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Supporting Information

Self-assembling Nitrilotriacetic Acid Nanofibers for Tracking or Enriching His-tagged Proteins in Living Cells

Li-Song Zhang, [a] Yi-Lun Yin, [a] Lei Wang, [a] Ying Xia, [a] SungJu Ryu, [a,c] Zhen Xi,[b] Lu-Yuan Li, [a,*] and Zhi-Song Zhang [a,*]

[a] State Key Laboratory of Medicinal Chemical Biology, College of Pharmacy and Tianjin Key Laboratory of Molecular Drug Research, NanKai University, Tianjin 300350, China.

[b] State Key Laboratory of Elemento-Organic Chemistry and College of Chemistry, Nankai University, 94 Weijin Road, Tianjin 300071, China

[c] Institute of Microbiology, State Academy of Sciences, Pyongyang, D.P.R. Korea

Corresponding Authors:

*E-mails: liluyuan@nankai.edu.cn and zzs@nankai.edu.cn

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Experimental Procedures

Experiment Materials and Instruments. Solvent preparations were carried according to described procedures. Trypsin-EDTA (0.25%), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dulbecco's Modified Essential Medium (DMEM), fetal bovine serum (FBS) and 10 × phosphate buffer saline (PBS, pH 7.4) were purchased from Thermo Fisher Scientific (Waltham, MA). Chemical reagents and solvents were used as receiving from commercial sources without further purification. 2-Cl-trityl chloride resin (1.0-1.2 mmol/g), Fmoc-OSu, O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU), Nepsilon-Benzyloxycarbonyl-L-lysine benzyl ester hydrochloride, naphthalene acetic acid, trifluoroacetate, triisopropylsilane and other Fmoc-amino acids were obtained from GL Biochem (Shanghai, China). Both the fluorophores ANA and biotin were purchased from Shanghai Beat Pharmaceuticals. N, N-diisopropylethylamine (DIPEA) and other chemical reagents and solvents were obtained from Thermo Fisher Scientific (Waltham, MA); Alkaline phosphatase was purchased from Biomatik (Cat. No. A1130, [ALP] >1300U/mg, in 50% glycerol). ^{1}H and ^{13}C NMR spectra were recorded at 25 °C on a Bruker AV400 NMR spectrometer, operating at 400 and 100 MHz, respectively, where the chemical shifts (δ in ppm) were determined using partially or non-deuterated solvent residues as internal references. DMSO-d6 was used as the solvents. Morphological analyses were conducted on a Thermo Scientific™ Talos™ F200C transmission electron microscopy. Cellular uptake and drug tracking images were taken by a confocal laser scanning microscopy (Leica TSC SP8, Germany).

Synthesis and Characterizations. The three NTA probes with self-assembly characteristics are all prepared by solid-phase peptide synthesis (SPPS) using 2-chlorotrityl chloride resin[1]. The first amino acid was loaded onto the resin at the C-terminal, followed by removal of the Fmoc protecting group by treatment with 20% piperidine. The next Fmoc-protected amino acid was coupled to the free amino group using N,N-Diisopropylethylamine/O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (DIPEA/HBTU) as the coupling reagent. Further deprotection and coupling steps followed established Fmoc-SPPS protocols. As the final step, the resin-bound peptide was cleaved using a cocktail of TFA/triisopropylsilane/water (95: 2.5: 2.5) for 2 hours under a nitrogen atmosphere[2], and then the cleavage solution was collected. The resin was further washed twice with TFA, and the filtrate was collected (Figure S1). Crude product was obtained after the addition of cold diethyl ether into concentrated filtrate and purified by reverse phase high performance liquid chromatography (HPLC) using a semi-prepare C18 column. HPLC solvents consisted of solvent A (0.05% TFA in water) and solvent B (0.05% TFA in methanol). The resulting peptide solution was frozen and lyophilized to afford purified compounds in around 45% yields after purification.

General Procedures for Hydrogel Preparation. Enzymatic gelation: To evaluate the *in vitro* self-assembling ability of NTA probes under the action of ALP and divalent metal ions, we dissolved the NTA probes in PBS (500 μL) solution at a concentration of 5 mmol/L. The pH of the solution was carefully monitored through a pH test paper by adding a 1 mol/L sodium carbonate solution. After the pH of the solution reached 7.4, we added an equal volume of 5 mmol/L nickel chloride solution (or copper chloride, cobalt chloride, zinc chloride) [3] to a final concentration of 2.5 mmol/L, and then added recombinant ALP(2.0 U/mL). Place in a constant temperature incubator at 37°C for 2 hours to form nano hydrogel.

TEM Sample Preparation. In this paper, a sample solution of NTA supramolecular hydrogel (3-5 μL) was added to a glow discharge thin carbon-coated copper grid (400 mesh, Pacific Grid-Tech). After 30 seconds, we carefully removed excess liquid from the edges of the grid with filter paper and rinsed the grid two or three times with deionized water. The grid was dried in air for 48 hours, and a TEM image was obtained by a transmission electron microscope[4].

Cell Culture and Cell Viability Assay. AD-293 and MCF-7 cells were purchased from the American Type Culture Collection (ATCC, USA) and cultured in a basic essential medium (MEM) supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. The incubation conditions for the cells are at 37 °C in a humidified atmosphere of 5% CO_2. For cell viability assay, AD-293 cells or MCF-7 cells in the logarithmic growth phase were seeded into 96-well plates with 1 × 10^4 cells per well and cultured for 12 hours. The medium was removed, and 200 μL of medium containing NTA probe (diluted from a freshly prepared stock solution of 10 mmol/L Nap-G/ANA/Biotin-FFpYGK-NTA(Ni^{2+})) at a gradient concentration (0 μmol/L as a control) was placed in each well. After 48 hours in a cell incubator, add 20 μL (5 mg/mL) of MTT to each well. 4 hours later, the medium was discarded and 200 μL of DMSO was added to each well. Measure the absorbance of each well at 595 nm with a microplate reader. The results are expressed as a percentage of cell survival relative to control group (Figure S11).

Western Blot and Immunoprecipitation. Using the RIPA buffer method to extract total protein from collected cells and subjected it to SDS PAGE. The samples were then transferred to a polyvinylidene difluoride membrane, blocked with 5% nonfat milk powder in TBS-T buffer (20 mmol/L Tris-HCl, pH 7.4, 137 mmol/L NaCl, and 0.1% Tween-20) for 1 hour at RT, incubated overnight at 4°C with specific primary antibody against the target proteins and then incubated with appropriate HRP-conjugated secondary antibodies. The films were developed with the ECL System (Millipore, Billerica, MA). Add 1 mL of cell lysis buffer (containing protease inhibitor) to a 10 cm cell culture dish at 4 °C for 30 minutes and centrifuge at 12,000 × g for 15 minutes. Mixed the lysates with 50 μL Streptavidin Agarose Resin 6FF resin was and slowly shaken at 4 °C for 16 hours. After the immunoprecipitation reaction, centrifuged at 3,000 × g for 5 minutes at 4 °C, and the supernatant was carefully discarded. Streptavidin Agarose Resin 6FF resin was washed 3-4 times with 1 mL of lysis buffer and 30 μL of 2 × SDS loading buffer was added. The mixture was boiled for 10 minutes in a metal bath and then the Western blot analysis was performed as described above.

Sample Preparation for Confocal Microscopy. MCF-7 cells and AD-293 cells in log phase growth were seeded at the density of 1 × 10^5 cells/well in a dedicated confocal dish. Incubate cells at 37ºC incubator for 12 hours. Change the medium and add 100 μmol/L of ANA-FFpYGK-NTA(Ni^{2+}) probe. After another 8 hours, cells were stained with 1.0 μg/mL Hoechst 33342 at 37 °C for 5 minutes. Rinse 3 times with PBS buffer and store in live cell imaging solution for confocal imaging.

Preparation of Expression Vectors. The coding sequence of human ARNT was obtained by PCR-based method from MCF-7 cDNA and ligated with a PTOPO-Blunt Simple Vector (Aidlab, China). Restriction sites BamHI and HindIII and the coding sequences of 6xHis tag are introduced with specifically designed primers by PCR followed by a topo-cloning into the PTOPO vector. Then the specific coding sequence for ARNT, N6His-ARNT or C6His-ARNT were cloned into pcDNA3.0 eukaryotic expression vector between the BamHI/HindIII sites to obtain pcDNA3.0-ARNT, pcDNA3.0-N6His-ARNT and pcDNA3.0-C6His-ARNT. All the inserted sequence of the constructs was confirmed by Sanger DNA sequencing. Also, the coding sequences of mCherry or 6His-mCherry were cloned downstream of the SV40 promoter to construct the eukaryotic expression vector by similar procedure. The corresponding sequences of the vectors are listed below:

mCherry vector (SV40 promoter-Purple, mCherry-Red)
GTGTGTCAGTTAGGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACC
AGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCATAGTCCCGCCC
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AGGCCGAGGCCGCCTCGGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAAAGCTTG
CATGCCTGCAGGTCGGCCGCCACGACCGGTGCCGCCACCATCCCCTGACCCACGCCCCTGACCCCTCACAAGGAGACGACCT
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GAGGACTACACCATCGTGGAACAGTACGAACGCGCCGAGGGCCGCCACTCCACCGGCGGCATGGACGAGCTGTACAAG

C6His-mCherry vector (SV40 promoter-Purple, mCherry-Red, 6×His tag-Green)
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TCCGCCACCATGGAGAGCGACGAGAGCGGCCTGGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTTCATGC
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AGCTGCGCGGCACCAACTTCCCCTCCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGAT
GTACCCCGAGGACGGCGCCCTGAAGGGCGAGATCAAGCAGAGGCTGAAGCTGAAGGACGGCGGCCACTACGACGCTGAGGTC
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GAGGACTACACCATCGTGGAACAGTACGAACGCGCCGAGGGCCGCCACTCCACCGGCGGCATGGACGAGCTGTACAAGCATC
ATCATCATCATCAC

pcDNA3.0-ARNT vector (CMV promoter-Purple, ARNT-Orange)
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AATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTA
GGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATAC
GACTCACTATAGGGAGACCCAAGCTTATGGCGGCGACTACTGCCAACCCCGAAATGACATCAGATGTACCATCACTGGGTCCAG
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CAGCAACCTGGCCAGCCTGAGGTCTTCCAGGAGATGCTGTCCATGCTGGGAGATCAGAGCAACAGCTACAACAATGAAGAATTC
CCTGATCTAACTATGTTTCCCCCCTTTTCAGAA

pcDNA3.0-C6His-ARNT vector (CMV promoter-Purple, ARNT-Orange, 6×His tag-Green)
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pcDNA3.0-N6His-ARNT vector (CMV promoter-Purple, ARNT-Orange, 6×His tag-Green)
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Results and Discussion

