Dox into both target chemosensitive cancer cells and target MDR cells. NFs protected drugs from efflux and enhanced drug accumulation and retention in MDR cells. As a result, NF-Dox induced potent cytotoxicity in both target chemosensitive cancer cells and MDR cells, but not nontarget cells, thus circumventing MDR and reducing side effects in nontarget cells. These results have demonstrated NFs as a potential platform for the circumvention of drug resistance during targeted anticancer drug delivery.

2. Experimental details

2.1 Self-assembly of DNA NFs using RCR

Phosphorylated DNA templates (0.6 μM) and the corresponding primers (1.2 μM) were mixed and annealed in DNA ligation buffer (5 mM Tris-HCl, 1 mM MgCl_2 , 0.1 mM ATP, and 1 mM Dithiothreitol) by heating at 95 $^{\circ}\text{C}$ for 2 min and then gradual cooling down to room temperature over 3 h. Linear templates were circularized using T4 DNA ligase (10 U/ μL , New England Biolabs, Ipswich, MA; reaction at room temperature, 3 h). To prepare NFs, circularized templates (0.3 μM) were incubated with $\Phi\text{T}29$ DNA polymerase (2 U/ μL), dNTP (each with 2 mM/ μL), and BSA (1X) in buffer solution (50 mM Tris-HCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 10 mM MgCl_2 , and 4 mM Dithiothreitol) (New England Biolabs, Ipswich, MA) at 30 $^{\circ}\text{C}$ for a specified time period. Reaction was terminated by heating at 75 $^{\circ}\text{C}$ for 10 min. NFs were washed with Dulbecco's PBS, precipitated by centrifugation, and stored at 4 $^{\circ}\text{C}$ for future use.

2.2 Drug accumulation and retention in cancer cells by flow cytometry

Drug delivery was also evaluated using flow cytometry. Specifically, cells were again treated with free Dox or NF-Dox, as in the confocal microscopy study. After incubation for 3 hours, cells were trypsinized (for MCF7 cells and MCF7/MDR cells) to single cells, washed and analyzed by flow cytometry to determine Dox fluorescence intensities. To study drug retention in cancer cells, the above treated cells were washed to remove Dox or NF-Dox, followed by further cell culture for different time lengths. The resultant cells were again trypsinized (for MCF7 cells and MCF7/MDR cells), washed twice with Dulbecco's PBS, and analyzed using flow cytometry to determine Dox fluorescence intensities to determine the Dox retention in cells.

2.3 Cytotoxicity assays

The cytotoxicity was evaluated using a CellTiter 96 cell proliferation assay (MTS assay) (Promega, Madison, WI, USA). Cells were treated with NFs, free Dox, or NF-Dox complexes in FBS-free medium. After incubation for 3 h in a cell culture incubator, supernatant medium was removed, and fresh medium (with FBS, 150 μL) was added to cells for further cell growth (48 h). Afterwards, medium was again removed, and CellTiter reagent (20 μL) diluted in fresh FBS-free medium (100 μL) was added to each well and

incubated for 1–2 h. The absorbance (490 nm) of the resultant solution was recorded using a microplate reader (Tecan Safire microplate reader, AG, Switzerland), and cell viability was determined according to the manufacturer's description. Results were fitted to a dose-response model using software Origin 8 (Northampton, MA).

3. Results and discussion

3.1 Self-assembly of Multifunctional NFs

The programmability of DNA and the sequence-independence of NF assembly allowed us to incorporate functional DNA moieties into NFs simply by designing the RCR DNA templates. For targeted drug delivery, NFs were modularly incorporated with DNA aptamers for specific recognition of cancer cells and drug-binding DNA motifs for drug delivery (Fig. 1). In this study, aptamers KK1B10 (KK) [16] and sgc8 [15] were chosen for leukemia and breast cancer cell models, respectively, and Dox, which is widely used in cancer therapy but often suffers from drug resistance, was used [5]. We first studied aptamer KK to assemble KK-integrated nanoflowers (KK-NFs) for Dox delivery into leukemia K562 cells (chemosensitive) and the corresponding K562/MDR cells. The RCR template, T-KK, was designed to encode DNA with alternative KK and Dox-binding DNA motifs, which are tandem GC or CG in dsDNA for Dox intercalation (Table S1 and Fig. S1) [38].

