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DNA Nano Trihedron Exhibits an Inhibitory Effect on Breast Cancer Cells

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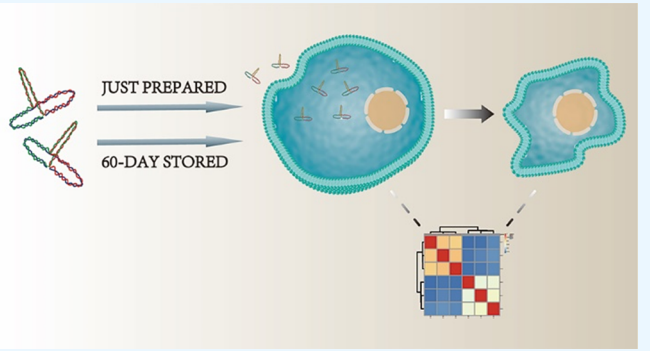
ABSTRACT: As a new type of nanomaterial, DNA nanomaterials show great potential in biomedical applications because of their high precision, high controllability, and high biocompatibility among other characteristics. Therapeutic drugs based on DNA nanomaterials have been shown to have beneficial therapeutic effects on a variety of diseases. The application of DNA nanomedicines in the treatment of diseases has become a rapidly developing area of study. However, the instability of DNA nanomaterials greatly limits their clinical application. Therefore, we designed and synthesized a stable topological DNA nanostructure: DNA Nano Trihedron (DNT). We demonstrated that DNT could enter MCF-7 cells without the transfection agent. In addition, DNT could induce dramatic changes in gene expression and produce significant inhibitory effects on MCF-7 cells. DNT after two months of storage still had an inhibitory effect on MCF-7 cells.



INTRODUCTION

With increasing morbidity and mortality, cancer has become a major public health problem worldwide.[1,2] The methods used in the clinical treatment of cancer (surgery, radiotherapy, chemotherapy, targeted therapy, and immunotherapy) generally have many disadvantages.[3,4] For example, chemotherapy can be expensive, time consuming, and hazardous.[5−8] Therefore, we need to continue to develop more effective and optimized anticancer therapies and drugs for cancer treatment.

Nucleic acid therapeutics have been widely studied as a new type of treatment.[9−11] Common types of nucleic acid therapeutics include antisense oligonucleotides,[12,13] ligand-modified small interfering RNA conjugates,[14,15] and lipid nanoparticles.[16] Nucleic acid-based drugs are advantageous for cancer treatment because of their high specificity, versatility, repeatable production, and adjustable immunogenicity.[17] Nucleic acid drugs have the ability to directly target pathogenic genes or mRNAs, and they can treat diseases at the genetic level, thereby achieving long-lasting therapeutic effects.[18]

However, nucleic acid-based drugs still face many challenges as therapeutic agents. First, they are easily degraded by nucleases in the body, which makes it difficult to ensure the integrity of structure and function in vivo.[14,19,20] Moreover, nucleic acid drugs have difficulty entering the cell through the cell membrane, since the cell membrane with a negative charge will repel the nucleic acid therapeutic carrying a negative charge.[21,22] Furthermore, many nucleic acid therapeutics have poor stability in vitro, such as unmodified mRNA-based drugs, which are broken down by readily available RNase.[23] As a result, these types of drugs require a sophisticated mechanism of transportation and storage, which will cause the cost of treatment to increase and further intensify the treatment burden patients experience.

In previous studies, we designed and synthesized DNA Nano Trihedron (DNT), a kind of DNA nanostructure with a topological structure. The experiments indicated that DNT has strong stability and can maintain its structural integrity and biological activity at room temperature for two months, which leads to great convenience for storage and transportation. Furthermore, we have demonstrated that DNT is able to enter the cells, even corneal cells (both corneal epithelial and endothelial cells) surrounded by an extracellular matrix without a transfection agent, which indicates that DNT has the potential to be a nucleic acid-based drug.

