ORIGINAL RESEARCH

Tumour-Targeted and Redox-Responsive Mesoporous Silica Nanoparticles for Controlled Release of Doxorubicin and an siRNA Against Metastatic Breast Cancer

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Methods: The tumour-taneted and repx-responsive mesoporous silica nanoparticles (MSNs) functionalised with D to aptamors (AS1411) as a co-delivery system was developed and investigated for a motential against metastatic breast cancer. Doxorubicin (Dox) was loaded onto the MrNs, where the 411 and a small interfering RNA (siTIE2) were employed as gately previous a moment to the MSNs with redox-sensitive disulfide bonds.

Reputs: The control of release of Dox and siTIE2 was associated with intracellular glutacione. As a fill mediated the targeted delivery of Dox by increasing its cellular uptake in meta the breast cancer, ultimately resulting in a lower IC50 in MDA-MB-231 cells (human breast cancer cell line with high metastatic potency), improved biodistribution in tumourbearing mic, and enhanced in vivo anti-tumour effects. The in vitro cell migration/invasion way and in vivo anti-metastatic study revealed synergism in the co-delivery system that suppresses cancer cell metastasis.

Conclusion: The tumour-targeted and redox-responsive MSN prepared in this study are promising for the effective delivery and controlled release of Dox and siTIE2 for improved treatment of metastatic breast cancer.

Keywords: drug delivery, mesoporous silica nanoparticles, DNA aptamer, controlled release, metastatic breast cancer

Introduction

Metastatic breast cancer accounts for a majority of deaths from breast cancer worldwide.¹ Whether metastatic breast cancer is the first diagnosis or a recurrence after treatment for early-stage breast cancer, it is rarely curable. Extensive evidence has shown that metastatic breast cancer response rates and response durations progressively decrease with increasing chemotherapy treatment.² Despite the remarkable development of novel diagnostic methods and therapeutic approaches, effective treatments for metastatic breast cancer are still

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© 2021 Zhuang et al. This work is published and licensed by Dove Medical Press Limited. The full terms of this license are available at https://www.dovepress.com/terms. by and incorporate the Creative Commons Attribution — Non Commercial (unported, v3.0) License (http://creativecommons.org/licenses/by-nc/3.0/). By accessing the work you hereby accept the Terms. Non-commercial uses of the work are permitted without any further permission for Dove Medical Press Limited, provided the work is properly attributed. For permission for commercial use of this work, please see paragraphs 4.2 and 5 of our Terms (https://www.dovepress.com/terms.php). limited.^{3,4} Recently, combinatorial therapeutic approaches that integrate anticancer drugs with RNA interference (RNAi) have drawn increasing attention for metastatic breast cancer therapy,⁵ and have posed significant challenges that need to be overcome in clinical practice. In clinical settings, combination chemotherapy, or the grouping of different therapeutic entities to exploit additive or synergistic effects, is commonly employed. This combination strategy should not only enhance therapeutic efficiency but also treat metastatic breast cancer using mechanistically different approaches, thereby enhancing therapeutic efficiency.^{6–9}

Doxorubicin (Dox) is a chemotherapy drug that is commonly used to treat both early-stage and metastatic breast cancer. Similarly, small interfering RNAs (siRNAs) have been widely explored for use in combination therapy over the last few decades.¹⁰ Recent studies have suggested that the expression level of TIE2, a tyrosine kinase receptor for angiopoietin-1 and -2, is associated with the metastasis of breast cancer.^{11–13} We previously reported that inhibiting TIE2 significantly reduces Dox-resistant breast cancer metastases.¹⁴ Hence, the co-delivery of Dox and siTIE2, an siRNA that targets TIE2 and inhibits its function, may provide an effective approach for the treatment of metastatic breast cancer by inhibiting the growth of tumours and reducing metastasis.

Owing to the low cytotoxicity and high rug-lo ding ability of mesoporous silica nanoparticle (MSN) that have been investigated as a nano-pictorn, the codelivery of drugs and siRNA.¹⁵⁻¹ Quring the ast few years, a variety of MSNs have been ccessfully developed for the co-deliver of anticant drugs and siRNA.¹⁹⁻²³ However, since studies confronted Dox leakage, which is problema because it decreases the delivery efficiency of the errso.²⁴ therefore scientists have also focused significant a potion eventing this problem, with the next company used method involving coating the MSN survey to prevent Dox leakage. Inspired by a study that used x as a gatekeeper of the MSN delivery system,¹⁸ we speculated that nucleic acids can also be employed to block cargo leakage. Moreover, MSN surfaces are readily functionalised with various molecules that can encapsulate therapeutic compounds or provide receptor recognition units.^{26,27} Consequently, in this study, we explored modifying MSN surfaces with two kinds of nucleic acid, including RNA and DNA, as a coating and for the concurrent controlled release of MSNs.

