Supporting Information

For

Multifunctional Metallo-Organic Vesicles Displaying Aggregation-Induced Emission: Two-Photon Cell-Imaging, Drug Delivery, and Specific Detection of Zinc Ion

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Experimental Section

Materials

Zinc nitrate hexahydrate ($Zn(NO_3)_2 \cdot 6H_2O$) and potassium hydroxide (KOH) were purchased from Xilong Chemicals with purity above 99.0%. All chemicals were of reagent-grade quality obtained from commercial sources and used without further purification. Aqueous solutions were prepared using Milli-Q water of 18 MΩ.

Synthesis

4-Formyltriphenylamine (TPA-CHO) was synthesized as follow.[1] Phosphorus oxychloride (9.5 mL, 101.9 mmol) was added dropwise at 0 °C under N_2 to DMF (7.26 mL, 93.8 mmol) and the reaction mixture was stirred for 1 h. Triphenylamine (1.00 g, 4.08 mmol) was added, and the resulting mixture was stirred at 45 °C for 4 h. After cooling to r.t., the mixture was poured into ice-water (200 mL), and basified with 1 M NaOH. After extraction with CH_2Cl_2 (200 mL), the organic layer was washed with water (3×50 mL), dried (Na_2SO_4) and filtered over a short pad of silica gel. The solvent was then removed under reduced pressure and then the crude product was purified by column chromatography using petroleum ether/ethyl acetate (5:1 v/v) as eluent. TPA-CHO was obtained as yellow solid in 74% yield (0.74 g).^{1}H NMR (400 MHz, DMSO), δ(TMS, ppm): 9.77(s, 1H), 7.78-7.66 (m, 2H), 7.48-7.37 (m,4H), 7.27-7.15(m, 6H), 6.93-6.85(m, 2H).

4-carboxytriphenylamine (TPA-COOH) was synthesized as follow.[2] In a dried flask, a sample of 4-formyltriphenylamine (TPA-CHO) (500 mg, 1.8 mmol) was dissolved

in 60 mL of acetone. Separately, 40 mL of aqueous solution of $KMnO_4$ (0.157 M, 3.5 equiv.) was prepared, filtered, and then added dropwise to the solution containing the aldehyde. The reaction mixture was left at room temperature for 72 h, and then filtered on celite to remove MnO_2. The filtered solution was collected in an open vessel and left at room temperature until excess MnO_4^- was decomposed and the solution becomes yellow and cloudy. Then it was filtered again on celite. The yellow solution was treated with 2.5 mL of HCl/H_2O 1:1, a pale yellow precipitate appears, then stirred for 30 minutes in an ice bath, and filtered again. The resulting solution was dried under vacuum affording 270mg (0.93 mmol) of pure microcrystalline product (isolated yield 50%). ^{1}H NMR (400 MHz, DMSO), δ (TMS, ppm): 7.93-7.90 (m, 2H), 7.38-7.28 (m, 4H),7.19-7.10 (m, 6H), 7.02-6.94 (m, 2H). Elemental analysis: Ana. Cal. for $C_{19}H_{15}NO_2$ (%): C, 77.32%; H, 5.52%; N, 4.77%. Found: C, 78.87%; H, 5.23%; N, 4.84%.

TPA-1 was used after TPA-COOH alkalizing.

Methods

Fluorescent Spectroscopy (FL). FL measurements were carried out using a Hitachi F-7000 instrument. Other conditions: slit 5 nm; scanning speed: 1200 nm/min; photomultiplier tube (PMT) voltage: 500 V.

Ultraviolet−Visible (UV−vis) Spectra. UV−vis were performed on a Shimadzu UV-1800 spectrophotometer. Absorbance was recorded in the range of 280~700 nm with a scanning rate is 10 nm / min.

^{1}H NMR. ^{1}H NMR spectra were measured on a Bruker-500 MHz NMR spectrometer.

The solvent is D_2O.

Fourier Transform Infrared (FT-IR) Spectroscopy. FT-IR spectra were recorded with a Bruker Vector-22 spectrophotometer. The samples were frozen in liquid nitrogen and subsequently lyophilized for 48 h before FT-IR measurements.