To generate DNA by RCR reaction, phosphorylated linear templates were circularized using DNA ligase and a ligation template, which then also served as the primer in downstream RCR catalyzed by Φ 29 DNA polymerase. Efficient production of DNA by RCR, as verified by gel electrophoresis (Fig. S2), resulted in increasing local DNA mass concentration and eventually led to DNA liquid crystallization when reaching a critical DNA concentration. By scanning electron microscopy (SEM), monodispersed spherical NFs were observed with diameters of about 200 nm after RCR reaction for 3 hours (Fig. 2a). Hierarchical pores, which are expected to encapsulate drugs and protect the drugs in NFs, were also observed in KK-NFs. Dynamic light scattering (DLS) confirmed the diameter of KK-NFs in bulk solution to be 230 ± 30 nm (mean $\pm$ SD) (Fig. 2b). The larger size shown in DLS compared to SEM images is most likely a result of the larger hydrodynamic size than dry NFs for SEM imaging. By EPR effect, nanocarriers with this size are expected to penetrate leaky blood vasculature and be retained in tumor for a prolonged time. We observed that NF size could be influenced by the activity of Φ 29, and the size was confirmed for each batch of enzyme.

To quantify DNA concentration in NFs, we measured the absorbance of remnant dNTP at 260 nm, calculated the amount of consumed dNTP, and determined that 52.6% of dNTP was consumed after RCR for 3 h. This means that, on average, 76 copies of DNA were produced for each template (Table S2), based on the amount of consumed dNTP and the number of nucleotides in one template (92 nucleotides in KK-T). NF sizes increased progressively with longer RCR time (Fig. S3), with diameters of about 1 μ m after RCR reaction for 10 h (Fig. 2c). The products from RCR for n hours are denoted as RCR _{n} , and NFs with diameters of m nm are denoted as NF _{m} . Within the same time length of RCR, NF sizes were larger than those observed previously using another RCR template, presumably due to less topological constraint or higher enzymatic activity in the present study [37]. Under polarized optical microscopy (POM), birefringent spherulite NFs were observed (Fig. 2d), verifying that NFs

were anisotropic and liquid crystalline and that NF self-assembly was driven by liquid crystallization. Furthermore, NFs showed high resistance to nuclease cleavage, as indicated by the morphological integrity of NFs treated with DNase I at 5 U/mL, a concentration considerably higher than that in human blood (< 1 U/mL) [39] (Fig. 2e). Moreover, we previously demonstrated that DNA NFs were also resistant to degradation by human serum or denaturation by urea, heating, or extreme dilution mimicking the situation of drug administration into blood circulation. The excellent stability makes NFs promising for drug delivery where NFs would encounter ubiquitous nucleases and are diluted in circulatory system. Owing to the small size, KK-NF₂₀₀ was used for subsequent studies.

Similarly, aptamer sgc8 was integrated into NFs (S-NFs) for chemosensitive breast cancer MCF7 cells, as well as MCF7/MDR cells developed by transducing MCF7 cells with *MDR1* gene for the overexpression of P-gp [40]. Flow cytometry confirmed sgc8 for selective recognition of both MCF7 and MCF7/MDR cells. Again, by the design of the RCR template, S-T, (Table S1), aptamers and drug-binding motifs were incorporated into S-NFs. SEM imaging revealed the diameters of S-NFs (~200 nm) after RCR reaction for 6 h and the stability under DNase I treatment (Fig. S4).