Herein, we report a tumour-targeted and redoxresponsive functionalised MSN nanosystem for the treatment of metastatic breast cancer. Specifically, we exploited novel Dox-loaded MSNs functionalised with disulfide bonds and with two different types of nucleic acid on their surfaces (Scheme 1): a DNA aptamer (AS1411) that serves as a cancer-targeting ligand,^{28,29} and an siRNA to inhibit TIE2 (siTIE2) and induce anti-metastatic effects.³⁰ AS1411, which was found to have a high affinity for nucleolin, was used to improve cancer-targeting ability.^{31,32} Moreover, functionalisation of the MSNs with both nucleic acids provides dox-responsive gatekeepers for the triggered releas of Dox with a cancer cells. Hence, functionalised MSNs n bind the cell surface of nucleolin – a *p* mbrane prote, the can migrate to the nucleus, and no vere ressed on the surface of rapidly proliferating cance ells - freeptor-mediated drug delivery the function sed MSNs are internalised by cancer cell high concentrations of intracellular redox redox glutathione (GSH), trigger the releve of siTIE2 and Dox. We demonstrate that the function lised MSN efficiently inhibit the progression of meta, tic breast ancer.

Maccials and Methods Materials

traethyl orthosilicate (TEOS, 99%), cetyltrimethylammonium bromide (CTAB, BioXtra, \geq 99%), (3-Mercaptopropyl) trimethoxysilane (MPTMS, 95%), and Pluronic[®] F-127 (F127, \geq 99%), 4, 4'-dithiodipyridin (DTDP, 98%), dithiothreitol (DTT, \geq 98%) were purchased from Sigma-Aldrich. And the other chemicals were purchased from Aladdin Chemical manufacturers (shanghai). The recombinant human nucleolin protein was purchased from Abcam. Doxorubicin hydrochloride (Dox) was purchased from Merck. The sequence of TIE2 siRNA was as following, 5'- CCAGGUAUAUAG GAGGAAATTUUUCCUCCUAUAUACCUGGTT-3'. The sequence of Aptamer AS1411 was as following, 5'-GGTGGTGGTGGTGGTGGTGGTGGTGGTGGTT-3'. All the thiol oligonucleotides were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China).

Cell Culture

MDA-MB-231 and HEK293T cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA). These cells were grown in DMEM (Gibico), which was supplemented with 10% (vol/vol)



Scheme I Schematic representation of planer-based cancer-targeted and redox-responsive MSN co-delivery Dox and siRNA. (A) The synthesis procedure for MSNsiRNA/Apt@Dox. (B) The pracelly trafficking of the nanoparticles and the release of siRNA and Dox within the cancer cells.

FBS (Gibi o), 1 pend line reptomycin, and 2 μ g/mL blasticit n at 37 C in a humidified atmosphere of 5% CO₂. Cell where harvested by treatment with 0.25% trypsin-EDTA (c. bico).

Synthesis of the Nanoparticles

The MSNs were synthesized via the method reported by Bouchoucha et al.²⁷ Briefly, 1.0 g of CTAB and 8 g of F127 were mixed in 85 mL of 100% EtOH and 213 mL of 2.9 wt % NH4OH solution in 500 mL conical flask. A 3.86 mL of TEOS was added slowly for 4 min by stirring at 500 rpm at room temperature. Then, the mixture was aged for 24 h under static conditions at room temperature. The surfactant was removed by methanol/ HCL (500:19 v/v) at 40°C for 12 h to remove the CTAB template. Then, the product was washed three times in methanol and water sequentially and dried in vacuum at 80°C overnight. The resulting NPs are designated as MSN. Next, 1.0 mL of MPTMS was added to bare particles (100 mg dissolved in 30 mL EtOH) for the production of MSN-SH. The reaction mixture was stirred for a further 24 h at room temperature. Then, the particles were separated by centrifugation (10,000 rpm, 5 min), washed with EtOH and water sequentially and dried in vacuum at 80°C overnight. Then, the DTDP (20 mg, 50 mg/mL, in EtOH) was added to MSN-SH (10 mg, 10 mg/mL, in EtOH) and the mixture was stirred at room temperature for 24 h. The pyridine-activated particles (MSN-Py) were collected by centrifugation and washed three times in methanol and water sequentially and dried in vacuum at 80°C overnight. Then, the 10 mg of MSNs-Py was dispersed in 1 mL of Dox solution (1 mg/mL) and stirred for 12 h at room temperature. Finally, thiol-functionalized oligonucleotides including siRNA and DNA aptamer were added into the solution to immobilized on the surface of MSN-Py@Dox to block the mesopores by a disulfide exchange reaction. The oligonucleotides were freshly activated before usage by the addition of DTT (60 µmol, 1 M), followed by subsequent purification with dextran desalting columns. The exchange reaction was carried out at room temperature for 24 h, followed by centrifugation at 10,000 rpm for 3 min and washed with water. The DNA-conjugated particles are denoted as MSN-siRNA/Apt@Dox.

Quantification of Immobilized Oligonucleotides

First, different amounts of aptamer-SH and siRNA-SH solution (0.1 mg/mL) and MSN solution (0.5 mg/mL) were mixed to obtain different weight ratios (MSN:Apt:siRNA) such as 5:1:1, 10:1:1, 15:1:1, 20:1:1 and 25:1:1. Gel retard tion assay was used to determine the best binding between MSN-Py and oligonucleotides. Then, the result arose gel electrophoresis were obtained and the or .mal re tion ratio was used to produce MSN-siRNA/Apt nd the of unreacted oligonucleotides was que fried from the supernatant by UV/Vis (260 nm) after dia the exchange action. At last, the amount of oligonucreotides onjugated on the particle surface was calculate a by equation:

Amount of oligonucleotide populate $n_{o} = \frac{n_{added} - n_{supernatant}}{n_{added}} \times 100\%$.

Where n_{added} is called a the radiust of added oligonucleotides, aring diallide exchange reaction.

