Supplementary Information

Array-based Protein Sensing Using an Aggregation-induced Emission (AIE) Light-up Probe

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Table of Contents

S1. Synthesis and characterization
  - S1.1 Materials and characterization
  - S1.2 Synthesis of AIEgens (AIE 1-4)

S2. Spectrometer experiment
  - S2.1 Characterization of AIEgens (AIE 1-4)
  - S2.2 Method for AIE fluorescence in presence of five proteins

S3. Determination of the binding stoichiometry and binding constant

S4. Classification proteins in the protein mixtures

S5. Reference
S1. Synthesis and characterization

S1.1 Materials and characterization

2,4-dihydroxy benzaldehyde, 1,3-dibromopropane, dimethylamine solution 40% in water and tert-butyl bromoacetate were purchased from Alfa Aesar (USA). Hydrazine monohydrate (N₂H₄.H₂O), trimethylamine 45% aqueous solution in water and all proteins (Bovine serum albumin, Esterase, Fibrinogen, transferrin and β-galactosidase) were purchased from Sigma Aldrich (USA). Trifluoroacetic acid (TFA) was purchased from TCI (Japan). Ethyl acetate (EtOAc) and hexane were obtained from SK Chemical (Korea). Potassium carbonate (K₂CO₃), potassium bicarbonate (KHCO₃), sodium hydroxide (NaOH), sodium chloride (NaCl), and sodium sulfite anhydrous (Na₂SO₃) was obtained from Samchun pure chemical Co.,Ltd (Korea). HPLC grade of tetrahydrofuran (THF ) and acetonitrile (ACN) were purchased from Burdick & Jackson Honeywell (USA). Dichloromethane (DCM) HPLC was purchased from Daejung Co.,Ltd (Korea). PBS 1X, pH 7.4 was obtained from Gibco, ThermoFisher SCIENTIFIC (USA)

AIEgens (AIE 1-4) were characterized using 400MHz FT-NMR (Agilent Technologies) and MALDI-TOF/TOF (Bruker Ultraflex III) with MALDI matrix made of 50:50 water/acetonitrile with 0.1% TFA and 2,5-dihydroxybenzoic acid (2,5-DHB). UV –vis spectroscopy (Jasco V-670 spectrophotometer), Fluorometer (Hitachi F-7000), Microplate reader (Molecular devices SpectraMax i3x)

S1.2 Synthesis of AIEgens (AIE1-4)

1) Synthesis of compound 2

![Compound 2](image)

Compound 2 was synthesized by adding hydrazine monohydrate (90 µL, 1.81 mmol) into compound 1, 2,4-dihydroxy benzaldehyde (0.5 g, 3.65 mmol) in 10 mL of methanol. The mixture was refluxed for 4 h at 75°C. The solution was cooled to room
temperature then yellow solid was formed which was isolated by filtration and dried over under vacuum. Yield (72%, 0.357 g) δ_H (400 MHz, DMSO) 6.29 (2H, s), 6.37 (2H, s), 7.39 (2H, s), 8.73 (2H, s), 10.13 (2H, s) and 11.38 (2H, s). δ_C (400 MHz, DMSO) 102.91, 108.65, 110.71, 133.39, 161.11, 162.20 and 162.51 Calculated [M], C_{14}H_{12}N_{2}O_{4}, molecular weight: m/z calcd 272.26; measured 272.60

2) Synthesis of AIE-1

![Structure of AIE-1](image)

a. Synthesis of t-butyl-protected AIE molecule

To synthesize AIE-1, compound 2 (500 mg, 1.836 mmol) and K_2CO_3 (507.6 mg, 3.673 mmol) were dissolved in 10 mL of acetonitrile. After 30 min, t-butyl bromoacetate (542 µL, 3.673 mmol) was added dropwise into mixture. The reaction was refluxed at 60 °C for overnight. After finishing the reaction, the reaction was then cooled to room temperature. The reaction mixture was treated with water and extracted with ethyl acetate (EA). Organic layer was washed with brine solution. The organic layer was dried over Na_2SO_4 and concentrated in vacuo. Purification was done by silica flash column chromatography with hexane: EA = 4:1. Yield (21%, 187 mg) δ_H (400 MHz, DMSO) 1.46 (18H, s), 4.68 (4H, s), 6.43 (2H, s), 6.55 (2H, s), 7.39 (2H, s) 7.52 (2H, s), 8.91 (2H, s). δ_C (400 MHz, DMSO) 27.82, 65.59, 82.79, 102.17, 107.28, 111.44, 133.58, 158.84, 161.76, 163.03, 167.42

