Supporting Information

**Fluorogenic Ag⁺–Tetrazolate Aggregation Enables Efficient Fluorescent Biological Silver Staining**

Sheng Xie, Alex Y. H. Wong, Ryan T. K. Kwok, Ying Li, Huifang Su, Jacky W. Y. Lam, Sijie Chen,* and Ben Zhong Tang*

anie_201801653_sm_misellaneous_information.pdf
Table of Contents

General procedures ........................................................................................................... S2
Synthesis and characterization .......................................................................................... S2
Figure S1. Synthesis of TPE-4TA .................................................................................... S2
Protocol for the cytotoxicity evaluation .......................................................................... S3
Gel electrophoresis ............................................................................................................ S3
Silver nitrate stain ........................................................................................................... S4
SYPRO Ruby protein gel stain ........................................................................................ S4
Fluorescent Silver-AIE in-gel stain .................................................................................. S4
Gel image analysis ............................................................................................................ S4
NMR characterization of TPE-4TA ................................................................................... S5
Figure S2. Cell viability assay of TPE-4TA ..................................................................... S6
Figure S3. UV-vis characterization of the fluorogenic silver sensing process ............. S6
Figure S4. Size distributions of aggregates in figure 3d (a) and 3e (b) ......................... S6
Figure S5. FTIR characterization of Probe-Ag⁺ complexes, TPE-4TA and AgNO₃ .......... S7
Figure S6. TEM element mapping of the TPE-4TA & Silver complexes .................... S7
Figure S7. Fluorescent intensity of TPE-4TA with different metal ions ....................... S7
Figure S8. Interference tests with Ag⁺-binding reagents ................................................ S8
Figure S9. Comparison of the brightness of the gels under the same imaging condition S8
Figure S10. Plot of arbitrary signal intensity (volume) against the protein amount of a band S9
Figure S11. A comparison study on the signal-quantity linearity by the three stains .... S10
Table S1. DLS characterization at varied concentrations of silver ion & TPE-4TA ...... S12
Table S2. Summary of the values regarding the signal-quantity linearity ....................... S12
**General procedures.** All reagents and solvents were used as received from Energy-Chemical, Sigma-Aldrich, J&K used without further purification. Solvents and other common reagents were obtained from Sigma-Aldrich. $^1$H NMR and $^{13}$C NMR spectra were measured on a Bruker ARX 400 MHz spectrometer. Chemical shifts are reported as δ values (ppm) with CDCl$_3$ ($^1$H: δ = 7.26, $^{13}$C: δ = 77.16) or DMSO-d$_6$ ($^1$H: δ = 2.50, $^{13}$C: δ = 39.52) as the internal standard. High-resolution mass spectra (HRMS) were recorded on a GCT Premier CAB 048 mass spectrometer operating in MALDI-TOF mode. UV-vis absorption spectra were recorded on a Rarian50 Conc UV-Visible spectrophotometer. Photoluminescence (PL) spectra and absolute fluorescence quantum yield were measured on Fluorolog®-3 spectrofluorometer with an integrating sphere. Particle size measurements were performed on Malvern Zetasizer Nano ZS with a backscattering angle of 173°, using polystyrene latex (RI: 1.59, Abs: 0.010) as the parameters. Scanning electron microscopy (SEM) images were obtained on a JSM-6390 scanning electron microscope. Transmission electron microscopy (TEM) images were obtained on a JEM 2010 transmission electron microscope. The fluorescence life-time was measured using an Edinburgh FLSP920 fluorescence spectrophotometer equipped with a xenon laser arc lamp (Xe900), a microsecond flash lamp (uF900), and a picosecond pulsed diode laser (EPL-375), and a closed-cycle cryostate (CS202*1-DMX-1SS, Advanced Research Systems).

