Supporting Information

Cytosolar delivery of large proteins using nanoparticle-stabilized nanocapsules

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Materials & Methods

Preparation of GIPA Ligand

Synthesis of Compound 1

1-(Triphenylmethyl)-L-histidine (1.50 g, 3.77 mmol) was dispersed in 20 mL methanol and mixed with triethylamine (382 mg, 0.53 mL, 3.77mmol). The suspension was stirred at room temperature for 10 min. Ethyl trifluoroacetate (698 mg, 0.58 mL, 4.91mmol) was added into the white suspension dropwise. The suspension turned into clear brownish solution after 1 h stirring at room temperature. The stirring was continued for another 5 hrs. Then the pH of the solution was adjusted to 1-2 with 1 M HCl at 0 °C. The aqueous solution was extracted with chloroform for 5 times. The combined organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The white solid was dissolved with a minimal amount of methanol and diethyl ether mixture (v/v = 1:1) and further recrystallized with *n*-hexanes at 4 °C. The product was filtered and dried under vacuum as white solid (1.55 g, 3.14 mmol, 83.3%) (Compound **1**, Figure 2). ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.57 (s, 1H), 7.44 – 7.33 (m, 9H), 7.28 (d, J = 1.4 Hz, 1H), 7.10 – 7.00 (m, 6H), 6.66 (d, J = 1.3 Hz, 1H), 4.48 (s, 1H), 3.03 (dd, J = 14.7, 4.1 Hz, 1H), 2.90 (dd, J = 14.6, 10.3 Hz, 1H) (Figure S1). MALDI-MS *m/z* calculated for $C_{27}H_{22}F_3N_3O_3$ $[M + H]^+$ 493.16, found 493.53 (Figure S2).

Synthesis of Compound 2

Compound **4** (Figure 2) was synthesized according to previously reported procedure.[1] Compound **1** (650 mg, 1.32 mmol), Compound **4** (982 mg, 1.58 mmol), and diisopropylamine (DIPEA, 511 mg, 0.69 mL, 3.95 mmol) were dissolved in 15 mL dimethylformamide (DMF) and stirred for 10 min at 0 °C. 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU, 601 mg, 1.58 mmol) was dissolved in 3 mL DMF and added into the previous mixture dropwise. Then the solution was warmed up to room temperature and stirred for 2 hrs. The reaction was quenched with 5 mL deionized water and diluted with 100 mL ethyl acetate. The organic layer was washed with 0.1 M HCl for 3

times, saturated $NaHCO_3$ solution for 3 times, and saturated NaCl solution for 5 times. Then the organic layer was separated and dried over Na_2SO_4. The mixture was concentrated and purified by column chromatography over silica gel with Ethyl acetate and subsequently ethyl acetate–methanol (95:5). The product was concentrated *in vacuo* and obtained as pale yellow oil (1.19 g, 1.08 mmol, 81.8%) (Compound **2**, Figure 2). ^1H NMR (400 MHz, $CDCl_3$): δ 8.67 (d, J = 7.0 Hz, 1H), 7.56 (s, 1H), 7.47 – 7.38 (m, 7H), 7.38 – 7.33 (m, 9H), 7.33 – 7.26 (m, 5H), 7.25 – 7.18 (m, 3H), 7.16 – 7.07 (m, 6H), 4.72 (q, J = 6.0 Hz, 1H), 3.69 – 3.60 (m, 6H), 3.60 – 3.55 (m, 6H), 3.55 – 3.48 (m, 2H), 3.48 – 3.35 (m, 4H), 3.10 (dd, J = 15.0, 4.8 Hz, 1H), 3.02 – 2.94 (m, 1H), 2.14 (t, J = 7.4 Hz, 2H), 1.63 – 1.52 (m, 2H), 1.40 (p, J = 7.2 Hz, 2H), 1.35 – 1.08 (m, 14H) (Figure S3). MALDI-MS *m/z* calculated for $C_{65}H_{75}F_3N_4O_6S$ $[M + Na]^+$ 1119.54, found 1119.63 (Figure S4).

Synthesis of Compound 3

Compound **2** (1.19 g, 1.08 mmol) was dissolved in 2 mL methanol. $LiOH{\cdot}H_2O$ (228 mg, 5.42 mmol) was dissolved in 2 mL deionized water and added into the methanol solution in one portion at room temperature. The mixture was stirred for 15 hrs. $CHCl_3$ was used to extract the solution for 6 times. The combined organic layer was dried over $MgSO_4$ and concentrated *in vacuo* without further purification. The deprotected product (680 mg) was directly used for the next step. The product after deprotection (272 mg, 0.27 mmol), *N,N*'-di-(tert-butoxycarbonyl)thiourea (Bis-Boc-thiourea, 63 mg, 0.23 mmol), and DIPEA (0.1 mL, 0.57 mmol) were dissolved in 2 mL dichloromethane (DCM) while being flushed with N_2. *N*-Iodosuccinimide (NIS, 51 mg, 0.23 mmol) [2] was dispersed in 2 mL DCM and added into the mixture in one portion at 0°C. The reaction was then warmed up to room temperature and stirred for 15 hrs. $Na_2S_2O_4$ (10 mL 1 M) solution was added to quench the reaction and stirred for another 15 min. Afterwards, the mixture was diluted with 100 mL ethyl acetate and washed with saturated NaCl solution for 3 times. The organic layer was separated and dried over Na_2SO_4. The mixture was concentrated and purified by column chromatography over

