DOI: 10.1002/cbic.200700627

Artificial Receptors Designed for Intracellular Delivery of Anionic Phosphate Derivatives

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Efficient delivery of functional materials, such as biological substances, bioactive drugs, and molecular probes, from the exterior to the interior of living cells is of critical importance in a variety of research areas such as cell biology and medicinal chemistry. Despite successful examples of the transportation systems for the biopolymers such as plasmid DNA, RNA fragments, or functional protein/peptides, methods available for the intracellular delivery of cell impermeable small molecules, in particular highly anionic short peptides or organic molecules of biological interest, have been scarcely developed.[1,2] This is partially because useful artificial receptors capable of capturing such anionic species have not been sufficiently developed, which could potentially serve as a career vector for intracellular transportation.[3] Therefore, the anionic molecules are inevitably subjected to laborious premodification such as acetoxymethyl esterification to gain cell permeability by neutralizing their anionic characters. We report herein a new intracellular delivery method for anionic phosphorylated derivatives using the artificial metalloreceptor as a carrier. The designer multinuclear zinc complex can act as a molecular machine in several critical processes of cellular transportation, including capture of a phosphate derivative outside living cells, translocation through the cellular membrane, and release of the phosphate derivatives inside the cells (Figure 1). This method allows the rapid delivery of a variety of phosphorylated peptides as well as the phosphorylated fluorogenic probes without any modification, thus facilitating the exploration of their biological activities and functions in the cellular context.

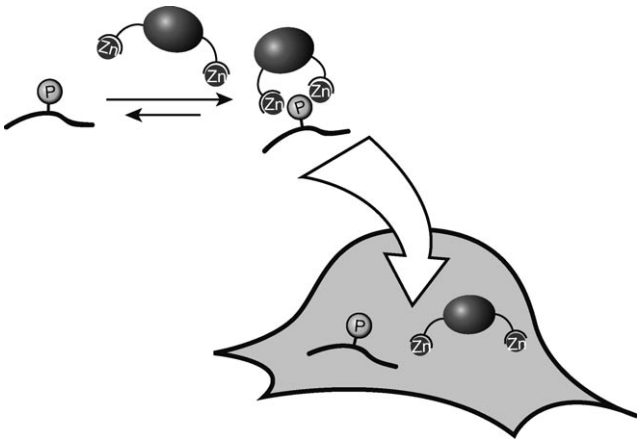

Figure 1. Intracellular delivery of an anionic phosphate derivative using the Dpa–Zn^{II} complex as a carrier vector.

We have recently revealed that the binuclear 2,2′-dipicolylamine (Dpa)–Zn^{II} complex is an excellent binding motif for phosphate anions in neutral aqueous solution.[4] Taking advantage of its selective binding, various binuclear Dpa–Zn^{II} receptors have been developed as the fluorescent chemosensors for phosphorylated peptides/protein and nucleoside pyrophosphate such as ATP (Scheme 1 A).[5] During the course of these studies, we found that a metalloreceptor **2**–2Zn^{II} possessing a pyrene fluorophore smoothly penetrated the cell membrane and was present in the cytosol of living cells (Figure 2 A). This observation prompted us to investigate whether the Dpa–Zn^{II} derivatives can serve as a carrier for phosphorylated anion derivatives. Prior to the intracellular transportation study, the binding ability of **1**–2Zn^{II} with various phosphate species was confirmed by isothermal titration calorimetry (ITC). The binding affinity for the phosphate species such as phenyl phosphate and the phosphorylated peptide **a** was determined to be 2.2×10^5 and $6.3\times10^4\ M^{-1}$, respectively, under the neutral aqueous conditions (50 mM HEPES, pH 7.2).