In this study, we used MCF-7 cells as a representative to investigate the effect and mechanism of DNT on cancer cells. The experimental results indicated that DNT was able to enter and significantly inhibit the activity of MCF-7 cells. Moreover, DNT at room temperature for two months was still able to inhibit the activity of MCF-7 cells. Transcriptome sequencing

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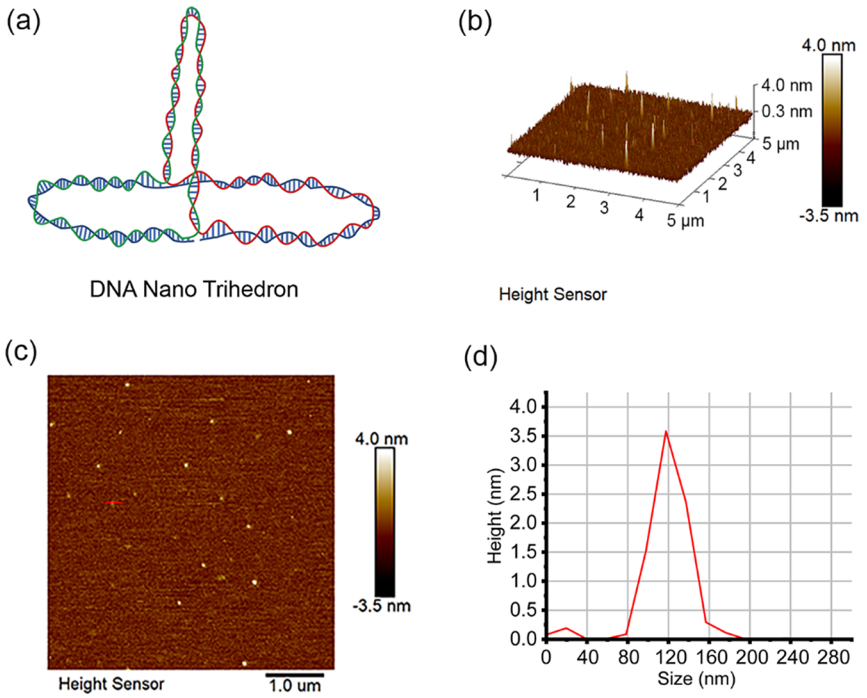

Figure 1. Characterization of DNT. (a) Pattern of DNT. (b) 3D image of DNTs with AFM. (c) 2D image of DNTs with AFM. (d) Diameter and height of a DNT particle. Measurement line is marked in Figure 1c.

results showed that DNT was able to affect the expression of many kinds of genes in MCF-7 cells that are involved in many signaling pathways. This indicates that DNT can grossly interfere with the biological processes of MCF-7 cells and exert an inhibitory effect on the cells.

MATERIALS AND METHODS

Synthesis and Characterization of DNT. First, five single-stranded linear DNA sequences were designed, and denoted S1, S2, S3, L1, and L2. We used the software, Primer Premier version 5, to design and evaluate DNA sequences. The sequences ssDNAs are listed in Table S1. In the presence of L1, the 5′ and 3′ ends of S1 can be ligated together by the T4 DNA ligase to form single-stranded circular DNA, C1. Similarly, S2 can be ligated by the T4 DNA ligase with the help of L2 to form single-stranded circular DNA, C2. We mixed C1, C2, and S3 in a ratio of 1:1:1 and carried out gradient cooling treatment to form a pro-DNT solution. After that, DNA Topoisomerase I was added to the pro-DNT solution. DNA Topoisomerase I can introduce a natural DNA double helix into the pro-DNT structure to form DNT, and the DNT structure is more stable than the pro-DNT structure. We used atomic force microscopy (AFM) (Bruker Co., Berlin, Germany) to characterize the DNT. First, we purified the DNT with isopropanol. Then, we dissolved the DNT powder with ddH_2O to produce a 200 ng/μL solution. Next, we mixed the DNT solution and a 10 mM $MgSO_4$ solution at a ratio of 1:1. We uniformly dispersed 10 μL of the mixed solution on a newly peeled mica plate and washed the sample surface with 600 μL of ddH_2O three times after drying. Finally, we dried the sample using clean nitrogen blowing observed using AFM.

Cell Culture. We used high glucose DMEM containing 10% (v/v) FBS and 1% (v/v) double antibody to grow MCF-7 human breast cancer cells. The cell culture temperature was 37 °C, and the CO_2 concentration was 5%.

