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Supplementary Information

A Fluorescent Molecular Rotor Probe for Tracking Plasma Membranes and Exosomes in Living Cells

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Content

S1. Supplementary material and methods

General information. All organic solvents and chemicals were purchased from commercial companies and used without further purification, unless indicated otherwise. The CellMask™ Plasma Membrane Stains (Cat. No. C10046) was purchased from Thermo Fisher Scientific Inc. The cell culture dishes and plates were from Corning (Corning, New York, USA). Hydrogenated phosphatidylcholine (hsPC) and cholesterol for liposomes preparation were purchased from Sigma-Aldrich. Thin layer chromatography (TLC) for Reaction monitoring was performed using pre-coated silica plates (Merck 60 F254 nm, 250 μm thickness), and spots were visualized by UV. Flash column chromatography was carried out with silica gel (Merck 60 F254 nm, 70-200 mesh). ^{1}H-NMR, ^{13}C-NMR were recorded on Bruker 300 MHz/400 MHz NMR spectrometers. The spectra were referenced against the NMR solvent peaks (CD_3OD = 3.31 ppm, $CDCl_3$ = 7.26 ppm, DMSO = 2.5 ppm) and reported as follows: ^{1}H: m (multiplet), t (triplet), s (singlet). ^{13}C: chemical shift δ (ppm).

*p*H value was measured with a Mettler-Toledo FiveEasy F20 *p*H meter. Mass spectra were obtained on PC Sciex API 150 EX ESI-mass spectrometers. Analytical high-performance liquid chromatography (HPLC) was carried out on a Waters 1525 Binary HPLC Pump and Waters 2489 UV/Visible Detector with a reverse-phase Phenomenex Luna® Omega 5 μm Polar C18 100 Å 250 × 4.6 mm column at a flow rate of 1 mL/min. Acetonitrile and Water were used as eluents. A scanning electron microscope (SEM) was performed on a Philips XL30 ESEM. Fluorescence measurement was performed with a FluoroMax-4 fluorescence photometer. Cell imaging was performed with a Leica SP5 laser scanning confocal microscope.

Preparation of liposomes. The liposomes were prepared from hydrogenated phosphatidylcholine (hsPC), mixed with cholesterol at various mole fractions cholesterol. Stock solutions of hydrogenated phosphatidylcholine (hsPC) and cholesterol were made up in chloroform and mixed to offer the desired mole ratio. Chloroform was removed by nitrogen flush. The dried liposomes were resuspended and sonicated in PBS at a total lipid concentration of 2 mg/mL. The liposomes prepared were characterized by dynamic light scattering (DLS) and scanning electron microscope (SEM). Liposomes were then stored at 4 ºC before use.

Cell Culture and Fluorescence Imaging. MCF-7, LO2 and Hela cells were seeded in confocal dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with sodium pyruvate (1 mM) (Thermo Scientific), 100 μg / mL streptomycin, 100 units/mL penicillin, 10% heat-inactivated fetal bovine serum (FBS, Invitrogen) at 37 °C in a humidified incubator with 5% CO_2 for 24 h.

Cell imaging was performed with a Leica SP5 laser scanning confocal microscope. Before experiments, cells were washed with PBS buffer three times. The cells were then incubated with probe **MRMP-1** or the CellMask™ Plasma Membrane Stain at 37 °C for 20 or 10 mins in cell culture DMEM medium. Before the fluorescence

imaging experiments were performed, cells were washed with PBS buffer 3 times. The samples were excited at 440 nm (**MRMP-1)** or 650 nm (CellMask™ Plasma Membrane Stain). For the fluorescence quenching analysis, cells were continuously laser-irradiated for 9 min. Images were taken every three minutes.

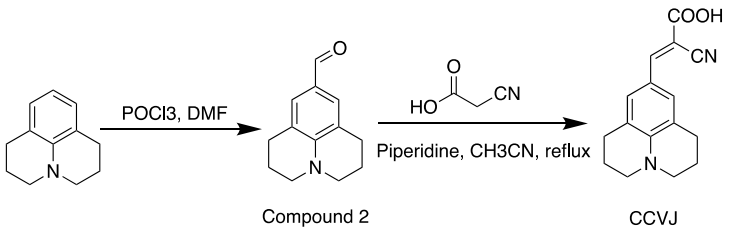
H_2O_2 stimuli experiment. 50 mM hydrogen peroxide was added to the cells in the confocal dish and incubated further at 37 °C in a humidified incubator with 5% CO_2 for indicated time before cell imaging experiment.

Exosome isolation and characterization. L cells were passaged from frozen stock with Dulbecco's modified Eagle's medium (DMEM) (Gibco) with 10% FBS. After a week of recovery, L cells are starved with DMEM and no serum for 48h to produce activated conditioned medium (ACM).

Exosome were isolated using XE-90 Beckman sequential ultracentrifugation with modification from Thery et al. 2006.[1] First harvest ACM and centrifuge 1000 x g for 10 mins, 10,000 x g for 60 and 100,000 x g for 2 hours. Then subsequently washed once in PBS, and isolated with 10000 x g for 70 mins.

The characterization of nanoparticle tracking analysis (NTA) were performed using Malvern NS300 Nanosight instrument equipped with a 488 nm laser. The samples were introduced manually using a 1 mL syringe. Three replicates of particle movement videos were taken and recorded. The camera level was set at 20 and analyzed with Nanosight Assistant Software at detection threshold of 10.

