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Tumor-Triggered Drug Release with Tumor-Targeted Accumulation and Elevated Drug Retention to Overcome Multidrug Resistance

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ABSTRACT: Multidrug resistance (MDR) is one of the main causes of the failure in cancer chemotherapy. To address this challenge, this work develops a tumor-triggered nanomedicine (HA-MSDOX-KLA) with switchable morphology transformation that significantly enhances tumor-specific accumulation and retention for overcoming MDR. Morphology changes could be triggered by tumor overexpressed matrix metalloproteinases (MMPs), achieving elevated drug accumulation and prolonged intracellular drug retention. Additionally, the adjunctive release of proapoptotic peptide KLA would targetedly induce the dysfunction of mitochondria with decreased ATP generation to further inhibit the drug efflux. *In vitro* and *in vivo* studies demonstrate that, compared with free doxorubicin (DOX), the functionalized HA-MSDOX-KLA shows a 6.1-fold and 4.5-fold increase in anti-MDR efficacy on MCF-7/ADR cancer cells and MCF-7/ADR tumor-bearing mice, respectively. We believe that this strategy will open a new window of designing transformable nanomedicine for combating drug resistance in tumor treatments.
INTRODUCTION

Multidrug resistance (MDR), a general phenomenon in chemotherapy, always leads to the failure of cancer therapy.\textsuperscript{1,2} In general, MDR arises from the overexpression of adenosine triphosphate ATP-binding cassette transporters such as P-glycoprotein (P-gp), which decrease the drug influx but promote its efflux, thereby reducing the intracellular drug level with limited therapeutic effect of chemotherapy.\textsuperscript{3,4} To date, development of effective strategy for overcoming MDR is still a critical challenge.

Recently, nanoparticle-based drug delivery systems with tailor-made properties have been proposed to reverse MDR by increasing high local drug bioavailability at tumor lesion via enhanced permeation and retention (EPR) effect and/or active targeting effect.\textsuperscript{5,6} However, drugs delivered by these nanosystems are commonly released in the cytoplasm, which still face the efflux fate by P-gp, and thus result in greatly reduced effectiveness of chemotherapy. An available strategy to anti-MDR is the nuclear-targeted drug delivery,\textsuperscript{7,9} in which drugs are directly delivered into nuclei where are the final intracellular target of most chemotherapeutic molecules. Unfortunately, this nuclear-targeted delivery system suffers from the serious obstacle of nuclear pore (about 9-40 nm).\textsuperscript{10,11} To address this dilemma, small-sized nanoparticles (< 50 nm) are usually adopted, which in turn undergo rapid clearance with restricted accumulation in tumor tissues due to their re-entering effect to the bloodstream.\textsuperscript{12,13} While, fibrous nanostructures have been proofed to exhibit more advantages than their spherical counterparts, such as the prone of trapping in tumor tissue and preferable cellular association.\textsuperscript{14-17} Moreover, fibers are difficult to pump out by P-gp, leading to enhanced intracellular drug retention to bypass the P-gp-mediated drug efflux for circumventing MDR. Nonetheless, delivery anticarcinogens by nanofibers are still far from satisfactory therapeutic efficacy, arising from the easy capture of
fibers by the reticuloendothelial system (RES).\textsuperscript{18} To integrate the advantages of both preferable tumor accumulation of spherical nanomaterials and enhanced intracellular retention of fibrous ones, changeable nanomaterial pattern would be an efficient strategy to deal with the MDR issue. This strategy utilizes endogenous stimuli in specific tumor microenvironments, such as proteases, hypoxia and mildly acidic, to trigger the mutation of morphology or physicochemical properties of drug delivery systems for promoting the drug accumulation in tumor tissues, namely “tumor-triggered transformation”.\textsuperscript{19-27}

Here, a tumor-triggered transformable nanomedicine with programmed therapeutic effect was designed to overcome multidrug resistance. This nanodrug delivery system undergoes morphology transition from spherical micelles to nanofibers as a function of tumor overexpressed matrix metalloproteinases (MMPs).\textsuperscript{28} As shown in Figure 1, the designed transformable nanomedicine (HA-MSDOX-KLA) is comprised of a tumor active targeting segment (hyaluronic acid, HA),\textsuperscript{29-31} and two programmed therapeutic agents, MMP-substrate peptide (CPLGLAGG) conjugated doxorubicin (designated as MSDOX), and proapoptotic peptide (KLAKLAK)\textsubscript{2} (designated as KLA). HA-MSDOX-KLA can self-assemble into micellar nanoparticles (HA-MSDOX-KLA NPs) at physiological condition due to its inherent amphiphilicity nature. Upon exposure to tumor overexpressed MMP, MMP-substrate peptide is specifically cleaved, which enhances the hydrophobicity of the molecules and decreases their steric hindrance, resulting in morphology switch from nanoparticles to drug nanofibers and expeditious release of adjunctive KLA. This transformable nanomedicine exhibits following unique merits for overcoming MDR: i) the passive and active tumor targeting effects of HA-MSDOX-KLA significantly enhance cellular uptake of anticancer drug; ii) tumor-triggered morphology transformation from nanoparticles to drug nanofibers not only improves the cellular
concentration of DOX but also enhances the intracellular drug retention effect; iii) the adjunctive KLA is a mitochondria-targeted biodrug that can selectively damage subcellular mitochondria and induce the downregulation of ATP and subsequently inhibit drug efflux (drug efflux is ATP-dependent). Collectively, the increased drug retention and decreased drug efflux would be a two-pronged strategy to collaboratively overcome multidrug resistance.

