Facile Synthesis of a H$_2$O$_2$-Responsive Alternating Copolymer Bearing Thioether Side Groups for Drug Delivery and Controlled Release

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ABSTRACT: A novel amphiphilic alternating copolymer with thioether side groups (P(MSPA-EG)) was synthesized through an amine-epoxy click reaction of 3-(methylthio)propylamine (MSPA) and ethylene glycol diglycidyl ether. P(MSPA-EG) was characterized in detail by nuclear magnetic resonance (NMR), gel permeation chromatography, Fourier transformed infrared, differential scanning calorimeter, and thermogravimetric analysis to confirm the successful synthesis. Due to its amphiphilic structure, P(MSPA-EG) could self-assemble into spherical micelles with an average diameter of about 151 nm. As triggered by H$_2$O$_2$, these micelles could disassemble because hydrophobic thioether groups are transformed to hydrophilic sulfoxide groups in MSPA units. The oxidant disassemble process of micelles was systematically studied by dynamic light scattering, transmission electron microscopy, and $^1$H NMR measurements. The MTT assay against NIH/3T3 cells indicated that P(MSPA-EG) micelles exhibited good biocompatibility. Furthermore, they could be used as smart drug carriers to encapsulate hydrophobic anticancer drug doxorubicin (DOX) with 4.90% drug loading content and 9.81% drug loading efficiency. In vitro evaluation results indicated that the loaded DOX could be released rapidly, triggered by H$_2$O$_2$. Therefore, such a novel alternating copolymer was expected to be promising candidates for controlled drug delivery and release.

INTRODUCTION

In the past decades, various stimuli-responsive polymeric nanocarriers have been developed to overcome the disadvantages of traditional chemotherapy agents, including poor bioavailability, nonspecific selectivity, low accumulation in tumor tissue, adverse side effects, etc.1–3 Up to now, a wide range of stimuli, such as redox,4,5 pH,6,7 oxidation,8–10 temperature,11–14 overexpressed enzymes,15 ultrasound,16 magnetic field,17 and light,18,19 have been introduced into polymeric nanocarriers to achieve the controlled drug delivery and release in targeted cancer cells while minimizing the toxicity to normal cells. Among these stimuli, reactive oxygen species (ROS) have been widely used to develop various ROS-responsive polymers for target-specific drug delivery on the basis of the different ROS content between the pathological sites and their surroundings.20–24 Usually, there are four ROS including hydrogen peroxides (H$_2$O$_2$), hydroxyl radicals (OH$^•$), superoxides (O$_2^•$), and peroxynitrites (ONOO$^−$). Among them, H$_2$O$_2$ is the most major ROS factor in biological microenvironment. Thus, a lot of H$_2$O$_2$-responsive polymeric materials have been designed and widely applied in drug delivery systems for cancer therapy in recent years.25–27 However, most H$_2$O$_2$-responsive polymeric materials were still prepared through the complicated synthetic process.28–30 Therefore, it is still a challenge to develop a facile synthetic method of H$_2$O$_2$-responsive polymers.

Alternating copolymers (ACPs) are an important kind of linear copolymers with two different structure units arranged alternately in their main chains and widely used as plasticizing agents,31 chemical sensors,32 photoelectricity,33,34 and so on. Recently, Khan et. al. reported a facile method to synthesize ACPs by the robust, efficient, and orthogonal click chemistry under mild reaction conditions.35 Our group also synthesized a serial of amphiphilic ACPs through the click reactions of amine-epoxy/amine-thiol and the obtained ACPs could self-assemble into various architectures, such as nanotubes,36 vesicles,37 and sea urchin like assemblies.38 In addition, some of them were used as electrode materials.39,40 Thus, we want to use this facile method of thiol-epoxy/amine-epoxy click reactions to further design and synthesize ACPs with excellent biocompatibility and stimulus-responsive properties for biomedical applications.

Herein, we reported a novel H$_2$O$_2$-responsive amphiphilic ACP P(MSPA-EG) with thioether side groups that was
conveniently synthesized by the amine-epoxy click reaction from 3-(methylthio)propylamine (MSPA) and ethylene glycol diglycidyl ether (EGDE) at room temperature. The self-assembly of P(MSPA-a-EG) in water and the corresponding oxidant disassembly process were studied in detail by dynamic light scattering (DLS), transmission electron microscopy (TEM), and \(^1\)H nuclear magnetic resonance (NMR) measurements. In addition, we also investigated the potential of this \(\text{H}_2\text{O}_2\)-responsive alternating copolymer as a smart carrier for controlled drug release (Scheme 1).

