Fabrication of magnetic nanochains linked with CTX and curcumin for dual modal imaging detection and limitation of early tumour

Yuedi Yang¹ | Zhongbing Huang¹ | Ximing Pu¹ | Guangfu Yin¹ | Lei Wang² | Fabao Gao²

¹College of Materials Science and Engineering, Sichuan University, Chengdu, China

²Department of Radiology, Molecular Imaging Center, West China Hospital of Sichuan University, Chengdu, China

Correspondence

Zhongbing Huang, College of Materials Science and Engineering, Sichuan University, No. 24, South 1st Section, 1st Ring Road, Chengdu, 610065, China.

Email: zbhuan@scu.edu.cn

and

Fabao Gao, Molecular Imaging Center, Department of Radiology, West China Hospital of Sichuan University, No. 2, 4th Keyuan Road, Chengdu, 610093, China. Email: gaofabao@yahoo.com

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Abstract

Objective: Five-year survival rate at early lung tumour was about 70%; however, its early diagnosis rate was still at a low level, so the enhancement of diagnosis level for early lung tumour is the key factor to increase the survival rate. Diagnosis and therapy of early lung tumour are still challenged.

Methods: The magnetic nanochains (NCs) with biocompatibility and transverse relaxivity ($r_2 = 231 \text{ Fe mmol l}^{-1} \text{ s}^{-1}$) were fabricated through a co-precipitation method in the assistance of dextran, and then, linked with chlorotoxin (CTX) and curcumin (Cur) via the PEGylation and carbodiimide technique (named as CTX-NCs-Cur).

Results: The results of cell test indicated that CTX-conjugated NCs could obviously target non-small-cell lung cancer cells and limit their growth. The *in vivo* results of magnetic resonance imaging and fluorescence imaging indicated that the CTX-NCs-Cur significantly targeted the tumour site and enhanced images contrast of the small-size tumour. Moreover, the results of everyday tail-vein injection confirmed that CTX-NCs-Cur could significantly limit the growth of early tumour, due to blocking Cl ion channels from CTX-NCs-Cur-MMP-2 composite and intracellular ROS increase from Cur treatment.

Conclusions: We provided a mechanism about the effect of CTX-NCs-Cur on the targeting and limiting early tumour, and these results indicated the application foreground of CTX-NCs-Cur in tumour diagnosis and therapy.

1 | INTRODUCTION

Lung cancer is the most general tumour with highest morbidity and mortality,¹ and 5-year survival rate of the lung cancer patients is less than 15%.² Although the traditional surgical resection, radiotherapy and chemotherapy could cure some patients, lung cancer was difficult to be cured due to its high invasive nature, rapid growth rate and the incomplete resection.³⁻⁵ Recently, drug-loaded magnetic nanoparticles (MNPs), as the T_2 -enhanced agent of magnetic resonance imaging (MRI), were used for the tumour therapy,⁶ because MNPs could passively target the *in vivo* tumour site to enhance the

contrast of MRI.⁷ The surface modification of MNPs could improve their biocompatibility and change their bio-distribution.⁸ Dextran (Dex)-coated iron oxide nanochains (NCs) could further enhance the T_2 -weighted contrast of MRI, compared with Dex-coated MNPs, due to higher accumulation effect of MNPs from Dex-coated NCs on the targeting site.⁹

In general, more than 75% of MNPs were removed by reticuloendothelial system from the blood and some organs, and about 80%-90% of MNPs were accumulated in the liver,¹⁰ leading to their lower efficiency of targeting tumour. So it is necessary to enhance their targeting efficiency via increasing their *in vivo* circulation time.

Polyethylene glycol (PEG), a hydrophilic macromolecule chain, could increase the circulation time of NMPs in blood through decreasing the protein adsorption from the blood.¹¹ Moreover, Dex-coated NCs were linked with the functionalized PEG, thus increasing the NCs ability to conjugate other functional molecules.¹²⁻¹⁴

Matrix metallo-proteinases-2 (MMP-2) was a kind of highly conserved enzyme, and several cancer cells could significantly high-expressed MMP-2, such as melanoma,¹⁵ glioma,¹⁶ lung cancer and breast cancer.¹⁷ It has been found that human lung adenocarcinoma cells (A549) in early tumour could excrete MMP-2 into the local extracellular matrix.¹⁸ Chlorotoxin (CTX), as Cl-substituted toxin of a small peptide (containing 36 amino acids and 4 disulphide bonds) from the venom of the scorpion *Leiurus quinquestriatus*,^{19,20} was specifically bound to MMP-2, forming the complex of CTX-MMP-2, which could block the chloride ion channels of tumour cells to limit the tumour activity,^{21,22} due to the Cl ion channel affinity of this Cl-substituted toxin.²³ However, there was only lower MMP-2 expression from normal cells, so the Cl ion channel affinity of CTX hardly affected the activity of normal cells.

