Received: 7 January 2022 | Revised: 26 April 2022 | Accepted: 5 May 2022

DOI: 10.1002/jbm.a.37410

RESEARCH ARTICLE

A new self-immolative colistin prodrug with dual targeting functionalities and reduced toxicity for the treatment of intracellular bacterial infections

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Funding information
National Key Research and Development Program, Grant/Award Number: 2021YFC2102300; National Natural Science Foundation of China, Grant/Award Number: 32071384

Abstract

Colistin is a potent antibiotic but its severe side effects including nephrotoxicity and neurotoxicity are the roadblock for their wide use in clinics. To solve this problem, we synthesized a new prodrug, mannose-maltose-colistin conjugate, termed MMCC that can reversibly mask the five amines of colistin that are primarily responsible for the toxicity. The deliberated design of disulfide-based self-immolative linker warranted the reversibly release of the pristine amines of colistin on demand without sacrificing antimicrobial efficacy. Once MMCC was delivered in cells, reducing agents cleaves the disulfide bond and release the pristine amines. The targeting ligands of maltose and mannose were grafted on colistin conjugate for targeting delivery of colistin to bacteria and macrophages, respectively. Taken together, MMCC as a new class of antimicrobial biomaterials, demonstrates its great potential for the treatment of intracellular bacterial infections.

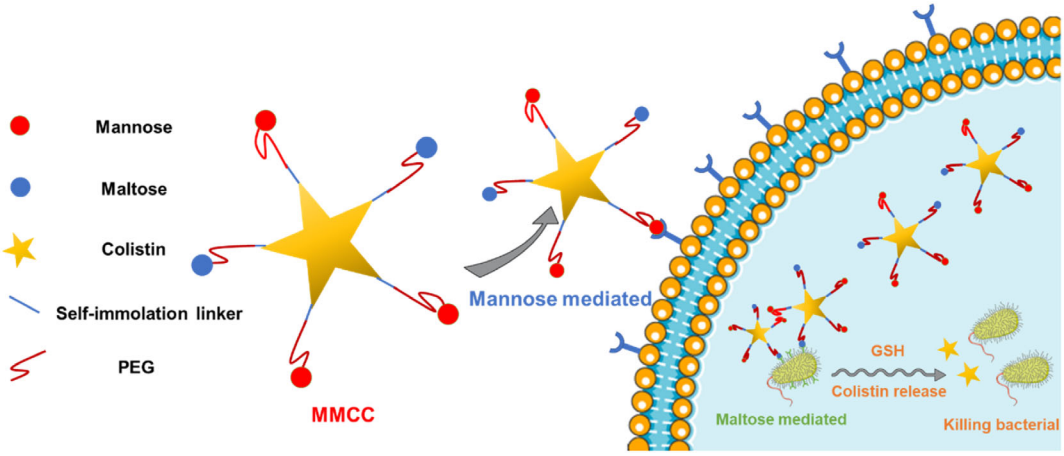
KEYWORDS
antibacterial, bacteria target, colistin, drug delivery, self-immolation

1 | INTRODUCTION

Bacterial infections have always been posing a serious threat to human health.[1] Common clinical manifestations of bacterial infections include sepsis, pneumonia,[2] inflammation[3] or joint symptom[4]. Since the discovery of penicillin in 1928, antibiotics have become the main tool for bacterial infections treatment.[5] However, due to the abuse of antibiotics and the retarded development of new antibiotics, multidrug-resistant (MDR) bacteria is spreading rapidly,[6–9] causing nearly 700,000 patients' death each year.[10] Particularly, those intracellular bacteria associated with the multidrug-resistance can lead to recurrent and chronic infections by residing in macrophages to evade immune clearance.[11]

Colistin is a polymyxin antibiotic, discovered in the late 1940s for the treatment of gram-negative bacterial infections.[12] The bactericidal mechanism of colistin relies on the destruction of the integrity of the cell wall induced by the positively charged pristine amines of colistin. The antibacterial efficacy for the treatment of the MDR-bacteria is concentration-dependent,[13] and meanwhile it is crucially important to control the colistin concentration in blood to avoid severe side effects, which presents a bottleneck to guarantee enough drug concentration in serum for bacterial killing.[14] Colistin can cause renal failure and tubular necrosis, increased oxidative stress, impaired mitochondrial function, and respiratory arrest,[15–18] thereby giving rise to serious nephrotoxicity and neurotoxicity. The neurological adversely side effects caused by colistin include dizziness, seizures, apnea, and ataxia[19,20] In addition, the side effect to the kidneys includes acute tubular necrosis, proteinuria, cylindrical, and oliguria.[21–23] Some commercial colistin prodrugs could reduce toxicity such as colistimethate sodium (CMS), which is made by shielding the amine groups,[24–26] but the colistin release from CMS is unpredictable, complicating the injection dose.[27,28] In addition, in our previous work, we designed colistin cross-linked micelles by cross-linking the Pluronic F127 block copolymers with colistin itself to reduce toxicity of colistin.[29,30]

Gengqi Liu and Di Lu contributed equally to this study.



SCHEME 1 Components and drug release mechanism of MMCC

Self-immolative linkers (SILs) can be used for the chemical modification of pristine drugs and triggered release them in a responsive and controlled manner. Conventional crosslinkers are generally synthesized by the direct binding of active pharmaceutical ingredients (APIs) and trigger groups. However, the chemical modification of the chemical structure of APIs, leads to impaired therapeutic efficacy.[31,32] In order to reversibly release amine groups of colistin in specific sites such as infection sites or intracellular conditions, SILs provide a good strategy to make new colistin prodrugs. The SILs are composed of a reaction site, a self-immolation spacer, and a stimuli responsive moiety.[33] In responsive to external stimuli, stimulus-responsive moiety is cleaved, triggering the self-immolation spacer to undergo a "domino" cleavage reaction, eventually releasing the pristine APIs.[34–36]

Herein, we designed a new colistin prodrug, termed MMCC (Mannose- and maltose-mediated based colistin conjugate) consisting of four parts: (1) colistin as the API, (2) mannose and maltose as the targeting ligands for macrophages and bacteria, respectively. (3) (ethylene glycol) (PEG) to increase water solubility, and (4) a SIL that can reversibly mask and on-demand release amine groups in colistin[37] (Scheme 1). Since the glutathione (GSH) concentration inside the cells is significantly higher than that outside the cells,[38] MMCC can quickly release colistin in the presence of higher concentration of GSH inside macrophages where intracellular bacteria most hide themselves and proliferate. Owing to the dual targeting capabilities to macrophages and bacteria, MMCC could deliver colistin to macrophages where most intracellular bacteria colonize. Because of the reversibly chemical modification of the free amine groups by the SIL, MMCC has significantly reduced toxicity compared with free colistin and can release pristine colistin in cells.