**Synthesis and characterization**

![Figure S1. Synthesis of TPE-4TA](image)

**Tetrphenylethylene (TPE).** Zinc dust (7.2 g) and dry THF (80 mL) were added into a two-necked round-bottom flask, which was vacuumed and purged with dry nitrogen for 3 times. TiCl$_4$ (6 mL) was then injected slowly into the flask over a period of 30 min. The ice-water bath was removed and the reaction mixture was refluxed for about 2 h. Benzophenone (5.0 g) was dissolved in dry THF (20 mL) and was added into the mixture slowly with a syringe. The mixture was refluxed overnight under nitrogen. After cooling to room temperature, the reaction mixture was quenched with (2 w %) HCl aqueous solution and was extracted with ethyl acetate and water (2 x 200 mL). The combined organic solution was washed with distilled water, dried with anhydrous magnesium sulfate and filtrated. The solvent was removed and the crude product was washed with ethanol and filtered to yield a white crystalline solid (3.7 g, 80 %). $^1$H NMR (CDCl$_3$, 400 MHz): δ (ppm) 7.05-7.08 (m, 8H), 7.10-7.13 (m, 12H); $^{13}$C NMR (CDCl$_3$, 100 MHz): δ (ppm) 126.6, 127.8, 131.5, 141.1, 143.9.

**Tetra(4-bromophenyl)ethylene (TPE-4Br).** In a two-necked round bottom flask, tetraphenylethylene (2 g) was dissolved with glacial acetic acid (30 mL) in an ice-bath. Bromine (5 mL) was injected into the solution with a syringe over a 10 min period followed by addition of dichloromethane (20 mL). After 15 min, the ice-water bath was removed and the resulting mixture was heated at 50 °C for about 15 min. The reaction mixture was added to 200 mL ice water, and the precipitated solid was filtered and washed repeatedly with water and ethanol until the appearance of a light yellow color. The yield of crude product was 1.65 g (43%). The product
was used directly without further purification. $^1$H NMR (CDCl$_3$, 500 MHz): $\delta$ (ppm) 7.27 (d, J = 8.5 Hz, 8H) and 6.85 (d, J = 8.5 Hz, 8H).

**Tetra(4-cyanophenyl)ethylene (TPE-4CN).** TPE-4Br (6.67 g), CuCN (5.0 g, 56 mmol), and DMF (50 mL) were added into a two-necked round bottom flask. The mixture was heated at reflux for 60 h under nitrogen condition and then suspended into 300 mL water. After ethylenediamine (10 mL) was added, the resulting mixture was stirred at 100°C for 1 h and was then filtered. The precipitated solid was extracted with dichloromethane (3 × 150 mL) and the combined organic phase was dried with anhydrous magnesium sulfate. After filtration and solvent evaporation, the residue was repeatedly purified by silica gel column chromatography with hexane and dichloromethane (v/v, 1/1) as the eluent to give TPE-4CN 3.1 g in 70% yield as a white solid. $^1$H NMR (CDCl$_3$, 500 MHz): $\delta$ (ppm) 7.48 (t, J = 5.0 Hz, 8H) and 7.08 (t, J = 5.0 Hz, 8H).

