Supplementary Information

Supramolecular assembly of hybrid Pt(II) porphyrin/tomatine analogues with different nanostructures and cytotoxic activities

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References
1. Materials and instrumentation

All chemicals were purchased from commercial suppliers (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan; FUJIFILM Wako Pure Chemical Co., Osaka, Japan; Kanto Chemical Co., Inc., Tokyo, Japan; Sigma-Aldrich Co. LLC, St. Louis, MO, USA) and were used without further purification unless otherwise noted. Pyrogen-free deionized water (DI) was obtained using Direct-Q3-UV (Merck KGaA, Darmstadt, Germany) purification units. NMR spectra were measured with a JEOL ECA 500 NMR spectrometer. ¹H NMR spectra were measured with an ECA 500 NMR spectrometer (JEOL Ltd., Tokyo, Japan). Ultraviolet (UV)-vis, circular dichroism (CD), and fluorescence spectra were acquired with RF-2500PC (Shimazu Co., Ltd., Kyoto, Japan), J-820 (JASCO, Tokyo, Japan) and RF-5300PC (Shimazu Co., Ltd.) spectrophotometers, respectively. Emission quantum yield was determined using a C9920-02 quantum yield spectrometer (excitation: 405 nm) (Hamamatsu Photonics KK, Shizuoka, Japan), and phosphorescence lifetimes were acquired using the time-correlated single-photon counting (TCSPC) method using a FluoroCube 3000U (Horiba, Ltd., Kyoto, Japan).
2. Preparation of SAG/PtTCPP hybrids

Synthesis of Pt(II) meso-tetrakis(4-carboxyphenyl)porphyrin (PtTCPP). All solvents and reagents were obtained from commercial sources and used as received. Tetramethoxycarbonylphenylporphyrin (H₂TMCPP) was synthesized as described previously. To a refluxing mixture of meso-tetrakis(4-methoxycarbonylphenyl)porphyrin (182 mg, 0.215 mmol) in benzonitrile (25 mL), PtCl₂ (69.2 mg, 0.260 mmol) was added under a N₂ atmosphere. The resulting mixture was refluxed for about 12 h, and the reaction was stopped when the fluorescence of the free-base porphyrin (λ_max = 645 nm) disappeared. After removing benzonitrile, the residue was purified by column chromatography (silica gel, 2% THF in CH₂Cl₂, followed by 5% THF in CH₂Cl₂).

Five KOH pellets were added to a well-stirred mixture of PtTMCPP (193 mg, 0.186 mmol) in 1 mL EtOH and 100 mL THF (100 mL). The mixture was stirred at r.t. for 1 h, then the mixture was concentrated and water (ca. 10 mL) was added dropwise to dissolve the precipitate. The solution was stirred for 1 h and then THF was removed by rotary evaporation. The mixture was filtered and acidified with conc. HCl (ca. 4 mL). The precipitate was isolated by centrifugation and washed with water three times to give pure PtTCPP as a dark red-purple solid.

Yield: 99.7 mg, 51.6%

¹H NMR (500 MHz, DMSO-d₆, δ): 8.73 (s, 8H), 8.35 (d, J = 7.5 Hz, 8H), 8.26 (d, J = 7.5 Hz, 8H);

Elemental Analysis: Calcd for C₄₈H₂₈N₄O₈Pt+H₂O: C, 57.55; H, 3.02; N, 5.59. Found: C, 57.51; H, 3.03; N, 5.53.
Purification of Tomatine (1) and Dehydrotomatine (2). All steroidal alkaloid glycosides (SAGs) were purified according to previously published procedures. The steroid alkaloid glycosides were isolated from fresh leaves of tomato (*Lycopersicon esculentum*) after harvesting the fruit. A 0.01% yield (w/w wet plant body) was obtained. All SAGs were purified and verified by high-performance liquid chromatography (HPLC) and $^1$H NMR analyses, especially with/without the olefin of the B ring in the aglycone (Figure S1).