Quantification of Loaded Dox

To determine the ding capacity of MSN-Py for Dox, the supernatant was collected after Dox loading and the amount of cargo remaining in the supernatant was quantified by UV/Vis (480 nm). The loading capacity of loaded Dox was calculated by equation:

Loading capacity (%) =
$$\frac{m_{added} - m_{supernatant}}{m_{added}} \times 100\%$$

Where m_{added} is calculated by the amount of added Dox during Dox loading.

Characterization of the Nanoparticles

The morphologies of MSN and MSN-siRNA/apt were investigated by transmission electron microscope (TEM) (JEM-2100F, JEOL, Japan) at an acceleration voltage of 90 kV.

The effective hydrodynamic diameters and the zeta potentials of MSN, MSN-SH, MSN-siRNA/apt were measured by DLS using the ZetaPlus' Zeta Potential Analyzer (Brookhaven Instruments, Santa Barbara, CA, USA).

Fourier transform infrared (FT-IR) spectra of MSN, MSN-SH, MSN-siRNA/apt were received by Nicolet 6700 FT-IR Spectrometer (Thermoreisher, UN).

The Raman spectra of MSN, M. I-SH, MSN, RNA/apt were measured by LabRAM, RAMIS NORIB, Scientific, France).

The porosity of N.N. was assessed by a nitrogen adsorption-desorption metarement. The measurement was operated to 77 K basis a TriStar II 3flex (Micromeritics, USA). The pore size distribution was determined from the mitrogen adsorption isotherms through the Barrett–Joyner–Halenda method.

the amount of siRNA and aptamer on the MSN surface were evaluated as following.

First, o.e. mg MSN-siRNA/Apt was resuspended with D⁺ compared water. After, the NPs were treated with 5 mM oSH for 6 h, the supernatant was separated by centrifugation. Then, the sample was divided into two parts and incubated with DNase I and RNase A separately for 30 min in 37°C. At last, the amount of nucleic acid adsorbed was calculated from the concentration of the nucleic acid of the supernatant, which was measured by UV–vis spectroscopy at 260 nm.

Release of the Cargos

The release profiles of Dox from the developed nanoparticles were obtained as follows. First, MSN-siRNA/apt@Dox were dispersed in PBS (pH 7.4) without or with different stimuli (20 mM GSH, and/or 1 µg Nuleolin protein). Subsequently, the mixture was incubated at 37°C and defined aliquots of the mixture (150 µL) were removed from the reaction tube at various time points (1, 2, 3, 5, 7, 9, 10 and 24 h). The removed aliquots were centrifuged and the concentration of Dox in the supernatant was measured by UV/Vis (480 nm). The cumulative release of Dox was calculated by the equation:

Cumulative Dox release(%) = $\frac{c_{supperant} \times V}{m_{tested}} \times 100\%$.

For control-release of siRNA from the developed nanoparticles was obtained as followed. First, GSH was added to MSN-siRNA/apt (PBS, pH 7.4) to reach a final GSH concentration of 0, 0.2 and 5 mM, and incubated for 12 h. Then, the samples were electrophoresed to detect the redox-triggered release of oligonucleotides. The anti-sense RNA siTIE2 and DNA aptamer AS1411T were used as controls.

Serum Stability of siRNA in MSN-siRNA/ Apt@Dox

For the assay, naked siRNA (300 ng/well siRNA), 6 mg (equivalent to 300 ng/well siRNA) MSN-siRNA/apt@Dox were incubated with 10% FBS at 37°C for 6 and 24 h. After treatment, the samples were collected and centrifuged. At last, the stability of siRNA was assessed by gel electrophoresis.

Cellular Uptake of MSN-siRNA/ Apt@Dox

For the cell-selectivity study, HEK293T cells and MDA-MB-231 cells were seeded in 12-well plate before the assay. After the adherence to the plate, the cells were incubated with different Dox-loaded MSNs (1 μ g Dox/well, by weight of Dox) for 8 h. After washing with PBS, the samples were fixed and stained with DAPI. The treated cell samples were observed by Olympus IX81. for each sample, the fluorescence was quarafied using the mean value of n > 3 pictures.

For the flow cytometry analysis, M. 4.4 B-231 certs were seeded in a 6-well plate refore the as w. After the adherence to the plate, the cent were incurated with different Dox-loaded M Ns (5 μ g u. x/well, by weight of Dox) for 8 h. The created cells were insed with PBS twice, trypsinized and fixed. The cells were re-suspended with PBS and determined by a PD FACSVerse flow cytometer (PD). The results were analyzed with Flowjo.

For the cells at accumulation study of MSN-siRNA /apt@Do. M. A-MB-251 cells were seeded in 12-well plate before the assay. After the adherence to the plate, the cells were acubated with MSN-siRNA/apt@Dox (5 µg Dox/well, by weight of Dox) for 2, 4, 8 h. The treated cells were stained with Hoechst 33342 for 20 min, rinsed with PBS twice and observed by Olympus IX81.

Pharmacokinetic Study

Pharmacokinetic date of Dox in female BALB/c mice (n=30, per group) using free Dox, MSN@Dox and MSN-siRNA/apt@Dox, which the dose of Dox is about 1 mg/kg,

via the tail vein. The treated animals were euthanized in indicated time points (at 0.166, 0.25, 0.5, 1, 2, 3, 4, 6, 8, and 24 h, after injection, 3 animals for each time point/ group). Blood plasma was collected and pre-treated by protein precipitation before LC-MS/MS detection. The pharmacokinetic parameters were calculated via PKSolver.