S2. Synthesis and characterization of MRMP-1

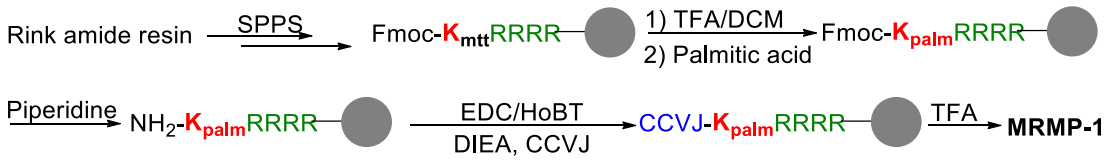


Scheme S1. Synthesis of CCVJ.

Synthesis of Compound 2. To anhydrous DMF (1.67 g, 22.87 mmol), phosphorous oxychloride (1.17 g, 7.62 mmol) was added dropwise at 0℃ under nitrogen protection. After stirring at room temperature for 30 min, the mixture was transferred to a flask containing julolidine (1.2 g, 6.93 mmol). The reaction was further heated at 90℃ for 4 h. After completion of the reaction, the mixture was quenched by adding cold water (100 mL) and neutralized with sodium bicarbonate. By extraction with ethyl acetate, the product was isolated and dried by evaporation under vacuo as a light yellow solid. ^{1}H NMR ($CDCl_3$, 300 MHz) (ppm): 9.60 (s, 1H), 7.29 (s, 2H), 3.32 – 3.28 (m, 4H),

2.79-2.75 (m, 4H), 2.0-1.93 (m, 4H). ^{13}C-NMR ($CDCl_3$, 75 MHz) (ppm): 190.13, 147.91, 129.46, 123.95, 120.31, 50.03, 27.66, 21.25. ESI-MS calcd for $[M+H]^+$ 202.27; Found 202.30.

Synthesis of CCVJ. To the solution of compound 2 (142 mg, 0.7 mmol) and cyanoacetic acid (119 mg, 1.4 mmol) in CH_3CN, piperidine (59.6 mg, 0.7 mmol) was added. The reaction mixture was heated to reflux for 2 h. After completion of the reaction, the *p*H of reaction mixture was adjusted to be acid. Following extraction with CH_2Cl_2 and purification by flash chromatography, the desired product CCVJ was obtained.[2] ^{1}H NMR (DMSO, 300 MHz) (ppm): 7.85 (s, 1H), 7.49 (s, 2H), 3.34-3.3 (m, 4H), 2.66 (t, *J* = 6.0 Hz, 4H), 1.90-1.82 (m, 4H). ^{13}C-NMR (DMSO, 75 MHz) (ppm): 165.73, 153.63, 147.82, 131.35, 120.98, 118.99, 117.82, 92.08, 49.87, 27.49, 21.00. ESI-MS calcd for $[M-H]^-$ 267.32; Found 267.30.



Scheme S2. Synthesis of probe **MRMP-1**.

Synthesis of MRMP-1. To synthesize **MRMP-1**, the peptide (sequence Fmoc-K_{mtt}RRRR) was firstly assembled by standard Fmoc solid phase peptide synthesis (SPPS) method. The Mtt group in the peptide was then deprotected with 3% trifluoroacetic acid in dichloromethane to allow further installation of a palmitoyl group. Subsequently the molecular rotor dye CCVJ was introduced to the peptide with O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluorophosphate (HBTU) and Hydroxybenzotriazole (HOBT) as the coupling reagent (Scheme S2). The crude product was obtained by cleavage from the resin using cleavage cocktail reagents (95% TFA, 2.5% TIS and 2.5% H_2O). Further purification by HPLC yielded the pure product **MRMP-1**, which was characterized by LC-MS. ^{1}H NMR (MeOD, 300 MHz) (ppm): 7.92 (s, 1H), 7.50 (s, 2H), 4.38-4.30 (m, 5H), 3.39-3.37 (m, 4H), 3.25-3.21 (m, 10H), 2.78-2.73 (m, 4H), 2.19-2.16 (m, 2H), 2.00-1.27 (m, 55H). ESI-MS calcd for $[M+2H]^{2+}$ 630.68; Found 630.4.

Photophysical parameters of MRMP-1 in the glycerol/water mixtures of various viscosity.

The radiative (K_r) and non-radiative (K_{nr}) decay constants were calculated using experimentally measured fluorescence quantum yields (Φ_f) and fluorescence lifetimes (τ_f) of **MRMP-1** in different solvents according to the following equations:

$$\Phi_f = K_r /(K_r + K_{nr}) \qquad \tau_f = 1/(K_r + K_{nr})$$

Table S1. Fluorescence quantum yield, lifetimes, radiative and non-radiative decay constants of **MRMP-1** in different water/glycerol mixtures.

Solvent	Φ_f	τ_f (ns)	K_r (ns^{-1})	K_{nr} (ns^{-1})
100% Glycerol	0.394	0.97	0.406	0.625
80% Glycerol	0.212	0.56	0.378	1.408
70% Glycerol	0.141	0.47	0.299	1.828
60% Glycerol	0.092	0.43	0.214	2.111
50% Glycerol	0.079	0.42	0.188	2.193
40% Glycerol	0.080	0.4	0.200	2.300
Water	0.041	0.38	0.107	2.525

Φ_f: Fluorescence quantum yield. τ_f: Fluorescence lifetime. K_r: Radiative decay constant. K_{nr}: Nonradiative decay constant.

