We demonstrate a novel DNAzymes-based nanocomposite that can simultaneously silence three types of genes in living cells and in vivo. The synergetic strategy for silencing three different genes can significantly enhance the knockdown efficacy and effectively inhibit the cancer cells’ progression.

Cancer has been the leading cause of death, which has led to cancer detection and treatment sparking many research frontiers in the creation of various platforms for diagnosis and therapy. There has been a tremendous amount of work done on radiotherapy, chemotherapy and photodynamic therapy. Besides these methods, gene silencing has been rapidly developed, which promotes inhibition of the expression and function of genes. This technology shows promising pre-clinical results, and is currently being applied in clinical trials for a variety of diseases, including many forms of cancer genetic disorders, and macular degeneration. Moreover, there have been multiple gene silencing strategies including short interfering RNA, aptamers, antisense oligonucleotides, decoys, ribozymes, and DNAzymes. Among these, DNAzymes have attracted our attention because of their high endonuclease activity, lower production cost and relative serum stability, which enable them to be therapeutic candidates for clinical use.

DNAzymes, which are derived through an in vitro selection process, are also considered as scissors to cut gene-specific molecules. DNAzymes have been extensively used to detect and image metal ions, small molecules, and biomacromolecules. Meanwhile, DNAzymes can also catalyze sequence-specific RNA cleavage in a similar manner to the hammerhead ribozyme. The most widely investigated DNAzymes for RNA cleavage are 10–23 DNAzymes. For instance, Tan’s group developed a C6–DNAzyme–MnO2 nanosystem combining gene silencing with photodynamic therapy. Kim et al. utilized the T315I DNAzyme to cleave relevant mRNA and combined it with imatinib treatment for overcoming imatinib resistance in leukemic cells. Remarkably, the therapeutic effect of merely silencing single type tumor-related gene still needs to be significantly improved. It is known that cancer is generally correlated with multiple tumor-related genes. Therefore, developing a novel nanocomposite with multiple DNAzymes is highly desirable to enhance the therapeutic effect of cancer therapies by simultaneously silencing multiple tumor-related genes.

Herein, we report a novel DNAzymes-based nanocomposite that can simultaneously silence three different tumor-related genes. Based on our study, cobalt oxyhydroxide (CoOOH) as an oxidizing agent can be reduced by intracellular GSH, and the Co2+ as a reaction product and cofactor catalyzes DNAzymes to cleave relevant mRNAs for gene silencing. Therefore, amino-functionalized cobalt oxyhydroxide nanoflakes (COHN) were chosen as genetic vectors to deliver DNAzymes. We selected three tumor-related genes: thymidine kinase 1 (TK1), survivin (SUR) and serine–threonine protein kinase AKT1. TK1 has been shown to be a marker of tumor growth and correlated with cell division. SUR is a bifunctional protein that regulates cancer cell division and apoptosis. Moreover, AKT1 regulates cancer cell migration, invasion and metastasis. As the nanocomposites were uptaken by the cancer cells, CoOOH was reduced by intracellular GSH to generate Co2+ ions and release three kinds of DNAzymes. Meanwhile, the Co2+ ions could be employed as cofactors of the 10–23 DNAzymes for multiple gene knockdowns (Scheme 1). Compared to the traditional single gene silencing method, the nanocomposite regulates the cancer's migration, invasion, proliferation and apoptosis through the synergetic effect of all three DNAzymes.

The CoOOH nanoflakes were synthesized using a one-step ultrasonic method by oxidizing CoCl2 with NaClO in the presence of NaOH. Subsequently, to effectively absorb DNAzymes, a significant amount of surface hydroxyl groups on CoOOH react with 3-aminopropyltriethoxysilane via a silanization reaction.
to yield COHN. According to the HRTEM pattern in Fig. 1a, the COHN displays a sheet structure with a large surface area and fine layers. In addition, the assay of ninhydrin further proves that the amino group was successfully modified on CoOOH (Fig. S1, ESI†). Moreover, the DNAzymes–COHN was fabricated through a strong electrostatic interaction. The sequences of the DNA oligonucleotides are shown in Table S1 (ESI†). The different zeta potentials in Fig. 1b demonstrate the successful assembly of the nanocomposite. Next, the optical properties of COHN and the DNAzymes–COHN nanocomposite were evaluated. In Fig. 1c, the UV-Vis absorption spectra demonstrate the maximum absorption of the CoCl₂ at 510 nm, which was blue-shifted to ~410 nm for the COHN. In addition, DNAzymes–COHN has an intense absorption at ~260 nm compared to COHN, which correlates with the characteristic absorption of DNA, implying the successful modification of DNAzymes on the COHN.