In vivo Biodistribution Study of MSN-siRNA/Apt@Dox

A 0.1 mL of MDA-MB-231 cell suspension $(1 \times 10^7/$ anate in the back of mouse) was orthotopically in female BALB/c nude mice sing 50% (v) Matrigel. The animals were purchase from Sharphai Model Organisms Center, In The experimental protocols were conducted within Shenzen University guidelines for animal research and per approve by the First Affiliated Hospital of denzhen Usersit Institutional Animal Care and Use com. ttee (IACU . Approval Number IACUC-DD-2019-07-07. mor volumes were monitored with ernier caliper even three days and calculated according b the formula: tumor volume (mm3) = $0.52 \times \text{length} \times$ idth2. When the tumor size reached around 200 mm3, the pimal were randomly assigned into 3 groups (n=3) and administrated with free Dox, MSN@Dox and MSNsin A/apt(a) Dox, which the dose of Dox is about 1 mg/kg, via the tail vein. Then, the mice were anesthetized for imaging by the IVIS spectrum (Xenogen, USA) at 0.5 h and 8 h postinjection. After 24 h treatment, all animals were sacrificed and the organs (liver, spleen, kidney, heart, lungs, and brain) and tumors were collected for ex vivo imaging. Finally, images were analyzed using Aura Imaging Software (https://spectralinvivo.com/software/).

Cytotoxicity Analysis and in vitro Tumor Inhibition Assay

The cytotoxicity of the developed nanoparticles was determined in HEK293T cells by a Cell Counting Kit (CCK-8) assay. The cells were seeded in 96-well plates before the assay. Then, the cells were treated with an increasing level of MSN-siRNA/apt@Dox for 24 h. The cell viability was measured as a percentage relative to untreated control cells.

CCK-8 assay was conducted to study the proliferation inhibitory efficacy of different Dox formulations on MDA-MB-231 cells. The cells were seeded in 96-well plates before the assay. Then, the cells were treated with a series of different concentrations of free dox, free dox+siRNA, MSN@Dox+siRNA and MSN-siRNA/Apt@Dox for 24 h. The cell viability was measured as a percentage relative to untreated control cells. Fifty percent cell growth inhibition (IC50) was calculated from curves constructed by plotting cell survival (%) versus dox concentration (µg/mL).

In vivo Antitumor Activity Study with MSN-siRNA/Apt@Dox

Female BALB/c nude mice were purchased from Shanghai Model Organisms Center, Inc. The experimental protocols were conducted within the Shenzhen University guidelines for animal research and were approved by the First Affiliated Hospital of Shenzhen University Institutional Animal Care and Use Committee (IACUC): Approval Number IACUC-DD-2019-07-24. The xenograft model bearing MDA-MB-231 tumors were established and randomized into four groups (n=5) when the tumors reach around 100 mm3. Then, the mice were treated with saline, free dox+ siRNA, MSN@Dox+siRNA and MSN-siRNA /Apt@Dox, which the dose of Dox is about 1 mg/kg and the dose of siRNA is about 0.5 mg/kg, every 4 days for 5 times via the tail vein. After the administration, the body weight of mice and tumor size were recorded. The orga (liver, spleen, kidney, heart, lungs, and brain) and turk tissues of treated-mice were collected at 20 days. Then, the tumors were weighed and all samples were .cu formalin for 24 h, followed by hematoxyli eosin &E) staining.

Knock-Down of TIE2 Cene Analysis

MDA-MB-231 cells were seried into a 6-hell plate. After the adherence to the place, the cells were treated with serum-free medium. Sen, the cells were treated with PBS, naked siRMA LipersiRNA Lipofectamine2000, d M. J-siRN /2, which the dose of Invitrogen), h and then the cells were replaced siRNA is for / with comple. culture medium. After another 36 h incubation, the pells were harvested and the protein samples were extracted for Western blot analysis. Total protein extracts were subjected to SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, the proteins were transferred to poly (vinylidene fluoride) (PVDF) membranes (Millipore). To block non-specific binding sites, the membranes were treated for 1 h with TBST (a mixture of Tris-buffered saline and Tween-20) containing 5% milk. Subsequently, the membranes were incubated with the primary antibody against TIE2 (ab221154, Abcam) or GAPDH (14C10, CST) for 1 h overnight at 4°C. After washing, signals were detected by HRPconjugated secondary anti-rabbit antibody and were visualized using ProteinSimple software.

In vitro Migration Analysis

The transwell migration and wound healing assays were used to evaluate the impact of the developed nanoparticles on cell migration. For transwell migration assay, MDA-MB -231 cells were seeded in a 6-well plate and pre-treated with PBS, naked siRNA, Lipo@siRNA //imfectamine2000, Invitrogen) and MSN-siRNA/apt which dose of siRNA is 1 µg for 24 h. Then, Cells were collected, resuspended in a serum-free medium and transferred into the upper transwell chaminers. The lower Ser was filled 97 with 10% FBS-containing cult c medium as the chemoattractant. After increation 1, 24 h, the digrated cells in the lower chamber are fixed here fixed here paraformaldehyde, stained with crystal jolet for 10 min, and then observed by an microsco. For the quantitative assay, the crys 1 violet staining cells were dissolved in 33% acetic acid and their all prbance was measured at 570 nm. For would healing a say, MDA-MB-231 cells were seeded in and cultured to form a tight cell monolayer. 6-well Tþ be cells were washed with serum-free medium after e cell monolayer scratching with a -µL sterile plastic pipette tip and treated with PBS, naked siRNA, ipo@siRNA (Lipofectamine2000, Invitrogen) and MSNsiRNA/apt, which the dose of siRNA is 1 µg for 24 h. Migrating cells at the wound front were analyzed using an inverted microscope at the indicated time.

