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Water-soluble doubly N-confused hexaphyrin: A near-IR fluorescent Zn(II) ion sensor in water

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A water-soluble doubly N-confused hexaphyrin (N₂CH) having two octa-arginine peptide arms displays an enhanced near-infrared (NIR) emission around 1050 nm in the presence of Zn²⁺ in aqueous solution.

In the biomedical/bioanalytical fields, NIR-light technology has been of growing importance since the interference by biomolecules could be minimized in the NIR region.¹ Among a variety of targets for NIR-biosensing, Zn²⁺ ion gathers wide attention because of its importance as one of the most essential metal ions in the human body and the interest for the neurochemical functions.² Previously, in a series of studies on N-confused porphyrinoids,³ we have shown doubly N-confused hexaphyrin (N₂CH, **1**, Chart 1), a kind of expanded porphyrin, forms bis-metal complexes with various divalent and trivalent metal ions such as Cu²⁺, Ni²⁺, Zn²⁺, Mn³⁺, and Fe³⁺, and displays an intensified near-infrared (NIR) emission with $\lambda_{\text{em}} > 1000$ nm in CH₂Cl₂ when Zn²⁺ ions are coordinated.⁴ Since then, we have been interested in synthesizing a water-soluble derivative, which serves as a NIR fluorescent sensor molecule, especially, for Zn²⁺ in aqueous media. Herein, we report the synthesis of a water-soluble derivative of N₂CH possessing two highly hydrophilic octa-arginine peptides (N₂CH-R8, **4**) and its emission behaviour in the presence of various metal ions in aqueous media. A largely enhanced NIR emission around 1050 nm in water by Zn²⁺ coordination was demonstrated for the first time.

Synthesis of N₂CH-R8 was performed by conjugation of two peptides and N₂CH via a Cu(I)-catalyzed "click reaction" between azide groups and terminal alkynes (Scheme 1),⁵ which are installed in the peptide and N₂CH, respectively.⁶ At first, N₂CH derivative possessing two ethynylaryl groups (**3**), which was derived from *meso*-aryl N₂CH having two 2,3,5,6-tetrafluoro-4-iodophenyl groups, was synthesized through Sonogashira coupling reactions with Pd(PPh₃)₂Cl₂ and CuI in 49% yield.⁷ The counterpart, octa-arginine (R8) peptide derivative (**5**), in which the arginine side-chains were protected with 2,2,4,4,6,7-pentamethyldihydro-benzofuran-5-sulfonyl (Pbf) groups and its N-terminus bears an azide

Scheme 1 Synthesis of N₂CH-R8 via conjugation of N₂CH (**3**) and protected octa-arginine peptide (**5**) by a Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition.

moiety, was prepared by the solid-phase synthesis, which started from fluorene-9-ylmethoxycarbonyl (Fmoc) protected aminoxanthene-3-yloxy-polystyrene resin (Sieber Amide resin).^{6,7} Then, N₂CH derivative **3** was conjugated with peptide **5** by Cu(I)-catalyzed Huisgen 1,3-dipolar cycloadditions between the terminal alkynes in **3** and the azide moiety at the N-terminus of **5** (Scheme 1).⁷ The reaction mixture was treated with trifluoroacetic acid (TFA) to remove Pbf groups and Cu²⁺ ions from the arginine side-chains and the macrocycle, affording the desired freebase N₂CH-peptide

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conjugate, **N₂CH-R8**. The resulting conjugate was purified by a reverse phase HPLC with CH₃CN/H₂O and obtained as a TFA salt (46% yield based on **3**). By partition experiments between ultrapure water and CH₂Cl₂, **N₂CH-R8** was selectively extracted to the aqueous phase.⁸

At first, we examined Zn²⁺ complexation of **N₂CH 1** in organic solvent by spectroscopic methods. In CH₂Cl₂, UV-vis-NIR spectrum of **1** with excess Zn(OAc)₂ (ca. 500 equiv) was nearly identical to that of isolated **2**.⁶ For 4.0 μM of **1**, completion of the spectral changes required 200 μM (50 equiv) of Zn(OAc)₂. The observation of isosbestic points at 591, 652, 764 and 830 nm suggests the coordination of second Zn²⁺ is rather fast to afford bis-Zn²⁺ complex, spontaneously.⁶ NIR-fluorescence spectra of **1**, **2**, and **1** treated *in situ* with excess Zn(OAc)₂ were compared at ambient temperature. Upon adding Zn(OAc)₂, fluorescence of **1** was enhanced approximately 10-fold,⁶ exhibiting a sharp emission band around 1047 nm, which was nearly identical to that of **2**. This result indicates that *in situ* formation of bis-Zn²⁺ complex of **1** proceeds almost quantitatively in CH₂Cl₂ at ambient temperature.