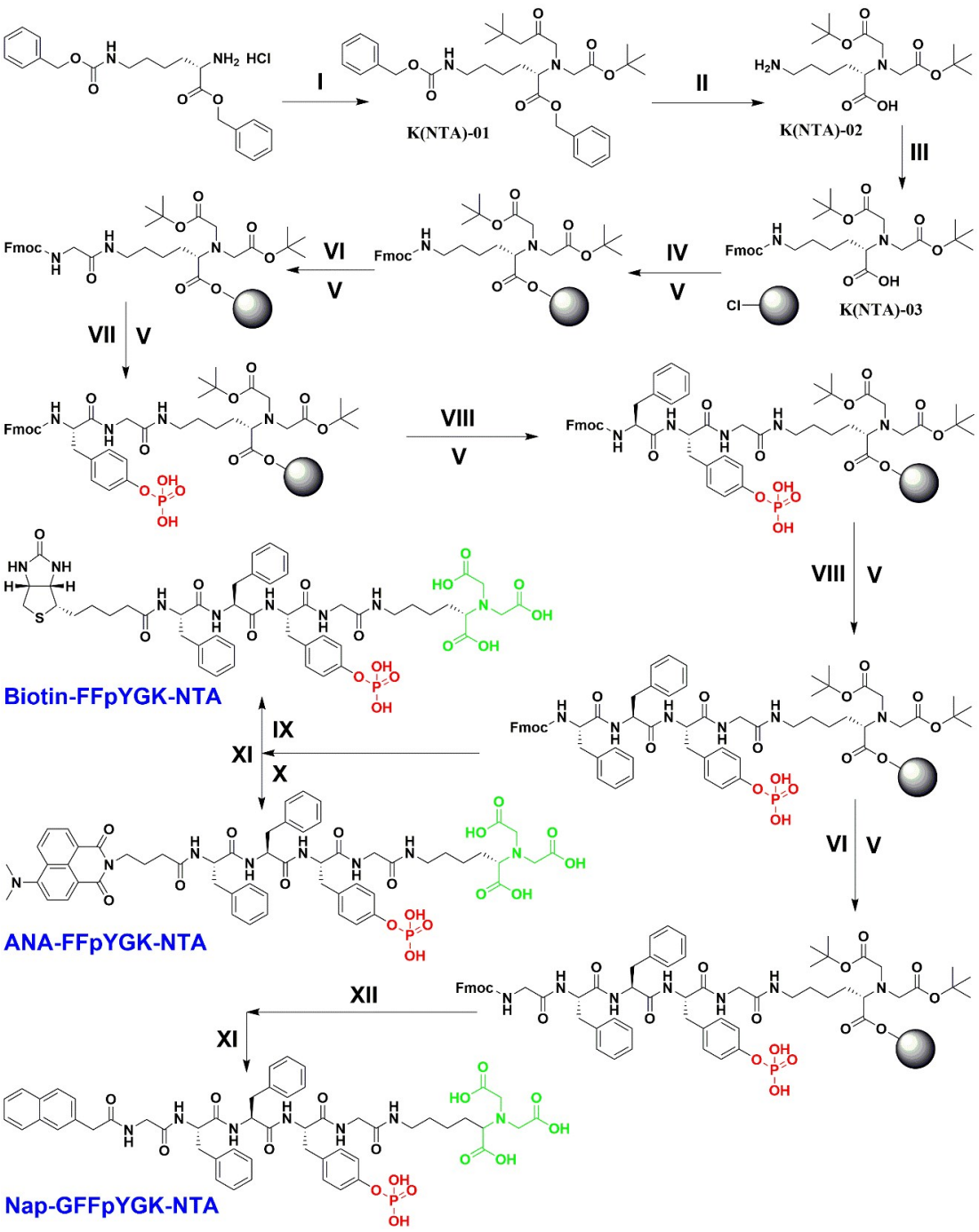

Figure S1. Synthesis route of Nap-GFFpYGK-NTA, ANA-FFpYGK-NTA and Biotin-FFpYGK-NTA. I) DMF K_2CO_3; II) 10% Pa-C H_2 MeOH; III) Fmoc-OSu MeCN $NaHCO_3$; IV) Fmoc-K(NTA)-OH DIPEA DCM; V) 20% piperidine DMF;VI) Fmoc-G-OH HBTU DIPEA DMF; VII) Fmoc-pY-OH HBTU DIPEA DMF; VIII) Fmoc-F-OH HBTU DIPEA DMF; IX) Biotin HBTU DIPEA DMF; X) ANA-NHS DIPEA DMF; XI) 95% TFA, 2.5% TIS, 2.5% H_2O; XII) 2-Naphthaleneacetic Acid HBTU DIPEA DMF.

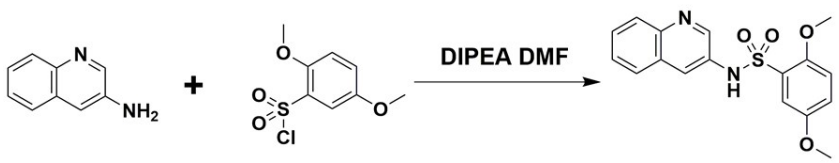

Figure S2. Synthesis routes of ALP inhibitor (DQB = 2,5-dimethoxy-N-(quinolin-3-yl)benzenesulfonamide).

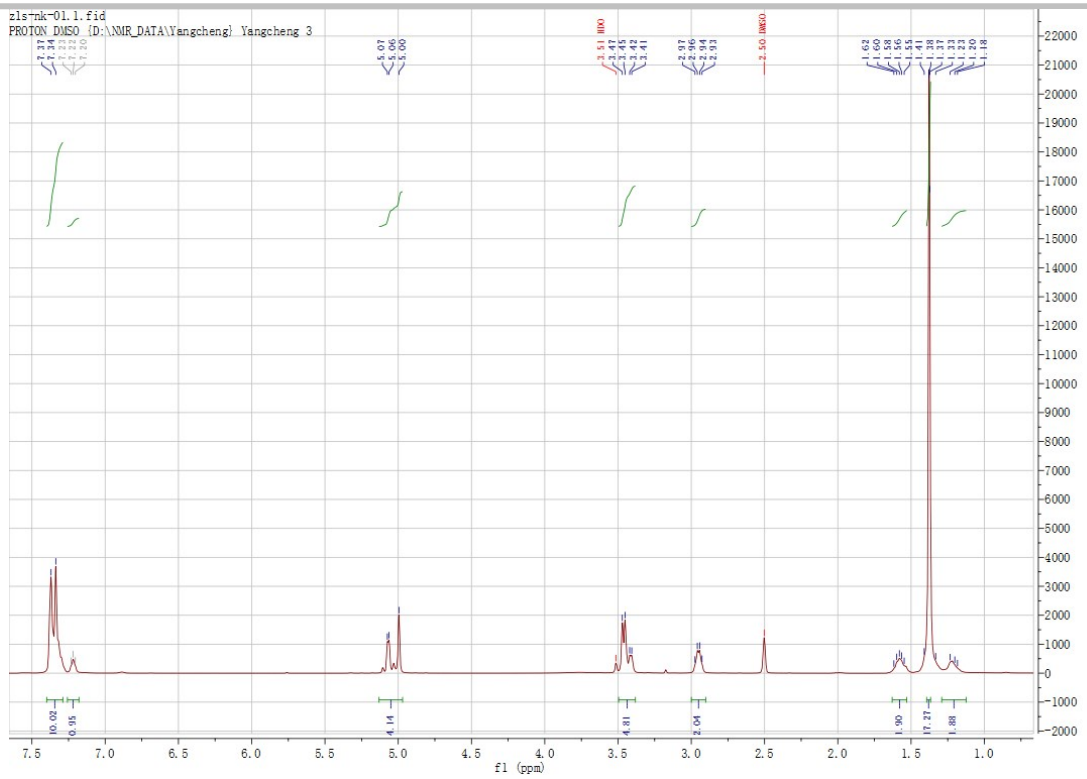

Figure S3A. [1]H NMR spectrum (400 MHz) of K(NTA)-01 in DMSO-d6 at 25 ºC.

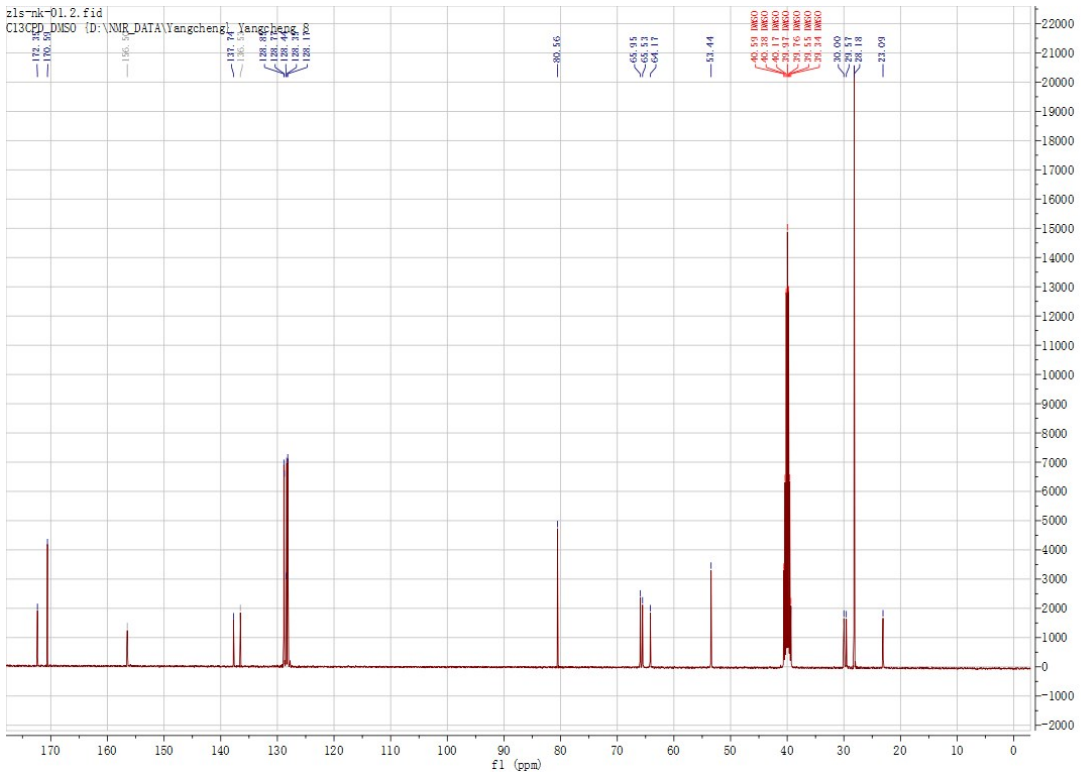

Figure S3B. [13]C NMR spectrum (100 MHz) of K(NTA)-01 in DMSO-d6 at 25 ºC

.

