Sequential co-delivery of miR-21 inhibitor followed by burst release doxorubicin using NIR-responsive hollow gold nanoparticle to enhance anticancer efficacy

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ABSTRACT

Previous literature and our study showed the delivery sequence of microRNA inhibitor and chemotherapeutic compounds achieve distinct therapeutic anticancer efficacy. Yet, it is challenging to use nanoparticle to achieve sequential drug delivery. In the current study, we designed sequential co-delivery system using a near-infrared-radiation (NIR) responsive hollow gold nanoparticle (HGNPs) to achieve sequential release of microRNA inhibitor (miR-21i)/doxorubicin(Dox) in order to achieve synergistic efficacy. PAMAM modified HGNPs was used to encapsulate miR-21i and Dox. Upon entering tumor cells, miRNA-21i was released first to sensitize the cancer cells, the subsequent burst release of Dox was achieved by NIR triggered collapse of HGNPs. This sequential delivery of miRNA-21i and Dox produced a synergistic apoptotic response, thereby enhancing anticancer efficacy by 8-fold and increasing anti-cancer stem cell activity by 50-fold. The sequential delivery of miR-21i and Dox using HGNPs under NIR after intravenous administration showed high tumor accumulation and significantly improved efficacy, which was 4-fold compared to free Dox group. These data suggested that the sequential co-delivery of miR-21i followed by burst release Dox using NIR-responsive HGNPs sensitized cancer cells to chemotherapeutic compound, which provided a novel concept for co-delivery miRNA inhibitors and chemotherapeutic compounds to enhance their efficacy.

Keywords

Co-delivery; Controlled sequential release; MicroRNA-21; Doxorubicin; Gold nanoparticle

1. Introduction

Since cancer cell growth is regulated by multiple signaling pathways, inhibition of one particular targeting molecule or single pathway is inadequate to eliminate cancer cells [1, 2]. Therefore, co-delivery of multiple drug components using nanoparticle, which simultaneously blocks multiple oncogenic pathways, provides advantages in cancer therapeutics [3, 4]. The roles of microRNAs (miRNAs) in the regulation of tumor progression and drug resistance have been widely recognized, thus miRNA-based therapies, such as miRNA-21 (miR-21), renders cancer cells more sensitive towards the chemical treatments, thereby broadly promoting the response to the chemotherapy [5, 6]. Increasing studies have demonstrated that co-delivery of miRNA/chemotherapeutic compounds had synergistic cell killing efficacy against cancer cells [7, 8].

In addition, recent clinical investigation has suggested that cancer is highly heterogeneous, which consists of differentiated cancer cells and cancer stem cells (CSCs) [9]. Conventional chemotherapy shrinks the bulk tumor mass, but fails to eliminate CSCs, which may be a cause for recurrence and metastasis [10, 11]. Previous study proved that high expression of miR-21 was required for maintenance of the pluripotency of CSCs, which was associated with drug resistance [12, 13]. Therefore, combination of miRNA gene therapy and chemotherapeutic compounds provided a novel approach to eliminate both cancer cells and CSCs [14, 15].

It has been noted that sequential delivery of miRNA and anti-cancer agents is critical to rewire cancer cells to exhibit sensitization effect to chemotherapeutic compounds, while simultaneous co-delivery of these agents failed to show synergistic efficacy [16, 17]. Our previous study also demonstrated that, pretreatment cancer cells with miRNA-21 inhibitor (miR-21i) for 4h followed by temozolomide treatment resulted in a far more synergistic effect, while co-delivery, reverse sequence or other dosing interval achieved additive or even antagonistic effect [18]. In addition, an interval of 4h administration exhibited the best killing efficacy for miR-21i plus doxorubicin (Dox) therapy in breast cancer (Figure S1). Therefore, it is critical to develop a drug delivery to precisely control the release sequence of miR21i and chemotherapeutic compound in order to achieve synergistic efficacy. The sequential delivery system will release miR-21i first to sensitize the cancer cells, and subsequent release of chemotherapeutic compounds will show better efficacy to eliminate both cancer cells and CSCs.

In this study, we designed sequential co-delivery system using a near-infrared-radiation (NIR) responsive hollow gold nanoparticle (HGNPs) to co-deliver miR-21i/Dox, termed as D-P-HGNPs/21i (Fig.1). HGNPs were employed as they stable, biocompatible, having plasmon absorption in the NIR region. In particular, the hollow interior of HGNPs increased drug payload compared with solid gold nanoparticle with same surface charge and weight [19]. As illustrated in Figure 1, this designed nanocarrier was fabricated by modifying HGNPs with thiolated PAMAM dendrimers through the strong Au-S linkage (Step I). Then, the

positive-charged Dox was successfully absorbed onto the negatively charged surface of HG NPs (Step II). Finally, miR-21i was condensed to PAMAM on HG NPs through the electrostatic interactions (Step III). D-P-HG NPs/21i entered the cells through the endocytic pathway. Upon entering the tumor cells (Step IV), miR-21i escaped from the endosome and released into the cytoplasm (Step V), due to the proton sponge mechanism of PAMAM dendrimer [20, 21], modulating the intrinsic state of cancer cells and CSCs to a more chemosensitive state (Step VI). In four hours, application of NIR triggered collapse of HG NPs (Step VII), thereby realizing a burst release of Dox (Step VIII). The efficacy of the sequential co-delivery miR21i/Dox system was tested to eliminate cancer cells and CSCs in vitro and in vivo. The data suggested that sequential release of miR-21i followed by burst release of Dox, not the simultaneous co-administration, produced a synergistic effect to exhibit a superior anti-cancer efficacy.

Fig.1. Schematic illustration of gold nanoparticle-based, NIR triggered sequential miR-21 inhibitor/Dox release with precisely time interval for optimal combination therapy. (I-III) Formation of HG NPs based co-delivery system. D-P-HG NPs/21i entered cells through endocytosis (IV). Upon entering the tumor cells, miR-21i was released first (V), modulating the intrinsic state to a more chemosensitive state (VI). At the desire time, application of NIR laser triggered collapse of HG NPs and a burst release of doxorubicin (VII), activating two apoptosis signaling pathway, thereby inducing the synergistic apoptosis response (IX).

2. Materials and methods

2.1. Materials and Cell lines.

Gold chloride trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$), poly amidoamine (G5-PAMAM), Dox were purchased from Sigma-Aldrich. The breast cancer cell lines MDA-MB-231 and MCF-7 were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) with high glucose, 10% v/v fetal bovine serum (FBS), 1% v/v penicillin/streptomycin. MCF-10A cells were cultured in DMEM medium supplemented with 10% FBS, 10ng/mL epidermal growth factor (EGF, Sigma-Aldrich), 500ng/mL hydrocortisone (Sigma-Aldrich), and 10 μ g/mL insulin (Sigma-Aldrich). MDA-MB-231 CSCs were cultured in serum-free DMEM/F12 (1:1) medium containing 2% B27 (Gibco-BRL), 20 ng/ml EGF and 20 ng/ml basic fibroblast growth factor (bFGF; Sigma-Aldrich).

2.2. Synthesis of hollows gold nanoparticle (HGNPs).

HGNPs were prepared as described previously [19]. Silver seed nanoparticles were prepared by reducing a well-stirred solution of 0.1 mL of 100mM AgNO_3 with 0.6 mL of 1.0 M NaBH_4 in the presence of 0.5 M sodium citrate in deionized water. The solution was stirred for several hours to allow NaBH_4 to fully hydrolyze. Larger silver nanoparticles to be used as sacrificial templates for the HGNPs were grown from the silver seed solution by adding 0.6 mL of 2.0 M $\text{NH}_2\text{OH HCl}$ and 150 μ L of 0.1M AgNO_3 and stirring overnight. Galvanic replacement of silver with gold was performed by quickly mixing 300 μ L HAuCl_4 with the silver nanoparticles at 60°C. The solution was cooled to room temperature and kept in the dark for at least several days before use. The concentration of HGNPs was estimated by the previously reported method [22].

2.3. Synthesis of PAMAM modified HGNPs (P-HGNPs).

Thiol-terminated PAMAM dendrimer was synthesized via ring opening reaction, based on previous reports [23]. Briefly, 2mM 2-iminothiolane was added to the G5-PAMAM solution. The mixture was reacted with stirring for 2 h to complete the formation of the thiol-terminated dendrimer. The residual 2-iminothiolane was removed by dialysis. HGNPs (2mL, 6×10^{10} particle/mL) and thiol-terminated dendrimer aqueous solutions (0.5mL, 1% w/v) were mixed and incubated for 24 h at room temperature to form stable aqueous solutions.

The average number of PAMAM conjugated onto the HGNPs was determined using a fluorescamine assay [24]. Briefly, fluorescamine (Sigma-Aldrich) was first dissolved in anhydrous dimethyl sulfoxide (DMSO) to make a 3 mg/mL stock solution. PAMAM dendrimer were then diluted with 0.1 M phosphate buffer (pH=7) to make a series of solutions; pipette 100 μ L of each solution to an opaque 96-well plate, respectively. 30 μ L of the fluorescamine solution was then added into each well; the plate was then incubated for 1 h at room temperature. After the incubation, fluorescence intensity ($\text{Ex}=360$ nm, $\text{Em}=465$ nm) was read with a plate reader. The

number of residual free amino group was then estimated by comparing the fluorescent intensity of the native PAMAM.

2.4. Dox loading

Aliquots of free Dox in double distilled water (DDW, 1.0 mM, 100 μ L) were added into an aqueous solution of P-HGNPs (6×10^{10} particle/mL, 0.5mL), and the mixtures were stirred at room temperature for 24 h. After centrifugation (14,000 rpm for 20 min), the precipitate was washed with DDW and centrifuged, and the washing cycle was repeated for six times. The final drug concentration was determined by high-performance liquid chromatography (HPLC).

2.5. Evaluation of the drug release

To determine the kinetics of drug release behavior, 2 mL Dox-loaded P-HGNPs (D-P-HGNPs) was placed in a dialysis bag (molecular mass cut off 1000 Da) and incubated in 150mL of phosphate-buffered saline buffer (PBS, pH7.4 or pH5.8) at 37°C at the stirring speed of 100 ± 5 rpm.

A femtosecond laser system (Tianjin Biotech) was used to trigger the Dox release. The laser pulses were guided through an automatized attenuator and a polarizing beam splitter. The generated laser pulses were fixed at the wavelength of 964nm, with a total fluence rate of 2.0 W/cm². After exposure to laser for 120s, D-P-HGNPs was centrifuged at 14,000rpm for 15min at predetermined time points, and supernatant was collected. The content of drug released was fluorimetrically determined using reverse-phase HPLC with a C18 column, with acetic acid, methanol, and 10 mM Na₂HPO₄ (13:19:68, vol/vol/vol, pH 4.0) as the eluant solution. And the detection wavelength was set at 480 nm.

2.6. D-P-HGNPs/miR-21inhibitor assembly.

The 2'-O-methyl (2'-OMe) miR-21 inhibitors (miR-21i) were chemically synthesized by Shanghai Gene Pharma, as miR-21i was specially binding with the mature miRNA-21 in base complementary manner, thereby blocking the miRNA mediated downstream inhibition of mRNA translation. 2'-OMe oligos were composed entirely of 2'-O-methyl bases and had the following sequences: miR-21 inhibitor: 5'-GTCCAC TCT TGT CCT CAA TG-3'; scrambled sequence: 5'-AAG GCA AGC UGA CCC UGA AGU-3'. The oligonucleotides were purified by a high-pressure liquid chromatography system, dissolved in diethylpyrocarbonate (DEPC)-treated water, and frozen at -20°C. MiR-21i (20 μ M) was added to vials containing appropriate amounts of D-P-HGNPs solution, and then incubated at room temperature for 20 min. A series of miR-21i bound D-P-HGNPs (D-P-HGNP/21i) were formed at different N/P (the number of PAMAM terminal amino groups in the D-P-HGNPs to the number of phosphate groups in the miR-21i) ratios. The condensation ability of D-P-HGNPs was assessed by agarose gel electrophoresis. Electrophoresis was carried out on a 1% agarose gel with a current of 80 V for 15 min in a Tris-acetate-EDTA (TAE) buffer solution (40mM Tris-HCl, 1% v/v acetic acid, and 1 mM EDTA). The

migration of the complexes was visualized by staining with ethidium bromide. The images were acquired with a Gel Doc system.