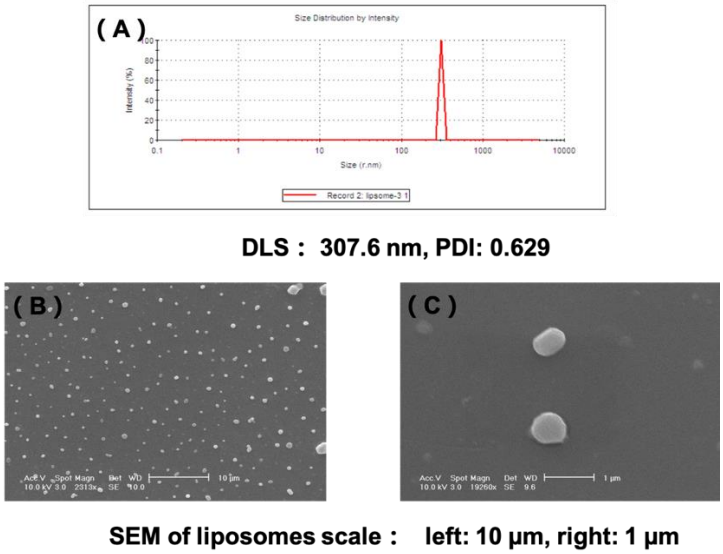


Figure S1. Dynamic light scattering (DLS, A), scanning electron microscope (SEM, B & C) of the liposomes prepared.

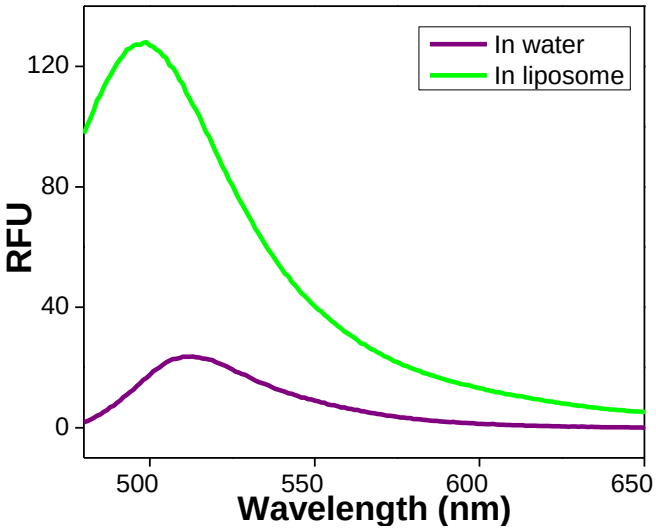


Figure S2. Fluorescence measurement of **MRMP-1** (50 μM) in water and liposomes. λex = 440 nm. The results indicated that the probe can bind to the liposomes and become highly emissive.

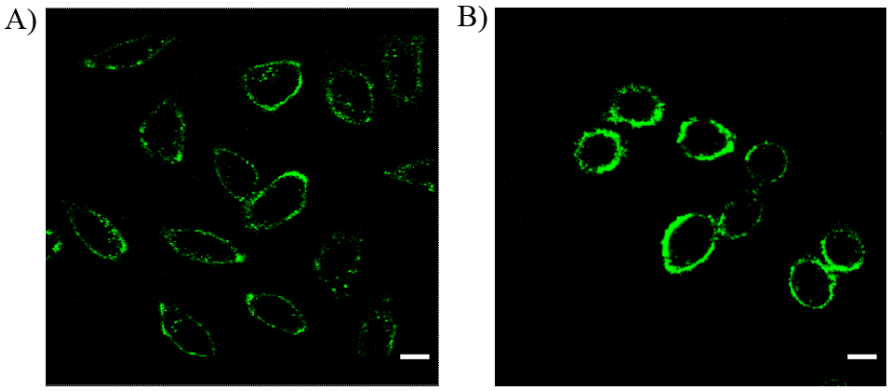


Figure S3. CLSM imaging of living Hela cells (A) and LO2 cells (B) incubated with **MRMP-1** (green channel) at concentrations of 20 μM for 20 min. The results indicated that **MRMP-1** can bind to the plasma membranes specifically, λex = 440 nm. Scale bar: 30 μm.