As one component from spice turmeric, curcumin (Cur) is natural polyphenolic compound with low toxicity and could produce the green fluorescence under the excitation light of 425 nm of wavelength.²⁴ Cur was used as the anti-inflammatory, antioxidant or anti-cancer drug, because it could induce cells to produce reactive oxygen species (ROS) in their mitochondria,²⁵ leading to the apoptosis of tumour cells.²⁶ However, the clinical application of Cur was still limited due to the low aqueous solubility ($<1 \mu\text{g mL}^{-1}$), rapid metabolism/removal of Cur.²⁴⁻³⁰ If CTX can be combined with Cur, under the help of special targeting effect of CTX to the early lung tumour, Cur will enhance the level of ROS in the mitochondria of the cancer cells, which limits cell activity. So it is necessary to improve the Cur bio-availability to achieve its potential application in the early tumour treatment.

In this work, the novel PEG-linked NCs were prepared by a co-precipitation and PEGylation methods, and then conjugated with Cur (named as Cur-NCs) via carbodiimide technique to render their anti-cancer; subsequently, Cur-NCs were connected with CTX by N-succinimidyl-4-maleimidomethyl cyclohexane-1-carboxylate (SMCC) to render NCs the active targeting behaviour. The cytotoxicity, targeting ability, imaging enhancement and anti-cancer of the prepared CTX-NCs, Cur-NCs and CTX-NCs-Cur were investigated.

2 | MATERIALS AND METHODS

2.1 | Materials

Ferrous chloride ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$), ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), *N,N'*-dicyclohexyl carbodiimide (DCC), 4-dimethylaminopyridine (DMAP), ethylene diamine tetra-acetic acid and dimethylsulphoxide (DMSO) were purchased from Kelong Chemical Reagent Factory (Chengdu, China). SMCC was from Highfine Biotech (Suzhou, China), and dextran (molecular weight 40 000 Da) was from Aladdin (Shanghai, China). Carboxymethyl- and

succinimide-modified PEG (SC-PEG-CM, $M_w = 3400$ Da) was purchased from JenKem Technology Co. Ltd. Cur was from Sichuan Victory Biological Technology Co. Ltd. (Chengdu, China). CTX (molecular weight 4602.5) was got from GL Biochem (Shanghai, China) and labelled with fluorescein isothiocyanate (FITC) in order to analyse the conjugation efficiency of CTX on NCs.

2.2 | Preparation of magnetic NCs

Nanochains were prepared via the co-precipitation, according to the previous report.⁹ Three grams of dextran, 0.25 g of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ and 0.63 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ were totally dissolved in 10 mL of de-ionized water; subsequently, the ammonia of 1 mL (~26%) was slowly added into the above solution, then the mixed solution was cured at 75°C for 1 hour in N_2 . The obtained suspension was centrifuged at 7000 g and rinsed 3 times with de-ionized water to remove fine MNPs. Finally, the obtained NCs were re-dispersed in de-ionized water and ultracentrifuged 3 times with 100 kDa of molecular weight filtering column (Amicon, Millipore Corporation Darmstadt, Germany) to remove the unbound dextran.

2.3 | Conjugation of CTX and Cur

Nanochains were aminated with epichlorohydrin (ECH) and ammonia water (~26%), respectively, as described in previous report.³¹ Briefly, 3 mL of NCs suspension (~40 w/v %), 5 mL of NaOH solution (5 mol L^{-1}) and ECH were mixed completely under vigorous agitation for 8 hours; then, 20 mL ammonia water was slowly added into the above suspension and reacted for 16 hours at room temperature. Finally, the suspension was centrifuged at 3000 g for 30 minutes, and rinsed 3 times with de-ionized water to obtain the aminated NCs (NH_2 -NCs), and these NCs were re-dispersed in de-ionized water of 15 mL.

In order to link CTX, 2 mg of SMCC was first dissolved in 1 mL of PBS buffer with 5 mmol L^{-1} of ethylene diamine tetra-acetic acid, and then 200 μL of NH_2 -NCs suspension (~40 w/v %) was added into above SMCC solution under magnetic agitation and cured at 4°C for 2 hours. Subsequently, the suspension was ultra-filtrated 3 times to remove the un-reacted SMCC, and SMCC-NCs were obtained. One milligram of CTX was dissolved in 0.5 mL of DMSO, and then DMSO with CTX was added into SMCC-NCs suspension at 4°C, and reacted for 24 hours to obtain CTX-linked NCs (CTX-NCs).