\section*{RESULTS AND DISCUSSION}

\textbf{Synthesis and Characterization of P(MSPA-a-EG).} As shown in Scheme 2, P(MSPA-a-EG) was synthesized from MSPA and EGDE by one-step amine-epoxy click polymerization at room temperature without any catalyst. The resulting ACP was characterized by NMR, Fourier-transform infrared spectroscopy (FTIR), and gel permeation chromatography (GPC) techniques. The \(^1\)H NMR spectra of EGDE, MSPA, and P(MSPA-a-EG) are displayed in Figure 1A. Compared with their \(^1\)H NMR spectra in CDCl\(_3\), the peaks at 2.6 ppm (a), 2.79 ppm (a’), and 3.16 ppm (b) belonging to the epoxy protons of EGDE disappeared completely, and two new peaks at 2.5 ppm (a) and 3.83 ppm (b) belonging to methylene protons (–\(\text{N}\)–\(\text{CH}_2\)–\(\text{CH(OH)}\)) and methyne protons (–\(\text{N}\)–\(\text{CH}_2\)–\(\text{CH(OH)}\)), respectively, appeared in the \(^1\)H NMR spectrum of P(MSPA-a-EG). The peak at 1.47 ppm (5) belonging to the amine protons of MSPA also disappeared completely in the \(^1\)H NMR spectrum of P(MSPA-a-EG).

Furthermore, the peak area integral ratio (\(S_b/S_a/S_c/S_{2+4+a}/S_1/S_3\)) of all protons in P(MSPA-a-EG) was approximately equal to its theoretical value of 2:4:4:8:3:2, which also confirmed the chemical structure of the resulting alternating copolymer. In addition, the \(^1\)C NMR spectrum of P(MSPA-a-EG) in Figure 1B further verified its chemical structure. The GPC curve, FTIR spectrum, thermogravimetric analysis (TGA) curve, and differential scanning calorimetry (DSC) curve of P(MSPA-a-EG) are shown in Figure 2. As shown in Figure 2A, the number-averaged molecular weight (\(M_n\)) and polydispersity index (PDI) of P(MSPA-a-EG) were 2110 and 2.66, respectively, which indicated that the amine-epoxy click polymerization was successful. The FTIR spectrum of P(MSPA-a-EG) is exhibited in Figure 2B. The strong broad peak at 3382 cm\(^{-1}\) was attributed to the stretching vibration of \(\text{–OH}\), which indicated that strong multiple hydrogen bonds would form among these hydroxyl groups.\(^{31}\) The peaks at 2903 and 2846 cm\(^{-1}\) could be ascribed to the asymmetric and...
Symmetric stretching vibrations of $-\text{CH}_2-$ respectively. The peak at 1447 cm$^{-1}$ belonged to the bending vibration of $-\text{CH}_2-$. The strong peak at 1110 cm$^{-1}$ was assigned to the stretching vibration of C=O. The thermal properties of P(MSPA-a-EG) were studied by TGA and DSC measurements, and the results are shown in Figure 2C,D. The thermal stability of P(MSPA-a-EG) was relatively high, with the initial decomposition temperature of around 304 °C (Figure 2C). The glass transition temperature of P(MSPA-a-EG) was about −36.8 °C (Figure 2D). All the above experimental results confirmed that P(MSPA-a-EG) was synthesized successfully.

