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 (N<sub>2</sub>CH) having two octa-arginine peptide arms displays an enhanced near-infrared (NIR) emission around 1050 nm in the presence of Zn<sup>2+</sup> in aqueous solution.

In the biomedical/bioanalytical fields, NIR-light technology has been of growing importance since the interferance by biomolecules could be minimized in the NIR region. Among a variety of targets for NIR-biosensing, Zn<sup>2+</sup> 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. Particularly, in a series of studies on N-confused porphyrinoids, we have shown doubly N-confused hexaphyrin (N<sub>2</sub>CH, 1, Chart 1), a kind of expanded porphyrin, forms bis-metal complexes with various divalent and trivalent metal ions such as Cu<sup>2+</sup>, Ni<sup>2+</sup>, Zn<sup>2+</sup>, Mn<sup>3+</sup>, and Fe<sup>3+</sup>, and displays an intensified near-infrared (NIR) emission with <i>λ</i><sub>em</sub> > 1000 nm in CH<sub>2</sub>Cl<sub>2</sub> when Zn<sup>2+</sup> ions are coordinated. Since then, we have been interested in synthesizing a water-soluble derivative, which serves as a NIR fluorescent sensor molecule, especially, for Zn<sup>2+</sup> in aqueous media. Herein, we report the synthesis of a water-soluble derivative of N<sub>2</sub>CH possessing two highly hydrophilic octa-arginine peptides (N<sub>2</sub>CH-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 Zn<sup>2+</sup> coordination was demonstrated for the first time.

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

Scheme 1 Synthesis of N<sub>2</sub>CH-R8 via conjugation of N<sub>2</sub>CH (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 fluoren-9-ylmethoxycarbonyl (Fmoc) protected aminooxanthen-3-yloxy-polystyrene resin (Sieber Amide resin). Then, N<sub>2</sub>CH 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). The reaction mixture was treated with trifluoroacetic acid (TFA) to remove Pbf groups and Cu<sup>2+</sup> ions from the arginine side-chains and the macrocycle, affording the desired freebase N<sub>2</sub>CH-peptide.

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<sup>†</sup> Electronic Supplementary Information (ESI) available: Details of the experiments, theoretical calculations and spectroscopic measurements.
conjugate, \( \text{N}_2\text{CH-R8} \). The resulting conjugate was purified by a reverse phase HPLC with \( \text{CH}_3\text{CN/H}_2\text{O} \) and obtained as a TFA salt (46% yield based on 3). By partition experiments between ultrapure water and \( \text{CH}_3\text{Cl}_2 \), \( \text{N}_2\text{CH-R8} \) was selectively extracted to the aqueous phase.

At first, we examined \( \text{Zn}^{2+} \) complexation of \( \text{N}_2\text{CH 1} \) in organic solvent by spectroscopic methods. In \( \text{CH}_3\text{Cl}_2 \), UV-vis-NIR spectrum of 1 with excess \( \text{Zn(OAc)}_2 \) (ca. 500 equiv) was nearly identical to that of isolated 2.\(^5\) For 4.0 \( \mu \text{M} \) of 1, completion of the spectral changes required 200 \( \mu \text{M} \) (50 equiv) of \( \text{Zn(OAc)}_2 \). The observation of isosbestic points at 591, 652, 764 and 830 nm suggests the coordination of second \( \text{Zn}^{2+} \) is rather fast to afford bis-\( \text{Zn}^{2+} \) complex, spontaneously.\(^6\) NIR-fluorescence spectra of 1, 2, and 1 treated in \textit{in situ} with excess \( \text{Zn(OAc)}_2 \) were compared at ambient temperature.

Upon adding \( \text{Zn(OAc)}_2 \), fluorescence of 1 was enhanced approximately 10-fold,\(^5\) exhibiting a sharp emission band around 1047 nm, which was nearly identical to that of 2. This result indicates that \textit{in situ} formation of bis-\( \text{Zn}^{2+} \) complex of 1 proceeds almost quantitatively in \( \text{CH}_3\text{Cl}_2 \) at ambient temperature.

The structure of bis-\( \text{Zn}^{2+} \) complex 2 was revealed by X-ray crystallography (Fig. 1).\(^3\) The complex shows a slightly distorted structure with a mean plane deviation of 0.0798 \( \text{Å} \) defined by 36 heavy atoms without oxygen in the macrocycle. The displacement of \( \text{Zn}^{2+} \) ion is 0.0572 \( \text{Å} \) and the Zn-Zn distance is 4.806 \( \text{Å} \). One \( \text{H}_2\text{O} \) molecule is coordinated at the axial position of each \( \text{Zn} \) atom with a \( \text{Zn}-\text{O} \) distance of 2.038 \( \text{Å} \). 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. Pentfluorophenyl groups are omitted for clarity in (b). Thermal ellipsoids are shown at the 30% probability level.](image)

