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Protein Transduction

Collagen-like Cell-Penetrating Peptides**

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The introduction of bioactive macromolecules, such as peptides and proteins, into living cells as cell-penetrating peptide (CPP)-conjugated forms has been established as a standard method in current cell biology, thereby replacing the use of electroporation or semi-permeabilized cells.[1] Typical CPPs are Arg-rich peptides such as oligoarginine and the HIV-1 Tat (49–57) peptide (RKKRRQRRR). These CPPs can transport various types of cargos into cells, including low-molecular-weight compounds, proteins, and nanoparticles, which otherwise do not penetrate cell membranes.[2] An Arg cluster on the peptides is crucial for their efficient cellular uptake.[3,4] However, there are two critical drawbacks of the Arg-rich CPPs for applications in vivo. One is the instability against attack by proteases in the bloodstream.[5] The other is the significant binding to serum proteins,[6] which lowers the availability of the CPP to target cells.

Herein, we aimed to overcome the drawbacks of the conventional CPPs by employing a rigid collagen-like triple-helical scaffold. The collagen triple helix is a unique tertiary structure found in the collagen family of proteins. In the triple helix, the three peptide strands adopt a polyproline-II-like left-handed helix and are intertwined with a one-residue stagger.[7] By virtue of this unique structure, native collagen shows extreme stability against attack by general proteases other than collagenases.[8]

We have designed four covalently tethered heterotrimeric peptides with collagenous Gly-Pro (or D-Pro)-Xaa sequences (Figure 1a). Xaa is L-4-hydroxyproline (abbreviated as O) or L-Arg. Because oligoarginine, a typical CPP, effectively crosses the cell membrane especially when the number of Arg residues is 7–9, peptides **1**, **2**, and **3** were designed to possess nine Arg residues per molecule.[9]

To tether three peptide strands, we employed Moroder's cysteine-knot system, which can control the one-residue staggering found in the native collagen triple helix.[10] Heterotrimer **1** was designed so as to axially align all the Arg residues to one side of the triple helix. In contrast, peptide **2** was designed to have Arg residues radiate in all directions from the axis of the triple helix. In peptide **3**, six D-Pro residues were introduced. The introduction of D-Pro was expected to destabilize the triple-helical conformation. A similar trimeric peptide **4** possessing only one Arg residue and a conventional CPP with the octaarginine sequence (**R8**) were also synthesized as controls.

The constituent peptide chains were synthesized using a 9-fluorenylmethoxycarbonyl (Fmoc)-based protocol. Heterotrimer formation was accomplished by stepwise and regioselective disulfide bond forming reactions.[10,11] Briefly, the peptides containing both *S*-pyridinesulfenyl (Pys)- and *S*-acetamidomethyl (Acm)-cysteine residues were reacted with the second set of thiol-containing peptides to yield heterodimers. The *S*-Acm groups in the heterodimers were directly converted to the *S*-3-nitro-2-pyridinesulfenyl (Npys) group by using NpysCl. Afterward, the heterodimers with Npys-activated Cys residues were reacted with the third set of peptides carrying a free thiol group to yield the desired heterotrimers (see Scheme S1 in the Supporting Information).

The conformational states of the peptides **1**–**4** in aqueous solution were analyzed by circular dichroism (CD) spectroscopy. Peptides **1**, **2**, and **4** showed positive maxima around 225 nm at 4 °C, thus indicating that these peptides adopt the collagen-like triple-helical conformation (Figure 1b). The melting temperatures (T_m) for the triple helices of **1**, **2**, and **4** were estimated to be 57.5, 58, and > 60 °C, respectively. This result indicated that the peptides were fully triple-helical at 37 °C, at which the mammalian cells were cultured (Figure 1c). As expected, peptide **3** containing D-Pro residues did not show the positive signal around 225 nm, thus indicating a random-coil conformation (Figure 1b).

Cellular uptake of **1** to **4** and **R8** labeled with fluorescein at the N terminus (named **flu-1** to **flu-4**, and **flu-R8**) by HeLa cells was examined by using confocal laser scanning microscopy (CLSM) and fluorescence-activated cell sorting (FACS) analysis (Figure 2). Significant intracellular fluorescent signals were observed in the cells treated with **flu-1** and **flu-R8** by CLSM (Figure 2a). Weaker but significant intracellular fluorescent signals were also detected in the cells treated with **flu-2**. Conversely, the intracellular fluorescence signals of **flu-3** and **flu-4** were very weak and nearly undetectable, respectively.