$^1$H NMR (500 MHz, Pyridine-$d_5$, $\delta$) 1, 5.51 (d, $J = 8.1$ Hz, 1H), 5.15 (d, $J = 8.0$ Hz), 5.11 (d, $J = 7.5$ Hz, 1H), 4.84 (d, $J = 7.5$ Hz, 1H), 2.91 (m, 2H, -NH-CH-), 1.10 (d, $J = 6.3$ Hz, 3H), 0.79 (s, 3H), 0.74 (d, $J = 5.7$ Hz, 3H), 0.57 (s, 3H); 2, 5.57 (d, $J = 7.4$ Hz, 1H), 5.32 (br d, $J = 3.6$ Hz, 1H, -CH=CH-), 5.19 (d, $J = 7.5$ Hz), 5.15 (d, $J = 8.0$ Hz, 1H), 4.91 (d, $J = 8.0$ Hz, 1H), 2.94, 2.89 (each m, 1H, -NH-CH-), 2.69 (br d, $J = 13.8$ Hz, 1H, -CH-CH=CH-), 2.45 (t-like, $J = 12.0$, -CH=C-CH-), 1.13 (d, $J = 6.9$ Hz, 3H), 0.88 (s, 6H), 0.80 (d, $J = 6.3$ Hz, 3H).
Figure S1. $^1$H-NMR of 1 ($\alpha$-tomatine, (a)) and 2 (dehydrotomatine, (b)). $^1$H-NMR spectra were recorded in pyridine-$d_5$ solution. The chemical shifts ($\delta$) are reported in parts per million (ppm) and $J$ values in Hz, using pyridine-$d_5$ for $^1$H-NMR (7.20 ppm) as an internal standard.
Preparation of SAG/PtTCPP hybrids. Ternary composites ([PtTCPP] = 0.025 mM. (SAG:PtTCPP = 4:1), 4 mL) were prepared by mixing and diluting solutions of SAGs 1 to 4 (0.2 mM, 2 mL) and solutions of PtTCPP (0.2 mM, 0.5 mL) with 1.8 mL DI water at room temperature. All composite solutions prepared in this manner were stable in oxygen-free DI water for one month, without the formation of oxidation and hydrolysis products and a concurrent loss of photoluminescence. The stabilization observed upon adding the SAGs indicates that aggregation of the composites following SAG addition prevents PtTCPP from reacting with oxygen and water.
3. TEM images of pure SAG and PtTCPP

Transmission electron microscopy (TEM) with energy-dispersive X-ray spectroscopy (TEM-EDX), and high-angle annular dark field scanning TEM (HAADF-STEM) were performed using a Titan Themis 200 (FEI Co., Hillsboro, OR, USA) operating at 200 kV. TEM samples were prepared by transferring the surface layers of dispersions to carbon-coated Cu grids.

Figure S2. Transmission electron micrographs of samples prepared from pure SAG 1 (a), and pure SAG 2 (b). Samples were not stained. (SAG = 0.2 mM).
4. Small-angle X-ray scattering (SAXS) measurements of SAG-Pt hybrids

SAXS measurements were carried out at BL40B2 of the synchrotron radiation facility SPring-8, Japan. The exposure time was 360 s, the wavelength was 0.1 nm, and the camera length was 1 m. The scattering data were recorded using a Pilatus detector. A quartz capillary cell (2 mm diameter; Hilgenberg GmbH (Malsfeld, Germany)) was used for all measurements. The scattering intensity \( I(q) \) was expressed as a function of the scattering vector \( q = 4\pi\sin(\theta)/\lambda \), where \( 2\theta \) is the scattering angle and \( \lambda \) is the incident wavelength.

The SAXS profile of \( 1/\text{PtTCP} \) indicated the presence of vesicular structures. Vesicles are composed of regions with four distinct average electron densities: the outer SAG, PtTCP, the inner SAG, and a water droplet. Accordingly, the profile was fitted with a four-layer spherical model expressed by the following equation:\(^6\)

\[
I(q) \propto \left[ \sum_{i=1}^{4} (\rho_i - \rho_{i+1}) V_i F_{sp}(qr_i) \right]^2
\]

\[
F_{sp}(q, r) = 3 \frac{\sin(qr) - qrcos(qr)}{(qr)^3}
\]  

(1)

Here, \( \rho_i \), \( V_i \), and \( r_i \) are the electron density, the volume, and the radius of the \( i \)-th sphere, respectively. The electron density \( \rho_{\text{sol}} \) of the water was fixed at 334.4 e\cdot\text{nm}^3. Furthermore, \( \rho_3 > \rho_2 = \rho_1 > \rho_{\text{sol}} \) was held owing to their consistent elements. The obtained parameters are shown in Table S1. The width of the \( i \)-layer \( (t_i) \) is given by \( r_i - r_{i-1} \). This nanostructure presumably formed as a result of the self-assembly of vesicular and tubular structures (formed from amphiphilic bilayers) composed of the SAG/Pt complex.