In vivo Anti-Metastasis Study

Female BALB/c nude mice were purchased from Shanghai Model Organisms Center, Inc. The experimental protocols were conducted within the Shenzhen University guidelines for animal research and were approved by the First Affiliated Hospital of Shenzhen University Institutional Animal Care and Use Committee (IACUC): Approval Number IACUC-DD-2019-07-24. The female BALB/c nude mice were intravenously injected with 1×10⁶ MDA-MB-231 cells (containing luciferase), and allowed to establish metastatic tumors primarily in lungs for 2 weeks. Then, the mice were randomly divided into four groups, and treated with saline, free dox+siRNA, MSN@Dox+siRNA and MSN-siRNA/Apt@Dox, which the dose of Dox is about 1 mg/kg and the dose of siRNA is about 0.5 mg/kg, every 3 days for 6 times via the tail vein. The luciferase activity in each mouse was measured with an in vivo imaging system every 12 days and quantified using Aura Imaging Software (<u>https://spectralinvivo.</u> <u>com/software/</u>). Mice were killed on day 38 after the initial cell injection. The lung of the mice was collected, weighed and photographed on day 38. The number of metastatic nodules on the surface of the lungs was counted and the lungs were sliced and stained with H&E.

Results and Discussion Synthesis and Characterisation of MSN-siRNA/Apt@Dox

To successfully construct nanocarriers for the targeted intracellular co-delivery of siRNA and drugs, we designed a strategy to encapsulate siTIE2 and Dox within MSNs and improve nanoparticle targeting ability, as illustrated in Scheme 1. MSNs were synthesised based on previous reports.33,34 (3-Mercaptopropyl) trimethoxysilane (MPTMS), a thiol-bearing organosilane, was then introduced to the MSNs to modify their surfaces with SH groups to obtain MSN-SH. Next, Dox was loaded into the porous structure of MSN-SH through incubation prior to disulfide bond exchange. Finally, Dox-loaded M was capped with siTIE2 and AS1411 via disulfide h kers to obtain MSN-siRNA/Apt.

The successful preparations of MSNs and MSN-siRNA /Apt were confirmed by transmission electron microscopy (TEM) (Figure 1A and B), which revealed that the MSNs are spherical in shape with an average diameter of 90-110 nm and with nucleic acid nanoshells surrounding the MSN cores. Then, we investigated the nitrogen adsorption-desorption isotherms of the nanoparticles and found that the samples exhibited typical IV features according to IUPAC nomenclature (Figure 1C), indicating the well-defined mesoporous structure. And the pore size distribution curve, which was calculated according to the Barrett-Joyner (BJH) method (Figure 1D), suggested that the werage port izes of 2.9, 2.4 and 1.7 nm for MSN, MSN-SH, MSN-siR, A/Apt, respectively. The MSN-siRM Apt parties are larger than the unmodified MSNs s observer by dynaic light scattering (DLS), confirming the surjessful conjugation of siTIE2 and AS1411 (Figure 1E and ble St. Moreover, the negatively charged KNA od DNA ap. ner conjugated with MSN also contribute to the lower zeta potential of MSN-siRNA/Apt. The urier-transform init red (FT-IR) spectra of unmodified ISNs, MSI SH, and MSN-siRNA/Apt are shown in ure 1D. urface functionalisation was confirmed by , the Si-OH band at 971 cm^{-1} after incubation a a th MPTMS. The appearance of minor peaks at approximatery 2875 and 1420 cm⁻¹ is attributed to C-H asymmetric



Figure I Characterization of MSN-siRNA/Apt. (A) Transmission electron microscopy (TEM) images of the MSN, MSN-SH and MSN-siRNA/Apt. (B) Particle size distribution of the nanoparticles. (C) Nitrogen adsorption-desorption isotherms of the nanoparticles. (D) The pore size distributions of the nanoparticles. (E) FTIR spectra of the nanoparticles. (F) Raman spectra of the nanoparticles.

stretching and rocking vibrations, respectively, in MSN-SH and MSN-siRNA/Apt, respectively. The existence of -SH groups was identified by the characteristic Raman peak at 2580 cm⁻¹, which suggests that the -SH groups from MPTMS had been grafted onto the MSN surfaces. No -SH Raman signal was detected after conjugation with thiolated SH-siTIE2 and the SH-AS1411 (Figure 1F). The successful synthesis of MSN-siRNA/Apt is supported by the abovementioned results.