b. Deprotection of t-butyl group

To synthesize AIE-1, boc-protected AIE molecules (244mg, 0.487 mmol) was dissolved in 5 mL of DCM. TFA (0.2 mL, excess amount) was added into boc-protected AIE in DCM. The reaction was stirred at room temperature for 12 hours. After reaction, DCM and TFA were removed using rotary evaporator and dried using high vacuum pump. To make negatively charged AIE-1, 2 equivalent of NaOH was
3) Synthesis of compound 3

![Image of compound 3]

Compounds 3 was synthesized through reference [1]. Compound 1, 2,4-dihydroxy benzaldehyde (2 g, 14.48 mmol) and potassium bicarbonate (KHCO₃, 1.448 g, 14.48 mmol) were dissolved in dry acetone and then 1,3-dibromopropane (1.47 mL, 14.48 mmol) was added dropwise into mixture. The reaction was refluxed at 60 °C for 60 h. After the reaction, solvent was evaporated under pressure. 20 mL of water was added and extracted with 30 mL of chloroform for three times. The organic layer was dried over Na₂SO₄ and concentrated in vacuo. Purification was done by silica flash column chromatography with hexane: EA = 1:1. Yield (17%, 1.2 g) δ_H (400 MHz, CDCl₃) 2.36 (4H, m), 3.57 (4H, t), 4.12 (4H, t), 6.48 (2H, s), 6.59 (2H, s), 7.48 (2H, s), 9.85 (2H, s), and 11.50 (2H, s). δ_C (400 MHz, CDCl₃) 29.47, 31.90, 65.79, 101.27, 108.50, 115.30, 135.30, 164.41, 165.81, 194.37. Calculated [M]⁺, C₁₀H₁₁BrO₃, molecular weight: m/z calcd 259.10; measured 258.36

4) Synthesis of compound 4

![Image of compound 4]

Compound 4 was synthesized by adding hydrazine monohydrate (7.5 µL, 0.09 mmol) into compound 3 (92.6 mg, 0.18 mmol) in 3 mL of acetonitrile. The mixture was
refluxed for 12 h at 60 °C. After the reaction, solvent was dried over under vacuum. Yield (91%, 168 mg) \( \delta_H \) (400 MHz, CDCl\(_3\)) 2.34 (4H, m), 3.60 (4H, t), 4.15 (4H, t), 6.53 (4H, s), 8.61 (2H, s), and 11.73 (2H, s). \( \delta_C \) (400 MHz, CDCl\(_3\)) 29.65, 32.11, 50.86, 65.52, 101.82, 107.72, 111.28, 133.52, 161.71, 162.80, 16.89 Calculated [M], C\(_{20}\)H\(_{22}\)Br\(_2\)N\(_2\)O\(_4\), molecular weight: m/z calcd 514.21; measured 515.12

5) Synthesis of AIE-2

![AIE-2](image)

Compound 4 (20 mg, 0.039 mmol) and dimethylamine 40% solution in water (17 µL, 0.155 mmol) in THF and water co-solvent was stirred and heated up to 60 °C for overnight. After the reaction, the solvent (THF and water) was unreacted trimethylamine removed by evaporation. The 16 mg (98%) of product AIE-3 was obtained. \( \delta_H \) (400 MHz, D\(_2\)O) 1.98 (4H, m), 2.68 (4H, t), 4.13 (4H, t), 6.38 (4H, s), 6.62 (4H, s), 7.16 (2H, s), and 7.85 (2H, s), 11.70 (2H, s). \( \delta_C \) (400 MHz, D\(_2\)O) 22.44, 52.84, 63.74, 64.60, 101.62, 107.58, 111.80, 133.88, 159.85, 161.87, 162.89 Calculated [M]\(^+\), C\(_{26}\)H\(_{40}\)N\(_4\)O\(_4\)\(^{2+}\), molecular weight: m/z calcd 472.63; measured 471.39