The scattering intensity for \( 2/\text{PtTCP} \) essentially obeys a power law of \( q^2 \), suggesting the presence of a network. For a network system, the SAXS intensity \( I(q) \) is calculated by the Lorentz (Ornstein-Zernike, denoted by OZ) function\(^9\):
\[ I_{\text{sol}}(q) \propto \frac{1}{1 + \zeta_q^2} \]  

(2)

where \( \zeta \) is related to the mesh size of the network (blob size). We obtained \( \zeta = 10 \text{ nm} \) for 2/PtTCPP (Table S1). The results suggest that the aggregated structure observed by TEM contains a network structure with mesh sizes of 10 nm for 2/PtTCPP.

**Figure S3.** SAXS profiles from 1/PtTCPP and 2/PtTCPP in DI water (grey dots), their theoretical curves (black lines), and schematic illustration of their profile.

**Table S1.** The best-fit parameters from SAXS profiles of 1/PtTCPP and 2/PtTCPP in DI water.

| Sample     | Model  | Best-fit parameters                  |
|------------|--------|--------------------------------------|
| 1/PtTCPP   | Vesicle| \( t_1 = 45 \text{ nm}, t_2 = t_4 = 1.5 \text{ nm}, t_3 = 0.5 \text{ nm}, \rho_2 = \rho_4 = 350 \text{ e} \cdot \text{nm}^{-3}, \rho_3 = 550 \text{ e} \cdot \text{nm}^{-3} \) |
| 2/PtTCPP   | OZ     | \( \zeta = 10 \text{ nm} \)           |
5. Dynamic light scattering (DLS) measurements of SAG-Pt hybrids

The nanostructure sizes of the SAG/PtTCPP complexes were determined by dynamic light scattering (DLS-8000HL, Otsuka Electronics Co., Ltd., Tokyo, Japan, equipped with a 10 mW He–Ne laser operating at 632.8 nm). The size distributions of the nanostructures in water were analyzed (Figure S4) by acquiring data for 1/PtTCPP and 2/PtTCPP solutions at 25 °C. The results show at least two peaks in the volume-based mean nanostructure size distributions, at 200-500 nm and at 5 μm or more, consistent with the TEM images (Figure 2). In addition, the intensities of the peaks changed from smaller size (200-500 nm) and larger size (5 μm or more) between pH 4 to 10. PtTCPP complexes therefore interacted electrostatically with the cationic nitrogen units of the SAG molecules, promoting aggregation of the PtTCPP ions.

![Figure S4](image-url)

**Figure S4.** Dynamic light scattering for 1/PtTCPP (a) and 2/PtTCPP (b) in DI water. [PtTCPP] = 0.025 mM. (SAG:PtTCPP = 4:1).
6. Luminescence spectra of 1/PtTCPP and stoichiometric ratio of the hybrid solution

The effect of varying the stoichiometric ratio of the hybrid solution was investigated by adding SAG 1 to a 0.025 mM PtTCPP solution in DI water (Supplementary Figure S5). The addition of SAG 1 (1 to 4 molar equivalents of 1) to PtTCPP increased the emission intensity at 660 nm (ex. 405 nm). Further addition of 1 (4 to 40 molar equivalents) led to a gradual increase in the emission intensity at 669 nm (Figure 3) along with a slight blue-shift of the emission band to 655 nm (Figure 3). The luminescence band resulting from intermolecular interactions can be attributed to self-assembly of PtTCPPs resulting from J-aggregates. The red-shifted emission band at 669 nm (upon adding SAG 1) indicates longer polynuclear PtTCPP complexes than were present in the initial solution of the complex, whereas the blue-shift (at 4 to 40 molar equivalents of SAG) suggests a decrease in the content of aggregate species due to disordered intermolecular interactions (Figure S5).

**Figure S5.** (a) Variations in the luminescence intensity at 669 nm for 1/PtTCPP with changes in the 1:PtTCPP molar ratio in DI water. (b) Variations in the luminescence wavelength for 1/PtTCPP with changes in the SAG:PtTCPP molar ratio in DI water. PtTCPP = 0.025 mM.
7. Cancer cell viability against SAGs

Cells were seeded in 96-well plates at a density of $3.0 \times 10^4$ cells/well. After growing overnight, the cells were incubated with SAGs for 24 h. The study was conducted with the same SAG concentration range as used in Fig 4, i.e., $[\text{SAGs}] = 0 – 20 \mu\text{M}$. The cells were washed with PBS and the cell viability was detected using PrestoBlue reagent according to the manufacturer’s protocol. The viability of the cells was calculated as a ratio (%) compared with cells that were not treated with the samples.