The intracellular delivery of phosphate anion species was subsequently conducted using the pyrene-appended **2**–2Zn^{II} as a carrier (Figure 2). A mixture of **2**–2Zn^{II} (3 μM) and the fluorescein-labeled phosphorylated peptide **b** (1 μM) in HBS buffer was preincubated with HeLa cells for 10 min, and the cellular uptake of peptide **b** was evaluated by the fluorescence detection using confocal laser scanning microscopy (CLSM). Unfortunately, both of the fluorescence of peptide **b** and **2**–2Zn^{II} were scarcely observed inside the living cells (data not shown). This result implies that the cell permeability of **2**–2Zn^{II} is greatly suppressed by the formation of the binding complex with the phosphorylated peptide **b**. To improve the carrier ability of the Dpa–Zn^{II} derivatives, the tetranuclear zinc complex **3**–4Zn^{II} that is more cationic compared to the binuclear **2**–2Zn^{II} was redesigned (Scheme 1 A).[6] The hydrophilic ethylene glycol side chain of **3**–4Zn^{II} was also introduced to suppress the aggregation of **3**-4Zn^{II} binding to phosphate derivatives.[7] As shown in Figure 3 B, **3**–4Zn^{II} also showed cell permeability, similar to **2**–2Zn^{II}. In the delivery experiment in HBS buffer, the strong fluorescence of peptide **b** was clearly detected in cytosol (Figure 2 C) in sharp contrast to the case of the binuclear **2**–2Zn^{II}, indicating that peptide **b** was successfully delivered into living cells by **3**–4Zn^{II}.[8] The delivery was strictly inhibited by addition of pyrophosphate anion ($H_2P_2O_7^{2-}$, data not shown), a strong binder for the Dpa–Zn^{II} unit,[9] and the corresponding nonphosphorylated peptide **c** was scarcely delivered by **3**–4Zn^{II} (data not shown). These results suggest that the binding of **3**–

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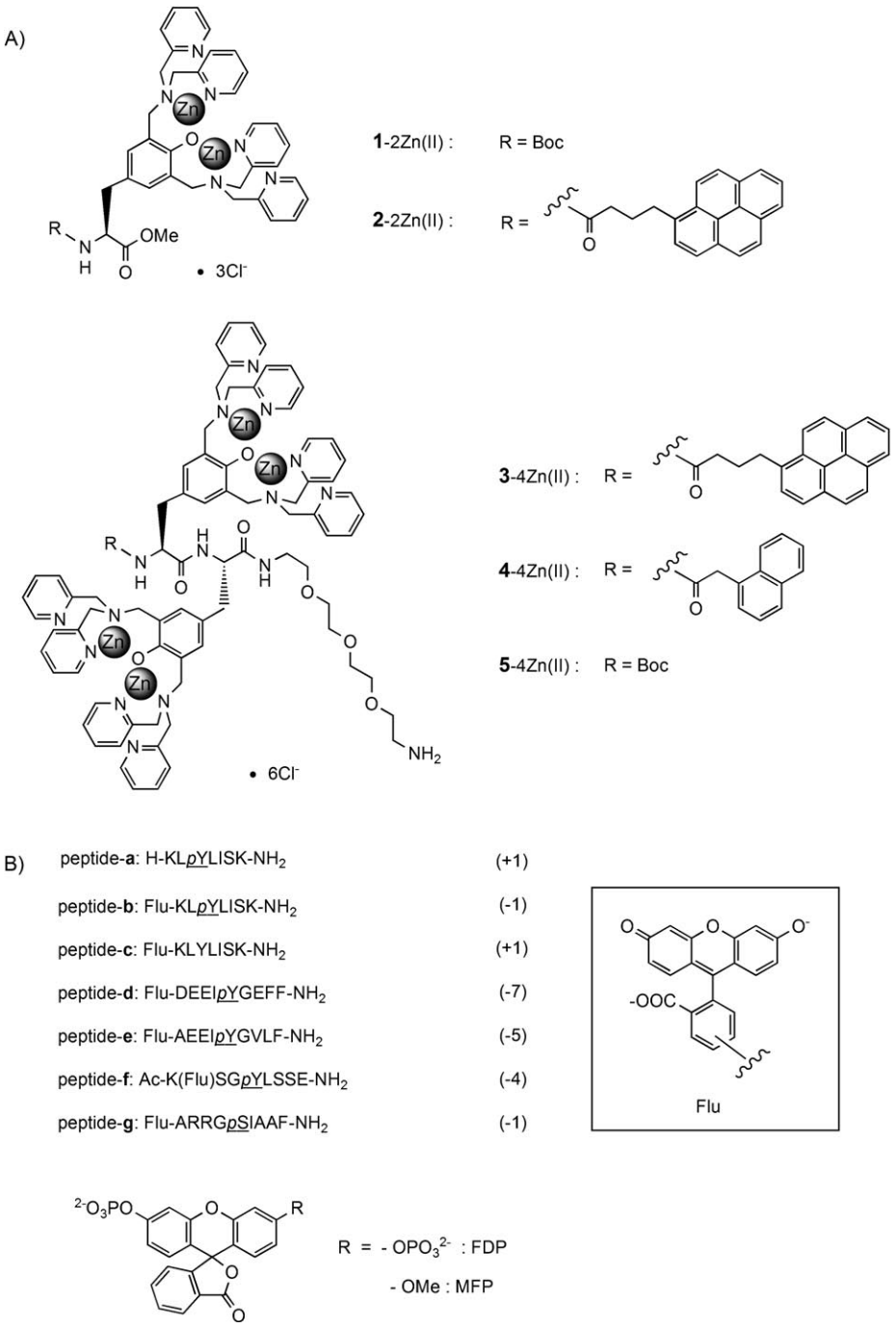