6) Synthesis of AIE-3

![AIE-3](image)

Compound 4 (20 mg, 0.039 mmol) and dimethylamine 40% solution in water (17 µL,
0.155 mmol) in 1 mL of THF and water co-solvent was stirred and heated up to 60 °C for overnight. After the reaction, the solvent (THF and water) and unreacted dimethylamine was removed by evaporation. The 16 mg (98%) of product AIE-3 was obtained. \(\delta_H\) (400 MHz, DMSO) 1.98 (4H, m), 2.68 (4H, t), 4.13 (4H, t), 6.53 (4H, s), 7.51 (2H, s), and 8.80 (2H, s). \(\delta_C\) (400 MHz, DMSO) 34.41, 42.99, 54.68, 65.55, 101.82, 107.84, 112.01, 133.06, 160.84, 162.32, 162.73 Calculated [M], \(C_{24}H_{34}N_4O_4\), molecular weight: m/z calcd 442.56; measured 443.32

7) Synthesis of AIE-4

Compound 4 (10 mg, 0.019 mmol) and sodium sulfite (NaSO₃. 6 mg, 0.042 mmol) in 2 mL of THF and water co-solvent was stirred and heated up to 100 °C under nitrogen atmosphere for overnight. After the reaction, the mixture kept at room temperature. Acetone (diethyl ether) was added to get white crystals. The 4 mg (80%) of product AIE-4 was obtained. \(\delta_H\) (400 MHz, D₂O) 1.98 (4H, m), 2.68 (4H, t), 4.13 (4H, t), 6.53 (4H, s), 7.51 (2H, s), and 8.80 (2H, s). \(\delta_C\) (400 MHz, D₂O) 24.10, 47.67, 66.48, 101.39, 107.51, 111.99, 133.25, 159.80, 162.05, 162.50 Calculated [M]⁺, \(C_{26}H_{40}N_4O_4^{2+}\), molecular weight: m/z calcd 514.52; measured; 514.42

S2. Spectrometer experiment

S2.1 Characterization of AIEgens (AIE 1-4)
Figure S1. UV-Vis absorption spectra of (a) AIE-2, (b) AIE-3 and (c) AIE-4 in water (black line) and in a mixture of H$_2$O /THF (v/v, 5:95) (red line). [AIEgens] = 40 µM

Figure S2. Fluorescence spectra of (a) AIE-1, (b) AIE-2, (c) AIE-3, and (d) AIE-4 in gradual addition (0%, 30%, 60%, 90% and 95%) of THF to water. $\lambda_{ex} = 365$ nm [AIEgens] = 40 µM

S2.2 Method for AIE fluorescence in presence of five proteins
Figure S3. Schematic illustration of fluorescence experiments with four AIEgens and five proteins

All four AIEgens solution in water were prepared 42.1 μM stock solution using 3rd distilled water. Also, five proteins were prepared 10 uM in 1X PBS pH 7.4. In individual 96 wells, 95 μL of 42.1 μM AIE stock solution was added followed by addition 5 μL of 10 uM protein solutions. We measured fluorescence using microplate reader right after adding proteins.

