Electronic Supplementary Information of

Verteporfin-loaded supramolecular micelles for enhanced cisplatin-based chemotherapy via autophagy inhibition

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Materials

4-Cyanopentanoic acid dithiobenzoate, 1-adamantanecarbonyl chloride, and 2-hydroxyethyl methacrylate (HEMA) were purchased from Aladdin Reagent. Cisplatin (CDDP, 99.5%) was supplied by Shandong Boyuan Chemical Company, China. Mono-(6-(triethylenetetraamine)-6-deoxy)-beta-Cyclodextrin (β-CD-NH2) was bought from Zhiyuan Biotechnology Co., Ltd (Shandong, China). 2-(Methacryloyloxy)ethyl phosphorylcholine (MPC) was bought from GY Biomaterials (Anqing, China). Dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) were provided by Energy Chemical Co., Ltd. (Shanghai, China). CDDP conjugated β-cyclodextrin (CD-CDDP) was synthesized according to our previous publication (ACS Nano 2021, 15, 8663−8675). Human hepatoma cell line LM3 was obtained from KeyGen BioTECH. LC3 primary antibody (anti-LC3) was bought from Cell Signaling Technology (CST, 3868 and 4108). Monodansylcadaverine (MDC) was provided by Solarbio. Rat anti-mouse CD31 antibody was provided by Sigma-Aldrich. Cell Counting Kit-8 (CCK-8) was purchased from Beyotime Biotechnology.

Characterizations

^{1}H NMR spectra were recorded on a Bruker DMX500 spectrometer. The hydrodynamic diameter (Dh) and size distribution of the micelles were measured by dynamic light scattering (DLS) measurements using Zetasizer Nano-ZS from Malvern Instruments equipped with a He-Ne laser (633 nm) at room temperature. The sizes and morphologies of the micelles were characterized by JEM-1200 transmission electron

microscopy (TEM) at an accelerating voltage of 80 kV without negative staining. UV-vis spectra were recorded on a Shimadzu UV-2550 UV-vis spectrometer. Inductively coupled plasma-mass spectrometry (ICP-MS, Ultimate3000-ICAP RQ, Thermo Fisher, USA) was used for the determination of Pt content. Fluorescence images were observed using a confocal laser scanning microscope (CLSM, Leica TCS SP5).

Cell lines and cell culture

Hepatoma carcinoma cells LM3 were incubated in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. NIH3T3 cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were cultivated at 37 °C in a 5% CO_2 environment. The culture medium was replaced every 3 days. Cultured cells at 80–90% confluence were used for further experiments.

Western blot assay

For the Western blot assay, LM3 cells were incubated in a 6-well plate at 2×10^5 cells per well for 48 h. CDDP MCs (10 μM CDDP), VTPF MCs (5 μM VTPF) or CDDP/VTPF MCs (10 μM CDDP, 5 μM VTPF) were then added and incubated for another 12 h. After that, LM3 cells were lysed to lead proteins extracted from cells. The total protein was quantified using BCA Protein Quantification Kit (Beyotime Biotechnology, P0010). The LC3 and p62 proteins of each sample were then separated by 10% SDS-PAGE followed by transferred onto PVDF membrane (Millipore, IPVH00010) according to the protocols as mentioned above with relevant primary

antibody: anti-LC3 (1:1000 dilution, Cell Signaling Technology, CST, 4108) or p62 (1:200 dilution, Abcam, ab56416) and secondary antibody: Goat anti-mouse IgG(H+L) secondary antibody (1:5000 dilution, Thermo Pierce, 31160).[50] The membranes were visualized on X-ray films and detected by chemiluminescence using SuperSignal® West Dura Extended Duration Substrate (Thermo Pierce, 34075). Band intensities were quantitated using Image Pro Plus 6.0 software and protein expression was normalized to β-actin (1:1500 dilution, Santa Cruz, SC-47778).