2.7. *Disassociation of D-P-HGNP/21i*

Chicken egg white lysozyme (hydrochloride form) was purchased from Sigma-Aldrich. The disassociation of D-P-HGNP/21i was carried out by adding 150 μ g/mL lysozyme at 37 °C for 1min.

2.8. *Cell transfection and cellular distribution in vitro*

2.8.1. *Cell transfection*

After seeding cells onto 6-well plates at a density of 3×10^5 /well for 24 h in DMEM containing 10% FBS, cells were starved for 4 h at 37°C before transfection. For D-P-HGNPs/21i group, 400 μ L D-P-HGNPs (6×10^{10} particle/mL, with the drug concentration 0.2 μ g/mL) was mixed with 5 μ L miR-21i (20 μ M) at a N/P ratio of 4:1 in DMEM culture medium and incubated for 15 min at room temperature for transfection. For lipofectamine 2000 (Invitrogen) group, 10 μ L lipofectamine 2000 incubated with 5 μ L miR-21i (20 μ M) for 15min in DMEM culture medium.

Co-administration therapy: Cells was transfected with D-P-HGNPs/21i, followed by immediate NIR laser exposure at 2.0 W/cm² for 2min, designated as D-P-HGNP/21i-R.

Sequential release therapy: Cells was transfected with D-P-HGNPs/21i for 4h, followed by NIR laser exposure at 2.0 W/cm² for 2min, designated as D-P-HGNP/21i-4R.

2.8.2. *Cellular distribution*

To investigate the cellular fate of the co-delivery system, the P-HGNPs were first labeled with an amine reactive dye, Cy5.5 (GE Healthcare). Briefly, P-HGNPs (6×10^{10} particle/mL) was reacted with Cy5.5 (5mg/mL) in a pH 7.4 PBS at a volume ration of 1: 100. The reaction was allowed overnight at room temperature by avoiding light. Excess dye molecules were removed by centrifugation filtration through 3 kDa MWCO Amicon filters and washed away with water for over 8 times until no noticeable color in the filtrate solution.

FITC-labeled miR-21i was chemically synthesized by Shanghai Gene Pharma, and Dox was loaded on Cy5.5 labeled P-HGNPs. After cell transfection for 1h or 4h, drug/miR-21i distribution was observed by fluorescence microscopy FV-1000.

The ability of D-P-HGNPs to transfer miR-21i into cancer cells were detected by fluorescence activated cell-sorting (FACS) analysis. Both MDA-MB-231 and MCF-7 cells were treated with D-P-HGNPs/21i-FITC for various time. Cells were trypsinised and washed three times with PBS. Then, the FITC fluorescence was detected by Verse flow cytometer (Becton Dickinson).

2.9. MiR-21 real-time PCR

Total RNA was extracted from cells using Trizol reagent (Invitrogen). The real-time PCR was carried out with the mirVana qRT-PCR miRNA detection kit (Ambion) in a 10 μ L reaction, comprising 2 μ L of mirVana, 5 $\times$ RT buffer, 1 μ L of mirVana, 1 $\times$ RT primer, 25ng of total miRNA, 0.4 μ L of ArrayScript enzyme mix, and DDW up to 10 μ L. Amplification reaction was performed using 7500 fast real-time PCR (Agilent) and the protocol was performed for 40 cycles at 95 $^{\circ}$ C for 3 min, 95 $^{\circ}$ C for 15 s, and 60 $^{\circ}$ C for 30 s. Both RT and PCR primer were purchased from Ambion. Relative quantification was conducted using amplification efficiencies derived from cDNA standard curves and obtained relative gene expression. U6 was used as the internal control. Data are presented as fold changes ($2^{-\Delta\Delta Ct}$) and were analyzed using Opticon Monitor Analysis Software V 2.02 (MJ Research).

2.10. Cytotoxicity assay.

Cells were seeded at 10^4 /well in 96-well cell culture plates. Cell viability was tested using a cell counting kit 8 (CKK-8). After 48 h of incubation with Dox, D-P-HGNPs/21i-R, D-P-HGNPs/21i-4R (with the drug concentration at 0.2 μ g/mL), the medium was removed, and fresh medium with 10% CKK-8 was incubated for 4 h. The absorbance at 450 nm was read using a microplate reader.

2.11. Analysis of the miRNA/chemotherapeutics combination treatment.

To analyze the combinatorial effect between the miR-21 inhibitor and Dox, the Jin method was used [25]. This method provides a “Q” value, allowing the effect of combining of two drugs to be classified as an antagonistic ($Q < 0.85$), an additive ($0.85 < Q < 1.15$), or a synergistic ($Q > 1.15$). The formula is $Q = Ea + b / (Ea + Eb - Ea \times Eb)$, where $Ea + b$, Ea and Eb are the average effect of the combination treatment, miR-21 inhibitor alone and Dox alone, respectively.

2.12. Evaluation of cell apoptosis

To quantify sequential release therapy induced apoptosis, Annexin V/PI staining was performed and further evaluated by FACS. Briefly, after various treatments, cells were collected and subjected to 5 μ L Annexin V and PI staining for 10min using an Annexin V-FITC Apoptosis Detection Kit (Becton Dickinson), according to the manufacturer’s protocol. The resulting fluorescence was measured by Verse flow cytometer (Becton Dickinson).

2.13. Subcellular distribution of cytochrome c.

Cells were labeled with MitoTracker green (Molecular Probes) for 30 min at 37 $^{\circ}$ C, then fixed with 4% paraformaldehyde for 10 min, followed by blocking with 10% bovine serum albumin (BSA) for 1 h. Sections were incubated with cytochrome c (ABcam) at a 1:1,000 dilution overnight. The secondary antibodies used were

Alexa-647-conjugated goat anti-mouse at a 1:300 dilution (Molecular Probes). The nuclear dye Hoechst 33342 was used at a 1:1,000 dilution.

2.14. Protein extraction and western blot analysis

Cell lysates were separated in an analytical 10% SDS- PAGE gel and transferred onto the polyvinylidene fluoride (PVDF) membrane (Invitrogen), followed by blocking in PBS with 5% bovine serum albumin (BSA) for 1 h. Sections were incubated with primary antibodies overnight. Primary antibodies were diluted in PBS with 1% BSA: p53, Bax, Bcl-2, cleaved caspase-3, cleaved caspase-8, cleaved caspase-9, AIF, and FasL (Cell Signaling) at 1:1000 dilutions, Cytochrome c (ABcam) at 1:1000 dilutions. Rabbit or mouse IgG antibody coupled with horseradish peroxidase was used as a secondary antibody (Santa Cruz). The density of the immunoblots was determined by Image J analysis software.

2.15. Animal studies.

All animal studies were approved by the Tianjin Medical University Animal Care and Use Committee and performed in accordance with national guidelines and regulations. The subcutaneous MDA-MB-231 xenograft mouse model was used to evaluate the therapeutic efficacy of different treatments. When tumor volume reached 400 mm³, mice were intravenously administrated with PBS, HG-NPs-R, Dox, D-P-HG-NPs/21i, D-P-HG-NPs/21i-R, or D-P-HG-NPs/21i-4R, at the dose of 1 mg/kg Dox equivalent, (n = 6-8). The treatment was given every two days for two weeks.

Tumor illumination was performed vertical to the largest protuberance of the tumor. The tumor was situated in the center of the light cone. The area to be irradiated was chosen with a peritumoral distance of healthy tissue of 0.5 cm. Tumor tissues were irradiated at 2.0 W/cm² for 3min immediately after D-P-HG-NPs/21i-R injection or 4h after D-P-HG-NPs-21i-4R injection.

2.16. Statistical Analysis.

All experiments were repeated at least three times. Data are presented as mean $\pm$ standard deviation. Statistical analysis was conducted using variance tests (ANOVA). Data sets were compared using two-tailed, unpaired t-test. *P* value of < 0.05 was regarded statistically significant.

3. Results

3.1. Formation of gold nanoparticle based sequential miR-21i/Dox release system

The sequential drug delivery system was based on hollow gold nanoparticle (HGNPs). As sphere shaped gold nanoparticle with much larger size possessed longer half-life of cellular uptake [26]. The PAMAM dendrimer was selected to modify HGNPs because it could form a stable complex with nucleic acid by electrostatic interaction. Also, PAMAM had the ability to escape from endosome via a process called the proton sponge effect [20, 21]. Transmission electron microscopy (TEM) images indicated that the HGNPs exhibited small, spherical, narrowly dispersed structure (Fig. 2A), with an average diameter of 61.3 ± 1.2 nm, as determined by dynamic light scattering (DLS) (Fig. 2B). Thiol-modified PAMAM dendrimer (PAMAM-SH) was successfully conjugated to HGNPs as the result of the Au-S absorption effect. P-HGNPs showed an increased in zeta potential from -14.64 to +14.59 mV after PAMAM modification (Figure S2). The average number PAMAM dendrimer conjugated to per gold nanoparticle was 10, as determined by a fluorescamine assay [22, 24].

Dox was effectively loaded on P-HGNPs to form nanoparticles at 91.6 ± 2.7 nm, designated as D-P-HGNPs (Fig. 2B). The amino group of Dox was the key for its high payload on to the HGNPs [19]. And approximately 7% surface of the HGNPs was occupied by PAMAM dendrimer according to the fluorescamine assay, thus P-HGNPs was efficient for drug loading. The color of the P-HGNPs changed from light blue to rose upon absorption of Dox (Fig.2C inset). UV-vis spectrum demonstrated that a broad NIR plasmon absorption peak at 964 nm characteristic of HGNPs, and a weak absorption peak at 480 nm characteristic of Dox, confirmed the loading of Dox to P-HGNPs (Fig.2C). The appearance of characteristic absorption peaks of nitrogen in the energy dispersive spectroscopy (EDS) further indicated the successfully drug loading (Figure S3).

The amount of Dox incorporated into P-HGNPs was 32.1%, as determined by high-performance liquid chromatography (HPLC) analysis. According to the high resolution TEM images (Fig.S4A), the shell of HGNPs was porous, with an average thickness of 4nm, which made it possible for drug molecules to diffuse into the core and bind to the inner surface of HGNPs. We also compared the drug loading efficiency between HGNPs and solid gold nanoparticle (AuNPs) having similar size. It was estimated that HGNPs should have three times payload of AuNPs if all Dox molecules were just coated on the outer surface of HGNPs. As shown in Fig.S4B, HGNPs reached a 4.8-fold increase drug payload compared to that in AuNPs, suggesting that the both the inner and outer surface of HGNPs were coated with Dox, which may explain the high drug loading capacity of HGNPs.

According to the drug release profile (Figure S5), only less than 15% Dox was release from D-P-HGNPs in PBS at pH 7.4 or cell culture medium containing 10% FBS after 2 days incubation without NIR excitation, indicating that Dox was stably

absorbed to P-HGNPs. As shown in Fig.S6, there were no significant changes in the particles size during the 7 day interval, as determined by DLS measurements, which further confirmed the stability of our delivery system.