**Self-Assembly Behavior of P(MSPA-a-EG).** Based on the amphiphilic structure of P(MSPA-a-EG) with alternating hydrophobic MSPA units and hydrophilic EG units in the main chain, it could self-assemble spontaneously in water. The formation of P(MSPA-a-EG) micelles was confirmed by fluorescence technique with Nile red as a fluorescent probe. The fluorescence intensity of Nile red increased dramatically when the concentration of P(MSPA-a-EG) increased to a certain value, which verified the formation of micelles and the encapsulation of Nile red into the hydrophobic core of micelles (Figure 3A). In addition, the maximum emission wavelength of Nile red in the P(MSPA-a-EG) solution exhibited about 26 nm blue shift from 659 to 633 nm when the concentration of P(MSPA-a-EG) was increased from $3.9 \times 10^{-3}$ to 0.5 mg/mL (Figure 3B). This further confirmed that the Nile red was transferred from the water into the hydrophobic environment as a result of the formation of micelles, which was consistent with previous report. Accordingly, the critical micelle concentration (CMC) of P(MSPA-a-EG) was calculated to be about 63.4 μg mL$^{-1}$ (Figure 3C). The size and morphology of P(MSPA-a-EG) micelles were measured by DLS and TEM measurements, respectively. As shown in Figure 4A, the DLS curve indicated that P(MSPA-a-EG) was able to assemble into nanoparticles with an average size of about 151 nm and the particle-size distribution index (PDI) of 0.249. The TEM image in Figure 4B exhibits that P(MSPA-a-EG) micelles were spherical with an average diameter of approximately 130 nm, which was slightly smaller than that measured by DLS. This was attributed to the dry state of micelles in the TEM measurement but the wet state of micelles in DLS measurement.

**H$_2$O$_2$ Responsiveness of P(MSPA-a-EG) Micelles.** Generally, hydrophobic thioether groups are easy to transform into hydrophilic sulfoxide groups in an oxidative environment. Here, H$_2$O$_2$ was employed as the oxidant to study the oxidation responsiveness of P(MSPA-a-EG) micelles. The aqueous dispersions of P(MSPA-a-EG) micelles were incubated with H$_2$O$_2$ at various concentrations from 0 to 166.6 mM for 12 h, and the final photographs are shown in Figure 5A. With the increasing concentration of H$_2$O$_2$, the turbid aqueous dispersions of P(MSPA-a-EG) micelles were gradually changed into transparent solutions, which indicated the disassembly of P(MSPA-a-EG) micelles in the oxidative process. The UV−vis spectrophotometer was used to detect the transmittance of aqueous dispersions of P(MSPA-a-EG) micelles, and the results are shown in Figure 5B. When the concentration of H$_2$O$_2$ was improved to 22.3 mM, the transmittance was increased to 98.6%, which confirmed that almost all the micelles dissociated at this concentration of H$_2$O$_2$. Meanwhile, the diameter changes of P(MSPA-a-EG) micelles with different amounts of H$_2$O$_2$ were measured by DLS and TEM. Both results exhibited the disassembly of...
P(MSPA-a-EG) micelles after oxidation. In detail, the diameter of P(MSPA-a-EG) micelles decreased from 151 nm to 6 nm by DLS with the increase in concentration of H₂O₂ from 0 to 166.6 mM (Figure 5C). Meanwhile, the TEM images in Figure 5D indicate that the size of P(MSPA-a-EG) micelles decreased continuously with the increase in concentration of H₂O₂. When the concentration of H₂O₂ was increased to 166.6 mM, lots of tiny fragments of several nanometers were observed. The disassembly of P(MSPA-a-EG) micelles was ascribed to the oxidation of hydrophobic thioether groups into hydrophilic sulfoxide groups or even sulfone groups, which endowed the change of P(MSPA-a-EG) from amphiphilic to hydrophilic.

To further verify the oxidation of thioether groups into sulfoxide groups or even sulfone groups, all the above samples were freeze-dried and then characterized by ¹H NMR measurement. As shown in Figure 6A, the proton signals at 2.06 and 2.42 ppm belonging to methyl (−SOC₃H₃) and methylene (−CH₂SOC₃H₃) adjacent to the sulfur atom in the thioether group gradually disappeared with the increase in concentration of H₂O₂. Meanwhile, some new proton signals appeared at 2.59, 2.62, and 1.91 ppm, which could be attributed to methyl (−SOC₃H₃), methylene (−CH₂SOC₃H₃), and methylene (−CH₃CH₂SOC₃H₃). When the concentration of H₂O₂ increased to 22.3 mM, the proton signals adjacent to the sulfur atom in the thioether groups disappeared completely, which indicated that all thioether groups were oxidized into sulfoxide groups. The oxidation extent of P(MSPA-a-EG) micelles was estimated by the following equation, and the results are shown in Figure 6B.

\[
\text{oxidation extent} = 1 - \left[ I(\text{−SOC₃H₃}) / I(\text{−OCH₂CH₂O − }) \times 1.333 \right] \times 100\%
\]

The oxidation extent of P(MSPA-a-EG) micelles was calculated as 22 and 85.3% after treatment with H₂O₂ at concentrations of 2.3 and 8.9 mM, respectively. Furthermore, the oxidation extent of P(MSPA-a-EG) micelles reached 100% when the concentration of H₂O₂ was 22.3 mM or higher. This H₂O₂-concentration-dependent behavior was consistent with the results of UV−vis and DLS measurements.

