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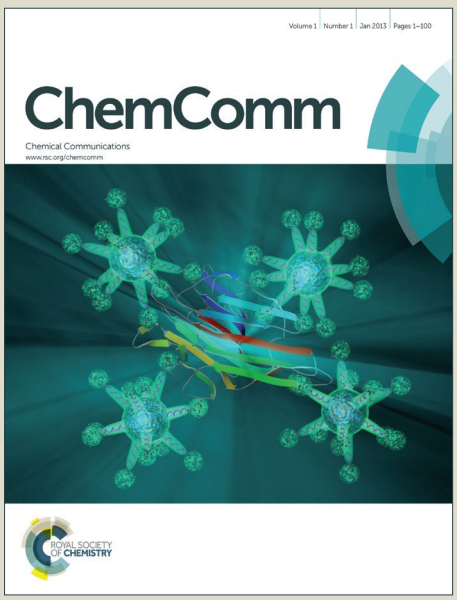

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Supramolecular Intracellular Delivery of an Anionic Porphyrin by Octaarginine-conjugated Per-*O*-methyl-β-cyclodextrin

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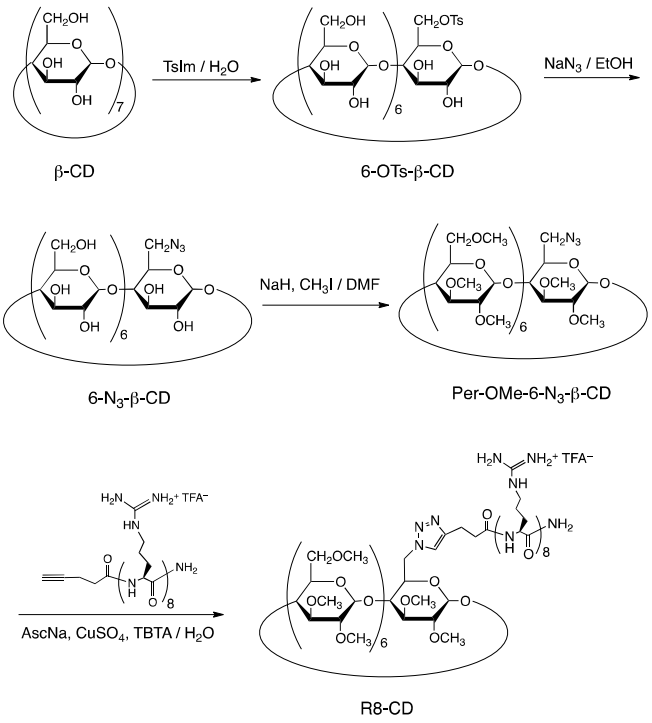
A convenient and efficient method for intracellular delivery of a water-soluble anionic porphyrin has been developed by utilizing its supramolecular interaction with per-*O*-methyl-β-cyclodextrin bearing an octaarginine chain as a cell-penetrating peptide.

Porphyrins and their related compounds have been utilized in mammalian cells as photosensitizers (PS) in photodynamic tumor therapy (PDT),[1] contrast agents for magnetic resonance imaging (MRI)[2] and luminescent sensors for metal ions[3] or oxygen.[4] A variety of synthetic tetrapyrrole derivatives have been prepared to achieve these functions.[1-4] An inevitable problem in these studies is delivery of the synthetic compounds into the cell. Some hydrophobic tetrapyrroles can penetrate cell membranes without any chemical modifications.[1b,1d,4] In these cases, however, an organic solvent such as dimethylsulfoxide (DMSO) is required to prepare a stock solution. Although a small amount of DMSO does not affect cell viability, this method occasionally causes low cellular uptake efficiency and unexpected cytotoxicity due to the formation of self-aggregates when the hydrophobic compound is dispersed from the organic solvent into an aqueous medium. In addition, non-specific hydrophobic interactions with biological components, such as serum proteins, must be considered. In contrast, water-soluble cationic porphyrins, such as 5,10,15,20-tetrakis(*N*-methyl-4-pyridinium)-porphyrin (TMPyP), are known to be efficiently transferred into cells without the use of organic solvents.[4,5] Therefore, TMPyP has often been used as the PS, although it tends to be accumulated in the nucleic acids of cells. In addition, self-assembled nanoparticles, including micelles and liposomes, have also been used for intracellular delivery of PS molecules.[6]