Ability of DNT To Enter Cells. We labeled four confocal culture dishes as A, B, C, and D. Then, we inoculated MCF-7 cells in the four plates with 2×10^3 cells per plate and cultured them for 24 h. After that, we added ssDNA loaded with Cy3 fluorophore to the dishes labeled A and C. The final concentration of ssDNA was 20 ng/μL. Additionally, we added DNT loaded with Cy3 fluorophore to the dishes labeled B and D. The final concentration of DNT was 20 ng/μL. Then, we cultured four groups of cells under the same conditions. We removed the supernatant of groups A and B, washed the cells with PBS three times, and exposed the cells to 0.2% Triton X-100 for 1 min. Then, the cells were fixed in 4% cold paraformaldehyde solution for 10 min, washed with PBS, and treated with 0.5% Triton X-100 for 3 min. After three washes with PBS, the cells were blocked with 1% BSA at 37 °C for 30 min. The cells were incubated overnight with antibody against TUBA1A (1:200) at 4 °C and then incubated with a relevant fluorescent secondary antibody at 37 °C for 90 min. Next, the cell samples were treated with DAPI for 10 min to stain the nuclei. Finally, images were captured using an inverted fluorescence microscope (Olympus, Shinjuku City, Tokyo, Japan). The treatment of groups C and D was essentially the same as that of groups A and B. The only difference is that the supernatants of groups C and D were removed after 48 h of culture.

Effect of DNT on the Morphology of MCF-7 Cells. We inoculated MCF-7 cells in a 6-well plate with 8×10^4 cells per well and cultured them for 24 h. Then, we divided the six wells into group A and group B. Group A was the control group, and group B was the experimental group. The three wells in each group were treated the same. We added 100 μL of ddH_2O to each of the three wells in group A and 100 μL of DNT solution at a concentration of 700 ng/μL to each of the three wells in group B. After 48 h, we placed the cells under an inverted microscope to observe their morphology.

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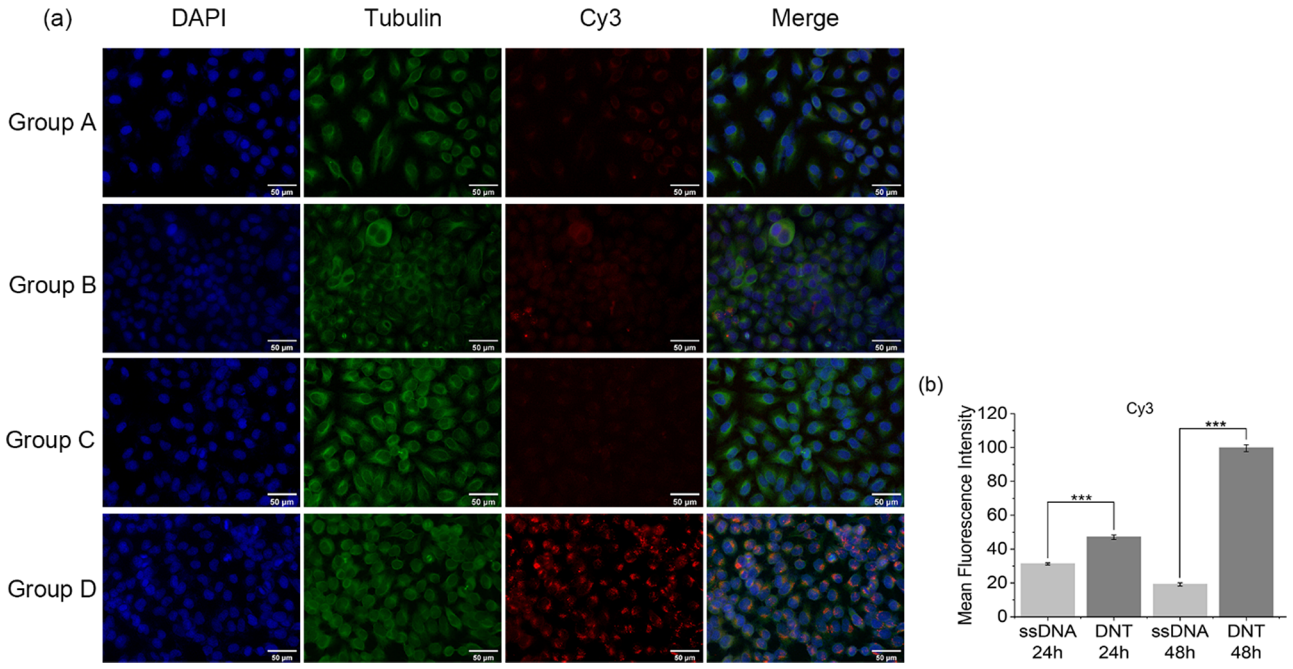