Cell Cytotoxicity Assay. As a nanocarrier for drug delivery, the cytotoxicity to normal cells or biocompatibility is a key parameter for the biomedical applications of P(MSPA-a-EG). Thus, the in vitro cytotoxicity of P(MSPA-a-EG) was evaluated by MTT assay against the NIH/3T3 cell line. The NIH/3T3 cells were incubated with aqueous solutions of P(MSPA-a-EG) at different concentrations from 0.005 to 1 mg mL⁻¹ for 24 h and the results are displayed in Figure 7.

Obviously, the cell viability even remained 80.0% when the concentration of P(MSPA-a-EG) increased to 1 mg mL⁻¹. Therefore, P(MSPA-a-EG) had good biocompatibility and could be used as materials for drug delivery.

DOX-Loaded and in Vitro H₂O₂-Responsive Drug Release. Here, anticancer drug DOX was selected as a model drug to evaluate the potential of H₂O₂-responsive P(MSPA-a-EG) micelles as a smart drug delivery system for drug control release. According to the standard curve of DOX, drug-loading content (DLC) and drug-loading efficiency (DLE) of P(MSPA-a-EG) micelles loaded with DOX were calculated as 4.9 and 9.81%, respectively. Then, the in vitro drug release of DOX-loaded P(MSPA-a-EG) micelles was investigated in pure phosphate-buffered saline (PBS) and PBS with 5 or 20 mM H₂O₂. As shown in Figure 8, the cumulative release of drug from DOX-loaded P(MSPA-a-EG) micelles in PBS was only about 23.4% for 24 h. When the concentration of H₂O₂ was 5 mM in PBS, the cumulative release of drug from DOX-loaded P(MSPA-a-EG) micelles was just increased to 31.4% within the same time. This indicated that a small part of hydrophobic thioether in P(MSPA-a-EG) was oxidized to hydrophilic sulfoxide at 5 mM H₂O₂ and resulted in the partial dissociation of the micelles. When the concentration of H₂O₂ in PBS was further improved to 20 mM, the cumulative release of drug from DOX-loaded P(MSPA-a-EG) micelles was significantly increased to 55.6%, which was ascribed to the rapid dissociation of P(MSPA-a-EG) micelles under the higher concentration of H₂O₂. Thus, H₂O₂-responsive drug release can be realized through the transition from amphiphilic to hydrophilic of P(MSPA-a-EG) on the basis of thioether groups.

Figure 6. (A) ¹H NMR spectra of P(MSPA-a-EG) micelles after treatment with different concentrations of H₂O₂. (B) Oxidation extent of P(MSPA-a-EG) micelles at various concentrations of H₂O₂.

Figure 7. Cell cytotoxicity of P(MSPA-a-EG) against NIH/3T3 cells after 24 h incubation. The data are presented as average ± standard deviation (n = 6).

Figure 8. In vitro drug release behavior of DOX-loaded P(MSPA-a-EG) micelles with different concentrations of H₂O₂ at 37 °C.
oxidized to sulfoxide groups. Overall, P(MSPA-α-EG) micelles could be used as a potential biomaterial for drug delivery and controlled release.

**CONCLUSIONS**

Novel amphiphilic ACP P(MSPA-α-EG) was synthesized successfully by amine-epoxy click copolymerization of MSPA and EGDE. H₂O₂-responsive micelles with an average diameter of about 151 nm were constructed by the self-assembly of P(MSPA-α-EG). Triggered by H₂O₂, hydrophobic thioether groups in the micelle cores were oxidized into hydrophilic sulfoxide groups and resulted in the rapid dissociation of P(MSPA-α-EG) micelles. In vitro cell cytotoxicity assay indicated that P(MSPA-α-EG) micelles exhibited good biocompatibility against normal cells. Anticancer drug DOX could be loaded into P(MSPA-α-EG) micelles effectively and realize H₂O₂-triggered rapid drug release. Such H₂O₂-responsive ACP P(MSPA-α-EG) micelles based on an amphiphilic-to-hydrophilic transition would be promising candidates as smart drug carriers for drug delivery and controlled release.

