Supporting Information

Confined Chromophores in Tobacco Mosaic Virus for the Mimic of Green Fluorescent Protein

Quan Zhou,ab Fengchi Wu,ab Man Wu,a Ye Tiana and Zhongwei Niu*a

a Key Laboratory of Photochemical Conversion and Optoelectronic Materials, Technical Institute of Physics and Chemistry, Chinese Academy of Sciences, Beijing 100190, China. E-mail: niu@mail.ipc.ac.cn
b University of Chinese Academy of Sciences, Beijing 100049, China.

General materials and methods

N-acetylglycine and N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC·HCl) were purchased from Aladdin Industrial Inc. 1-Hydroxybenzotriazole (HOBt) was obtained from J&K Chemical, Ltd. 4-hydroxybenzaldehyde was bought from Acros Organics. Other chemical reagents were analytical grade and purchased from Beijing Chemical Works. All the reagents were used without further purification.

TMV was isolated and purified according to the method reported previously.1 UV-Vis spectra were acquired on Hitachi UV-3900 spectrometer. Fluorescence emission spectra were obtained on Hitachi F-4600 spectrophotometer. TEM measurements were carried out by JEOL JEM-2100 transmission electron microscope at 200 KV accelerating voltage. NMR spectra were recorded on Bruker Avance 400 instrument. MALDI-TOF MS analysis was performed by AB Sciex 5800 MALDI-TOF/TOF mass spectrometer.

Synthesis of HBI-en chromophore
(Z)-4-acetoxybenzylidene-2-methyloxazol-5(4H)-one (1)

This compound was synthesized using a reported protocol. N-acetylglycine (9.597 g, 82 mmol), 4-hydroxybenzaldehyde (10.002 g, 82 mmol) and Sodium acetate anhydrous (6.726 g, 8 mmol) were dissolved in acetic anhydride (50 ml). The mixture was refluxed for 4 h at 100 °C and cooled to room temperature before 240 ml of ice water was added to produce a red precipitate. The precipitate was collected by suction filtration, washed by ethanol twice and dried under vacuum to yield a yellow powder (15.079 g, 75 %). \(^1\)H-NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) (ppm): 8.24(d, 2H), 7.29(t, 2H), 3.31(s, 1H), 2.40(s, 3H), 2.30(s, 3H).

(Z)-2-(Acetylamino)-N-(2-aminoethyl)-3-(4-hydroxyphenyl) acrylamide (2)

Compound 1 (4 g, 16.273 mmol) was dissolved in absolute ethanol (100 ml), then added ethylenediamine (19.06 ml, 0.285 mol) resulting in a red solution. After stirring for 30 min at room temperature, the mixture was evaporated in vacuo at 30 °C to get red oil with minor impurities (2.896 g, 67.6 %). Another method is that the product is no need to separate and the mixture could proceed directly to the next reaction. \(^1\)H-NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) (ppm): 9.79(s, 1H), 7.75(d, 2H), 7.39(d, 2H), 6.94(d, 2H), 6.76(t, 1H), 3.86(s, NH\(\_2\)), 3.25(m, 2H), 2.35(m, 2H), 2.02 (s, 3H).

(Z)-1-(2-Aminoethyl)-4-(4-hydroxybenzylidene)-2-methyl-imidazol-5(4H)-one (3)

Compound 2 (2 g, 7.60 mmol) was dissolved in absolute ethanol (100 ml), added molecular sieves (4 Å, 4 g) and refluxed overnight. The mixture was filtered and evaporated in vacuo. After that the product was purified by silica gel chromatography (EtOH/\(\text{CH}_2\text{Cl}_2\), \(\nu/\nu=1/5\)) to get a yellow powder (1.025 g, 55%).
Hydrochloride (HBI-en)

Compound 3 (1.0 g, 4.08 mmol) was dissolved in ethanol (10 ml), added 6 M HCl until ca. pH 2 and precipitated by Et₂O (90 ml). After filtration, the precipitate was washed with Et₂O and dried under vacuum to yield yellow powder with trace ethanol (1.14 g, ca. 100 %). ¹H-NMR (400 MHz, D₂O) δ (ppm): 7.84(d, 2H), 7.18(s, 1H), 6.92(d, 2H), 3.98(t, 2H), 3.27(t, 2H), 2.42(s, 3H). ¹³C-NMR (DMSO-d₆) δ = 167.37, 163.87, 161.06, 134.65, 129.04, 127.52, 123.62, 116.24, 38.54, 37.05, 14.74.