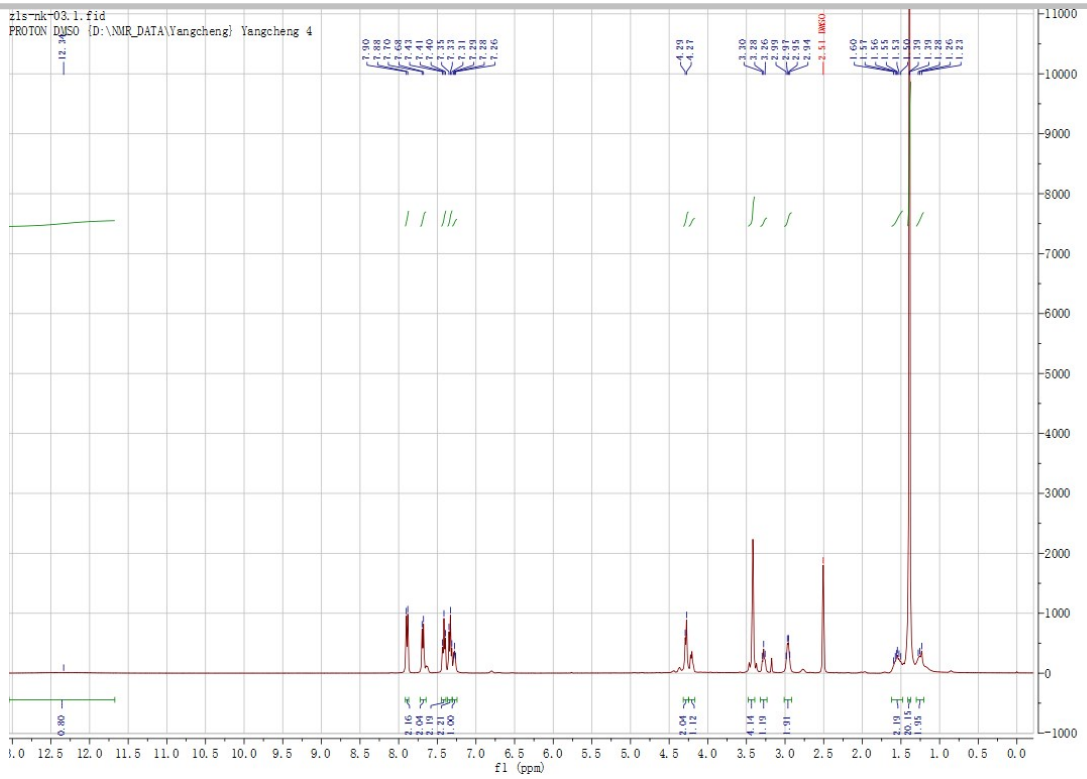

Figure S4A. [1]H NMR spectrum (400 MHz) of K(NTA)-03 in DMSO-d6 at 25 ºC.

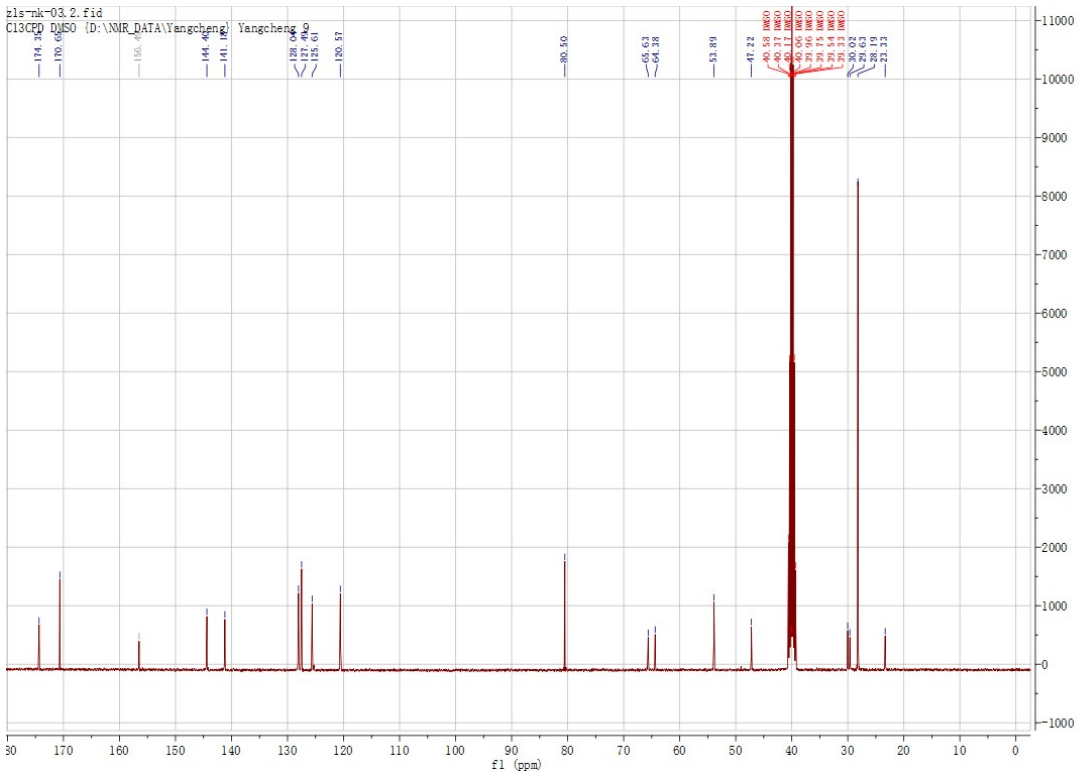

Figure S4B. [13]C NMR spectrum (100 MHz) of K(NTA)-03 in DMSO-d6 at 25 ºC.

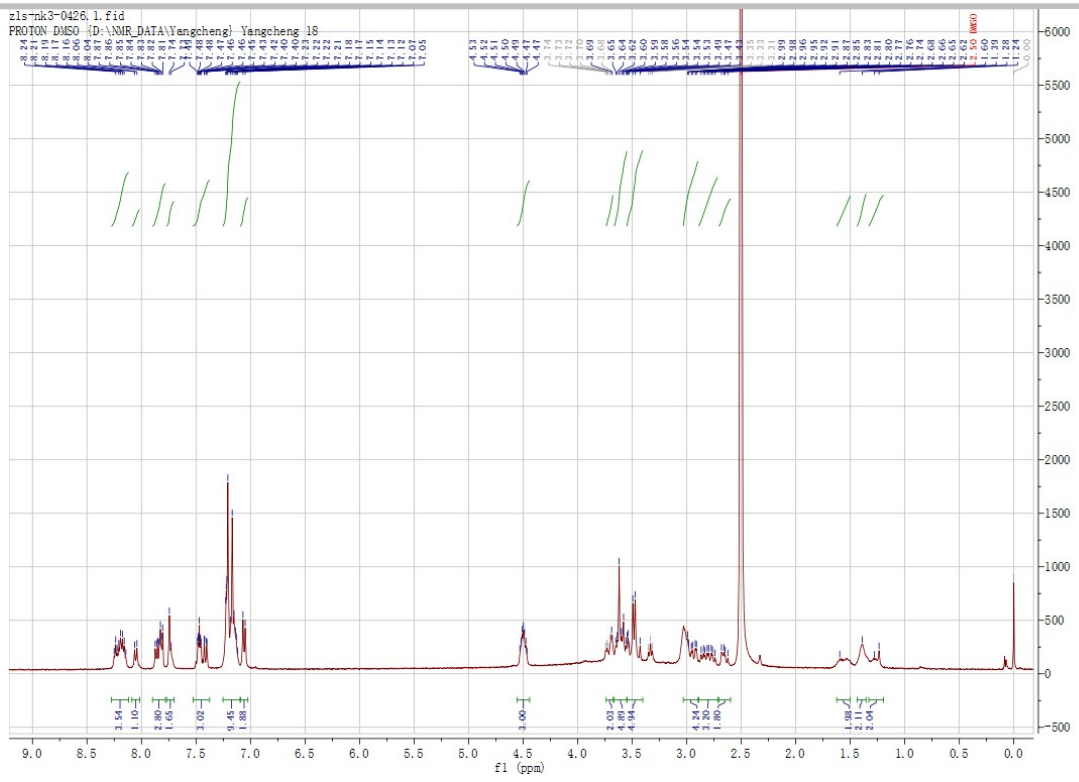

Figure S5A. ^{1}H NMR spectrum (400 MHz) of Nap-GFFpYGK-NTA in DMSO-d6 at 25 ºC.