3.2 Drug Loading into NFs and Controlled Drug Release from NFs

NFs were then studied *in vitro* as nanocarriers for drug loading and conditionally release drugs from NFs. To load drugs into NFs, Dox was incubated with NFs, followed by centrifugation to remove free Dox and quantification of Dox loaded into NFs (Fig. S5a). Dox was rapidly loaded into NFs, reaching a plateau within 30 min (Fig. S5b for KK-NFs as an example). SEM imaging verified the morphological integrity of Dox-loaded KK-NFs (KK-NF-Dox) (Fig. S5c). Dox loading capacity was determined to be 71.4% (wt/wt) (see calculation in Table S3), which is exceptionally high. The high drug loading capacity was attributed to both densely packed drug-binding DNA motifs in NFs and the internal porous structures in NFs for physical encapsulation. Since maximally 12 Dox-binding sites could be formed in one RCR replicate, by calculation, a maximum of 34.3% Dox in NF-Dox was loaded by specific DNA-binding, and the remainder of Dox was likely loaded via physical encapsulation into porous structures in NFs (Table S3). The high drug loading capacity by physical encapsulation in NFs makes it possible to load and deliver non-DNA-binding drugs as well using NFs. Overall, such high drug loading capacity makes NFs excellent drug carriers.

NF-Dox was then evaluated for stability under physiological conditions and controlled drug release. Given the central role of pH in physiological regulation, we studied the influence of pH on the stability and drug release of NF-Dox using dialysis. Since the trace amount of released Dox during a relatively short time could not be accurately quantified by Dox absorbance, we sought to quantify released Dox by measuring Dox fluorescence intensities. And since Dox fluorescence could vary at different pH conditions or after incubation in buffer solution for different time periods, we calibrated Dox fluorescence intensities vs Dox concentrations by measuring the fluorescence intensities of Dox in a series of concentrations at different pH (5, 7.4, and 9) and after incubation in buffer for different time periods. Fluorescence intensities were then plotted as a function of Dox concentrations for different

pH and different incubation time periods, respectively (Fig. S6), which showed negligible influence of Dox fluorescence intensities by incubation time. A linear relationship between Dox concentrations and fluorescence intensities was found at the Dox concentration range of 0.1 μM – 10 μM , where Dox concentrations and fluorescence intensities for each pH condition were fit into functions, by which the amount of released Dox was determined by measuring Dox fluorescence intensities.

Dox release kinetics was then studied. KK-NF-Dox, as an example, was dialyzed at pH 5, pH 7.4, and pH 9, respectively, followed by quantifying released Dox at a series of time points. Dox release was well fit into a drug release model for each pH (Fig. 2f). At pH 7.4, KK-NF-Dox was stable and Dox release from NF-Dox was relatively slow, in contrast to rapid release of free Dox at the equivalent concentrations (as a positive control) from dialysis units. At both pH 5 and 9, Dox release was greatly enhanced, at rates approximately half that of free Dox diffusion. The facilitated drug release was likely because acidic or basic conditions induced changes in DNA conformations, DNA-drug interactions, and the structures of NFs. The plateaux of drug release were lower than 100%, most likely because drug release reached equilibrium or the dialysis membrane was partially blocked after immersing in drug solution for a long time. Since most extra-/intracellular fluid is regulated at or near pH 7.4, under which the relatively high stability of NF-Dox was expected to 1) ensure the integrity of NF-Dox and prevent Dox from being rapidly released before diffusing away from the vicinity of cell membrane during a relative short time period, thus avoiding efflux of Dox released from NFs by P-gp on adjacent cell membrane, and 2) allow the controlled (gradual) release [41] of Dox from NFs when NF-Dox stays away from cell membrane in cytosol during a relatively long time period, and the gradually released Dox would avoid efflux by P-gp. Furthermore, Dox release could be facilitated if transported into acidic subcellular organelles, such as the endosome and the lysosome.