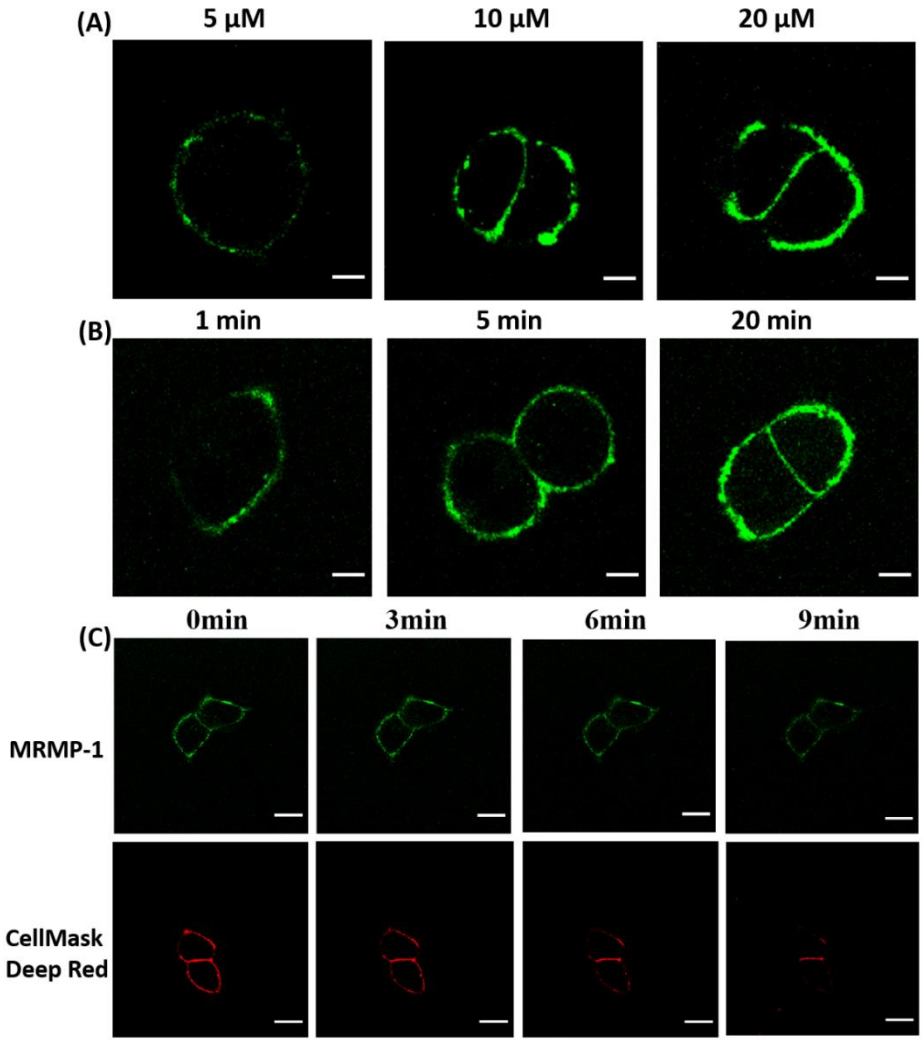


Figure S4. (A) CLSM images of MCF-7 cells incubated with **MRMP-1** (green channel) at concentrations of 5, 10 and 20 μM for 20 min. Scale bar: 10 μm. (B) CLSM images of MCF-7 cells incubated with **MRMP-1** (20 μM, green channel) for 1, 5 and 20 min. Scale bar: 10 μm. (C) CLSM images of MCF-7 cells treated with **MRMP-1** (green channel) or CellMask Deep Red (red channel) with increasing scanning time (0−9 min). **MRMP-1**, λex = 440 nm; CellMask Deep Red, λex = 650 nm. Scale bar: 30 μm.

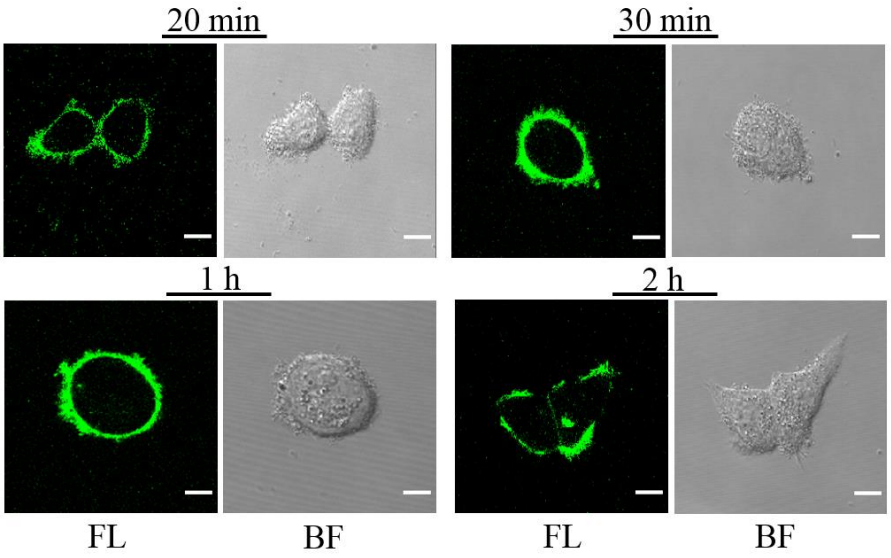


Figure S5. CLSM images of MCF-7 cells incubated with **MRMP-1** (20 μM, green channel) for 20 min, 30 min, 1 h and 2 h, λex = 440 nm. The result showed that the probe could migrate into the cells when extending the incubation time for 2 h. Scale bar: 10 μm.

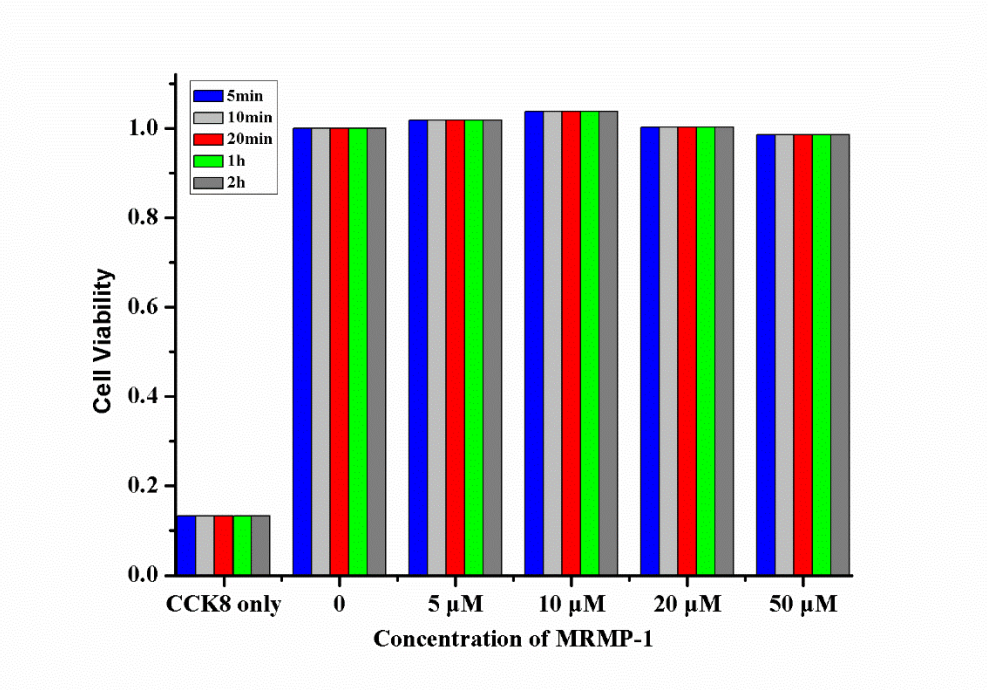


Figure S6. In vitro cytotoxicity of **MRMP-1** in MCF-7 cells determined by CCK8 assay.