The use of cell-penetrating peptides (CPP) can effectively deliver a large amount of PS molecules into cells.[7,8] CPPs are small peptides consisting of less than 30 amino acid residues with a high density of basic amino acids (arginine or lysine). CPPs are capable of assisting in the intracellular delivery of various biomolecules and synthetic compounds.[9] In general, the target molecule is covalently attached to the CPP for effective delivery. However, covalent conjugation of PS with CPP is time-consuming.[7,8] In this study, we describe a simple methodology for non-covalent delivery of PS into cells using a CPP-conjugated artificial host molecule, per-*O*-methylated β-cyclodextrin (CD), which forms a stable inclusion complex with 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrin (TPPS) in an aqueous culture medium even in the presence of serum proteins.



Scheme 1 Synthetic route of R8-CD.

We chose the arginine octamer (R8) as a CPP because of its structural simplicity and well-characterized cell-penetrating ability.[10] The R8 peptide with a terminal alkyne was conjugated with mono-6-azido-6-deoxy-per-*O*-methyl-β-CD (Per-OMe-6-N_3-β-CD)[11] by a copper(I)-catalyzed azide-alkyne cycloaddition reaction (CuAAC) (Scheme 1). Using tris(3-hydroxypropyltriazolylmethyl)amine (TBTA) ligand,[12] which can suppress unfavorable oxidation of Cu^+ to Cu^{2+}, the CuAAC reaction was performed in an aqueous solution

under aerobic conditions and ambient temperature. Pure R8-conjugated per-*O*-methylated β-CD (R8-CD) was obtained after purification by reverse-phase liquid chromatography in a 52 % yield. R8-CD was confirmed by elemental analysis, analytical chromatography and mass spectroscopy (Fig. S1, ESI†).

The intermolecular interaction between R8-CD and TPPS in aqueous solution was studied by UV-vis and NMR spectroscopy and size exclusion chromatography (SEC). It is known that 2,3,6-tri-*O*-methyl-β-cyclodextrin (TMe-β-CD) strongly interacts with TPPS to form a trans-type 2:1 inclusion complex, where two TMe-β-CD molecules interact with the 4-sulfonatophenyl groups at the 5- and 15-positions.[13] In contrast to the simple UV-vis spectral changes observed when TPPS was titrated with TMe-β-CD,[13b] a biphasic spectral change was observed in the TPPS/R8-CD complex formation (Fig. 1). In the first phase, tetraanionic TPPS forms a water-insoluble ion-pair complex with octacationic R8-CD, resulting in a weakening in the absorption band. Further addition of R8-CD caused dissociation of the ion-pair complex, resulting in the formation of the 2:1 inclusion complex. As shown in Fig. 1b, the titration curve was almost saturated after the addition of 2 equiv of R8-CD, and the final spectrum was nearly identical to that of the TPPS/TMe-β-CD complex (Fig. S2, ESI†). Therefore, R8-CD forms a 2:1 inclusion complex with TPPS in the same manner as TMe-β-CD. The structural similarity of the R8-CD/TPPS complex to the TMe-β-CD/TPPS complex was also confirmed by the pH titration, NMR titration, 2D NMR and the SEC analysis (Fig. S3–5, ESI†).

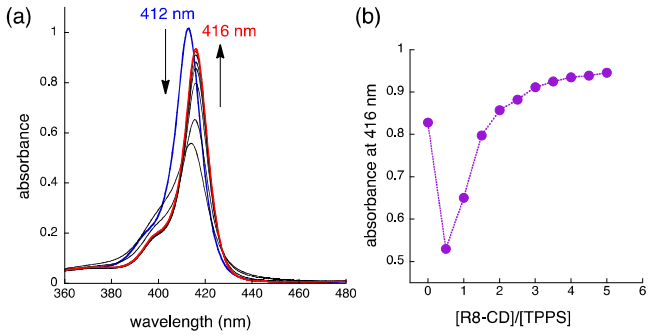


Fig. 1 (a) UV-vis spectral changes in TPPS (1.3×10^{-6} M) upon addition of R8-CD in phosphate buffer saline (PBS) at pH 7.4 and 25°C. (b) The changes in absorbance at 416 nm of TPPS as a function of [R8-CD]/[TPPS]