Then, \( \text{N}_2\text{CH-R8} \) was subjected to the spectroscopic measurements in aqueous solution (Fig. 2). The absorption \( \lambda_{\text{max}} \) 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 \( \text{N}_2\text{CH} \) nor aqueous media intrinsically perturbs the absorption property of \( \text{N}_2\text{CH} \) macrocycle. Furthermore, in the fluorescence spectrum of \( \text{N}_2\text{CH-R8} \) in aqueous solution, an emission band at 1047 nm was observed similar to 1 in \( \text{CH}_3\text{Cl}_2 \) (1053 nm) as well as 1 and \( \text{N}_2\text{CH-R8} \) in DMF (1053 nm). The fluorescence efficiency of \( \text{N}_2\text{CH-R8} \) in \( \text{H}_2\text{O} \) appears similar or even better than 1 in \( \text{CH}_3\text{Cl}_2 \),\(^6\) indicating that the aqueous media do not affect the fluorescence property of \( \text{N}_2\text{CH} \) macrocycle adversely.

With addition of an excess \( \text{Zn(OAc)}_2 \) to the aqueous solution of \( \text{N}_2\text{CH-R8} \), the absorption \( \lambda_{\text{max}} \) of the Soret-like band shifted to 600 nm, which is identical to that of 1 with \( \text{Zn(OAc)}_2 \) in \( \text{CH}_3\text{Cl}_2 \). Relative fluorescence quantum yield increased 14-fold without shifting the emission maximum \( (\lambda_{\text{em}}) \). Therefore, both the changes in the absorption and fluorescence spectra indicate that the \( \text{N}_2\text{CH} \) macrocycle spontaneously captures \( \text{Zn}^{2+} \) 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 \( \text{N}_2\text{CH-R8} \) is significantly responsive to \( \text{Zn}^{2+} \) in a \( \text{pH} \) region under neutral to weakly alkaline conditions (Fig. 2c). An excess amount of \( \text{Zn(OAc)}_2 \) (800 \( \mu \text{M} \), 200 equiv) was insufficient for rapid completion of the spectral changes with \( \text{N}_2\text{CH-R8} \) (4.0 \( \mu \text{M} \) (Fig. 2d), suggesting that the sensitivity of \( \text{Zn}^{2+} \) complexation of \( \text{N}_2\text{CH-R8} \) in \( \text{H}_2\text{O} \) is several fold lower than that of 1 in \( \text{CH}_3\text{Cl}_2 \).

Next, to gain insights into the complexation of other metals, absorption spectra of \( \text{N}_2\text{CH-R8} \) were investigated in aqueous solution upon addition of 19 metal ions. Besides \( \text{Zn}^{2+} \), eight metal ions \( \text{[Cd}^{2+}, \text{Co}^{2+}, \text{Cr}^{3+}, \text{Cu}^{2+}, \text{Fe}^{3+}, \text{Hg}^{2+}, \text{Pd}^{2+}, \text{Sn}^{2+}] \) were coordinated by the macrocycle judging from the changes of the absorption spectra.\(^6\) Among coordinated metals, only \( \text{Zn}^{2+} \) showed a marked enhancement in emission. On the other hand, the fluorescence spectra of \( \text{N}_2\text{CH-R8} \) in the presence of \( \text{Cd}^{2+}, \text{Co}^{2+}, \text{and Fe}^{3+} \) were similar to that of freebase. In contrast, remaining five metal ions, \( \text{Cu}^{2+}, \text{Cr}^{3+}, \text{Hg}^{2+}, \text{Pd}^{2+}, \text{and Sn}^{2+} \) quenched the emission (Figs. 2b and 3a).\(^6\)
Then, the effect of metal ions on the fluorescence property of Zn$^{2+}$ bis[CH-R8] complex was examined. Metal ions (1000 equiv) that cause no effect on the absorption spectra of bis[CH-R8] also did not show any change in the fluorescence spectra of bis[CH-R8] with 1000 equiv Zn$^{2+}$. 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 Pb$^{2+}$, suggesting that the affinity of the last three metal ions to bis[CH-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 bis[CH-R8] complex nearly completely within 30 min (Fig. 3b, Fig. S13). This observation suggests that the Zn$^{2+}$ complex of bis[CH-R8] is also capable of serving as a "switch-off" fluorescent sensor to detect Cu$^{2+}$ ions in aqueous environment.

![Fig. 3](image-url) (a) Relative fluorescence intensity of bis[CH-R8] (0.53 μM, λ$_{ex}$ = 600 nm) in H$_2$O with 1000 equiv of metal ions of interest. (b) Fluorescence quenching of bis[CH-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 (bis[CH-R8]), which exhibits enhanced NIR fluorescence around 1050 nm only by Zn$^{2+}$ ion coordination in aqueous solution. Thus, bis[CH-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 bis[CH-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. 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, in vivo application of bis[CH-R8] would be of interest. Because bis[CH-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