**EXPERIMENTAL SECTION**

**Materials.** 3-(Methylthio)propylamine (MSPA, 98%, TCI), Nile red (99%, Acros), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma), triethylamine (TEA, 99%, Adamas), acetone (99.5%, Adamas), and ethanol (99.5%, Adamas) were used as received. Ethylene glycol diglycidyl ether (EGDE) was purchased from Adamas and purified by distillation under reduced pressure.

**Measurements.** GPC measurement was performed on an HLC-8320GPC (TOSOH, EcoSEC GPC System) system at 40 °C with dimethylformamide as the mobile phase at a flow rate of 0.6 mL min⁻¹. ¹H NMR and ¹³C NMR spectra were obtained on a Varian Mercury Plus 400 MHz spectrometer with deuterium chloroform (CDCl₃) as a solvent at 20 °C. Tetramethylsilane was used as an internal standard. The DSC measurement was carried out on a Thermal Advantage DSC Q2000 autosampler (TA Instruments) equipped with a refrigerating cooling system. The polymer (9.1 mg) was placed in aluminum pans (nonhermetic) (30 μL) scanned at a heating rate of 10 °C min⁻¹ from −80 to 80 °C under dry nitrogen atmosphere. The data were treated using Universal Analysis 2000 V 4.3 software from TA Instruments. FTIR spectrum was recorded on a PerkinElmer Spectrum 100 FTIR spectrometer by KBr sample holder method. TGA was measured on a PerkinElmer Q5000IR thermobalance by using nitrogen as the purging gas at a heating rate of 20 °C min⁻¹. Dynamic light scattering (DLS) measurement was performed under a 3,000 HS (Malvern Instruments, Ltd.) equipped with 125 mW laser light operating at λ = 633 nm with a scattering angle of 90°. Transmission electron microscopy (TEM) studies was observed under a JEOL 1010 instrument operated at 200 kV. One little drop of the micelle solution (0.2 mg mL⁻¹) was dropped onto a carbon-coated copper grid. Then, the grid was immersed into liquid nitrogen and freeze-dried in vacuum at −50 °C before measurement. Fluorescent spectra were measured on a Q-4 CW spectrometer, made by Photon Technology International, Int. USA/CAN. The excitation wavelength was set at 550 nm, and the emission was monitored from 570 to 750 nm. Ultraviolet–visible (UV–vis) absorption of the sample solutions was measured at room temperature by using a Thermo Electron-EV300 UV–vis spectrophotometer. The slit-width was set as 1 nm with a scan speed of 480 nm min⁻¹.

**Synthesis of P(MSPA-α-EG).** P(MSPA-α-EG) was synthesized from MSPA and EGDE as monomers by the amine-epoxy click reaction. Typically, MSPA (2.613 g, 15 mmol) and EGDE (1.578 g, 15 mmol) were added into a 25 mL round flask. After stirring for 48 h at room temperature, the polymer was purified by dialysis against ethanol for 48 h (MWCO = 3500 g mol⁻¹). By rotary evaporation to remove ethanol, the yellowish viscous liquid P(MSPA-α-EG) was obtained.

**Preparation of P(MSPA-α-EG) Micelles.** Briefly, 10.0 mg P(MSPA-α-EG) was dissolved in 0.5 mL ethanol completely. Then, the solution was added dropwise into 5 mL deionized water under slight stirring for 10 min. Subsequently, the solution was dialyzed in deionized water for 12 h (MWCO = 1000 g mol⁻¹), during which the deionized water was renewed every 4 h. Finally, P(MSPA-α-EG) micelle aqueous solution was obtained.

**Critical Micellization Concentration (CMC) of P(MSPA-α-EG).** To determine the CMC value of P(MSPA-α-EG), Nile red was used as a fluorescent probe. Twenty-five microliters of Nile red acetone solution (1.6 × 10⁻⁴ mol L⁻¹) was added into 4 mL of aqueous solution of P(MSPA-α-EG) with different concentrations (from 0.00753 to 1 mg mL⁻¹), while the final concentration of Nile red in each solution was kept at 1.6 × 10⁻⁶ mol L⁻¹. Then, the samples were exposed to air overnight to remove acetone completely. The fluorescence emission spectra of all samples were recorded on a fluorescence spectrometer at the excitation wavelength of 550 nm.

