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Synthesis and protonation behavior of a water-soluble N-fused porphyrin: Conjugation with an oligoarginine by click chemistry

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ABSTRACT

A water-soluble derivative of N-fused porphyrin (NFP) possessing a nona-arginine (R9) peptide tail was synthesized for the first time by a Cu(I)-catalyzed azide-alkyne 'click' reaction. In aqueous solution, at pH 8, the conjugated molecule (**NFP-R9**) exists as free base and protonated below pH < 6.5 to form monoprotonated species dominantly, and diprotonated one below pH < 2.3, while such clear two-step protonation behavior was not observed in the DMF solution.

In recent years, application of near-infrared (NIR) light has been expanding in various fields involving material science,[1] information technology,[2] and also biomedical science/technology.[3] In the biomedical fields, NIR has been recognized as wavelength most suitable for diagnostic and therapeutic applications because interference by water and bio(macro)molecules can be minimized at NIR region.[4] However, only limited numbers of organic compounds applicable for NIR science/technology have been reported to date,[5] presumably because of the difficulty in satisfying stability and synthetic accessibility required for practical use.

N-Fused porphyrin (NFP) is a porphyrin analogue with an 18π aromaticity derived from a tetrapyrrolic macrocycle (Chart 1).[6a] A notable feature of NFP is its optical property that absorbs NIR light over 1000 nm.[6b] Such unusual long wavelength absorption of NFP is supposed to originate from its characteristic tri-pentacylic unit, by which a HOMO–LUMO gap of the macrocycle would become smaller than ordinary porphyrins.[6b] NFP can be readily prepared in two steps from a porphyrin isomer, N-confused porphyrin (NCP, Chart 1),[6b] which is synthesized by a one-pot condensation of pyrrole and aromatic aldehyde.[7] Owing to these features, NFP is expected as a promising candidate applicable to NIR bioscience/technology. However, due to the lack of water-soluble derivative, the property of NFP in aqueous media has been totally veiled. Herein, we report a first synthesis of a water-soluble derivative of NFP, which possesses a hydrophilic nona-arginine peptide tail (R9), and its protonation behavior in aqueous solution.

Synthesis of the title conjugated molecule **NFP-R9** consists of three parts. The first part covers a selective introduction of an ethynylaryl group to the C(21) position of NFP (Scheme 1).[6c] At first, N-confused tetraphenylporphyrin (**1**) was converted to the C(21)-brominated N-fused tetraphenylporphyrin (**2**) in two steps.[6b] Successively, **2** was treated with 4-(triisopropylsilylethynyl)phenylboronic acid in the presence of $Pd(PPh_3)_4$ and Cs_2CO_3, affording **3** in 68% yield.[6c] Triisopropylsilyl group of **3** was then removed by treating with tetrabutylammonium fluoride (TBAF) to afford **4** in 99% yield (see Supplementary data online).

In the second part, a nona-arginine peptide (R9) possessing an azidoglycine moiety at the N-terminus was synthesized by Fmoc-solid phase synthesis (Scheme 2).[8] Starting from fluoren-9-ylmeth-

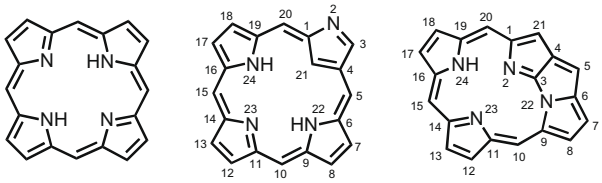

Chart 1. Basic structures of porphyrin (left), N-confused porphyrin (middle), and N-Fused porphyrin (right).