Figure 2. DNT enters MCF-7 cells. (a) DNT and control ssDNA were incubated with MCF-7 for 24 and 48 h. Scale bar is 50 μm. Group A, MCF-7 cells incubated with Cy3-ssDNA for 24 h; Group B, MCF-7 cells incubated with Cy3-DNT for 24 h; Group C, MCF-7 cells incubated with Cy3-ssDNA for 48 h; Group D, MCF-7 cells incubated with Cy3-DNT for 48 h. (b) Mean fluorescence intensity of Cy3-ssDNA and Cy3-DNT. Data are presented as mean ± SD (n = 3). Statistical analysis: *P < 0.05, **P < 0.01, ***P < 0.001.

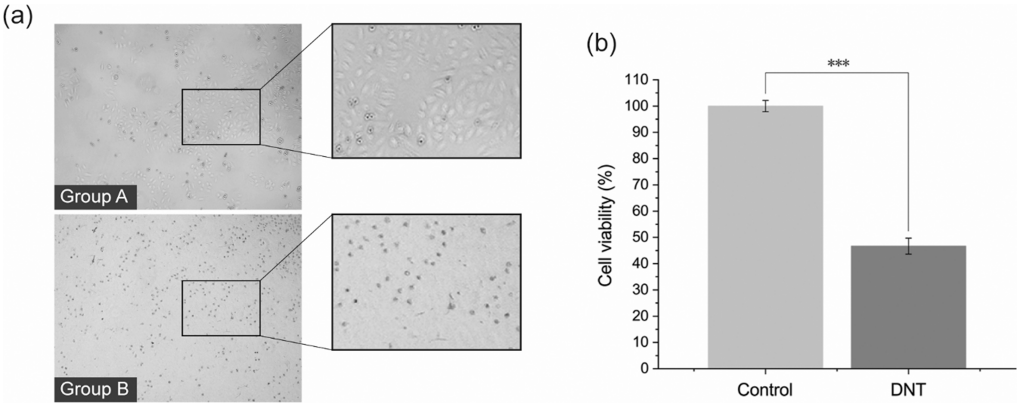

Figure 3. Effect of DNT on MCF-7 cells. (a) Morphology of MCF-7 cells. Group A is the MCF-7 cells before DNT treatment. Group B is the MCF-7 cells after DNT treatment. (b) Cell viability of MCF-7 cells treated with DNT. Data are presented as mean ± SD (n = 3). Statistical analysis: *P < 0.05, **P < 0.01, ***P < 0.001.

CCK-8 Assay. MCF-7 cells were cultured in 24-well plates, and the effect of DNT on MCF-7 cells was investigated by the Cell Counting Kit-8 (CCK-8). First, we inoculated MCF-7 cells in a 6-well plate with 2 × 10^3 cells per well and cultured them for 24 h. Then, we divided the six wells into group A and group B. Group A was the control group, and group B was the experimental group. The three wells in each group were treated the same. We added 100 μL of ddH_2O to each of the three wells in group A and 100 μL of DNT solution to each of the three wells in group B. The concentration of DNT solution was 700 ng/μL. After 48 h of culture, 30 μL of CCK-8 solution was added to each well, and the reaction was carried out for 1 h at 37 °C. Then, we removed 100 μL of supernatant from each well and determined the absorbance at 450 nm.