Dynamic Light Scattering (DLS). DLS measurements were conducted on an ALV/DLS/SLS5022F light scattering-apparatus, equipped with a 22 mW He−Ne laser (632.8 nm wavelength) with a refractive index matching bath of filtered toluene. The samples were filtered by 450 nm filters to remove dusts. The scattering angle was 90°.

Transmission Electron Microscopy (TEM) and Energy Dispersive X-ray Spectroscopy (EDX). A Tecnai G2 F20 TEM (120 kV) and EDS were employed to observe the morphology of assemblies. An aberration corrected Titan FEI instrument operated at 300 kV was used for high resolution high angle annular dark field (HAADF) imaging in the scanning transmission electron microscrope (STEM) mode. Drops of samples were put onto 230 mesh copper grids coated with a carbon film. Excess water was removed by filter paper, and the samples were placed at room temperature to dry before TEM observation.

X-ray Photoelectron Spectroscopy (XPS). A droplet of sample solution was placed on silicon wafer and dried in air. The X-ray photoelectron spectroscopy (XPS) experiments were performed on ESCALAB 250 X-ray photoelectron spectrometer with an Mg Kα excitation source of 1.0×10^{-6} Pa and a resolution of 0.4 eV.

Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FT-MS). ESI-MS measurements were carried out on an APEX IV FT-MS (Bruker, USA). The

operating conditions of the ESI source: positive ion mode; spray voltage -3300 V; capillary voltage -3800 V, capillary temperature 200 C; skimmer1 33.0 V, skimmer2 28.0 V; sheath gas nitrogen pressure 0.3 bar. The initial TPA-1/Zn^{2+} samples for MS measurements: concentration of 0.5 mM TPA-1 with different concentration of Zn^{2+} (0.1-0.3 mM), pH 7.4. The samples were diluted 10 times by methanol and introduced *via* direct infusion at a flow rate of 3.00 mL/min.

Powder X-ray Diffraction (XRD). For XRD measurements, the samples of TPA-1/Zn^{2+} were vacuum-dried on clean glass slides. All samples were tested by an instrument (Rigaku Dmax-2000, Ni-filtered Cu Kα radiation) under ambient conditions at room temperature.

Atomic Force Microscopy (AFM). AFM measurements were conducted under tapping mode at ambient conditions using a D3100 AFM (VEECO, USA). One drop of the solution was spin-coated on silicon surface, and dried at room temperature before AFM observation.

Confocal Laser Scanning Microscopy (CLSM). The samples were carried out using a LeicaTcs-sp A1R-si inverted confocal laser scanning microscope (Nikon, Japan). A drop of TPA-1/Zn^{2+} solution was sealed between two slides at room temperature and ready for CLSM observation.

Two-Photon Fluorescence Measurements. The excitation femtosecond (fs) pulse laser (λ = 700-880 nm, 100 fs, SP-5W, Spectra physics, USA) was applied onto the crystals on quartz substrate, and the up-conversion PL spectra were collected by an Omni-λ300 monochromator/spectrograph (Zolix instruments Co., LTD, China)

equipped with a PMT (PMTH-S1C1-CR131, Zolix instruments). For two photo-excited fluorescence measurements, quartz-glass sample with 1.0 cm thickness were used. The samples were prepared in aqueous solution.

Two-Photon Fluorescence Microscopy Imaging of the Vesicles. Two-photon fluorescence microscopy (FV1200MPE-M) images of the vesicles were obtained by exciting the probes with a mode-locked titanium-sapphire laser source set at wavelength 800 nm. A drop of TPA-1/Zn^{2+} solution was sealed between two slides at room temperature and ready for CLSM observation.

Determination of Two-Photon Fluorescence Cross-section (δ)

Two-Photon Fluorescence spectra were measured using femtosecond laser pulse and Ti: sapphire system (680–1080 nm, 80 MHz, 140 fs, Chameleon II) as the light source. All measurements were carried out in air at room temperature. Two-Photon absorption cross sections were measured using two-photon-induced fluorescence measurement technique. The Two-Photon absorption cross sections (δ) are determined by comparing their Two-Photon Fluorescence to that of fluorescein in aqueous solution, according to the following equation[3]:

$$\delta = \delta_{ref}\frac{\Phi_{ref}}{\Phi}\frac{c_{ref}}{c}\frac{n_{ref}}{n}\frac{F}{F_{ref}}$$