DNAzymes–COHN cutting mRNAs in vitro was studied using a RhB labeled target. When Co²⁺ catalyzed the DNAzyme, cleaving the RhB labeled target, the cleaved single-stranded RhB labeled target was absorbed by graphene oxide (GO) and the fluorescence of RhB was quenched. The RhB labeled target was hybridized with DNAzyme to form double strands which cannot absorb on GO without Co²⁺ (Fig. S2, ESI†). These results demonstrate that DNAzymes–COHN can cut mRNAs in vitro. The stability of DNAzymes–COHN was evaluated under physiological conditions. CoOOH nanoflakes, as a fluorescence quencher, can test the stability of the nanocomposite by evaluating the fluorescence changes, due to the broad absorption band of COHN (Fig. 1c) overlapping with the emissions of the RhB-labeled AKT1 DNAzyme. As shown in Fig. S3 (ESI†), the RhB-labeled AKT1 DNAzyme–COHN nanocomposite treated with deoxyribonuclease I (DNase I) or ribonuclease A (RNase A), exhibits a slight fluorescence change compared to the case without DNase I or RNase A. However, when GSH was added into the solutions, the fluorescence intensities were greatly enhanced (Fig. S3 inset, ESI†). The results indicate that the fluorescence increase is due to the reduction of COHN by GSH, and the nanocomposite possesses high nuclelease stability. Moreover, the amounts of every DNAzyme adsorbed on the surface of COHN were quantified by a nanodrop experiment.²¹ The results show that 1 μg of COHN carries 8.6 × 10⁻¹² mol SUR DNAzyme, 9.3 × 10⁻¹² mol AKT1 DNAzyme and 7.9 × 10⁻¹² mol AKT1 DNAzyme (Fig. S4, ESI†).

The response of the nanocomposite to GSH was investigated. The decomposition of COHN was caused by the reduction of COHN to Co²⁺ in the presence of GSH. Fig. 1d shows UV-Vis absorption spectra of the supernatant after the reduction of COHN, with an UV absorption peak of Co²⁺ at 510 nm compared with the COHN. The data reveals that COHN was reduced to generate Co²⁺ in vitro. Compared with untreated MCF-7 cells, the cobalt content in the MCF-7 cells treated with COHN show a 5.6-fold increase (Fig. S5, ESI†). These results reveal that COHN would be reduced by GSH within living cells. In confocal fluorescence images (Fig. S6a–f, ESI†), MCF-7 cells treated with the AKT1 (RhB)–COHN nanocomposite show stronger fluorescence signals in the cytoplasm than those treated with free DNAzymes, demonstrating that the COHN can efficiently deliver DNAzymes into the cells. Further studies were performed in MCF-10A cells, as shown in Fig. S7a–f (ESI†); the cells incubated only with AKT1 (RhB)–COHN nanocomposite show faint fluorescence. The results clearly confirm that high levels of GSH²² can be applied to selectively release DNAzymes from COHN in MCF-7 cells better than in MCF-10A cells. Fig. S8 (ESI†) shows that the cobalt contents in the MCF-7 cells treated with 10, 20 and 40 μg mL⁻¹ COHN are 0.237, 0.538 and 0.782 pg per cell respectively. The result confirms that the nanocomposite successfully entered the cells.

Once the DNAzymes were internalized, the Co²⁺ generated by GSH can catalyze 10–23 DNAzymes to cleave relevant mRNAs. To optimize the dosage of the DNAzymes–COHN nanocomposite, a quantitative real-time polymerase chain reaction (RT-PCR) was used to analyze the expression of three mRNAs in MCF-7 cells treated with different concentrations of nanocomposites. As shown in Fig. S9 (ESI†), the expression of TK1, SUR and AKT1 mRNAs of the cells treated with more than 10 μg mL⁻¹ DNAzymes–COHN was only modestly decreased. Thus, 10 μg mL⁻¹ of DNAzymes–COHN was chosen for the following experiments. GO and COHN were chosen as vectors to investigate Co²⁺ catalyzed DNAzymes cleaving mRNAs in vivo. In Fig. S10 (ESI†),
compared with MCF-7 cells incubated with DNAzymes–GO, the expression of TK1, SUR and AKT1 mRNAs in MCF-7 incubated with DNAzymes–COHN was decreased. The results indicate that the DNAzymes–COHN can cleave mRNAs in living cells. Next, the reduction in TK1, SUR and AKT1 mRNAs expression level following treatment with nanocomposite in MCF-7 cells was evaluated. Fig. 2a shows that TK1, SUR and AKT1 mRNAs expression in MCF-7 was decreased to 25.88%, 20.88% and 15.82%, respectively. However, the expression of TK1, SUR and AKT1 mRNAs in MCF-7 cells incubated with equivalent quantities of free DNAzyme, control DNA, and control DNA–COHN was not obviously different. Western blot was performed to verify the impact of the TK1, SUR and AKT1 silencing on the protein level, as shown in Fig. 2b. This further confirms that the TK1, SUR and AKT1 protein expression were significantly down-regulated, which is consistent with RT-PCR.