In order to link Cur with NCs, 3 mg of SC-PEG-CM ($M_w = 3400$ Da) was first dissolved in 1 mL of DMSO; then NCs (or CTX-NCs) were added into SC-PEG-CM solution under the mild agitation and reacted for 6 hours at 4°C to obtain PEG-linked NCs (or PEG-linked CTX-NCs) suspension. Second, 2 mg of DCC, 0.4 mg of DMAP and 1 mg of Cur were dissolved in 1 mL of DMSO; subsequently, the above DMSO solution was added into PEG-linked NCs (or PEG-linked CTX-NCs) suspension and reacted at 4°C for 12 hours. Finally, the obtained suspension was centrifuged at 3000 g for 30 minutes to obtain Cur-NCs (or CTX-NCs-Cur).

2.4 | Characterization of NCs

The shape of NCs was observed with transmission electron microscope (TEM, JEOL JEM-100CX, Japan). To determine their iron content, the NC samples were dispersed into the hydrochloric solution (5 mol L^{-1}), and then solution were analysed with inductively coupled plasma atomic emission spectrometer (ICP-AES, SPECTRO ARCOS, Germany). The chemical composition of NCs was analysed by Fourier transform infrared (FT-IR) spectra (Thermo Nicolet 81 Wyman Street, Waltham, Massachusetts, USA Smart-380 FTIR spectrometer). The crystal structure of NCs was characterized with a X-ray diffraction analyser (Dandong Fangyuan Dandong, Liaoning, China DX-1000) following Cu K α radiation ($k = 1.5418 \text{ \AA}$, 40 kV, 80 mA) with a step size of 0.06° . The dextran percentage in NCs was analysed with the thermo-gravimetry by TG/SDTA851e analyser (METTLER TOLEDO Co, Zurich, Switzerland) in N_2 at the heating rate of $10^\circ\text{C min}^{-1}$ in the range of 20–1000°C. The magnetization curve of NCs was investigated with vibrating sample magnetometer (VSM, Lake Shore-7410, Columbus, Ohio, USA). The conjugations of CTX and Cur with NCs were analysed with a fluorescence spectrophotometer (F-7000, Hitachi Tokyo, Japan).

2.5 | Cytotoxicity assays

Human non-small-cell lung cancer (A549 cells) or human umbilical vein endothelial cells (HUVECs) were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco Co., 81 Wyman Street, Waltham, Massachusetts, USA), supplemented with 10% newborn calf serum (Gibco) and antibiotics penicillin (100 IU mL^{-1})/streptomycin (100 mg mL^{-1}) in a humidified atmosphere with 5% CO_2 at 37°C . To analyse the cytotoxicity of NCs, Cur-NCs, CTX-NCs and CTX-NCs-Cur, cells were seeded into 96-well plate (BD Biosciences, Franklin Lakes, NJ, USA) at the density of 4×10^4 cells per well, and incubated with different concentrations (from 25 to $200 \mu\text{g mL}^{-1}$) of these NCs for 12, 24 and 36 hours. Subsequently, $20 \mu\text{L}$ of MTT solution was added into these cell suspensions for further incubation of 4 hours. After the cells were washed 3 times with PBS, $150 \mu\text{L}$ of DMSO was added into each wells; finally, the absorbance of solubilized formazan at 490 nm was measured under Microplate Reader 3550 (Bio-Rad Hercules, California, USA). Cell viability was calculated as follows:

$$\text{Cell viability(\%)} = \frac{\text{optical density (OD) of the treated cells}}{\text{OD of the control cells}} \times 100$$

All experiments were performed in triplicate.

2.6 | In vitro and in vivo MR imaging

A 7.0 T of MR imaging system (Bruker biospec 70/30 USR Billerica, Massachusetts, USA) was used to obtain T_2 -weighted MR imaging and relaxation time *via* the multi-slice and multi-echo sequence. NCs suspension was diluted with PBS solution into the 1.5 mL centrifuge tubes with different concentration of iron ions (0.12, 0.25, 0.50,

1.00, 2.00 and 4.00 mmol L^{-1}), and then these tubes were scanned in the MR imaging system with the following observation conditions: a field of view of $4 \times 4 \text{ cm}^2$, the slice thickness of 2.0 mm, the number of excitation of 4, the image size of 256×256 , a value of repetition time of 180 ms and echo time of 6.0 ms.