3.3 Selective Recognition and Internalization of NFs to Target Cancer Cells

With high drug loading capacity, NFs were tested for specific cancer cell recognition for targeted drug delivery. Specifically, we incorporated Cy5 into NFs by supplementing dNTP with Cy5-dUTP that can be enzymatically incorporated into NFs during RCR. Flow cytometry verified that Cy5-labeled KK-NFs specifically recognized target leukemia K562 and K562/MDR cells, but not nontarget Ramos cells (Fig. 3a, c). Similarly, Cy5-labeled S-NFs were specifically bound to target human breast cancer MCF7 and MCF7/MDR cells, but not nontarget Ramos cells (Fig. 3b, c). To study recognition specificity of NFs, we prepared another NF using a control DNA template, in which a non-aptamer control DNA substituted the aptamer-encoding sequences in KK-T (Table S1). The resultant ctrl-NFs (SEM images in Fig. S7a) did not specifically bind to K562, K562/MDR, MCF7, or MCF7/MDR cells (Fig. S7b, c). These results demonstrated that the aptamer in NFs maintained the specific recognition ability to cancer cells, allowing NFs to serve as nanocarriers for targeted drug delivery.

For efficient drug delivery, it is also desirable for nanocarriers to be internalized into cells. We thus studied the internalization of Cy5-labeled NFs into cancer cells using confocal laser scanning microscopy. After incubation of NFs with the corresponding target cancer cells for

3 h, strong Cy5 fluorescence intensity was identified in both chemosensitive cells and MDR cells (KK-NFs for K562 cells and K562/MDR cells (Fig. 4a); and S-NFs for MCF7 cells and MCF7/MDR cells (Fig. 4b)). Compared to DNA nanostructures without aptamers that can also be internalized into cells over a relatively long time (*e.g.*, 24 h) [22], we believe the aptamer moieties in NFs enhanced the internalization of NFs, indicating the potential of NFs for efficient drug delivery.

3.4 Intracellular Drug Delivery, Accumulation and Retention in Cancer Cells

With the high drug payload capacity of NFs, high stability of NF-Dox complexes and controlled drug release from NF-Dox, NFs were then studied as nanocarriers for targeted drug delivery into both chemosensitive and the corresponding MDR cancer cells. The overexpression of P-gp, which has been reported to pump out many types of drugs from MDR cells, was verified by flow cytometry (Fig. S8). Confocal microscopy was used to visualize the fluorescence of intracellular Dox. Specifically, K562 and K562/MDR were treated with free Dox and KK-NF-Dox, respectively, followed by incubation for 3 h. Hoechst 33342 was used to identify cell nucleus. Strong Dox fluorescence intensity was observed in chemosensitive K562 cells treated with either free Dox or KK-NF-Dox (Fig. 5a), indicating that free Dox rapidly entered cells by passive diffusion and accumulated in K562 cells and that KK-NF-Dox also efficiently delivered Dox into these cells. In contrast, when K562/MDR cells (Fig. 5b) were treated with the same concentration of free Dox, only weak fluorescence intensity of Dox was observed, indicating the reduced ability of K562/MDR cells to retain Dox inside cells. However, strong Dox fluorescence intensity was observed in K562/MDR cells treated with KK-NF-Dox, demonstrating that the KK-NF nanocarriers dramatically increased the accumulation and retention of Dox in MDR cells. Similarly, free Dox rapidly entered and accumulated in breast cancer MCF7 cells, but not in MCF7/MDR cells, in contrast to efficient delivery and retention of Dox by S-NFs in both MCF7 and MCF7/MDR cells (Fig. 5c, d).

To further investigate Dox retention in cancer cells, we examined the amount of Dox retained in cells at different time points after removal of treatment. Again, chemosensitive cells and MDR cells (K562 and K562/MDR cells in Fig. 6a and b; MCF7 and MCF7/MDR cells in Fig. 6c and d) were treated with free Dox and the corresponding NF-Dox, respectively, for 3 h, and cells were washed to remove treatment, followed by further incubation. Dox fluorescence intensities of the resultant cells were analyzed by flow cytometry at different time points. When treated with free Dox, chemosensitive cells exhibited nearly constant fluorescence intensities over 4 h after removal of extracellular drugs (Fig. 6a for K562 cells, and Fig. 6c for MCF7 cells), whereas Dox fluorescence intensities in MDR cells treated with free Dox were gradually decreased (Fig. 6b for K562/MDR cells, and Fig. 6d for MCF7/MDR cells). In contrast, when treated with the corresponding aptamer-NF-Dox, both chemosensitive and MDR cells maintained high Dox fluorescence intensities, and importantly, the Dox fluorescence intensities in MDR cells decreased relatively slowly, compared to these cells treated with free Dox. The enhanced accumulation and retention of Dox delivered by NFs in MDR cells is most likely a result of protection against efflux by P-gp at physiological pH (7.4).