Effect of DNT on MCF-7 Gene Expression. We performed transcriptome sequencing to investigate the effect of DNT on MCF-7 gene expression. First, we inoculated MCF-7 cells in a 6-well plate with 8 × 10^4 cells per well and cultured them for 24 h. Then, we divided the six wells into group A and group B. Group A was the control group, and group B was the experimental group. The three wells in each group were treated the same. We added 100 μL of ddH_2O to each of the three wells in group A and 100 μL of DNT solution to each of the three wells in group B. The concentration of the DNT solution was 700 ng/μL. The cells in group A and group B were cultured under the same conditions. After 48 h of culture, 1 mL of TRIZOL solution was added to each of the six wells. We lysed the cells by repeatedly blowing the cell suspension. Then, we sequenced the transcriptome of cells with Illumina sequencing technology.

Effect of DNT on MCF-7 Cells after Storage. We stored the DNT solution at room temperature (20−25 °C) for 2 months. We inoculated MCF-7 cells in a 6-well plate with 2 × 10^3 cells per well and cultured them for 24 h. The three wells

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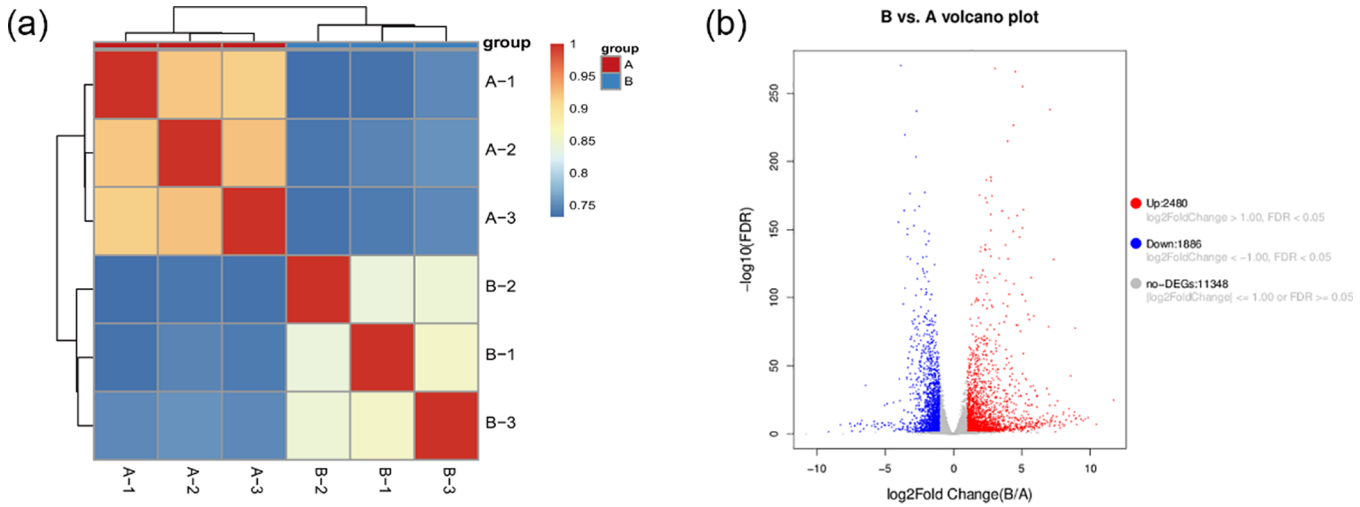

Figure 4. Transcription results of MCF-7 cells. (a) Sample correlation heatmap of known genes at the group scale. Group A is the MCF-7 cells without DNT treatment. Group B is the MCF-7 cells after DNT treatment. (b) Volcano plots of genes with significant differences. Red dots, upregulated genes; blue dots, downregulated genes; gray dots, genes with no significant difference.