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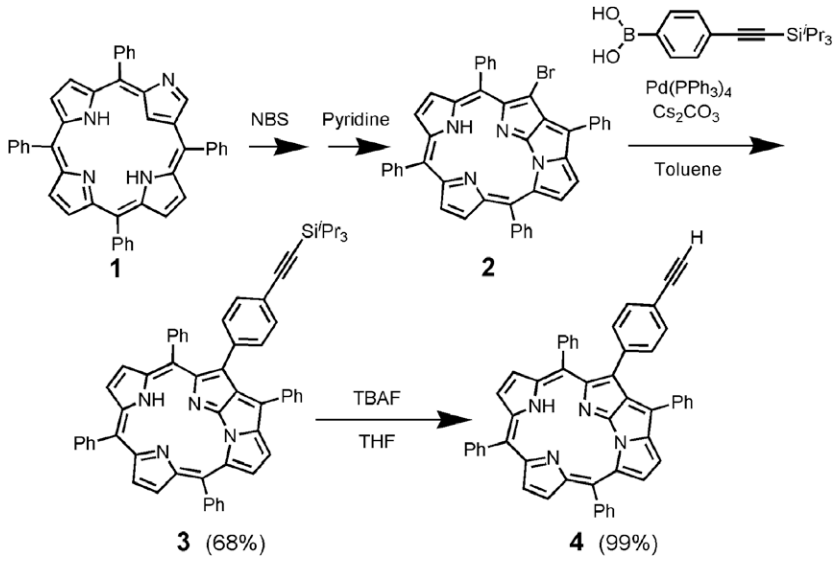
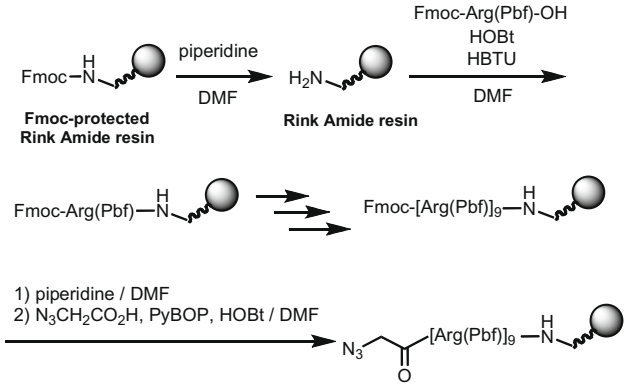


Scheme 1. Synthesis of a terminal alkyne-attached NFP derivative **4**.



Scheme 2. Solid phase synthesis of protected R9 peptide possessing an azide moiety at the N-terminus on the Rink Amide resin.

yloxycarbonyl (Fmoc) protected 4-(2′,4′-dimethoxyphenylaminomethyl)-phenoxy resin (Rink Amide resin), Fmoc group of the resin was removed and then nona-arginine peptide was synthesized stepwise by solid-phase methods. With arginine monomer whose α-amino moiety and side-chain were protected by Fmoc and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) groups, respectively, coupling reactions were carried out using *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uroniumhexafluorophosphate (HBTU)-mediated hydroxybenzotriazole (HOBt) ester activation protocols. The terminal azidoglycine was introduced to the protected nona-arginine peptide on the Rink Amide resin by using HOBt and benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) as coupling reagents. The resulting resin, on which the protected R9 tail peptide (abbreviated as N_3-Gly-[Arg(Pbf)]$_9$) was loaded, was used for the next part reactions.

In the final part, the N_3-Gly-[Arg(Pbf)]$_9$ peptide attached on the resin was linked with the NFP derivative (**4**) by a Cu(I)-catalyzed cycloaddition between the azide moiety of peptide N-terminus and the terminal alkyne in **4** (Scheme 3).[9] In the presence of 0.1 mmol CuI, the resin, which possesses N_3-Gly-[Arg(Pbf)]$_9$ peptide prepared from 0.05 mmol Fmoc-protected Rink Amide resin, was reacted with 0.1 mmol of **4** in CH_2Cl_2 at ambient temperature. After extensive washing with CH_2Cl_2 to remove excess **4**, the resin was treated with trifluoroacetic acid (TFA) containing *m*-cresol (v/v 5%) and thioanisol (v/v 5%) to release the NFP-peptide conjugate (**NFP-R9**) from the resin and to remove protective Pbf groups from arginine side-chains.[8]