Scheme 1. A) Molecular structures of the Dpa–Zn^{II} complexes. B) Amino acid sequences of the peptides (top) and the structures of the phosphorylated fluorescein derivatives (bottom). The number in the parenthesis indicates the net charge of each peptide. pY and pS represent *O*-phosphorylated tyrosine and serine, respectively. FDP = fluorescein diphosphate tetraammonium salt, MFP = 3-*O*-methylfluorescein phosphate cyclohexylammonium salt.

$4Zn^{II}$ with peptide **b** through the metal–phosphate interactions is crucial for the intracellular delivery of the phosphorylated peptide. The cellular uptake of peptide **b** was quantitatively evaluated by fluorescence activated cell sorting (FACS) analysis. As shown in Figure 3A, the cellular uptake of peptide **b** was largely enhanced by **3**–$4Zn^{II}$. Such enhancement was effectively suppressed by pyrophosphate anion (PPi, Figure 3B), and the cellular uptake of the non-phosphorylated peptide **c** was not significantly enhanced by **3**–$4Zn^{II}$ (Figure 3C). These agree well with the results obtained in the CLSM studies. The cellular uptake of peptide **b** was also enhanced by the carrier **3**–$4Zn^{II}$ in other cell lines such as CHO and L6 (Figure S1), indicating the general utility of this method. Interestingly, the hydrophobic substituent of the carrier greatly influenced the delivery efficiency (Figure 3D). That is, the pyrene-appended **3**–$4Zn^{II}$ delivered peptide **b** most efficiently, whereas **4**–$4Zn^{II}$ and **5**–$4Zn^{II}$ which possesses a less hydrophobic naphthyl and *tert*-butoxycarbonyl (Boc) group on the N terminus showed moderate and weak delivery efficiency, respectively. These results suggest that the sufficiently lipophilic character and the highly cationic property of the carrier is important for the phosphate anion transport.[10] We observed that the treatment of HeLa cells with **3**–$4Zn^{II}$ under the delivery conditions did not affect cell viability, which was confirmed using the staining experiment with propidium iodide (PI) by FACS analysis (Figure S2). We also confirmed that the cells normally proliferated and kept a healthy state after the treatment of **3**–$4Zn^{II}$. Thus it is clear that **3**–$4Zn^{II}$ dose not exert serious cell toxicity.

The intracellular delivery was carried out by **3**–$4Zn^{II}$ for other phosphorylated peptides **d**, **e**, **f**, and **g**, each of which possesses a distinct number of net negative charges from −7 to −1 under neutral aqueous conditions (Scheme 1B). Figure 3E summarizes the mean fluorescence of the cells treated with the series of peptides in the presence or absence of the carrier **3**–$4Zn^{II}$. The fluorescence intensity of the cells in the presence of the carrier was four- to sixfold greater than that in the absence of the carrier in all cases. The enhanced uptake of these peptides by **3**–$4Zn^{II}$ was again confirmed by CLSM and FACS analysis (Figures S3 and S4). These data clearly demonstrate the good substrate accept-

