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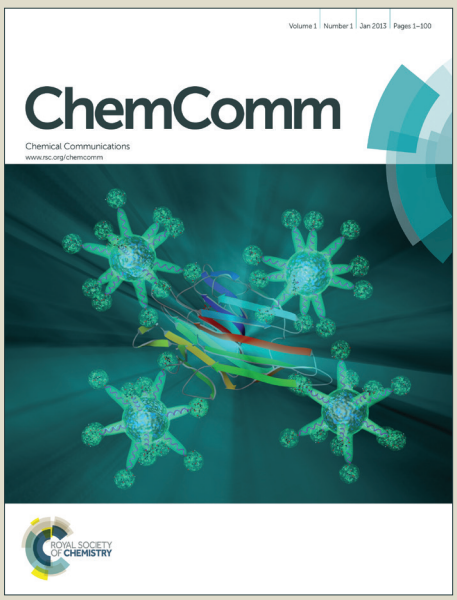

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Confined Chromophores in Tobacco Mosaic Virus for the Mimic of Green Fluorescent Protein†

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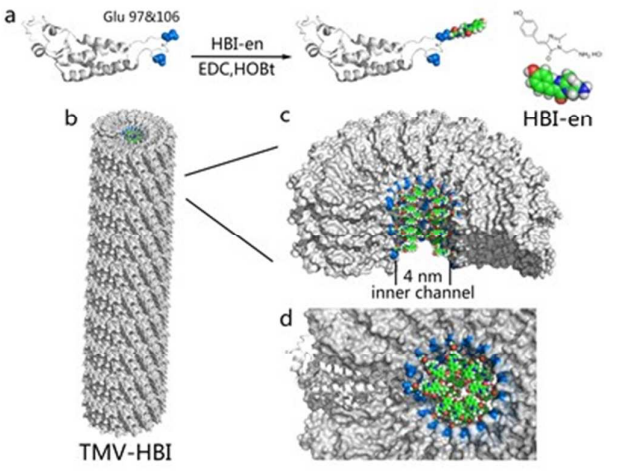
Due to nano confinement effect, grafting green fluorescent protein like chromophores in the interior surface of tobacco mosaic virus can greatly enhance its fluorescence emission in various solvents.

Green fluorescent protein (GFP) is widely used as a biomarker in genetic and molecular biology, enlightening a new way to study the function of proteins inside living system.[1] The chromophore in GFP is 4-hydroxybenzylidene imidazolinone (HBI),[2] formed via a post-translational autocatalytic cyclization and autoxidation,[3] surrounded by 11-stranded β-barrel structure.[4] The fluorescent properties of HBI are closely related to the surrounding environment and molecular conformation.[5] The β-barrel structure of GFP offers a hydrophobic environment and restricts intrarmolecular vibration to enhance the fluorescence emission of HBI.[6] The unconfined chromophore is almost non-fluorescence emission by partly destroying the β-barrel structure in denatured GFP,[7] and the fluorescence emission can be restored by recovering the β-barrel structure,[8] which is suggested that the confinement effect of protein-chromophore interactions is very important for the fluorescence emission pathway of energy release.[9] This confined emission property of GFP has always been an object for mimicking. As a famous RNA mimic of GFP, the fluorogenic aptamer Spinach[10] has immobilized GFP-like chromophore in a G-quadruplex binding site by restricting the small molecule to planar conformation.[11] Polymer scaffold[12] and supramolecular host-guest system[13] can also supply a hydrophobic environment for blocking molecular vibration of GFP-like chromophores. Even the rigid porous scaffold of metal-organic frameworks[14] could be utilized to mimic the GFP β-barrel behavior by coordinatively trapping GFP-like chromophore in confined nano pore structures. For artificial GFP β-barrel mimics, nanoparticles, especially viral nanoparticles (VNPs) based on protein assembly, raise the possibility of structure-driven design of fluorescent materials by modulating nano pore aperture and confined microenvironment.

Tobacco mosaic virus (TMV) is a classic rod-like plant virus, which consists of 2130 capsid proteins stacked in helix around 6395 nucleotides single-strand RNA genome, constituted monodisperse nanotube with 300 nm long, 18 nm in diameter and 4 nm diameter of inner channel.[15] The well-defined inner channel structure of TMV may provide an ideal confined space for mimicking the β-barrel structure of GFP.