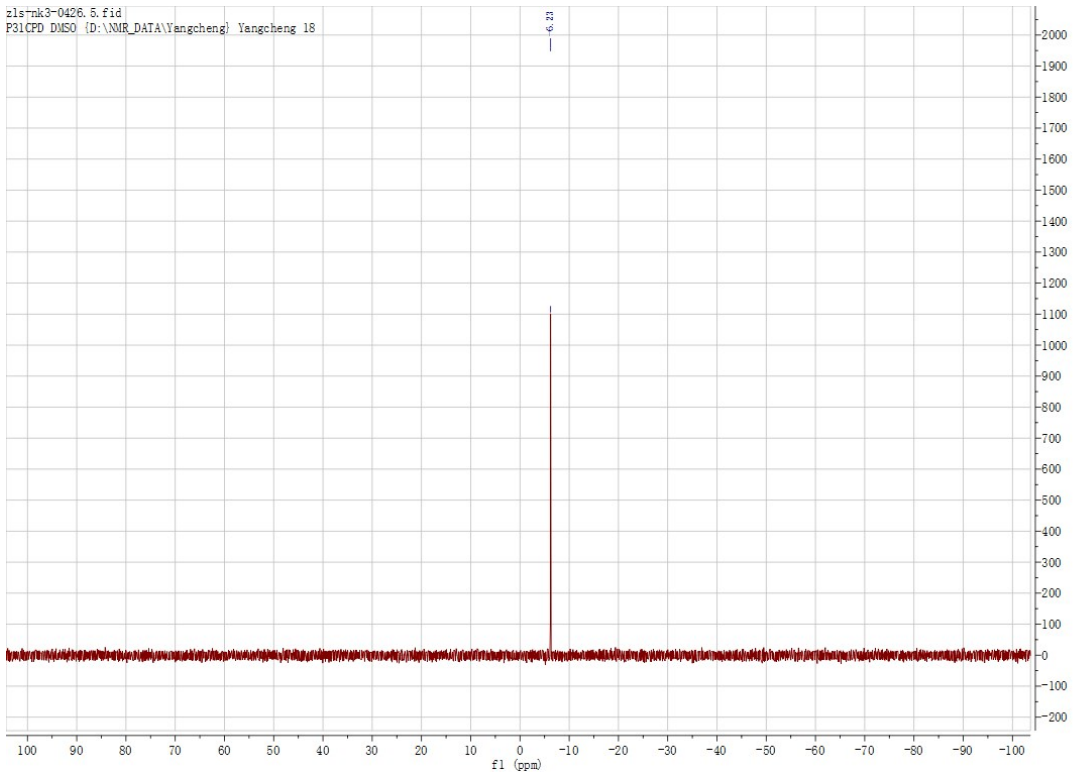

Figure S5B. ^{31}P NMR spectrum of Nap-GFFpYGK-NTA in DMSO-d6 at 25 ºC.

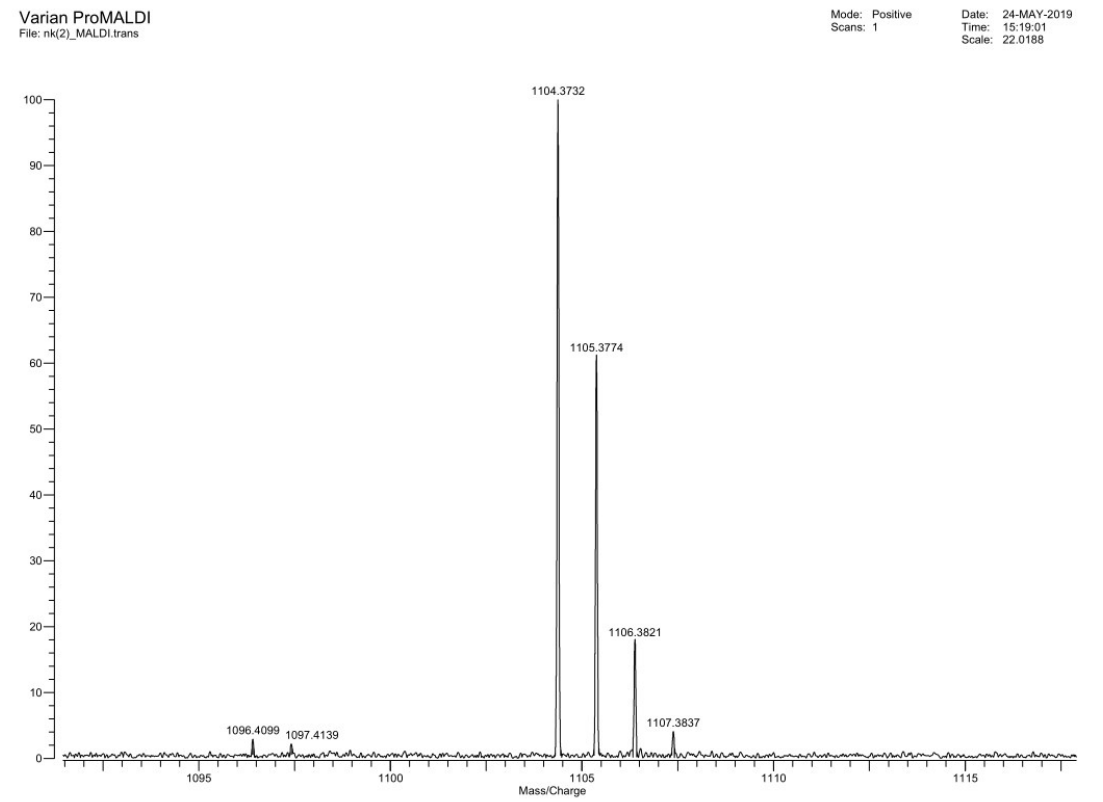

Figure S5C. HR-MS spectrum of Nap-GFFpYGK-NTA. M+Na$^+$=1104.3732.

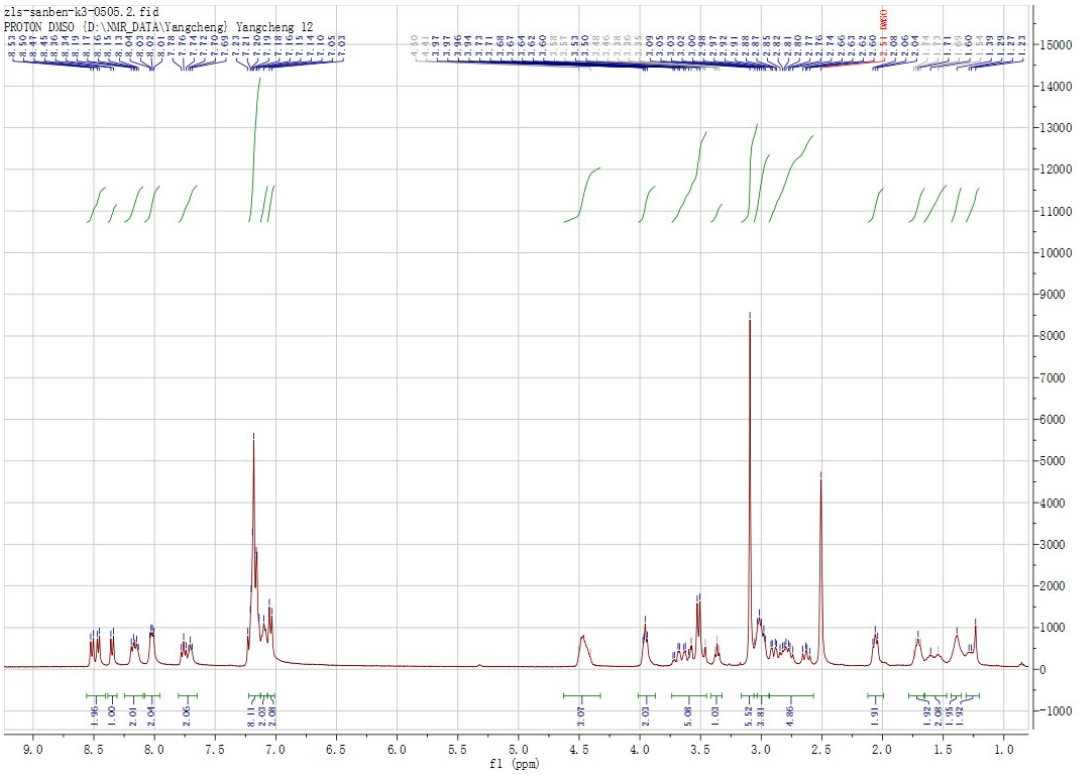

Figure S6A. ^{1}H NMR spectrum (400 MHz) of ANA-FFpYGK-NTA in DMSO-d6 at 25 ºC.

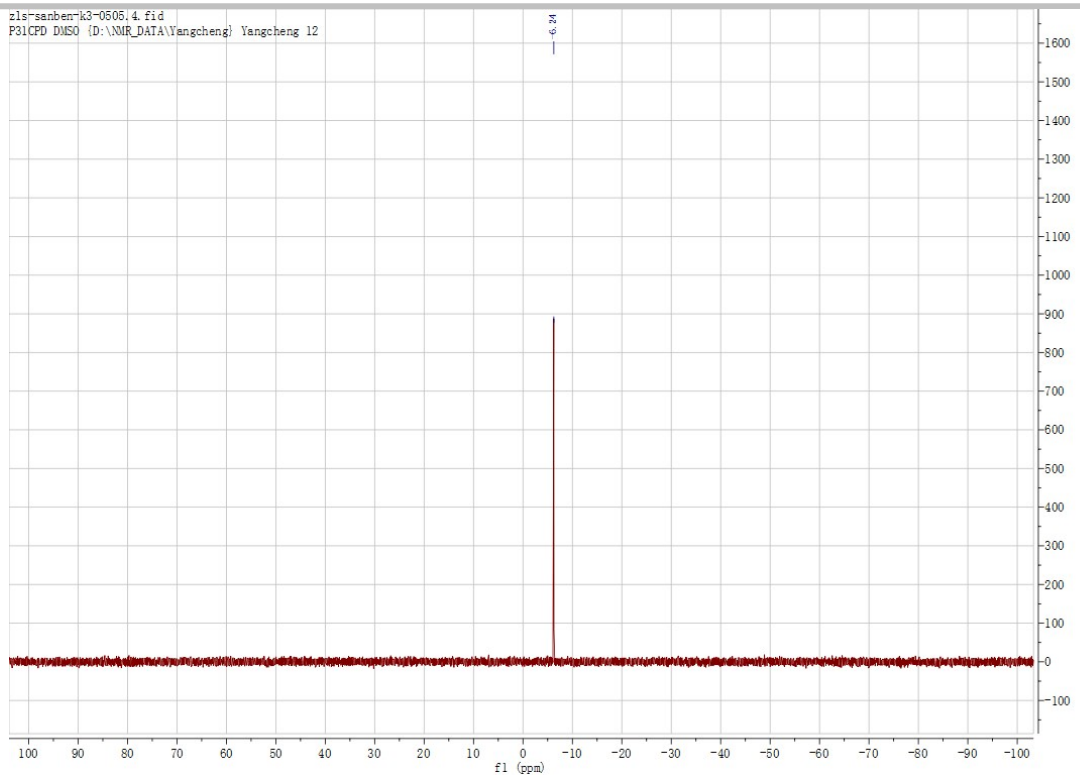

Figure S6B. ^{31}P NMR spectrum of ANA-FFpYGK-NTA in DMSO-d6 at 25 ºC.

