Electronic Supplementary Information

A naphthalimide-aminal-based pH-sensitive fluorescent donor for lysosome-targeted formaldehyde release and fluorescence turn-on readout

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General Information

Fluorescence spectra were collected on a FluoroMax-4 (Horiba Scientific) fluorescence spectrophotometer with slit widths were set at 2 nm both for excitation and emission. The pH measurements were carried out with a FE20 plus (Mettler Toledo) pH meter.

All aqueous solutions were prepared using double distilled water. **NAP-FAD-1** stock solution (1 mM in dry DMSO) was prepared and stored at -20 °C. All fluorescence spectroscopic measurements were performed in PBS buffer (pH 7.4, purchased from Shanghai Bioscience Co. Ltd.) at 25 °C unless otherwise stated. PBS solution (pH 5.0) was obtained by addition of 1 M HCl to pH 5.0 monitored by a pH-meter. Samples for fluorescence measurements were contained in 1 cm×1 cm quartz cuvettes (3.5 mL volume).

Part I: Additional Absorption and Fluorescence Studies of NAP-FAD-1

1.1 Normalized Fluorescence Spectra of NAP-FAD-1 in PBS Buffer and the Fluorescent Product 4.

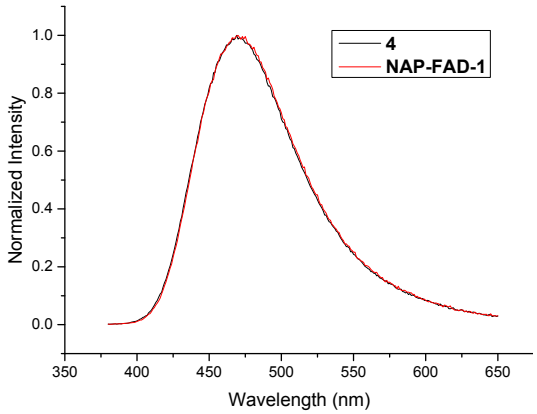


Figure S3 Normalized fluorescence emission spectra (λ_{ex} = 359 nm) of **NAP-FAD-1** (60 μM) incubated in PBS buffer for 5 h and the fluorescent product **4** (1 μM) in PBS buffer (pH=7.4).