Detection of LC3 dots by fluorescent microscopy

The formation of LC3 dots was detected using immunofluorescence technique. Typically, LM3 cells were incubated with CDDP MCs (10 μM CDDP), VTPF MCs (5 μM VTPF) or CDDP/VTPF MCs (10 μM CDDP, 5 μM VTPF) for 8 h. After that, the cells were fixed for 20 min by 4% paraformaldehyde. After washed by PBS for three times, cells were treated with Triton X-100 (0.2%) for 15 min. The cells were then washed by PBS for another three times and treated with 1% bovine albumin (BSA) for 1 h. After that, KB cells were incubated with the primary antibody (anti-LC3, CST, 3868) for 12 h at 4 °C according to the specification. After cells were washed by PBS for three times, the secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG (H+L)) (Sigam-Aldrich, SAB4600234) was added and incubated for 2 h at room temperature in dark. After washed by PBS for another three times, the LC3 dots were monitored by fluorescent microscopy.

MDC staining assay

For the MDC staining assay, LM3 cells were incubated with CDDP MCs (10 μM CDDP), VTPF MCs (5 μM VTPF) or CDDP/VTPF MCs (10 μM CDDP, 5 μM VTPF) for 8 h. Afterthat, the cells were incubated with MDC (Solarbio, G0170) according to the specification for 30 min. After washed by PBS for three times, the LC3 dots were monitored by fluorescent microscopy.

Observation of autophagosome by TEM

In order to observe autophagosome by TEM, CDDP MCs (10 μM CDDP), VTPF MCs (5 μM VTPF) or CDDP/VTPF MCs (10 μM CDDP, 5 μM VTPF) for 8 h. The cells were then fixed with 2.5% glutaraldehyde for 1 h. After washed by PBS for three times, LM3 cells were fixed by osmium tetroxide for 2 h. The cells were then dehydrated using graded acetone and embedded in epoxy resin to form ultrathin sections. Finally, the autophagosomes in these cell ultrathin sections were detected by TEM.

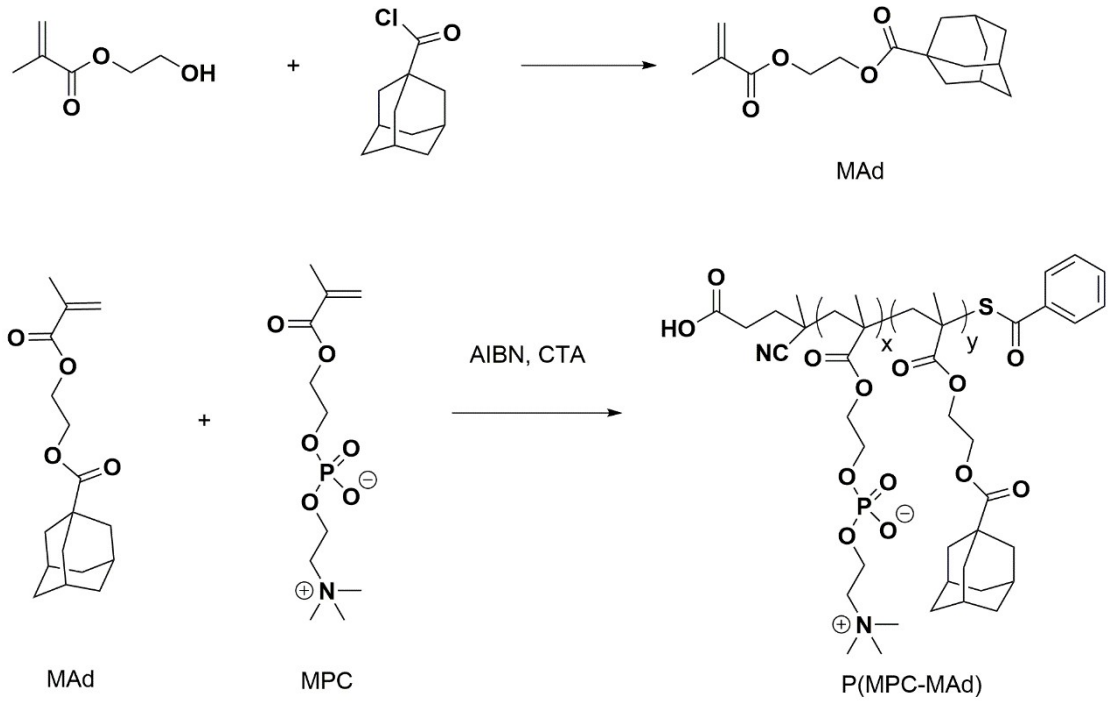
***In vivo* assays**

All animal experiments were performed according to the “Principles of Laboratory Animal Care” (NIH publication no.86-23, revised 1985) and the guidelines for Animal Care and Use Committee, Zhejiang University. Healthy BALB/c nude mice (4−6 weeks old) were purchased from the animal center of Zhejiang Academy of Medical Sciences.