Here, the subscripts *ref* stands for the reference molecule. δ is the Two-Photon absorption cross-section value, c is the concentration of solution, n is the refractive index of the solution, F is the Two-Photon Fluorescence integral intensities of the solution emitted at the exciting wavelength, and Φ is the fluorescence quantum yield. The δ_{ref} value of reference was taken from the literature.[4]

Determination of binding constant (Ka) measurements

The binding constant (Ka) was calculated on the basis of titration of the probes with Zn^{2+}. The binding modes with Zn^{2+} assigned as 2:1 for TPA-1. As a result, the binding constant (Ka) of TPA-1 with Zn^{2+} was determined by a nonlinear least-squares fitting of the titration data with the following equation for a 2:1 binding model (Figure S9)[5-6] :

$$c_{Zn^{2+}} = \frac{x}{2k_a(1-x)^2 c_{TPA-1}} + \frac{xc_{TPA-1}}{2}$$

where x is the fluorescence intensity ratio, $(I-I_0)/(I_{max}-I_0)$ observed at the corresponding emission wavelength during addition of Zn^{2+}, $c_{Zn^{2+}}$ is the concentration of total added Zn^{2+}, k_a is the binding constant, and c_{TPA-1} is the concentration of the probe. Finally, the binding constant of TPA-1 with Zn^{2+} was calculated to be 3.9×10^6 M^{-2} .

Cell Imaging. HeLa cells were grown overnight in 35 mm Petri dishes. The fluorescent vesicles (TPA-1/Zn^{2+}) were respectively transferred into the medium, immediately incubated with cells at 37 °C at a dose of 15 μg/mL. Wells with cells without test compound (but with equal proportion of sterile PBS) were included as controls. After 12 h incubation, two-photon fluorescence microscopy (FV1200MPE-M) images of the HeLa cells were obtained by exciting the probes with a mode-locked titanium-sapphire laser source set at wavelength 800 nm.

Drug Loading. 5-Fluorouracil (5-Fu) was added into the aqueous solution of $Zn(NO_3)_2$, which was dropped into the aqueous solution of TPA-1 to allow formation

of vesicles. The system was thermostatically incubated at 30 °C for 30 min to allow 5-Fu loading sufficiently. The 5-Fu loaded vesicles was placed in a dialysis bag (MWCO = 3500) against water for 6 h to reach equilibration. To determine the encapsulation efficiency, several 5-Fu water solutions in the 0–10 μg/mL concentration range were prepared to obtain a calibration curve. The 5-Fu loaded vesicles was placed in a dialysis bag (MWCO = 3500) against water for 6 h to reach equilibration. The amount of free drug 5-Fu containing in the excess of solvent was measured by UV-Vis absorption at its wavelength of maximum absorbance (λmax = 265 nm) and subsequently calculated on the basis of the calibration curve. The drug loading efficiency was calculated by the following equation[7]: loading efficiency (%) = $(m_1-m_2)/m_1$, where m_1, and m_2 represent the initial weight of 5-Fu and the weight of 5-Fu present in the excess of solvent respectively. The loading efficiency of 5-Fu on 2TPA-1@Zn^{2+} vesicles was 53.4%.

In vitro Drug Release Study. The drug release of 5-Fu from TPA-1/Zn^{2+}/5-Fu is conducted in different pH solutions (pH = 7.4 and pH = 5.8) at 37 °C. In short, 1mL TPA-1/Zn^{2+}/5-Fu (5-Fu relevant to 2.6 mg) was introduced into a dialysis bag and then immersed into 20 mL aqueous solution in a 25 mL glass beaker at 37 °C. At predetermined time intervals, 1 mL of solution was withdrawn, and the amount of 5-Fu released from the TPA-1/Zn^{2+}/5-Fu was examined at 265 nm by recording the UV absorbance of the solution. The cumulative release percentages of 5-Fu from TPA-1/Zn^{2+}/5-Fu were calculated as follows and plotted against time: Cumulative 5-Fu release = (amount of released 5-Fu/amount of total 5-Fu) × 100%. The

concentration of 5-Fu released from TPA-1/Zn^{2+}/5-Fu is obtained according to the standard equation using UV-Vis. Through the curve fitting, we calculated the standard equation of 5-Fu as $A = 0.0525c + 0.0034$, $R^2 = 0.9999$, where A is the absorbance, c is the concentration (μg/mL) of 5-Fu solution and R is the correlation coefficient.