1.2 Time-dependent Absorption Spectra of NAP-FAD-1 at pH 5.0 and pH 7.4

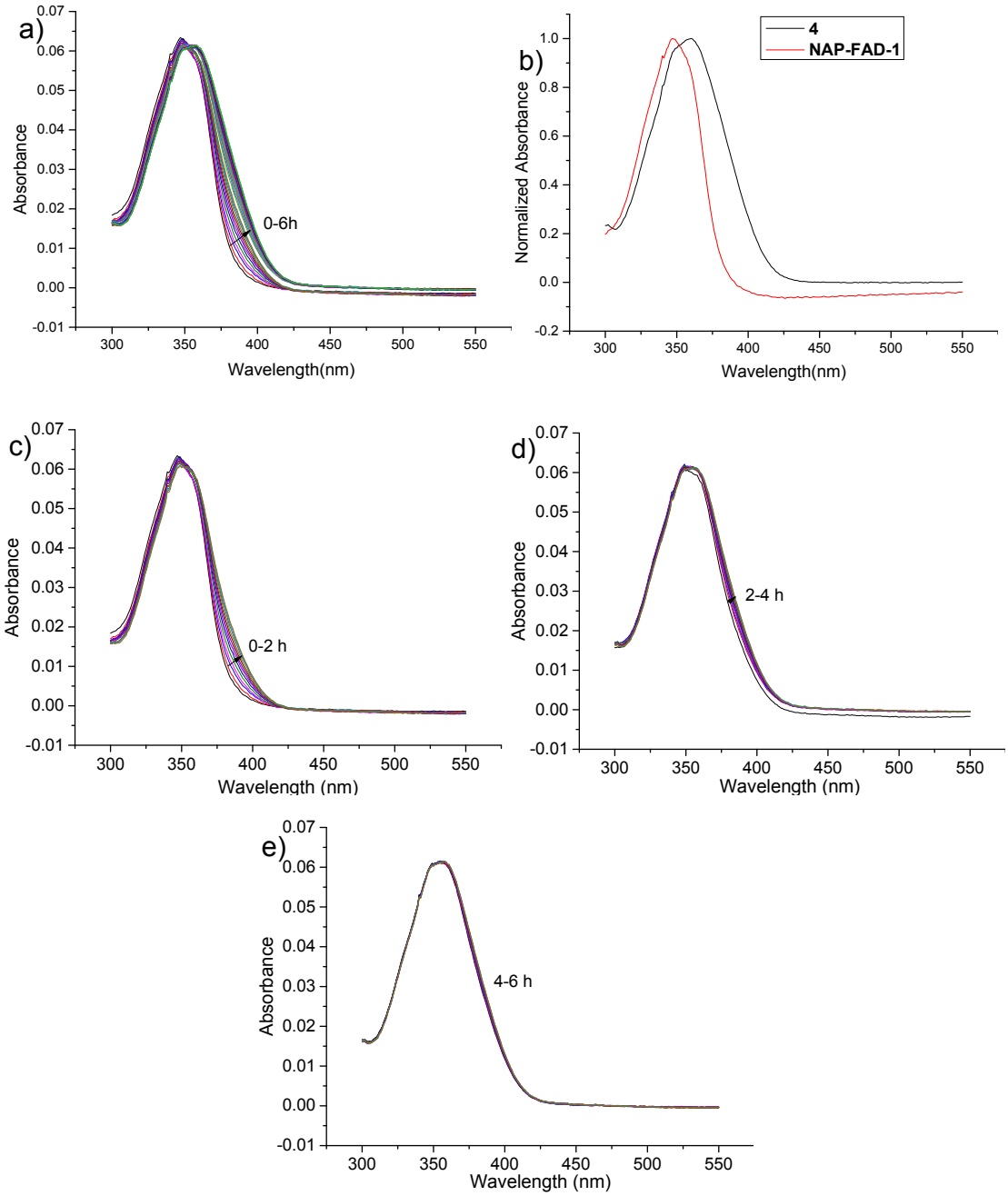


Figure S2 a) Time-dependent absorption spectra of **NAP-FAD-1** (5 μM) in PBS solution (pH 5.0); b) Comparison of normalized absorption spectra of **NAP-FAD-1** and the fluorescent product **4** in PBS solution (pH 5.0); c-e) Time-dependent absorption spectra of **NAP-FAD-1** (5 μM) in PBS solution (pH 5.0) from 0-2 h, 2-4 h, and 4-6 h, respectively.