2 | MATERIALS AND METHODS

2.1 | Synthesis of MMCC

The synthetic route of MMCC was shown in Figure 1(A). The synthesis and characterization of the SIL was previously reported by our group.[37] The amine-modified maltose was kindly provided by Xiaojiang Wang's group at Nanjing Tech University. For the synthesis of MMCC, Colistin E sulfate (40 mg, 0.034 mmol) (Shanghai yuanye Bio-Technology Co., Ltd) was first dissolved in 3 ml distilled water, and self-immolation linker (87.567 mg, 0.1734 mmol) and *N*,*N*-Diisopropylethylamine (10 μl) were dissolved in DMSO (1 m). Then the DMSO solution was added into the colistin aqueous solution. The reaction was stirred overnight at room temperature. After the reaction, the solvent was removed by dialysis (MW: 2000 Da) and then subjected to lyophilization to obtain intermediate product as the white power (compound 1). Then, compound 1 (90 mg, 0.03 mmol) was dissolved in 1 ml DMSO. NHS-PEG-SH (346.8 mg, 0.1734 mmol) (Shanghai Yare biotech company) was dissolved in 2 ml water, and then was added to the above compound 1 DMSO solution, and then was stirred at room temperature overnight. After complete reaction, the solvent was removed by dialysis (MW: 3000 Da) and then subjected to lyophilization to obtain intermediate product as the white power (compound 2). The intermediate product, compound 2 (300 mg, 0.023 mmol) obtained above was dissolved in 2 ml PBS solution (pH = 9), and then 1-(3-DiMethylaMinopropyl)-3-ethylcarbodiiMide hydrochloride (22 mg, 0.1173 mmol) was added for activation for 10 min. After activation, the mannose of 4-Aminophenyl-α-D-mannopyranoside (12.4 mg, 0.046 mmol) (Shanghai Xianding Biotechnology Co., Ltd) and maltose (47.8 mg, 0.069 mmol) were added at room temperature and stirred overnight. After the reaction, the solvent was removed by dialysis (MW: 3000 Da) and then subjected to lyophilization to obtain MMCC as the white power (compound 3). Free amines were quantified by the TNBSA method.

2.2 | In vitro drug release

In order to characterize the in vitro colistin release kinetics from MMCC, we labeled colistin with Fluorescein Isothiocyanate (FITC) (Beijing Solarbio Science & Technology Co., Ltd) by directly adding FITC in colistin aqueous solution for 4 h, and FITC-labeled colistin conjugate was synthesized similarly using the above method by

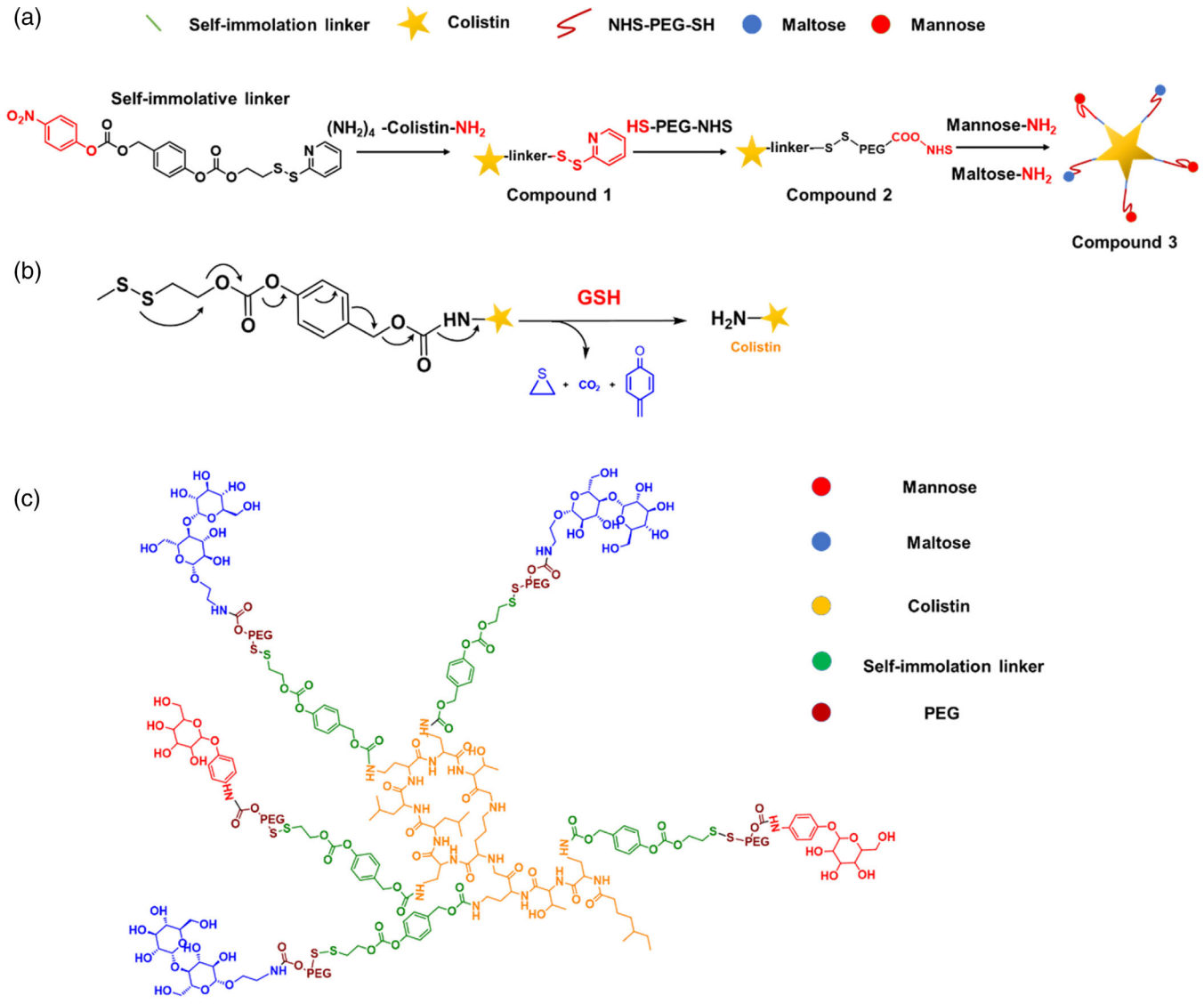


FIGURE 1 (A) The synthetic route of MMCC, (B) self-immolation process triggered by GSH, and (C) the chemical structural of MMCC