The structure of bis-Zn²⁺ complex **2** was revealed by X-ray crystallography (Fig. 1).[‡] The complex shows a slightly distorted structure with a mean plane deviation of 0.0798 Å defined by 36 heavy atoms without oxygen in the macrocycle. The displacement of Zn²⁺ ion is 0.0572 Å and the Zn-Zn distance is 4.806 Å. One H₂O molecule is coordinated at the axial position of each Zn atom with a Zn-O distance of 2.038 Å. The water molecule might come from the adventitious moisture in the solvent and such coordination would be usual for a water-soluble derivative of **2** in aqueous media.

Fig. 1 ORTEP structure of **2**: a) top view, b) side view. Pentafluorophenyl groups are omitted for clarity in (b). Thermal ellipsoids are shown at the 30% probability level.

Then, **N₂CH-R8** was subjected to the spectroscopic measurements in aqueous solution (Fig. 2). The absorption λ_{max} values of the Soret-like (566 nm) and Q-like bands (730, 807, 902, 1040 nm) were similar to the spectrum of **1** in DMF, indicating that neither attachment of octa-arginine peptides via triazole linkers to **N₂CH** nor aqueous media intrinsically perturbs the absorption property of **N₂CH** macrocycle. Furthermore, in the fluorescence spectrum of **N₂CH-R8** in aqueous solution, an emission band at 1047 nm was observed similar to **1** in CH₂Cl₂ (1053 nm) as well as **1** and **N₂CH-R8** in DMF (1053 nm). The fluorescence efficiency of **N₂CH-R8** in H₂O appears similar or even better than **1** in CH₂Cl₂,⁶ indicating that the aqueous media do not affect the

fluorescence property of **N₂CH** macrocycle adversely.

With addition of an excess Zn(OAc)₂ to the aqueous solution of **N₂CH-R8**, the absorption λ_{max} of the Soret-like band shifted to 600 nm, which is identical to that of **1** with Zn(OAc)₂ in CH₂Cl₂. Relative fluorescence quantum yield increased 14-fold without shifting the emission maximum (λ_{em}). Therefore, both the changes in the absorption and fluorescence spectra indicate that the **N₂CH** macrocycle spontaneously captures Zn²⁺ ions in aqueous solution and its coordination mode is very similar to that of **1** in organic solvent (Fig. 2b, see also Supporting Information). The fluorescence spectra also indicate that **N₂CH-R8** is significantly responsive to Zn²⁺ in a pH region under neutral to weakly alkaline conditions (Fig. 2c). An excess amount of Zn(OAc)₂ (800 μM, 200 equiv) was insufficient for rapid completion of the spectral changes with **N₂CH-R8** (4.0 μM) (Fig. 2d), suggesting that the sensitivity of Zn²⁺ complexation of **N₂CH-R8** in H₂O is several fold lower than that of **1** in CH₂Cl₂.

Fig. 2 (a) UV-vis-NIR spectra of **N₂CH (1)** in DMF and **N₂CH-R8** in H₂O. [**1**] = [**N₂CH-R8**] = 0.27 μM. The inset shows partition experiments of **1** and **N₂CH-R8** (6.5 μM). i: **1** in DMF, ii: **1** in H₂O-CH₂Cl₂, iii: **N₂CH-R8** in H₂O-CH₂Cl₂, iv: **N₂CH-R8** in DMF. (b) NIR fluorescence spectra of **N₂CH-R8** (0.53 μM, λ_{ex} = 588 nm) in H₂O in the absence or presence of 1000 equiv of metal ions. (c) pH dependent fluorescence intensity changes of **N₂CH-R8** (0.53 μM, λ_{ex} = 600 nm) with 1000 equiv Zn²⁺. pH values were adjusted by aqueous HCl or NaOH. **N₂CH-R8** became insoluble when pH exceeded 11. (d) Time dependent Zn²⁺ coordination of **N₂CH-R8** (4.0 μM) in H₂O determined from absorption spectra.