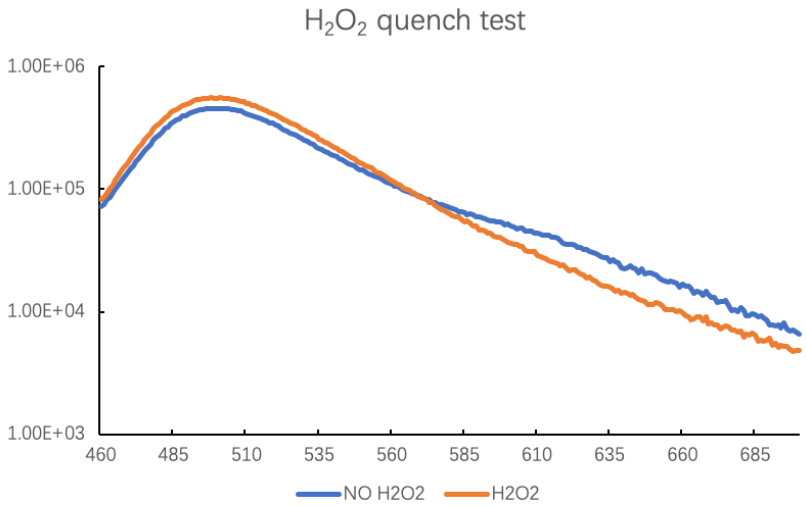


Figure S7. Fluorescence analysis of **MRMP-1** (20 μM) in the conditions of DMSO only or DMSO containing 50 mM hydrogen peroxide. The probe was incubated for 1 h before fluorescence measurement. The results indicated that hydrogen peroxide would not quench the probe.

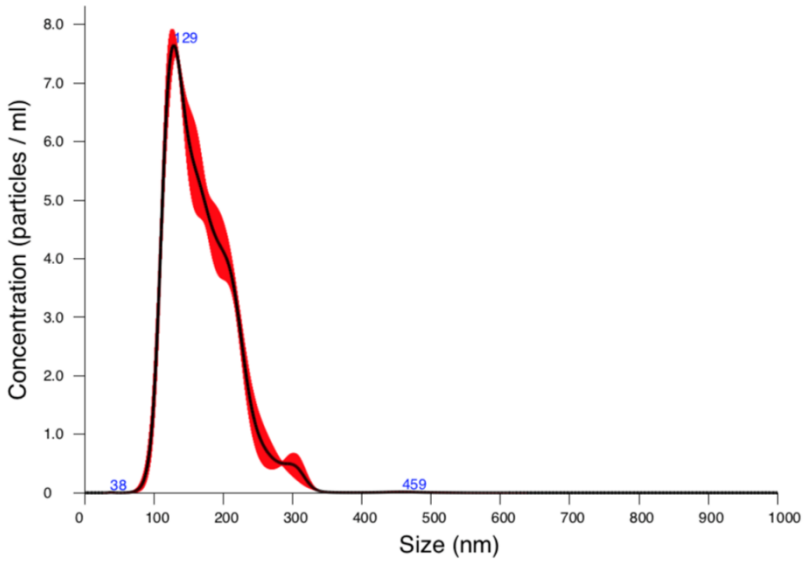


Figure S8. Nanoparticle tracking analysis (NTA) of exosomes extracted from L cells. It indicated that the mode size of isolated exosomes is 129 nm and the concentration was determined as 7.3×10^8 particles/mL.

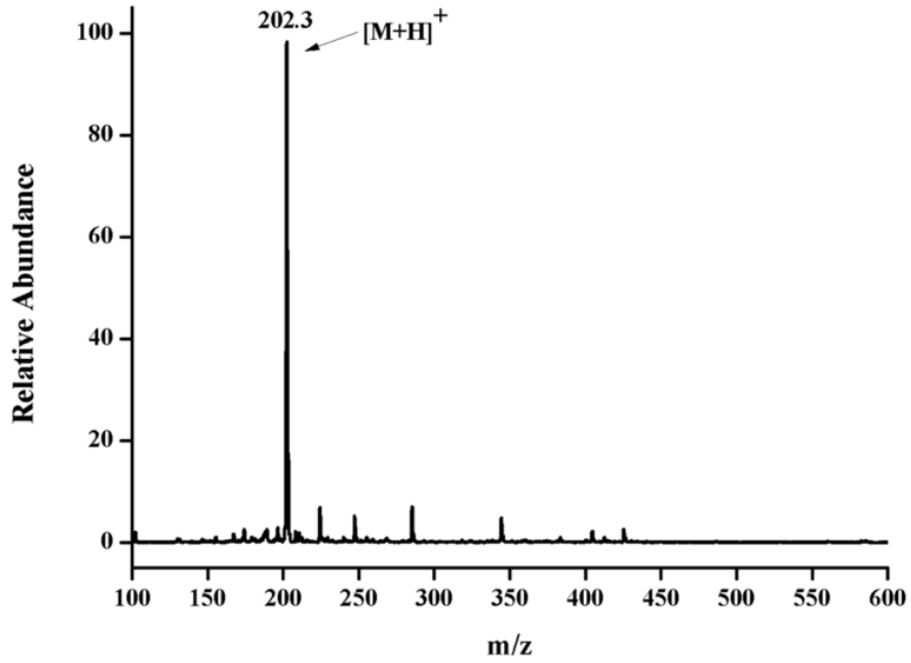


Figure S9. MS analysis of compound 2.