**HRMS (MALDI-TOF), m/z calcd. for C$_{30}$H$_{16}$N$_4$: 432.1375; found 432.1379.**

**TPE-4TA.** Into a 25 mL round-bottomed flask were added sodium azide (1.12 g, 16 mmol), zinc bromide (450 mg, 2 eq.) and 2 mL of water. TPE-4CN (2 mmol) was dissolved in 10 mL of N-Methylpyrrolidone and injected into the solution. The reaction mixture was refluxed for 24 h with vigorous stirring at 150°C. The mixture was acidified to pH 1 with aqueous HCl solution (3 M) and was extracted into organic later with ethyl acetate (20 mL). The organic phase was washed with 3 M HCl (2 x 10 mL) and solvent was evaporated to yield a crude product. This crude product was added into NaOH solution (0.25 M, 40 mL) and stirred vigorously until a white precipitate of zinc hydroxide was observed. The resulting suspension was filtered to remove zinc hydroxide. The filtrate was washed with ethylacetate (2 x 10 mL) and acidified to pH 1 with 3 M HCl. The tetrazole product precipitated upon stirring, which was again extracted into 20 mL ethyl acetate and the organic layer was separated. The aqueous layer was washed with ethyl acetate (2 x 20 mL). The organic layers were combined, concentrated and dried under vacuum to yield TPE-4TA (71%) as a yellowish solid. $^1$H NMR (400 MHz, DMSO): $\delta$ (ppm) 7.89 (d, 8H, J = 8.2 Hz), 7.31 (d, 8H, J = 8.2 Hz). $^{13}$C NMR (100 MHz, DMSO): $\delta$ (ppm) 155.0, 144.9, 140.9, 131.8, 126.8, 122.9. HRMS (MALDI-TOF), m/z calcd. for C$_{30}$H$_{20}$N$_{16}$Na$: 627.1949; found 627.1986 (M + Na$^+$).

**Protocol for the cytotoxicity evaluation.**

The cytotoxicity of TPE-4TA to HeLa cells was measured by a methylthiazolyldiphenyltetrazolium (MTT) assay according to the manufacturer’s Method (n = 6). For each cell line, the cells were seeded in 96-well plates (Costar, IL, USA) at a density of 6 × 10$^3$ cells/well. After 24 h culture, the medium was replaced by a series of different concentrations of materials (100 µL/well), further incubated for 24 h. The medium was then changed with 100 µL of fresh medium containing 10 µL of MTT solution in each well and incubated at 37 °C for 4 h, and the removed the MTT medium solution carefully and added 100 µL of DMSO to dissolve the purple crystals. The absorption was measured at 570 nm with a microplate reader (Perkin-Elmer Victor3™). Cell viability was expressed by the ratio of absorbance of the cells incubated with TPE-4TA to that of cells incubated with culture medium only.

**Gel electrophoresis.** PageRuler™ Unstained Protein Ladder was purchased from Thermo Fisher Scientific. The ladder contained a mixture of 14 recombinant proteins ranging from 10 kDa to 200 kDa. SYPRO™ Ruby Protein Gel Stain, NuPAGE™ 4-12% Bis-Tris Protein Gels (1.0 mm thick gels), NuPAGE™ MES SDS Running Buffer (20X) and NU PAGE™ sample reducing agent (10X) were purchased from Invitrogen. Other reagents were commercially sourced and of analytical grade. In the gel, the first lane was loaded with double the amount of stock (10 µL) followed by the normal stock amount (5 µL) and a series of two-fold dilutions of
of Millipore water, LDS buffer (4X) and NUPAGETM sample reducing agent (10X). Each stock protein had a concentration of 0.02-0.05 mg/mL in buffers (62.5 mM Tris-H3PO4, pH 7.5 at 25 °C, 1 mM EDTA, 2% SDS, 10 mM DTT, 1 mM NaN3, 0.01% bromophenol blue and 33% glycerol).

SDS-PAGE was performed with NuPAGE™ 4-12% Bis-Tris Protein Gels (1.0 mm thick gels) using the Mini Gel Tank (Invitrogen), at a constant voltage of 200 V for 30 min with the PowerEase® 300W power supply (Thermos Fisher Scientific).

**Silver nitrate stain.** The silver stain was carried out by following a reported protocol.18 After electrophoresis, the gels were first fixed for 1 h in a 40% MeOH/10% Acetic acid (HAc) solution and then subsequently rinsed in 100 mL of ultra-pure water for 3 x 10 min. The gels were impregnated with 100 mL 0.2% silver nitrate solution for 20 min in a sealed glass container and washed with 100 mL of Millipore water for 2 x 15 s before development in 100 mL 3% sodium carbonate and 0.046% formaldehyde (1.25 mL 37% formaldehyde/L). Development was terminated by repeatedly washing in 100 mL 1% HAc solution. The gels were imaged by an Azure c600 gel documentation system.