silica gel with ethyl acetate–methanol (95:5). The product was obtained as yellow oil (198 mg, 0.16 mmol, 44.0%) (Compound **3**, Figure 2). ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.00 (d, *J* = 7.2 Hz, 1H), 7.44 (t, *J* = 1.8 Hz, 2H), 7.43 – 7.40 (m, 3H), 7.34 (td, *J* = 4.4, 1.8 Hz, 9H), 7.31 (q, *J* = 1.9, 1.5 Hz, 2H), 7.30 – 7.26 (m, 6H), 7.24 (t, *J* = 1.3 Hz, 1H), 7.22 (d, *J* = 2.2 Hz, 1H), 7.20 (t, *J* = 1.3 Hz, 1H), 7.16 – 7.10 (m, 5H), 3.70 – 3.61 (m, 6H), 3.59 (dq, *J* = 6.2, 4.0, 2.9 Hz, 5H), 3.51 (t, *J* = 5.4 Hz, 2H), 3.45 (t, *J* = 6.9 Hz, 3H), 2.15 (t, *J* = 7.3 Hz, 2H), 1.58 (p, *J* = 6.9 Hz, 3H), 1.52 (s, 7H), 1.46 (d, *J* = 1.6 Hz, 8H), 1.39 (q, *J* = 7.5 Hz, 2H), 1.25 (d, *J* = 23.0 Hz, 16H) (Figure S5). ESI-MS *m/z* calculated for $C_{74}H_{94}N_6O_9S$ $[M + H]^+$ 1243.7, $[M + H + CH_3OH]^+$ 1275.7, found 1243.5, 1275.5 (Figure S6).

Synthesis of GIPA Ligand

Compound **3** (198 mg, 0.16 mmol) was dissolved in 2 mL anhydrous DCM and stirred at room temperature with N_2 flushed. A mixture containing 9.25 mL trifluoroacetic acid (TFA), 0.25 mL 1,2-ethanedithiol (EDT), 0.25 mL triisopropylsilane (TIPS), and 0.25 mL deionized H_2O was made and added into the DCM solution. The mixture was then stirred at room temperature for 1 hr under N_2 protection. The solvent was evaporated afterwards and the residue was washed with *n*-hexanes for 2 times, *n*-hexanes–diethyl ether (v/v = 4:1) for 4 times. The residue was dried under vacuum and the product was obtained as pale yellow oil (105 mg, 0.13 mmol, 83.1%) (**GIPA Ligand**, Figure 2). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.82 – 8.46 (m, 2H), 8.28 (d, *J* = 63.0 Hz, 2H), 7.38 (s, 2H), 7.27 (s, 2H), 4.77 (s, 1H), 3.61 (t, *J* = 13.6 Hz, 16H), 3.43 (p, *J* = 21.3, 17.8 Hz, 8H), 2.51 (q, *J* = 7.4 Hz, 2H), 1.56 (dp, *J* = 20.4, 6.6 Hz, 5H), 1.43 – 1.15 (m, 19H) (Figure S7). MALDI-MS *m/z* calculated for $C_{26}H_{50}N_6O_5S$ $[M + H]^+$ 559.36, found 559.50 (Figure S8).

Gold nanoparticle (AuNP) synthesis and functionalization with GIPA Ligand

The AuNPs (*ca.* 2 nm core size) were synthesized according to previously reported procedures. [3] The 1-pentanethiol-stablized AuNPs were functionalized with GIPA Ligand via place-exchange reaction.[4] Briefly, 20 mg AuNP stabilized with 1-pentanethiol was dissolved

in 2 mL anhydrous DCM and stirred at room temperature with N_2 flushed. GIPA Ligand (60 mg) was dissolved in 2 mL DCM–methanol mixture (v/v = 9:1) and added into the AuNP solution. The mixture was then stirred at room temperature for 96 hrs under N_2 protection. The solvent was evaporated afterwards and the residue was washed with *n*-hexanes–DCM (v/v = 9:1) for 5 times. Then the functionalized AuNPs were dispersed in *Mili-Q* water and dialyzed using 10,000 MWCO SnakeSkin Dialysis Tubing (Thermo Scientific, USA) for 120 hrs. The concentration of the AuNP solution was measured according to a reported method by UV spectroscopy on a Molecular Devices SpectraMax M2 at 506 nm.[5] Transmission electron microscopy (TEM) of AuNPs was performed on a JEOL 2000FX electron microscope (Figure S9). Dynamic light scattering (DLS) profiles (Figure S10) and zeta potential were carried out on a Malvern Zetasizer Nano ZS. The surface zeta potential of GIPA-AuNPs was (14.6 ± 0.4) mV.

Cell culture

HeLa cells were cultured in a humidified atmosphere (5% CO_2) at 37 °C, and grown in Dulbecco's modified eagle's medium (DMEM, low glucose) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (100 $U\cdot mL^{-1}$ penicillin and 100 $\mu g\cdot mL^{-1}$ streptomycin).

Fluorescence titration

In the fluorescent titration experiment between nanoparticles and dsRed, the change of fluorescence intensity at 585 nm was measured with an excitation wavelength of 561 nm at various concentrations of AuNPs from 0 to 200 nM on a Molecular Devices SpectraMax M2 microplate reader (at 25 °C). Decay of fluorescence intensity arising from 100 nM dsRed was observed with increasing NP concentration. Nonlinear least-squares curve fitting analysis was carried out to estimate the binding constant (K_s).[6]

Protein-NPSC complex formation

To make the protein-NPSC complex, 2.5 μM GIPA AuNPs were incubated with 1.5 μM of protein in 60 μL of phosphate buffer (5 mM, pH = 7.4) for 10 min. Then, 1 μL of linoleic acid was mixed with 500 μL of phosphate buffer (5 mM, pH = 7.4) containing 1 μM GIPA AuNPs and agitated with an amalgamator (Yinya New Materials Co. Ltd, Hangzhou, China) at 5000 rpm for 100 s to form emulsions. Finally, the mixture of the protein and GIPA AuNPs were diluted to 135 μL with phosphate buffer (5 mM, pH = 7.4) followed by the addition of 15 μL of the emulsion. The protein-NPSC complexes were ready to use after 10 min of incubation at room temperature. The final concentrations of GIPA AuNPs and the protein were 1.5 μM and 600 nM, respectively.