EXPERIMENTAL SECTION

Materials. N-Fluorenyl-9-methoxycarbonyl (Fmoc) protected amino acids, 2-chlorotrityl chloride resin (100-200 mesh, loading: 0.4 mmol/g, 1%DVB), 1-hydroxybenzotriazole (HOBt), o-benzotriazole-N,N,N’,N’-tetramethyluroniumhexafluorophosphate (HBTU), trifluoroacetic acid (TFA), and diisopropylethylamine (DIEA) were purchased from GL Biochem. Ltd. (Shanghai, China) and used as received. 6-Maleimido caproic acid, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC•HCl), and N-hydroxysuccinimide (NHS) were provided by Shanghai Chemical Co. (China). Hyaluronic acid (HA, 35 kDa) was purchased from Freda Biochem Co. Ltd. (Shandong, China). Dulbecco’s Modified Eagle’s Medium (DMEM), adenosine 5’-triphosphate (ATP) bioluminescent assay kit, Annexin V-FITC/PI apoptosis detection kit, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT), fetal bovine serum (FBS), trypsin, penicillin-streptomycin, molecular probe (Hoechst 33342), and Dulbecco’s phosphate buffered saline (PBS) were purchased from Invitrogen. All other reagents and solvents were of analytical grade and used directly.

Synthesis of HA-Mal. Briefly, 6-maleimidocaproic acid (0.63 g, 3 mmol), NHS (0.69 g, 6.0 mmol) and EDC (1.15 g, 6.0 mmol) were dissolved in 20 mL N,N-dimethylformamide (DMF) followed by addition of 30 mL HA (0.5 g) solution. The reaction mixture was stirred at room
temperature for 24 h. Then the resulting HA-Mal was obtained by dialyzing (MWCO: 14000 Da) the reaction solution against deionized water for three days and followed by lyophilization. The $^1$H NMR spectra of HA and HA-Mal are shown in Figure S1.

**Synthesis of MSDOX and KLA.** MSDOX and KLA were synthesized according to the solid phase peptide synthesis (SPPS) technique. Briefly, peptide chains were conjugated on 2-chlorotriyl chloride resin. The different amino acid couplings were implemented with Fmoc-protected amino acid and HBTU/HOBt/DIEA for 2 h. During the synthesis, Fmoc-protected groups were deprotected with 20% piperidine/DMF (v/v) for twice and every time for 15 min. At the end of the synthesis, the resin was finally washed with DMF (four times), CH$_3$OH (four times) and CH$_2$Cl$_2$ (four times), respectively, and dried under vacuum. Peptides were cleaved from resin in a cleavage cocktail containing TFA (95%), TIS (2.5%), and H$_2$O (2.5%) for 2 h. After precipitated in cold ether, the crude product was collected and vacuum dried, then freeze-dried. The molecular weights were characterized by matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) and ESI-MS, respectively.

**Synthesis of HA-MSDOX-KLA.** MSDOX and KLA were conjugated with HA-Mal to prepare HA-MSDOX-KLA via thiol-Michael chemistry.$^{32}$ Briefly, a mixture solution containing MSDOX (112.7 mg, 0.09 mmol) and KLA (48.8 mg, 0.03 mmol) were added dropwise into HA-Mal (500 mg) and stirred at room temperature for 24 h. In this study, the molar ratio of MSDOX and KLA was fixed at 3:1 and reacted with HA-Mal. The data of molar ratio at 2:1 and 1:1 were not showed here. Then the mixture solution was dialyzing against deionized water (MWCO: 14000 Da) for three days. Finally, the prodrug HA-MSDOX-KLA was yielded after lyophilized as red solid.
Measurement of Critical Micelle Concentration (CMC). The CMC of HA-MSDOX-KLA micelles was examined by fluorescence spectra, which was measured on an RF-530/PC spectrofluorophotometer (Shimadzu) by using pyrene as a hydrophobic fluorescent probe. Briefly, 50 µL of pyrene solutions (6 × 10^{-7} M in acetone) were added to containers firstly. After evaporation of acetone, 1 mL aqueous solution of HA-MSDOX-KLA at predetermined concentration varying from 1 × 10^{-4} to 1 mg/mL was added to the container. Then the mixtures were kept at room temperature for 24 h to reach the equilibrium of pyrene partition between water and micelles. For the pyrene excitation spectra measurement, the emission wavelength was performed at 390 nm, and the excitation spectra of samples were collected at a wavelength range from 300 nm to 360 nm. The fluorescence intensity ratio of the third and first vibronic bands (I_3/I_1) were plotted against the logarithm of HA-MSDOX-KLA concentrations, and the CMC value was determined from the intersection of the tangent to the curve at the inflection with the horizontal tangent through the points at low concentration.

Size and Morphology Characterization. HA-MSDOX-KLA was dissolved in PBS buffer solution in the presence or absence of MMP. The morphology was observed by transmission electron microscopy (TEM, JEM-2100), and the hydrodynamic diameter was determined by dynamic light scattering (DLS, Nano-ZS ZEN3600, Malvern Instruments). The stability of the HA-MSDOX-KLA micelles in the presence of serum proteins at physiological condition was also detected by DLS.

Cell Culture. African green monkey SV40-transformed kidney fibroblast (COS7) cells were cultured in DMEM medium supplemented with 10% FBS and 1% antibiotics (penicillin-streptomycin, 10000 U/mL) in a humidified atmosphere containing 5% CO_2. Human breast cancer MCF-7 and MCF-7/ADR cell lines were served as drug-sensitive and drug-resistant
cancer cells, respectively, and cultured in DMEM medium supplemented with 10% FBS and 1% antibiotics (penicillin-streptomycin, 10000 U/mL) in a humidified atmosphere containing 5% CO₂.