replacing colistin with FITC-labeled colistin. 30 ml of PBS (pH = 7.4, with or without 10 m*M* GSH) was prepared. Then, FITC-labeled colistin conjugate was added in the PBS solution and put in dialysis bag (MW: 2000 Da). At the predetermined time points, 100 μl solution was taken out from outside dialysis bag for fluorescence measurement by microplate reader (λex = 480 nm, λem = 520 nm), and then 100 μl of the corresponding fresh PBS (with or without 10 m*M* GSH) was added back in the tube.

2.3 | Minimum inhibitory concentration

Escherichia coli (Strain CICC 10003) was purchased from China Center of Industrial Culture Collection (CICC). Minimum inhibitory concentration (MIC) values of colistin and MMCC were determined by the broth microdilution method. The bacteria were cultured to logarithmic phase (O. D600 = 0.5) with LB liquid medium (0.5 ml) in constant temperature shaker (37°C, 180 rpm), and bacteria solutions were diluted 100 folds with normal saline. Then *E. coli* was incubated with colistin or MMCC (with 0.5 m*M* or 40 μM GSH) or MMCC (without GSH) in 96-well at 37°C overnight. The concentration range of colistin were 256 to 0.25 μg/ml. The MIC value is recorded as the minimum concentration where the O. D 600 is lower than 0.1.

2.4 | Maltose-mediated uptake

In this experiment, 7-amino-4-methylcoumarin (AMC) (Shanghai Meryer Chemical Technology Co., Ltd) was used to replace colistin as a model dye to synthesize maltose-PEG-Linker-AMC (denoted as maltose-AMC conjugate), and the conjugate without grafting maltose ligand as control is denoted as AMC conjugate (see supporting information for the synthesis methods and routes). 10 μl of 1 m*M* maltose-AMC conjugate or AMC conjugate solution was added into the 500 μl

suspension of *E. coli* (O. D600 = 0.5). Then the above samples were incubated at 37°C for 2 h, followed by centrifugation at 10,000 rpm for 5 min. The supernatant was discarded, and the *E. coli* precipitate was washed three times with 0.5 ml PBS. The *E. coli* precipitate was resuspended with 0.5 ml PBS solution, and *E. coli* was lysed by ultrasonic disintegrator. Then the AMC fluorescence intensity of each sample was measured by microplate reader.

Similarly, 1×10^5 macrophage cells were incubated with 1 m*M* maltose-AMC conjugate and AMC-conjugate for 2 h on a 24-well plate. After incubation, the cells were lysed with 0.5 ml TX-100 solution (1%), and the fluorescence intensity of AMC in each well was measured.

2.5 | Targeting to macrophages by mannose

The murine lung epithelial cells (J774A.1 macrophages) was used as a cell model. In order to characterize the targeting ability of mannose to macrophages, mannose-colistin conjugate was prepared and colistin was labeled with FITC, but no mannose whereas another control group was colistin conjugate without mannose or maltose modification (See supporting information for the synthesis methods and routes). 500 μl of cell suspension (J774A.1) was added to each well in a 24-well plate to ensure that each well contains about 20,000 cells. And the cells were cultured for 24 h. The original culture medium in the each well was removed after incubation for 0.5, 1, 2, 4, and 8 h, and the culture medium containing mannose-colistin conjugate or colistin conjugate was added. The fresh culture medium was added in blank group wells. After incubation, the original culture medium was removed, the wells were thoroughly washed with PBS solution, and 500 μl of TX-100 solution (0.1%) was added to lyse the cells. We then analyzed FITC fluorescence intensity (λem = 520 nm, λex = 480 nm) using a microplate reader.

2.6 | Intracellular antibacterial efficacy in vitro

The cell model used in this experiment is mouse lung epithelial cells (J774A.1), and the bacteria model used are multidrug resistant *Klebsiella Pneumoniae* (MDR-KP) gifted by Prof Pengfei Wang at Shanghai Jiaotong University. The experimental group was MMCC and the five control groups include PBS group, free colistin group, mannose-colistin conjugate group (modified with mannose only), maltose-colistin conjugate group (modified with maltose only), and colistin conjugate group (without modification by maltose or mannose). 500 μl of cell suspension (J774A.1) was added in a 96-well plate and each well contains about 20,000 cells. And we cultured the cells for 24 h. At the same time, MDR-KP was cultured to the logarithmic phase (O. D 600 = 0.5). Bacteria solution was centrifuged at 3000 g for 10 min, the medium was discarded, and the pellet at the bottom was resuspended with PBS solution. Next, the resuspension solution was diluted 20 folds with DMEM medium for subsequent infection of the J774A.1 cells. Then the cultured medium was replaced in each well with a bacterial dilution. After the cells were infected with MDR-KP for 1 h, we removed the bacterial dilution from each well, washed each well with PBS solution, and then added DMEM medium containing gentamicin (100 μg/ml) to kill extracellular bacteria in each well. After incubation for 1 h, we then removed the original medium in each well, washed each well with PBS solution, and then added DMEM medium at a concentrations of 128 μg/ml colistin formulation to each well. After the incubation for 12 h, we removed the original medium in each well and washed each well with PBS solution. Then TX-100 solution (0.1%) was added to each well for lysis of the cells. After 40 min, 100 μl solution in each well was evenly applied on the LB agar plate, followed by further incubation at 37°C for 24 h. After incubation, individual bacterial colony on each plate was counted.