Cytotoxicity Measurements. In vitro cytotoxicity of the 2TPA-1@Zn^{2+} vesicle, vesicle loaded 5-Fu (TPA-1/Zn^{2+}/5-Fu), and 5-Fu were assessed in HeLa cells by the MTT method.[8] In brief, HeLa cells were added to each well of a 96-well plate and incubated for 24 h. Then the culture medium was removed and cells were subject to blank vesicles，TPA-1/Zn^{2+}/5-Fu and 5-Fu solutions in serum free medium at equivalent 5-Fu concentrations of 0, 1, 2.5, 5, 7.5,10 and 12.5 μg/mL to investigate the cytotoxic effect of different 5-Fu formulations for another 24 h. After the previous nutrient solution was removed, 20 μ L of MTT solutions was added to each well and incubated for another 4 h. All media were then removed, and 100 μ L of DMSO was added to each well. After shaking the plates for 2 min, absorbance values per wells at 520 nm were read by a microplate reader. The cell viability rate (VR) was calculated by the equation of $VR = A/A_0 \times 100\%$ (A is the absorbance of the experimental groups, and A_0 is the absorbance of the control group.). Each assay was repeated six times.

Supplemental Figures

Figure S1

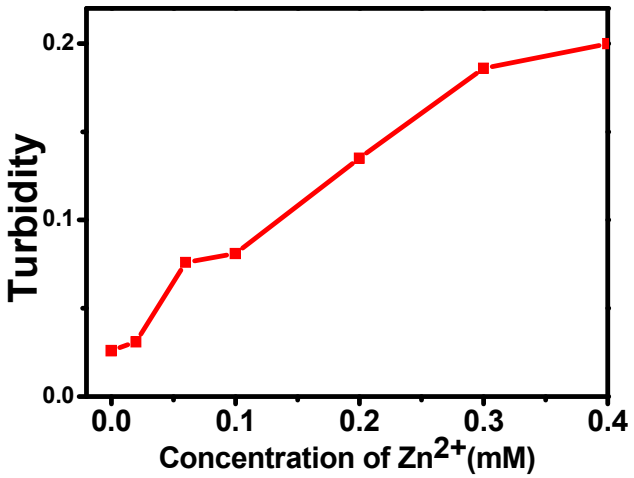


Figure S1 Turbidity changes of 0.5 mM TPA-1 with varied Zn^{2+} concentrations (0-0.3 mM) at pH 7.4.

Figure S2

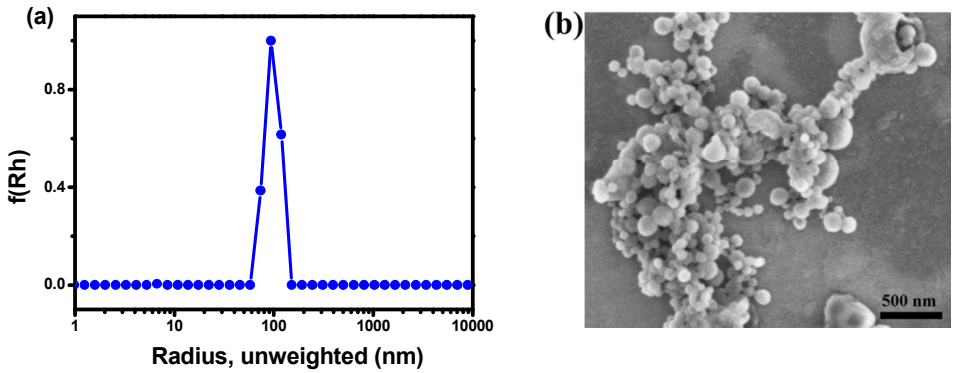


Figure S2 (a)DLS size distribution of the vesicles formed in the TPA-1/Zn^{2+} systems at the concentration of 0.5 mM TPA-1 and 0.05 mM $Zn(NO_3)_2$**; (b)** SEM image of TPA-1/Zn^{2+} (0.5 mM/0.4 mM).