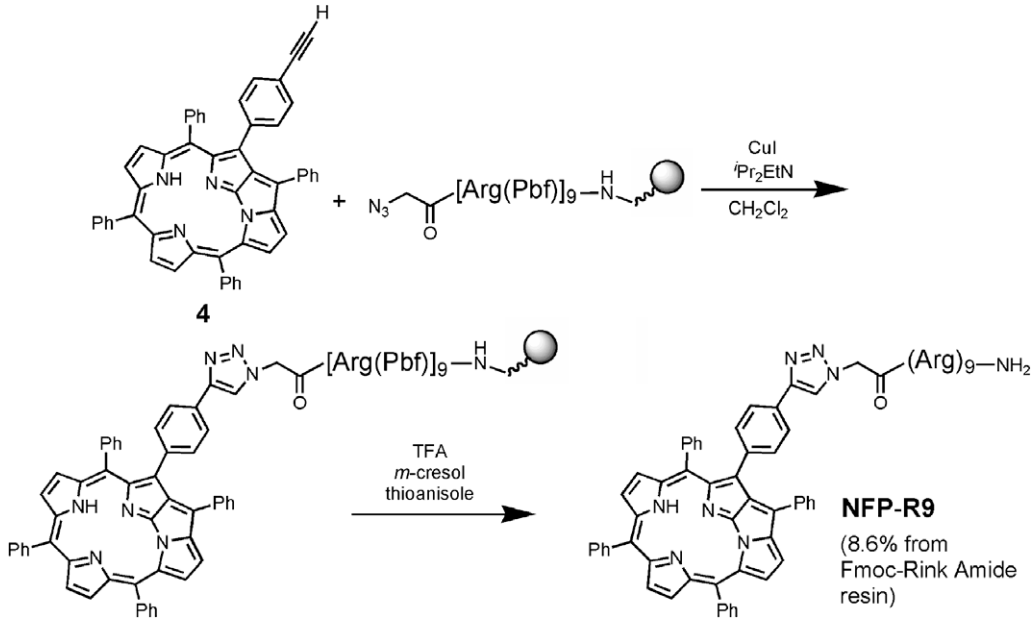
The conjugated **NFP-R9** was purified by a reverse phase HPLC with CH_3CN/H_2O and stored as a TFA salt. **NFP-R9** was characterized by MALDI-TOF mass and UV–vis–NIR spectroscopy. Separately, NFP derivative **5**, which possesses an ester-linked triazole group, was synthesized in 85% yield by the reaction between **4** and azidoacetic acid ethyl ester as a control compound (see Chart 2 and Supplementary data online).

Water-solubility of **NFP-R9** and **5** was tested by partition experiments between ultrapure water and CH_2Cl_2 (Fig. 1).[10] As expected, ester derivative **5** was only soluble in CH_2Cl_2. In contrast, **NFP-R9** remained in water phase, indicating that the nona-arginine peptide tail is hydrophilic enough to solubilize the hydrophobic NFP macrocycle in aqueous media.

The UV–vis–NIR absorption spectra of tetraphenyl NFP (**NFTPP**, Chart 2), **5**, and **NFP-R9** were measured in DMF. **NFTPP** showed the Soret-like bands at 362, 498, and 545 nm and Q-like bands at 648, 705, 858 and 942 nm (Fig. 2A) [6b], which are virtually identical to those measured in CH_2Cl_2. The NFP ester **5** also showed three Soret-like bands at 348, 503, and 548 nm and, characteristically, the third band was largely intensified in comparison with the spectrum of **NFTPP**. Furthermore, **5** showed an additional small band at 414 nm, which was not observed in **NFTPP**. Q-like transitions of **5** were observed at 646, 699, 871, and 961 nm (Fig. 2A). The UV–vis–NIR spectrum of **NFP-R9** in DMF was essentially identical to that of **5** (Fig. 2A), indicating that the attachment of nona-arginine peptide to NFP does not cause any significant effect on its absorption property in the DMF solution.

Next, UV–vis–NIR absorption spectrum of **NFP-R9** was measured in aqueous solution (pH 8.0) (Fig. 2B). The λ_{max} values of the Soret-like and Q-like bands were virtually identical to the spectrum in DMF but the third Soret-like transition at 548 nm was weaker in aqueous solution than that in DMF.

In CH_2Cl_2, **NFTPP** undergoes protonation by the addition of TFA, causing a significant increase of the second Soret-like transition with a blue-shift from 499 to 487 nm and a modest increase of Q-like bands.[6b] In DMF, similar spectral change of **NFTP**, which also showed a blue-shift of second Soret-like transition to 487 nm, was observed by addition of TFA (Fig. S1). When TFA was added to a DMF solution of **5**, the spectral change observed was significantly



Scheme 3. Conjugation of NFP and nona-arginine peptide by a Cu(I)-catalyzed azide-alkyne cycloaddition on the solid support, followed by acid cleavage from the resin.

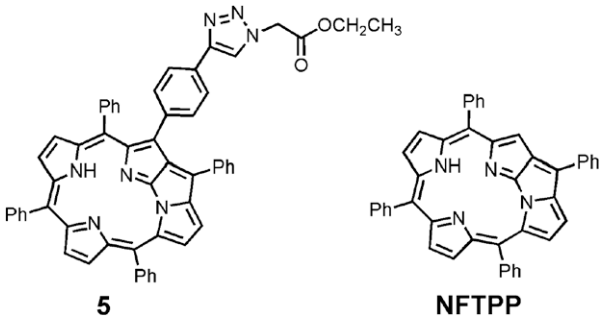


Chart 2.