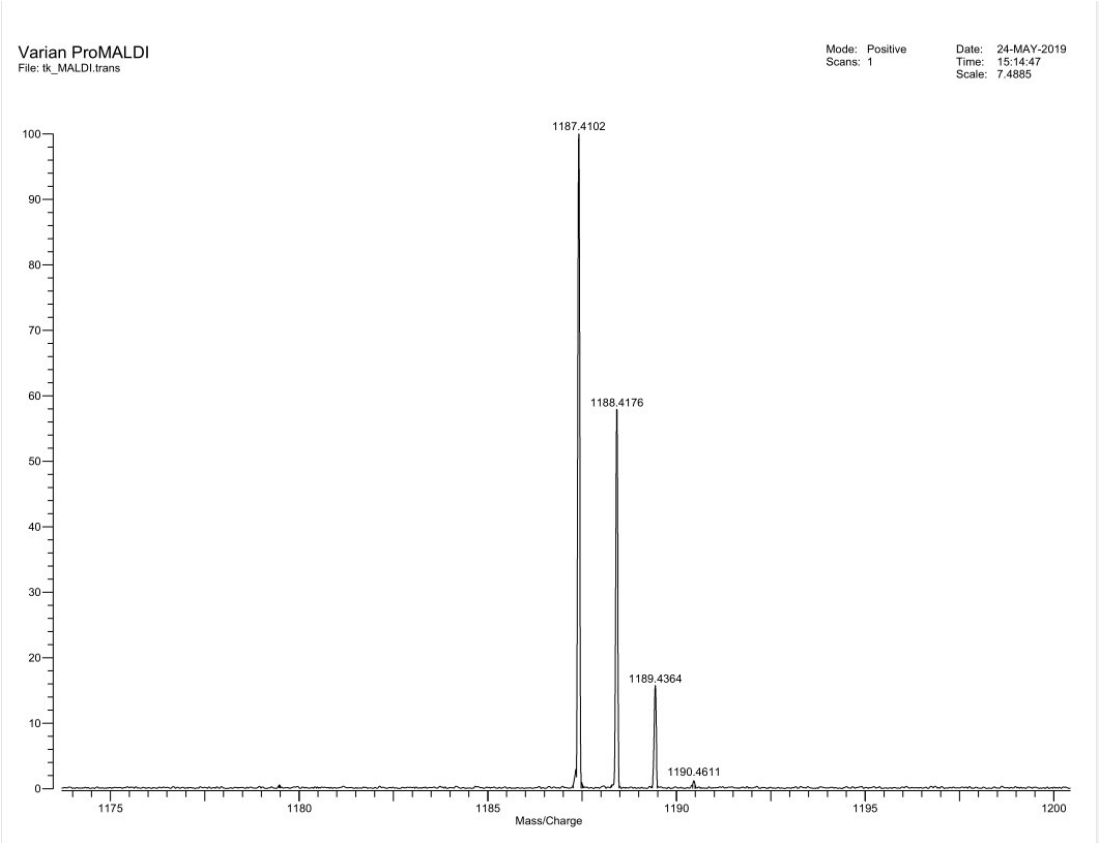

Figure S6C. HR-MS spectrum of ANA-FFpYGK-NTA. M+Na$^+$=1187.4102.

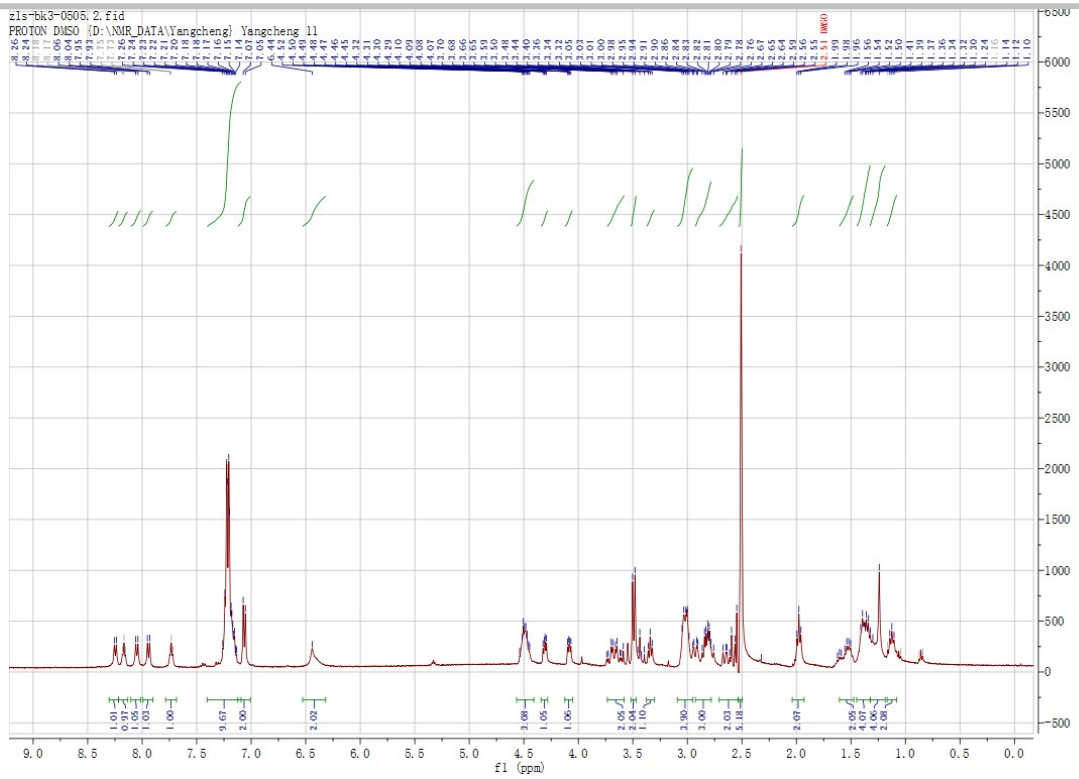

Figure S7A. ^{1}H NMR spectrum (400MHz) of Biotin-FFpYGK-NTA in DMSO-d6 at 25 ºC.

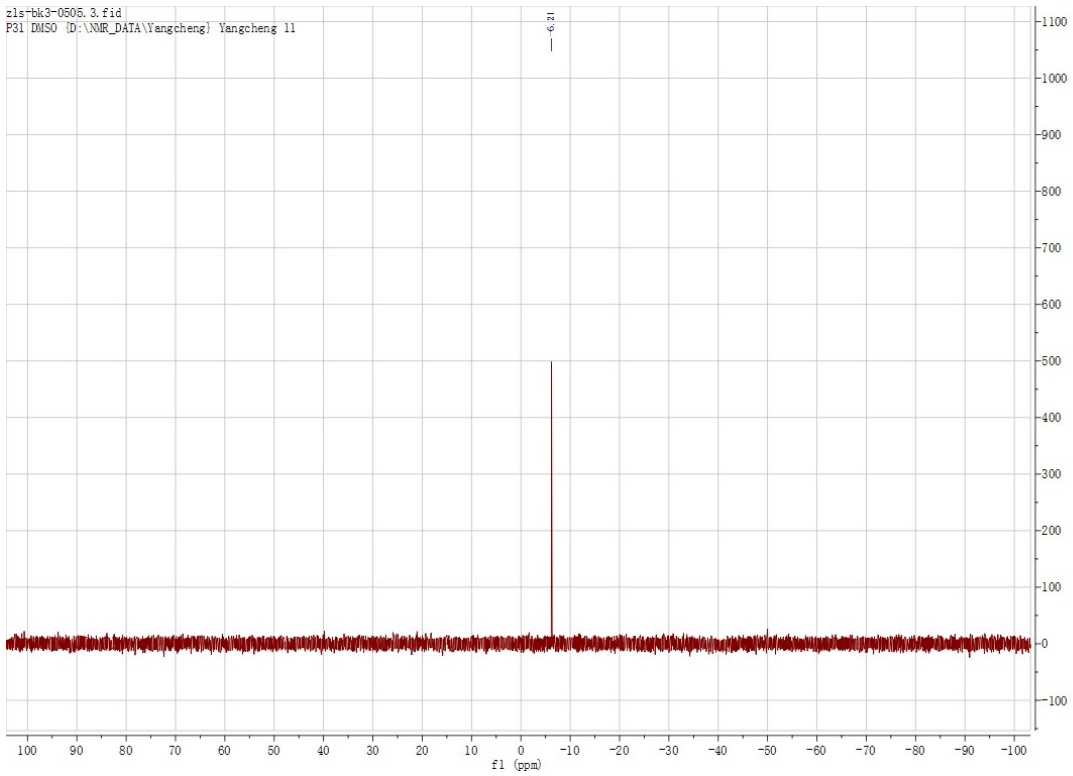

Figure S7B. ^{31}P NMR spectrum of Biotin-FFpYGK-NTA in DMSO-d6 at 25 ºC.

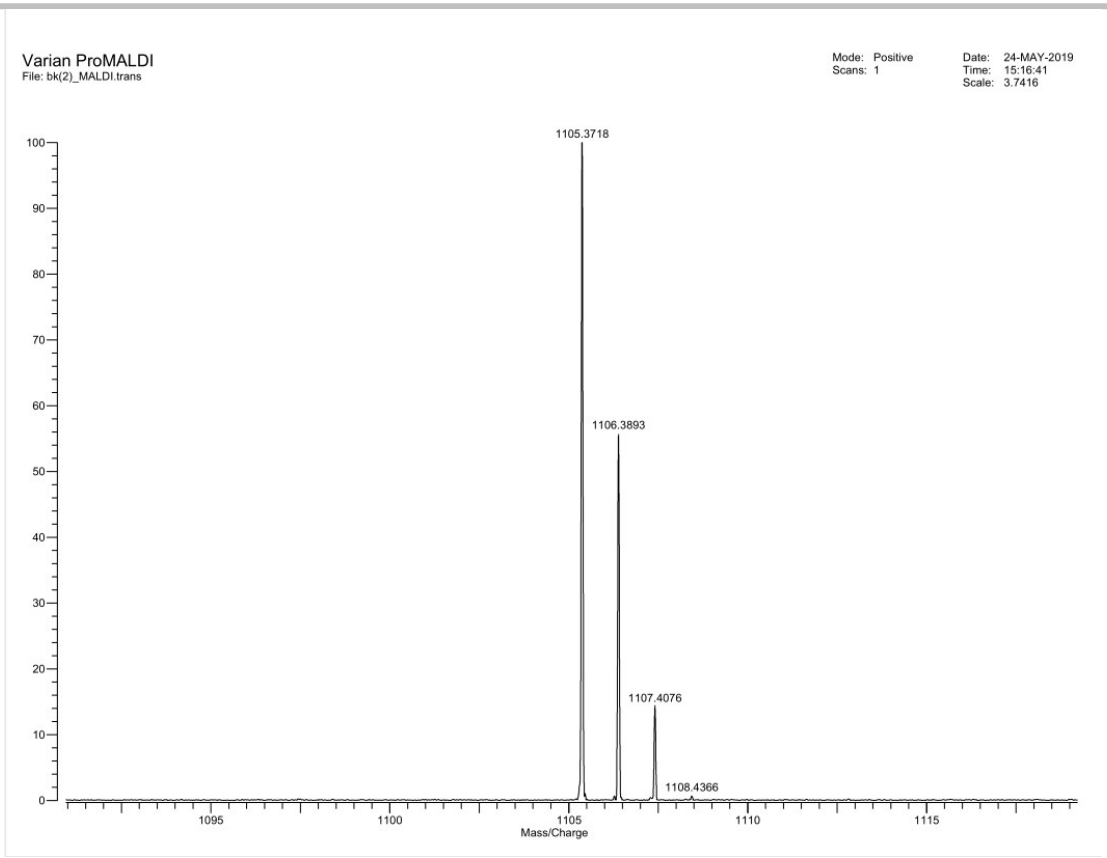

Figure S7C. HR-MS spectrum of Biotin-FFpYGK-NTA. M+Na$^+$=1105.3718.