Protein delivery

A total of 240,000 HeLa cells were cultured in a confocal dish for 24 hrs prior to delivery. The cells were washed with cold phosphate buffer saline (PBS) thrice right before delivery. After preparation, the cells were incubated in protein-NPSC complex solution (150 μL of the complex diluted by 1.35 mL of the DMEM without FBS) for 1 hr, followed by incubating with fresh DMEM (with 10% FBS) for 10 min, unless otherwise mentioned. The cells were then kept in PBS and imaged by a laser scanning confocal microscope (LSM 510, Zeiss, Germany).

Cell viability assay (Alamar Blue)

15,000 HeLa cells were cultured in a 96-well plate for 24 hr prior to the experiment. The cells were washed by cold phosphate buffer saline (PBS) three times before the delivery, then different amounts of the NPSC complex (prepared as mentioned above) were diluted by DMEM and incubated with the cells for 1 hr followed by the incubation with DMEM containing 10% FBS and 1% antibiotics for 23 hr. After washing with PBS three times, the cells were then incubated with 200 μL DMEM containing 10% Alamar Blue for 3 h. Cell viability was calculated by measuring the fluorescence intensity of Alamar Blue at 590 nm, with an excitation of 535 nm.

X-gal staining

Cells were stained according to the assay kit (Genlantis, USA). Briefly, cells were washed with PBS once and fixed with the fixation solution followed by 4 hrs of staining. Cells were then washed once with PBS and observed under an optical microscope. Experiments were performed in triplicate.

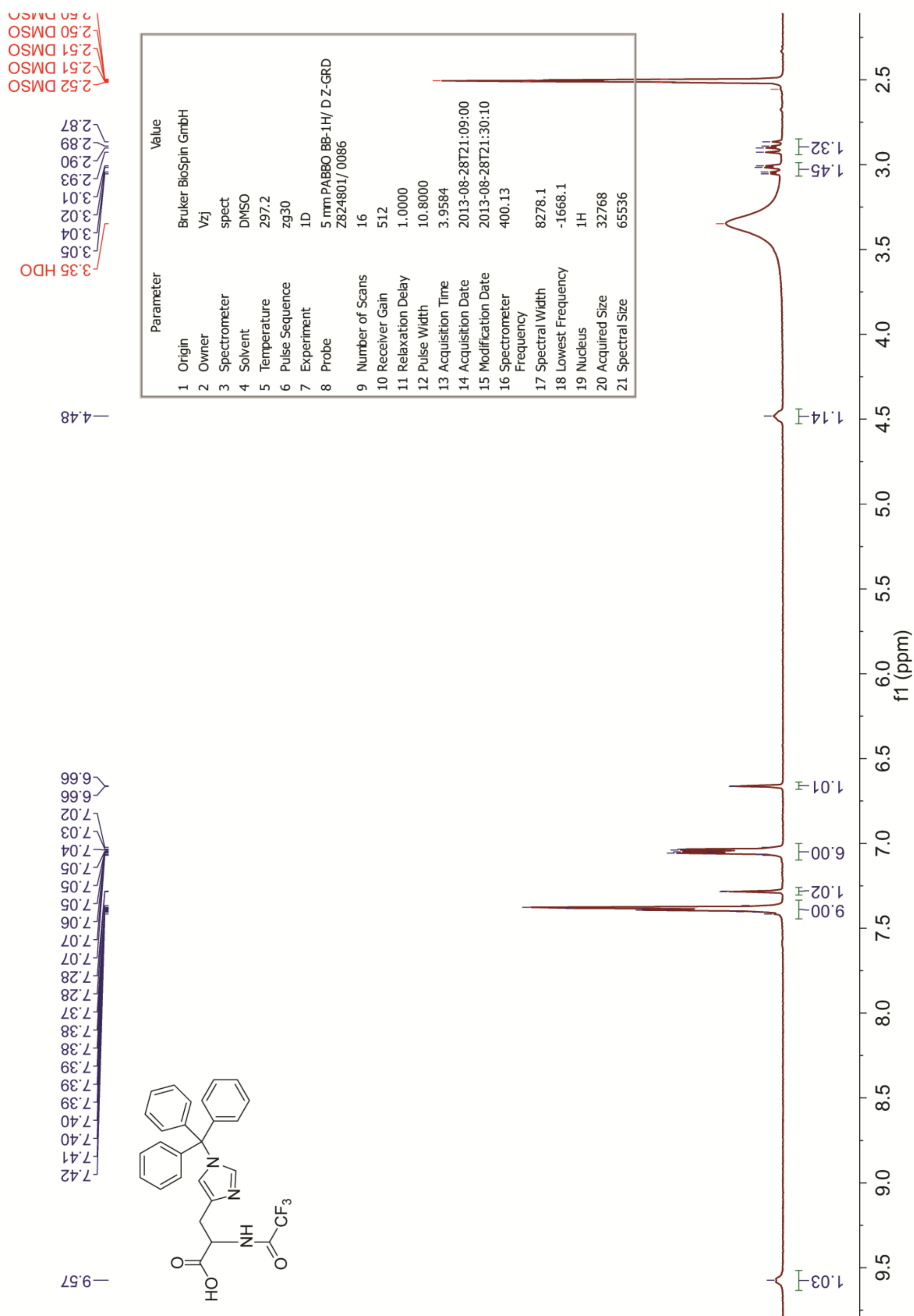

Figure S1. ^{1}H NMR spectra (400 MHz) of Compound **1** in DMSO-d_6.