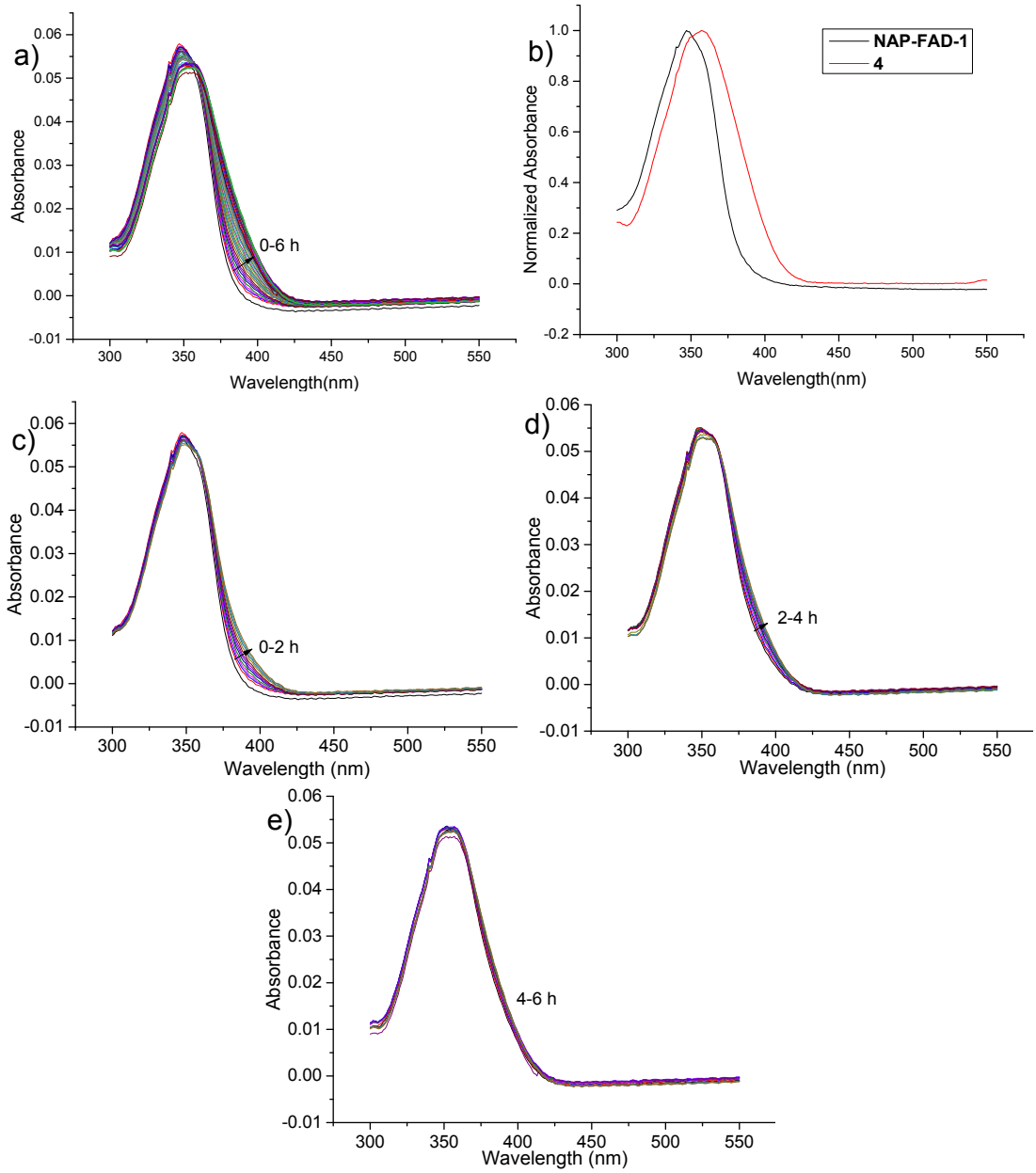


Figure S3 a) Time-dependent absorption spectra of **NAP-FAD-1** (5 μM) in PBS buffer (pH 7.4); b) Comparison of normalized absorption spectra of **NAP-FAD-1** and the fluorescent product **4** in PBS buffer (pH 7.4); c-e) Time-dependent absorption spectra of **NAP-FAD-1** (5 μM) in PBS buffer (pH 7.4) from 0-2 h, 2-4 h, and 4-6 h, respectively.

Part II Reaction Kinetics Analysis of FA Release from NAP-FAD-1.

2.1 The *pseudo*-First-Order Reaction Constants of NAP-FAD-1 Hydrolysis at pH 7.4 and 5.0.

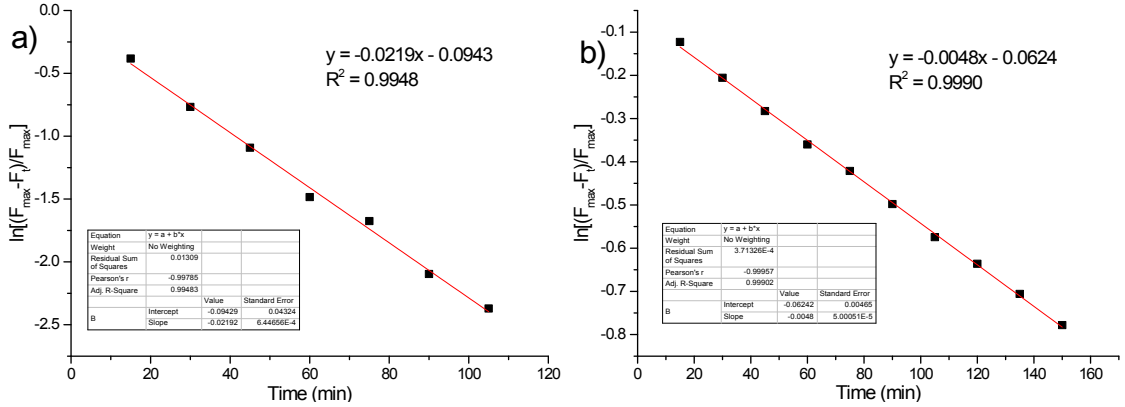


Figure S4 a) Linear regression analysis of $Ln[(F_{max}-F_t)/F_{max}]$ versus t (0-105 min) of the time-dependent fluorescence intensity at 470 nm of **NAP-FAD-1** (1 μM) in PBS buffer at pH 7.4 with 500 equiv. FA; (b) Linear regression analysis of $Ln[(F_{max}-F_t)/F_{max}]$ versus t (0-150 min) of the time-dependent fluorescence intensity at 470 nm of **NAP-FAD-1** (1 μM) in PBS buffer at pH 7.4 without FA. The *pseudo*-first-order reaction constants were calculated as 0.0219 min^{-1} (half-time 31.7 min) and 0.0048 min^{-1} (half-time 144 min), respectively for with and without 500 equiv. FA.