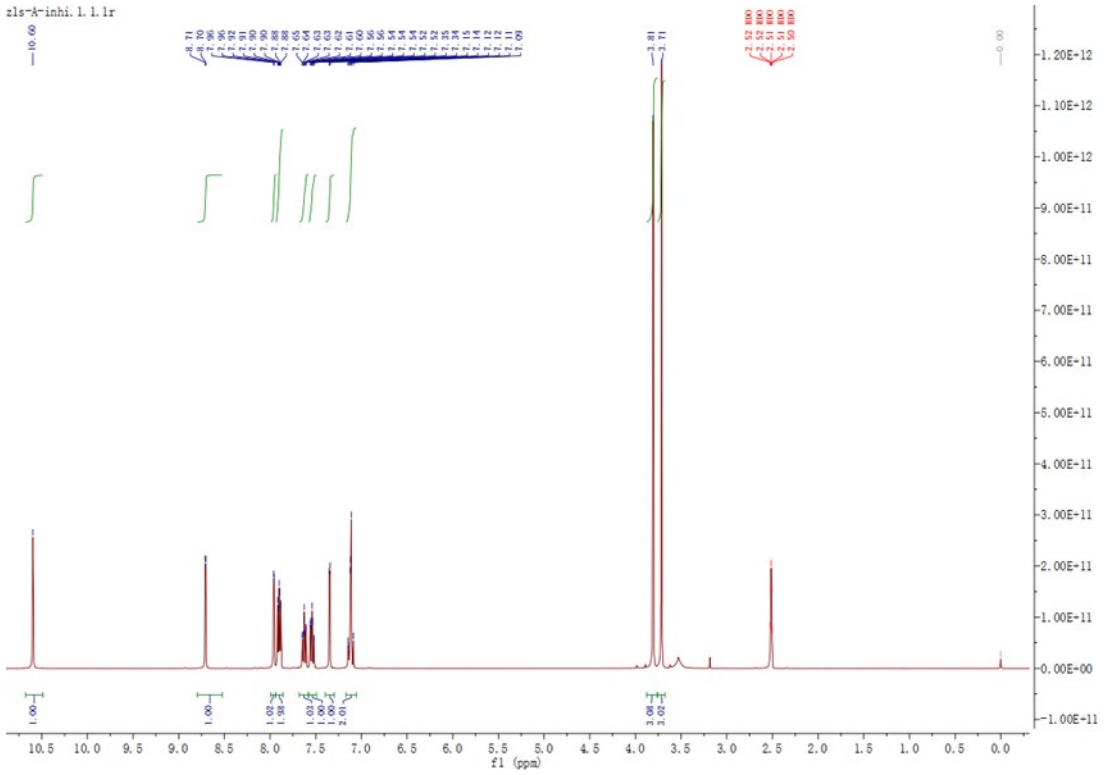

Figure S8A. ^{1}H NMR spectrum (400 MHz) of ALP inhibitor (DQB) in DMSO-d6 at 25 ºC.

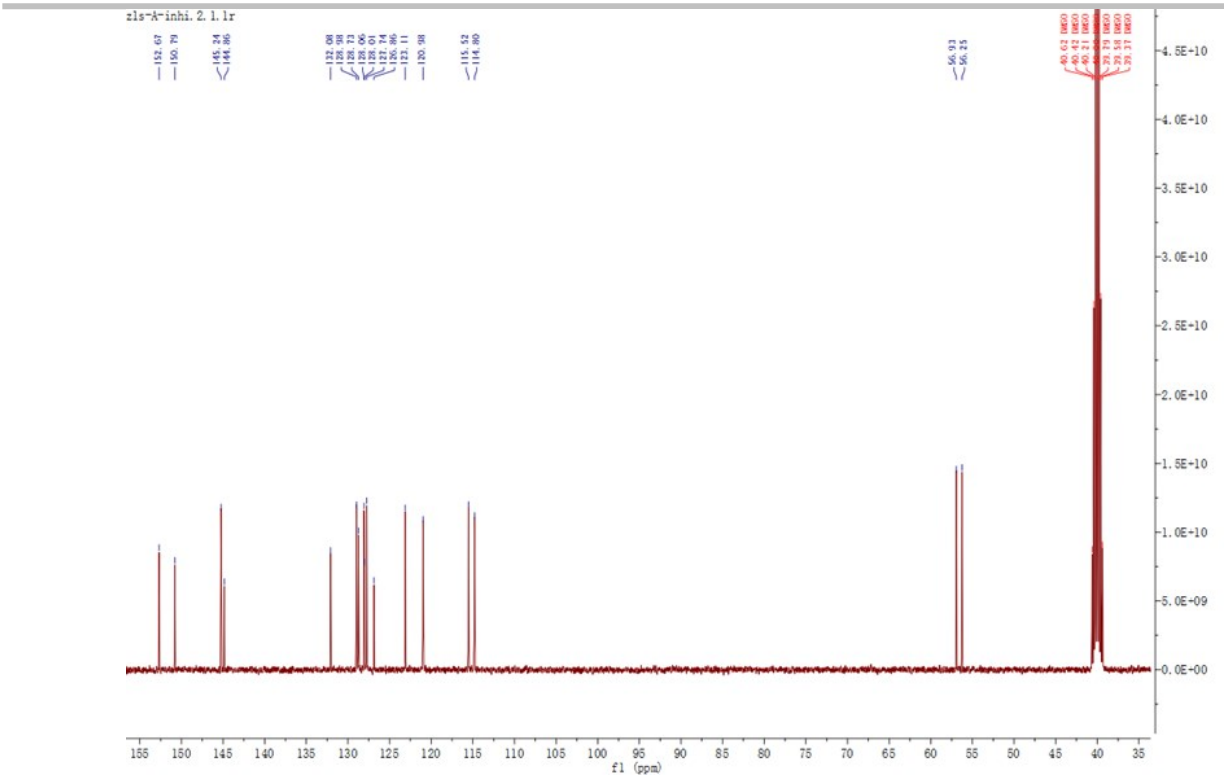

Figure S8B. ^{13}C NMR spectrum (100 MHz) of ALP inhibitor (DQB) in DMSO-d6 at 25 ºC.