The cellular uptake of the TPPS/R8-CD complex was studied using HeLa cells. The TPPS solution with or without CD (2.4 equiv of TMe-β-CD or R8-CD) in phosphate buffer saline (PBS) was added to cells soaked in the culture medium. To investigate the effect of serum, medium containing 10% serum (D-10) and serum-free medium (OPTI) were used as the culture media.[14] After 2 h incubation, the cells were washed several times with PBS, followed by monitoring with fluorescence microscopy (Fig. 2).[15] Unsurprisingly, no fluorescence due to the free-base porphyrin was observed from the cells in the absence of R8-CD. In the presence of R8-CD, clear fluorescence signals due to the free-base porphyrin were detected in the cells. In both the serum-containing and serum-free media, fluorescence signals were detected in endosomal structures mainly located at the peripheries of the cell nuclei. In addition, clear cytosolic dispersion was also observed for the sample incubated in the serum-free medium (Fig. 2f). Similar observations were reported by Futaki et al.,[10d] who found that the R12 and R16 peptides in serum-free medium showed cytosolic dispersion through an ATP-independent, direct transmembrane mechanism. The arginine oligomers strongly adhered to the cell surface, and could be directly translocated into the cytosol due to potential differences across the cell membrane (the cell interior is relatively negative).[16] Indeed, the cytosolic dispersion was also observed when the cells were incubated with the TPPS/R8-CD complex at 4°C in serum-free medium (Fig. S7, ESI†), which indicates that the porphyrin was dispersed from the serum-free medium into the cytosol as a result of the direct transmembrane mechanism because ATP-dependent endocytosis should be blocked at 4°C.[10d,17] Non-specific interactions of the oligoarginine chain with albumin, a major acidic protein in serum, seem to reduce the effective concentration of the peptide that participates in the direct transmembrane pathway. As a result, the endocytosis mechanism is the major cellular uptake pathway in serum-containing medium.

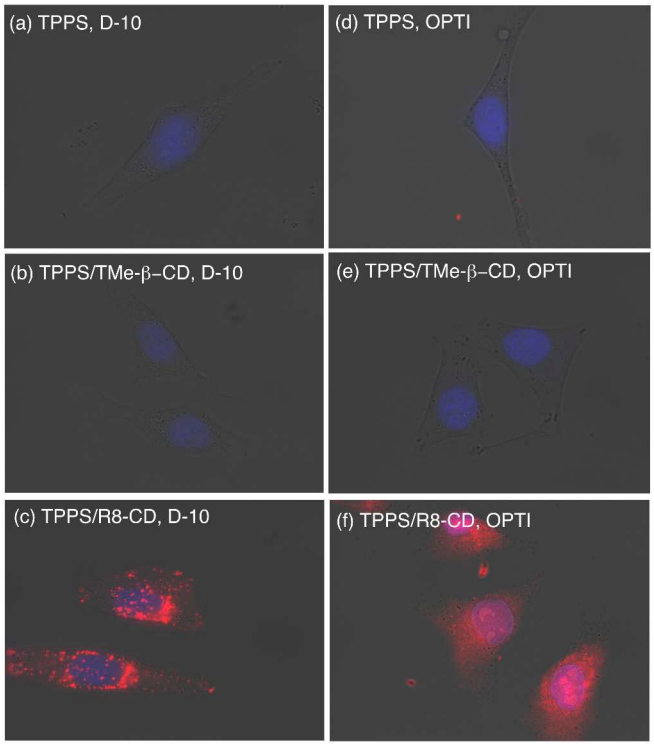


Fig. 2 The overlaid fluorescence/phase-contrast images of the HeLa cells after treatment with (a, d) TPPS (5.0×10^{-6} M), (b, e) TPPS (5.0×10^{-6} M) plus TMe-β-CD (1.2×10^{-5} M), and (c, f) TPPS (5.0×10^{-6} M) plus R8-CD (1.2×10^{-5} M) in different culture media (a-c: D-10; d-f: OPTI). Luminescence was shown in red (excited at 435 ± 25 nm for porphyrin) and blue (excited at 360 ± 40 nm for DAPI). The images were obtained using an oil-immersion objective lens (100×).