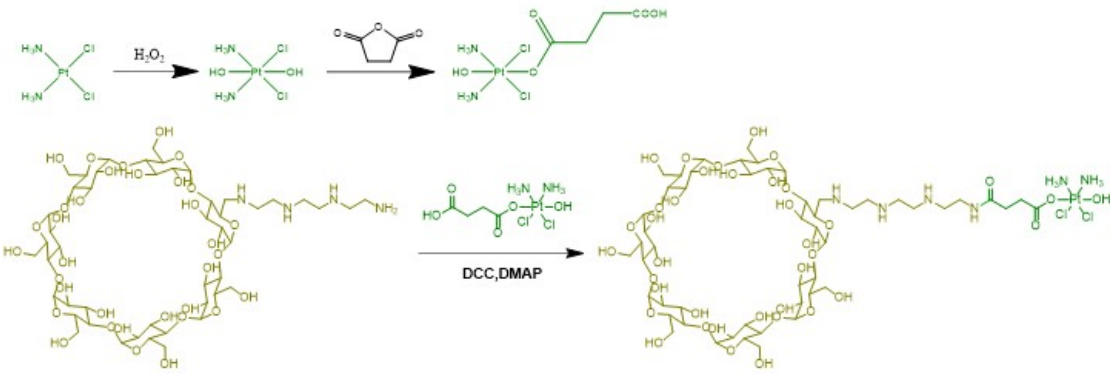
Statistical Analysis

Data were expressed as mean ± SD. Analyses were performed using GraphPad Prism version 8.0 software. Student’s t test was used to make two-group comparisons.

Comparisons of parameters among three or more groups were made using one-way analysis of variance (ANOVA) for single-factor variables followed by Tukey's posthoc tests or two-way ANOVA for two-factor variables with repeated measurements over time, followed by Bonferroni posthoc tests. No significant difference was denoted n.s., and statistical significance was denoted as *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.



Scheme S1. Synthetic procedure of MAd and P(MPC-MAd).



Scheme S2. Synthetic procedure of CD-CDDP.

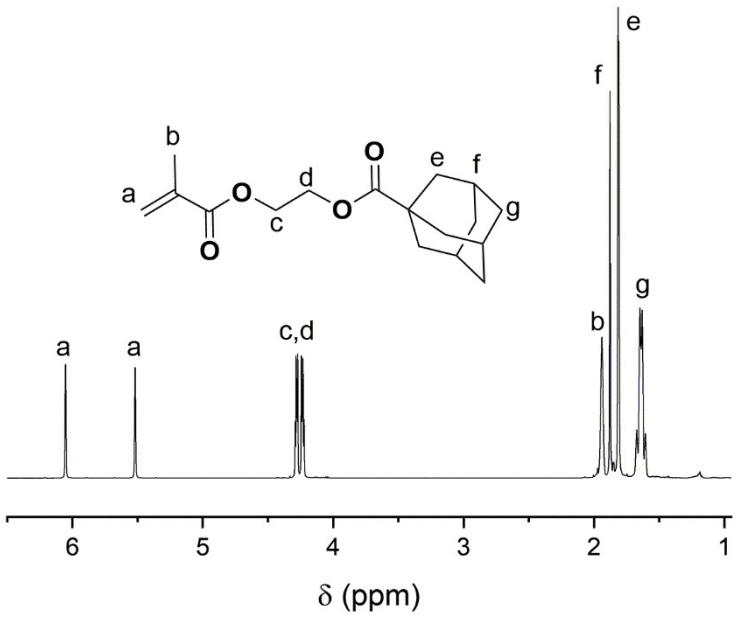


Figure S1. ^{1}H NMR spectrum of MAd in $CDCl_3$.