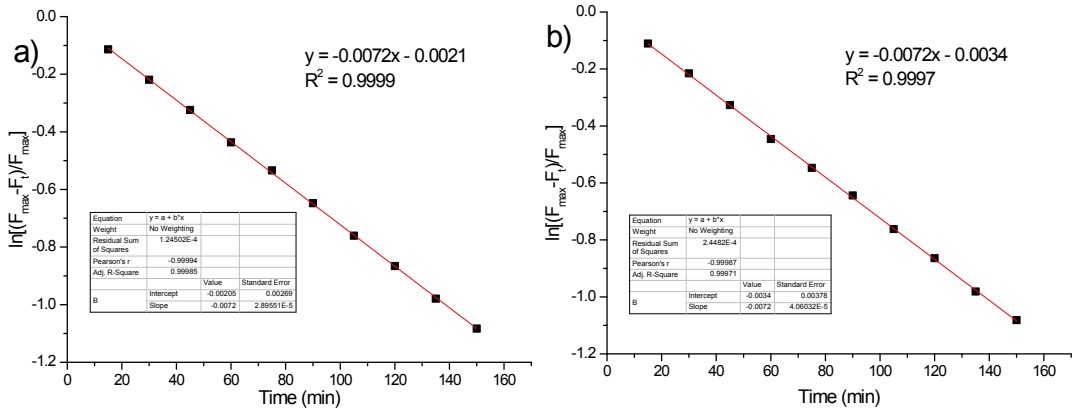
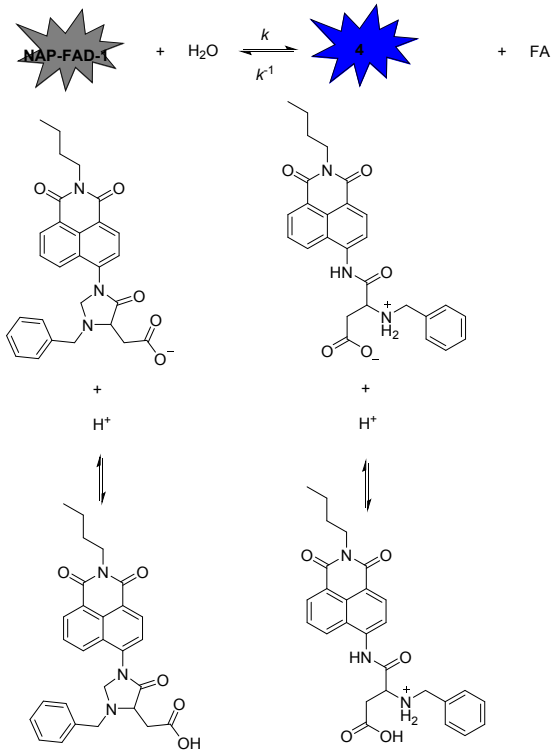


Figure S5 Linear regression analysis of $Ln[(F_{max}-F_t)/F_{max}]$ versus t (0-150 min) of the time-dependent fluorescence intensity at 470 nm of hydrolysis of **NAP-FAD-1** (1 μM) in PBS solution at pH 5.0 with 500 equiv. FA (a) and without FA (b). The reactions were assumed to be irreversible at pH 5.0 and the *pseudo*-first-order reaction constants were about the same value of 0.0072 min^{-1} (half-time 96 min).

2.2 Reaction Kinetics Analysis of FA Release from NAP-FAD-1.



Scheme S1 Conversion of **NAP-FAD-1** to **4** in equilibrium with their own acid-conjugate base equilibrium.

From **the Scheme S1**, we could deduce:

$$d[\mathbf{4}]/dt=k[H_2O][\mathbf{NAP\text{-}FAD\text{-}1}]-k^{-1}[FA][\mathbf{4}] \quad (1)$$

At pH=5.0, there were almost no difference in *pseudo*-first-order reaction constant whether incubated with 500 equiv. or without FA (**Figure S5**). It was only possible when the $k^{-1}[FA][\mathbf{4}]$ in equation (1) can be omitted. Then, from equation (1), we got the equation (2) at pH 5.0:

$$d[\mathbf{4}]/dt\approx k[H_2O][\mathbf{NAP\text{-}FAD\text{-}1}] \quad (2)$$

According to the equation (2), the *pseudo*-first-order reaction constant is $k[H_2O]$. The experimental value of $k[H_2O]$ at pH=5.0 was 0.0072 min^{-1} (half-time 96 min, **Figure S5**).