To understand the inclusion state of the TPPS/R8-CD complex in cells, the cells containing the complex were lysed by a freeze-thaw cycle in water, and the cell lysate was analyzed by fluorescence spectroscopy. In the sample incubated in D-10 and OPTI, the cell lysate showed a characteristic fluorescence spectrum resulting from the complex of TPPS with two R8-CD molecules (λ_{em} = 643 and 704 nm). The spectrum was quite distinguishable from free TPPS (Fig. S8, ESI†). These results indicate that the inclusion phenomenon is sustained, even in the cell-interior. The fluorescence intensity of the lysate was increased as a function of incubation time and reached plateau in incubations more than 120 min (Fig. S8, ESI†).

Flow cytometry was applied to quantify the amount of TPPS taken up by the cells. After incubation with the TPPS/R8-CD complex, the number of highly fluorescent cells increased, as a result of internalization of TPPS in the cells (Fig. 3). Owing to the direct transmembrane pathway that occurs in serum-free medium, the cells incubated with the complex in OPTI showed a wider distribution in the high fluorescence region than those in serum-containing

medium. However, as compared to the previous intracellular delivery systems,[8a,10d] serum-induced inhibition was remarkably weak, and serum proteins did not substantially interfere with cellular uptake of the TPPS/R8-CD complex. Flow cytometry also revealed that bare TPPS weakly interacted with the cells in OPTI (green line in Fig. 3a), whereas complexation with TMe-β-CD suppressed the interaction (red line in Fig. 3a). Because TPPS strongly interacts with serum proteins, primarily serum albumin,[18] the interaction of TPPS with the cells was inhibited in serum-containing medium (Fig. 3b). Competitive titration experiments indicated that R8-CD interacts more strongly with TPPS than BSA (Fig. S9, ESI†). On the basis of these results, it can be concluded that R8-CD can be widely used for intracellular delivery of water-soluble tetraarylporphyrins, such as TPPS, even in the presence of serum proteins.

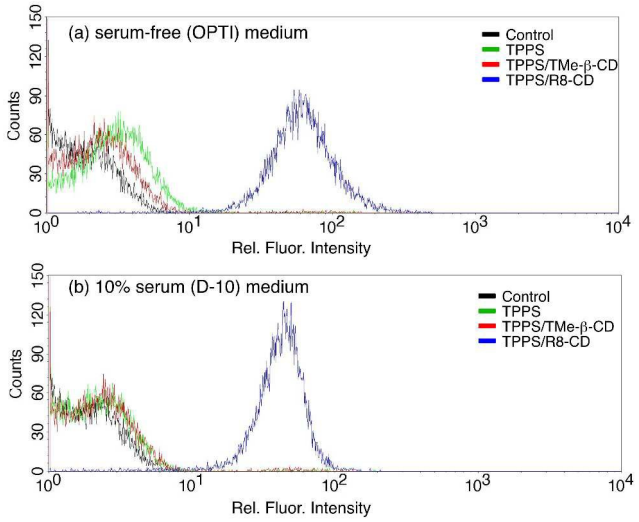


Fig. 3 Flow cytometry histograms of HeLa cells (10^4 cells per measurement). The cells were pre-treated with TPPS (5.0×10^{-6} M; green lines), TPPS (5.0×10^{-6} M) plus TMe-β-CD (1.2×10^{-5} M; red lines), and TPPS (5.0×10^{-6} M) plus R8-CD (1.2×10^{-5} M; blue lines) in the different culture media (a: OPTI; b: D-10). Untreated control cells are represented by the black lines. The cells were individually excited at 643 nm and luminescence signals were detected at 667 nm.