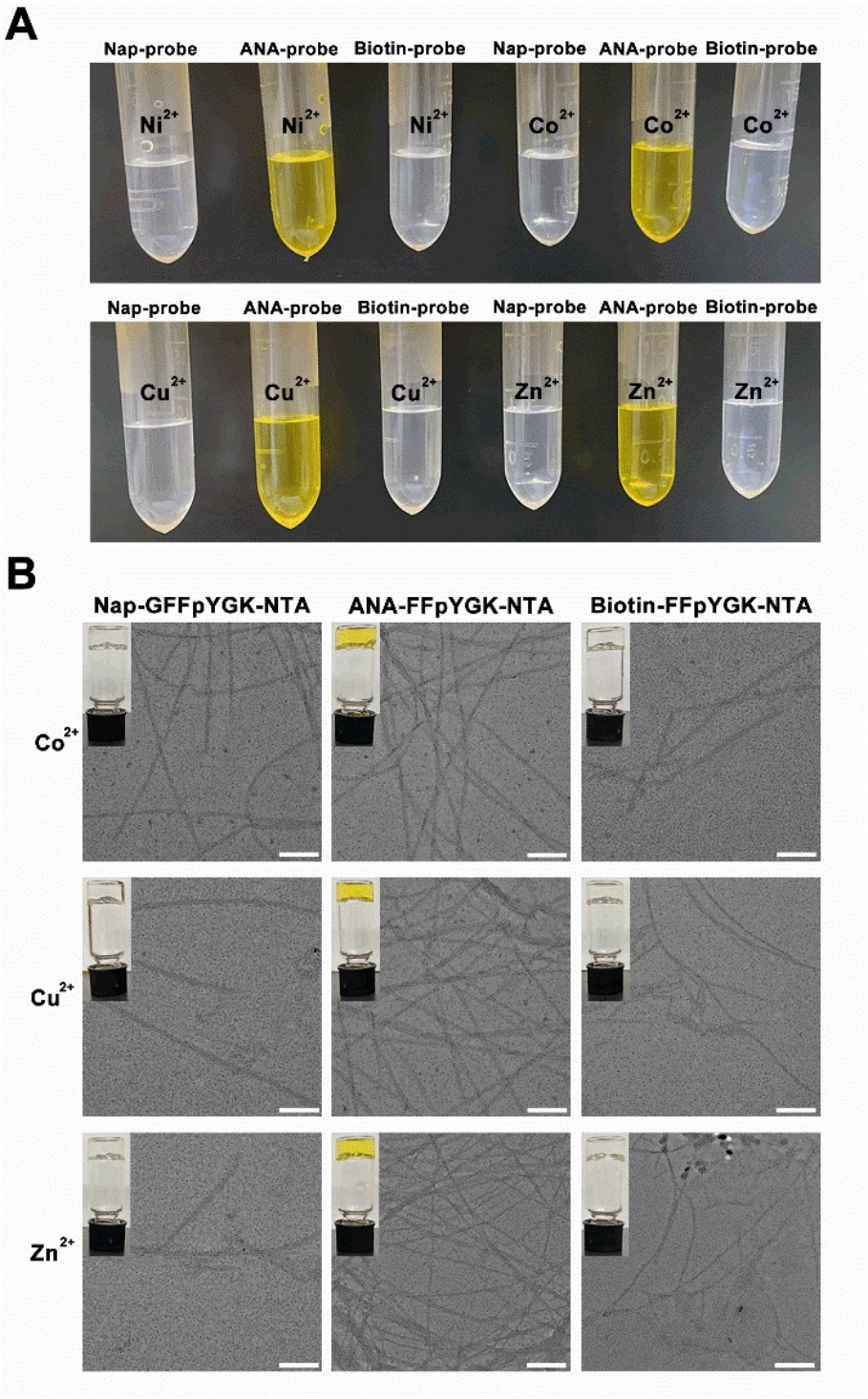

Figure S9. Solubility and self-assembly of the probes (Nap-G/ANA/Biotin-FFpYGK-NTA(Ni^{2+})) with divalent ions (Ni^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}). (A) The probes (Nap-GFFpYGK-NTA, ANA-FFpYGK-NTA and Biotin-FFpYGK-NTA) and divalent metal ions (Ni^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}) form a clear and transparent solution (5 mmol/L) in PBS solution at pH=7.4. (B) TEM images of the hydrogels formed using ALP (2.0 U/mL) to treat Co^{2+} (Cu^{2+} or Zn^{2+}) with probes at PH=7.4 and concentrations of 5 mmol/L. Inset: optical images. Scale bar: 100nm.

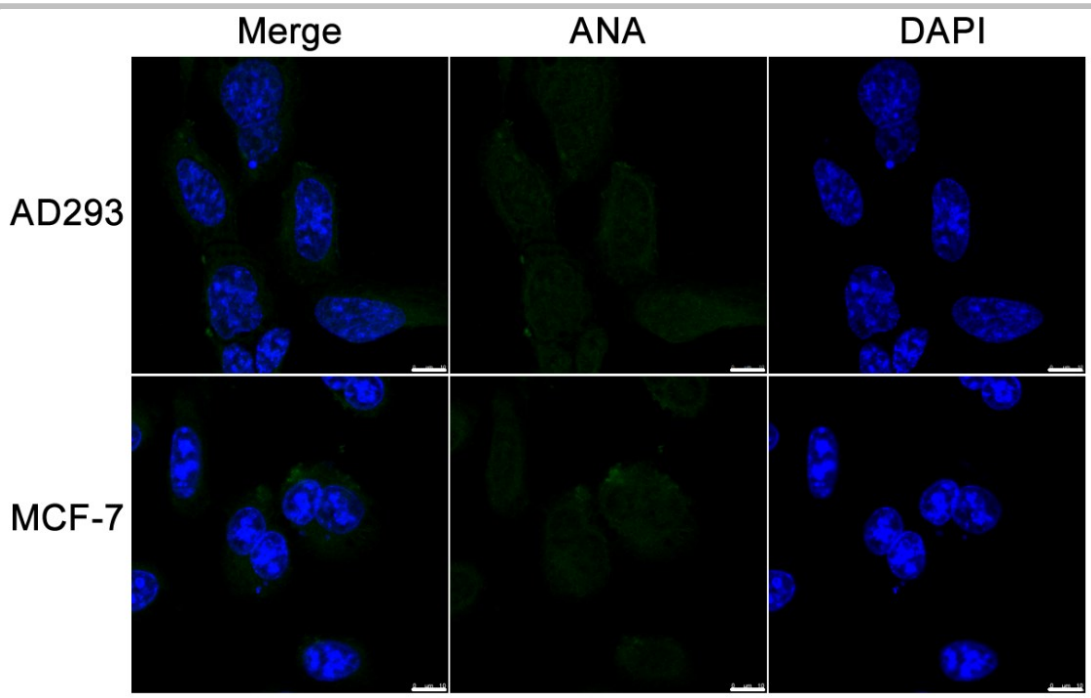

Figure S10. Cell uptake efficiency of ANA-FFpYGK-NTA(Ni^{2+}) (100 μmol/L, 4h) in AD293 cells and MCF-7 cells with ALP inhibitor (2,5-dimethoxy-N-(quinolin-3-yl)benzenesulfonamide) (50 μmol/L, 6h). ANA: λex = 488nm, λex = 530nm; Scale bar = 10μm.

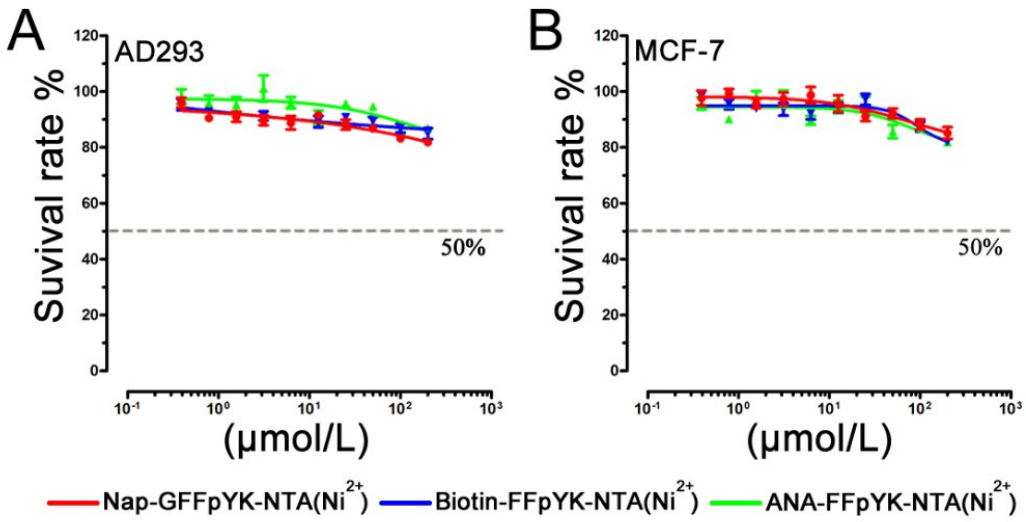

Figure S11. IC_{50} values of Nap-GFFpYGK-NTA(Ni^{2+}) (red), ANA-FFpYGK-NTA(Ni^{2+}) (green), and Biotin-FFpYGK-NTA(Ni^{2+}) (blue) incubated with AD-293 cells and MCF-7 cells after 48 hours.

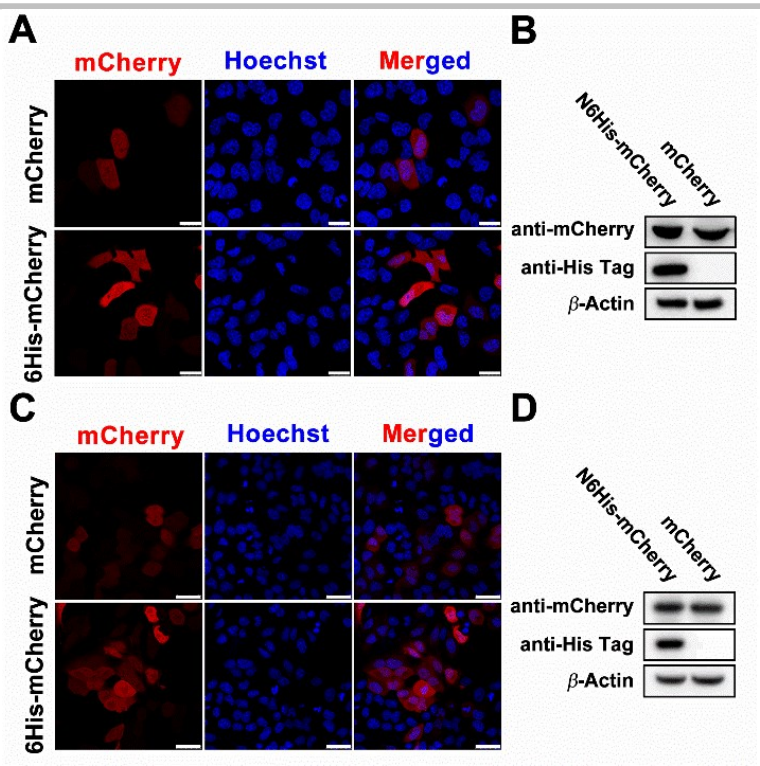

Figure S12. 6His-tagged mCherry and mCherry protein expressed in AD-293 cells (A, B) and MCF-7 cells (C, D). Red fluorescence distribution of AD-293 cells (A) and MCF-7 cells (C) transfected with 6His-tagged mCherry and mCherry plasmids 48 hours later. The confocal images were captured using a confocal microscope. Excitation of 561 nm and emission between 600 nm to 700 nm was collected. Scale bar: 10 μm. Western Blotting (anti-His antibody, anti-mCherry antibody) analyses of the labeling of the 6His-tagged mCherry protein and mCherry protein in AD-293 cells (B) and MCF-7 cells (D).