While at pH=7.4, we first consider the condition without added FA at the beginning. Then, at each reaction time point, [FA]=[**4**]. From equation (1), we got equation (3):

$$\begin{aligned} d[\mathbf{4}]/dt&=k[H_2O][\mathbf{NAP\text{-}FAD\text{-}1}]-k^{-1}[FA][\mathbf{4}] \\ &=k[H_2O](C_0-[\mathbf{4}])-k^{-1}[\mathbf{4}]^2 \end{aligned} \quad (3)$$

In this case, as the observed time-dependent increase of **[4]** also followed the *pseudo*-first-order

reaction (**Figure S4b**). Then the contribution of k^{-1}[**4**]^2 in the equation (3) must be very small. Therefore, the equation (2) was still viable at pH 7.4 in this condition. From equation (2), the *pseudo*-first-order reaction constant is $k[H_2O]$.The experimental value of $k[H_2O]$ at pH=7.4 was 0.0048 min^{-1} (half-time 144 min, **Figure S4b**). From the value $k[H_2O]$, the hydrolysis reaction rate constant of **NAP-FAD-1** at pH 5.0 is about 1.5-fold of the value at pH=7.4.

In case of pH=7.4 with 500 equiv. of FA, from equation (1) we got the following equation (4):

$$d[\mathbf{4}]/dt=k[H_2O](C_0-[\mathbf{4}])-k^{-1}(500\ C_0-[\mathbf{4}])[\mathbf{4}] \quad (4)$$

As [**4**]<C_0, (500 C_0-[**4**]) ≈500 C_0, from equation (4), we got the following equation (5):

$$d[\mathbf{4}]/dt \approx k[H_2O](C_0-[\mathbf{4}])-k^{-1}500\ C_0[\mathbf{4}]$$
$$=-(k[H_2O]+k^{-1}500\ C_0)\ [\mathbf{4}]+k[H_2O]C_0 \quad (5)$$

Then, the observed the *pseudo*-first-order reaction constant should be $k[H_2O]+k^{-1}500\ C_0$ with the experimental value 0.0219 min^{-1} (half-time 31.7 min). From the experimental value of $k[H_2O]$ at pH=7.4 (0.0048 min^{-1}) and C_0=1 μM, the value of k^{-1} was calculated as 34.2 M^{-1} min^{-1}= 0.57 M^{-1} s^{-1}.

When the reaction reaches in equilibrium,

$$k[H_2O][\mathbf{NAP\text{-}FAD\text{-}1}]=k^{-1}[FA][\mathbf{4}] \quad (6)$$

The equilibrium constant for the reaction shown in the **Scheme S1** at pH 7.4 was calculated as below:

$$K=[FA][\mathbf{4}]/([H_2O][\mathbf{NAP\text{-}FAD\text{-}1}])=k/k^{-1}$$
$$= k[H_2O]/\ k^{-1}[H_2O]=0.0048/(34.2\times55.6)=2.5\times10^{-6} \quad (7)$$

When an equal mole ratio of [**4**] and [**NAP-FAD-1**] mixed together to reach equilibrium at pH 7.4, [FA] can be calculated as below:

$$[FA]=K[H_2O]= 1.4\times10^{-4}\ M = 140\ \mu M \quad (8)$$

Part III: UPLC-MS Studies of Hydrolysis Reaction of NAP-FAD-1.

UPLC-MS analysis was performed in Q-Exactive™ plus equipped with ZORBAX Eclipse Plus C18 (Agilent) 1.8 μm, 100 mm×2.1 mm column. All the samples were eluted using a gradient mixture from 30:70 to 95:5 CH_3CN : H_2O containing 0.1 % formic acid at a flow rate of 0.2 mL/min in 10 min. LC peaks were monitored by UV absorption at 254 nm and MS was detected in ESI positive mode.