Fig.2. Synthesis and characterization of HGNPs-based precisely scheduled gene/drug release system. (A) TEM images of HGNPs, D-P-HGNPs, D-P-HGNPs with NIR laser (D-P-HGNPs-R). (B) Particle size measured by DLS.(C) UV-vis absorbance spectra of free Dox (red), P-HGNPs (blue), and D-P-HGNPs (rose). (D) Gel mobility shift assay of D-P-HGNPs/21i at different N:P ratios. Lane M: Marker. Lane 1: naked miR-21i. Lane 2-3: various N:P ratios with D-P-HGNPs/21i. (E) Disassociation of D-P-HGNPs/21i complexes at a N:P ratio of 4:1 (lane 2), after digestion with lysozyme (Lane 3). Lane 1: naked miR-21i. (F) Fluorescence absorption of Dox, D-P-HGNPs and D-P-HGNPs-R at 580nm, with equal Dox concentrations. (G) Dox

release profiles in the presence and absence of NIR laser. (H) Dox release profile at pH7.4 and pH5.8 PBS in the absence or presence of laser radiation.

MiR-21i was bounded to D-P-HGNPs via electrostatic interaction. To investigate the ability of miR-21i to complex with D-P-HGNPs, we performed electro mobility shift assays on 1% agarose gels. As shown in Figure 2D, the free miR-21i band at 22bp (lane 1) was barely visible when combined with D-P-HGNPs above the N:P ratio of 4:1 (lane 5), indicating that miR-21i has strong affinity for D-P-HGNPs. Following digestion with lysozyme, the miR-21i was released from the D-P-HGNPs, as evident from the bright band at 22bp (Fig.2E, lane 3).

The NIR triggered drug release was confirmed by the fluorescence spectrum. As shown in Figure 2F, the fluorescence emission of free Dox peaked at 580nm, while the fluorescence of Dox was quenched in D-P-HGNPs, probably due to fluorescence resonance energy transfer (FRET) [27]. Fluorescence at 580nm reappeared after D-P-HGNPs exposure to the NIR laser, indicating the release of drug. Moreover, D-P-HGNPs undergo shape transformation and size decreased after absorption of NIR laser, which might further facilitate the drug released (Fig.2A, 2B) [28].

The controlled release behavior of D-P-HGNPs was further tested in cell-free solutions. After an initial release of 7-10% over the first 4 hours, no further release of Dox from D-P-HGNPs was observed, demonstrating the stable incorporating capability of D-P-HGNPs (Fig.2G). A burst release of drug (from 4.1% to 30.3%) occurred within 15 min when the suspension of D-P-HGNPs was exposed to a NIR laser at 2.0 W/cm² output power. Only a slow release of approximately 5% of Dox was detected between 4h to 16 h, indicating only a small quantity of drug was release when laser was switched off. Little drug release was detected during the second and third NIR laser treatment cycle (Figure S7). These data demonstrate that Dox release from D-P-HGNPs could be triggered by the external NIR laser. At pH5.8, which mimics the acidic environment of tumor sites, only 18% drug was release during the 16h, further confirmed drug stability of D-P-HGNPs (Fig.2H). The cumulative drug release increased from 15.1% to 51.4% with NIR exposure at 2.0 W/cm² output power, indicating that Dox could be triggered by NIR at the desired time.

3.2. Effect of D-P-HGNPs/21i mediated programmed release at cellular level

Next, sequential drug release behavior of D-P-HGNPs/21i was evaluated in two breast cancer cell lines, MDA-MB-231 cells and MCF-7 cells. Dox was loaded on Cy5.5 labeled P-HGNPs, which was further conjugated to FITC-labeled miR-21i. As shown in Figures 3A, after incubating 1h with D-P-HGNPs-Cy5.5/21i-FITC, confocal microscopy revealed dissociation between FITC and Cy5.5 fluorescence signals, suggesting miR-21i was quickly released from the D-P-HGNPs to the cytoplasm. Meanwhile, minimal red fluorescence at 580nm was observed, indicating little Dox leakage. When exposed to NIR laser, increased accumulation of fluorescence signal at 580nm was detected in nucleus, suggesting release of Dox. A similar pattern was observed in MCF-7 cells (Fig.3B). When the incubation time was extended to desired

time interval, only weak fluorescence indicative of Dox release could be observed in D-P-HGNPs treatment 4h without NIR exposure (Fig.3C, 3D). These data demonstrate that our delivery system could achieve the precisely sequential gene/chemotherapeutics release at cellular level, with miR-21i being released first, followed by a controlled release of Dox at the desired time in response to NIR exposure.

Fig.3. D-P-HGNPs/21i controlled gene/drug release in a precisely scheduled manner at the cellular level. Intracellular distribution of miR-21i (20 μ M, green) and Dox (0.2 μ g/ml red,) after incubation with D-P-HGNPs-Cy5.5/21i-FITC (6×10^{10} particle/mL, orange) for 1h in MDA-MB-231(A) and MCF-7 cells (B).When the incubation time extended to 4h, only weak red fluorescence could be observed in

MDA-MB-231(C) and MCF-7 cells (D), without NIR exposure. (E) Uptake of the miR-21i analyzed by FACS. (F) Real-time PCR analysis detected relative miR-21 expression levels. All of the bar size was 20 μ m.

Specific miR-21 inhibition is critical to enhance the drug sensitivity and impair the stemness of CSCs. Thus, we also evaluated the gene transfection efficiency of the D-P-HGNPs in both cell lines (Fig.3E). Cells were incubated with FITC-labeled miR-21i. FACS analysis showed that D-P-HGNPs facilitated efficient gene delivery in both cell lines. Our previous study revealed that naked miR-21i couldn't enter the cells [29]. FITC positive cells reached almost 100% within 4h post-transfection. To further confirm the transfection efficiency of D-P-HGNPs, suppression of miR-21 expression was measured by real-time PCR. As shown in Figure 3F, miR-21 expression was reduced by 60% after treatment with D-P-HGNPs, which showed similar transfection efficiency to that of commercially available transfection reagent lipofectamine 2000. These data further indicate that D-P-HGNPs provided efficient delivery of miR-21i at cellular levels.

3.3. Cytotoxicity of D-P-HGNPs/21i based sequential release system

The killing efficacy of sequential miR-21i/Dox treatment was first tested in breast cancer cells. In order to avoid the photothermal effect of gold nanoparticle, the laser setup we used in this study is operating with a femtosecond laser system. No significantly variation of temperature was detected after NIR exposure (Figure S8), further confirmed the cell killing effect is not due to the thermal effect.

Free Dox has an IC₅₀ value of 1.1 μ g/mL in MDA-MB-231 cells (Fig.4A). The co-administration therapy D-P-HGNPs/21i-R, appeared to enhance drug efficacy (IC₅₀: 0.27 μ g/mL). In the sequential D-P-HGNPs/21i-4R treatment, the survival rate of cancer cells was further reduced, resulting in a total of 8-fold decrease in IC₅₀ values (0.13 μ g/mL) for MDA-MB-231 cells (Fig.4B). D-P-HGNPs/21i-4R sequential therapy also decreased the IC₅₀ value by 12 folds compared to free drug treatment in MCF-7 breast cancer cells (Figure S9). These results suggested that the sequential therapy achieved better synergistic efficacy.

Fig.4. D-P-HGNPs/21i based sequential release therapy enhanced cytotoxicity compared to simultaneously co-administration therapy. (A) IC₅₀ value of MDA-MB-231 cells treated with free Dox, D-P-HGNPs/21i-R and D-P-HGNPs/21i-4R. (B) D-P-HGNPs/21i-4R reduced the 8-fold IC₅₀ value compared with free Dox treatment. (C) Cytotoxicity of Dox, D-P-HGNPs/21i-R and D-P-HGNPs/21i-4R on MDA-MB-231 cells, at a drug dose of 0.2μg/mL. (D) Combination therapy effect analysis indicated that sequential therapy achieved a marked synergistic effect, which is more effective than simple co-administration therapy.

To further confirm the efficacy of time-staggered miR-21i/Dox release therapy, cell survival rates upon treatment with 0.2μg/ml of Dox were monitored for 5 days in MDA-MB-231 cells (Fig.4C). Dox and D-P-HGNPs/21i-R resulted in cell death initially by 29% and 45%, respectively. Meanwhile, D-P-HGNPs/21i-4R exhibited a marked increase in drug response, the cellular killing rate reaching 69%. In contrast, the control groups did not show any significant reduction in viability when cells were incubated with HGNPs with or without exposure to the NIR laser, suggesting that HGNPs were non-toxic at the cellular level.

The combination efficacy of the time-sequenced miR-21i/Dox treatment was further analyzed by using the Z.J. Jin method (Figure 4D). Briefly, this method supplied a “Q” value. The combination effect between two drugs can be classified as a synergistic effect ($Q > 1.15$), an additive effect ($0.85 < Q < 1.15$), or an antagonistic effect ($Q < 0.85$). In the 5 days period post-treatment, Q value varied from 0.76 to 1.24 in D-P-HGNPs/21i-R simple combination therapy group, suggesting that

co-administration achieved modest synergistic effect at day 5. When Dox was released 4h after nanoparticle entered the cells, the Q value of D-P-HGNPs/21i-4R reached 1.87, indicating the sequential therapy achieved a marked synergistic effect throughout the 5-day treatment. Taken together, these data indicated that time-staggered miR-21i/Dox sequentially release therapy is more effective at killing cancer cells than simple co-administration.

3.4. Synergized two apoptotic signaling pathways by sequential delivery system

The molecular mechanism of the sequential therapy was investigated. It had been reported that inhibition of miR-21 increased drug sensitivity and promoted apoptosis [30, 31]. FACS analysis showed that, comparing to the free drug, simple co-administration by D-P-HGNPs/21i-R increased apoptotic cell numbers from $7.9\pm0.8\%$ to $21.6\pm1.3\%$ in MDA-MB-231 cells, from $6.0\pm0.7\%$ to $15.8\pm1.4\%$ in MCF-7 cells (Figure 5A). D-P-HGNPs/21i-4R sequential therapy further enhanced the cell apoptosis response, reaching approximately $32.5\pm2.4\%$ and $25.5\pm1.8\%$ apoptotic cells, respectively.

Fig.5. D-P-HGNPs/21i based sequential release therapy enhanced cancer cell apoptotic response to chemotherapy. (A) Cell suspensions were stained by Annexin V-PI and measured by FACS. (B) Cytochrome c distribution in MDA-MB-231 cells. Results were further proved by spectral intensity analysis. (C) Results of western blotting indicated that both mitochondrial apoptosis and death receptor-related apoptosis pathways were activated by sequential therapy. Bar size was 20μm for all images.

Confocal fluorescence analysis of cytochrome c release further confirmed FACS results. As shown in Figure 5B, cytochrome c localized on mitochondrial in untreated cells. Co-administration by D-P-HGNPs/21i-R induced modest redistribution of cytochrome c into cytosol, a hallmark of apoptosis [32]. The sequential therapy D-P-HGNPs/21i-4R induced further dissociation of cytochrome c signals from mitochondrial. Western blotting results revealed that the sequential therapy increased the cytosolic levels of cytochrome c and apoptosis inducing factor (AIF), which further confirm the microscopy results. Furthermore, p53, Bax, cleaved caspase-3 and caspase-9, which played a central role in the mitochondrial apoptotic pathway, were dramatically increased in D-P-HGNPs/21i-4R-treated cells [33-35]. The anti-apoptotic protein Bcl-2, a downstream target of miR-21 [36], was substantially decreased, indicating activation of the mitochondrial apoptosis pathway. FasL played a central role in programmed cell death by activation of caspase-8 through the receptor-mediated pathway to trigger the classic apoptosis cascade [37, 38]. After D-P-HGNPs/21i-4R treatment, cells exhibited significantly elevated levels of FasL and cleaved caspase-8 (Fig.5C), indicating the activation of the death-receptor-mediated apoptosis pathway. Taken together, these results indicated that the sequential gene/chemotherapy achieved a marked, 8-fold increase in killing efficacy against normal cancer cells by synergizing two apoptotic signaling pathways.