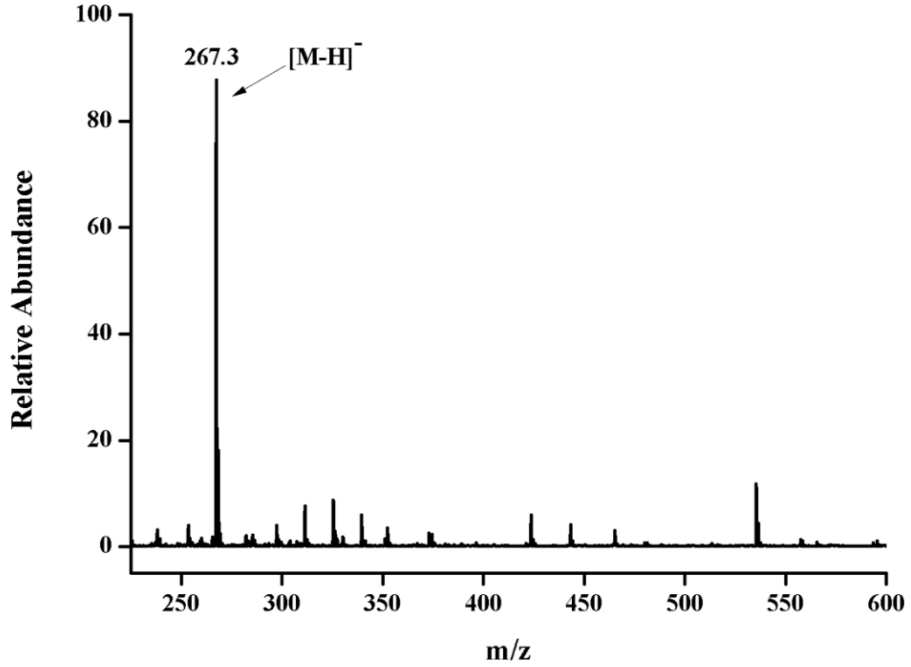


Figure S10. MS analysis of CCVJ

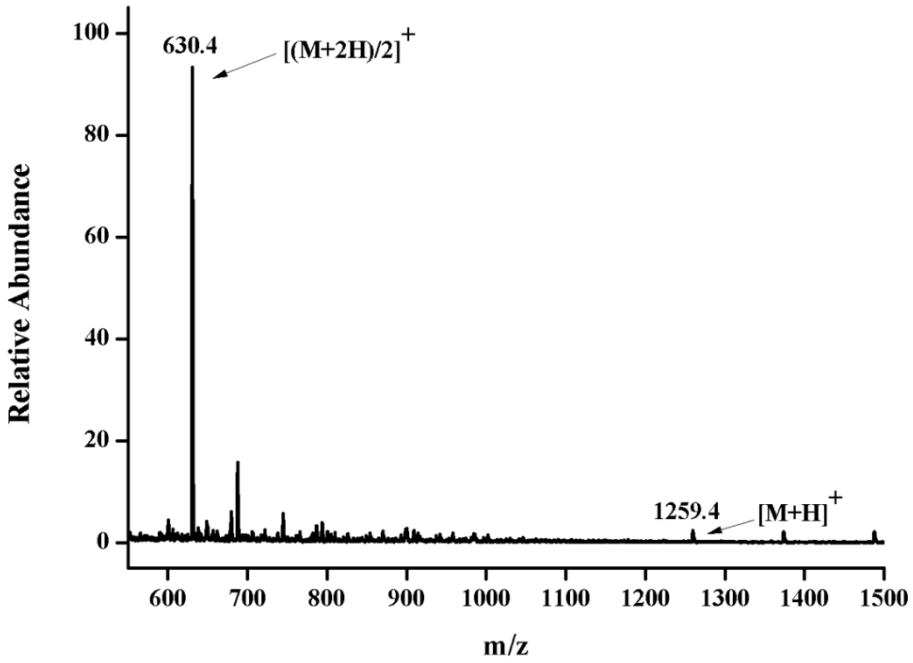
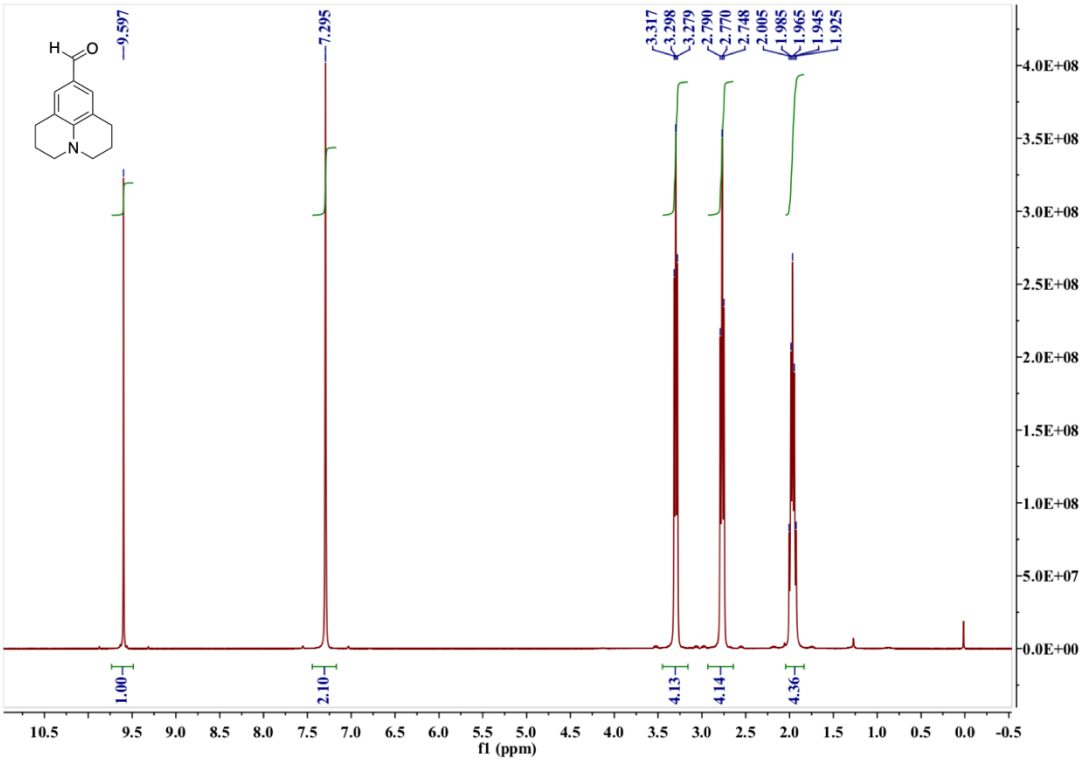