3.5 Circumvention of MDR by NF-mediated Targeted Drug Delivery

We then evaluated the above prepared NFs for circumvention of drug resistance in targeted therapy. Both chemosensitive cells and the corresponding MDR cells were treated with 1) free Dox, 2) ctrl-NF-Dox, and 3) the corresponding aptamer-NF-Dox, respectively. After incubation for 3 hours to allow permeation or internalization of Dox or NF-Dox into cells, treatment was removed and cells were cultured for two days, followed by MTS assay to determine cell viability. In chemosensitive cancer cells, both free Dox and aptamer-NF-Dox induced potent cytotoxicity (Fig. 7a for K562 cells, and Fig. 7d for MCF7 cells). In contrast, in MDR cells, while free Dox induced only slight cytotoxicity, the corresponding aptamer-NF-Dox dramatically decreased cell viability (Fig. 7b for K562/MDR cells, and Fig. 7e for MCF7/MDR cells). In particular, in K562 chemosensitive cells, the IC₅₀ of free Dox, Ctrl-NF-Dox, and S-NF-Dox are 4.5 μ M, >50 μ M, and 5.1 μ M, respectively; in K562/MDR cells, the IC₅₀ of free Dox, Ctrl-NF-Dox, and S-NF-Dox are >50 μ M, >50 μ M, and 13.2 μ M, respectively; in MCF7 chemosensitive cells, the IC₅₀ of free Dox, Ctrl-NF-Dox, and S-NF-Dox are 4.2 μ M, >50 μ M, and 3.7 μ M, respectively; in MCF7/MDR cells, the IC₅₀ of free Dox, Ctrl-NF-Dox, and S-NF-Dox are >200 μ M, >200 μ M, and 42.7 μ M, respectively. With increasing treatment time, the cytotoxicity of NF-Dox increased and always outperformed free Dox (Fig. 7c for K562/MDR cells as an example). However, in nontarget Ramos cells, both KK-NF-Dox and S-NF-Dox induced dramatically less cytotoxicity than free Dox, indicating the selectivity of cytotoxicity mediated by aptamer-NF-Dox (Fig. 7f). Furthermore, ctrl-NF-Dox, which did not specifically recognize cancer cells, induced only slight toxicity at high dosages, further demonstrating the selectivity of NF-mediated drug delivery. Pure NFs did not induce apparent cytotoxicity, verifying their biocompatibility at the cellular level (Fig. S9). Overall, NF-Dox mediated targeted drug delivery to both chemosensitive cells and MDR cells and circumvented drug resistance in MDR cells, making NFs promising for circumvention of MDR in targeted drug delivery.

4. Conclusion

We have demonstrated that multifunctional DNA NFs for circumventing drug resistance in targeted anticancer drug delivery in both leukemia and breast cancer cell models. DNA NFs were self-assembled through DNA liquid crystallization driven by efficient DNA production in RCR. NF assembly avoids the otherwise complicated sequence design in conventional assembly of DNA nanostructures and makes it easy to incorporate functionalities including aptamers for specific cancer cell targeting, fluorophores for molecular imaging, and drug-binding DNA sequences for specific drug intercalation during drug delivery. Therapeutic functionalities, such as DNA antisense [42] and immunomodulatory DNA, [43] could also be simultaneously integrated into NFs and will be reported elsewhere. Particularly in this study, NFs were incorporated with different aptamers for specific recognition of both chemosensitive cancer cells and MDR cells during targeted drug delivery. Combining high density of drug-binding DNA motifs and porous DNA structures in NFs, NFs were endowed with dual-mode high drug loading capacity. Specifically, the Dox loading efficiency of KK-NFs was as high as 71.4% (wt/wt). Furthermore, we have estimated that up to 34.3% Dox in NF-Dox could be loaded by intercalation into drug-binding motifs, and the remainder of Dox was likely loaded via physical encapsulation into the porous structures in NFs, implying