Herein, we demonstrated a simple method to mimic the confinement effect of GFP β-barrel structure by grafting GFP-like chromophores in the inner channel of TMV (Scheme 1). HBI-ethylamine (HBI-en) was synthesized as GFP-like chromophore by following literature protocols (Scheme S1, see



Scheme 1 (a) Coupling reaction of HBI-en chromophore and TMV capsid protein with glutamate residues 97&106 emphasized (blue). (b-d) Structural illustration of TMV-HBI conjugates in side view (b), cross section view (c) and close-up top view (d). Images generated by the PyMOL Molecular Graphics System, Version 1.7, Schrödinger, LLC.

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ESI†).[16] The structure of HBI-en was characterized by nuclear magnetic resonance spectrum (NMR) (Fig. S1-S4, see ESI† for more experimental details). The interior surface of TMV is covered with glutamate residues 97&106 (Glu 97&106), which are easy to be conjugated with the amino group of HBI-en activated by N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and 1-hydroxybenzotriazole (HOBt) (Scheme 1a and ESI†).[17,18] GFP β-barrel has a diameter of 2.4 nm with a height of 4.2 nm, and HBI chromophore is located in the centre of β-barrel.[19] Similar to GFP, TMV inner channel is 4 nm in diameter with 300 nm in length, and HBI-en chromophores are immobilized inside the inner channel, which can simulate the essential character of GFP β-barrel structure in geometry.

The conjugation of HBI-en chromophores in the inner channel of TMV was confirmed by UV-Vis spectra. As shown in Fig. 1A, a new absorption band at 366 nm[20] indicated that HBI-en was successfully conjugated onto TMV. From UV-Vis data, the grafting efficiency of TMV interior surface modification is 85% according to a reported method[21] (Fig. S5 see ESI† for more details). The conjugation was also identified by MALDI-TOF MS (Fig. S6, see ESI†). After conjugation, TMV-HBI still maintains its original structure (Fig. 1C). The photographs of TMV-HBI solution were shown in quartz cuvette under daylight (Fig. 1D) and UV lamp at λ_{ex}=365 nm (Fig. 1E). The fluorescence intensity at 447 nm of TMV-HBI (Fig. 1B) was significantly enhanced comparing to TMV and HBI-en chromophore background. PH value is a key factor in TMV assembly & disassembly process.[22] In harsh pH environment at 2.30 or 9.56, TMV was disassembled. As a result, without nano confinement effect, the fluorescence emission of TMV-HBI was dramatically decreased (Fig. S7, see ESI† for more details).

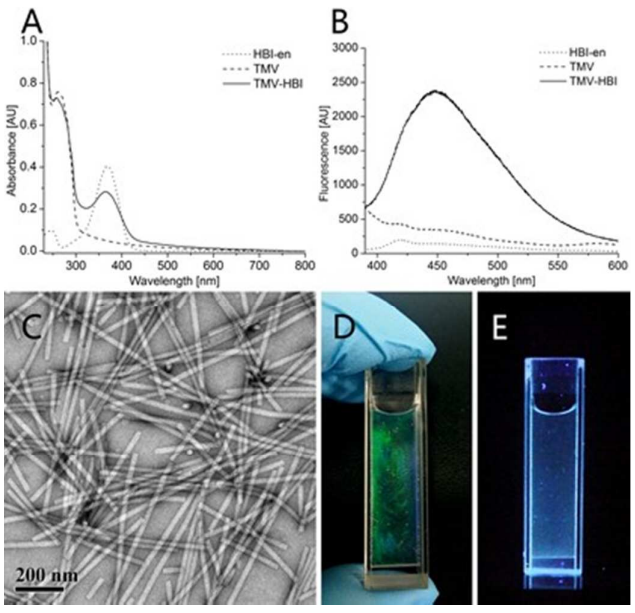

Fig. 1 (A) UV-Vis spectra of HBI-en (5 μg·ml^{-1}), TMV (0.242 mg·ml^{-1}) and TMV-HBI (0.238 mg·ml^{-1}). (B) Fluorescence emission spectra of HBI-en, TMV and TMV-HBI at λ_{ex}=365 nm. (C) TEM image of TMV-HBI after uranyl acetate stained. The photographs of TMV-HBI solution (1.0 mg·ml^{-1}) under daylight (D) and UV lamp at λ_{ex}=365 nm (E).