**H₂O₂-Responsiveness of P(MSPA-α-EG) Micelles.** The blank P(MSPA-α-EG) micelles (1.5 mL, 10 mg mL⁻¹) were mixed with 0.5 mL H₂O₂ solutions at different concentrations. After treatment for 12 h at room temperature, the appearance and transmittance of these samples were recorded by using a digital camera and a UV–vis spectrophotometer at the wavelength of 500 nm. In addition, the diameter changes of P(MSPA-α-EG) micelles was monitored by DLS. Finally, all the samples were lyophilized and characterized by ¹H NMR in CDCl₃.

**Preparation of DOX-Loaded P(MSPA-α-EG) Micelles.** The typical preparation of DOX-loaded P(MSPA-α-EG) micelles was as follows: a predetermined amount of DOX-HCl and one molar equivalent of trimethylamine (TEA) were dissolved in 1 mL of DMSO solution containing 15 mg of P(MSPA-α-EG) completely. Then, the mixture was slowly added into 8 mL of deionized water under slight stirring at room temperature for 20 min. Subsequently, the mixture was transferred to a dialysis bag (MWCO = 1000 g mol⁻¹) and dialyzed against deionized water for 24 h. To determine the loading amount of DOX, 1 mL of DOX-loaded micelle solution was lyophilized and then redissolved in DMSO. The total loading amount of DOX was determined by the UV absorbance of the solution at 500 nm. The drug-loading content (DLC) and drug-loading efficiency (DLE) were calculated according to the following equations

\[
\text{DLE} (\%) = \frac{W_{\text{loaded}}}{W_{\text{total}}} \times 100\%
\]

\[
\text{DLC} (\%) = \frac{W_{\text{loaded}}}{W_{\text{polymer}} + W_{\text{loaded}}} \times 100\%
\]

Here, \(W_{\text{loaded}}\), \(W_{\text{total}}\), and \(W_{\text{polymer}}\) represent the weight of the loaded DOX, total DOX, and P(MSPA-α-EG), respectively.
In Vitro H$_2$O$_2$-Triggered Drug Release. Two milliliters of DOX-loaded P(MSPA-a-EG) micelles solution (1 mg mL$^{-1}$) was transferred into a dialysis bag (MWCO = 1000 g mol$^{-1}$). Then, the dialysis bag was immersed in 30 mL PBS, or PBS with 5 mM, or 20 mM H$_2$O$_2$ in a shaking water bath at 37 °C. At predetermined time intervals, 3 mL of the external buffer solution was withdrawn and replaced with 3 mL of fresh PBS, or PBS with 5 mM or 20 mM H$_2$O$_2$. The cumulative released amount of DOX was determined by using the fluorescence measurement by QC-4-CW spectrometer at the excitation wavelength of 485 nm. All DOX-released experiments were carried out in triplicate, and the results are shown as the average data with standard deviations.

Cell Culture. NIH/3T3 normal cells (a mouse embryonic fibroblast cell line) were cultivated in Dulbecco’s modified Eagle’s medium (DMEM) supplied with 10% FBS and antibiotics (50 U mL$^{-1}$ penicillin and 50 U mL$^{-1}$ streptomycin) at 37 °C in a humidified atmosphere containing 5% CO$_2$.

MTT Assay. The biocompatibility of P(MSPA-a-EG) was evaluated by MTT assay. NIH/3T3 cells were seeded in 96-well plates at 8 x 10$^3$ cells per well in 200 µL of DMEM. After incubation overnight, the DMEM was removed and fresh DMEM with P(MSPA-a-EG) at different concentrations (0.005 to 1 mg mL$^{-1}$) added. The cells without the treatment were used as control. The cells were incubated for another 24 h. Then, 20 µL of 5 mg mL$^{-1}$ MTT solution in PBS was added to each well. After the cells were incubated for 4 h, the DMEM containing unreacted MTT was carefully removed. Then, 200 µL of DMSO was added into each well to dissolve the blue formazan crystal, and the absorbance at a wavelength of 490 nm was measured by a BioTek Synergy H4 hybrid reader. The blank was subtracted to the measured optical density values, and the cell viability was expressed as percentage of the values obtained for the untreated control cells.

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