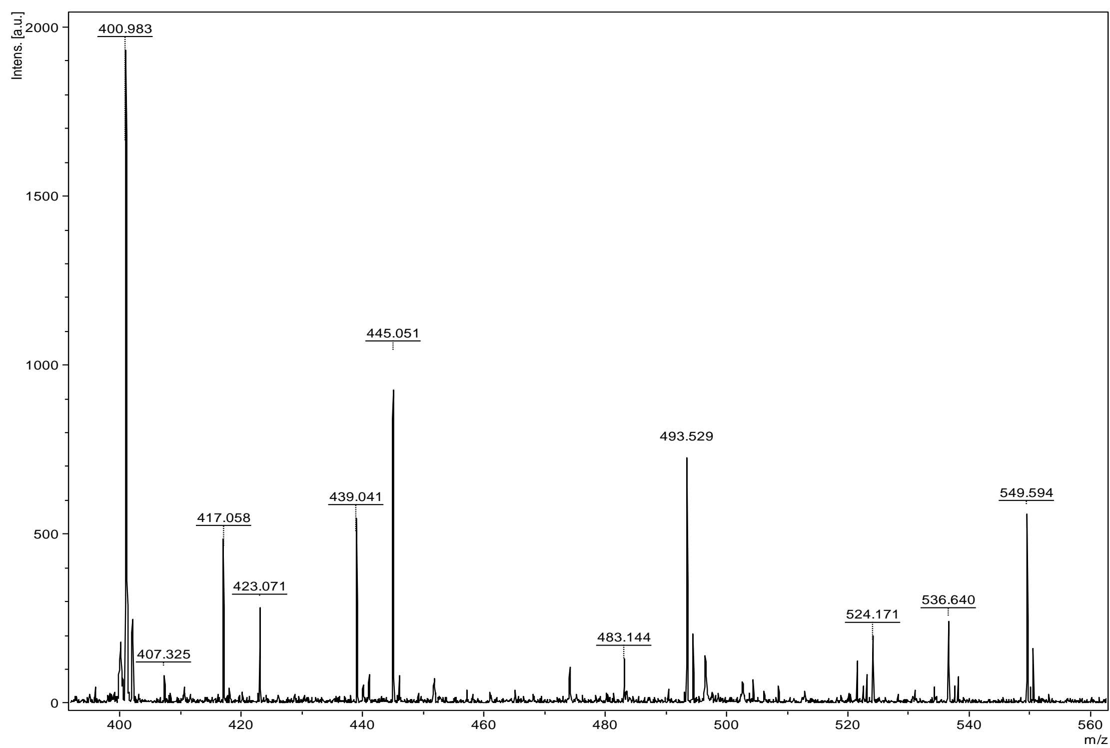

Figure S2. Representative MALDI-MS spectra of Compound **1**.

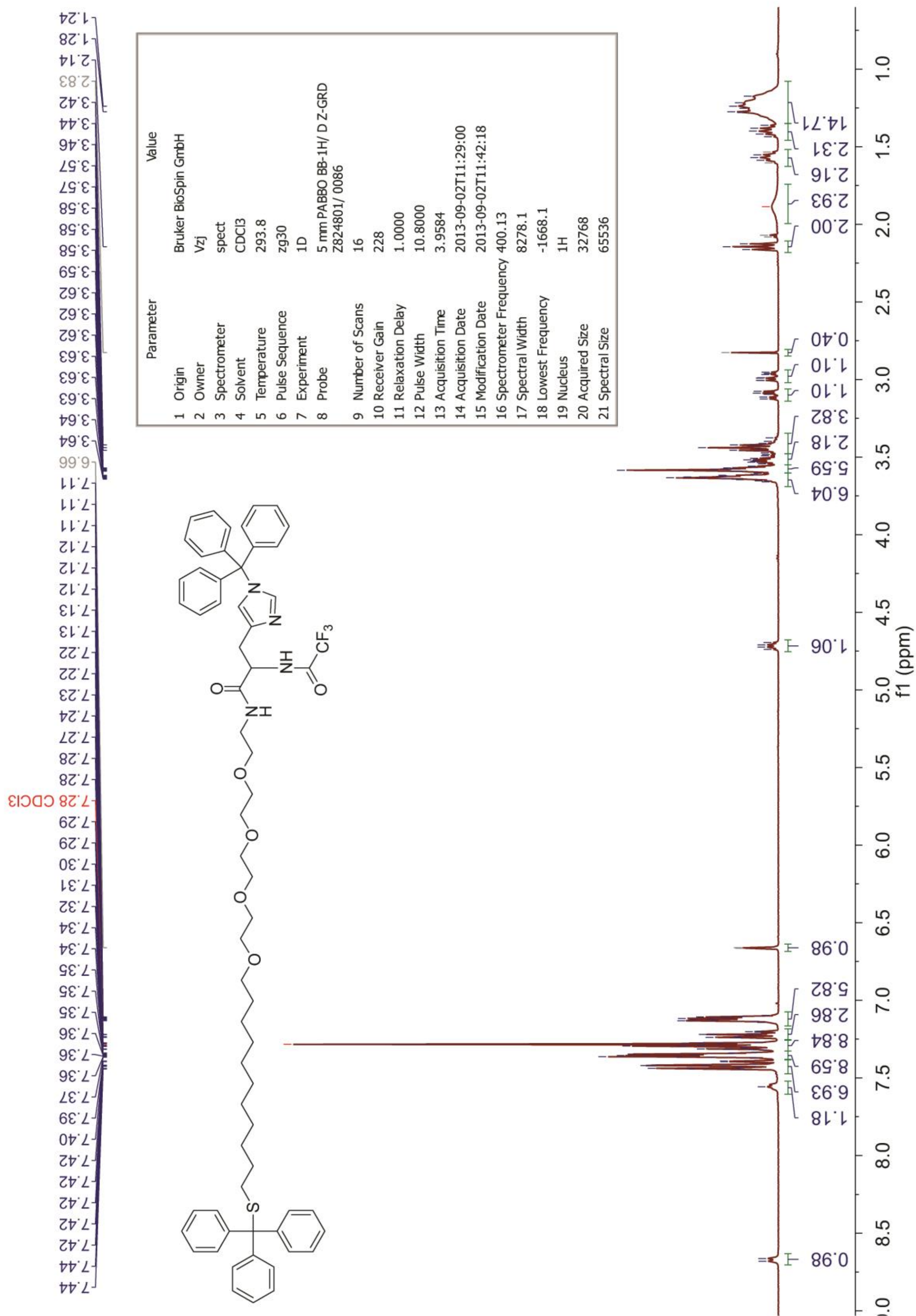

Figure S3. ^{1}H NMR spectra (400 MHz) of Compound **2** in $CDCl_3$.