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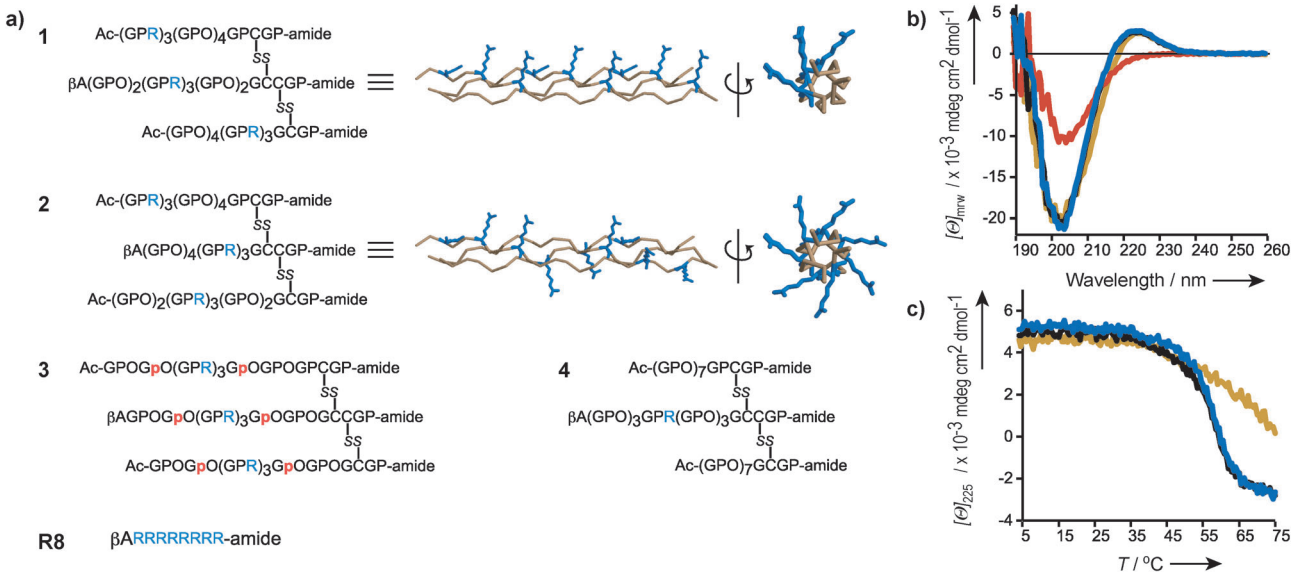

Figure 1. Triple-helical peptides with different arrangements of Arg residues and their conformational analysis. a) The sequences and 3D models of the peptides. Each peptide has an N-terminal β-alanine for conjugation of fluorescein or biotin. The 3D models were created by processing the PDB file 1k6f with the Swiss-PdbViewer software. "O" and "p" (in red) denote L-4-hydroxyproline and D-proline residues, respectively. In the 3D models, Arg residues are shown in blue. b) CD spectra recorded at 4 °C. $[\theta]_{mrw}$ = mean residue ellipticity. c) Thermal melting curves of the triple helices. Blue: **1**, black: **2**, red: **3**, yellow: **4**.

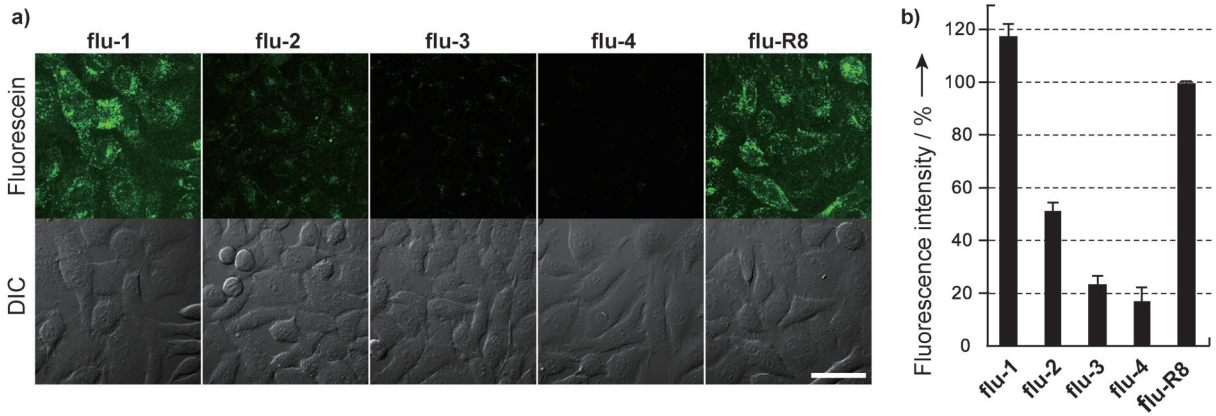

Figure 2. Cellular uptake of fluorescein-labeled Arg-rich heterotrimers. a) CLSM images of HeLa cells after treatment with 10 μM of fluorescein-labeled Arg-rich heterotrimers and **R8** in minimum essential medium (α-MEM) containing 10% bovine serum (BS) for 3 h at 37 °C. Scale bar is 50 μm. DIC = differential interference contrast. b) FACS analysis of the total cellular uptake of the peptides. The cells were treated with peptides (10 μM) for 30 min at 37 °C in the same medium as in (a) and the total cellular uptake was quantified (mean ± standard deviation (S.D.), $n = 3$).