3.5. D-P-HGNPs/21i based sequential release therapy enhanced drug efficacy in CSCs

Next, we tested the hypothesis that pretreated miR-21 inhibitor would impair the stemness and enhance the killing efficacy of CSCs upon the sequential therapy. The numbers of primary cancer stem cells isolated from patients were too low for this study, thus MDA-MB-231-derived stem cells were used for evaluation. As shown in Fig.6A, MDA-MB-231 cells formed stem cell spheres after two weeks of culture in suspended conditions with serum-free stem cell culture medium, consistent with previous reports [39]. Morphology change could be observed in the present of miR-21i, suggesting cell differentiation upon treatment with D-P-HGNPs/21i-R or D-P-HGNPs/21i-4R. Q-PCR revealed that stem cells expressed high level of miR-21 and Dox did not alter its levels (Fig. 6B). MCF-10A, mammary epithelial cells, expressed even lower level of miR-21 than that in MDA-MB-231 cells. Treatment with nanoparticle loaded with miR-21 inhibitors significantly decreased the levels of miR-21 in stem cells to that similar in MDA-MB-231 cells. The stemness of CSCs was monitored by the levels of three stem cell-specific transcriptional factors, Nanog, Oct4, and Sox2. As shown in Fig.6C, the sequential therapy, better than co-administration, significantly reduced the level of these three transcriptional factors. Confocal microscopy analysis showed that cancer stem cells resist cellular uptake of Dox, which partially accounted for the drug resistance (Fig.6D). The D-P-HGNPs/21i-4R mediated sequential therapy appeared to facilitate drug entry to CSCs, increasing the cellular levels of Dox in stem cells to that similar in MDA-MB-231 cells. These results indicated that the sequential therapy could impair the stemness of CSCs and dramatically improve the uptake of drugs.

Fig.6. D-P-HGNPs/21i-based sequential release therapy effectively killed CSCs. (A) DIC image of MDA-MB-231 stem cells. Morphology change could be detected in D-P-HGNPs/21i-4R treated cells. (B) MiR-21 expression in MCF-10A, MDA-MB-231 cells and CSCs. (C) RT-PCR detected the stem cell transcription markers Nanog, Oct4, and Sox2 in MDA-MB-231 cells and stem cells. (D)

Intracellular drug distribution of Dox in MDA-MB-231 cells and stem cells. (E) IC₅₀ value of MDA-MB-231 stem cells treated with Dox, D-P-HGNPs/21i-R, and D-P-HGNPs/21i-4R. (F) D-P-HGNPs/21i-4R reduced the IC₅₀ value by 50-fold compared with free Dox.

We further examined the killing efficacy of sequential therapy in CSCs by CCK-8 assays. Single Dox treatment was ineffective in killing stem cells, with an IC₅₀ value of 20.7 µg/mL, indicative drug resistance of CSCs (Fig.6E, 6F). D-P-HGNPs/21i-R treatment reduced IC₅₀ values to 1.3 µg/mL. D-P-HGNPs/21i-4R further enhanced the drug effect, resulting in a total of 50-fold decrease in IC₅₀ value (0.39 µg/mL). The killing potency of sequential therapy against CSCs, was even higher than that in free Dox treatment against normal cancer cells, (IC₅₀ of 1.1 µg/mL) (Fig. 4A). These data suggested that sequential release therapy might modulate the stemness of CSCs and rewire the intrinsic state of CSCs to a more chemosensitive form, thereby improving drug accumulation and reducing the IC₅₀ value. Taken together, sequential release therapy achieved a striking killing efficacy against CSCs.

3.6. In vivo behavior of D-P-HGNPs/21i based sequential release system

To study the *in vivo* behaviors of sequential release therapy, mice bearing tumors derived from MDA-MB-231 and MCF-7 breast cancer cell lines were administered with Cy5.5 labeled D-P-HGNPs intravenously. Our previous study revealed that majority of free Cy5.5 is excreted within 4 h by renal clearance [40]. Thus, in order to monitor the organ distribution of nanoparticles, we set to detect fluorescence signals in various animal organs at 4 h and 24 h. Strong fluorescent signals were detected in area of tumors 4 h after D-P-HGNPs-Cy5.5/21i injection (Fig.7A). Weak fluorescent signals were also observed in liver, but not in other organs, indicating D-P-HGNPs/21i displayed a superior biodistribution pattern. After 24 h, the fluorescent levels in the liver were reduced, but the signal from the tumor remained at a similar intensity, suggesting that D-P-HGNPs/21i could accumulate and be retained within tumor. Moreover, the similarly high tumor uptake was also observed in MCF-7 tumor-bearing mice (Fig.7A).

Fig.7. *In vivo* behavior of D-P-HGNPs/21i based sequential release therapy. (A) Distribution of D-P-HGNPs/21i in MDA-MB-231 and MCF-7 tumor-bearing mice at 4h and 24h post-injection with Cy5.5 labeled D-P-HGNPs/21i. And the injection site was marked with white arrow. The average radiant efficiency was acquired via an in

vivo imaging system. (B) Tumor growth curves for nude mice bearing MDA-MB-231 human breast tumors. (C) Body weight curves revealed that D-P-HGNPs/21i-4R caused no significant body weight change in treated mice. (D) H&E stained images of major organs.

Motivated by the high tumor targeting of D-P-HGNPs/21i, we further explored the anti-tumor effect of the sequential drug release therapy in vivo, using the subcutaneous MDA-MB-231 tumor model in nude mice. Mice were treated with intravenous injection with 200 μ L of D-P-HGNPs/21i (Dox at 1mg/kg). Then the tumor of each mouse was exposed to the NIR laser at the power density of 2 W/cm² simultaneously or 4 h post-injection. Dox at 1mg/kg or HGNPs with radiation failed to constrain the tumor growth (Fig.7B). Simple co-administration therapy displayed a modest suppression on the tumor growth, with the relative tumor volume at 4.07. D-P-HGNPs/21i-4R sequential release therapy showed a potent inhibition in tumor growth, with the relative tumor volume at 1.73, suggesting that time-programmed drug release system was more effective in vivo.

Potential in vivo toxicity is always a great concern for nanomaterials used in biomedicine. In this work, we did not notice any apparent signs of toxic side effects in mice injected with D-P-HGNPs/21i-4R over 20 days. Furthermore, we did not observe death or significant body weight change in the D-P-HGNPs/21i-4R treated group (Fig.7C). Hematoxylin and eosin (H&E) staining revealed no detectable pathological effects or myocardial injury in the sequential release group (Fig.7D). Meanwhile, remarkable nucleus atypia, increased necrotic cells, and lower chromatin staining were observed in D-P-HGNPs/21i-4R treated cells.

4. Discussion

Sequential application of various targeted therapies may be a better strategy to block tumor progression than simultaneous application of all anticancer agents in the course [16, 17]. Thus, well-organized multi-release systems must be established to produce effective combination therapies. Recently, several delivery systems have been reported for sequential drug delivery. However, PEI-grafted graphene oxide (PEI-GO) based nanoparticle has to be given twice [41]. Although sequential release from a single carrier was accomplished in PLGA [42], polyacylhydrazone (HPAH) [43], chitosan [44] based delivery system by one administration, while none of them could precisely control the time interval between individual drugs.

To meet this important challenge, we developed and tested sequential applications of various targeted therapies using HGNPs based platform for precisely programmed delivery with only one administration. Indeed, studies of NIR triggered delivery of macromolecules using Au cluster based capsule enlightened us in our design of the precisely controlled sequential release system [45, 46]. MiR-21i was first released upon reaching the tumor. After 4h transfection, NIR exposure triggered

Dox release due to HGNPs lost their spherical integrity and started to shrink, which facilitated the quick drug release (Fig.2A &B). Not only can the release order of miR-21inhibitor and doxorubicin be controlled easily, the time interval between individual drugs can be precisely managed. And we speculate that the key for a sequential controlled release system is to take advantage of various molecular interactions and explore the subcellular micro-environment.

In addition, this sequential delivery platform is easy to fabricate, thiol-terminated PAMAM was prepared by ring opening reaction and then linked to the citrate gold nanosphere via the strong Au-S absorption. Dox and miRNA were just added to the dispersion and incubation for 24h at room temperature. The formation of complexes between drug molecules and nanocarriers is advantageous compared to covalent conjugation approach owing to easy fabrication and scale up, low cost, and predictable release profile [47]. In our study, D-P-HGNPs condensed miR-21i at the small N/P ratio, thus the moderate positive charge making it low uptake in reticuloendothelial system, including the liver and spleen [48]. Furthermore, the small size of D-P-HGNPs/21i might also favor the distribution of the drugs to tumor sites, probably due to the enhanced permeability and retention (EPR) effect [49].

Both cellular and animal experiments suggested that a controlled sequential release mechanism was critical for improving drug efficacy, especially against CSCs (Fig. 4A, 6D, & 7). It has been documented that CSCs are more resistant to drug treatment than bulk cancer cells [11]. Indeed, in our system, IC_{50} of Dox for breast cancer stem cells was $20.7\mu\text{g/mL}$, much higher than that for bulk cancer cells ($1.1\mu\text{g/mL}$), demonstrating the drug resistance of cancer stem cells (Figure 4A &6E). High expression of miR-21 played an important role to maintain the stemness of and drug resistance of cancer cells [12, 13]. Our drug delivery system first reduce the stemness by disrupt miR-21 expression, as indicated by reduction of stemness marker Nanog, Oct4, and Sox2 (Figure 6B&C). Four hours later, the altered cancer stem cells became sensitive towards Dox treatment with IC_{50} at $0.39\mu\text{g/mL}$ (Fig 6E). The same approach is also effective against drug resistant tumor cell because those cells also express high level of miR-21 [31]. Thus, our drug delivery system was designed specifically to eliminate CSCs, which showed promising efficacy both in cell and animal models.

As the only FDA approved nanomaterial for clinic trails [50], gold nanoparticle based sequential delivery system could induce two distinct apoptosis signaling pathway, both mitochondrial apoptosis pathway and death-receptor mediated apoptosis pathway, which is approved to be a promising strategy to restore the drug sensitivity. Therefore, our sequential delivery system modulated the intrinsic state of the cells to a more chemosensitive form, thereby achieving a robust efficacy against cancer stem cells and cancer cells, which may be a promising technology for advanced combination therapy.

5. Conclusions

To date, developing the capability to deliver miRNA/chemotherapeutics with programmed release order and precisely time interval has been of great demand but remains highly challenging. We have developed a well-organized co-delivery platform (D-P-HGNPs/21i), based on biocompatible materials, which was able to efficiently release miR-21i and Dox in a site-specific and precisely scheduled manner. And we speculate that the key for a precisely controlled release system is to take advantage of various molecular interactions, explore the subcellular microenvironment and utilize the external NIR radiation. The sequential delivery of miR-21i/Dox was critical for improving drug efficacy by synergizing two apoptotic signaling pathways, instead of simultaneously, which might account for the exciting efficacy against CSCs and normal cancer cells. Most importantly, HGNPs based co-delivery platform could be easily fabricated, achieving the enhanced synergist killing effect without inducing toxicity and side effects, which may be translated into clinic to combat drug resistant tumors.