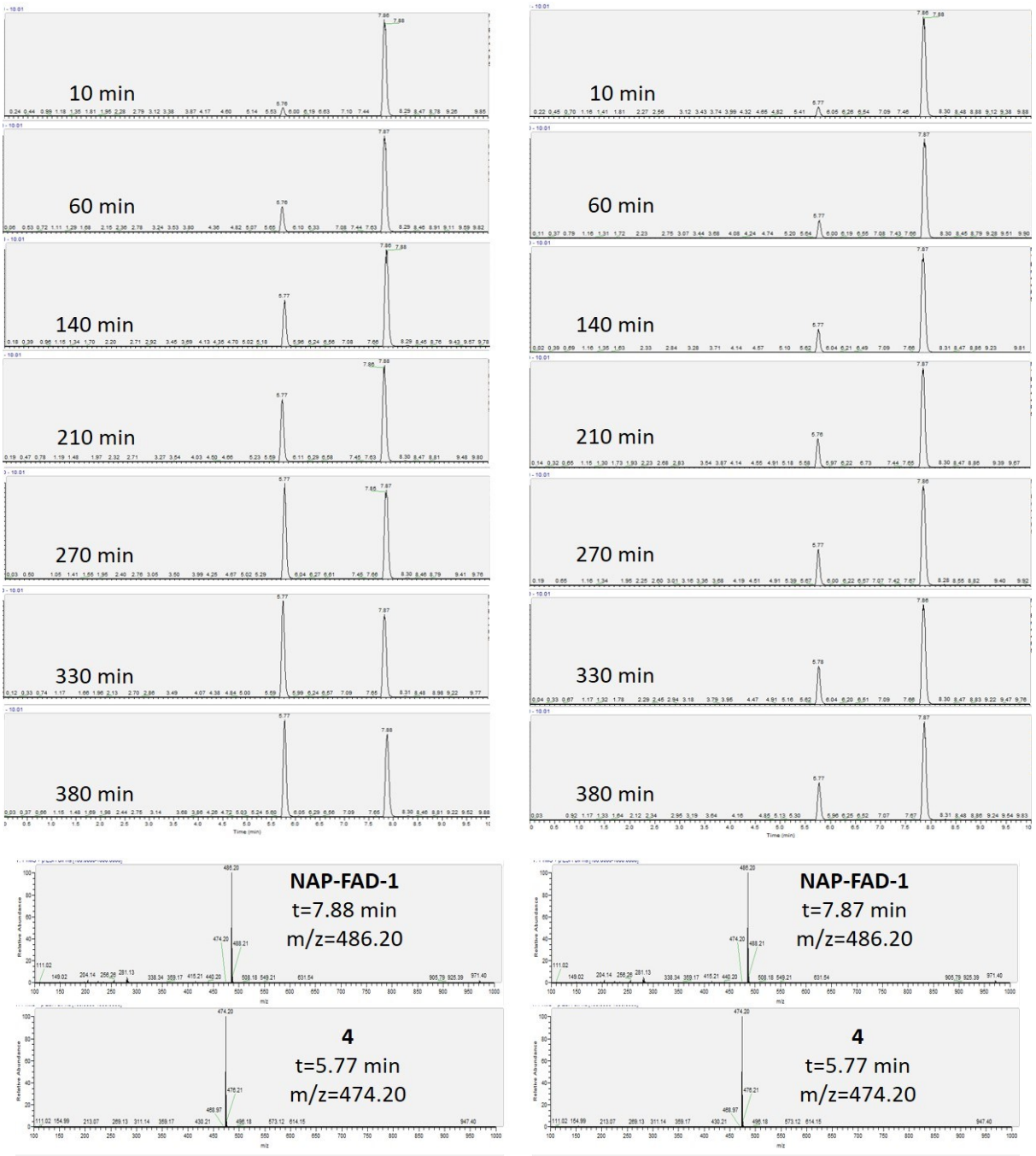


Figure S6 UPLC-MS studies of **NAP-FAD-1** (4.94 mM) hydrolysis in THF : PBS = 1:1 (V/V) at pH 5.0 (left column) and pH 7.4 (right column) at different time-points from 10 to 380 min.

Part IV: Cytotoxicity and Cell Imaging Studies of the FA Donor NAP-FAD-1

Cell culture: The HeLa human cervix cell line and Jurkat human T lymphocyte cell line were kindly provided by Stem Cell Bank, Chinese Academy of Sciences. HeLa cells were maintained in DMEM medium (Gibco) supplied with 10% Fetal Bovine Serum (FBS, Gibco) and 1% Glutamine (Gibco) at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. Jurkat cells were maintained in RPMI 1640 medium (Hyclone) supplied with 10% FBS and 100 U/ml Pen/Strep (Thermo-Fisher Scientific), maintained at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2.