Next, to gain insights into the complexation of other metals, absorption spectra of **N₂CH-R8** were investigated in aqueous solution upon addition of 19 metal ions. Besides Zn²⁺, eight metal ions [Cd²⁺, Co²⁺, Cr³⁺, Cu²⁺, Fe³⁺, Hg²⁺, Pd²⁺, Sn²⁺] were coordinated by the macrocycle judging from the changes of the absorption spectra.⁶ Among coordinated metals, only Zn²⁺ showed a marked enhancement in emission. On the other hand, the fluorescence spectra of **N₂CH-R8** in the presence of Cd²⁺, Co²⁺, and Fe³⁺ were similar to that of freebase. In contrast, remaining five metal ions, Cu²⁺, Cr³⁺, Hg²⁺, Pd²⁺, and Sn²⁺ quenched the emission (Figs. 2b and 3a).⁶

Then, the effect of metal ions on the fluorescence property of Zn^{2+} **N₂CH-R8** complex was examined. Metal ions (1000 equiv) that cause no effect on the absorption spectra of **N₂CH-R8** also did not show any change in the fluorescence spectra of **N₂CH-R8** with 1000 equiv Zn^{2+} .⁶ The fluorescence was not affected by Cd^{2+} or Co^{2+} , whereas it was partially quenched by Cr^{3+} , Fe^{3+} , or Sn^{2+} and strongly quenched by 1000 equiv Cu^{2+} , Hg^{2+} , or Pd^{2+} ,⁶ suggesting that the affinity of the last three metal ions to **N₂CH-R8** is comparable to or higher than that of Zn^{2+} . The strong inhibitory effect of Cu^{2+} ion is demonstrated by the experiment, in which 0.53 μM Cu^{2+} quenched the fluorescence of same molar concentration of **N₂CH-R8** complex nearly completely within 30 min (Fig. 3b, Fig. S13). This observation suggests that the Zn^{2+} complex of **N₂CH-R8** is also capable of serving as a “switch-off” fluorescent sensor to detect Cu^{2+} ions in aqueous

Fig. 3 (a) Relative fluorescence intensity of **N₂CH-R8** (0.53 μM , λ_{em} = 600 nm) in H_2O with 1000 equiv of metal ions of interest. (b) Fluorescence quenching of **N₂CH-R8** (0.53 μM , λ_{ex} = 600 nm) with 1000 equiv Zn^{2+} ions by 1.0, 2.0 or 20 equiv of metal ions.

In summary, we have synthesized a water-soluble derivative of doubly N-confused hexaphyrin (**N₂CH-R8**), which exhibits enhanced NIR fluorescence around 1050 nm only by Zn^{2+} ion coordination in aqueous solution. Thus, **N₂CH-R8** is a promising platform to develop a “switch-on” NIR fluorescent sensor for Zn^{2+} in aqueous solution albeit further improvement of coordination affinity and specificity, as well as emission efficiency would be necessary. Furthermore, Zn^{2+} complex of **N₂CH-R8** can also serve as a promising platform for a “switch-off” NIR fluorescent sensor for Cu^{2+} ion, a similarly important metal ion involved in a number of biological processes in living cells.¹¹ As octa-arginine (R8) is known as a member of peptides showing a cell penetrating property that enables various molecules to be introduced into mammalian cells,¹² *in vivo* application of **N₂CH-R8** would be of interest. Because **N₂CH-R8** would be applicable directly to *in vivo* analysis, investigation of its photophysical and coordination properties in cultured cells is now underway.

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Notes and references

- Crystal data for **2**: $\text{C}_{66}\text{H}_{10}\text{F}_{30}\text{N}_6\text{O}_2 \cdot 6(\text{C}_4\text{H}_8\text{O}) \cdot 4(\text{O})$, M_r = 2116.20, monoclinic, space group $P2_1/n$ (no. 14), a = 20.43(3), b = 8.939(11), c = 25.15(3) Å, β = 97.89(6)°, V = 4550(10) Å³, Z = 2, ρ_{calcd} = 1.545 g cm⁻³, $\mu(\text{MoK}\alpha)$ = 0.650 cm⁻¹, T = 123.1 K; Rigaku RAXIS-RAPID; 55° > 2 θ > 6°, 23026 measured reflections, 10446 unique reflections, 5121 with $I > 2\sigma(I)$ used in F^2 refinement, 632 parameters, R = 0.0666, wR = 0.1920 (all data), GOF = 0.955. CCDC 759874 contains the supplementary crystallographic data for **2**.
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