BALB/c nude mice were purchased from the laboratory animal centre of Sichuan University. Animals were well tended based on the guidelines of the Institution Animal Care and Use Committee at Sichuan University. These experiments about animal were carried out according to the related laws, ethical committee and guidelines of the Institution Animal Care and Use Committee at the laboratory animal central. In order to analyse the contrast enhancement of the in vivo MR imaging in the tumour site, tumour-bearing nude (TBN) mice of A549 models were fabricated *via* the subcutaneous injection of A549 cell suspension in the pygal and 1 week feeding until the tumour size was close to 5 mm, which was as the early tumour model.⁵ To evaluate the targeting tumour property of different NCs (the concentration was $2.5 \text{ mg Fe mL}^{-1}$ PBS), TBN mice were injected with a dose of $5 \text{ mg[Fe]/kg[mice]}$ *via* tail vein, then T_2 -weighted images of these mice were obtained in 7.0 T MR imaging system at different time of post-injection of 4 NCs (NCs, Cur-NCs, CTX-NCs and CTX-NCs-Cur), including 0 hour (pre-injection), 3 and 8 hours. The mice were anesthetized *via* 1.5%–2.5% isoflurane mixed with pure oxygen, and the MRI image of pre-injected tumours was used as the controls. The brightness of tumour site in a serial of MRI images was measured by the soft of MATLAB (Newark, Massachusetts, USA) 7.0, and the lower the tumour brightness is, the stronger the T_2 -weight signal in tumour site.

2.7 | Bio-distribution of NCs

Bio-distribution test of the NC samples was performed with 20 male nude mice of 4-week-old, and these mice were randomly divided into 5 groups, of which 4 groups injected *via* tail vein of an equivalent dose of $5 \text{ mg[Fe]/kg[mice]}$ for 25 days with pure NCs, Cur-NCs, CTX-NCs and CTX-NCs-Cur, respectively. Saline were daily injected in the mice of control group *via* tail vein. After 25 days, mice of each group were killed, and the heart, liver, spleen, lung, kidney, brain and tumour were collected, then a part of different organ were treated with hydrochloric solution (5 mol L^{-1}) of 5 mL, and finally the iron ion content in each treated solution was measured with ICP-AES. The solution of treated organs from the nude mice without the injection of NCs was used as the control group, and inherent iron ion content of each organ was calculated as the control value, and the percentage of test groups was from injected NCs/g wet tissue (% ID/g).

2.8 | Cellular target in A549 cells

A549 cells co-cultured with NC samples were observed under a fluorescent microscope after Prussian blue stain, which could react with the iron ions to show the blue. A549 cells were planted in 96-well plate (BD Biosciences) and cultured for 24 hours to reach the

confluence of 70%-80%, and rinsed 3 times with PBS. Subsequently, cells were co-cultured with the different NCs ($200 \mu\text{g mL}^{-1}$ of NCs dispersed in DEME) for 24 hours, then washed 3 times with PBS and incubated with 4',6-diamidino-2-phenylindole (DAPI, Sigma, $10 \mu\text{g mL}^{-1}$) for 3 hours, and finally cells were observed under the inverted microscopy (IX-71, Olympus Shinjuku Monolith, 3-1 Nishi-Shinjuku 2-chome, Shinjuku-ku, Tokyo, Japan). The fluorescence intensity of A549 cells images were analysed with Matlab system, and the averaged intensity value was calculated from the 20 points in images of each group.

2.9 | ROS assay

The cellular ROS level in A549 was analysed with the fluorescent probe 20, 70-dichlorofluoresce in diacetate (DCFH-DA), according to the following steps. A549 cells were planted in 96-well plate and cultured with 4 NCs of different NCs concentration

(from 25 to $200 \mu\text{g mL}^{-1}$) for 6 and 24 hours, respectively; then cells were washed 3 times with PBS, subsequently each well was added with DCFH-DA ($10 \mu\text{mol L}^{-1}$) and cultured for 20 minute at 37°C in the dark. After the cells were washed 3 times with fresh culture medium, the cellular ROS was quantitatively analysed by enzyme-linked immunosorbent assay (ELISA kit, R&D Minneapolis, Minnesota, USA), through measuring the absorbance at 405 nm of wavelength with Microplate Reader 3550 (Bio-Rad).