that NFs are also likely to serve as carriers for drugs that can not specifically bind to DNA. NFs have high biostability and dramatically resisted to nuclease degradation. NF-Dox complexes were relatively stable at physiological pH, with Dox slowly released from NFs, avoiding rapid drug efflux by P-gp on adjacent cell membrane during the short time period before NF-Dox diffuses from the vicinity of cell membrane to cytosol and allowing for controlled drug release during a relatively long time period when NF-Dox stays in inner cytosol. Furthermore, Dox release from NF-Dox was facilitated in either an acidic or basic condition, making it possible to facilitate drug release when transported into acidic subcellular organelles, such as the lysosome and the endosome. In contrast to free Dox which can only efficiently diffuse into chemosensitive cells, NFs efficiently delivered and retained Dox in both chemosensitive cells and MDR cells, and consequently selectively inhibited cell proliferation in both target chemosensitive cells and target MDR cells, but not in nontarget Ramos cells or by non-targeting ctrl-NFs. Overall, DNA NFs are promising for circumvention of MDR in targeted drug delivery and present tremendous clinical significance as drug resistance and drug toxicity are two major hurdles in cancer therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Schematic illustration of multifunctional DNA NFs as nanocarriers to circumvent MDR in targeted drug delivery. NFs were self-assembled by liquid crystallization of DNA generated from RCR, for which the DNA templates encoded aptamers and drug-binding DNA motifs. Drugs (Dox) were loaded into NFs via both DNA-binding motifs and physical encapsulation. NFs mediated the specific recognition, internalization, and targeted drug delivery to both chemosensitive and MDR cancer cells. NF-Dox was relatively stable at physiological pH, thus avoiding rapid drug release and allowing for controlled drug release. In target MDR cells (depicted), NF-Dox was internalized and transported to cytosol, during which NFs protected Dox from efflux by P-gp, in contrast to rapid efflux of free Dox by adjacent P-gp. In cytosol, gradually-released drugs evaded efflux due to the absence of P-gp. Consequently, NF-Dox enhanced drug delivery into subcellular target organelles (e.g., nucleus), promoting the cytotoxicity in MDR cells and circumventing drug resistance in targeted cancer therapy.

Figure 2.

Structural characterization and drug release from biostable NFs. (A) SEM images displaying KK-NFs self-assembled from RCR reaction for 3 h (diameters: ~ 200 nm). (B) DLS results verifying the diameter of the above NFs in solution to be 230 ± 30 nm (mean $\pm$ SD). (C) SEM images displaying KK-NFs from RCR reaction for 10 h (diameters > 1 μm). (D) A POM image displaying disc-shaped optical textures of spherulite NFs when observed between two crossed polarizers, indicating birefringent and liquid crystalline NFs. (E) SEM images displaying the morphological integrity of KK-NFs treated with DNase I (5 U/mL, 37 °C, 24 h), indicating high biostability. Insets in (A, C, and E) are individual NFs. (F) Drug release curves showing slow Dox release from NFs at pH 7.4, indicating high stability of NF-Dox at physiological pH, and dramatically facilitated Dox release at pH 5 and pH 9. Dox release was fit into a drug release model. (Free Dox: control; solid lines: fitted curves; R²: correlation coefficient).

Figure 3.

Flow cytometry results demonstrating NFs for specific recognition of both chemosensitive cancer cells and MDR cancer cells, but not to nontarget cells. (A) KK-NFs bound to chemosensitive K562 cells and K562/MDR cells. (B) S-NFs bound to chemosensitive MCF7 cells and MCF7/MDR cells. (C) Neither KK-NFs nor S-NFs specifically bound to nontarget Ramos cells. (lib: random DNA sequences; lib, KK, sgc8: labeled with Cy5)

Figure 4.