Quantitative analysis by FACS showed that **flu-1** has a comparable cell-internalizing activity to **flu-R8**. The cellular uptake of **flu-2** was approximately 50% lower than that of **flu-1** (Figure 2b). These data indicate that both the number of Arg residues and the triple-helical conformation were critical for the cellular uptake of the collagen-like peptides. The high density or clustering of Arg side chains on the triple-helix surface was also strongly suggested to be important for the efficient cellular uptake.

The cells treated with **flu-1**, **flu-2**, and **flu-R8** for three hours exhibited punctate fluorescence in the cells (Figure 2a). This suggested that these peptides were taken up by endocytosis and the majority of them were trapped in the endosomes before translocating to the cytosol. Because octaarginine was reported to accumulate on plasma membranes by interaction with heparan sulfate glycosaminoglycans (HSPGs), thereby leading to cellular uptake by endocytosis (or macropinocytosis),[12] we next investigated the correlation between cell internalization efficacy and heparin-binding affinity of the peptides. The affinity was estimated by using heparin affinity chromatography. The retention times obtained by HPLC [15.2 min (**1**), 12.1 min (**2**), 11.5 min (**3**), 2.8 min (**4**), 23.8 min (**R8**)] reflect the heparin affinities of the different collagen-like CPPs. The affinities of heterotrimers **1**–**4** to heparin positively correlated with the internalization efficacy, thus indicating the contribution of cell-surface-HSPG binding to the initial step of the cellular uptake. In addition, the most efficient uptake of **flu-1** was

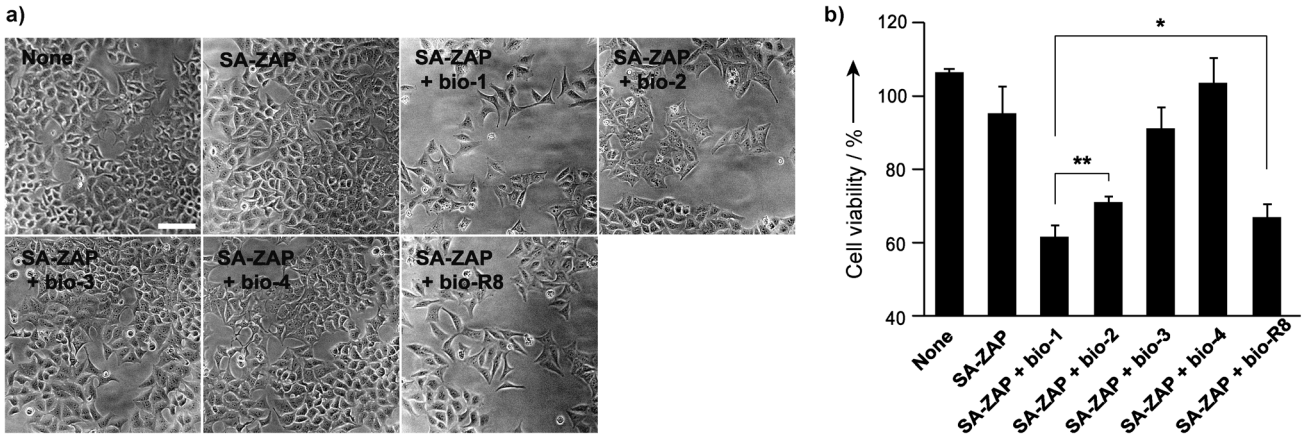

Figure 3. Macromolecular-cargo delivery by Arg-rich heterotrimers. a) Light microscopy images of HeLa cells treated with biotinylated peptide/**SA-ZAP** complexes. After preparing complexes of the **SA-ZAP** and the biotinylated peptides in phosphate-buffered saline (PBS), cells were incubated with the complexes of each in Dulbecco's modified Eagle's medium (DMEM) containing 2% fetal bovine serum (FBS) for 48 h at 37°C. Final concentrations of the **SA-ZAP** and the biotinylated peptides were 10 and 40 nM, respectively. Scale bar is 250 μm. b) Cell viability was assessed by using the Cell Counting Kit-8 after the treatment of the complexes (mean ± S.D., $n = 5$). * and ** denote $p < 0.05$ and $p < 0.001$, respectively, determined by the two-tailed paired Student's t-test.

attributed to the spatial organization of the Arg residues on the peptide that led to optimal interaction with HSPGs on the cell membrane.