2.7 | In vivo toxicity induced by free colistin and MMCC

Eight-week-old female CD-1 mice were purchased from Charles River Beijing Co., Ltd (Beijing, China). Animal experiments were performed in accordance with Tianjin University Institutional Animal Care and Use Committee. In order to evaluate the nephrotoxicity and neurotoxicity of MMCC, we divided the mice into PBS group, free colistin group and MMCC group. All mice were given PBS or colistin formulations intravenously daily for 7 days (on day 1–7). Then we collected blood samples and cerebrum on the day 8 for further toxicity analysis. Before taking blood samples and cerebrum on the day 8, neurobehavioral damage was evaluated by a so-called beam score experiment. Briefly, a wood beam with a length of 150 cm (and a diameter of 6 mm) was marked into three parts and was placed about 60 cm above the ground. Mice were placed on a wooden beam to let them move from one end of the beam to another. Scores were recorded by the following rules: mice that fell in the first part (d < 50 cm, d is the distance that mouse moved on the beam before it fell), in the second part (50 < d < 100 cm), in the third part (100 < d < 150 cm) or were able to cross the beam without falling were scored as 0, 1, 2, and 3, respectively.

To evaluate the nephrotoxicity, the blood samples taken from mice given PBS, free colistin or MMCC were centrifuged at 1000 g in 4°C, the supernatant was collected, and analyzed using biochemical assay kits [NGAL (Neutrophil gelatinase-associated lipocalin) and BUN (Blood urea nitrogen)] according to the protocols provided by kit manufacturers. In order to determine the neurotoxicity caused by the MMCC, the cerebrum samples were homogenized and the supernatant was collected after centrifuging at 1000 g in 4°C, then the neurotoxicity was analyzed using caspase-3 kit according to the protocols provided by kit manufacturers.

2.8 | Statistical analysis

Data were analyzed with Prism 8.0 (GraphPad Software) using the tests described in the figure captions. Statistical significance was

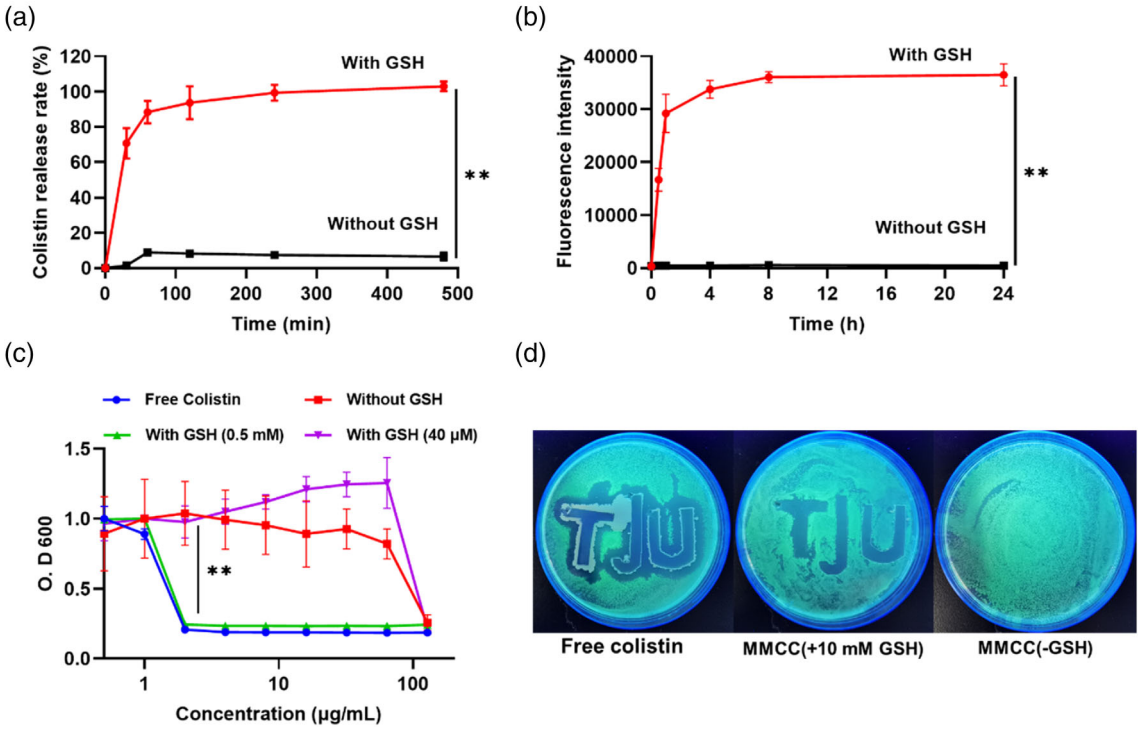


FIGURE 2 Colistin release mediated by GSH. (A) Colistin release kinetics from colistin conjugate with/without GSH buffer quantified by HPLC. (B) Release kinetics of FITC-labeled colistin from colistin conjugate at presence/absence of 10 m*M* GSH in PBS, quantified by measuring fluorescence of FITC using microplate reader. (C) Minimum inhibitory concentrations (MICs) of different formulations of colistin against *E. coli*. (D) The fluorescence images of GFP-expressing *E. coli* treated by different colistin formulations. Error bars show mean ± std dev for n = 3 experiments for release and MIC studies. $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$, analyzed by two-tailed unpaired Student's *t*-test.

analyzed by two-tailed unpaired Student's *t*-test or one-way ANOVA followed by Bonferroni's post hoc analysis or nonparametric test by Kruskal–Wallis analysis. *P* values less than .05 were considered statistically significant. Values were reported as mean ± SD with the indicated sample size.

3 | RESULTS

3.1 | Synthesis of mannose and maltose-grafted colistin conjugate

The chemical structure of MMCC consists of five moieties: colistin as active therapeutic ingredient; a SIL for traceless release of colistin; PEG for improved water solubility; Maltose and mannose as targeting ligand for bacteria and for the delivery of colistin to macrophages, respectively. The intermediate and final products were characterized by ^{1}H-NMR (Figures S1–S3). Colistin, as the last-resort of antibiotic, however, induced serious nephrotoxicity and neurotoxicity, which are caused by the primary amines on colistin with positive changes. Therefore, we designed a SIL to mask the amines to prevent the positive changed amines from directly interacting cell membranes. In addition, PEG was grafted in order to improve the water solubility and biocompatibility of MMCC. For targeting delivery of colistin to macrophages where intracellular bacteria usually reside, amine-containing mannose and amine-modified maltose were conjugated on the terminal NHS-activated carboxyl groups to target macrophages and bacteria, respectively. According to the ^{1}H-NMR characterization, all the five free amino groups of colistin were chemically modified, and the graft ratio of maltose to mannose was 3:2. The synthetic route was described in Figure 1(A). And a proposed self-immolation process triggered by GSH and the chemical structure of MMCC were shown in Figure 1(B,C), respectively.