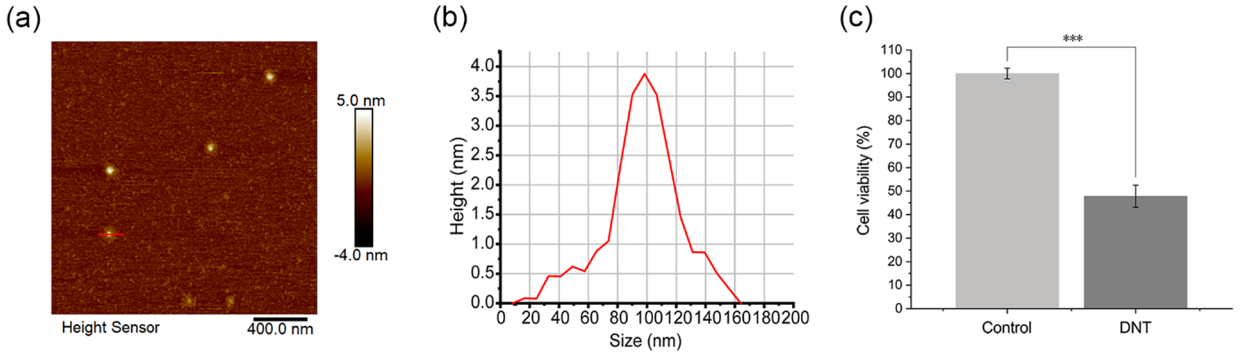

Figure 5. Characterization and bioactivity of DNT after storage. (a) 2D image of DNTs with AFM. (b) Diameter and height of a DNT particle. The measurement line is marked in Figure 5a. (c) Cell viability of MCF-7 cells treated with DNT after storage. Data are presented as mean ± SD (n = 3). Statistical analysis: *P < 0.05, **P < 0.01, ***P < 0.001.

in each group were treated the same. We added 100 μL of ddH_2O to each of the three wells in group A and 100 μL of DNT solution to each of the three wells in group B. The concentration of DNT solution was 700 ng/μL. The cells in group A and group B were cultured under the same conditions. After 48 h of culture, 30 μL of the CCK-8 solution was added to each well, and the reaction was carried out for 1 h at 37 °C. After that, we removed 100 μL supernatant from each well and determined the absorbance at 450 nm.

RESULTS AND DISCUSSION

Synthesis and Characterization of DNT. The pattern of DNT and the AFM characterization results of DNT are shown in Figure 1. Many particles of the uniform size are seen in Figure 1b,c. The analysis result shows that these particles have a diameter of 20−30 nm and a height of approximately 3 nm.

Ability of DNT To Enter Cells. To explore the mechanism by which DNT inhibits the activity of MCF-7 cells, we added DNT loaded with the Cy3 fluorophore to the culture medium of MCF-7 cells. After different culture times, we observed the cells by staining, and the results are shown in Figure 2. After 24 h of culture, red fluorescence appeared in the cytoplasm of the cells of group B. This phenomenon indicated that DNT could enter MCF-7 cells within 24 h in the absence of the transfection reagent and was retained in the cytoplasm. After 48 h of culture, we stained the cells in group D. Compared with the cells in group B, the red fluorescence in the cells of group D was more intense, indicating that more DNT entered the cytoplasm with the extension of the culture time. We eliminated the influence of fluorescently labeled ssDNA on the experimental results, as no obvious red fluorescence existed in group A and group C cells.

Effect of DNT on MCF-7 Cells. We used inverted microscopy to observe the effect of DNT on the morphology of MCF-7 cells, and the results are shown in Figure 3a. Compared with the cells in group A, the cells in group B had obvious morphological changes, which manifested as cell shrinkage, deformation, and significantly smaller volume. Overall, the results showed that DNT had a significant negative effect on the normal growth of MCF-7 cells. We explored the effect of DNT on MCF-7 growth with a CCK-8 assay, and the results are shown in Figure 3b. The absorbance of group A was significantly higher than that of group B. The results indicated that DNT exerts a significant inhibitory effect on the growth of MCF-7 cells.

Effect of DNT on MCF-7 Gene Expression. Transcriptome analysis was performed to identify changes in the gene expression of DNT-treated MCF-7 cells. The results of the sample correlation heatmap (Figure 4a) showed that there were significant differences in gene expression between group

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A cells and group B cells. A total of 2480 genes were upregulated and 1886 genes were downregulated in MCF-7 cells after DNT treatment (Figure 4b). The genes with significant differences in expression were involved in various cellular activities, such as apoptosis, cell aggregation, and biomineralization (Figure S1). In addition, we found that multiple signaling pathways (Figure S2) were involved in DNT-treated MCF-7 cells. This suggests that the inhibitory effect of DNT on MCF-7 cells is not achieved through a single pathway, and its effect on MCF-7 cells is more extensive.