**SYPRO Ruby protein gel stain.** The Ruby stain was carried out by following the manufacturer’s protocols (Molecular Probes™, 2007). Two protocols were evaluated: 1) Basic protocol with an overnight incubation, 2) Rapid stain protocol with the assistance of microwave-assisted dye staining. Both methods gave comparable results. The basic protocol we used is described here. Fixation was performed with incubation of gels in 100 mL 50% MeOH/7% HAc solution for 2 x 15 min, agitated on an orbital shaker at 50 rpm. Subsequently, the gel was immersed in 60 mL of SYPRO Ruby gel stain and agitated on an orbital shaker overnight. Next, the gel was transferred to a new container with 100 mL of 10% MeOH/7% HAc washing solution, allowing the destaining to proceed for 30 min. After a rinse in ultra-pure water for 2 x 5 min, the gel was imaged by an Azure c600 gel documentation system at 302 nm UV channel.

**Fluorescent silver-AIE in-gel stain.** After electrophoresis, the gels were first fixed for 2 x 30 min in a 40% Ethanol/10% HAc solution on an orbital shaker at 50 rpm and then subsequently rinsed in 100 mL of ultra-pure water for 3 x 10 min. The gels were impregnated with 100 mL 0.0001% AgNO3 solution at room temperature for 1 h in a sealed glass container and then washed with 100 mL of Millipore water for 2 x 60 s. Subsequently, the gel was transferred to a new staining box and immersed in 100 mL of TPE-4TA staining solution (10 µM) stain on a shaker for 2 h (With comparable sensitivity to that of SYPRO Ruby stain) to 12 h (Long incubation time further increased the sensitivity). In the final washing step, the gel was transferred to a new container with 100 mL washing solution (10% Ethanol) for 30 min. After a quick rinse in water, the gels were imaged by an Azure c600 gel documentation system at 302 nm or 365 nm UV channel.

**Gel image analysis.** The gel images were analyzed and compared using the commercial AzureSpot software. In order to compare the three stain methods, common criteria were chosen as relevant measures of effectiveness. Lowest limit of detection (LLD) is defined as the lowest concentration of proteins that delivers a pixel volume three standard deviations greater than that of the measured background. Linear dynamic range (LDR) is determined by evaluating the strictly linear relationship (R2>0.80) between quantity and signal with optimal deviation (<5%), and measures the quantitative performance of the stains.
NMR characterization (1H, 13C) of TPE-4TA
Figure S2. Effect of different doses of TPE-4TA on the viability of Hela cells assessed by the MTT assay.

Figure S3. UV-vis characterization of the fluorogenic silver sensing process.

Figure S4. Size distributions of aggregates in figure 3d (a) and 3e (b).
Figure S5. FTIR characterization of (TPE-4TA)-Ag⁺ complexes, TPE-4TA and AgNO₃.

Figure S6. TEM element mapping of the TPE-4TA & Silver complexes.

Figure S7. Fluorescent intensity of TPE-4TA with different metal ions.

Referring to Fig. 2c. TPE-4TA (5 µM) in phosphate aqueous solution (pH 7.4) was treated without different metal ions (20 µM). Y values showed the fluorescence intensity at 504 nm (I / Iₘₙ) of the corresponding mixture, which is normalized to that of the mixture (Blank group) without the addition of metal ions.
Figure S8. Inteference tests with Ag⁺-binding reagents.

Gray bars indicate the fluorescence by mixing of TPE-4TA (5 µM) with Ag⁺-binding reagents (100 equiv.). Red bars show the fluorescence of the mixture when TPE-4TA was added to Ag⁺ solutions pre-mixed with Ag⁺-binding reagents (10 equiv.). Y values (I502 nm) are normalized against the fluorescence of TPE-4TA (I0) in phosphate aqueous solutions (pH 7.4). Blk: blank group without any additive. Cys: cysteine. Asp: aspartic acid. His: histidine. Glu: glutamic acid. Lys: lysine. A: adenosine. T: thymidine. G: guanosine. C: cytidine.