3.2 | In vitro drug release

We first investigated the colistin release kinetics triggered by GSH, given that the concentration of GSH in cells is higher than extracellular environment. As demonstrated in Figure 2(A), after incubation with 10 m*M* GSH, the disulfide in colistin conjugate was cleaved, followed by the self-immolation process proposed in Figure 1(B), eventually giving rise to the complete and traceless release of free colistin (for simplicity, colistin conjugate without mannose or maltose grafted, which is named colistin conjugate throughout the text, was used instead of MMCC for the self-immolation and drug release studies). By contrast, almost no free colistin was released when no GSH was added. Similarly, FITC-labeled colistin conjugate was dialyzed against PBS solution (pH = 7.4) with or without 10 m*M* GSH, and then the in vitro release kinetics of colistin was measured. As shown in

TABLE 1 Minimum inhibitory concentrations (μg/ml) of various colistin formations

Sample	Free colistin	+GSH (0.5 mM)	+GSH (40 μM)	Without GSH
MIC (μg/ml)	2	2	128	128

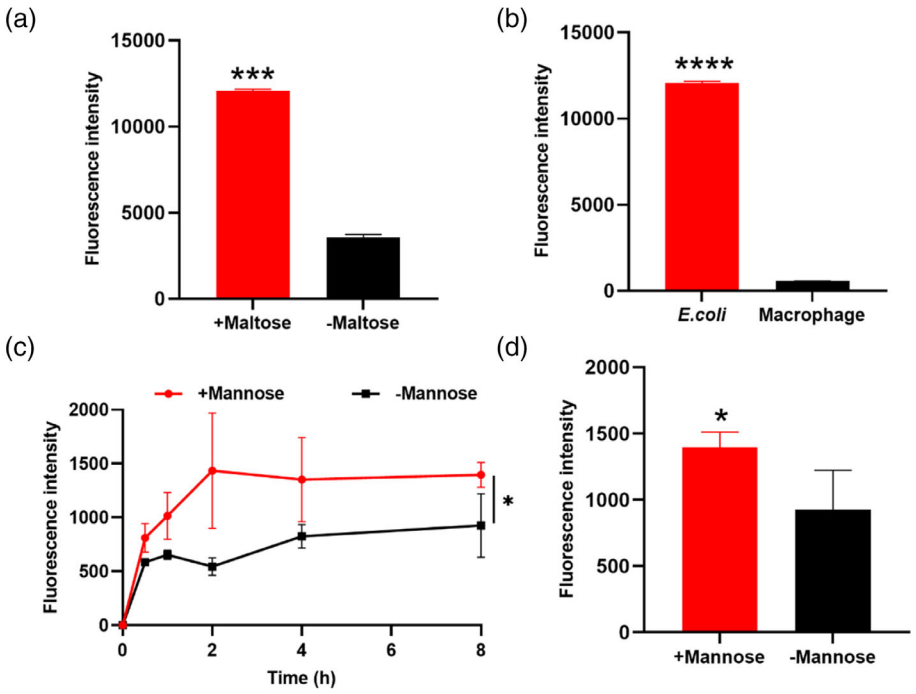


FIGURE 3 Maltose and mannose mediated targeting delivery of colistin. (A) Uptake of maltose-AMC conjugate in *E. coli* was significantly higher than that of AMC conjugate without maltose. (B) Maltose-mediated cellular uptake of maltose-AMC conjugate was specifically observed in *E. coli*, but not in macrophages. (C) Cellular uptake of colistin conjugate with and without mannose after incubation with macrophage for 8 h. (D) Cellular uptake kinetics of mannose-colistin conjugate in macrophages. Error bars show mean ± std dev for n = 3 experiments. *$p < .05$, **$p < .01$, ***$p < .001$, and ****$p < .0001$, analyzed by two-tailed unpaired Student's *t*-test.

Figure 2(B), colistin conjugate has a fast drug release rate with a half-life of around 10 min at the presence of 10 m*M* GSH. However, without GSH added, no FITC-labeled colistin was released within 24 h, showing its good stability in PBS.

3.3 | Minimum inhibitory concentrations

To evaluate the antimicrobial efficacy of MMCC, we measured the MICs of colistin in different forms under different GSH concentrations against *E. coli* by checkerboard dilution method. As seen from Figure 2(C) and Table 1, colistin and colistin conjugate incubated with 0.5 m*M* GSH have the same MIC value (2 μg/ml), suggesting MMCC could release free colistin even when the concentration of GSH is as low as 0.5 m*M*. At presence of no GSH or 40 μM GSH (This concentration of GSH is chosen because the GSH concentration in serum is around 40 μM), the MIC of MMCC increased by about 64-fold (around 128 μg/ml). In order to present the process above more intuitively, we incubated GFP (green fluorescence protein)-expressing *E. coli* on a plate medium containing ampicillin, and three pieces of filter paper with the TJU shape were soaked with the following different solutions (Colistin, MMCC +10 m*M* GSH, MMCC) and were then applied on the plate medium for 4 h, followed by further incubation at 37°C for 12 h. As shown in Figure 2(D), after incubation, the growth of GFP-expressing *E. coli* in the colistin group and the MMCC +GSH group was effectively inhibited in the filter paper-covered area, while the GFP *E. coli* in the MMCC group continued to grow on the filter paper-covered area.

3.4 | Targeted delivery capacity of maltose and mannose

In the studies of the targeting ability of maltose, we synthesized maltose-AMC conjugate by simply replacing colistin with the 7-amino-4-methylcoumarin (AMC) because colistin can kill bacteria so that uptake could not be evaluated. After incubation of maltose-AMC conjugate with bacteria or macrophages, cells were lysed and the fluorescence intensity was measured to quantify the uptake of the conjugate by the bacteria and macrophages. As demonstrated in Figure 3(A), the uptake of maltose-AMC conjugate in *E. coli* was significantly enhanced compared with AMC conjugate without attaching maltose. In addition, the targeting is specifically for the bacteria, rather than the normal cells such as macrophages (Figure 3B).