Cell migration, invasion and proliferation were studied using wound-healing assays, cell invasion assays and an MTT assay. In the wound-healing assays, it was found that the wound of the control group was almost closed (~100%), while cells separately treated with SUR–COHN, AKT1–COHN or TK1–COHN exhibited partial closure of the scratched region (42%, 47% and 45%). Notably, the wound of cells treated with DNAzymes–COHN was wider than the scratched region of other samples (~29%). Similar effects were observed in the cell invasion analysis, and cells treated with DNAzymes–COHN displayed a minimum quantity of invasive cells. The results demonstrate that the cells treated with DNAzymes–COHN had a greater capability of inhibiting tumor migration and invasion. We then proceeded to examine the therapeutic efficacy of the DNAzymes–COHN via an MTT assay. In the MTT assay, the viability of the DNAzymes–COHN nanocomposite treated cells was much lower than other samples under the same conditions. However, a slight growth inhibition in MCF-10A cells was observed, and the inhibition of MCF-10A cells after incubation with the concentration of COHN less than 40 μg mL⁻¹ was negligible. Thus, the DNAzymes–COHN nanocomposite-treated cells showed a significant decrease in proliferation of the MCF-7 cells compared with other samples. When the concentration of COHN was less than 40 μg mL⁻¹, it slightly inhibited the growth of normal cells. Significantly increased caspase-3 expression was found due to apoptosis of MCF-7 cells.

In immunofluorescence assays, the activity of caspase-3 was detected when MCF-7 cells were treated with the DNAzymes–COHN nanocomposite. The production of caspase-3 further confirmed the apoptosis of MCF-7 cells by simultaneously silencing TK1, SUR and AKT1 genes. The in vivo therapeutic efficacy of the DNAzymes–COHN nanocomposite was subsequently assessed in subcutaneous MCF-7 cells of tumor-bearing mice. We intratumorally administered DNAzymes–COHN (0.75 mg mL⁻¹, 40 μL) and compared this with equivalent amounts of PBS and DNA–COHN as controls. After two weeks, the tumors were formed and grew rapidly in the PBS and control DNA–COHN groups, but extremely slowly in the DNAzymes–COHN treated group due to the multiple gene silencing. As is shown in Fig. 4b, the change in the tumor volume of the mice treated with DNAzymes–COHN is smallest, whereas the tumor sizes of the mice treated with PBS and control DNA–COHN were found to increase about 6-fold and 12-fold during the course, respectively. The body weights of all treatment groups did not show any noticeable change over the period of the therapy, suggesting that the treatments were nontoxic. According to the histological analysis, the images of hematoxylin and eosin (H&E)-stained tumor tissue showed large-scale tissue damage after the mice were treated with DNAzymes–COHN. In contrast, only little tissue damage was observed in the tumor tissue of the mice treated with PBS and control DNA–COHN. Additionally, H&E stained images of the major organs showed no significant tissue damage or

Fig. 2 (a) PCR analysis and (b) Western blot analysis of TK1, SUR and AKT1 expression in MCF-7 cells treated with control DNA, control DNA–COHN, DNAzyme or DNAzymes–COHN for two days.

Fig. 3 (a) Wound-healing assay of cells treated with SUR–COHN, AKT1–COHN, TK1–COHN or SUR + AKT1 + TK1–COHN at different time points after wounding. The black arrows indicate the wound edge. (b) A chamber invasion assay was conducted in MCF-7 cells with different treatments. Cell viability of MCF-7 (c) and MCF-10A (d) treated with different samples for 48 h.
generated Co\(^{2+}\) ions can be served as cofactors of 10–23 DNAzymes genes in living cells and three kinds of DNAzymes, which can silence three different and synthesis of a nanocomposite based on CoOOH nanoflakes normal tissue at experimental doses. The CoOOH nanoflakes can be reduced to Co\(^{2+}\) ions in the presence of cellular GSH. The † (Fig. S14, ESI†) These preliminary results suggested that DNAzymes–COHN nanocomposites were not obviously toxic to normal tissue at experimental doses.

In conclusion, we have reported a new strategy for the design and synthesis of a nanocomposite based on CoOOH nanoflakes and three kinds of DNAzymes, which can silence three different genes in living cells and \textit{in vivo}. The CoOOH nanoflakes can be reduced to Co\(^{2+}\) ions in the presence of cellular GSH. The generated Co\(^{2+}\) ions can be served as cofactors of 10–23 DNAzymes for gene knockdowns. The nanocomposite possesses significant biostability, high resistance to nuclease cleavage, high selectivity and excellent biocompatibility. The nanocomposite can simultaneously cleave three different tumor-related mRNAs in living cells and perform outstandingly towards the inhibition of cancer cells’ migration, invasion and proliferation compared to the traditional single gene silencing method. Moreover, the formation of a tumor can be enormously inhibited in a mouse model. We anticipate that this approach can provide new routes for developing multiplexed gene silencing platforms for cancer therapy.

This work was supported by 973 Program (2013CB933800), National Natural Science Foundation of China (21390411, 21535004, 21422505, 21375081, 21505087), and Natural Science Foundation for Distinguished Young Scholars of Shandong Province (JQ201503).

Notes and references