**Figure S6.** Cell toxicity of SAG1 and SAG2 towards A549 cells (a) and HeLa cells (b).

Each point represents the mean ± S.E. of 4 experiments.
8. Near-infrared luminescence spectra showing $^1$O$_2$ generation by SAGs/PtTCPP

$^1$O$_2$ generation by the hybrid solutions was investigated by adding SAG 1 to a 0.025 mM PtTCPP solution in DI water, which increased the emission intensity at 1273 nm (ex. 405 nm) (Figure S7a). The luminescence band resulting from near-infrared wavelengths can be attributed to energy transfer from PtTCPP to $^1$O$_2$.\textsuperscript{10-12} Excitation spectra at 1273 nm (upon adding SAG 1) shows that PtTCPP complexes affected $^1$O$_2$ generation in hybrid solutions of the complex (Figure S7b).

**Figure S7.** Near-infrared luminescence spectra (a) and excitation spectra (b) of 1/PtTCPP, 2/PtTCPP, and pure PtTCPP in DI water. [PtTCPP] = 0.02 mM. (SAG:PtTCPP = 4:1). Excitation wavelength = 405 nm and emission wavelength = 1273 nm.
References

(1) Vinogradov, S. A.; Lo, L. -W.; Wilson, D. F. Dendritic Polyglutamic Porphyrins: Probing Porphyrin Protection by Oxygen-Dependent Quenching of Phosphorescence. *Chem. Eur. J.* **1999**, *5*, 1338–1347.

(2) Briñas, R. P.; Troxler, T.; Hochstrasser, R. M.; Vinogradov, S. A. Phosphorescent Oxygen Sensor with Dendritic Protection and Two-Photon Absorbing Antenna. *J. Am. Chem. Soc.* **2005**, *127*, 11851–11862

(3) Toohara, S.; Tanaka, Y.; Sakurai, S.; Ikeda, T.; Tanaka, K.; Gon, M.; Chujo, Y.; Kuroiwa. K. Self-assembly of [Au(CN)₂⁻] Complexes with Tomato (*Solanum lycopersicum*) Steroidal Alkaloid Glycosides to Form Sheet or Tubular Structures. *Chem. Lett.* **2018**, *47*, 1010–1013.

(4) Ikeda, T.; Yamauchi, K.; Nakano, D.; Nakanishi, K.; Miyashita, H.; Ito, S.; Nohara, T. Chemical Trans-glycosylation of Bioactive Glycolinkage: Synthesis of an α-Lycotetraosyl Cholesterol. *Tetrahedron Lett.* **2006**, *47*, 4355–4359.

(5) Ikeda, T.; Tsumagari, H.; Honbu, T.; Nohara, T. Cytotoxic Activity of Steroidal Glycosides from Solanum Plants. *Biol. Pharm. Bull.* **2003**, *26*, 1198–1201.

(6) Sakuragi, M.; Zushi, T.; Seguchi, R.; Arai, T.; Taguchi, K.; Kusakabe, K.; Locational Analysis of Glutathione in Liposomes by Using Small-angle X-ray Scattering. *Chem. Lett.* **2017**, *46*, 185–187.

(7) Fujii, S.; Sakuragi, M.; Sakurai, K. Characterizing PEG Chains Tethered onto Micelles and Liposomes Applied as Drug Delivery Vehicles Using Scattering Techniques. In *Control of Amphiphile Self-Assembling at the Molecular Level: Supra-Molecular*
Assemblies with Tuned Physicochemical Properties for Delivery Applications, Vol. 1271, 115–129 (American Chemical Society, 2017).

(8) Hirai, M.; Iwase, H.; Hayakawa, T.; Koizumi, M.; Takahashi, H. Determination of Asymmetric Structure of Ganglioside-DPPC Mixed Vesicle Using SANS, SAXS, and DLS. *Biophys. J.* 2003, 85, 1600–1610.

(9) Shibayama, M. Structure-mechanical Property Relationship of Tough Hydrogels. *Soft Matter* 2012, 8, 8030–8038.

(10) Amao, Y.; Asai, K.; Okura, I. Photoluminescent Oxygen Sensing Using Palladium Tetrakis(4-carboxyphenyl)porphyrin Self-assembled Membrane on Alumina. *Anal Commun.* 1999, 36, 179–180.

(11) Amao, Y.; Okura, I. An Oxygen Sensing System Based on the Phosphorescence Quenching of Metalloporphyrin Thin Film on Allumina Plates. *Analyst* 2000, 125, 1601–1604.

(12) Lo, L. -W.; Koch, C. J.; Wilson, D. F. Calibration of Oxygen Dependent Quenching of the Phosphorescence of Pd-meso-tetra (4-carboxyphenyl)porphine: a Phosphor with General Application for Measuring Oxygen Concentration in Biological Systems. *Anal. Biochem.* 1996, 236, 153–160.