Then, we synthesized mannose-colistin conjugate with colistin labeled with FITC, followed by incubation with J774A.1 macrophage for different times. J774A.1 can be found in lung epithelial cells and are commonly colonized by intracellular bacteria causing lung infection. As shown in Figure 3(C), the cellular uptake of mannose-colistin conjugate and the colistin conjugate (which has no mannose conjugation, as a control group) gradually increased over time and reached a plateau after around 2 h. Next, we compared the cellular uptake of these two groups at the timepoint of 8 h. As demonstrated in Figure 3(D), the cellular uptake of mannose-colistin conjugate was significantly enhanced than the uptake of colistin conjugate without mannose, indicative of the targeting capability of mannose to macrophages.

3.5 | Intracellular antibacterial efficacy in vitro

After separately demonstrating the targeting capability of maltose and mannose to bacteria and macrophages, respectively, we then conjugated both maltose and mannose to colistin conjugate, forming MMCC. To show the potential use of MMCC for the targeting treatment of intracellular bacterial infection, we evaluated the antimicrobial efficacy of MMCC on J774A.1 macrophages that were pre-infected by MDR-KP. Then MMCC and controls including PBS, free colistin, mannose colistin conjugate (without maltose targeting ligands), maltose-colistin conjugate (without mannose targeting ligand), and colistin conjugate (without any targeting ligands) were used to treat MDR-KP infected macrophages. And CFUs in macrophages after treatment were counted for the characterization of the intracellular antibacterial efficacy of each sample. As shown in Figure 4, at the colistin concentration of 128 μg/ml, MMCC has a significant therapeutic effect compared with other control groups. Mannose colistin conjugate has better antimicrobial effect than maltose-colistin conjugate, further demonstrating that the cell targeting ability of mannose could improve the cellular uptake. The MMCC group showed better antimicrobial effect than the mannose-colistin conjugate, indicative of the bacteria targeting capability of maltose. The intracellular antibacterial effect of MMCC was significantly better than that of the free colistin group, which further demonstrated the potential of MMCC for the treatment of intracellular bacterial infections with reduced toxicity but improved efficacy owing to the deliberated chemical design of MMCC.

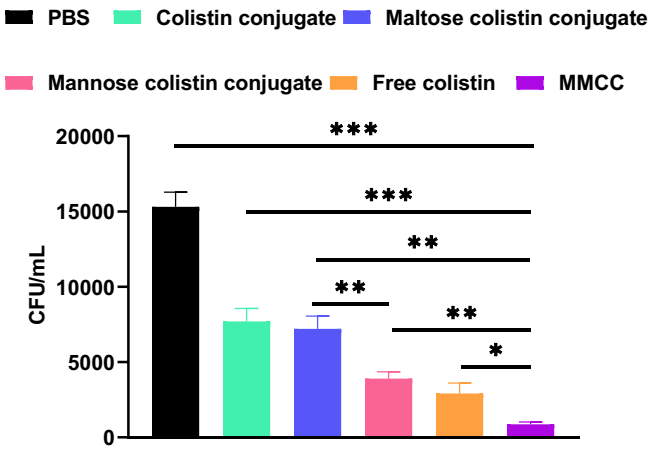


FIGURE 4 Intracellular antibacterial effects of MMCC. The CFUs of MDR-KP bacteria left intracellularly after treatment with 128 μg/ml of various drug formations. Error bars show mean ± std dev for n = 3 experiments. Significance shown with $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$, as analyzed by one-way ANOVA followed by Bonferroni's post hoc analysis

3.6 | Toxicity evaluation in vivo

We evaluated the in vivo toxicity of MMCC including nephrotoxicity and neurotoxicity that are commonly found when using free colistin. 5 mg/kg free colistin or MMCC to intravenously injected in mice every day for seven consecutive days. Blood and cerebrum were collected on the day 8 for the evaluation of toxicity. Colistin-induced kidney damage is related to the filtration capacity of the glomerulus and

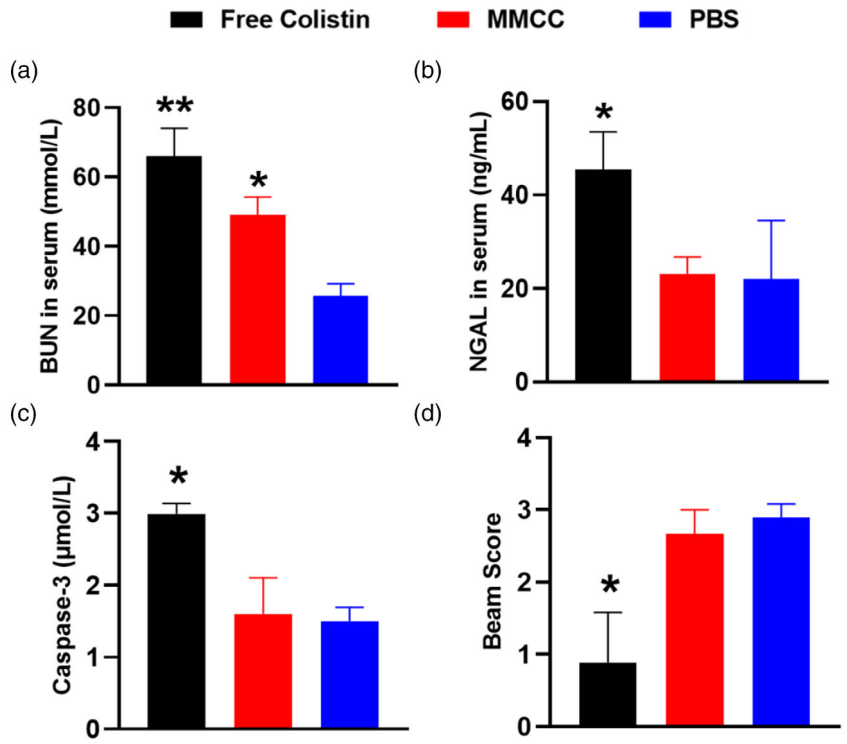


FIGURE 5 In vivo toxicity evaluation of MMCC. Mice were intravenously given PBS, MMCC or free colistin daily for seven consecutive days, and the mice were sacrificed 12 h after the last injection. The following biomarkers or parameter were measured: (A) BUN and (B) NGAL in serum. (C) Caspase-3 in cerebrum. (D) Beam score: Walking capability by recording the walking distance on a beam. Error bars show mean ± std dev for n = 3 experiments. Significance shown with $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$, as analyzed by one-way ANOVA followed by Bonferroni's post hoc analysis (A, B, C) and nonparametric analysis by Kruskal–Wallis test (D).

the increased permeability of the tubular epithelial cell membrane. Drug elimination by tubular cells in kidney can lead to the accumulation of colistin and the reabsorption of renal tubules. BUN is a biomarker for the evaluation of glomerular damage. As shown in Figure 5(A), no significant difference of the BUN concentration between MMCC group and the PBS group was observed, while the BUN level of the free colistin group increased significantly. In addition, we also assessed the level of NGAL in serum, which is a more sensitive and specific biomarker for the diagnosis of kidney injury. As shown in Figure 5(B), colistin caused a significant increase in serum NGAL levels, but there was no significant difference between MMCC group and the PBS group.