Fig. S1 ¹H-NMR of 1
Fig. S2 $^1$H-NMR of 2

Fig. S3 $^1$H-NMR of HBI-en
Preparation of TMV-HBI conjugates

TMV (10 mg, 0.57 \( \mu \)mol) was added in 5 ml HEPES buffer (100 mM, pH 7.4) to get the TMV stock solution (2 mg·ml\(^{-1}\)). Then HOBt (11.55 mg, 85.5 \( \mu \)mol), EDC (4.37 mg, 22.8 \( \mu \)mol) and HBI-en (10.48 mg, 42.75 \( \mu \)mol) were added successively to the TMV stock solution. Additional EDC (4.37 mg, 22.8 \( \mu \)mol) was added twice in 6 h and 12 h, and the mixture was stirred at room temperature for 18 h. After that, the mixture was centrifuged at 9,500 rpm for 10 min and the supernatant was dialyzed against water 6 times in 4 \( ^\circ \)C for 48 h to remove small molecule reagents and denatured proteins.

We hypothesize that the UV-Vis absorbance of TMV-HBI at 370 nm is the linear superposition of the absorbance of HBI-en and TMV at 370 nm in Fig. 1A (i.e. \( A_{\text{TMV-HBI at 370 nm}} = A_{\text{TMV at 370 nm}} + A_{\text{HBI-en at 370 nm}} \)). Fig. S6 showed that the slope of calibration curve for HBI-en chromophore was 0.07956 A·\( \mu \)g\(^{-1}\)·ml, and the square of the standard deviation was 0.999978. The Beer–Lambert law’s equation is:

\[
A_{\text{HBI-en at 370 nm}} = 0.079559 \times C_{\text{HBI-en}}
\]
C_{HBI-en} = \frac{A_{TMV-HBI} \text{ at } 370 \text{ nm} - A_{TMV} \text{ at } 370 \text{ nm}}{0.079559} \\
= \frac{0.266-0.041}{0.079559} = 2.8281 \mu g \cdot ml^{-1} \\
C_{TMVCP} = \frac{A_{TMV-HBI} \text{ at } 260 \text{ nm}}{3} = 0.714/3 = 0.238 \text{ mg} \cdot ml^{-1} \\
Molecule \ Ratio_{HBI-en vs TMVCP} = \frac{C_{HBI-en}}{M_{HBI-en}} / \frac{C_{TMVCP}}{M_{TMVCP}} \\
= \frac{2.8281 \cdot 10^{-6}}{244.27} / \frac{2.38 \cdot 10^{-4}}{17515} = 0.8520 \\
Molecule \ Ratio_{HBI-en vs Disk} = \text{Mol ratio}_{HBI-en vs TMVCP} \times 16.33 = 13.91 \\
Molecule \ Ratio_{HBI-en vs TMV} = \text{Mol ratio}_{HBI-en vs TMVCP} \times 2130 = 1814.76 \\
So \ the \ grafting \ efficiency \ of \ TMV \ interior \ surface \ modification \ is \ 85.20\%. \ The \ results \ indicate \ that \ 0.852 \ HBI-en \ chromophore \ is \ grafted \ to \ one \ capsid \ protein, \ 13.91 \ HBI-en \ chromophores \ per \ helical \ layer \ or \ 1814.76 \ HBI-en \ chromophores \ per \ TMV \ nanoparticle.