4.1 Cytotoxicity Evaluation of the FA Donor NAP-FAD-1 and the Fluorescent Product 4

Cell survival was evaluated by the MTS assay (CellTiter 96 AQueous One Solution Reagent), based on the conversion of a tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS), to a colored formazan product by living cells.[1] Absorbance was read by a microplate reader (Molecular Devices SpectraMax I3) at 490 nm. The quantity of formazan product, as measured by the amount of absorbance, was directly proportional to the metabolic activity of viable cells in the culture.

$$\text{Cell viability (\% of control)} = (OD_{EG}-OD_{ZG})/(OD_{CG}-OD_{ZG})*100\% \quad (9)$$

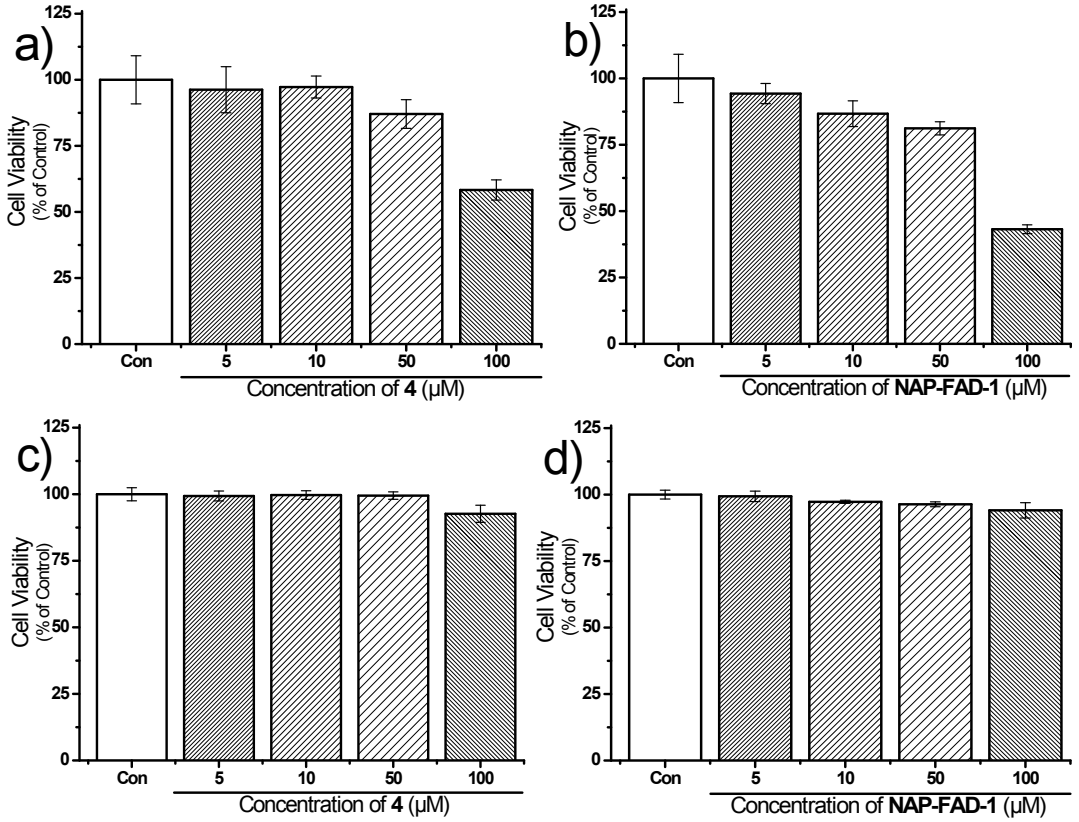


Figure S7 a-b) Cell viabilities of Jurkat cells after 8 h incubation in fluorescent product **4** and **NAP-FAD-1** at various concentrations; c-d) Cell viabilities of HeLa cells after 24 h incubation in fluorescent product **4** and **NAP-FAD-1** at various concentrations.

4.2 Time-dependent Overall Fluorescence Intensity Study of Jurkat cells in NAP-FAD-1 Containing Cell Culture Media

Jurkat cells were grown in RPMI medium (Gibco) supplied with 10% Fetal Bovine Serum (FBS, Gibco). At least 1 d before the experiment, the cells (cell counts between 0.5×10^5 and 0.8×10^5) were transferred into 96-well cell culture plates and incubated for 24 h. Cells were then transferred to PE OptiPlate Black 96-well microplate and maintained in RPMI 1640 medium supplemented with 10% FBS. Then **NAP-FAD-1** were added to wells to a final concentration of 10 μM or 50 μM, and incubated at 37 °C for 8 hours, while blank media were added to the control wells containing Jurkat cells in DMEM supplemented with 10% FBS to the same volume. Fluorescence intensity at for 2, 4, 6, and 8 h were obtained with a SpectraMax i3x microplate reader (Molecular Devices) at excitation wavelength of 359 nm and the emission wavelength of 470 nm. The results were shown in Fig. 4b.