Effect of DNT on MCF-7 Cells after Storage. We explored the structural integrity of DNT after storage, using AFM. The results are shown in Figure 5. The DNT was uniform in size. Its diameter and height were similar to those of freshly prepared DNT. The results showed that DNT can maintain its structural integrity after two months at room temperature. Then, we explored the effect of DNT on MCF-7 growth after storage with a CCK-8 assay, and the results are shown in Figure 5c. The OD450 value of group A was significantly higher than that of group B. The results indicated that the number of cells in group A was significantly higher than that in group B. This finding indicates that DNT still has the ability to significantly inhibit the growth of MCF-7 cells after two months of storage at room temperature.

With the continuous elucidation of the nucleic acid molecular structure and function, nucleic acid drugs have gradually become a hot spot of disease treatment. In contrast with antibody drugs, nucleic acid drugs demonstrate better efficacy and safety and can be used in the treatment of cancer and some genetic diseases. Among various nucleic acid-based drugs, small nucleic acid drugs have gained popularity for their simple structures and batch-to-batch reproducibility. At present, a popular small-size nucleic acid therapeutic is a tetrahedral DNA nanostructure (TDN) composed of three single strands of DNA. It has been proven that TDN can enter the cell without the help of a transfection agent.[24−26] In this study, we simplified the design of TDNs and synthesized DNT, a type of three-sided DNA nanostructure whose size is similar to that of a TDN. DNT consists of two circular DNA and one ssDNA. The two ends of ssDNA can provide natural binding sites, on which we can connect fluorescent groups for localization. In addition, we can try to connect the adapter for targeted transport or connect the drug for drug delivery. In this study, MCF-7 cells were used to investigate the ability of DNT to enter the cells. The experimental results indicated that DNT was able to enter the cells without a transfection agent and was mainly found in the cytoplasm of the MCF-7 cells. These results demonstrated that DNT could surpass the cell membrane barrier and may be a candidate drug carrier.

In addition, the extracorporeal stability of DNA nanostructures is directly related to the preparation, storage, and transport conditions of DNA nanostructures, and it is also an important basis to evaluate whether DNA nanostructures can be used as nucleic acid drug delivery systems. The less stable the DNA nanostructures, the more demanding and costly the storage condition can be. It is well known that TDNs rely on only hydrogen bonds between base pairs to maintain the integrity of the structure, and the rupture of hydrogen bonds leads to the collapse of the structure. As a result, the strength of the hydrogen bonds determines the strength of a TDN and, more importantly, the cost of TDN. It has been shown that the detection ability of the molecular detection device prepared using TDNs decreased substantially after 15 days of placement.[27] The instability of TDN greatly limits its application.[28] We used DNA Topoisomerase I to introduce the natural topological structure into the double-strand region of DNT. The presence of covalent bonds makes DNA nanostructures more stable compared to relying only on hydrogen bonds. Previous studies showed that DNT in solution still contains structure integrity and the bioactivity to enter the cells after being stored in solution for two months.

CONCLUSIONS

The emergence of nucleic acid drugs has introduced a novel treatment for cancer treatment.[29−31] In this study, we used MCF-7 cells as an example to explore the effect of DNT on MCF-7 cells by CCK-8 assay and transcriptome analysis. The experimental results indicated that DNT could significantly inhibit the growth of MCF-7 cells, which shows that DNT has the potential to be a therapeutic drug for cancer. To further investigate the mechanism by which DNT inhibits the growth of MCF-7 cells, transcriptome analysis was performed on MCF-7 cells treated with DNT. DNT treatment resulted in many abnormal signaling pathways in MCF-7 cells, including but not limited to the signaling pathways regulating apoptosis, cell aggregation, and biomineralization. This indicates that the presence of DNT can grossly interfere with the normal physiological activities of MCF-7 cells and finally leads to an inhibitory effect on MCF-7 cells.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c07859.

Sequences of the ssDNAs of DNT and transcriptome analysis results of MCF-7 cells after DNT treatment (PDF)

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Author Contributions

Y.W. and S.C. contributed equally. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

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■ ABBREVIATIONS

AFM	atomic force microscopy
DMEM	Dulbecco's modified eagle medium
DNT	DNA nano trihedron

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