2.10 | In vivo fluorescence imaging

In order to obtain the *in vivo* fluorescence imaging, TBN mice were injected with 4 NCs (NCs, Cur-NCs, CTX-NCs and CTX-NCs-Cur, 2.5 mgFe mL^{-1} of PBS, a dose of 5 mgFe kg^{-1} of mice weight), respectively, and the mice were anesthetized by the intra peritoneal injection of $200 \mu\text{L}$ chloral hydrate (10 wt%) at 10 hours of post-injection,

FIGURE 1 A, B, TEM images of NCs and PEG-NCs, and the inset is the image of magnified part. C, FT-IR spectrum of NCs. D, TG curves of NPs and NCs. E, X-ray diffraction pattern of NCs. F, Magnetization curve of NCs at room temperature, and the inset is the magnetization curve of zero field. G, A series of T_2 -weighted MR images of NCs with different Fe concentration. H, T_2 -relaxation rates as a function of iron concentration

and then the fluorescence images were obtained with a Maestro In-Vivo Imaging System in a dark room under the excitation of a blue light ($\lambda = 455$ nm). The fluorescence intensity of *in vivo* images was analysed with Matlab system, and the averaged intensity value was calculated from 10 points of images of each group.

2.11 | Limitation of NCs on tumour

Therapeutic effect of the as-prepared NCs was analysed with the *in vivo* experiments, in which TBN mice with 5 mm diameter of tumour were used as the animal model with early tumour.⁵ All mice were administrated for 25 days with pure NCs, Cur-NCs, CTX-NCs and CTX-NCs-Cur (100 μ L per day, 2.5 mg mL⁻¹), respectively. Anti-tumour effect of 4 kinds of NCs was evaluated *via* the measurement of the volume of tumour in 25 days.

Subsequently, the mice of each group were killed, and the heart, liver, spleen, lung and kidney were collected and stored at 4°C, then a half of each organ was digested through the mixed acids (nitric acid:perchloric acid, v:v = 3:1) for 1 hour, and another half of each organ was fixed in 4% phosphate-buffered paraformaldehyde overnight to prepare the haematoxylin-eosin (HE)-stained tissue samples, which were observed under the inverted microscopy (IX-71, Olympus).

3 | RESULTS

3.1 | Composition, structure and property of NCs

TEM images in Figure 1A,B show that NCs consisted of iron oxide cores with average diameter of 6 nm, and the mean lengths of NCs and PEG-NCs were 60 and 110 nm, respectively, indicating that the conjugation of PEG slightly increased the NCs length. HR-TEM images in Figure S1 show that 0.29 nm of the fringe spacing in one core was equivalent to the (2 2 0) lattice plane of Fe₃O₄. FT-IR spectra in Figure 1C show that the typical peaks at around 549 and 621 cm⁻¹ were assigned to Fe-O bond, and the peaks at 1654 and 3422 cm⁻¹ were the absorption peak of H₂O. The peaks at 2925 and 1370 cm⁻¹ were the absorption of C-H bond, and the obvious peaks at 1017, 1459 and 1154 were well matched with bending mode of C-O, C-H, C-OH bonds, indicating the existence of Dex. TG curves in Figure 1D show a 2-stage weight loss of the obtained NCs, which were contributed to the loss of adsorbed water in the range of 50–200°C and the decomposition of dextran at about 300°C, respectively. Compared with pure Fe₃O₄ NPs, it is calculated from Figure 1D that there was about 50 wt% of dextran in NCs. XRD pattern in Figure 1E shows that the peaks of 30.2°, 35.5°, 43.2°, 57.1° and 62.7° matched well with crystalline diffractions of Fe₃O₄ (JCPDS card, file no. 88-0315).³² The saturation hysteresis loops in Figure 1F indicate that the saturation magnetization of Fe₃O₄ NCs was 4.3 emu g⁻¹, and there was not coercivity and remanence in NCs (shown in inset pattern of Figure 1F), indicating their superparamagnetic behaviour. The results of MR imaging in Figure 1G,H show that with the increase of Fe ion concentration, the T₂ signal was gradually increased, and the transverse relaxivity value (r_2) of NCs was 231 Fe mM s⁻¹

(Figure 1H). Fluorescence curves in Figure S2 demonstrate the successful conjugation of CTX and Cur with NCs.