4.3 Time-dependent Fluorescence Intensity Study of NAP-FAD-1 in FBS or RPMI 1640 Medium

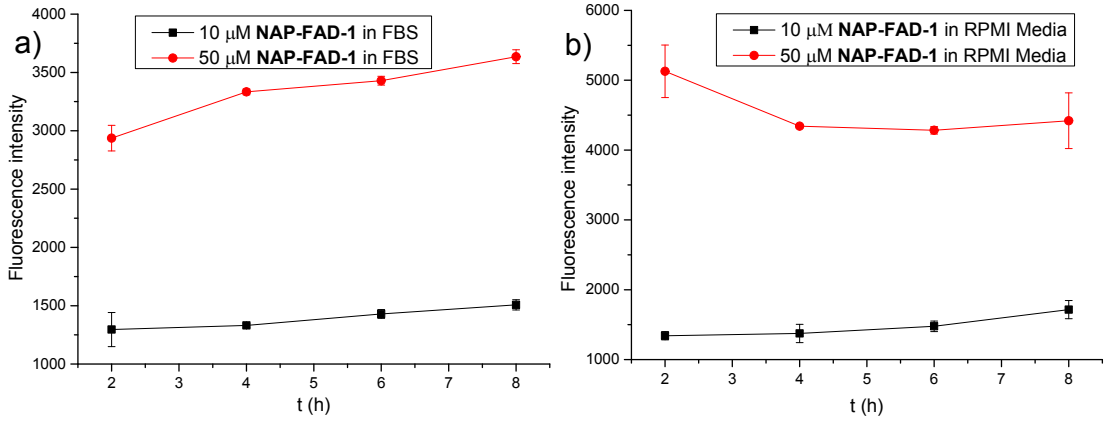


Figure S8 a) Time-dependent fluorescence intensity ($\lambda_{ex/em}$ = 359/470 nm) of **NAP-FAD-1** incubated in FBS at 37 °C at two different concentrations (10 and 50 μM) in FBS (Gibco); b) Time-dependent fluorescence intensity ($\lambda_{ex/em}$ = 359/470 nm) of **NAP-FAD-1** incubated in RPMI 1640 medium (Hyclone) at 37 °C at two different concentrations (10 and 50 μM).

4.4 Time-dependent Fluorescence Intensity Study of Jurkat cells Pretreated with NAP-FAD-1 Containing Cell Culture Media

Jurkat cells were grown in RPMI medium (Gibco) supplied with 10% Fetal Bovine Serum (FBS, Gibco). At least 1 d before the experiment, the cells (cell counts between 0.5×10^5 and 0.8×10^5) were transferred into 96-well cell culture plates and incubated for 24 h. Cells were then transferred to PE OptiPlate Black 96-well microplate and maintained in RPMI 1640 medium supplemented with 10% FBS. **NAP-FAD-1** were added to wells containing Jurkat cells in DMEM supplemented with 10% FBS to a final concentration of 10 μM or 50 μM, and incubated at 37 °C for 30 min. Then the **NAP-FAD-1** containing media were removed and replaced with RPMI medium supplemented with 10% FBS, and incubated at 37 °C for 8 h. Fluorescence intensity at for 2, 4, 6, and 8 h were obtained with a SpectraMax i3x microplate reader (Molecular Devices) at excitation wavelength of 359 nm and the emission wavelength of 470 nm. The results were shown in Fig. 4c.

4.5 Confocal Fluorescence Imaging Experiments of NAP-FAD-1

HeLa cells were seeded in a 6-well glass tissue culture dish at the cell culture facility of East China University of Science and Technology (ECUST) and cultured in DMEM medium (with phenol red, Gibco/Invitrogen) with 10% Fetal Bovine Serum (FBS, Gibco) and 1% Glutamine (Gibco) at 37 ˚C for 24 hours. The cells were incubated with 50 μM **NAP-FAD-1** in DMEM medium (with phenol red, Gibco/Invitrogen) with 10% Fetal Bovine Serum (FBS, Gibco) and 1% Glutamine (Gibco) for 8 h, and then incubated with 200 nM Lyso-Tracker Red in the same media for 10 min. Then the media was washed with PBS buffer three times, and replaced with fresh media and fluorescence images was obtained using a Leica TCS SP8 equipped with 40× objective lens and PMT gain of 800. The results were shown in Fig. 5.

References

1. A. H. Cory, T. C. Owen, J. A. Barltrop and J. G. Cory, *Cancer Commun.*, 1991, **3**, 207-212.