**Cellular Uptake.** In brief, cells were seeded in a glass bottom dish at a density of $1 \times 10^5$ cells per well for 24 h. Then, MCF-7/ADR cells, MCF-7 cells, and COS7 cells were incubated with free DOX (5 µg/mL) or HA-MSDOX-KLA (containing 5 µg/mL of DOX) in the cell culture media for 6 h or 12 h, respectively. After that, the cell culture media were removed and all the cells were washed with PBS three times. Then the nuclei were stained with Hoechst 33342 at 37°C for 15 min and all the cells were observed by CLSM (Nikon C1-si). Excitation of DOX and Hoechst 33342 were performed with lasers at 488 nm and 405 nm. The corresponding emission spectra were collected using two different ranges of wavelength at 510-540 nm for DOX and 450-490 nm for Hoechst 33342, respectively. Quantitative evaluation of intracellular DOX fluorescent intensity was measured by flow cytometry analysis. After treating cells with the same conditions as the CLSM observation, the intracellular uptake of DOX was determined by flow cytometry (BD FACSARia TM III). The cells without any treatment were detected as the blank control.

**Evaluation of Uptake Mechanism.** Briefly, MCF-7/ADR cells were seeded in a glass bottom dish at a density of $1 \times 10^5$ cells per well for 24 h. To evaluate the factor of temperature on the cellular uptake of HA-MSDOX-KLA, MCF-7/ADR cells were pre-incubated with HA-MSDOX-KLA (DOX dosage of 5 µg/mL) at 4°C for 6 h. To examine the effect of diverse inhibitors on the cellular uptake of the micelles, the cells were preincubated individually with three inhibitors (inhibitor of clathrin-mediated endocytosis: chlorpromazine hydrochloride (10 µg/mL); inhibitor of caveolin-mediated endocytosis: genistein (50 µg/mL); inhibitor of macropinocytosis:
amiloride (13.3 µg/mL) of endocytosis for 1 h at 37 °C. Then the cells were treated with HA-MSDOX-KLA (DOX dosage of 5 µg/mL) at 37 °C for another 6 h. The cells incubated with HA-MSDOX-KLA with no inhibitor treatment were used as the control. Afterwards, all the cells were washed with PBS three times and the cell nuclei were stained with Hoechst 33342 at 37°C for 15 min and then observed by CLSM (Nikon C1-si). Excitation of DOX and Hoechst 33342 were performed with lasers at 488 nm and 405 nm. The corresponding emission spectra were collected using two different ranges of wavelength at 510-540 nm for DOX and 450-490 nm for Hoechst 33342, respectively. Quantitative evaluation of intracellular DOX fluorescent intensity was measured by flow cytometry analysis. After treating cells with the same conditions as CLSM observation, the intracellular uptake of DOX was determined by flow cytometry (BD FACSAria TM III). The cells without any treatment were detected as the blank control.

**Investigation of Intracellular Retention Effect.** Briefly, MCF-7/ADR and MCF-7 cells were seeded in a glass bottom dish at a density of $1 \times 10^5$ cells per well for 24 h. To investigate the intracellular retention effect, MCF-7/ADR and MCF-7 cells were pre-incubated with free DOX (5 µg/mL) or HA-MSDOX-KLA (DOX dosage of 5 µg/mL) for 6 h. After that, the cell culture media were removed and all the cells were further cultured with new medium for 6 h, 12 h, 18 h, and 24 h, respectively. Subsequently, the cell nuclei were stained with Hoechst 33342 at 37 °C for 15 min and then observed by CLSM (Nikon C1-si). Excitation of DOX and Hoechst 33342 were performed with lasers at 488 nm and 405 nm. The corresponding emission spectra were collected using two different ranges of wavelength at 510-540 nm for DOX and 450-490 nm for Hoechst 33342, respectively. Quantitative evaluation of intracellular DOX fluorescent intensity was analyzed by using Image-J software.
Evaluation of Intracellular Morphology Transformation by Bio-TEM. The cellular uptake of HA-MSDOX-KLA micelles and in situ morphology transformation were observed by bio-TEM (TEM, JEM-2100). The MCF-7/ADR cells were cultured with HA-MSDOX-KLA (DOX dosage of 5 µg/mL) at 37 °C for 6 h or 24 h, respectively. Afterwards, the cells were washed with PBS three times, collected and fixed with 1 mL general fixative (containing 2.5% glutaraldehyde in 0.1 M PBS) at 4 °C for 24 h. Then the cells were treated with the standard procedures for bio-TEM observation and observed by using a electronic microscopy.

Evaluation of the Drop of Mitochondrial Membrane Potential by JC-1 Assay. MCF-7/ADR cells and COS7 cells were respectively cultured with HA-MSDOX-KLA (DOX dosage of 5 µg/mL) in cell culture dish. After incubation 12 h, 24 h or 48 h at 37 °C, the cells were stained with JC-1 (10 µg/mL) in DMEM for 30 min and the cell nuclei were stained with Hoechst 33342 at 37 °C for 15 min. Then all the cells were observed by CLSM (Nikon C1-si). Excitation of JC-1 monomer and Hoechst 33342 were performed with lasers at 488 nm and 405 nm. The corresponding emission spectra were collected using two different ranges of wavelength at 510-540 nm for green fluorescence of JC-1 monomer and 450-490 nm for Hoechst 33342, respectively.