3.2 | Cytotoxicity on A549 and HUVEC cells

The cytotoxicity of Cur-NCs, CTX-NCs, CTX-NCs-Cur was analysed with through MTT and cell fluorescence, according to the previous method.^{33,34} MMP-2 proteins were high expressed at the early stage of A549 cells,^{34,35} so the targeting ability of as-prepared NCs could be assessed with these cells test. As shown in Figure S3, the morphology of some A549 cells were changed when cells were cultured with pure NCs; moreover, the images in Figures S3 and S4 indicate that some cells became loose and smaller (marked by white arrows in Figure S3) when treated by Cur-NCs, CTX-NCs and CTX-NCs-Cur for 12 hours, and many cells changed round (marked by white arrows in Figure S4) at 36 hours of treatments, which might partly result from the stiffness effect of the prepared NCs.³⁶ The results of MTT in Figure S7E–H indicate that there was no obvious cytotoxicity of 4 NCs on HUVEC cells at their lower concentration (≤ 100 μ g mL⁻¹); however, the MTT values at 200 μ g mL⁻¹ of the as-prepared NCs in Figure S7F,H remarkably decreased ($P < .05$). Similarly, the results in Figure S7A,E also indicate that pure NCs did not obviously limit the proliferation of A549 and HUVEC cells until the concentration of 200 μ g mL⁻¹. Compared with pure NCs (Figure S7A), the results of Figure S7B–D indicate that Cur-NCs, CTX-NCs and CTX-NCs-Cur could obviously limit the proliferation of A549 cells, with the time dependence and the dose dependence from ≥ 50 μ g mL⁻¹ ($P < .05$), and the limitation of CTX-NCs-Cur was significantly higher ($P < .01$) than those of pure NCs, Cur-NCs and CTX-NCs at the concentration of 100 μ g mL⁻¹ at 12 hours incubation.

In Figure S8A, the activity of A549 cell co-cultured with the different NCs after 36 hours was significantly decreased with the increase of NCs concentration, especially CTX-NCs-Cur could obviously limit the proliferation of A549 cells at NCs concentration of ≥ 50 μ g mL⁻¹ ($P < .01$, Figure S8A). The cell activity of A549 and HUVECs cultured with CTX-NCs after 36 hours indicates that the CTX-NCs could significantly limit the proliferation of A549 cells with dose dependence from iron concentration of ≥ 50 μ g mL⁻¹ ($P < .01$, Figure S8B); however, there was no obvious change for HUVECs under different concentration of CTX-NCs-Cur (Figure S8B). However, there was no obvious effect of different concentration of CTX-NCs-Cur on HUVECs (Figure S8C).

3.3 | ROS production from various NCs

ROS level of A549 cells was determined by measuring the oxidative conversion of fluorescent probe of DCFH-DA into 20,70-dichlorofluoresce (DCF). As shown in Figure 2, compared with the control group, ROS in cells was increased with the increase in the time and the NCs concentration. The results in Figure 2A showed that ROS level of A549 treated with the different NCs for 6 hours did not obviously change, except for the CTX-NCs-Cur groups at the concentration of ≥ 100 μ g mL⁻¹ ($P < .05$);

and the results in Figure 2B indicated that after 24-hour treatment, Cur-NCs, CTX-NCs and CTX-NCs-Cur could obviously induced the ROS increase in A549 cells from NCs concentration of $\geq 50 \mu\text{g mL}^{-1}$ ($P < .05$), and ROS level of A549 cells in CTX-NCs-Cur group was obviously higher ($P < .01$) than those of pure NCs, Cur-NCs and CTX-NCs groups. The increase of ROS level of cells in CTX-NCs group should result from the special targeting effect of CTX, which could increase the endocytosis effect of the cells on CTX-NCs, leading to the similar ROS generation in A549 cells from Fe_3O_4 nanoparticles.³⁷

3.4 | In vivo MR imaging

T_2 -weighted MR images in tumour regions at the different time of the post-injection of various NCs (0, 3 and 8 hours) are shown in Figure 3A, and the average tumour brightness of MRI image of different condition are shown in Figure 3B. Before/after pure NCs injection, there was no obvious intensity change in the tumour region. It is found that the brightness of the tumour site in Cur-NCs, CTX-NCs and CTX-NCs-Cur groups obviously decreased at 3 hours of post-injection ($P < .01$), and the intensity value of each groups were 87 ± 3.61 , 63 ± 5.13 and 76 ± 4.16 . Especially brightness of the tumour in CTX-NCs group at 3 hours was the lowest, compared with those of other 3 hours groups, suggesting that CTX conjugation could accumulated NCs into tumour field due to the specific targeting effect of CTX to MMP-2 over-expressed by A549 cancer, leading to the increase of T_2 intensity of tumour. Compared with

CTX-NCs, the brightness of tumour sites in Cur-NCs and CTX-NCs-Cur groups were significantly decreased at 8 h of post-injection ($P < .01$, Figure 3B), resulting from the relatively lower conjunction amount of CTX on CTX-NCs-Cur and the block effect of PEG on the CTX targeting of CTX-NCs-Cur.