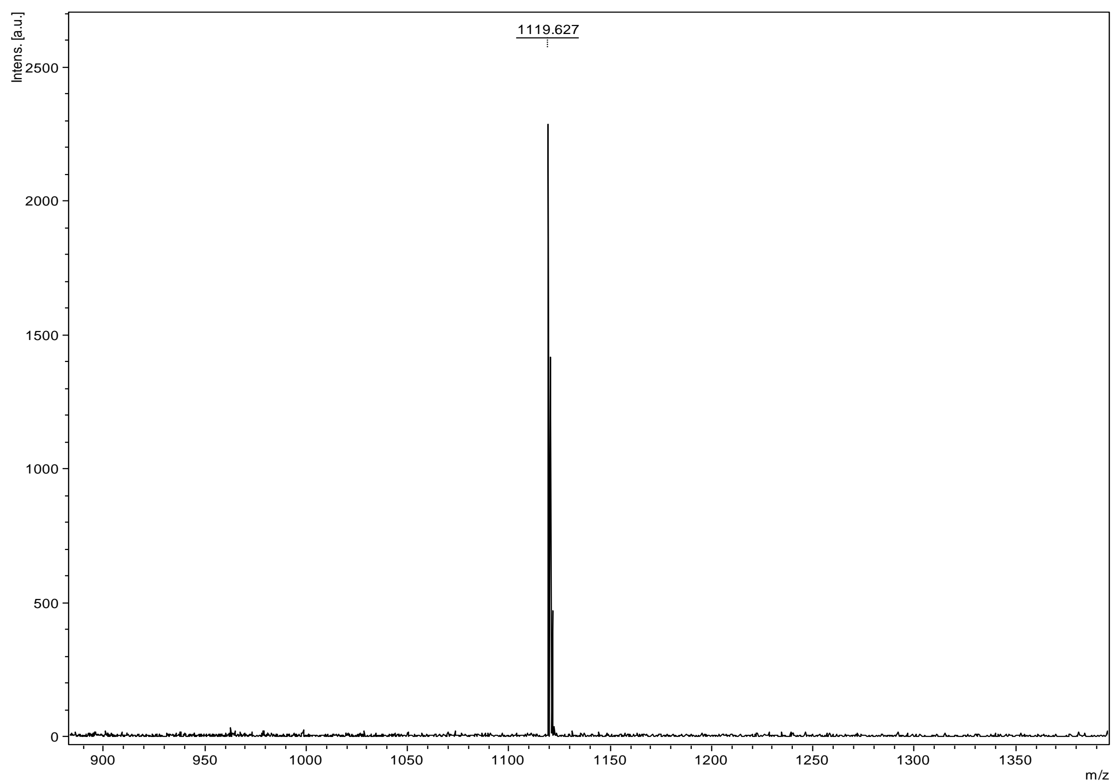

Figure S4. Representative MALDI-MS spectra of Compound **2**.