Evaluation of Intracellular ATP Level. To examine the therapeutic function of mitochondria-targeted biodrug KLA in HA-MSDOX-KLA, the intracellular ATP levels were measured. MCF-7/ADR cells, MCF-7 cells and COS7 cells were seeded in 24-well plates at a density of $5 \times 10^4$ cells per well for 24 h. Then the cells were treated with HA-MSDOX-KLA (DOX dosage of 5 µg/mL) or free DOX (5 µg/mL) for different incubation time (12 h, 24 h or 48 h), respectively, and then washed with PBS three times. Subsequently, the cells were trypsinized and counted, centrifuged and collected in centrifuge tube. And the cell pallets were lysis and the
ATP level was evaluated according to the protocol of adenosine 5’-triphosphate (ATP) bioluminescent assay kit.

**In vitro Cytotoxicity Assay.** The cytotoxicity of HA-MSDOX-KLA and free DOX were estimated in MCF-7/ADR cells, MCF-7 cells, and COS7 cells by MTT assay. In brief, MCF-7/ADR cells, MCF-7 cells, and COS7 cells were seeded in a 96-well plate at a density of 6000 cells per well and incubated in 100 μL DMEM containing 10% FBS and 1% antibiotics for 24 h. After that, HA-MSDOX-KLA and free DOX at different concentrations were added to each well. After co-incubation for 48 h, 20 μL MTT (5 mg/mL in PBS buffer solution) was added to each well and further incubated for another 4 h. Afterwards, the medium was removed and replaced with 150 μL DMSO. The absorbance of the DMSO solution in the wells at the wavelength of 570 nm was measured by a microplate reader (Model 550, Bio-Rad) to determine cell viability. The relative cell viability was calculated as $(OD_{570_{\text{sample}}}/OD_{570_{\text{control}}}) \times 100\%$, where $OD_{570_{\text{control}}}$ was obtained in the absence of therapeutic agents and $OD_{570_{\text{sample}}}$ was obtained for cells treated with HA-MSDOX-KLA and free DOX.

**Evaluation of Apoptosis by Flow Cytometry.** MCF-7/ADR cells and MCF-7 cells were respectively seeded in 24-well plates at a density of $5 \times 10^4$ cells per well. After 24 h incubation, the cells were treated with HA-MSDOX-KLA (DOX dosage of 5 μg/mL) or free DOX (5 μg/mL) for different incubation time (24 h or 48 h), respectively. Then the cells were washed with PBS three times, digested by trypsin (EDTA deplete), collected by centrifugation. After washing with PBS three times, the cells were resuspended in 0.5 mL annexin-binding buffer. After that, all the cells were stained in propidium iodide (PI) and Annexin-V-FITC contained binding buffer for 15 min, and finally detected by flow cytometry (BD FACSArria TM III). The cells without any treatment were detected as the blank control.
**Animals and Tumor Model.** BALB/c nude mice (3-4 weeks old) were bought from Wuhan University Animal Biosafety Level III Lab and used for animal experiments directly. All animal experiments were agreed with institutional animal use and care regulations from Wuhan University. The tumor-bearing mice were obtained by injecting MCF-7/ADR cancer cells (3 × 10^7 cells) into subcutaneous of female mice on the right armpit region.

**Pharmacokinetic Studies.** When the tumor volume reached to approximately 50 mm^3^, the mice were randomized into three groups (n = 3) and administered intravenously with free DOX, HA-MSDOX, or HA-MSDOX-KLA at the same DOX dosages (2 mg/kg). After injection, blood samples were harvested at predestined time intervals (0.5, 1, 2, 4, 6, 8, 12, and 24 h) using a heparinized tube. Plasma samples were immediately collected by centrifuging the blood samples at 3000 rpm for 15 min. After that, plasma samples were frozen at -20 °C for fluorescence analysis. The percent injected dose (% ID) values were calculated according to previous report.  

**In vivo Tumor Cellular Accumulation and Retention.** BALB/c nude mice bearing MCF-7/ADR tumors received one intravenous injection of free DOX, HA-MSDOX, or HA-MSDOX-KLA at an equivalent DOX dose of 2 mg/kg. Then the mice were sacrificed at 6 h, 12 and 24 h, and the tumor tissues were harvested. The tumor tissues were washed with PBS buffer, wiped with filter paper, weighed, and homogenized in 2 mL DMSO using a tissue grinder, followed by centrifugation at 3500 rpm for 15 min. The supernatants were collected and frozen at -20 °C for fluorescent analysis. The data were normalized to the tumor weights. For the visible evaluation of the drug distribution in tumor tissues, the mice were euthanized at 24 h after injection, and the tumors were excised and immediately frozen in -80 °C refrigerator for sectioning. The accumulation of DOX in the tumor tissues was observed by CLSM (Nikon C1-si) on frozen section of tumor tissues.
**In vivo Antitumor Study.** When the tumor volume reached about 40-50 mm$^3$, MCF-7/ADR tumor-bearing mice were randomly divided into four groups (n = 5 per group) and treated with PBS, free DOX, HA-MSDOX, or HA-MSDOX-KLA with an equivalent DOX dose of 2 mg/kg, respectively. All the mice were injected every other day. The weight of mice and tumor volume was measured every day. Tumor size was measured by a caliper and tumor volume was calculated by the following formula: $V = (\text{tumor width})^2 \times (\text{tumor length})/2$. Relative tumor volume was calculated as $V/V_0$ ($V_0$ was the tumor volume before therapy). When the treatment was fulfilled, the tumors were excised and weighed. Simultaneously, the main organs (heart, liver, spleen, lung and kidney) of mice were also harvested and used for histologic analysis.

Moreover, the tumor tissue was also stained by terminal deoxynucleotidyl transferased dUTP nick end labeling according to the manufacturer’s protocol (Roche, Penzberg, Germany) for investigating the apoptosis. Simultaneously, the tumor sections were further stained by cleaved caspase-3 (Asp175) antibody (1:200; CST, USA) for caspase-3 labeling and then Alexa Fluor 555 conjugated goat anti-rabbit secondary antibody (1:1000; CST, USA) was used for fluorescence microscope detection. After the nuclei stained by 4',6-diamidino-2-phenylindole (DAPI), all the samples were examined by fluorescence microscopy.