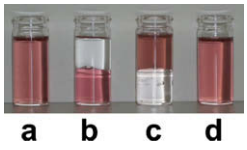


Figure 1. Partition experiments of hydrophobic and hydrophilic derivatives of NFP, (a) **5** in DMF, (b) **5** in H_2O + CH_2Cl_2, (c) **NFP-R9** in H_2O+CH_2Cl_2, (d) **NFP-R9** in DMF. [**5**] = [**NFP-R9**] = 10 μM.

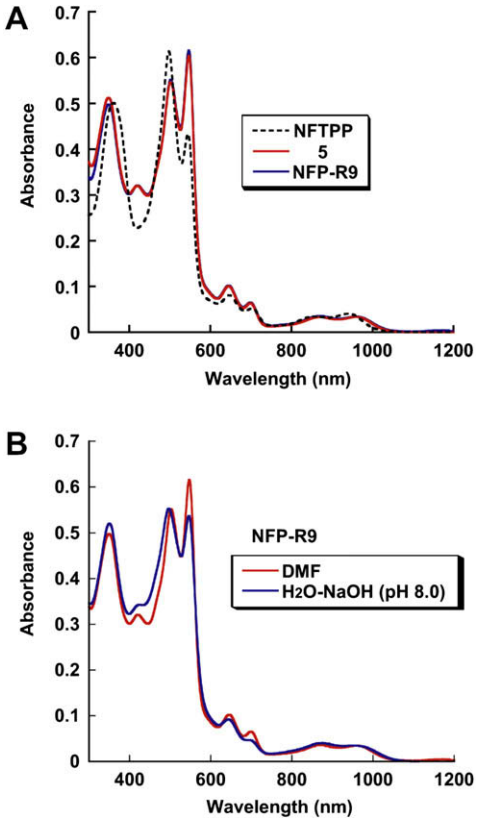


Figure 2. UV–vis–NIR absorption spectra of (A) **NFTPP**, **5** and **NFP-R9** in DMF, and (B) **NFP-R9** in DMF and aqueous solution (pH 8.0 adjusted with NaOH). Concentration: [**5**] = [**NFP-R9**] = 10 μM.

different from that of **NFTPP** in CH_2Cl_2. The second Soret-like transition of **5** was broadened and split with blue-shifts. The third Soret-like transition was decreased with slight red-shifts (Fig. 3A). The spectral change of **5** caused by TFA addition exhibited two isosbestic points at 492 and 560 nm (Fig. 3A), suggesting a two-state transition without stable intermediate(s). In the titration of **NFP-R9**, the spectral change was closely similar to that of **5** but a larger amount of TFA was required for the protonation of **NFP-R9** (Fig. 3B), presumably due to the influence of polycation charges of nona-arginine group nearby.

To clarify the protonation behavior of **NFP-R9** in aqueous solution, the pH was gradually decreased from 7.5 to 1.0 and followed the absorption spectral changes. As a result, three major changes were observed according to the pH range: (i) in the pH range from 7.5 to 5.1, the absorption of the second Soret-like transition was enhanced with a blue-shift of λ_{max} from 503 to 483 nm and that of Q-like bands was increased moderately. These changes afforded two isosbestic points at 492 and 553 nm (Fig. 4A); (ii) in the pH range from 5.1 to 3.5, the spectra were essentially unchanged (Fig. S2) and were similar to that of the protonated **NFTPP** which showed the second Soret-like band at 487 nm in CH_2Cl_2;[6b] (iii) in

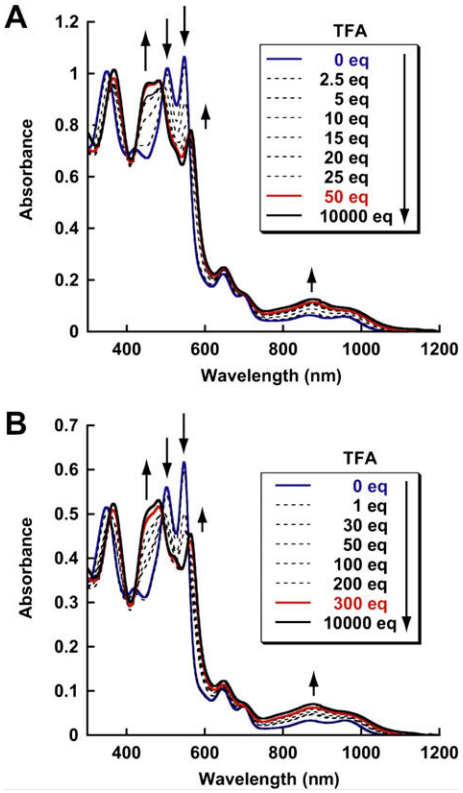


Figure 3. Protonation of NFP derivatives by TFA in DMF, (A) **[5]** = 20 μM, (B) [**NFP-R9**] = 10 μM.