3.5 | Cell and in vivo fluorescence imaging

Targeting effect of the as-prepared NCs on cells was analysed with Prussian blue and 4',6-diamidino-2-phenylindole staining. In Figure 4A, the images of A549 cells treated with CTX-NCs and CTX-NCs-Cur showed more blue than those with NCs and Cur-NCs at the same NCs concentration ($200 \mu\text{g mL}^{-1}$), indicating that MMP-2 receptor-mediated endocytosis could increase the adhesion of NCs. Due to FITC labelled on CTX and the inherent fluorescence of Cur, the images in Figure 4B shows that the fluorescence images of cell treated with Cur-NCs, CTX-NCs and CTX-NCs-Cur, and their fluorescence intensity was measured with MATLAB 7.0 (corresponding values were 61 ± 8.93 , 112 ± 13.49 and 95 ± 9.41 , respectively, $n = 20$), indicating that the green fluorescence of the cells treated with the CTX-NCs and CTX-NCs-Cur were obviously stronger than that of Cur-NCs, due to the specific targeting effect of CTX. However, the fluorescence of CTX-NCs-Cur was weaker than that of CTX-NCs, maybe due to the less CTX conjugated with Cur-NCs.

In order to further analyse the in vivo targeting property of different NCs, TBN mice injected with 4 kinds of NCs were photographed at 10 hours of post-injection. As shown in Figure S9A, pure NCs

FIGURE 2 The ROS generation of the A549 cells treated with pure NCs, Cur-NCs, CTX-NCs and CTX-NCs-Cur for 6 h (A) and 24 h (B), $n = 4$. * $P < .05$ and ** $P < .01$ show the significant difference with NCs group. # $P < .01$ shows the significant difference between 2 corresponding groups

FIGURE 3 (A) T_2 -weighted MR images of TBN mice at 0 (pre-injection), 3 and 8 h of post-injection of pure NCs, Cur-NCs, CTX-NCs and CTX-NCs-Cur, respectively. Green and white arrows point to the tumour interior and tumour periphery, respectively. (B) The intensity change of the tumour interior treated by the different NCs, $n = 8$. * $P < .05$ and ** $P < .01$ represent a significant difference between 2 corresponding groups

were not accumulated at the tumour region; however, the quantitative results in Figure S10 indicate that the fluorescence intensities of tumour area of NCs, Cur-NCs, CTX-NCs and CTX-NCs-Cur groups were 58.6 ± 11.33 , 133.7 ± 32.80 , 196 ± 36.35 and 219.7 ± 31.99 ($n = 20$), respectively. The significant intensity increase in Cur-NCs

groups resulted from the relatively longer blood circulation time of PEG and the passive targeting of enhance the permeability and retention (EPR) effect; and the increase of CTX-NCs groups resulted from the special targeting effect of CTX. The tumour site of mice treated with CTX-NCs-Cur (Figure S9D) showed the more intensive fluorescence, compared with those with Cur-NCs and CTX-NCs (Figure S9B,C), due to the special targeting effect of CTX on the MMP-2 over-expressed A549 lung cancer and the longer circulation effect of PEG on NCs. The image in Figure S11 confirmed that the luminescence imaging from CTX-NCs-Cur in the real lung tumour site should clearly appear, due to the conjugation of Cur with good luminescence, indicating that the prepared CTX-NCs-Cur material could be applied in the diagnose of lung tumour.

3.6 | Therapeutic effect

Therapeutic effect of various NCs was analysed by the *in vivo* injection experiment, in which nude mice with subcutaneous A549 tumour of 0.5 cm size were used as the animal model to measure the volume change of tumour.⁵ Images in Figure 5A show that, compared with those of saline-, Cur-NCs- and CTX-NCs-injected mouse, the tumour size of CTX-NCs-Cur-injected mice was hardly changed. Compared with CTX-NCs and CTX-NCs-Cur, the accumulation of Cur-NCs in the tumour was not significant, although the limitation of Cur-NCs to the tumour was also obvious, because the Cur in the cell could significantly enhance the ROS level and then limit the cellular activity, mainly due to the passive target ability from EPR effect of Cur-NCs. The results of Figure 5B show that the tumour volumes of pure NCs-, Cur-NCs- and CTX-NCs-injected TBN mice rapidly increased with the time; however, the tumour volume of CTX-NCs-Cur-injected mice at 25th day slightly increased (~10%).