For western blotting analysis, tumors in different treatment groups were lysed on ice by RIPA buffer for 30 min and then centrifuged 10 min (12000 rpm). The total protein in the supernatant was quantified by the BCA protein assay kit (KGPBCA, KeyGEN). 100 µg of protein was electrophoresed in 10% SDS-PAGE and transferred to a nitro-cellulose membrane, blocked and incubated overnight with mouse monoclonal anti-caspase-3 antibody (1:3000 dilution, Cell Signaling Technology), and mouse monoclonal anti-Bcl-2 antibody (1:3000 dilution, Cell Signaling Technology) and subsequently treated with the secondary antibody HRP-labeled goat
anti-rabbit IgG (1:3000 dilution, Santa Cruz Biotechnology) for 1 h. Each protein was detected by enhanced chem-iluminescence (ECL, Pierce). Mouse monoclonal anti-GADPH antibody (Santa Cruz Biotechnology) was used as protein loading control.

**In vivo Systemic Toxicity Study.** After all the treatments were finished, 5 mice in each group were used to detect alanine aminotransferase (ALT), aspartate aminotransferase (AST), and kidney function urea nitrogen (BUN) in the serum and hematological analysis. The blood samples of each groups were collected and detected at Union Hospital (Tongji Medical College, Wuhan, China).

**RESULTS AND DISCUSSION**

**Preparation and Characterization of the Transformable Nanomedicine.** In this study, 6-maleimidocaproic acid (Mal) was first reacted with HA via esterification to yield HA-Mal, and its structure was confirmed by \(^1\)H NMR (Figure S1). Then the remaining maleimide group of HA-Mal was further reacted with MSDOX (Figure S2) and KLA (Figure S3) to obtain HA-MSDOX-KLA via thiol-Michael chemistry.\(^{32}\) The prepared HA-MSDOX-KLA showed a critical micelle concentration (CMC) of 0.019 mg/mL (Figure S4). Once above this concentration, it readily self-assembled into micellar nanoparticles in aqueous solution with a surface comprised of tumor active targeting group of HA and a hydrophobic MSDOX core. As visualized by transmission electron microscopy (TEM) in Figure 2A, HA-MSDOX-KLA was well-dispersed and showed regular spherical shape with an average diameter of 38.2 ± 3.7 nm. With the stimulation of tumor overexpressed MMP, MMP-substrate peptide was specifically cut to trigger the expeditious release of DOX with peptide residue, which can self-assemble into drug nanofibers. This morphology changes was confirmed by TEM observation and dynamic light
scattering (DLS). As shown in Figure 2B, upon exposure to MMP, the spherical micellar nanoparticles were transformed to nanofibers with an average diameter of 30-40 nm and a length about 200-300 nm. Along with morphology change, the hydrodynamic diameter was increased remarkably (Figure 2C) and enlarged gradually with prolonged incubation time (Figure 2D). Particularly, HA-MSDOX-KLA nanoparticles were exceptionally stable at normal physiological condition (Figure 2D and Figure S5), which was beneficial to prolong circulation and enhance tumor accumulation as a result of active and passive tumor targeting effect. The above results demonstrated that HA-MSDOX-KLA showed specific MMP selectivity, and the morphology could be responsively transformed from micellar nanoparticles to fibrous nanostructure with the trigger of MMP.

Tumor-Targeted Accumulation and Morphology Transformation Triggered the Enhanced Drug Retention. To investigate the tumor active targeting capability and morphology transformation endowed long cellular retention effect, HA-MSDOX-KLA was incubated with MCF-7 (breast adenocarcinoma), MCF-7/ADR (multidrug resistant breast adenocarcinoma), and COS7 (kidney fibroblast) cell lines, respectively. As confirmed by western blotting analysis in Figure 3A and 3B, MCF-7/ADR cells exhibited much high expression of P-gp and MMP, while healthy COS7 cells had very low expression of P-gp and MMP. Since the overexpressed P-gp promoted chemotherapeutic drug efflux, nearly undetectable red fluorescence was found in free DOX incubated MCF-7/ADR cells (Figure 3C), but remarkable red fluorescence was observed in chemosensitive MCF-7 cancerous cells (Figure 3D) and COS7 healthy cells (Figure S6). In marked contrast, significant red fluorescence of DOX was detected in MCF-7 cells (Figure 3E) and MCF-7/ADR cells (Figure 3F) rather than COS7 cells (Figure S6) incubated with HA-MSDOX-KLA. The enhanced cellular uptake in CD44 receptor-positive tumor cells can be
attributed to the tumor active targeting moiety (HA) in HA-MSDOX-KLA. However, free DOX diffusively entered cells without selectivity and easily pumped out from MCF-7/ADR cells by the membrane protein of P-gp. Furthermore, flow cytometry was employed to evaluate the cellular accumulation of drug molecules. As shown in Figure 3G and Figure S7, the corresponding mean fluorescence intensity (MFI) of HA-MSDOX-KLA was increased 6.5-fold and 9.6-fold in MCF-7/ADR cells compared with that of free DOX for 6 h and 12 h, respectively. Conversely, MFI of free DOX was much higher than that of HA-MSDOX-KLA in COS7 cells, which were consistent with CLSM observations (Figure S6). These results validated that HA-MSDOX-KLA dramatically increased the endocytosis of DOX in MCF-7/ADR cells rather than in normal cells, which is beneficial to efficiently eliminate tumor cells while sparing normal cells.