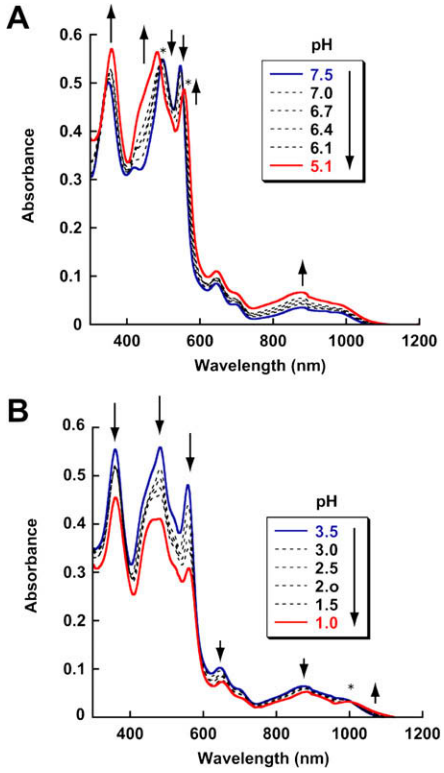


Figure 4. pH dependent spectral changes of **NFP-R9** in ultrapure water; (i) (A) between pH 7.5 and pH 5.1 and (B) between pH 3.5 and pH 1.0. Asterisks indicate isosbestic points. Titrations were performed with aqueous NaOH and HCl. [**NFP-R9**] = 10 μM.

the pH range from 3.5 to 1.0, the intensity of three Soret-like transitions decreased without shifting λ_{max}. Modest spectral change was also observed in the Q-like bands, which showed an isosbestic point at 1006 nm (Fig. 4B). The spectral feature of **NFP-R9** at pH 1.0 was closely similar to that of **5** in DMF solution containing excess TFA (Fig. 3A).

The absorption spectra of **NFP-R9** solution observed between pH 5.1 and 3.5 and at pH 1.0 could be assigned as those of monoprotonated species (**H^+-NFP-R9**) and diprotonated one (**$2H^+$–NFP-R9**), respectively. From the titration curve, the pK_a values of the deprotonation process [**$2H^+$-NFP-R9**]//[**H^+–NFP-R9**] and [**H^+–NFP-R9**]//[**NFP-R9**] were estimated to be 6.5 and 2.3, respectively. The corresponding monoprotonated species for **NFP-R9** and **5** were not observed in DMF. Such direct transition from freebase to diprotonated species is frequently seen in the porphyrin derivatives[10,11] and a water-soluble NCP derivative.[12] The polycation charges of R9 moiety might have largely raised the energy barrier of the second protonation at the **NFP-R9** core. At present, it is unclear which positions in NFP macrocycle accept proton(s) under acidic conditions. Like regular porphyrin, the first protonation might occur at the imino-form nitrogen, N(23). Because, the basicity of N(23) and N(24) does not seem largely different, judged by the structural data of various NFP derivatives, which show the inner hydrogen atom either at N(23) or N(24).[6b] The position of the second protonation is less certain. The imino-form nitrogen at N(2) is a candidate but its basicity is unknown because this pyrrolic moiety is integrated into the tri-pentacyclic ring. An alternative candidate is C(21) position, to which protonation by TFA occurs smoothly in the rhenium(I)-tricarbonyl complex of **NFTPP**.[13]

In summary, we have synthesized a water-soluble N-fused porphyrin-nona-arginine peptide conjugate (**NFP-R9**) by click chemistry. In aqueous solution, at pH 8, **NFP-R9** exists as free base and is easily protonated below pH < 6.5 to form monoprotonated species dominantly, and diprotonated one below pH < 2.3, while such explicit two-step protonation behavior was not observed in the DMF solution. The photophysical properties in the NIR region of NFP would be useful for the biological application. Furthermore, biological effects of NFP itself are of interest. Nona-arginine (R9) is a member of peptides showing cell penetrating ability.[14] Various molecules can be introduced into mammalian cell if they form covalent conjugation or noncovalent complex with cell penetrating peptides.[15] Because **NFP-R9** can be subjected to in vivo analysis without further modification, its biological effects to cultured cells are under investigation in our group.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.03.066.

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