3.7 | Bio-distribution of various NCs

Fe ion accumulations in tumour and normal organs were quantitatively analysed and shown in Figure 5C. It is found that many

FIGURE 4 (A) NCs internalized by A549 cells are illustrated by Prussian blue staining (blue). (B) Images of cells cultured for 1 h with Cur-NCs, CTX-NCs and CTX-NCs-Cur, respectively. The green presents NCs adhered to cells, and the blue are cell nucleus stained by DAPI

FIGURE 5 (A) Photographs of mice bearing A549 tumours treated for 25 days, (B) changes of tumour volume during the tumour-therapy assay, (C) the bio-distributions of iron ions in the heart, liver, spleen, lung, kidney, brain and tumour of TBN mice, respectively, after the NCs treatment of 25 days, $n = 4$. * $P < .05$ and ** $P < .01$ represent the significant difference between 2 corresponding groups

FIGURE 6 Schematic of synergistic mechanism of CTX and Cur linked with NCs (named as CTX-NCs-Cur). (A) CTX-NCs-Cur were injected into TBN mice via tail vein, (B) CTX-NCs-Cur specifically targeted and accumulated in tumour area by EPR effect, (C) the CTX-NCs-Cur-MMP-2 complex could block Cl⁻ ion channels of tumour cells, and Cur could obviously enhance the ROS amounts of mitochondrial in tumour cell

CTX-NCs were aggregated in the spleen and liver; however, the Fe ion concentrations in tumours of CTX-NCs and CTX-NCs-Cur groups were significantly higher than those of pure NCs and Cur-NCs ($P < .05$ and $P < .01$, respectively), and the result of CTX-NCs-Cur showed their obvious targeting and accumulation in the tumour. Especially the amount of Cur-NCs in tumour was lower than that of CTX-NCs, indicating that the special targeting effect of CTX to the tumours resulted in the more accumulation of CTX-NCs in tumour. Although the tumour interstitial pressure was higher,³⁸ the longer circulation time of modified NCs could enhance the permeability and retention effect via the open fenestrations of the imperfect tumour blood vessels.³⁹ However, the images of the morphology and inner structures of main organs of TBN mice (Figure S10) indicate that there was no toxicity of CTX-NCs-Cur on normal tissues.

4 | DISCUSSION

Based on the above results, the anti-cancer mechanism of CTX-NCs-Cur is proposed, and the schematic is shown in Figure 6. CTX-NCs-Cur are injected into the blood of TBN mice via tail vein, as shown in Figure 6A. Due to some MMP-2 on the nascent blood vessel in tumour,⁴⁰ more CTX-NCs-Cur could target the nascent blood vessel wall in the tumour site via their long-time circulation and could also enter into the tumour through EPR effect,⁴¹⁻⁴³ then CTX on the surface of

NCs could target and accumulate around tumour cells, where MMP-2 was over-expressed on the surface and intercellular substance of cells, as shown in Figure 6B. With the accumulation of CTX-NCs-Cur in the tumour site, T_2 -intensity of MR images in the tumour area was obviously increased, leading to the brightness decrease in tumour field, and the fluorescence of tumour region was also improved due to Cur fluorescence from accumulated CTX-NCs-Cur. Then, CTX (linked with NCs) and MMP-2 could specifically bind together, and CTX-MMP-2 complex could block Cl⁻ ion channels of tumour cells,²² leading to the fact that CTX-NCs-Cur-MMP-2 composite could limit the active of A549 cells, as shown in Figure 6C. Meanwhile, a part of CTX-NCs-Cur could enter into A549 cells by the endocytosis of lipid rafts, because the special shape of nanostructure favoured drug-load carrier to enter into lung cancer cells,⁴⁴ and the ROS of the mitochondria induced from the cellular Cur molecules could be obviously enhanced, leading to the damage of mitochondrial membrane and the cell apoptosis.³⁰ So the synergistic effect from blocking ion channels of CTX-NCs-Cur-MMP-2 composite and ROS increase in Cur-treated mitochondria could finally limit the proliferation of tumour cells and inhibit the growth of tumour body.

5 | CONCLUSIONS

The novel dual-functional NCs linked with CTX and Cur were fabricated via the co-precipitation and carbodiimide methods, and pure NCs presented higher T_2 relaxivity ($r_2 = 231 \text{ Fe mmol l}^{-1} \text{ s}^{-1}$) and lower cytotoxicity, and CTX-NCs-Cur could efficiently inhibit growth of A549 cells in vitro, and the accumulation and targeting ability of CTX-NCs and CTX-NCs-Cur on A549 cells was significantly enhanced due to the special conjugation between CTX (on NCs) and MMP-2 from A549 cell. The results of *in vivo* test showed the good targeting property of CTX-NCs-Cur due to their accumulation in the tumour regions, leading to the MRI contrast enhancement and good fluorescence imaging on tumour body of small size. A mechanism on the synergistic effect of blocking ion channels from CTX-NCs-Cur-MMP-2 composite and ROS increase from Cur treatment to inhibit the growth of tumour body is proposed. Based on these outstanding properties, CTX-NCs-Cur would be promising to be applied in the diagnosis and therapy of the early non-small-cell lung cancer.

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ORCID

Zhongbing Huang <http://orcid.org/0000-0001-9210-1842>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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