Furthermore, the uptake mechanism and intracellular retention capacity of HA-MSDOX-KLA were investigated. As depicted in Figure 4A-4E, significant decrease in the uptake of HA-MSDOX-KLA in MCF-7/ADR cells was observed at 4 °C, demonstrating that the endocytosis of HA-MSDOX-KLA was indeed an energy-dependent process. As shown in Figure 4F and 4G, genistein (an inhibitor of caveolae-mediated endocytosis) and amiloride (an inhibitor of micropinocytosis) showed little effect on the uptake of HA-MSDOX-KLA than chlorpromazine (an inhibitor of clathrin-mediated endocytosis), implying that the receptor-mediated endocytosis was the primary cellular uptake pathway for HA-MSDOX-KLA, which could successfully bypass the recognition of efflux P-gp, and thus contributed to the increased uptake of DOX in drug-resistant tumor cells. Once HA-MSDOX-KLA was internalized by MCF-7/ADR cells, the overexpressed MMP specifically cleaved the peptide substrate and triggered the expeditious release of drugs, leading to the formation of drug nanofibers in cytoplasm. The in situ formed fibrous nanostructures were difficult to pump out by P-gp, resulting in long drug retention.
capability. As shown in Figure 5E-5H, little DOX was expelled by MCF-7/ADR cells and above 80% of drug was continuously remained inside cells after 24 h (Figure 5K). More surprisingly, drug molecules were observed to enter into nuclei after further 24 h incubation (Figure 5I and 5J), which would be benefit to potentiate the therapeutic effect since nucleus is the final target of DOX. On the contrary, free DOX was dramatically eliminated by drug-resistant MCF-7/ADR cells within 6 h (Figure S8 and Figure S9), resulting in limited intracellular drug below the therapeutic window. Moreover, bio-TEM was utilized to investigate the cellular internalization and in situ morphology switch of HA-MSDOX-KLA directly.\textsuperscript{34} As shown in Figure 6A and Figure S11, a multitude of HA-MSDOX-KLA nanoparticles were observed in MCF-7/ADR cells after 6 h incubation. When incubated for 24 h (Figure 6B), the transformed nanofibers as well as undeformed nanoparticles were intuitively displayed in the cytoplasm. Meanwhile, the damaged mitochondria with abnormal cristae and destructive morphology were observed as expected due to the release of proapoptotic peptide KLA in the morphology transition of HA-MSDOX-KLA, which induced the mitochondria-targeted damage.

\textit{In Vitro Anti-MDR Evaluation.} It is well known that the drug efflux implemented by P-gp transporter is an ATP-dependent behavior.\textsuperscript{3,35} The decreased generation of ATP would inhibit the drug efflux and increase the intracellular drug retention. As demonstrated by TEM observation (Figure 6), the internalized HA-MSDOX-KLA underwent the morphology transition and triggered the release of KLA to destroy mitochondria. The unusual morphologies of mitochondria were found obviously. Together with the drop of mitochondrial membrane potential as confirmed by JC-1 analysis (Figure S12), mitochondria in drug-resistant tumor cells were specifically damaged by the released KLA. Such a serious damage of mitochondria would lead to the decrease of intracellular ATP level.\textsuperscript{36} As displayed in Figure 7A and 7B, after the
HA-MSDOX-KLA treatment, the cellular ATP levels of MCF-7 cells and MCF-7/ADR cells were significantly decreased, but the cellular ATP level of COS7 normal cells did not change apparently (Figure S13). In contrast, free DOX showed little influence on the ATP production. Clearly, HA-MSDOX-KLA could specifically damage mitochondria to induce lower ATP level, which are expected to suppress energy-dependent drug efflux, and therefore promote the intracellular drug retention for bypassing the drug-resistant barriers.

Furthermore, to evaluate the efficacy of HA-MSDOX-KLA for overcoming MDR, its antiproliferative efficiency against MCF-7 and MCF-7/ADR cells was tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. As show in Figure 7C and 7D, the half-inhibitory concentration (IC_{50}) of free DOX for MCF-7 cells was 0.9 µg/mL, which remarkably increased to 8.3 µg/mL for MCF-7/ADR cells since free drug molecules are easily pumped out by overexpressed P-gp to provide resistance in chemotherapy. While the IC_{50} value of HA-MSDOX-KLA for MCF-7 cells was 2.9 µg/mL and 4.2 µg/mL for MCF-7/ADR cells. Thus, the MDR factor of HA-MSDOX-KLA was 1.5 (calculated as 4.2/2.9), which was much lower than that of free DOX (9.2, calculated as 8.3/0.9), validating a robust anti-MDR capability of HA-MSDOX-KLA (6.1-fold increased than that of free DOX). In addition, HA-MSDOX-KLA showed the negligible cytotoxicity to COS7 normal cells and much lower cytotoxicity than that of free DOX (Figure S14), which was beneficial to selectively kill tumor cells while reduce off-target toxicity to normal tissues. Moreover, as displayed in Figure 7E, 7F and Figure S15, HA-MSDOX-KLA induced apoptotic cell death selectively in both MCF-7 and MCF-7/ADR cell lines, whereas free DOX showed visible apoptosis of MCF-7 cells but not for MCF-7/ADR cells. The overexpressed P-gp of MCF-7/ADR cells increased the drug efflux and promoted tolerability to DOX, resulting in insignificant therapeutic efficacy. In contrast, HA-MSDOX-
KLA increased tumor cells uptake via the tumor active targeting moiety and overcame the drug-resistance by following the two-pronged effects: i) tumor-triggered morphology transition which elevated the retention effect of chemotherapeutic drug intracellularly; ii) KLA induced the targeted damage of mitochondria, causing the dysfunction of mitochondria with decreased ATP generation to inhibit the drug efflux effectively.