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Sequential co-delivery of miR-21 inhibitor followed by burst release
doxorubicin using NIR-responsive hollow gold nanoparticle to enhance
anticancer efficacy

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ABSTRACT

Previous literature and our study showed the delivery sequence of microRNA inhibitor and chemotherapeutic compounds achieve distinct therapeutic anticancer efficacy. Yet, it is challenging to use nanoparticle to achieve sequential drug delivery. In the current study, we designed sequential co-delivery system using a near-infrared-radiation (NIR) responsive hollow gold nanoparticle (HGNPs) to achieve sequential release of microRNA inhibitor (miR-21i)/doxorubicin(Dox) in order to achieve synergistic efficacy. PAMAM modified HGNPs was used to encapsulate miR-21i and Dox. Upon entering tumor cells, miRNA-21i was released first to sensitize the cancer cells, the subsequent burst release of Dox was achieved by NIR triggered collapse of HGNPs. This sequential delivery of miRNA-21i and Dox produced a synergistic apoptotic response, thereby enhancing anticancer efficacy by 8-fold and increasing anti-cancer stem cell activity by 50-fold. The sequential delivery of miR-21i and Dox using HGNPs under NIR after intravenous administration showed high tumor accumulation and significantly improved efficacy, which was 4-fold compared to free Dox group. These data suggested that the sequential co-delivery of miR-21i followed by burst release Dox using NIR-responsive HGNPs sensitized cancer cells to chemotherapeutic compound, which provided a novel concept for co-delivery miRNA inhibitors and chemotherapeutic compounds to enhance their efficacy.

Keywords

Co-delivery; Controlled sequential release; MicroRNA-21; Doxorubicin; Gold nanoparticle

1. Introduction

Since cancer cell growth is regulated by multiple signaling pathways, inhibition of one particular targeting molecule or single pathway is inadequate to eliminate cancer cells [1, 2]. Therefore, co-delivery of multiple drug components using nanoparticle, which simultaneously blocks multiple oncogenic pathways, provides advantages in cancer therapeutics [3, 4]. The roles of microRNAs (miRNAs) in the regulation of tumor progression and drug resistance have been widely recognized, thus miRNA-based therapies, such as miRNA-21 (miR-21), renders cancer cells more sensitive towards the chemical treatments, thereby broadly promoting the response to the chemotherapy [5, 6]. Increasing studies have demonstrated that co-delivery of miRNA/chemotherapeutic compounds had synergistic cell killing efficacy against cancer cells [7, 8].

In addition, recent clinical investigation has suggested that cancer is highly heterogeneous, which consists of differentiated cancer cells and cancer stem cells (CSCs) [9]. Conventional chemotherapy shrinks the bulk tumor mass, but fails to eliminate CSCs, which may be a cause for recurrence and metastasis [10, 11]. Previous study proved that high expression of miR-21 was required for maintenance of the pluripotency of CSCs, which was associated with drug resistance [12, 13]. Therefore, combination of miRNA gene therapy and chemotherapeutic compounds provided a novel approach to eliminate both cancer cells and CSCs [14, 15].

It has been noted that sequential delivery of miRNA and anti-cancer agents is critical to rewire cancer cells to exhibit sensitization effect to chemotherapeutic compounds, while simultaneous co-delivery of these agents failed to show synergistic efficacy [16, 17]. Our previous study also demonstrated that, pretreatment cancer cells with miRNA-21 inhibitor (miR-21i) for 4h followed by temozolomide treatment resulted in a far more synergistic effect, while co-delivery, reverse sequence or other dosing interval achieved additive or even antagonistic effect [18]. In addition, an interval of 4h administration exhibited the best killing efficacy for miR-21i plus doxorubicin (Dox) therapy in breast cancer (Figure S1). Therefore, it is critical to develop a drug delivery to precisely control the release sequence of miR21i and chemotherapeutic compound in order to achieve synergistic efficacy. The sequential delivery system will release miR-21i first to sensitize the cancer cells, and subsequent release of chemotherapeutic compounds will show better efficacy to eliminate both cancer cells and CSCs.

In this study, we designed sequential co-delivery system using a near-infrared-radiation (NIR) responsive hollow gold nanoparticle (HGNPs) to co-deliver miR-21i/Dox, termed as D-P-HGNPs/21i (Fig.1). HGNPs were employed as they stable, biocompatible, having plasmon absorption in the NIR region. In particular, the hollow interior of HGNPs increased drug payload compared with solid gold nanoparticle with same surface charge and weight [19]. As illustrated in Figure 1, this designed nanocarrier was fabricated by modifying HGNPs with thiolated PAMAM dendrimers through the strong Au-S linkage (Step I). Then, the

positive-charged Dox was successfully absorbed onto the negatively charged surface of HGNPs (Step II). Finally, miR-21i was condensed to PAMAM on HGNPs through the electrostatic interactions (Step III). D-P-HGNPs/21i entered the cells through the endocytic pathway. Upon entering the tumor cells (Step IV), miR-21i escaped from the endosome and released into the cytoplasm (Step V), due to the proton sponge mechanism of PAMAM dendrimer [20, 21], modulating the intrinsic state of cancer cells and CSCs to a more chemosensitive state (Step VI). In four hours, application of NIR triggered collapse of HGNPs (Step VII), thereby realizing a burst release of Dox (Step VIII). The efficacy of the sequential co-delivery miR21i/Dox system was tested to eliminate cancer cells and CSCs in vitro and in vivo. The data suggested that sequential release of miR-21i followed by burst release of Dox, not the simultaneous co-administration, produced a synergistic effect to exhibit a superior anti-cancer efficacy.

2. Materials and methods

2.1. Materials and Cell lines.

Gold chloride trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$), poly amidoamine (G5-PAMAM), Dox were purchased from Sigma-Aldrich. The breast cancer cell lines MDA-MB-231 and MCF-7 were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) with high glucose, 10% v/v fetal bovine serum (FBS), 1% v/v penicillin/streptomycin. MCF-10A cells were cultured in DMEM medium supplemented with 10% FBS, 10ng/mL epidermal growth factor (EGF, Sigma-Aldrich), 500ng/mL hydrocortisone (Sigma-Aldrich), and 10 $\mu\text{g/mL}$ insulin (Sigma-Aldrich). MDA-MB-231 CSCs were cultured in serum-free DMEM/F12 (1:1) medium containing 2% B27 (Gibco-BRL), 20 ng/ml EGF and 20 ng/ml basic fibroblast growth factor (bFGF; Sigma-Aldrich).

2.2. Synthesis of hollows gold nanoparticle (HGNPs).

HGNPs were prepared as described previously [19]. Silver seed nanoparticles were prepared by reducing a well-stirred solution of 0.1 mL of 100mM AgNO_3 with 0.6 mL of 1.0 M NaBH_4 in the presence of 0.5 M sodium citrate in deionized water. The solution was stirred for several hours to allow NaBH_4 to fully hydrolyze. Larger silver nanoparticles to be used as sacrificial templates for the HGNPs were grown from the silver seed solution by adding 0.6 mL of 2.0 M $\text{NH}_2\text{OH HCl}$ and 150 μL of 0.1M AgNO_3 and stirring overnight. Galvanic replacement of silver with gold was performed by quickly mixing 300 μL HAuCl_4 with the silver nanoparticles at 60°C. The solution was cooled to room temperature and kept in the dark for at least several days before use. The concentration of HGNPs was estimated by the previously reported method [22].

2.3. Synthesis of PAMAM modified HGNPs (P-HGNPs).

Thiol-terminated PAMAM dendrimer was synthesized via ring opening reaction, based on previous reports [23]. Briefly, 2mM 2-iminothiolane was added to the G5-PAMAM solution. The mixture was reacted with stirring for 2 h to complete the formation of the thiol-terminated dendrimer. The residual 2-iminothiolane was removed by dialysis. HGNPs (2mL, 6×10^{10} particle/mL) and thiol-terminated dendrimer aqueous solutions (0.5mL, 1% w/v) were mixed and incubated for 24 h at room temperature to form stable aqueous solutions.

The average number of PAMAM conjugated onto the HGNPs was determined using a fluorescamine assay [24]. Briefly, fluorescamine (Sigma-Aldrich) was first dissolved in anhydrous dimethyl sulfoxide (DMSO) to make a 3 mg/mL stock solution. PAMAM dendrimer were then diluted with 0.1 M phosphate buffer (pH=7) to make a series of solutions; pipette 100 μL of each solution to an opaque 96-well plate, respectively. 30 μL of the fluorescamine solution was then added into each well; the plate was then incubated for 1 h at room temperature. After the incubation, fluorescence intensity (Ex=360 nm, Em=465 nm) was read with a plate reader. The

number of residual free amino group was then estimated by comparing the fluorescent intensity of the native PAMAM.

2.4. Dox loading

Aliquots of free Dox in double distilled water (DDW, 1.0 mM, 100 μ L) were added into an aqueous solution of P-HGNPs (6×10^{10} particle/mL, 0.5mL), and the mixtures were stirred at room temperature for 24 h. After centrifugation (14,000 rpm for 20 min), the precipitate was washed with DDW and centrifuged, and the washing cycle was repeated for six times. The final drug concentration was determined by high-performance liquid chromatography (HPLC).

2.5. Evaluation of the drug release

To determine the kinetics of drug release behavior, 2 mL Dox-loaded P-HGNPs (D-P-HGNPs) was placed in a dialysis bag (molecular mass cut off 1000 Da) and incubated in 150mL of phosphate-buffered saline buffer (PBS, pH7.4 or pH5.8) at 37°C at the stirring speed of 100 ± 5 rpm.

A femtosecond laser system (Tianjin Biotech) was used to trigger the Dox release. The laser pulses were guided through an automatized attenuator and a polarizing beam splitter. The generated laser pulses were fixed at the wavelength of 964nm, with a total fluence rate of 2.0 W/cm². After exposure to laser for 120s, D-P-HGNPs was centrifuged at 14,000rpm for 15min at predetermined time points, and supernatant was collected. The content of drug released was fluorimetrically determined using reverse-phase HPLC with a C18 column, with acetic acid, methanol, and 10 mM Na₂HPO₄ (13:19:68, vol/vol/vol, pH 4.0) as the eluant solution. And the detection wavelength was set at 480 nm.

2.6. D-P-HGNPs/miR-21inhibitor assembly.

The 2'-O-methyl (2'-OMe) miR-21 inhibitors (miR-21i) were chemically synthesized by Shanghai Gene Pharma, as miR-21i was specially binding with the mature miRNA-21 in base complementary manner, thereby blocking the miRNA mediated downstream inhibition of mRNA translation. 2'-OMe oligos were composed entirely of 2'-O-methyl bases and had the following sequences: miR-21 inhibitor: 5'-GTCCAC TCT TGT CCT CAA TG-3'; scrambled sequence: 5'-AAG GCA AGC UGA CCC UGA AGU-3'. The oligonucleotides were purified by a high-pressure liquid chromatography system, dissolved in diethylpyrocarbonate (DEPC)-treated water, and frozen at -20°C. MiR-21i (20 μ M) was added to vials containing appropriate amounts of D-P-HGNPs solution, and then incubated at room temperature for 20 min. A series of miR-21i bound D-P-HGNPs (D-P-HGNP/21i) were formed at different N/P (the number of PAMAM terminal amino groups in the D-P-HGNPs to the number of phosphate groups in the miR-21i) ratios. The condensation ability of D-P-HGNPs was assessed by agarose gel electrophoresis. Electrophoresis was carried out on a 1% agarose gel with a current of 80 V for 15 min in a Tris-acetate-EDTA (TAE) buffer solution (40mM Tris-HCl, 1% v/v acetic acid, and 1 mM EDTA). The

migration of the complexes was visualized by staining with ethidium bromide. The images were acquired with a Gel Doc system.