Figure S11. MS analysis of **MRMP-1**.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7.295
3.317
3.298
3.279
2.790
2.770
2.748
2.005
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1.965
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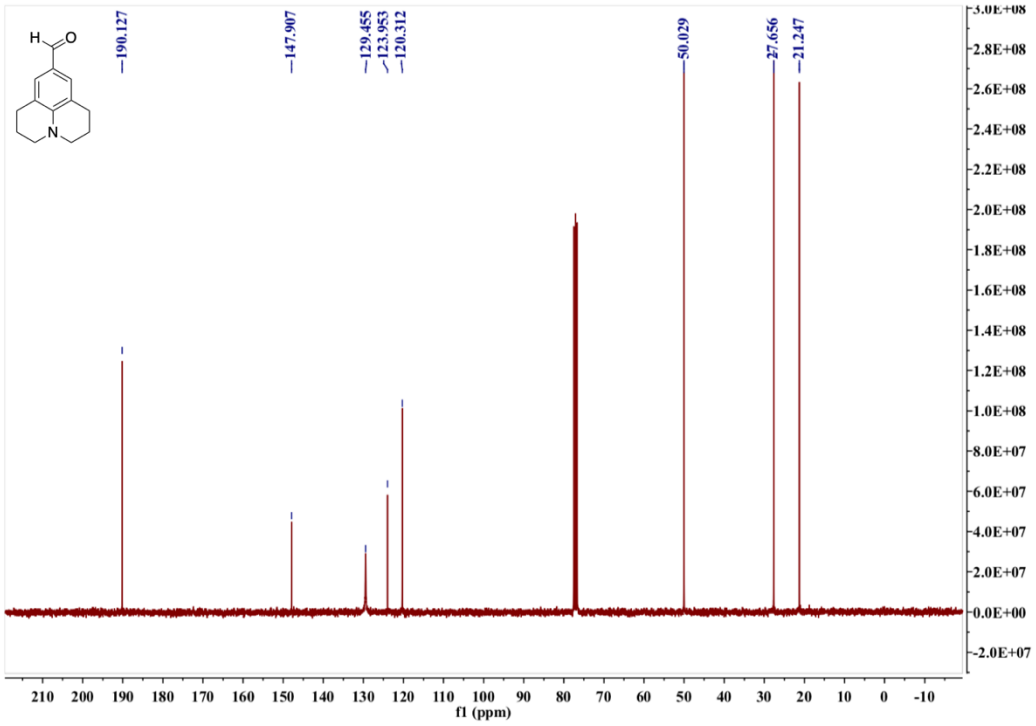
2.10
4.13
4.14
4.36
f1 (ppm)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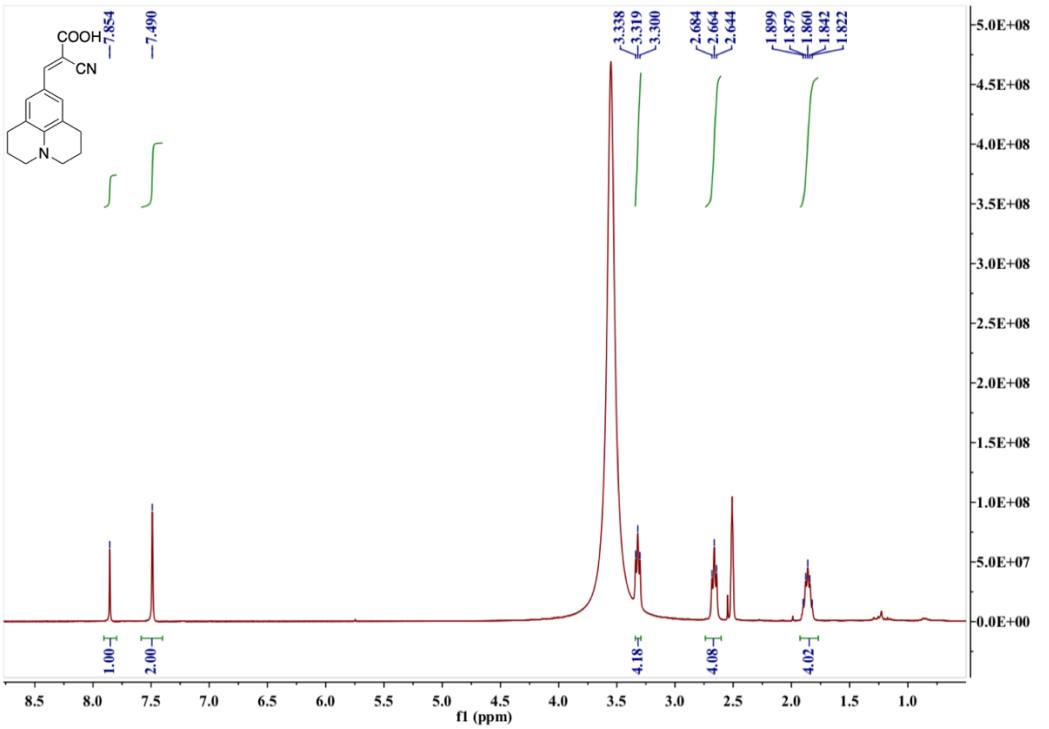
27.656
21.247
f1 (ppm)