Figure S3

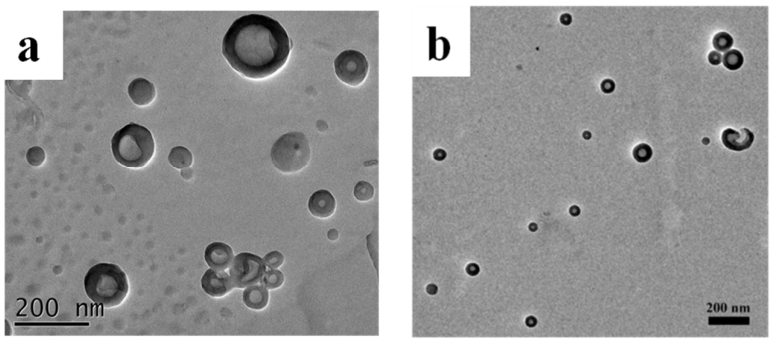


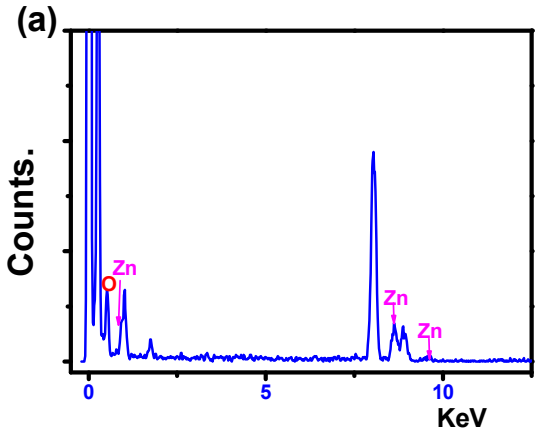
Figure S3 TEM images of the vesicles formed in the TPA-1/Zn^{2+} systems of 0.5/0.2 mM (a) and 0.5/0.3 mM (b).

Table S1

Table S1 Zeta Potential of 0.5 mM TPA-1 at different concentration of Zn^{2+} (0-0.3 mM), pH=7.4.

Concentration of Zn^{2+} (mM)	**0**	**0.10**	**0.20**	**0.30**
Zeta Potential (mV)	**-43.4**	**-41.9**	**-39.2**	**-33.4**

Figure S4



(b)

Sample	O(%)	Zn(%)	O/Zn ratio
TPA-1/Zn^{2+}	5.40	1.20	4.50

Figure S4 Energy dispersive X-ray (EDX) analysis of TPA-1/Zn^{2+}=0.5/0.3 mM (a) and compositions of the TPA-1/Zn^{2+} complex (b).

Figure S5

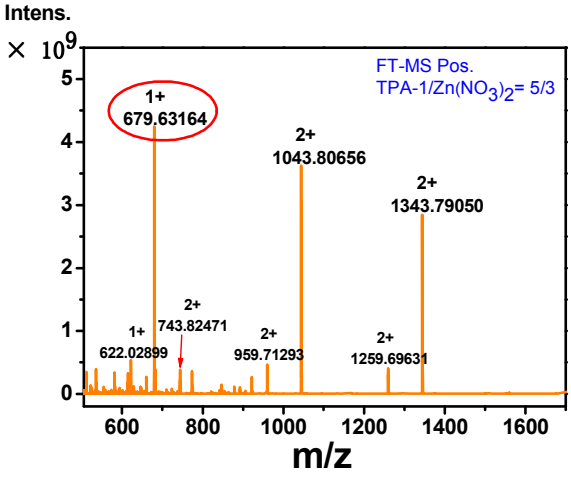


Figure S5. ESI-MS spectra of TPA-1/Zn^{2+} mixture at the ratio TPA-1/Zn^{2+}=5/3 in positive mode. The concentration of TPA-1 is 0.5 mM. The peak in red circle represent the species peak corresponding to $Zn(TPA\text{-}1)_2$.