2.7. Disassociation of D-P-HGNP/21i

Chicken egg white lysozyme (hydrochloride form) was purchased from Sigma-Aldrich. The disassociation of D-P-HGNP/21i was carried out by adding 150 μ g/mL lysozyme at 37 °C for 1min.

2.8. Cell transfection and cellular distribution in vitro

2.8.1. Cell transfection

After seeding cells onto 6-well plates at a density of 3×10^5 /well for 24 h in DMEM containing 10% FBS, cells were starved for 4 h at 37°C before transfection. For D-P-HGNPs/21i group, 400 μ L D-P-HGNPs (6×10^{10} particle/mL, with the drug concentration 0.2 μ g/mL) was mixed with 5 μ L miR-21i (20 μ M) at a N/P ratio of 4:1 in DMEM culture medium and incubated for 15 min at room temperature for transfection. For lipofectamine 2000 (Invitrogen) group, 10 μ L lipofectamine 2000 incubated with 5 μ L miR-21i (20 μ M) for 15min in DMEM culture medium.

Co-administration therapy: Cells was transfected with D-P-HGNPs/21i, followed by immediate NIR laser exposure at 2.0 W/cm² for 2min, designated as D-P-HGNP/21i-R.

Sequential release therapy: Cells was transfected with D-P-HGNPs/21i for 4h, followed by NIR laser exposure at 2.0 W/cm² for 2min, designated as D-P-HGNP/21i-4R.

2.8.2 .Cellular distribution

To investigate the cellular fate of the co-delivery system, the P-HGNPs were first labeled with an amine reactive dye, Cy5.5 (GE Healthcare). Briefly, P-HGNPs (6×10^{10} particle/mL) was reacted with Cy5.5 (5mg/mL) in a pH 7.4 PBS at a volume ration of 1: 100. The reaction was allowed overnight at room temperature by avoiding light. Excess dye molecules were removed by centrifugation filtration through 3 kDa MWCO Amicon filters and washed away with water for over 8 times until no noticeable color in the filtrate solution.

FITC-labeled miR-21i was chemically synthesized by Shanghai Gene Pharma, and Dox was loaded on Cy5.5 labeled P-HGNPs. After cell transfection for 1h or 4h, drug/miR-21i distribution was observed by fluorescence microscopy FV-1000.

The ability of D-P-HGNPs to transfer miR-21i into cancer cells were detected by fluorescence activated cell-sorting (FACS) analysis. Both MDA-MB-231 and MCF-7 cells were treated with D-P-HGNPs/21i-FITC for various time. Cells were trypsinised and washed three times with PBS. Then, the FITC fluorescence was detected by Verse flow cytometer (Becton Dickinson).

2.9. MiR-21 real-time PCR

Total RNA was extracted from cells using Trizol reagent (Invitrogen). The real-time PCR was carried out with the mirVana qRT-PCR miRNA detection kit (Ambion) in a 10 μ L reaction, comprising 2 μ L of mirVana, 5 $\times$ RT buffer, 1 μ L of mirVana, 1 $\times$ RT primer, 25ng of total miRNA, 0.4 μ L of ArrayScript enzyme mix, and DDW up to 10 μ L. Amplification reaction was performed using 7500 fast real-time PCR (Agilent) and the protocol was performed for 40 cycles at 95 $^{\circ}$ C for 3 min, 95 $^{\circ}$ C for 15 s, and 60 $^{\circ}$ C for 30 s. Both RT and PCR primer were purchased from Ambion. Relative quantification was conducted using amplification efficiencies derived from cDNA standard curves and obtained relative gene expression. U6 was used as the internal control. Data are presented as fold changes ($2^{-\Delta\Delta C_t}$) and were analyzed using Opticon Monitor Analysis Software V 2.02 (MJ Research).

2.10. Cytotoxicity assay.

Cells were seeded at 10^4 /well in 96-well cell culture plates. Cell viability was tested using a cell counting kit 8 (CKK-8). After 48 h of incubation with Dox, D-P-HGNPs/21i-R, D-P-HGNPs/21i-4R (with the drug concentration at 0.2 μ g/mL), the medium was removed, and fresh medium with 10% CKK-8 was incubated for 4 h. The absorbance at 450 nm was read using a microplate reader.

2.11. Analysis of the miRNA/chemotherapeutics combination treatment.

To analyze the combinatorial effect between the miR-21 inhibitor and Dox, the Jin method was used [25]. This method provides a “Q” value, allowing the effect of combining of two drugs to be classified as an antagonistic ($Q < 0.85$), an additive ($0.85 < Q < 1.15$), or a synergistic ($Q > 1.15$). The formula is $Q = Ea + b / (Ea + Eb - Ea \times Eb)$, where $Ea + b$, Ea and Eb are the average effect of the combination treatment, miR-21 inhibitor alone and Dox alone, respectively.

2.12. Evaluation of cell apoptosis

To quantify sequential release therapy induced apoptosis, Annexin V/PI staining was performed and further evaluated by FACS. Briefly, after various treatments, cells were collected and subjected to 5 μ L Annexin V and PI staining for 10min using an Annexin V-FITC Apoptosis Detection Kit (Becton Dickinson), according to the manufacturer’s protocol. The resulting fluorescence was measured by Verse flow cytometer (Becton Dickinson).

2.13. Subcellular distribution of cytochrome c.

Cells were labeled with MitoTracker green (Molecular Probes) for 30 min at 37 $^{\circ}$ C, then fixed with 4% paraformaldehyde for 10 min, followed by blocking with 10% bovine serum albumin (BSA) for 1 h. Sections were incubated with cytochrome c (ABcam) at a 1:1,000 dilution overnight. The secondary antibodies used were

Alexa-647-conjugated goat anti-mouse at a 1:300 dilution (Molecular Probes). The nuclear dye Hoechst 33342 was used at a 1:1,000 dilution.

2.14. Protein extraction and western blot analysis

Cell lysates were separated in an analytical 10% SDS- PAGE gel and transferred onto the polyvinylidene fluoride (PVDF) membrane (Invitrogen), followed by blocking in PBS with 5% bovine serum albumin (BSA) for 1 h. Sections were incubated with primary antibodies overnight. Primary antibodies were diluted in PBS with 1% BSA: p53, Bax, Bcl-2, cleaved caspase-3, cleaved caspase-8, cleaved caspase-9, AIF, and FasL (Cell Signaling) at 1:1000 dilutions, Cytochrome c (ABcam) at 1:1000 dilutions. Rabbit or mouse IgG antibody coupled with horseradish peroxidase was used as a secondary antibody (Santa Cruz). The density of the immunoblots was determined by Image J analysis software.

2.15. Animal studies.

All animal studies were approved by the Tianjin Medical University Animal Care and Use Committee and performed in accordance with national guidelines and regulations. The subcutaneous MDA-MB-231 xenograft mouse model was used to evaluate the therapeutic efficacy of different treatments. When tumor volume reached 400 mm³, mice were intravenously administrated with PBS, HG-NPs-R, Dox, D-P-HG-NPs/21i, D-P-HG-NPs/21i-R, or D-P-HG-NPs/21i-4R, at the dose of 1 mg/kg Dox equivalent, (n = 6-8). The treatment was given every two days for two weeks.

Tumor illumination was performed vertical to the largest protuberance of the tumor. The tumor was situated in the center of the light cone. The area to be irradiated was chosen with a peritumoral distance of healthy tissue of 0.5 cm. Tumor tissues were irradiated at 2.0 W/cm² for 3min immediately after D-P-HG-NPs/21i-R injection or 4h after D-P-HG-NPs-21i-4R injection.

2.16. Statistical Analysis.

All experiments were repeated at least three times. Data are presented as mean $\pm$ standard deviation. Statistical analysis was conducted using variance tests (ANOVA). Data sets were compared using two-tailed, unpaired t-test. *P* value of < 0.05 was regarded statistically significant.

3. Results

3.1. Formation of gold nanoparticle based sequential miR-21i/Dox release system

The sequential drug delivery system was based on hollow gold nanoparticle (HGNPs). As sphere shaped gold nanoparticle with much larger size possessed longer half-life of cellular uptake [26]. The PAMAM dendrimer was selected to modify HGNPs because it could form a stable complex with nucleic acid by electrostatic interaction. Also, PAMAM had the ability to escape from endosome via a process called the proton sponge effect [20, 21]. Transmission electron microscopy (TEM) images indicated that the HGNPs exhibited small, spherical, narrowly dispersed structure (Fig. 2A), with an average diameter of 61.3 ± 1.2 nm, as determined by dynamic light scattering (DLS) (Fig. 2B). Thiol-modified PAMAM dendrimer (PAMAM-SH) was successfully conjugated to HGNPs as the result of the Au-S absorption effect. P-HGNPs showed an increased in zeta potential from -14.64 to +14.59 mV after PAMAM modification (Figure S2). The average number PAMAM dendrimer conjugated to per gold nanoparticle was 10, as determined by a fluorescamine assay [22, 24].

Dox was effectively loaded on P-HGNPs to form nanoparticles at 91.6 ± 2.7 nm, designated as D-P-HGNPs (Fig. 2B). The amino group of Dox was the key for its high payload on to the HGNPs [19]. And approximately 7% surface of the HGNPs was occupied by PAMAM dendrimer according to the fluorescamine assay, thus P-HGNPs was efficient for drug loading. The color of the P-HGNPs changed from light blue to rose upon absorption of Dox (Fig.2C inset). UV-vis spectrum demonstrated that a broad NIR plasmon absorption peak at 964 nm characteristic of HGNPs, and a weak absorption peak at 480 nm characteristic of Dox, confirmed the loading of Dox to P-HGNPs (Fig.2C). The appearance of characteristic absorption peaks of nitrogen in the energy dispersive spectroscopy (EDS) further indicated the successfully drug loading (Figure S3).

The amount of Dox incorporated into P-HGNPs was 32.1%, as determined by high-performance liquid chromatography (HPLC) analysis. According to the high resolution TEM images (Fig.S4A), the shell of HGNPs was porous, with an average thickness of 4nm, which made it possible for drug molecules to diffuse into the core and bind to the inner surface of HGNPs. We also compared the drug loading efficiency between HGNPs and solid gold nanoparticle (AuNPs) having similar size. It was estimated that HGNPs should have three times payload of AuNPs if all Dox molecules were just coated on the outer surface of HGNPs. As shown in Fig.S4B, HGNPs reached a 4.8-fold increase drug payload compared to that in AuNPs, suggesting that the both the inner and outer surface of HGNPs were coated with Dox, which may explain the high drug loading capacity of HGNPs.

According to the drug release profile (Figure S5), only less than 15% Dox was release from D-P-HGNPs in PBS at pH 7.4 or cell culture medium containing 10% FBS after 2 days incubation without NIR excitation, indicating that Dox was stably

absorbed to P-HGNPs. As shown in Fig.S6, there were no significant changes in the particles size during the 7 day interval, as determined by DLS measurements, which further confirmed the stability of our delivery system.

MiR-21i was bounded to D-P-HGNPs via electrostatic interaction. To investigate the ability of miR-21i to complex with D-P-HGNPs, we performed electro mobility shift assays on 1% agarose gels. As shown in Figure 2D, the free miR-21i band at 22bp (lane 1) was barely visible when combined with D-P-HGNPs above the N:P ratio of 4:1 (lane 5), indicating that miR-21i has strong affinity for D-P-HGNPs. Following digestion with lysozyme, the miR-21i was released from the D-P-HGNPs, as evident from the bright band at 22bp (Fig.2E, lane 3).