Neurotoxicity can be attributed to the accumulation of colistin in the central nervous system, manifested as enhanced oxidative stress or mitochondrial dysfunction. In order to evaluate the neurotoxicity in vivo, we homogenized the mouse cerebrum and tested the level of caspase-3. As shown in Figure 5(C), the caspase-3 concentration in the colistin group was significantly higher than that of MMCC group or the PBS group. In addition, before sacrificing the mice, we also conducted a beam score experiment (see method for the protocol) to evaluate the walking ability and nerve damage of the treated mice. As shown in Figure 5(D), the mice of colistin group showed obvious dyskinesias. However, the mice of MMCC group could walk normally, similarly as the mice treated by PBS.

4 | DISCUSSION

In this work, a colistin prodrug conjugated by a SIL was made for targeted delivery of colistin with reduce drug toxicity. Given the fast proliferation rate of bacteria, it is required that antibiotics are needed to be fast released in infection sites. Amines can induce the nephrotoxicity and neurotoxicity but are also responsible for the antimicrobial effect; therefore, they have to be reversibly masked and released in a timely manner. The drug release experiment showed that colistin was released rapidly in the present of GSH, but there was almost no release in the physiological environment. These suggested that colistin conjugate could remain stable in physiological environment with free amines masked, but could release free colistin at infection sites characterized with higher concentration of GSH and other reducing agents.

Then MICs of colistin in different forms under different GSH concentrations were measured to explore the antibacterial properties of this colistin prodrug. When GSH concentration was 0.5 m*M*, the MIC of MMCC was the same as free colistin. However, in the simulated physiological environment, MIC was much higher than free colistin. Because the antimicrobial ability of colistin was deactivated when the five amine groups that are primarily responsible for the antimicrobial efficacy were masked by the self-immolation linker. Self-immolation process as shown in Figure 1(B) could be triggered after disulfide bonds are cleaved, leading to the reversible release of the amine groups and restorage of colistin's antimicrobial capability.

The maltose-mediated targeting delivery of colistin to bacteria is attributed to the specific maltose transport channel on the bacterial cell wall, which can be effectively taken up by the bacteria. Macrophages are one of the main host cells of intracellular bacteria.[39] Since mannose receptors (CD206) abundantly exist in the cell membranes of macrophages where intracellular bacteria persist and reside, mannose can be used as a cell-targeting ligand to chemically modify the prodrug for the enhanced cellular uptake of the colistin conjugate. In vitro uptake experiments showed that maltose significantly increased the uptake of *E. coli*. and mannose can effectively target macrophages to improve the uptake of colistin. Intracellular antibacterial experiments further showed that MMCC had great potential for the treatment of intracellular bacterial infections. In addition, compared with free colistin, MMCC has significantly reduced nephrotoxicity and neurotoxicity.

5 | CONCLUSION

Herein we synthesized a colistin prodrug with dual targeting moieties for targeting the intracellular bacteria. The five amine groups of colistin were masked by a self-immolative crosslinker and can be reversibly released, triggered by higher concentration of GSH in intracellular environment. We demonstrated that MMCC could be used for the treatment of intracellular bacterial infections by in vitro experiments. We also investigated its safety of MMCC in vivo and found that the nephrotoxicity and neurotoxicity were significantly reduced by the same dose of MMCC. Therefore, the newly synthesized prodrug, MMCC holds its great potential for the treatment of intracellular bacterial infections with reduced toxicity.

ACKNOWLEDGMENTS

We thank the funding sources mentioned above. We also Thank Prof. Pengfei Wang for gifting multidrug resistant *Klebsiella Pneumoniae* (MDR-KP).

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

REFERENCES

1. Travis J. Reviving the antibiotic miracle? *Science*. 1994;264(5157):360-363.
2. Yang ZQ, Huang YL, Zhou HW, Zhang R, Zhu K. Persistent carbapenem-resistant Klebsiella pneumoniae: a Trojan horse. *Lancet Infect Dis*. 2017;22:22-23.
3. Azoulay E, Russell L, Van de Louw A, et al. Diagnosis of severe respiratory infections in immunocompromised patients. *Intensive Care Med*. 2020;46(2):298-314.
4. Ryan M, Kavanagh R, Wall P, Hazleman B. Bacterial joint infections in England and Wales: analysis of bacterial isolates over a four year period. *Br J Rheumatol*. 1997;36(3):370-373.
5. Wright GD. Molecular mechanisms of antibiotic resistance. *Chem Commun*. 2011;47(14):4055-4061.
6. Li J, Nation RL, Turnidge JD, et al. Colistin: the re-emerging antibiotic for multidrug-resistant gram-negative bacterial infections. *Lancet Infect Dis*. 2006;6(9):589-601.