The Dox loading in the prepared MSNs was determined using fluorescence spectroscopy. The MSN loading was ~8.5% at a Dox incubation concentration of 5 mg/mL. Thiolated nucleic acid binding in MSN-SH@Dox was investigated by agarose gel electrophoresis (Figure S1), which revealed that an MSN/SH-siTIE2/SH-AS1411 ratio of 20:1:1 (w/w/w) resulted in almost complete nucleic acid binding. Most of the nucleic acids were typically immobilised on the surface of MSN-siTIE2/Apt@Dox with a high conjugation efficiency (90.7%). Moreover, we also measured the amounts of siRNA and aptamer on the MSN surface, which revealed that siRNA binds more easily to the MSN surface than the aptamer at the same mass ratio to form a denser nucleic acid coating (Figure S2). In addition, the stability of siRNA on the MSN surface was evaluated; however, the results show that MSN-siRNA /Apt only moderately protected siRNA from enzyme degradation over 6 h (Figure S3).

Release of siRNA and Dox

The microenvironments of tumour cells enable redoxresponsive nanocarriers to release loaded cargo in response to high levels of redox molecule over, redoxresponsive delivery systems are *r* in used to crease the concentration of a drug in the cytop, m, increas herapeutic efficacy, and reduce the oxicity of antincer drug. For example, redox-regionsive hivery systems with disulfide bonds facilities the rel se of ephapped drug molecules within two ur cells. ³⁶ The efore, the cleavable disulfide liph is attach side 2 and AS1411 to the MSNs should also fact the controlled release of Dox and si 1E2 in response to redox trigger from MSN-siRNA /apt Dox (Figure 2A). The functionalisation of MSNs with



Figure 2 Nanovalve gatekeeper of MSN-siRNA/Apt@dox and the control-release profiles of DOX and siRNA. (A) Schematic illustration of the nuclear acid nanovalve gatekeeper installed on MSN-siRNA/apt@Dox through cleavable disulfide bonds. Cargo can be released by either redox-responsive reductive cleavage or nucleolin-induced aptamer reconfiguration. (B) Release profiles of DOX from the cargo-loaded nanoparticles in the presence or absence of different stimuli. (C) Release of siRNA from the MSN-siRNA/Apt in the increasing level of GSH.

nucleic acids was found to efficiently block the release of Dox; ie, ~71% of Dox was released from MSN-SH@Dox after 24 h in phosphate-buffered saline (PBS), whereas only 8% of Dox was released from MSN-siRNA/Apt@Dox under these conditions. Dox was released from MSNsiRNA/Apt@Dox in the presence of nucleolin or GSH. AS1411 changes its conformation through interactions with cellular nucleolin, thereby enabling the release of Dox from MSNs and acting as a "nanovalve" gatekeeper. Although both GSH and nucleolin trigger the release of Dox (Figure 2B), only small amounts of Dox were released by nucleolin, which indicates that the encapsulated Dox cannot easily be released from the AS1411 gatekeeper by interacting with nucleolin. The amount of Dox released was significantly higher (80%) in the presence of 10 mM GSH and nucleolin; therefore, most of the Dox was only released in a cellular microenvironment with a high concentration of GSH and nucleolin. In addition, we investigated the drug release behaviour of MSN-siTIE2/Apt@Dox with different GSH levels (0, 0.1, 1, 10 mM). The release profiles reveal developed nanoparticles exhibit GSHthat our concentration-dependent Dox release behaviour (Figure <u>S4</u>). We also did not observe any cumulative release of siTIE2 in 0.2 mM GSH (Figure 2C). In contrast, the burst release of siTIE2 was observed in response to 5 mM GSH, which indicates that only high concentrations of GSH can cleave the disulfide bonds of the attached siTIE2 to obtain efficient siTIE2 release. With this in mind, we modified the disulfide bonds on the MSN surfaces to construct an appropriate redox-responsive delivery system for the controlled release of Dox and siRNA.

Cellular Uptake Study

The targeting specificity of the reveloped h. oparticles was investigated using MDA-MB-11 cells (nuc olin positive) and HEK293T cells (cleolin h ative) Recent studies have used the transport mechanism of cleolin (ie, migrance tion from the cell su the nucleus) to enhance intranuclear de¹ ery.^{37,38} Therefe , we speculated that the MSNs will specifically Jin ng AS1411 nucleolir direct the funct nalised MSNs toward cancer cells. mocal microscopy revealed that MSNs without S1411 functionalisation lacked cancer cell targeting abil-MSN-siTIE2/Apt@Dox and significantly ly



Figure 3 In vitro and In vivo evaluations of Dox delivery by developed MSN. (A) The cellular uptake of the nanoparticles between MDA-MB-231 cells (nucleolin positive) and HEK293T cells (nucleolin negative). (B) Intracellular fluorescence intensities of different Dox formulations determined by flow cytometry. (C) The merged images of MDA-MB-231 cells treated with MSN@Dox and MSN-siTIE2/Apt@Dox. (D) The cellular accumulation of Dox for MSN@Dox and MSN-siTIE2/Apt@Dox at 2, 4 and 8 h. (E) Plasma drug concentration (at a dose of 1 mg/kg body weight) of Free Dox, MSN@Dox and MSN-siTIE2/Apt@Dox Data show mean± SD (n=3). (F) In vivo fluorescent images of MDA-MB-231 tumor-bearing mice treated with different Dox-loaded nanoparticles via tail vein injection. Images were taken at 0.5 h and 8 h after injection. (G) The biodistribution of Dox in the organs after injection for 24 h in different groups.

increased the cellular uptake of MSNs in MDA-MB-231 cells (Figures 3A and <u>S5</u>). A flow cytometry assay was used to investigate the delivery of Dox in MDA-MB-231 using different Dox delivery groups (PBS, free Dox, MSN@Dox and MSN-siTIE2/Apt@Dox). Poor uptake of Dox by MDA-MB-231 cells was observed for free Dox (Figure 3B). MSN-siTIE2/Apt@Dox exhibited the strongest fluorescence in the MDA-MB-231 cells, suggesting that Dox is significantly better accumulated through incubation of the functionalised MSNs. In addition, MSN-siTIE2/Apt@Dox efficiently penetrated the MDA-MB-231 cells within 4 h and delivered Dox to the cell nuclei after treatment for 8 h (Figure 3C). Combined, these results indicate that MSN-siTIE2/Apt@Dox exhibited targeted delivery and high Dox accumulation in MDA-MB-231 cells.