The NIR triggered drug release was confirmed by the fluorescence spectrum. As shown in Figure 2F, the fluorescence emission of free Dox peaked at 580nm, while the fluorescence of Dox was quenched in D-P-HGNPs, probably due to fluorescence resonance energy transfer (FRET) [27]. Fluorescence at 580nm reappeared after D-P-HGNPs exposure to the NIR laser, indicating the release of drug. Moreover, D-P-HGNPs undergo shape transformation and size decreased after absorption of NIR laser, which might further facilitate the drug released (Fig.2A, 2B) [28].

The controlled release behavior of D-P-HGNPs was further tested in cell-free solutions. After an initial release of 7-10% over the first 4 hours, no further release of Dox from D-P-HGNPs was observed, demonstrating the stable incorporating capability of D-P-HGNPs (Fig.2G). A burst release of drug (from 4.1% to 30.3%) occurred within 15 min when the suspension of D-P-HGNPs was exposed to a NIR laser at 2.0 W/cm² output power. Only a slow release of approximately 5% of Dox was detected between 4h to 16 h, indicating only a small quantity of drug was release when laser was switched off. Little drug release was detected during the second and third NIR laser treatment cycle (Figure S7). These data demonstrate that Dox release from D-P-HGNPs could be triggered by the external NIR laser. At pH5.8, which mimics the acidic environment of tumor sites, only 18% drug was release during the 16h, further confirmed drug stability of D-P-HGNPs (Fig.2H). The cumulative drug release increased from 15.1% to 51.4% with NIR exposure at 2.0 W/cm² output power, indicating that Dox could be triggered by NIR at the desired time.

3.2. Effect of D-P-HGNPs/21i mediated programmed release at cellular level

Next, sequential drug release behavior of D-P-HGNPs/21i was evaluated in two breast cancer cell lines, MDA-MB-231 cells and MCF-7 cells. Dox was loaded on Cy5.5 labeled P-HGNPs, which was further conjugated to FITC-labeled miR-21i. As shown in Figures 3A, after incubating 1h with D-P-HGNPs-Cy5.5/21i-FITC, confocal microscopy revealed dissociation between FITC and Cy5.5 fluorescence signals, suggesting miR-21i was quickly released from the D-P-HGNPs to the cytoplasm. Meanwhile, minimal red fluorescence at 580nm was observed, indicating little Dox leakage. When exposed to NIR laser, increased accumulation of fluorescence signal at 580nm was detected in nucleus, suggesting release of Dox. A similar pattern was

observed in MCF-7 cells (Fig.3B). When the incubation time was extended to desired time interval, only weak fluorescence indicative of Dox release could be observed in D-P-HGNPs treatment 4h without NIR exposure (Fig.3C, 3D). These data demonstrate that our delivery system could achieve the precisely sequential gene/chemotherapeutics release at cellular level, with miR-21i being released first, followed by a controlled release of Dox at the desired time in response to NIR exposure.

Specific miR-21 inhibition is critical to enhance the drug sensitivity and impair the stemness of CSCs. Thus, we also evaluated the gene transfection efficiency of the D-P-HGNPs in both cell lines (Fig.3E). Cells were incubated with FITC-labeled miR-21i. FACS analysis showed that D-P-HGNPs facilitated efficient gene delivery in both cell lines. Our previous study revealed that naked miR-21i couldn't enter the cells [29]. FITC positive cells reached almost 100% within 4h post-transfection. To further confirm the transfection efficiency of D-P-HGNPs, suppression of miR-21 expression was measured by real-time PCR. As shown in Figure 3F, miR-21 expression was reduced by 60% after treatment with D-P-HGNPs, which showed similar transfection efficiency to that of commercially available transfection reagent lipofectamine 2000. These data further indicate that D-P-HGNPs provided efficient delivery of miR-21i at cellular levels.

3.3. Cytotoxicity of D-P-HGNPs/21i based sequential release system

The killing efficacy of sequential miR-21i/Dox treatment was first tested in breast cancer cells. In order to avoid the photothermal effect of gold nanoparticle, the laser setup we used in this study is operating with a femtosecond laser system. No significantly variation of temperature was detected after NIR exposure (Figure S8), further confirmed the cell killing effect is not due to the thermal effect.

Free Dox has an IC_{50} value of 1.1 μ g/mL in MDA-MB-231 cells (Fig.4A). The co-administration therapy D-P-HGNPs/21i-R, appeared to enhance drug efficacy (IC_{50} : 0.27 μ g/mL). In the sequential D-P-HGNPs/21i-4R treatment, the survival rate of cancer cells was further reduced, resulting in a total of 8-fold decrease in IC_{50} values (0.13 μ g/mL) for MDA-MB-231 cells (Fig.4B). D-P-HGNPs/21i-4R sequential therapy also decreased the IC_{50} value by 12 folds compared to free drug treatment in MCF-7 breast cancer cells (Figure S9). These results suggested that the sequential therapy achieved better synergistic efficacy.

To further confirm the efficacy of time-staggered miR-21i/Dox release therapy, cell survival rates upon treatment with 0.2 μ g/ml of Dox were monitored for 5 days in MDA-MB-231 cells (Fig.4C). Dox and D-P-HGNPs/21i-R resulted in cell death initially by 29% and 45%, respectively. Meanwhile, D-P-HGNPs/21i-4R exhibited a marked increase in drug response, the cellular killing rate reaching 69%. In contrast, the control groups did not show any significant reduction in viability when cells were incubated with HGNPs with or without exposure to the NIR laser, suggesting that HGNPs were non-toxic at the cellular level.

The combination efficacy of the time-sequenced miR-21i/Dox treatment was further analyzed by using the Z.J. Jin method (Figure 4D). Briefly, this method supplied a “Q” value. The combination effect between two drugs can be classified as a synergistic effect ($Q > 1.15$), an additive effect ($0.85 < Q < 1.15$), or an antagonistic effect ($Q < 0.85$). In the 5 days period post-treatment, Q value varied from 0.76 to 1.24 in D-P-HGNPs/21i-R simple combination therapy group, suggesting that co-administration achieved modest synergistic effect at day 5. When Dox was released 4h after nanoparticle entered the cells, the Q value of D-P-HGNPs/21i-4R reached 1.87, indicating the sequential therapy achieved a marked synergistic effect throughout the 5-day treatment. Taken together, these data indicated that time-staggered miR-21i/Dox sequentially release therapy is more effective at killing cancer cells than simple co-administration.

3.4. Synergized two apoptotic signaling pathways by sequential delivery system

The molecular mechanism of the sequential therapy was investigated. It had been reported that inhibition of miR-21 increased drug sensitivity and promoted apoptosis [30, 31]. FACS analysis showed that, comparing to the free drug, simple co-administration by D-P-HGNPs/21i-R increased apoptotic cell numbers from $7.9 \pm 0.8\%$ to $21.6 \pm 1.3\%$ in MDA-MB-231 cells, from $6.0 \pm 0.7\%$ to $15.8 \pm 1.4\%$ in MCF-7 cells (Figure 5A). D-P-HGNPs/21i-4R sequential therapy further enhanced the cell apoptosis response, reaching approximately $32.5 \pm 2.4\%$ and $25.5 \pm 1.8\%$ apoptotic cells, respectively.

Confocal fluorescence analysis of cytochrome c release further confirmed FACS results. As shown in Figure 5B, cytochrome c localized on mitochondrial in untreated cells. Co-administration by D-P-HGNPs/21i-R induced modest redistribution of cytochrome c into cytosol, a hallmark of apoptosis [32]. The sequential therapy D-P-HGNPs/21i-4R induced further dissociation of cytochrome c signals from mitochondrial. Western blotting results revealed that the sequential therapy increased the cytosolic levels of cytochrome c and apoptosis inducing factor (AIF), which further confirm the microscopy results. Furthermore, p53, Bax, cleaved caspase-3 and caspase-9, which played a central role in the mitochondrial apoptotic pathway, were dramatically increased in D-P-HGNPs/21i-4R-treated cells [33-35]. The anti-apoptotic protein Bcl-2, a downstream target of miR-21 [36], was substantially decreased, indicating activation of the mitochondrial apoptosis pathway. FasL played a central role in programmed cell death by activation of caspase-8 through the receptor-mediated pathway to trigger the classic apoptosis cascade [37, 38]. After D-P-HGNPs/21i-4R treatment, cells exhibited significantly elevated levels of FasL and cleaved caspase-8 (Fig.5C), indicating the activation of the death-receptor-mediated apoptosis pathway. Taken together, these results indicated that the sequential gene/chemotherapy achieved a marked, 8-fold increase in killing efficacy against normal cancer cells by synergizing two apoptotic signaling pathways.

3.5. D-P-HGNPs/21i based sequential release therapy enhanced drug efficacy in CSCs

Next, we tested the hypothesis that pretreated miR-21 inhibitor would impair the stemness and enhance the killing efficacy of CSCs upon the sequential therapy. The numbers of primary cancer stem cells isolated from patients were too low for this study, thus MDA-MB-231-derived stem cells were used for evaluation. As shown in Fig.6A, MDA-MB-231 cells formed stem cell spheres after two weeks of culture in suspended conditions with serum-free stem cell culture medium, consistent with previous reports [39]. Morphology change could be observed in the present of miR-21i, suggesting cell differentiation upon treatment with D-P-HGNPs/21i-R or D-P-HGNPs/21i-4R. Q-PCR revealed that stem cells expressed high level of miR-21 and Dox did not alter its levels (Fig. 6B). MCF-10A, mammary epithelial cells, expressed even lower level of miR-21 than that in MDA-MB-231 cells. Treatment with nanoparticle loaded with miR-21 inhibitors significantly decreased the levels of miR-21 in stem cells to that similar in MDA-MB-231 cells. The stemness of CSCs was monitored by the levels of three stem cell-specific transcriptional factors, Nanog, Oct4, and Sox2. As shown in Fig.6C, the sequential therapy, better than co-administration, significantly reduced the level of these three transcriptional factors. Confocal microscopy analysis showed that cancer stem cells resist cellular uptake of Dox, which partially accounted for the drug resistance (Fig.6D). The D-P-HGNPs/21i-4R mediated sequential therapy appeared to facilitate drug entry to CSCs, increasing the cellular levels of Dox in stem cells to that similar in MDA-MB-231 cells. These results indicated that the sequential therapy could impair the stemness of CSCs and dramatically improve the uptake of drugs.

We further examined the killing efficacy of sequential therapy in CSCs by CCK-8 assays. Single Dox treatment was ineffective in killing stem cells, with an IC_{50} value of $20.7\mu\text{g/mL}$, indicative drug resistance of CSCs (Fig.6E, 6F). D-P-HGNPs/21i-R treatment reduced IC_{50} values to $1.3\mu\text{g/mL}$. D-P-HGNPs/21i-4R further enhanced the drug effect, resulting in a total of 50-fold decrease in IC_{50} value ($0.39\mu\text{g/mL}$). The killing potency of sequential therapy against CSCs, was even higher than that in free Dox treatment against normal cancer cells, (IC_{50} of $1.1\mu\text{g/mL}$) (Fig. 4A). These data suggested that sequential release therapy might modulate the stemness of CSCs and rewire the intrinsic state of CSCs to a more chemosensitive form, thereby improving drug accumulation and reducing the IC_{50} value. Taken together, sequential release therapy achieved a striking killing efficacy against CSCs.

3.6. *In vivo* behavior of D-P-HGNPs/21i based sequential release system

To study the *in vivo* behaviors of sequential release therapy, mice bearing tumors derived from MDA-MB-231 and MCF-7 breast cancer cell lines were administered with Cy5.5 labeled D-P-HGNPs intravenously. Our previous study revealed that majority of free Cy5.5 is excreted within 4 h by renal clearance [40]. Thus, in order to monitor the organ distribution of nanoparticles, we set to detect fluorescence signals in various animal organs at 4 h and 24 h. Strong fluorescent signals were detected in area of tumors 4 h after D-P-HGNPs-Cy5.5/21i injection (Fig.7A). Weak fluorescent signals were also observed in liver, but not in other organs, indicating

D-P-HGNPs/21i displayed a superior biodistribution pattern. After 24 h, the fluorescent levels in the liver were reduced, but the signal from the tumor remained at a similar intensity, suggesting that D-P-HGNPs/21i could accumulate and be retained within tumor. Moreover, the similarly high tumor uptake was also observed in MCF-7 tumor-bearing mice (Fig.7A).