V_{TMV \ inner \ channel} = \pi r_{TMV}^2 L_{TMV} = 3.142 \times (2 \text{ nm} \times 2 \text{ nm}) \times 300 \text{ nm} = 3769.91 \text{ nm}^3 \\
\rho_{HBI-en} = \frac{N_{HBI-en}}{V_{TMV \ inner \ channel}} = 0.4814 \text{ molecule per nm}^3 \\
V_{GFP} = \pi R_{GFP}^2 L_{GFP} = 3.142 \times (1.2 \text{ nm} \times 1.2 \text{ nm}) \times 4.2 \text{ nm} = 19.0004 \text{ nm}^3 \\
\rho_{HBI} = \frac{N_{HBI}}{V_{GFP}} = \frac{1}{19.0004} = 0.0526 \text{ molecule per nm}^3 \\
So \ the \ grafting \ density \ of \ HBI-en \ chromophore \ in \ TMV \ inner \ channel \ is \ 0.4814 \ molecules \ per \ nm^3 \ or \ 2.0774 \text{ nm}^2 \text{ per \ molecule}. \ The \ grafting \ density \ of \ HBI \ chromophore \ in \ GFP \ is \ 0.0526 \ molecules \ per \ nm^3 \text{ or } 19.0004 \text{ nm}^3 \text{ per \ molecule}.

**Fig. S5** The calibration curve of HBI-en chromophore with the concentration rang is 1-20 $\mu g \cdot ml^{-1}$. 
Fig. S6 MALDI-TOF MS of the capsid protein of TMV (m/z 17515.41) and TMV-HBI (m/z 17742.82). The mass discrepancy between TMV and TMV-HBI (m/z 227.41) is consistent with the theoretical molar mass of HBI-en chromophore (m/z 227.11) after bioconjugation reaction. The mass peak (m/z 17672.10) was attributed to the side reaction of TMV-EDC conjugates.

**Fluorescence spectrum measurement**

The samples for fluorescence measurement were diluted TMV-HBI stock solution (2 mg·ml⁻¹, 0.5 ml) to 1.0 ml with H₂O and various organic solvents, *i.e.* for final 1.0 mg·ml⁻¹ working solution, TMV-HBI in 100% water was added 0.5 ml H₂O to 0.5 ml TMV-HBI stock solution, TMV-HBI in 20% DMSO was added 0.3 ml H₂O and 0.2 ml DMSO, TMV-HBI in 50% DMSO was added 0.5 ml DMSO.

The spectrophotometer in emission mode was collected the wavelength from 385 nm to 600 nm at excitation of 365 nm. The scan speed was 1200 nm·min⁻¹ and the slit of excitation and emission was 10 nm.

**Disassembly of TMV-HBI**

Similar to the denatured GFP, TMV can be denatured at low pH value. As a result, the HBI conjugated TMV was treated by double volume of acetic acid to be denatured into the HBI conjugated TMV coat proteins. TMV-HBI in neutral pH value was prepared by adding 0.5 ml TMV-HBI stock solution (2 mg ml⁻¹) into 0.5 ml 100 mM phosphate buffer solution at pH 7.39. TMV-HBI in harsh pH values were prepared by
adding 0.5 ml TMV-HBI stock solution into 0.5 ml 1 M HAc buffer at pH 2.30 or into 1 M Tris buffer at pH 9.56 for 30 min before fluorescence measurement.

![Fig. S7](image)

**Fig. S7** Fluorescence emission spectra of HBI-en chromophore, TMV, TMV-HBI in 1 M HAc (pH 2.30), 100 mM phosphate buffer solution (pH 7.39) and 1 M Tris (pH 9.56) buffer solution, respectively.

**Dependence of fluorescence emission on coupling efficiency**

TMV (2 mg, 0.114 μmol) was added in 2 ml HEPES buffer (100 mM, pH 7.4), then HOBt (2.3 mg, 17.1 μmol), EDC (1.98 mg, 10.3 μmol) and HBI-en (0.5, 1, 5, 25, 75 and 150 equiv., 0.057-17.1 μmol) were added to the solution. The solutions were stirred at room temperature for 18 h. After that, the solutions were centrifuged at 9,500 rpm for 10 min and the supernatants were dialyzed against water 6 times in 4 °C for 48 h.

![A](image)

![B](image)

**Fig. S8** (A) Dependence of the coupling number of HBI-en attached per virus on HBI-en equivalent in reaction. (B) Dependence of the fluorescence emission on the coupling number of HBI-en attached per virus.
References