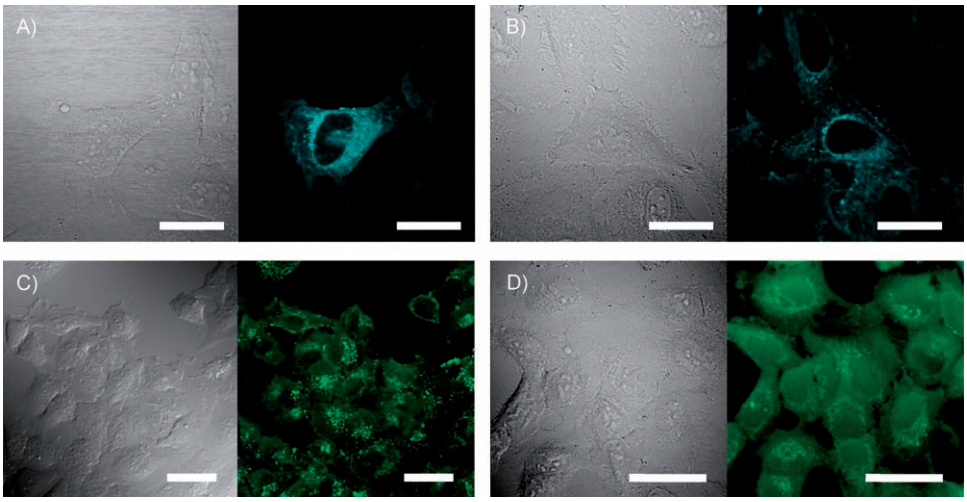
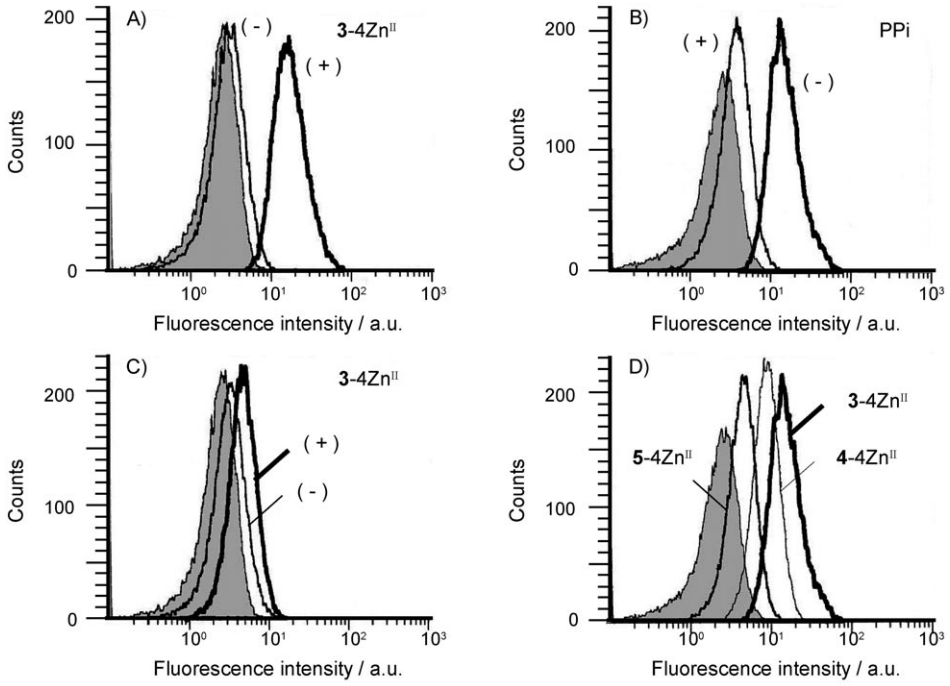


Figure 2. Fluorescence microscopy analysis of the intracellular transportation of the phosphate derivatives using the Dpa–Zn^{II} complexes as the carriers. The left and right panels show the differential interference contrast (DIC) and confocal laser scanning micrographs of HeLa cells, respectively. Pyrene emission of the cells incubated with A) **2**–$2Zn^{II}$ or B) **3**–$4Zn^{II}$ excited at 350 nm. Fluorescein emission of C) peptide **b** and D) FDP delivered into living cells excited at 488 nm. Scale bar: 30 μm in panels A and B;, 50 μm in panels C and D.