Motivated by the high tumor targeting of D-P-HGNPs/21i, we further explored the anti-tumor effect of the sequential drug release therapy *in vivo*, using the subcutaneous MDA-MB-231 tumor model in nude mice. Mice were treated with intravenous injection with 200 μ L of D-P-HGNPs/21i (Dox at 1mg/kg). Then the tumor of each mouse was exposed to the NIR laser at the power density of 2 W/cm² simultaneously or 4 h post-injection. Dox at 1mg/kg or HGNPs with radiation failed to constrain the tumor growth (Fig.7B). Simple co-administration therapy displayed a modest suppression on the tumor growth, with the relative tumor volume at 4.07. D-P-HGNPs/21i-4R sequential release therapy showed a potent inhibition in tumor growth, with the relative tumor volume at 1.73, suggesting that time-programmed drug release system was more effective *in vivo*.

Potential *in vivo* toxicity is always a great concern for nanomaterials used in biomedicine. In this work, we did not notice any apparent signs of toxic side effects in mice injected with D-P-HGNPs/21i-4R over 20 days. Furthermore, we did not observe death or significant body weight change in the D-P-HGNPs/21i-4R treated group (Fig.7C). Hematoxylin and eosin (H&E) staining revealed no detectable pathological effects or myocardial injury in the sequential release group (Fig.7D). Meanwhile, remarkable nucleus atypia, increased necrotic cells, and lower chromatin staining were observed in D-P-HGNPs/21i-4R treated cells.

4. Discussion

Sequential application of various targeted therapies may be a better strategy to block tumor progression than simultaneous application of all anticancer agents in the course [16, 17]. Thus, well-organized multi-release systems must be established to produce effective combination therapies. Recently, several delivery systems have been reported for sequential drug delivery. However, PEI-grafted graphene oxide (PEI-GO) based nanoparticle has to be given twice [41]. Although sequential release from a single carrier was accomplished in PLGA [42], polyacylhydrazone (HPAH) [43], chitosan [44] based delivery system by one administration, while none of them could precisely control the time interval between individual drugs.

To meet this important challenge, we developed and tested sequential applications of various targeted therapies using HGNPs based platform for precisely programmed delivery with only one administration. Indeed, studies of NIR triggered delivery of macromolecules using Au cluster based capsule enlightened us in our design of the precisely controlled sequential release system [45, 46]. MiR-21i was

first released upon reaching the tumor. After 4h transfection, NIR exposure triggered Dox release due to HGNPs lost their spherical integrity and started to shrink, which facilitated the quick drug release (Fig.2A &B). Not only can the release order of miR-21inhibitor and doxorubicin be controlled easily, the time interval between individual drugs can be precisely managed. And we speculate that the key for a sequential controlled release system is to take advantage of various molecular interactions and explore the subcellular micro-environment.

In addition, this sequential delivery platform is easy to fabricate, thiol-terminated PAMAM was prepared by ring opening reaction and then linked to the citrate gold nanosphere via the strong Au-S absorption. Dox and miRNA were just added to the dispersion and incubation for 24h at room temperature. The formation of complexes between drug molecules and nanocarriers is advantageous compared to covalent conjugation approach owing to easy fabrication and scale up, low cost, and predictable release profile [47]. In our study, D-P-HGNPs condensed miR-21i at the small N/P ratio, thus the moderate positive charge making it low uptake in reticuloendothelial system, including the liver and spleen [48]. Furthermore, the small size of D-P-HGNPs/21i might also favor the distribution of the drugs to tumor sites, probably due to the enhanced permeability and retention (EPR) effect [49].

Both cellular and animal experiments suggested that a controlled sequential release mechanism was critical for improving drug efficacy, especially against CSCs (Fig. 4A, 6D, & 7). It has been documented that CSCs are more resistant to drug treatment than bulk cancer cells [11]. Indeed, in our system, IC_{50} of Dox for breast cancer stem cells was $20.7\mu\text{g/mL}$, much higher than that for bulk cancer cells ($1.1\mu\text{g/mL}$), demonstrating the drug resistance of cancer stem cells (Figure 4A &6E). High expression of miR-21 played an important role to maintain the stemness of and drug resistance of cancer cells [12, 13]. Our drug delivery system first reduce the stemness by disrupt miR-21 expression, as indicated by reduction of stemness marker Nanog, Oct4, and Sox2 (Figure 6B&C). Four hours later, the altered cancer stem cells became sensitive towards Dox treatment with IC_{50} at $0.39\mu\text{g/mL}$ (Fig 6E). The same approach is also effective against drug resistant tumor cell because those cells also express high level of miR-21 [31]. Thus, our drug delivery system was designed specifically to eliminate CSCs, which showed promising efficacy both in cell and animal models.

As the only FDA approved nanomaterial for clinic trials [50], gold nanoparticle based sequential delivery system could induce two distinct apoptosis signaling pathway, both mitochondrial apoptosis pathway and death-receptor mediated apoptosis pathway, which is approved to be a promising strategy to restore the drug sensitivity. Therefore, our sequential delivery system modulated the intrinsic state of the cells to a more chemosensitive form, thereby achieving a robust efficacy against cancer stem cells and cancer cells, which may be a promising technology for advanced combination therapy.

5. Conclusions

To date, developing the capability to deliver miRNA/chemotherapeutics with programmed release order and precisely time interval has been of great demand but remains highly challenging. We have developed a well-organized co-delivery platform (D-P-HGNPs/21i), based on biocompatible materials, which was able to efficiently release miR-21i and Dox in a site-specific and precisely scheduled manner. And we speculate that the key for a precisely controlled release system is to take advantage of various molecular interactions, explore the subcellular microenvironment and utilize the external NIR radiation. The sequential delivery of miR-21i/Dox was critical for improving drug efficacy by synergizing two apoptotic signaling pathways, instead of simultaneously, which might account for the exciting efficacy against CSCs and normal cancer cells. Most importantly, HGNPs based co-delivery platform could be easily fabricated, achieving the enhanced synergist killing effect without inducing toxicity and side effects, which may be translated into clinic to combat drug resistant tumors.

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Figure Captions

Fig.1. Schematic illustration of gold nanoparticle-based, NIR triggered sequential miR-21 inhibitor/Dox release with precisely time interval for optimal combination therapy. (I-III) Formation of HGNNPs based co-delivery system. D-P-HGNNPs/21i entered cells through endocytosis (IV). Upon entering the tumor cells, miR-21i was released first (V), modulating the intrinsic state to a more chemosensitive state (VI). At the desire time, application of NIR laser triggered collapse of HGNNPs and a burst release of doxorubicin (VII), activating two apoptosis signaling pathway, thereby inducing the synergistic apoptosis response (IX).

Fig.2. Synthesis and characterization of HGNNPs-based precisely scheduled gene/drug release system. (A) TEM images of HGNNPs, D-P-HGNNPs, D-P-HGNNPs with NIR laser (D-P-HGNNPs-R). (B) Particle size measured by DLS.(C) UV-vis absorbance spectra of free Dox (red), P-HGNNPs (blue), and D-P-HGNNPs (rose). (D) Gel mobility shift assay of D-P-HGNNPs/21i at different N:P ratios. Lane M: Marker. Lane 1: naked miR-21i. Lane 2-3: various N:P ratios with D-P-HGNNPs/21i. (E) Disassociation of D-P-HGNNPs/21i complexes at a N:P ratio of 4:1 (lane 2), after digestion with lysozyme (Lane 3). Lane 1: naked miR-21i. (F) Fluorescence absorption of Dox, D-P-HGNNPs and D-P-HGNNPs-R at 580nm, with equal Dox concentrations. (G) Dox release profiles in the presence and absence of NIR laser. (H) Dox release profile at pH7.4 and pH5.8 PBS in the absence or presence of laser radiation.

Fig.3. D-P-HGNNPs/21i controlled gene/drug release in a precisely scheduled manner at the cellular level. Intracellular distribution of miR-21i (20 μ M, green) and Dox (0.2 μ g/ml red,) after incubation with D-P-HGNNPs-Cy5.5/21i-FITC (6*10¹⁰ particle/mL, orange) for 1h in MDA-MB-231(A) and MCF-7 cells (B).When the incubation time extended to 4h, only weak red fluorescence could be observed in MDA-MB-231(C) and MCF-7 cells (D), without NIR exposure. (E) Uptake of the miR-21i analyzed by FACS. (F) Real-time PCR analysis detected relative miR-21 expression levels. All of the bar size was 20 μ m.

Fig.4. D-P-HGNNPs/21i based sequential release therapy enhanced cytotoxicity compared to simultaneously co-administration therapy. (A) IC₅₀ value of MDA-MB-231 cells treated with free Dox, D-P-HGNNPs/21i-R and D-P-HGNNPs/21i-4R. (B) D-P-HGNNPs/21i-4R reduced the 8-fold IC₅₀ value compared with free Dox treatment. (C) Cytotoxicity of Dox, D-P-HGNNPs/21i-R and D-P-HGNNPs/21i-4R on MDA-MB-231 cells, at a drug dose of 0.2 μ g/mL. (D) Combination therapy effect analysis indicated that sequential therapy achieved a marked synergistic effect, which is more effective than simple co-administration therapy.

Fig.5. D-P-HGNPs/21i based sequential release therapy enhanced cancer cell apoptotic response to chemotherapy. (A) Cell suspensions were stained by Annexin V-PI and measured by FACS. (B) Cytochrome c distribution in MDA-MB-231 cells. Results were further proved by spectral intensity analysis. (C) Results of western blotting indicated that both mitochondrial apoptosis and death receptor-related apoptosis pathways were activated by sequential therapy. Bar size was 20 μ m for all images.

Fig.6. D-P-HGNPs/21i-based sequential release therapy effectively killed CSCs. (A) DIC image of MDA-MB-231 stem cells. Morphology change could be detected in D-P-HGNPs/21i-4R treated cells. (B) MiR-21 expression in MCF-10A, MDA-MB-231 cells and CSCs. (C) RT-PCR detected the stem cell transcription markers Nanog, Oct4, and Sox2 in MDA-MB-231 cells and stem cells. (D) Intracellular drug distribution of Dox in MDA-MB-231 cells and stem cells. (E) IC₅₀ value of MDA-MB-231 stem cells treated with Dox, D-P-HGNPs/21i-R, and D-P-HGNPs/21i-4R. (F) D-P-HGNPs/21i-4R reduced the IC₅₀ value by 50-fold compared with free Dox.

Fig.7. *In vivo* behavior of D-P-HGNPs/21i based sequential release therapy. (A) Distribution of D-P-HGNPs/21i in MDA-MB-231 and MCF-7 tumor-bearing mice at 4h and 24h post-injection with Cy5.5 labeled D-P-HGNPs/21i. And the injection site was marked with white arrow. The average radiant efficiency was acquired via an *in vivo* imaging system. (B) Tumor growth curves for nude mice bearing MDA-MB-231 human breast tumors. (C) Body weight curves revealed that D-P-HGNPs/21i-4R caused no significant body weight change in treated mice. (D) H&E stained images of major organs.

Graphic Abstract

Our controlled sequential release system, inducing drug sensitivity by gene therapy followed by chemotherapy, showed significantly improved efficacy against tumors than conventional co-delivery system, may be more effective against CSC.