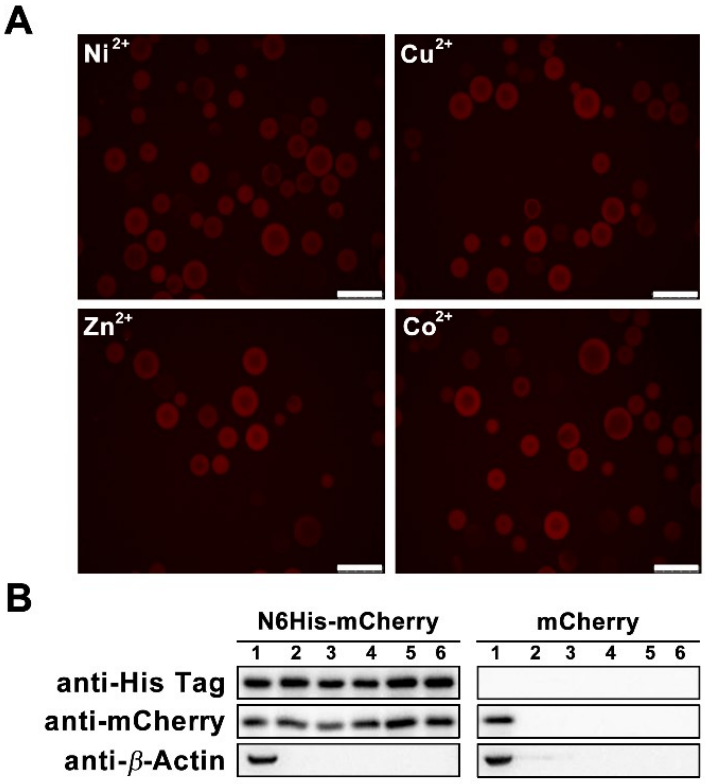

Figure S13. Affinity of Biotin-FFpYGK-NTA probe with 6His-tagged mCherry in cell lysates with divalent ions (Ni^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}). (A) Red fluorescence of Streptavidin agarose resin after the treatment of Biotin-FFpYGK-NTA with Ni^{2+}, Cu^{2+}, Zn^{2+} or Co^{2+} (100 μmol/L, 25 °C for 12 hours) in AD-293 cell lysate containing 6His-tagged mCherry protein. Scale bars: 50 μm. (B) Western Blotting analyses of the labeling of the 6His-tagged mCherry protein and mCherry protein. Lane 1 to 6: Input, Biotin-FFpYGK-NTA(Ni^{2+}), Biotin-FFpYGK-NTA(Cu^{2+}), Biotin-FFpYGK-NTA(Zn^{2+}), Biotin-FFpYGK-NTA(Co^{2+}), Ni bestarose FF.

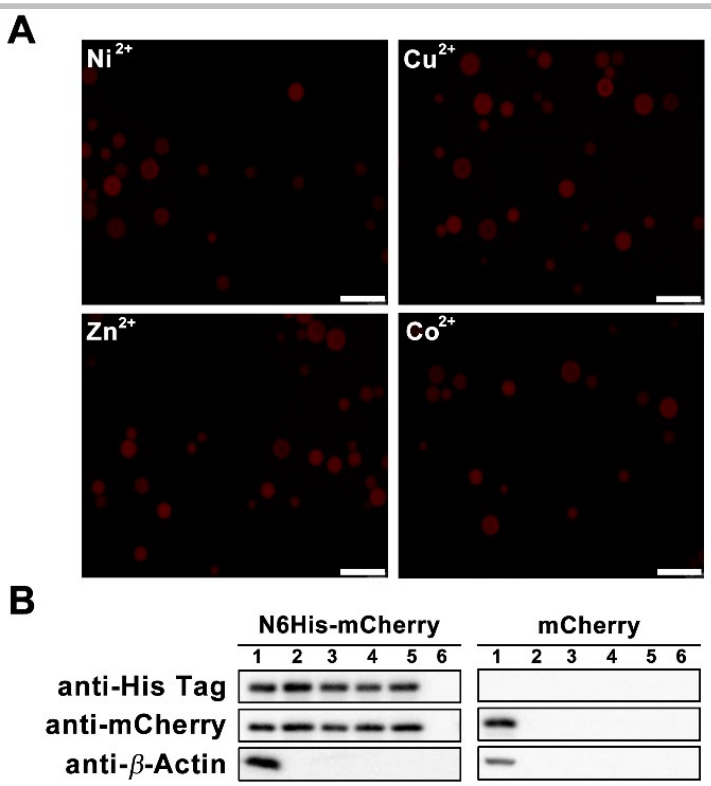

Figure S14. Affinity of Biotin-FFpYGK-NTA probe with 6His-tagged mCherry in living cells with divalent ions (Ni^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}). (A) Red fluorescence of Streptavidin agarose resin after the treatment of Biotin-FFpYGK-NTA with Ni^{2+}, Cu^{2+}, Zn^{2+} or Co^{2+} (100 μmol/L, 37 °C for 6 hours) in AD-293 cells expressing 6His-tagged mCherry protein. Scale bars: 100 μm. (B) Western Blotting analyses of the labeling of the 6His-tagged mCherry protein in living cells. Lane 1 to 6: Input, Biotin-FFpYGK-NTA(Ni^{2+}), Biotin-FFpYGK-NTA(Cu^{2+}), Biotin-FFpYGK-NTA(Zn^{2+}), Biotin-FFpYGK-NTA(Co^{2+}), Ni bestarose FF.

Figure S15. Divalent ions (either Ni^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}) are critical to the affinity of Biotin-FFpYGK-NTA probe interacting with 6His-tagged mCherry in cell lysates. No fluorescence was observed on the Streptavidin agarose resin if either Ni^{2+}, Cu^{2+}, Zn^{2+} or Co^{2+} is not included in the Biotin-FFpYGK-NTA treatment conditions of the AD-293 cell lysate containing 6His-tagged mCherry protein (25 °C for 12 hours). Scale bars: 50 μm.

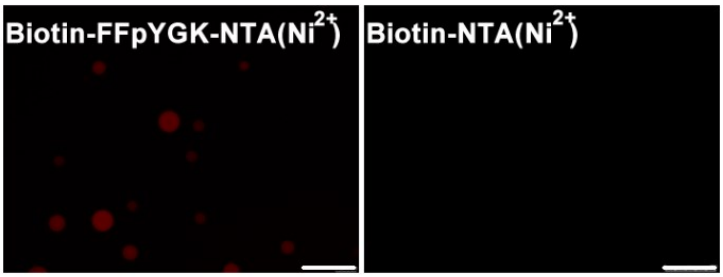

Figure S16. Affinity of Biotin-FFpYGK-NTA(Ni^{2+}) and Biotin-NTA(Ni^{2+}) probes with 6His-tagged mCherry in living cells. Red fluorescence of Streptavidin agarose resin after the treatment of Biotin-FFpYGK-NTA(Ni^{2+}) and Biotin-NTA(Ni^{2+}) probe in AD-293 cells expressing 6His-tagged mCherry protein. Probe concentration: 100 μmol/L. 37 °C for 6 hours. Scale bars: 100 μm.

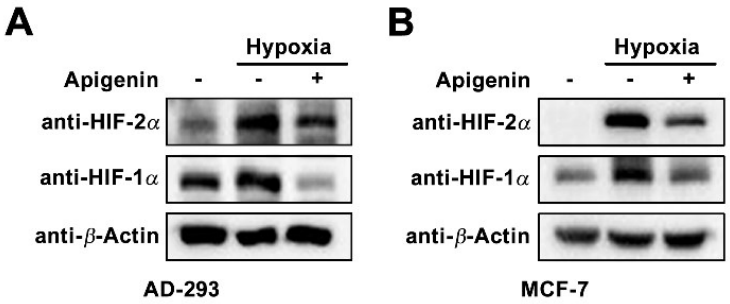

Figure S17. The inhibitory effect of apigenin on HIF-1α and HIF-2α in AD-293 cells and MCF-7 cells. AD-293 cells (A) and MCF-7 cells (B) were cultured for 6 hours in hypoxia (1% O_2, 5% CO_2, 94% N_2) and treated with apigenin (100 μmol/L). Western Blotting was then performed using anti-HIF-1α and anti-HIF-2α antibodies.

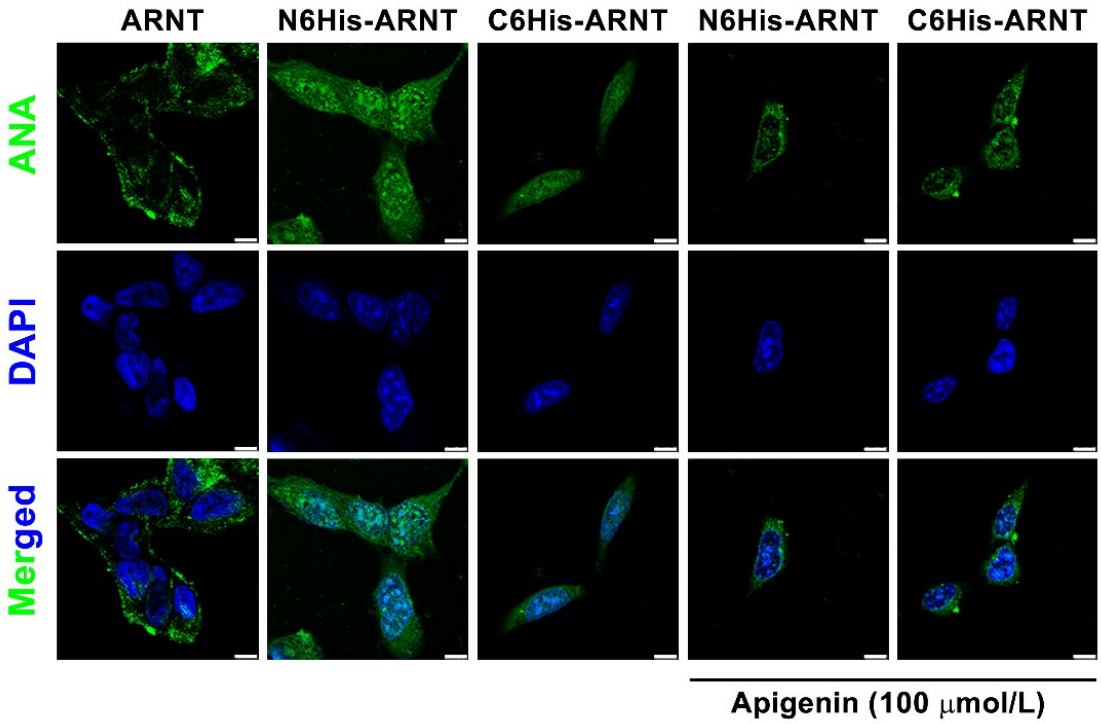

Figure S18. Intracellular 6His-tagged ARNT tracing by ANA-FFpYGK-NTA(Ni^{2+}) probe in AD-293 cells. Cells were transfected with vectors expressing C6His-ARNT, N6His-ARNT or wild-type ARNT. After 48 hours, ANA-FFpYGK-NTA(Ni^{2+}) probe (100 μmol/L) and apigenin (100 μmol/L) were added and cells were kept under hypoxic environment for another 6 hours. The confocal images were captured using a confocal microscope using excitation of 488 nm and emission between 500 nm to 540 nm. Scale bar: 10 μm.

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Author Contributions

L. Z. and Z. Z. designed and conducted the experiments and wrote the manuscript.
Y. Y. and L. W. helped with the plasmid design and construction.
Y. X. and S. J. R helped with the synthesis of peptides.
Z. X, L. L. and Z. Z. proposed and supervised the project, and co-wrote and manuscript with L. Z.