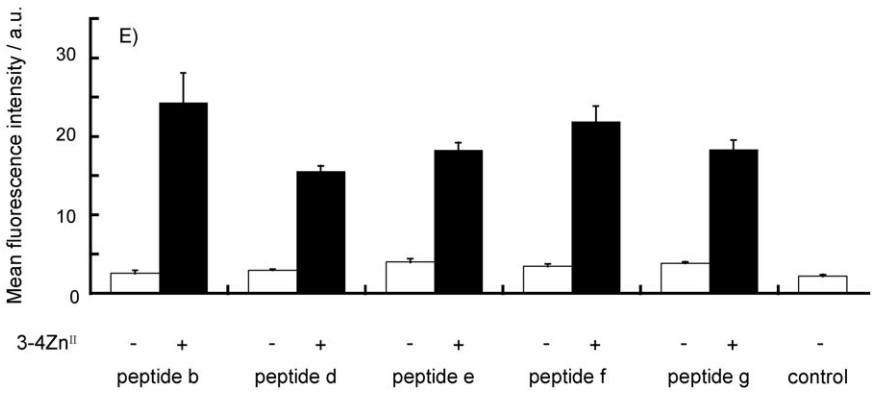


Figure 3. FACS analysis of the cellular uptake of the fluorescein-appended peptides into HeLa cells. The cells were incubated with A) peptide **b** in the presence or absence of **3**–$4Zn^{II}$, B) peptide **b** and **3**–$4Zn^{II}$ in the absence or presence of pyrophosphate anion (PPi), C) peptide **c** in the presence or absence of **3**–$4Zn^{II}$, D) peptide **b** in the presence of **3**-4-Zn^{II}, **4**–$4Zn^{II}$, or **5**–$4Zn^{II}$. The shaded area indicates the control cells treated with HBS buffer in each graph. E) Mean fluorescence of cells incubated with 1 μM of the peptide in the presence or absence of 3 μM of **3**–$4Zn^{II}$ for 10 min at 37 °C. The control cells were treated with HBS buffer before FACS analysis.

ability of this delivery method. The transportation experiments were also conducted with the phosphorylated organic molecules such as fluorescein diphosphate (FDP) and *O*-methyl-fluorescein monophosphate (MFP), both of which are cell impermeable fluorogenic probes for phosphatase enzymes (Figures 2D and S3E).[11] In these cases, the strong fluorescence due to the fluorescein gradually increased in cytosol over one hour after incubation. This observation indicates that the delivered nonfluorescent FDP or MFP in cytosol is hydrolyzed by intracellular phosphatases to generate a fluorescent species. This result also suggests that FDP and MFP were released from the binding complex with the carrier **3**–$4Zn^{II}$ inside the cells so that they serve as phosphatase substrates. The release may be caused by the competitive replacement of FDP or MFP with the intracellularly abundant phosphate species such as ATP.[12,13]

To determine whether endocytosis is predominant or not in the cellular uptake of the phosphorylated derivatives, the delivery experiment was conducted at 4 °C. The FACS analysis showed that the delivery efficiency of peptide **b** was significantly reduced at 4 °C as compared to room temperature (Figure S5). Furthermore, the CLSM observation revealed that the

fluorescent particles of the delivered peptide were similar in location to cytosolic endosome, which was fluorescently stained with SynaptoRed (Figure S6). These two results suggest that endocytosis is a major pathway for the cell entry of the phosphorylated derivatives enhanced by the Dpa–Zn^{II} carriers.[14]

In conclusion, we have developed a unique intracellular delivery method for the anionic phosphorylated derivatives using the Dpa–Zn^{II} metalloreceptor as a carrier vector. The designer carrier **3**–4Zn^{II} efficiently delivered a variety of phosphorylated peptides and fluoregenic organic probes in a rapid and convenient manner. The simple operation and rapid delivery of the present method demonstrate its utility in the transport of phosphorylated derivatives. Further study to address transportation ability for a wider variety of phosphorylated derivatives and application in biological research will be forthcoming.

Keywords: anions · drug delivery · molecular recognition · noncovalent interaction · phosphorylation

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[12] The binding affinity (K_{app}, M^{-1}) of **1**–2Zn^{II} for ATP was evaluated to be in the order of 10^6 by ITC measurement (data not shown), the value of which is larger than that of the monophosphorylated species such as peptide **b** ($K_{app} = 6.3 \times 10^4\ M^{-1}$) or phenyl phosphate ($K_{app} = 2.2 \times 10^5\ M^{-1}$).

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[14] The fluorescence of peptide **b** was also observed in the cytosol region (Figure 3C and Figure S4), suggesting that this peptide was effectively released from endosome. On the other hand, the endosome-like punctuates remained predominant in the case of peptides **d**–**g** (Figure S1), indicating that the escape from endosome was less effectively induced.

Received: October 22, 2007
Published online on February 5, 2008