Figure S9. Comparison of the brightness of the gels stained with SYPRO Ruby stain (left) or the fluorescent silver-AIE (right) stain under the same illumination and imaging condition.
Figure S10. Plot of arbitrary signal intensity (volume) against the protein amount of a band for all the 14 proteins stained by the three methods (n=3). Gel images in the right are representative samples. Error bars are omitted for clarity.
Figure S11. A comparison study on the signal-quantity linearity (linear dynamic range, slopes) for the 14 proteins stained by the three methods (n=3).
Table S1. DLS characterization of fluorescent colloidal solutions at varied concentrations of silver ion & TPE-4TA.\(^{(a)}\)

| Entry | [Ag\(^{+}\)] (µM) | [TPE-4TA] (µM) | [Ag\(^{+}\)] / [Tetrazolate] | Eff. Size (nm) | PDI | FL Peak\(^{(b)}\) (nm) | Zeta Potential (mV) |
|-------|-----------------|----------------|-------------------------------|----------------|-----|----------------------|---------------------|
| 1     | 5               | 5              | 10:40                         | 191 ± 14       | 0.34| 504                  | - 40 ± 2            |
| 2     | 10              | 5              | 10:20                         | 63 ± 17        | 0.35| 504                  | - 33 ± 2            |
| 3     | 25              | 5              | 10:8                          | 50 ± 15        | 0.32| 504                  | - 30 ± 1            |
| 4     | 50              | 5              | 10:4                          | 107 ± 11       | 0.32| 505                  | - 17 ± 1            |
| 5     | 100             | 5              | 10:2                          | 91 ± 13        | 0.34| 504                  | - 19 ± 3            |
| 6     | 50              | 12.5           | 10:10                         | 108 ± 23       | 0.34| 504                  | - 39 ± 4            |
| 7     | 50              | 25             | 10:20                         | 192 ± 14       | 0.34| 504                  | - 49 ± 2            |
| 8     | 50              | 50             | 10:40                         | 288 ± 29       | 0.36| 502                  | - 52 ± 1            |
| 9     | 100             | 100            | 10:40                         | 267 ± 70       | 0.31| 505                  | - 49 ± 2            |
| 10    | 1000            | 100            | 10:4                          | 633 ± 181      | 0.36| 505                  | - \(^{(c)}\)         |

\(^{(a)}\)The colloidal solutions were made by adding the equal volume of silver ion solutions into the TPE-4TA solutions under strong sonication. The DLS characterization were carried out when the solutions were stable. \(^{(b)}\)Fluorescence peak. \(^{(c)}\)Not consistent.
Table S2. Summary of the parameter values regarding the signal-quantity linearity of figures S7, S8.