Biodistribution of the MSNs in vivo

We first studied the pharmacokinetic characteristics of Dox (Figure 3D and <u>Table S2</u>), which revealed that Dox was rapidly released in all groups, and that the Dox level was predominantly higher in the Free Dox group. However, the concentration of Dox also decreased faster after free-Dox treatment compared with other groups. Our results demot strate that the AUC_{0-t} values of MSN@Dox and MSN siTIE2/Apt@Dox were higher than those of the other groups, which indicate that both MSN-based parver systems improve Dox accumulation. Moreover, the cloud

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circulation half-life of Dox in the MSN-siTIE2/Apt@Dox group was found to be 13.49 ± 3.68 h, which is longer than those of the free-Dox and MSN@Dox groups. Nude mice with MDA-MB-231 tumour xenografts were used to evaluate the in vivo biodistributions of free-Dox and Doxloaded MSN formulations. A significant difference in the Dox biodistribution was observed among the various delivery groups after intravenous administration (Figure 3E). A much higher fluorescence intensity of Dox was observed at primary tumour sites from MSN-siTIE2/Apt@Dox than from MSN-SH@Dox and free Dox 24 ulation in the bodies of mice for 8 h (Figure 3F The mice ere euthanized after 24 h, and the major vans (ie, h art, liver, spleen, lungs, and kidney and tunk rs we collected Ayse the accuand fluorescence image to quant atively mulation of Dox appng 20 Aerent groups. MSN-siRNA /Apt@Dox was vealed to accv fulated in tumours to a significant gr er extent win the other two groups (Figure <u>3G</u>), which demonstrates the high tumourng ability of our functionalised MSNs that enhance targe ng to cance cells and increase Dox accumulation in bin tably, quantitative analysis of the the umours. fluores isity of Dox present in the major organs led that MSN-siRNA/Apt@Dox led to less Dox accudation in the liver and kidneys than either free Dox or MSN-SH@Dox, both of which accumulated in the liver in oundance (Figure S6). MSN-siRNA/Apt@Dox enhanced



Figure 4 Cytotoxicity and the in vitro tumor inhibition effect of the developed nanoparticles. (A) The cytotoxicity of MSN-Apt/siRNA determined in HEK293T cells. (B) The viability of MDA-MB-231 cells treated with different Dox formulations.

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tumour permeability and redox-responsive release of Dox in tumours instead of vital organs.

In vitro Cytotoxicity Investigation

The viabilities of HEK293T cells treated with different concentrations of unmodified MSNs were investigated using the MTT assay, which revealed that MSN-siTIE2 /Apt displays little cytotoxicity against the normal cell line (Figure 4A). The IC₅₀ values of free Dox and Dox-loaded MSNs were evaluated in MDA-MB-231 to determine the proliferation inhibition of tumour cells in vitro. Treatment with different Dox formulations at various concentrations

for 48 h significantly affected the growth of MDA-MB-231 cells (IC50 of free Dox=1.901 µg/mL, IC50 of free Dox/free siTIE2 = 1.750 µg/mL; Figure 4B). Compared with free Dox and free Dox/free siTIE2, MSN-siTIE2/Apt@Dox exhibited enhanced antiproliferative activity, which may contribute to the larger accumulation of Dox in MDA-MB -231 cells by the MSNs. Furthermore, MSN-siTIE2 /Apt@Dox exhibited increased cytotoxicity compared to the other Dox formulations investigated, as illustrated by the IC₅₀ value of 0.433 µg/mL. A superior therapeutic effect on MDA-MB-231 cells was obtained by the MSN-siTIE2 /Apt@Dox nano-drug delivery ystem.



Figure 5 In vivo anti-tumor activity of different dox and siRNA-loaded nanoparticles on MDA-MB-231 tumor-bearing mice. (A) The tumor growth of the mice tested by different treatments. (B) The body weight of the mice tested by different treatments. (C) The images of solid tumors from the mice in each group after 20 days of treatment. (D) The tumor weight of the mice in different groups. (E) The H&E staining of the tumor in different groups.