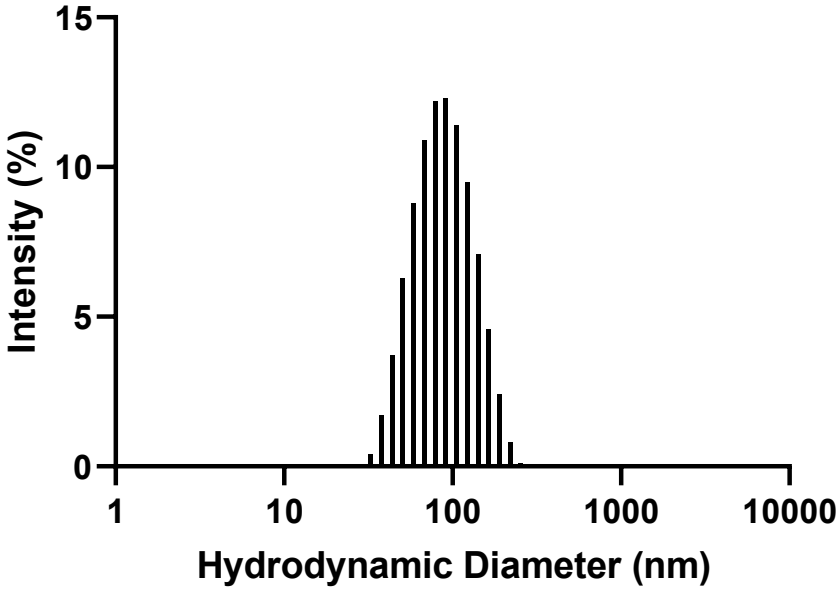


Figure S2. The intensity-average hydrodynamic diameter of CDDP MCs after incubation with 5 mM ascorbic acid for 48 h. The large aggregation was removed by a filter prior to measurement.

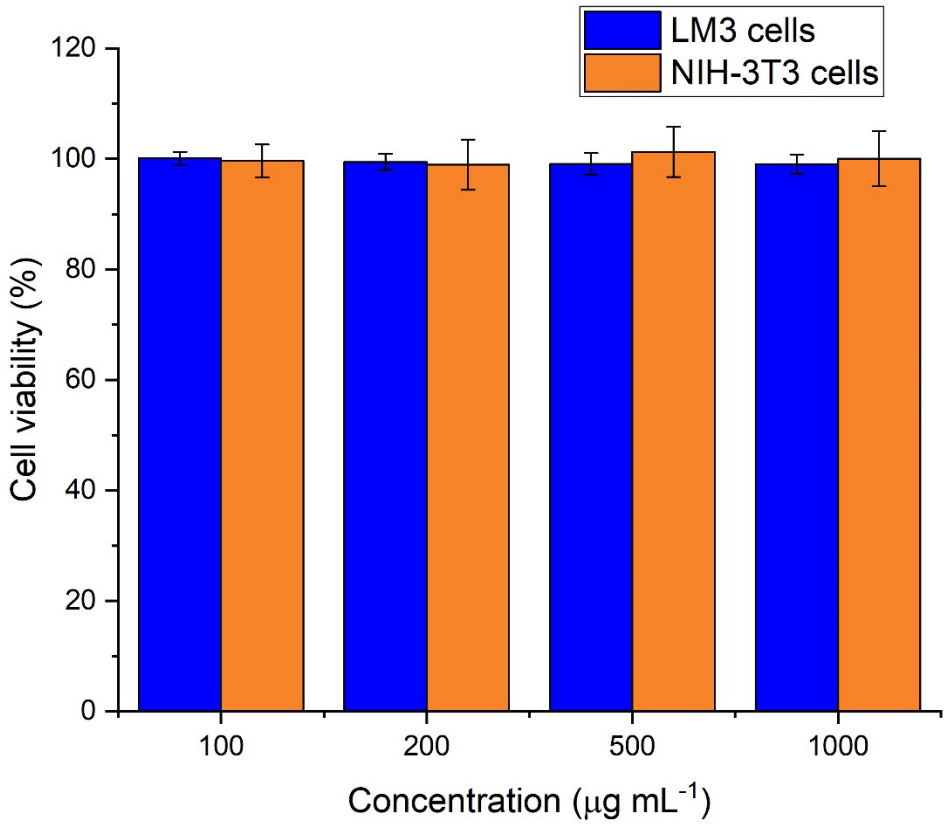


Figure S3. Viability curves of LM3 cells and NIH-3T3 cells after treatment with different concentrations of blank P(MPC-co-MAd) micelles.