7. Li X, Bai H, Yang Y, Yoon J, Wang S, Zhang X. Supramolecular antibacterial materials for combatting antibiotic resistance. *Adv Mater*. 2019;31(5):1805092.
8. Ray PC, Khan SA, Singh AK, Senapati D, Fan Z. Nanomaterials for targeted detection and photothermal killing of bacteria. *Chem Soc Rev*. 2012;41(8):3193-3209.
9. Tian S, Su L, Liu Y, et al. Self-targeting, zwitterionic micellar dispersants enhance antibiotic killing of infectious biofilms—An intravital imaging study in mice. *Sci Adv*. 2020;6(33):eabb1112.
10. Tagliabue A, Rappuoli R. Changing priorities in vaccinology: antibiotic resistance moving to the top. *Front Immunol*. 2018;9:1068.
11. Helaine S, Cheverton AM, Watson KG, Faure LM, Matthews SA, Holden DW. Internalization of salmonella by macrophages induces formation of nonreplicating persisters. *Science*. 2014;343:204-208.
12. Storm DR, Rosenthal KS, Swanson PE. Polymyxin and related peptide antibiotics. *Annu Rev Biochem*. 1977;46(1):723-763.
13. Li J, Nation RL, Kaye KS. Polymyxin antibiotics: from laboratory bench to bedside. *Adv Exp Med Biol*. 2019;1145:37-54.
14. John E, Bennett R, Blaser M. Polymyxins (polymyxin B and colistin). *Mandell Douglas Bennett's Princ Pract Infect Dis*. 2015;8:549-555.
15. Dai C, Xiao X, Li J, et al. Molecular mechanisms of neurotoxicity induced by polymyxins and chemoprevention. *ACS Chem Nerosci*. 2019;10(1):120-131.
16. Dai C, Ciccotosto GD, Cappai R, et al. Rapamycin confers neuroprotection against colistin-induced oxidative stress, mitochondria dysfunction, and apoptosis through the activation of autophagy and mTOR/Akt/CREB signaling pathways. *ACS Chem Nerosci*. 2018;9(4):824-837.
17. Dai C, Tang S, Biao X, Xiao X, Chen C, Li J. Colistin induced peripheral neurotoxicity involves mitochondrial dysfunction and oxidative stress in mice. *Mol Biol Rep*. 2019;46(2):1963-1972.
18. Nation RL, Li J. Colistin in the 21st century. *Curr Opin Infect Dis*. 2009;22(6):543.
19. Kelesidis T, Falagas ME. The safety of polymyxin antibiotics. *Expert Opin Drug Saf*. 2015;14(11):1687-1701.
20. Falagas ME, Kasiakou SK. Toxicity of polymyxins: a systematic review of the evidence from old and recent studies. *Crit Care*. 2006;10(1):R27.
21. Koch-Weser JAN, Sidel VW, Federman EB, Kanarek P, Finer DC, Eaton AE. Adverse effects of sodium colistimethate. *Ann Intern Med*. 1970;72(6):857-868.
22. Price D, Graham D. Effects of large doses of colistin sulphomethate sodium on renal function. *Br Med J*. 1970;4(5734):525-527.
23. Falagas ME, Fragoulis KN, Kasiakou SK, Sermaidis GJ, Michalopoulos A. Nephrotoxicity of intravenous colistin: a prospective evaluation. *Int J Antimicrob Agents*. 2005;26(6):504-507.
24. Florescu DF, Qiu F, McCartan MA, Mindru C, Fey PD, Kalil AC. What is the efficacy and safety of colistin for the treatment of ventilator-associated pneumonia? A systematic review and meta-regression. *Clin Infect Dis*. 2012;54(5):670-680.
25. Wallace SJ, Li J, Nation RL, Prankerd RJ, Boyd BJ. Interaction of colistin and colistin methanesulfonate with liposomes: colloidal aspects and implications for formulation. *J Pharm Sci*. 2012;101(9):3347-3359.
26. Barnett M, Bushby SRM, Wilkinson S. Sodium sulphomethyl derivatives of polymyxins. *Br J Pharmacol Chemother*. 1964;23(3):552-574.
27. Bergen Phillip J, Li J, Rayner Craig R, Nation Roger L. Colistin methanesulfonate is an inactive prodrug of colistin against *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 2006;50(6):1953-1958.
28. Plachouras D, Karvanen M, Friberg LE, et al. Population pharmacokinetic analysis of colistin methanesulfonate and colistin after intravenous administration in critically ill patients with infections caused by gram-negative bacteria. *Antimicrob Agents Chemother*. 2009;53(8):3430-3436.
29. Yang X, Ren H, Zhang H, et al. Antibiotic cross-linked micelles with reduced toxicity for multidrug-resistant bacterial sepsis treatment. *ACS Appl Mater Interfaces*. 2021;13(8):9630-9642.
30. Yang X, Qiu Q, Liu G, et al. Traceless antibiotic-crosslinked micelles for rapid clearance of intracellular bacteria. *J Control Release*. 2022;341:329-340.
31. Böhme D, Beck-Sickinger AG. Drug delivery and release systems for targeted tumor therapy. *J Pept Sci*. 2015;21(3):186-200.
32. Chen J, Zhao M, Feng F, Sizovs A, Wang J. Supramolecular antibacterial materials for combatting antibiotic resistance. *J Am Chem Soc*. 2013;135(30):10938-10941.
33. Liu G, Lovell JF, Zhang L, Zhang Y. Stimulus-responsive nanomedicines for disease diagnosis and treatment. *Int J Mol Sci*. 2020;21:17.
34. Rautio J, Kumpulainen H, Heimbach T, Oliyai R, Savolainen J. Prodrugs: design and clinical applications. *Nat Rev Drug Discov*. 2008;7(3):255-270.
35. Kratz F, Müller IA, Ryppa C, Warnecke A. Prodrug strategies in anticancer chemotherapy. *ChemMedChem*. 2010;3(1):20-53.
36. Bildstein L, Dubernet C, Couvreur P. Prodrug-based intracellular delivery of anticancer agents. *Adv Drug Deliv Rev*. 2011;63(1–2):3-23.
37. Liu G, Jiang Z, Lovell JF, Zhang L, Zhang Y. Design of a thiol-responsive, traceless prodrug with rapid self-immolation for cancer chemotherapy. *ACS Appl Bio Mater*. 2021;4(6):4982-4989.
38. Wu G, Fang Y-Z, Yang S, Lupton JR, Turner ND. Glutathione metabolism and its implications for health. *J Nutr*. 2004;134(3):489-492.
39. Xie S, Li S, Zhang Z, Chen M, Li X. Bacterial ghosts for targeting delivery and subsequent responsive release of ciprofloxacin to destruct intracellular bacteria. *Chem Eng J*. 2020;399:125700.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Liu G, Lu D, Zhu S, et al. A new self-immolative colistin prodrug with dual targeting functionalities and reduced toxicity for the treatment of intracellular bacterial infections. *J Biomed Mater Res*. 2022;110(9):1590-1598. doi:10.1002/jbm.a.37410