To evaluate cargo delivery into the cells by Arg-rich collagen-like CPPs, we prepared a macromolecular complex consisting of a streptavidin-saporin conjugate (**SA-ZAP**, approximately 120 kDa) and a biotinylated CPP by using the specific interaction between streptavidin and biotin (N-terminally biotinylated **1** to **4** and **R8** were named **bio-1** to **bio-4** and **bio-R8**, respectively). Because saporin induces cytotoxicity after entering into the cytosol, the efficacy of the cargo delivery to the cytosol can be estimated by counting viable cells.[13]

Accordingly, the complex of **SA-ZAP** and **bio-1** exhibited a cytotoxic effect comparable to that of **bio-R8**, thus indicating an efficient delivery of the macromolecular cargo (Figure 3). The complexes of **bio-3** and **bio-4** showed less cytotoxicity, and the cytotoxic effect of the complex of **bio-2** was lower than that of **bio-1**. These results indicated that the number of Arg residues and the triple-helical conformation were critical to the cargo delivery, and the density or spatial arrangement of Arg residues also contributed to the efficacy. This finding was in accord with the data shown in Figure 2.

The Arg-rich collagen-like peptides themselves showed negligible cytotoxicity for HeLa cells after treatment for 48 h at a peptide concentration of 20 μM in DMEM containing 2 or 10% FBS (Table S6 in the Supporting Information). The hemolytic response for all Arg-rich heterotrimers was also negligible up to 40 μM (Figure S4 in the Supporting Information).

The stability of the Arg-rich heterotrimers in human serum was examined in vitro by using reverse-phase (RP) HPLC analysis (Figure 4). The triple-helical heterotrimers **1**, **2**, and **4** exhibited a much higher stability (half-life > 1 day) than **R8**. In contrast, heterotrimer **3**, a single-chain counterpart, was almost completely degraded within 15 min. This

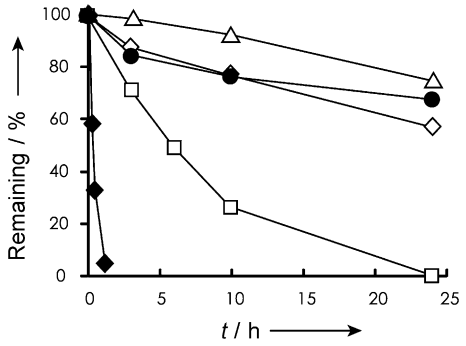

Figure 4. Stability of the Arg-rich heterotrimers in human serum. Intact-peptide concentrations were determined from corresponding peak areas on RP-HPLC chromatograms after incubation at 37°C in 90% serum in PBS at the time intervals indicated. **1** (●), **2** (◇), **3** (◆), **4** (△), **R8** (□).

result showed that the triple-helical CPPs were extremely resistant against degradation by serum proteases.

Because adsorption of the cationic CPPs on serum proteins is considered to be one of the major impediments for applications in vivo,[6] we further assessed the serum-protein binding properties of the peptides. The fluorescein-labeled peptides (**flu-1**–**flu-4** and **R8**) were incubated with BS in α-MEM, followed by passing the samples through an ultrafiltration unit with a molecular weight cutoff of 30 kDa.[6] The relative amounts of the peptides adsorbed to serum proteins were estimated by comparing filtered and unfiltered fractions. The protein binding values of **flu-1** and **flu-2** with cell-penetrating activity were lower than that of **flu-R8** (Table 1). This result could be accounted for by the backbone flexibility of the peptides: the positively charged guanidino groups of **flu-R8** could fit into the negatively charged surfaces of serum proteins, because this peptide has a flexible backbone, whereas some guanidino groups fixed on the rigid

Table 1: Serum-protein binding of fluorescein-labeled Arg-rich collagen-like CPPs [%].

flu-1	flu-2	flu-3	flu-4	flu-R8
41.4	37.5	29.1	2.2	61.6

triple-helical scaffold should stick out and interfere with binding to the surface of serum proteins, thereby leading to lower binding affinities.

In addition to the general peptidic backbone, some other particular backbone structures, such as β-peptides,[14] peptoids,[15] oligocarbamates,[16] and polyproline helices,[17] have been employed as scaffolds that construct cationic cell-penetrating molecular tools. Herein, we employed the collagen-like triple helix as the scaffold for cationic CPPs. The activity of the Arg-rich triple-helical CPPs in delivering a macromolecular cargo into cells was comparable to that of conventional Arg-rich CPPs, and negligible cytotoxicity and hemolytic activities were observed. By virtue of the rigid scaffold, two important requirements for the practical application of cationic CPPs have been fulfilled: sufficient HSPG-binding enabling efficient cellular up-take and low adsorption onto serum proteins. In addition, the triple-helical CPPs are extremely stable in animal serum. Such unique properties are expected to be advantageous to long-term applications in cell culture systems and to in vivo drug delivery.

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