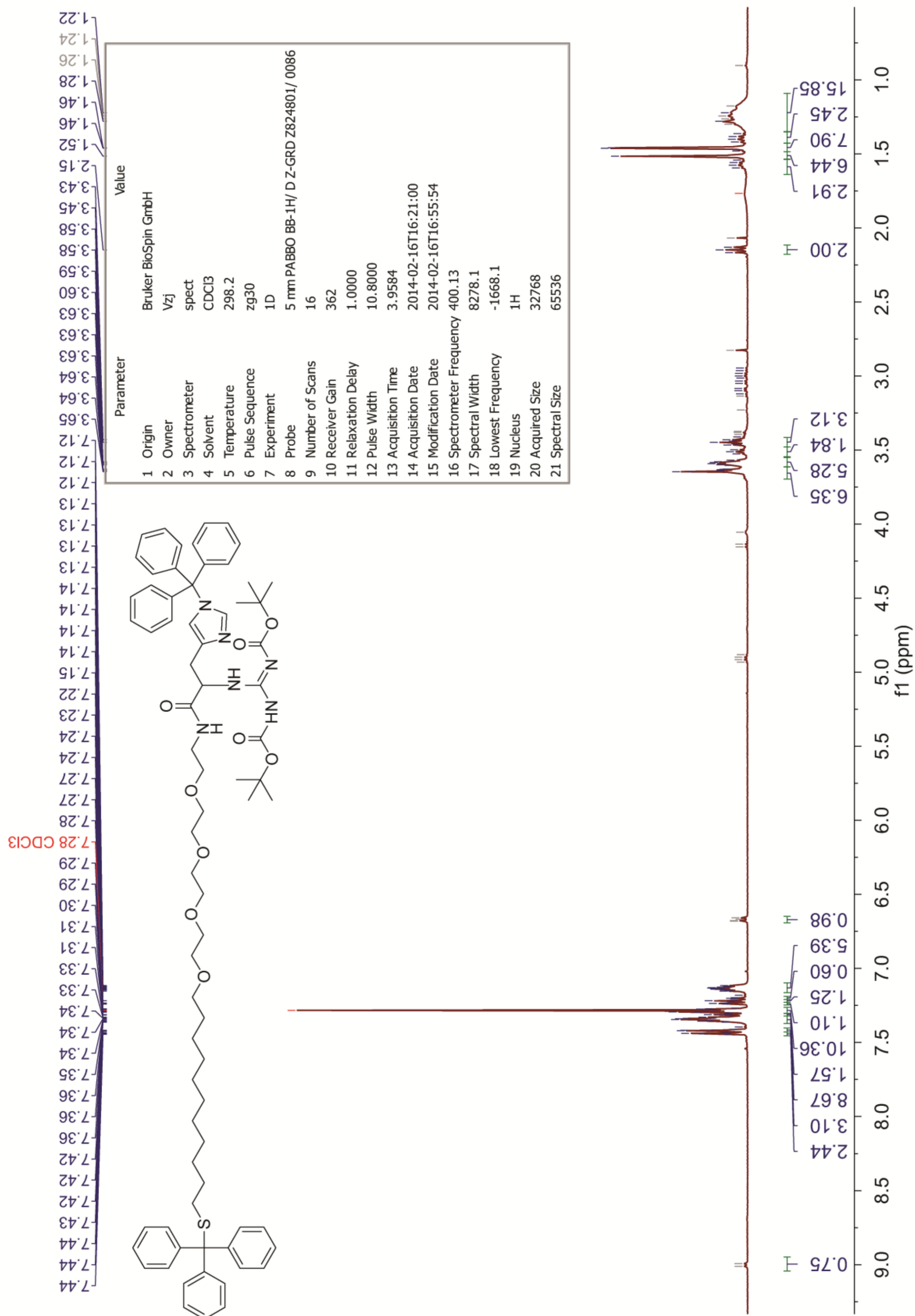

Figure S5. ^{1}H NMR spectra (400 MHz) of Compound **3** in $CDCl_3$.

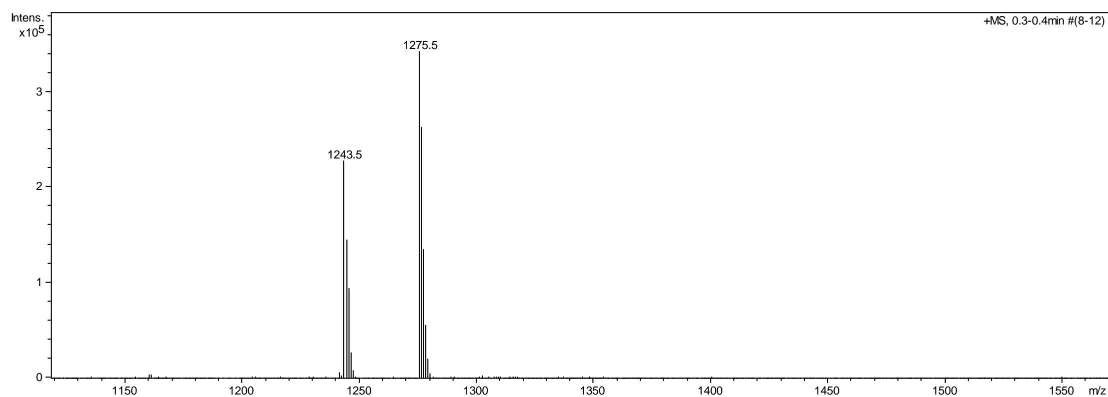

Figure S6. Representative ESI-MS spectra of Compound **3**.