| MW (kDa) | Rf   | Method       | LDR       | k   | R²     |
|---------|------|--------------|-----------|-----|--------|
| Band 1  | 200  | 0.052        | Silver    | Lane 5 ~ Lane 7 | 18.19 | 0.957  |
|         |      |              | Silver-AIE| Lane 2 ~ Lane 9 | 1.55  | 0.992  |
|         |      |              | Ruby      | Lane 2 ~ Lane 9 | 1.52  | 0.975  |
| Band 2  | 150  | 0.094        | Silver    | Lane 5 ~ Lane 8 | 12.72 | 0.996  |
|         |      |              | Silver-AIE| Lane 2 ~ Lane 9 | 1.61  | 0.990  |
|         |      |              | Ruby      | Lane 2 ~ Lane 7 | 1.84  | 0.961  |
| Band 3  | 120  | 0.134        | Silver    | Lane 2 ~ Lane 7 | 1.55  | 0.826  |
|         |      |              | Silver-AIE| Lane 1 ~ Lane 9 | 1.01  | 0.942  |
|         |      |              | Ruby      | Lane 2 ~ Lane 6 | 1.68  | 0.941  |
| Band 4  | 100  | 0.176        | Silver    | Lane 2 ~ Lane 9 | 1.72  | 0.878  |
|         |      |              | Silver-AIE| Lane 1 ~ Lane 10| 1.01  | 0.975  |
|         |      |              | Ruby      | Lane 2 ~ Lane 8 | 1.63  | 0.989  |
| Band 5  | 85   | 0.200        | Silver    | Lane 4 ~ Lane 9 | 4.31  | 0.910  |
|         |      |              | Silver-AIE| Lane 2 ~ Lane 10| 1.66  | 0.981  |
|         |      |              | Ruby      | Lane 2 ~ Lane 8 | 1.78  | 0.947  |
| Band 6  | 70   | 0.264        | Silver    | Lane 3 ~ Lane 9 | 1.80  | 0.553  |
|         |      |              | Silver-AIE| Lane 1 ~ Lane 11| 1.02  | 0.945  |
|         |      |              | Ruby      | Lane 1 ~ Lane 9 | 1.00  | 0.911  |
| Band 7  | 60   | 0.296        | Silver    | Lane 4 ~ Lane 7 | 4.04  | 0.835  |
|         |      |              | Silver-AIE| Lane 1 ~ Lane 11| 1.04  | 0.909  |
|         |      |              | Ruby      | Lane 1 ~ Lane 8 | 1.00  | 0.907  |
| Band 8  | 50   | 0.373        | Silver    | Lane 6 ~ Lane 11| 14.74 | 0.819  |
|         |      |              | Silver-AIE| Lane 3 ~ Lane 13| 2.23  | 0.960  |
|         |      |              | Ruby      | Lane 3 ~ Lane 9 | 2.59  | 0.957  |
| Band 9  | 40   | 0.456        | Silver    | Lane 3 ~ Lane 9 | 3.48  | 0.882  |
|         |      |              | Silver-AIE| Lane 1 ~ Lane 9 | 1.00  | 0.966  |
|         |      |              | Ruby      | Lane 3 ~ Lane 7 | 2.49  | 0.991  |
| Band 10 | 30   | 0.531        | Silver    | Lane 4 ~ Lane 9 | 3.97  | 0.909  |
|         |      |              | Silver-AIE| Lane 1 ~ Lane 9 | 1.04  | 0.951  |
|         |      |              | Ruby      | Lane 2 ~ Lane 7 | 1.85  | 0.942  |
| Band 11 | 25  | 0.638 | Silver | Lane 3 ~ Lane 9 | 3.05 | 0.951 |
|---------|-----|-------|--------|-----------------|------|-------|
|         |     |       | Silver-AIE | Lane 1 ~ Lane 10 | 1.04 | 0.949 |
|         |     |       | Ruby | Lane 2 ~ Lane 7 | 2.12 | 0.979 |
| Band 12 | 20  | 0.699 | Silver | Lane 3 ~ Lane 9 | 2.10 | 0.970 |
|         |     |       | Silver-AIE | Lane 1 ~ Lane 10 | 1.01 | 0.991 |
|         |     |       | Ruby | Lane 2 ~ Lane 7 | 1.58 | 0.983 |
| Band 13 | 15  | 0.772 | Silver | Lane 1 ~ Lane 9 | 0.84 | 0.941 |
|         |     |       | Silver-AIE | Lane 1 ~ Lane 11 | 1.01 | 0.995 |
|         |     |       | Ruby | Lane 1 ~ Lane 8 | 1.06 | 0.941 |
| Band 14 | 10  | 0.918 | Silver | Lane 6 ~ Lane 10 | 4.89 | 0.785 |
|         |     |       | Silver-AIE | Lane 3 ~ Lane 10 | 2.00 | 0.975 |
|         |     |       | Ruby | Lane 3 ~ Lane 8 | 2.50 | 0.981 |

MW: molecular weight. Rf: retardation factor. LDR: linear dynamic range. k: slopes of the linear fitting line over the LDR. $R^2$ is a statistical measure of the linear fitted regression line over the LDR.