Figure S6

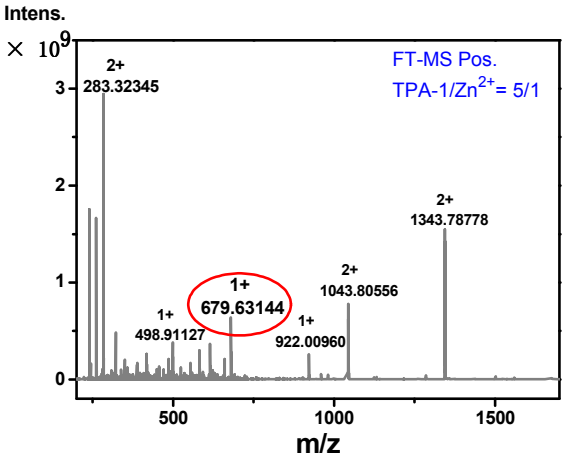


Figure S6. ESI-MS spectra of TPA-1/Zn^{2+} mixture at the ratio TPA-1/Zn^{2+}=5/1 in positive mode. The concentration of TPA-1 is 0.5 mM. The peak in red circle represent the species peak corresponding to $Zn(TPA\text{-}1)_2$.

Figure S7

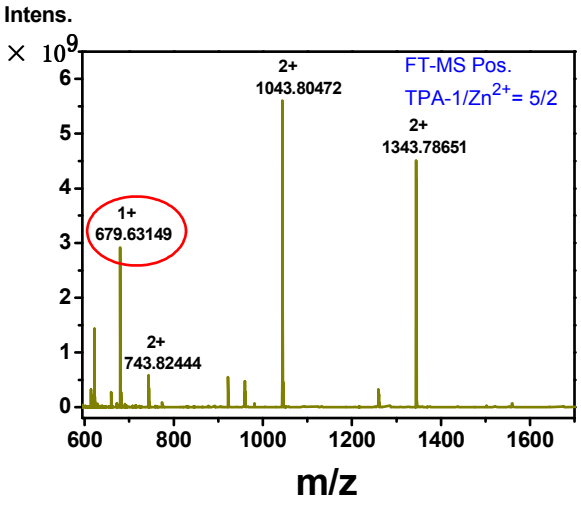


Figure S7. ESI-MS spectra of TPA-1/Zn^{2+} mixture at the ratio TPA-1/Zn^{2+}=5/2 in positive mode. The concentration of TPA-1 is 0.5 mM. The peak in red circle represent the species peak corresponding to $Zn(TPA\text{-}1)_2$.

Figure S8

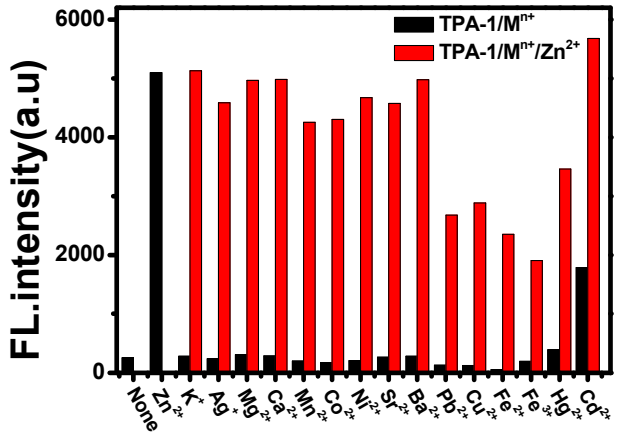


Figure S8. Fluorescence intensity of TPA-1 (0.5 mM) with different metal ions (0.2 mM) in the absence or presence of Zn^{2+} (0.2mM) at pH 7.4.

Figure S9

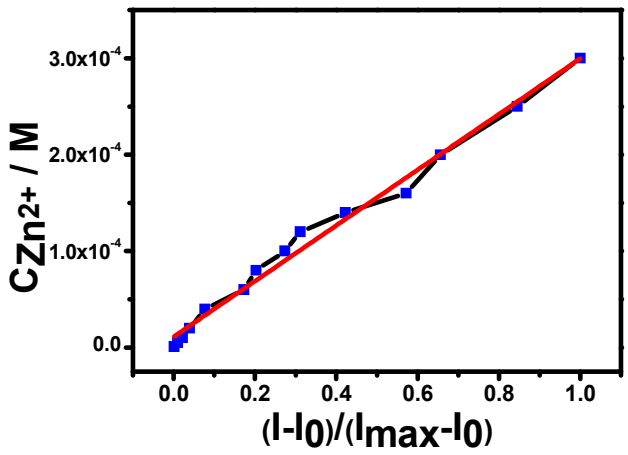


Figure S9. Plot of concentration of Zinc ion vs. fluorescence intensity ratio $(I-I_0)/(I_{max}-I_0)$ at 460 nm for TPA-1**.**

References

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