|       | BSA     | Esterase | transferrin | fibrinogen | β-galactosidase |
|-------|---------|----------|-------------|------------|----------------|
| AIE-1 | 1.239416| 1.056613 | 1.059014    | 0.818529   | 1.096484       |
|       | 1.216347| 1.04249  | 1.092795    | 0.864261   | 1.073643       |
|       | 1.262054| 1.037588 | 1.098455    | 0.873736   | 1.105933       |
|       | 1.233958| 1.047265 | 1.082385    | 0.910473   | 1.218116       |
|       | 1.179534| 1.018032 | 1.097318    | 0.929701   | 1.108485       |
|       | 1.33237 | 1.10467  | 1.112301    | 0.913101   | 1.11177        |
| AIE-2 | 0.95884 | 1.934575 | 1.039262    | 1.031855   | 1.290872       |
|       | 0.962982| 1.896937 | 1.060973    | 1.024958   | 1.28009        |
|       | 0.936983| 1.981135 | 1.10684     | 1.022423   | 1.271752       |
### S3. Determination of the binding stoichiometry and binding constant

Stoichiometry of the AIEgen-protein was determined by saturation point of fluorescence intensity with increasing AIEgens’ concentrations at fixed concentration of proteins. The fluorescence intensity was measured when protein concentration was fixed at 5 µM by increasing AIE-1 and AIE-2 from 0 to 64 µM. Normalized fluorescence intensity was calculated by deducting fluorescence intensity of only AIE-1 and AIE-2 solution from 0 to 64 µM.

The raw data of normalized fluorescence intensity (F/F₀) of AIE-1 to AIE-4 (40 µM) with 5 µM of five proteins are shown in Table S1.

|       | F/F₀       | F/F₀       | F/F₀       | F/F₀       |
|-------|------------|------------|------------|------------|
| AIE-1 | 0.951488   | 1.933937   | 1.076116   | 1.006586   |
|       | 0.962325   | 1.887742   | 1.051304   | 0.99564    |
|       | 0.926256   | 1.823174   | 1.069183   | 0.956779   |
|       | 1.100433   | 2.893602   | 1.246832   | 1.407829   |
|       | 1.013874   | 2.760429   | 1.244907   | 1.300993   |
|       | 1.192766   | 2.843424   | 1.340327   | 1.327254   |
|       | 1.147104   | 2.941761   | 1.353363   | 1.287405   |
|       | 1.116288   | 2.985459   | 1.274084   | 1.254207   |
|       | 1.211556   | 2.812856   | 1.125703   | 1.235092   |
| AIE-2 | 1.911631   | 1.699986   | 1.336614   | 1.206542   |
|       | 1.952883   | 1.641435   | 1.299267   | 1.167729   |
|       | 1.89577    | 1.62924    | 1.268687   | 1.190794   |
|       | 1.96701    | 1.595404   | 1.276893   | 1.127111   |
|       | 1.802932   | 1.585535   | 1.279092   | 1.082842   |
| AIE-3 | 1.945707   | 1.595178   | 1.243127   | 1.059213   |
|       | 1.911631   | 1.699986   | 1.336614   | 1.206542   |
|       | 1.952883   | 1.641435   | 1.299267   | 1.167729   |
|       | 1.89577    | 1.62924    | 1.268687   | 1.190794   |
|       | 1.96701    | 1.595404   | 1.276893   | 1.127111   |
|       | 1.802932   | 1.585535   | 1.279092   | 1.082842   |
|       | 1.945707   | 1.595178   | 1.243127   | 1.059213   |
| AIE-4 | 1.911631   | 1.699986   | 1.336614   | 1.206542   |
|       | 1.952883   | 1.641435   | 1.299267   | 1.167729   |
|       | 1.89577    | 1.62924    | 1.268687   | 1.190794   |
|       | 1.96701    | 1.595404   | 1.276893   | 1.127111   |
|       | 1.802932   | 1.585535   | 1.279092   | 1.082842   |
|       | 1.945707   | 1.595178   | 1.243127   | 1.059213   |

**Table S1** Raw data of normalized fluorescence intensity (F/F₀) of AIE-1 to AIE-4 (40 µM) with 5 µM of five proteins.
μM from proteins with AIE-1 and AIE-2.