In vivo Antitumour Efficacy

An MDA-MB-231 tumour-bearing mouse xenograft model was established to confirm the in vivo antitumour efficacies of the co-delivery systems. Because MDA-MB-231 cells are not sensitive to Dox treatment, free Dox/free siRNA did not suppress tumour growth when compared with the saline group (Figure 5A), and MSN@Dox+siTIE2 exhibited similar tumour inhibition to that of free Dox/free siTIE2. However, remarkable inhibition of tumour growth was observed with MSNsiTIE2/Apt@Dox. Furthermore, injection of MSNsiTIE2/Apt@Dox did not affect the body weights of the mice, whereas free Dox/free siTIE2 led to lower body weights during the early stages of treatment (Figure 5B), indicating that the functionalized MSNs are not systemically toxic. At the end of the animal study, the tumours were removed from the mice and weighed to further investigate tumour inhibition. MSNsiTIE2/Apt@Dox displayed the best tumour growth inhibition compared to the other groups (Figure 5C and D). Sections of the major organs and tumours were analysed by H&E staining; no obvious morphological damage to the major organs in most of the groups was observed, with only a minor change the liver observed in the free Dox+siTIE2 group, sug gesting that the functionalised MSNs improv bio-TE2 compatibility of free Dox (Figure S7) **⊿**SN-s /Apt@Dox presented large necrotic ions ler gro tumour when compared with the (Figure 5E). Considering these find MSN-TIE2/ 4 Apt@Dox is promising for tumour hibition against MDA-MB-231 cells and le suppression f breast cancer metastasis.

In vitro Celemeration and Invasion Experiments

Since inhibit per TIE2 was found to result in inhibition of cancer of migration and tumour invasion in our previous findings,^{39,40} the anti-metastatic effect of MSN-siRNA/Apt was assessed in vitro. Notably, Dox was found to impact cell migration and invasion in previous studies;^{41,42} consequently, Dox was not loaded into the siRNA formulations in our in vitro antimetastasis study. First, the TIE2 protein expression among different treatments was investigated to determine the knockdown effect of siTIE2. The negative control, naked siTIE2, did not impact the expression of TIE2, while positive siTIE2 transfection with Lipo 2000 effectively inhibited expression (Figure 6A). Furthermore, the expression of TIE2 was dramatically reduced by MSN-siRNA/Apt@Dox, which confirmed that the functionalised MSNs knockdown TIE2 expression. We found that the number of invading cells was sharply lower after MSN-siRNA/Apt treatment (Figure 6B and C). Furthermore, the migration capacity of MDA-MB-231 cells was significantly suppressed by Lipo@siTIE2 (liposomes carrying siTIE2) and MSN-siRNA/Apt (Figure 6D and E). Conservently, our functionalized MSNs exhibited both Liver cells wasion and migration.

In vivo Anti-Meta atic Effect

A pulmonary metasta, mode was established to evaluate the anti-r tastash outcom of MSN-siRNA the initia in avenous injection of /Apt@Dox. MDA-MB-231-Luc yells in mice, Dox was administered different ox formulations every three day from day 14 onwards. In vivo bioluminescence imating was the employed to monitor metastasis of each ox formy tion on days 14, 26, and 38 after cell found that the intensities of the biolumiinjection. signals of MSN@Dox+siTIE2 slightly ne ecreased during tumour metastasis in mice (Figure A and B); however, its anti-metastatic ability was milar to that of the free Dox+siTIE2 treatment. The difference in biodistribution between MSN-SH and MSN-siTIE2/Apt might contribute to the difference in anti-metastatic ability. Accordingly, since MSN-siTIE2 /Apt@Dox accumulated more in tumours, the functionalised MSNs exhibited a remarkable reduction in tumour metastasis. Metastatic nodules in the lungs were also identified to characterise the anti-metastatic ability of MSN-siRNA/Apt@Dox (Figures 7C and S8). Metastatic nodules were obvious in the saline and free Dox+siRNA groups, while MSN-siRNA/Apt@Dox displayed a lower degree of tumour metastasis. H&E staining further verified the anti-metastatic ability of MSN-siRNA/Apt@Dox, with fewer metastatic foci present in the lungs (Figure 7D). The functionalised MSNs clearly suppressed the pulmonary metastasis of MDA-MB-231.

Conclusion

We demonstrated that our functionalised MSNs are effective as a nanomedicine with both chemotherapeutic and





gene therapeutic actig against metas ic breast cancer, amour redoxthereby providing responsive release. ar design the tumour-targeted MS selectively transported delivery of onal pecific Dox to Ils, which greatly enhanced the therafcar against MDA-MB-231 cells. peutic -01 Furthermore the DNA aptamer AS1411 and the siRNA (siTIE2) nucle, acids attached to the surfaces of the MSNs through disulfide bonds act as gatekeepers that prohibit the leakage of Dox into circulation and prevent the degradation of siTIE2 molecules. Our co-delivery system was, therefore, able to inhibit the growth of MDA-MB -231 cells and downregulate TIE2 expression, and

exhibited outstanding synergism for treatment of metastatic breast cancer. The functionalized MSNs presented in this study provide a promising strategy that combines chemotherapy with gene therapy for the treatment of metastatic breast cancer, a type of cancer that is difficult to treat clinically and which benefits from multi-mechanistic treatment methods.

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Figure 7 Anti-metastasis effect of MSN-siRNA/Activity. (A) The benchminescent images of the pulmonary metastasis models after tail vein injection of MDA-MB-231 cells in different groups at day 14, 26, and 38. (B) 7 year ative bioluminescent intensity in different groups. (C) The images of lung collected from the pulmonary metastasis models treated with different formulations, the yellow are use indicate the metastatic nodules. (D) Typical histopathologic examination of lungs in different groups, the areas within the yellow circles are the area of cell necrosis, indicate the presence of tumor cell infiltration.

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Disclosure

The authors declare no competing interests.

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