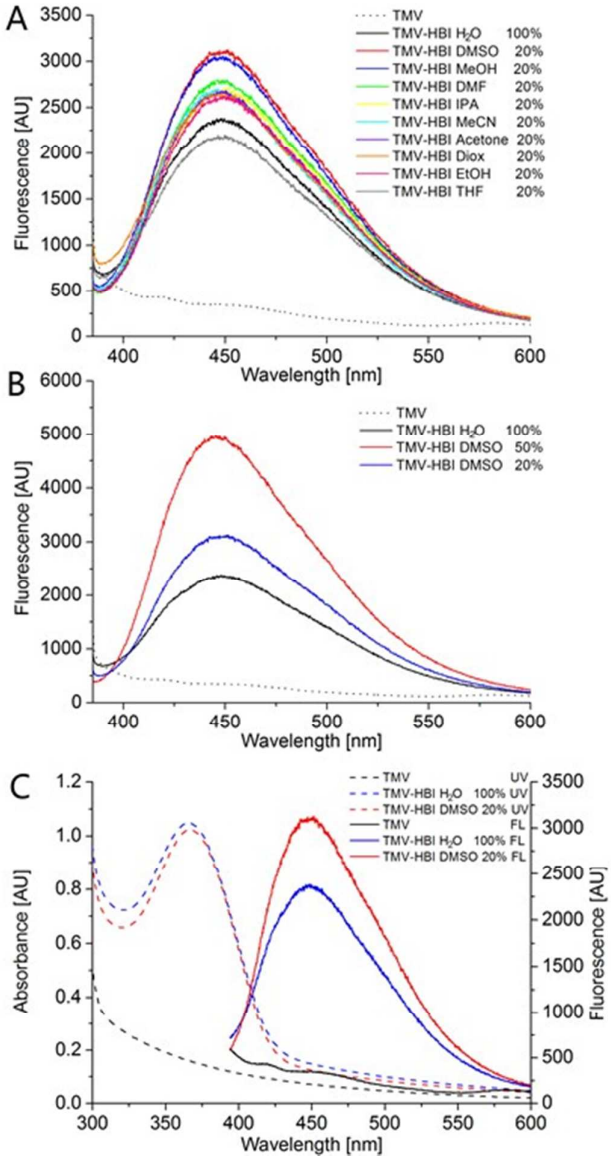

Fig. 2 (A) Fluorescence emission spectra of TMV-HBI in 100% water and various 20% organic mixed aqueous solution at λ_{ex}=365 nm, *i.e.* dimethyl sulfoxide (DMSO), methanol (MeOH), N, N'-dimethyl formamide (DMF), 2-propanol (IPA), acetonitrile (MeCN), acetone, 1,4-dioxane (Diox), ethanol (EtOH) and tetrahydrofuran (THF). (B) Fluorescence emission spectra of TMV-HBI in 100% water, 20% and 50% DMSO aqueous solution. (C) The bathochromic shift between UV-Vis absorbance and fluorescence emission spectra of TMV-HBI.

Excited state proton transfer (ESPT) is one of the basic ways of proton transfer in photochemical transformation and biological process.[23] The ESPT process of GFP-like chromophores in various solvents is obviously influenced by intermolecular hydrogen bond.[24] We have succeeded in further enhancing the fluorescence emission of TMV-HBI by adding different organic solvents into aqueous solution (Fig. 2A). The fluorescence enhancement was consistent with the increase of dimethyl sulfoxide (DMSO) proportion in the aqueous solution. As depicted in Fig. 2B, the fluorescence emission of TMV-HBI in 50% DMSO aqueous solution was

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increased 2.09 fold comparing with water. This phenomenon may come from the aprotic property of DMSO. DMSO can strongly attract the phenolic proton and easily stabilizes the anionic phenolate form of HBI-en chromophore in excited state, resulting in enhanced fluorescence emission. A bathochromic shift between UV-Vis absorbance and fluorescence emission spectra of TMV-HBI compared to TMV was clear visualized (Fig. 2C). The observed displacement of spectral line is red-shifted by 81 nm, which can be attributed to the ESPT process of TMV-HBI and the extended structure of HBI-en chromophore.

In summary, we have successfully demonstrated that TMV inner channel structure can be used to simulate GFP β-barrel behavior by nano confinement effect with grafting HBI-en chromophore on TMV interior surface. The observed fluorescence enhancement of HBI-en in the inner channel of TMV with high grafting density indicated that the molecular vibration of HBI-en chromophore was efficiently confined. The well-defined TMV-HBI conjugates promote the understanding of protein-chromophore interactions in confined nano tubular space of protein assembly and expand the application of TMV in protein-based fluorescent materials for future bioimaging.

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