Furthermore, binding constant was calculated from the emission intensity-titration plot of $1/(F-F_0)$ as a function of $1/[\text{protein}]$ in micromoles. $F$ and $F_0$ are the fluorescence intensities of AIEgens with or without proteins. Four AIEgens solutions with 100 μM solution in water and proteins solution (50 and 200 μM) in PBS were prepared for fluorescence titration studies. The concentration of five proteins was varied from 0 to 20 μM. The binding constant for the AIEgens and proteins was evaluated using a Benesi-Hildebrand plot.

![Graphs](a) AIE-1 and BSA (5 μM) (b) AIE-2 and Esterase (5 μM)

**Figure S4.** Normalized fluorescence intensity of (a) AIE-1 and BSA (b) AIE-2 and esterase [proteins] = 5 μM [AIE-1] and [AIE-4] = 0 to 64 μM.

|          | BSA       | Esterase  | Transferrin | Fibrinogen | β-galactosidase |
|----------|-----------|-----------|-------------|------------|----------------|
| AIE-1    | 2.524 x 10^5 M⁻¹ | 5.003 x 10^4 M⁻¹ | 3.009 x 10^3 M⁻¹ | 6.627 x 10^4 M⁻¹ | 9.454 x 10^4 M⁻¹ |
| AIE-2    | 3.615 x 10^6 M⁻¹ | 7.292 x 10^5 M⁻¹ | -           | -          | 9.690 x 10^5 M⁻¹ |
| AIE-3    | 1.129 x 10^6 M⁻¹ | 9.738 x 10^5 M⁻¹ | -           | -          | -              |
| AIE-4    | 3.125 x 10^5 M⁻¹ | 2.001 x 10^6 M⁻¹ | 2.539 x 10^6 M⁻¹ | 2.771 x 10^6 M⁻¹ | 2.079 x 10^6 M⁻¹ |

**Table S2.** Binding constant between four AIEgens and five proteins at 25 °C
Figure S5. Benesi-Hildebrand plot (emission at 520 nm) of (a) AIE-1, (b) AIE-2, (c) AIE-3, and (d) AIE-4 by plotting $1/(F-F_0)$ as a function of $1/[\text{BSA}]$ [AIEgens] = 40 µM.

Figure S6. Benesi-Hildebrand plot (emission at 520 nm) of (a) AIE-1, (b) AIE-2, (c) AIE-3, and (d) AIE-4 by plotting $1/(F-F_0)$ as a function of $1/[\text{Esterase}]$ [AIEgens] = 40 µM.
Figure S7. Benesi-Hildebrand plot (emission at 520 nm) of (a) AIE-1 and (b) AIE-4 by plotting $1/(F-F_0)$ as a function of $1/[\text{transferrin}]$ [AIE-1 and AIE-4] = 40 µM.

Figure S8. Benesi-Hildebrand plot (emission at 520 nm) of (a) AIE-1 and (b) AIE-4 by plotting $1/(F-F_0)$ as a function of $1/[\text{Fibrinogen}]$ [AIE-1 and AIE-4] = 40 µM.

Figure S9. Benesi-Hildebrand plot (emission at 520 nm) of (a) AIE-1, (b) AIE-2, (c) AIE-4 by plotting $1/(F-F_0)$ as a function of $1/[\beta\text{-galactosidase}]$ [AIE-1, AIE-2, and AIE-4] = 40 µM.

S4. Classification proteins in the protein mixtures
**Figure S10** Array-based sensing of proteins (1 µM) in 4% FBS solution using three AIEgens (40 µM) at room temperature. (a) Fluorescence pattern of the synthesized AIEgens (AIE-1–4) in the presence of the proteins (BSA, esterase, and β-galactosidase) for subsequent fluorescence experiments. Each value is an average of four parallel measurements. (b) LDA analysis using 2D with 95% ellipse confidence

**S5. Reference**

(1) Ali, A., Kamra, M., Roy, S., Muniyappa, K., Bhattacharya, S. Novel Oligopyrrole Carboxamide based Nickel(II) and Palladium(II) Salens, Their Targeting of Human G-quadruplex DNA, and Selective Cancer Cell Toxicity. *Chem. Asian. J.* 2016, 11, 2542-2254.