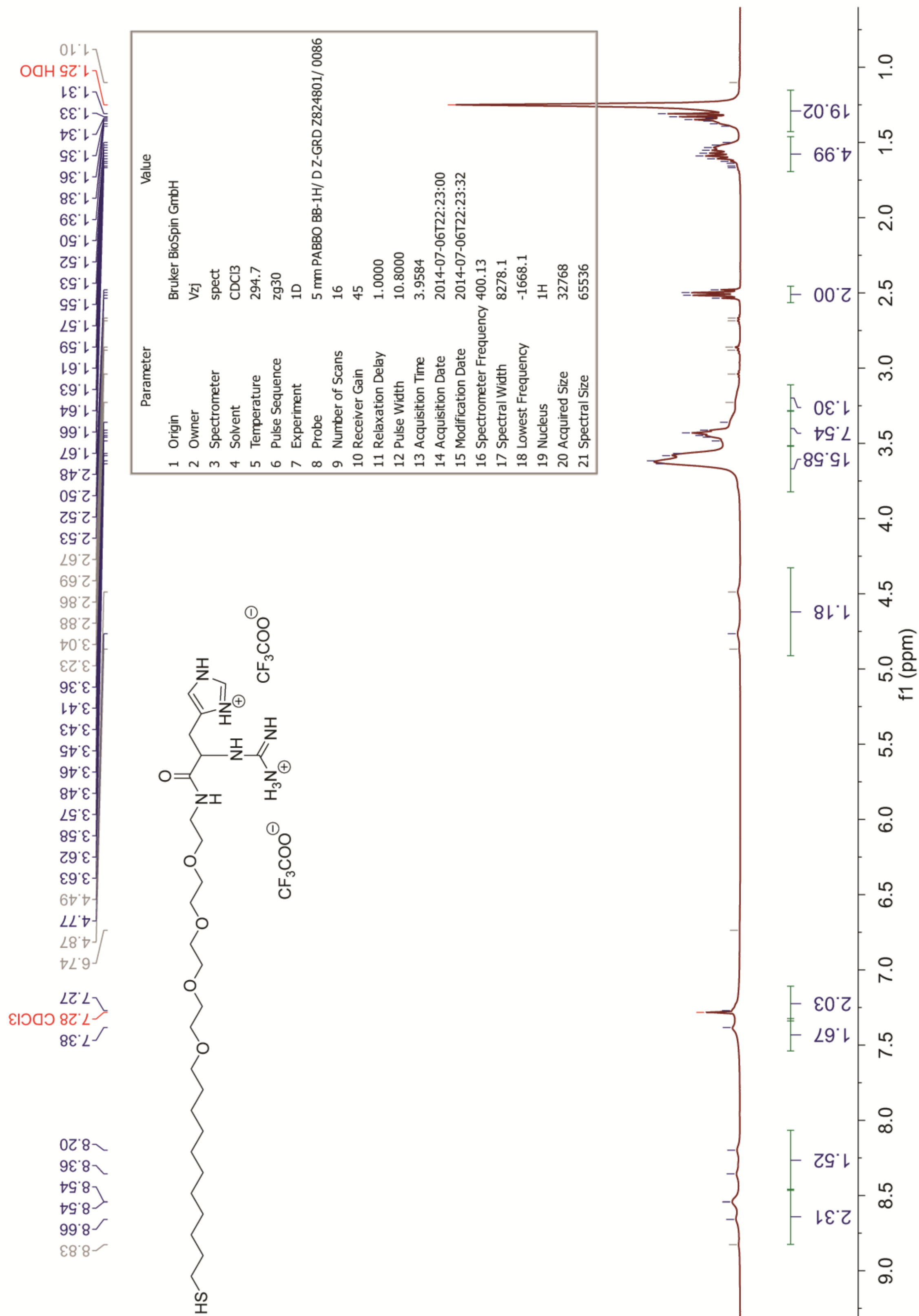

Figure S7. ¹H NMR spectra (400 MHz) of **GIPA Ligand** in $CDCl_3$.

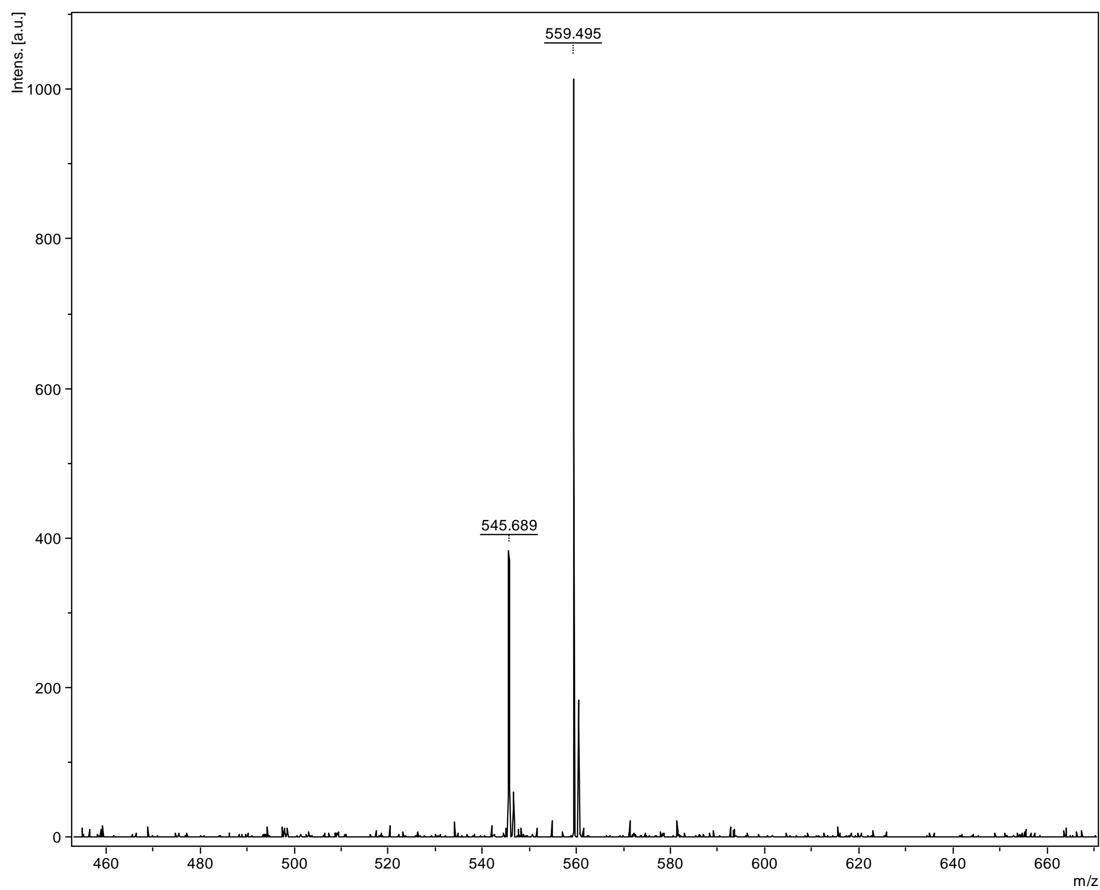

Figure S8. Representative MALDI-MS spectra of **GIPA Ligand**.

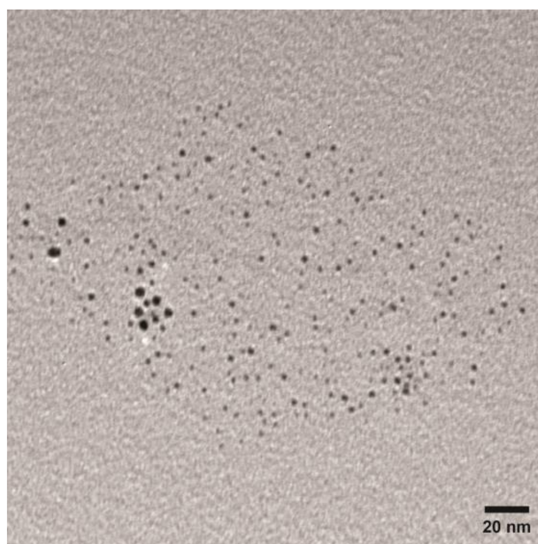

Figure S9. TEM image of GIPA-functionalized AuNPs. The black scale bar is 20 nm as denoted.