COOH
CN
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7.490
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3.319
3.300
2.684
2.664
2.644
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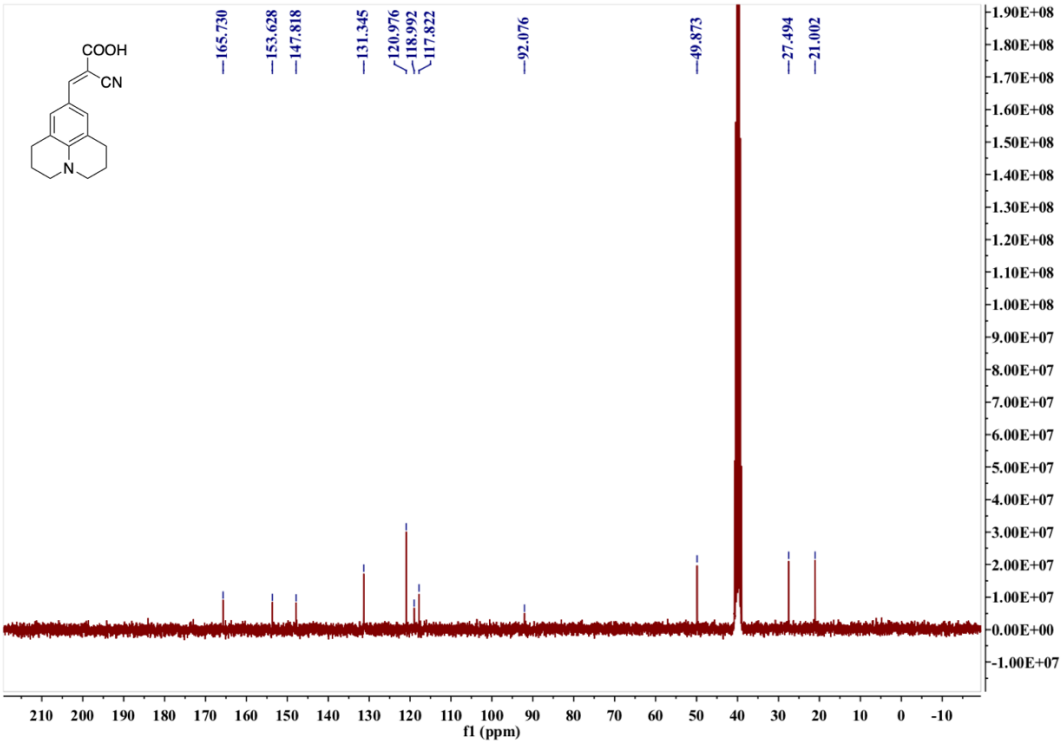
1.00
2.00
4.18
4.08
4.02
f1 (ppm)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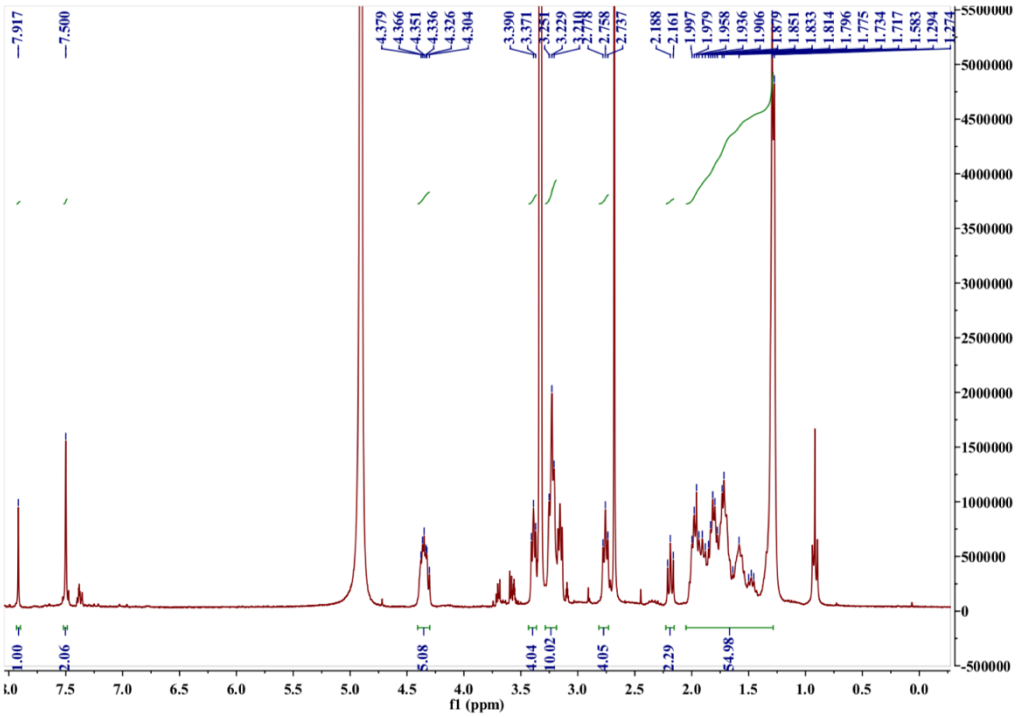

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1. C. Théry, S. Amigorena, G. Raposo and A. Clayton, *Curr. Protoc. Cell Biol.,* 2006, **30**, 3.22.21-23.22.29.
2. Rumble, C.; Rich, K.; He, G.; Maroncelli, M., *J. Phys. Chem. A,* 2012**,** ***116***, 10786-10792.