Internalization of fluorophore-labeled and aptamer-integrated NFs into both chemosensitive cancer cells and MDR cancer cells. (A, B) Confocal microscopy images showing that KK-NFs (A) were internalized into K562 cells and K562/MDR cells and that S-NFs (B) were internalized into MCF7 cells and MCF7/MDR cells. NFs were labeled with Cy5 using dUTP-Cy5, and cells were stained with Hoechst 33342 to identify cell nuclei. (Scale bar: 100 μm)

Figure 5.

Efficient drug delivery to both chemosensitive and MDR cancer cells by NF-Dox. (a, b) CLSM images displaying strong Dox fluorescence intensities in K562 cells (a) treated with either free Dox or KK-NF-Dox, in contrast to K562/MDR cells (b) that only displayed strong Dox fluorescence intensity when treated with KK-NF-Dox, but not free Dox, indicating efficient Dox delivery into both K562 cells and K562/MDR cells, and enhanced the efficiency of drug delivery to MDR cells by NFs. (c, d) Similarly, S-NF-Dox efficiently delivered Dox into both MCF7 cells and MCF7/MDR cells. Cells were stained with Hoechst 33342 to identify nuclei. (Scale bars: 100 μm (a, b), and 25 μm (c, d))

Figure 6.

Flow cytometric analysis of intracellular Dox accumulation and retention in chemosensitive cancer cells and the corresponding MDR cells. (a, c) Cells were treated with free Dox or the corresponding NF-Dox for 3 h, followed by washing to remove extracellular drugs and further incubation for 0 h, 1 h, 2 h, and 4 h, respectively. Both free Dox and Dox delivered by NF-Dox were well retained in chemosensitive K562 cells (a) and MCF7 cells (c), as shown by the nearly constant average fluorescence intensities over 4 h after removal of extracellular drugs. (b, d) Dox fluorescence intensities in the corresponding MDR cells treated with free Dox gradually decreased (K562/MDR in b; MCF7/MDR in d). Note the log scale of Dox fluorescence intensities. However, MDR cells treated with the corresponding aptamer-NF-Dox maintained Dox fluorescence intensities over the 4-h period after removal of extracellular drugs. The relatively wide distribution of Dox fluorescence intensities in MCF7/MDR is partially a result of the morphological heterogeneity of these cells. (Legends: cells only: untreated cells; 0–4 h: incubation time after removal of extracellular drugs. Dash lines: reference bars.)

Figure 7.

NF-Dox induced selective cytotoxicity in both target chemosensitive and MDR cancer cells, thus circumventing MDR in targeted drug delivery. (a, b) MTS assay results showing that both free Dox and KK-NF-Dox induced potent cytotoxicity in target K562 cells (a), whereas in target K562/MDR cells, only KK-NF-Dox induced potent cytotoxicity (b). Furthermore, ctrl-NF-Dox only induced slight cytotoxicity in these cells, indicating the specificity of aptamer-NF-Dox-mediated cytotoxicity. (c) Cell viability of K562/MDR cells treated with free Dox (8 μM) or KK-NF-Dox (8 μM Dox equivalent) showing increased cytotoxicity with increasing treatment time and enhanced cytotoxicity induced by KK-NF-Dox compared to free Dox. (d, e) Similarly, MTS assay results in target MCF7 cells (d) and MCF7/MDR cells (e) showed specific cytotoxicity induced by S-NF-Dox, which also circumvented drug resistance and induced potent cytotoxicity in MDR cells, in contrast to negligible cytotoxicity induced by ctrl-NF-Dox. (f) Compared to free Dox, KK-NF-Dox and S-NF-Dox induced dramatically less cytotoxicity in nontarget Ramos cells. (Solid lines: fitted curves to a drug response model, with correlation coefficient $R^2 > 0.95$ in all cases)