**In Vivo Anti-MDR Evaluation and Histological Analysis.** Encouraged by the favorable therapeutic effect of HA-MSDOX-KLA *in vitro*, *in vivo* experiments to assess the tumor-targeting ability and antitumor efficacy were performed in MCF-7/ADR tumor-bearing mice. To verify that the damage of mitochondria was beneficial to overcome drug resistance, HA-MSDOX without proapoptotic peptide KLA was used as a negative control. As displayed in Figure 8A, free DOX without selectivity was eliminated significantly after injection of 4 h. In contrast, both HA-MSDOX-KLA (t_{1/2} = 9.3 h) and HA-MSDOX (t_{1/2} = 8.6 h) showed remarkably prolonged blood circulation time, which provided the opportunity to accumulate in tumor sites. The quantitative analysis of DOX content in tumors demonstrated that HA-MSDOX-KLA significantly increased drug internalization and retention than that of HA-MSDOX and free DOX at each examined time point (Figure 8B). The accumulation of DOX in tumors after injection of HA-MSDOX-KLA were 3.9-fold, 11.5-fold, and 40.2-fold increased than that of free DOX at 6 h, 12 h, and 24 h, respectively. Moreover, the DOX content in tumors decreased over time in the free DOX treated mice, while the levels of DOX continuously increased in tumors treated with HA-MSDOX-KLA and HA-MSDOX. Particularly, the accumulated DOX in tumors of HA-MSDOX-KLA treated mice were notably more than that of HA-MSDOX, validating that the damage of mitochondria with reduced ATP is an effective strategy to decrease the drug efflux by drug-resistant tumor. As shown in Figure 8C, the highest fluorescence intensity of DOX was
found intuitively in the HA-MSDOX-KLA group by CLSM observation, indicating the outstanding capacity of HA-MSDOX-KLA to enhance drug accumulation and retention in the tumor site. As shown in Figure 8D, in comparison with PBS group, free DOX showed minimally suppressed tumor growth with a low tumor inhibition rate of 17.6%. HA-MSDOX treatment exhibited a moderate therapeutic effect with a tumor inhibition rate of 60.1%. In contrast, HA-MSDOX-KLA significantly inhibited tumor growth and dramatically improved antitumor efficacy with an optimal tumor inhibition rate of 79.6%. The superior treatment efficacy of HA-MSDOX-KLA was further confirmed by tumor weights at 16 d post-treatment (Figure 8E) and the representative tumor images (Figure S16). Taken together, HA-MSDOX-KLA preferably accumulated at tumor tissues and efficiently inhibited the drug-resistant tumor growth, demonstrating that the two-pronged strategy of nanomedicine morphology transformation and mitochondria dysfunction effectively reversed MDR in vivo.

Histological examination and immunohistochemistry analysis were further performed to investigate the in vivo antitumor mechanism. As shown in Figure 8F, no obvious destruction in tumor morphology was observed in either PBS control group or free DOX group, but a multitude of apoptotic and non-proliferative cells were detected in tumors treated with HA-MSDOX-KLA. The quantitative analysis of apoptotic cells (Figure 8G and Figure S17) in each group also verified that the tumor treated with HA-MSDOX-KLA had the maximum percentage of apoptosis (65.3%), and the therapeutic outcome was much superior to other groups (PBS: 2.6%; free DOX: 18.2%; HA-MSDOX: 45.3%). The analysis of activated caspase-3 in tumors by immunofluorescence assay suggested that HA-MSDOX-KLA kill tumor cells by activation of apoptotic cell death, which was 15.4-fold or 3.5-fold higher than that in PBS-treated or free DOX-treated tumors (Figure 8H and Figure S17). Additionally, western blotting was utilized to
evaluate the expression of apoptosis-related caspase-3 and Bcl-2 proteins in MCF-7/ADR tumors. The treatment of HA-MSDOX-KLA resulted in remarkably increased expression of caspase-3 and downregulation of Bcl-2, demonstrating that HA-MSDOX-KLA was highly effective at ablating drug-resistance tumor cells by triggering apoptosis (Figure 8I and Figure 8J). Collectively, these results confirmed that HA-MSDOX-KLA with programmed therapeutic effect had the ability to induce apoptosis of tumor cells and overcome multidrug resistance by enhancing the drug retention and inhibiting drug efflux.

**In Vivo Biosafety Evaluation.** In order to evaluate the potential in vivo toxicity of HA-MSDOX-KLA, the body weight of MCF-7/ADR tumor-bearing mice was measured. As shown in Figure S18, no significant changes of mice body weight was observed during the entire treatment period, revealing that HA-MSDOX-KLA did not cause detectable systemic toxicities to the mice. Moreover, no noticeable organ damages were detected by histological analysis (Figure S19), further confirming the favorable biocompatibility of HA-MSDOX-KLA. In addition, the examination of alanine aminotransferase, aspartate aminotransferase, and kidney function urea nitrogen validated that HA-MSDOX-KLA had almost no effects on liver and kidney functions (Figure 9A-9C). Hematological assessment (Figure 9D-9I) showed all the examined parameters after HA-MSDOX-KLA treatment were normal, with no significant difference compared with that of the control group. Altogether, HA-MSDOX-KLA displayed enhanced chemotherapeutic efficacy to overcome MDR with negligible side effects.