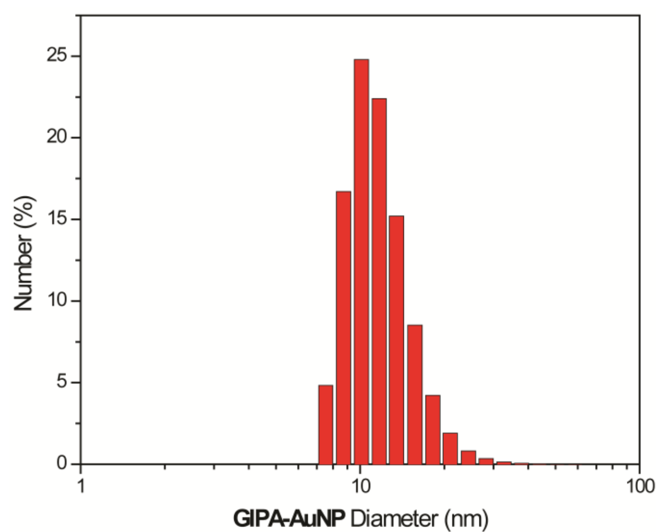

Figure S10. DLS histogram of GIPA-functionalized AuNPs, demonstrating the hydrodynamic diameter distribution of nanoparticles.

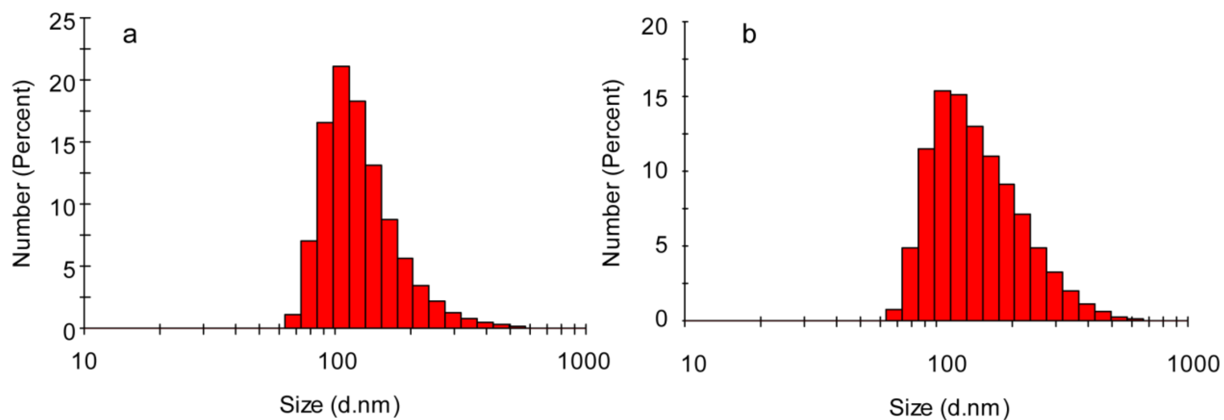

Figure S11. DLS histogram of dsRed-NPSCs. a) As-prepared. Average diameter: 130 ± 50 nm. b) After 24 hr. Average diameter 150 ± 70 nm.

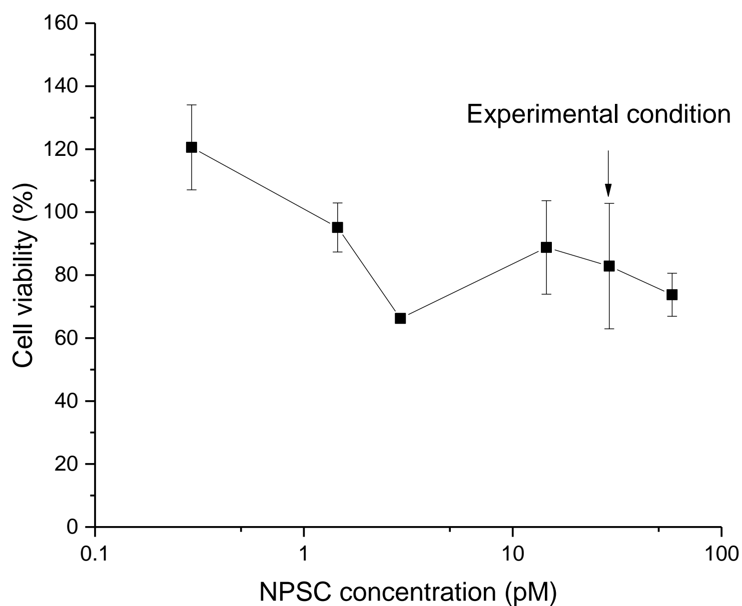

Figure S12. Cell viability assay after 24 hr culture of HeLa cells following dsRed delivery.

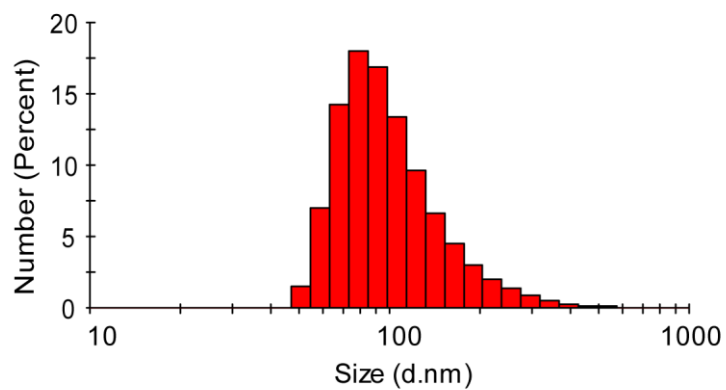

Figure S13. DLS histogram of β-Gal-NPSCs indicating an average diameter of 110 ± 50 nm.

References

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