We next studied the photo-induced cytotoxicity in this system. The TPPS solution with or without CD (TMe-β-CD or R8-CD) was added to cells in culture medium (OPTI or D-10), and filtered light (310-510 nm) was successively irradiated to the cell cultures for 15 min. After photo-irradiation, the cell viability was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay (Fig. 4). Corresponding to the flow cytometry data shown in Fig. 3, TPPS without serum showed weak toxicity due to weak interaction with the cells. The photo-induced cytotoxic function of TPPS was drastically reduced in the serum-containing medium. The TPPS/TMe-β-CD complex did not show any photo-induced cytotoxicity in both types of media. Meanwhile, the TPPS/R8-CD complex showed very high cell-killing ability, regardless of the presence of serum. Therefore, R8-CD is usable as an effective delivery tool for PDT, even in the presence of competitive biomolecules in the medium. Because R8-CD itself and the TPPS/R8-CD complex did not show any cytotoxicity without light irradiation (Fig. S10, ESI†), the photo-induced cytotoxic effect of the TPPS/R8-CD system must be due to the PDT effect, i.e. singlet oxygen generation from the excited porphyrin in the cells.[1] As reported previously,[19] the ability of TPPS to produce singlet oxygen upon photo-irradiation seems to be unaffected by complexation with R8-CD (Fig. S11, ESI†).

In conclusion, we have successfully synthesized an octaarginine-conjugated per-*O*-methyl-β-cyclodextrin (R8-CD) for the delivery of an anionic tetraarylporphyrin, TPPS, into mammalian cells. R8-CD forms a very stable 2:1 inclusion complex with TPPS in aqueous solution in the same manner as TMe-β-CD without the peptide chain. Because of the higher binding affinity of R8-CD to TPPS, compared to serum proteins, R8-CD can be used as a powerful molecular tool for intracellular delivery of tetraarylporphyrins *in vivo*.

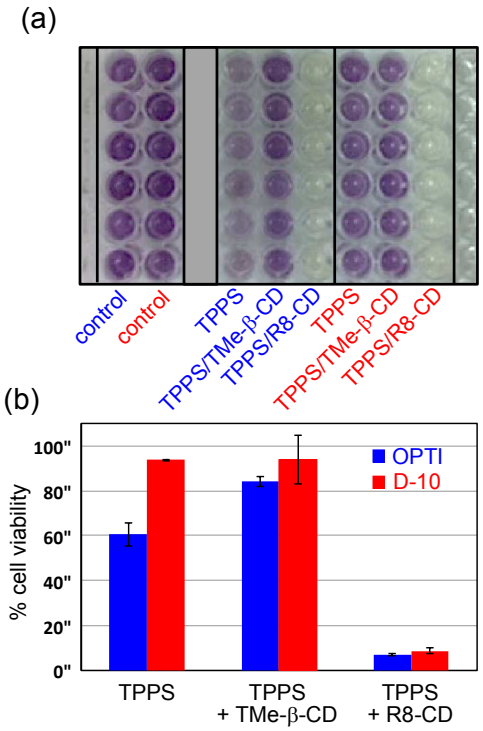


Fig. 4 Photo-induced cytotoxic effects of TPPS (5.0×10^{-6} M), TPPS (5.0×10^{-6} M) complexed with TMe-β-CD (1.2×10^{-5} M), and TPPS (5.0×10^{-6} M) complexed with R8-CD (1.2×10^{-5} M) in the different culture media (blue: OPTI; red: D-10). HeLa cells cultured in a 96-well plate were treated with filtered light at 310-510 nm for 15 min. The cell viability was measured by the MTT assay. (a) Photograph of the 96-well plate after the MTT assay. The purple color indicates the presence of living cells. (b) The histogram for the cell viability. Each bar represents the mean ± SD of the data obtained from at least three experiments.

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Notes and references

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†Electronic Supplementary Information (ESI) available: Experimental details and Figs. S1-S11. See DOI: 10.1039/c000000x/

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14 The culture media are abbreviated as follows; D-10: Dulbecco's minimal Eagle's medium (D-MEM, Wako) containing 10% (v/v) fetal bovine serum (GIBCO, heat inactivated at 56 °C before use) and 1% penicillin/streptomycin (Wako); OPTI: OPTI-MEM® (GIBCO), an optimized culture medium that keeps cells alive in a low or zero serum supplement condition.

15 To see the cell images in detail, the large, high-resolution images are shown in Fig. S6, ESI†.

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