**CONCLUSIONS**

In summary, a tumor-triggered transformable nanomedicine with programmed therapeutic effect was designed and fabricated to overcome multidrug resistance essentially. The HA-
MSDOX-KLA nanoparticulates were beneficial to potentiate the tumor-specific accumulation of chemotherapeutic drugs and subsequently responded to tumor overexpressed MMP to trigger the morphology switch from micellar nanoparticles to drug nanofibers, which significantly elevated the intracellular drug retention effect. Moreover, the adjunctive release of proapoptotic peptide KLA would targetedly damage mitochondria to decrease ATP generation to further inhibit the drug efflux and prolong the residence time of drugs. This two-pronged strategy we developed remarkably improves the bioavailability of anticancer drugs and shows the superior therapeutic efficacy for anti-MDR with limited off-target side effects, which opens a new paradigm of designing transformable nanomedicine for combating drug resistance in chemotherapy.

ASSOCIATED CONTENT

Supporting Information. Details for materials preparation and additional experimental results. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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REFERENCES


Figure 1. Schematic illustration of the two-pronged strategy to overcome multidrug resistance by tumor-triggered transformable nanomedicine.
Figure 2. TEM image of self-assembled HA-MSDOX-KLA NPs A) before and B) after exposure to MMP, validating enzyme-triggered morphology transformation. C) Size distribution of HA-MSDOX-KLA NPs with or without MMP. D) Hydrodynamic diameter changes of HA-MSDOX-KLA NPs with or without MMP.
Figure 3. Western blotting analysis of P-gp and MMP among COS7, MCF-7 and MCF-7/ADR cell lines (A) and (B) the corresponding gray values. CLSM images of MCF-7/ADR cells (C) and MCF-7 cells (D) treated with free DOX for 6 h, respectively. CLSM images of MCF-7 cells (E) and MCF-7/ADR cells (F) treated with HA-MSDOX-KLA for 6 h, respectively. Scale bar: 30 µm. (G) The quantitative mean fluorescence intensity of corresponding intracellular DOX assessed by flow cytometry.
Figure 4. A-E) CLSM images of MCF-7/ADR cells incubated with HA-MSDOX-KLA at 4 °C (A) and various endocytotic pathway inhibitors: chlorpromazine (B), genistein (C), amiloride (D), and without inhibitors treatment as the control (E), respectively. F) Flow cytometry analysis of intracellular uptake of DOX after MCF-7/ADR cells incubated with HA-MSDOX-KLA at 4 °C and various endocytotic pathway inhibitors. The cells without any treatment were used as the control. G) The relative uptake efficiency of HA-MSDOX-KLA in MCF-7/ADR cells with different treatments.
Figure 5. A-D) CLSM images of MCF-7 cells (the cells were pre-treated with HA-MSDOX-KLA for 6 h) for additional incubation with fresh culture medium of 6 h, 12 h, 18 h and 24 h, respectively. E-H) CLSM images of MCF-7/ADR cells (the cells were pre-treated with HA-MSDOX-KLA for 6 h) for additional incubation with fresh culture medium of 6 h, 12 h, 18 h and 24 h, respectively. The nuclei are stained by Hoechst 33342. Scale bar: 20 µm. I) Fluorescence signals based on the white line in D. J) Fluorescence signals based on the white line in H. The red fluorescence is DOX and the blue fluorescence is Hoechst 33342. K) Statistical quantification of MCF-7/ADR intracellular retention DOX after treated with HA-MSDOX-KLA via software Image-J.
Figure 6. A) and B) Bio-TEM images of MCF-7/ADR cells treated with HA-MSDOX-KLA for 6 h and 24 h, respectively. The green “M” represents mitochondria. HA-MSDOX-KLA nanoparticles are highlighted by red circles and the transformed nanofibers are highlighted by blue circles.
Figure 7. Relative ATP levels of MCF-7 cells (A) and MCF-7/ADR cells (B) after treatment with free DOX and HA-MSDOX-KLA. Cytotoxicity of free DOX and HA-MSDOX-KLA against MCF-7 cells (C) and MCF-7/ADR cells (D). Statistical quantification of the apoptosis of MCF-7 cells (E) and MCF-7/ADR cells (F) with the treatment of 1) blank control, 2) free DOX for 24 h, 3) free DOX for 48 h, 4) HA-MSDOX-KLA for 24 h, and 5) HA-MSDOX-KLA for 48 h, respectively.
Figure 8. A) Pharmacokinetic profiles of DOX after intravenous administration of different DOX formulations (mean ± SD, n=3). B) Content of DOX in tumor tissue after intravenous administration of different DOX formulations at a dosage of 2 mg/kg (mean ± SD, n=3). C) Representative CLSM images of tumor tissues from MCF-7/ADR tumor-bearing mice after intravenous administration of different DOX formulations at 24 h. D) Relative tumor sizes of MCF-7/ADR xenograft tumor after treatment by different formulations. E) Representative image of the harvested MCF-7/ADR tumors and the average tumor weight after different treatment. Evaluation of the antitumor efficacy of each treatment modality by histological analysis: F) H&E
staining; G) TUNEL staining; H) activated caspase-3 staining. I) The apoptosis-related protein expression (caspase-3 and Bcl-2) in tumors evaluated by western blotting analysis. J) Quantitative determination of the relative protein expression from western blotting results.
Figure 9. Biological safety evaluation. Blood biochemistry data of the mice after 16 d treatment:

A) alanine aminotransferase (ALT), B) aspartate aminotransferase (AST), and C) kidney function marker urea nitrogen (BUN), respectively. Hematology analysis of the mice after different treatments: D) red blood cell (RBC), E) white blood cell (WBC), F) platelet (PLT), G) hematocrit (HCT), H) mean corpuscular volume (MCV), and I) hemoglobin (HGB), respectively.
TOC Graphic

[Image of a TOC graphic showing molecular structures and labels for efflux and inhibition]
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