Synthesis and Transport Activities of HIV–Tat Peptide-modified Cholesterol Pullulan

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A complex in which the cell-penetrating peptide HIV–Tat and cholesterol pullulan were conjugated through a disulfide bond was synthesized. The complex showed marked cell penetrating activities as a vector.

Cell-penetrating peptides are useful tools for the delivery of nucleic acids, proteins, and drugs into cells. In particular, the peptide fragment of the Tat protein derived from HIV 1 is known as a cell-penetrating peptide that has a high introduction rate as a vector. The Tat protein consists of 86 amino acid residues that are divided into three regions: an acidic N-terminal region for transcriptional activity, a cysteine-rich region for DNA binding, and a basic region for nuclear transfer activity. The minimum basic region of residues 49–57 (Tat peptide) is involved in stimulating cell penetration.[1] The Tat peptide conjugates with an antisense oligonucleotide via an S–S bridge, which enhances the delivery to the nucleus without interfering with the base-pairing function of antisense oligonucleotides,[2] and the plasmid DNA that is incorporated into the liposome modified on its surface by the Tat peptide enhances the expression of the gene.[3]

Cholesterol pullulan (CP), in which maltotriose moieties are partially modified by cholesterol, is unique in forming self-assembled nanoparticles (20–30-nm diameter) in water.[4] The nanoparticles consist of a hydrogel structure that is bridged noncovalently at the hydrophobic cholesterol moieties. CP can encapsulate proteins as well as stabilize enzymes and carry drugs.[5] It has been reported that complexes of CP and antigenic proteins of tumor showed highly potent activities as antigens.[6]

The combined characteristics of the HIV–Tat peptide and CP are considered to be promising for the development of not only effective vectors for protein without toxicity but also non-viral vectors for genes. In this paper, a conjugate of HIV–Tat and CP was synthesized, and its ability to transfect genes and introduce a fluorescently labeled protein into cells was evaluated.

A fully protected HIV–Tat–(48–57)–Cys(Acm)–Gly–NH–Linker (Linker: 2,4-dimethoxy-4′-carboxymethoxybenzhydryl) **1** was synthesized by the Fmoc/*t*-Bu solid-phase method using {4-[amino(2,4-dimethoxyphenyl)methyl]phenoxy}acetoxy-2-chlorobenzhydryl resin (Rink–Bernatowicz-amide Barlos Resin)[7] followed by treatment with 25% 1,1,1,3,3,3-hexafluoro-2-propanol/dichloromethane for 1 h at room temperature. Peptide **1** was converted to the corresponding Cys(Snm) peptide **2** [Snm: (*N*-methyl-*N*-phenylcarbamoyl)sulfenyl] by treatment with (chlorocarbonyl)sulfenyl chloride (4 equiv) for 15 min followed by treatment with *N*-methylaniline (10 equiv) for 15 min in dichloromethane.[8] The sulfanyl group was introduced to the hydroxy groups of CP by acylation with Trt-3-sulfanylpropionic acid (1 equiv with respect to CP; Trt: trytyl) using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and 4-dimethylaminopyridine followed by treatment with TFA. The resulting 3-sulfanylpropionyl-CP (CP-Joint, **3**) was coupled with Cys(Snm) peptide **2** (1 equiv with respect to 3-sulfanylpropionyl-CP) in 1% 4-methylmorpholine/DMF to form the disulfide bridge, and the protecting groups of the peptide were removed by treatment with TFA to afford CP–Tat conjugate **4** (Figure 1). Amino acid analysis showed that Tat peptide was introduced to every 5th glucose residue of CP. Compound **4** was easily soluble in water. The dynamic light scattering and ζ-potential of compound **4** indicated a particle diameter of approximately 80 nm and a positive charge of 20 mV in water.

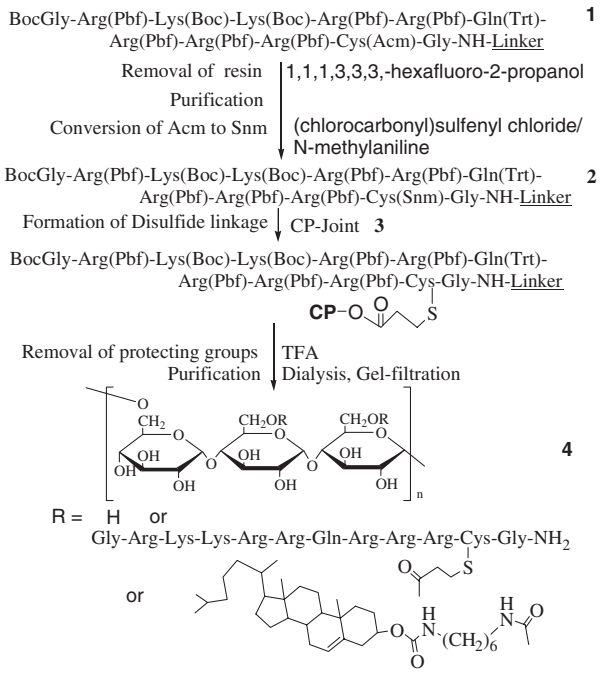

Figure 1. Synthetic scheme of CP–Tat. Pbf: 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl, Acm: acetamidomethyl.

CP is neutral and genes are anionic, and therefore, an efficient gene vector cannot be prepared by electrostatic force. Recently, Akiyoshi et al. reported that a cationic CP complex with oligo-DNA exhibits increased melting temperature and DNA strand exchange.[9] We expected that CP–Tat would be a good vector for genes, namely, that it would be safe and could penetrate into the cytoplasm and nucleus. In this paper, we investigate the transfection of plasmid CMV–Luc into COS7 cells with CP–Tat.

Various amounts of CP–Tat were mixed with the luciferase reporter plasmid pCMV–Luc (1 μg), and these were transfected to COS7 cells (2×10^5 cells/35-mm dish) in serum-free Opti-MEM I medium (Gibco^R, Invitrogen). Luciferase activities were measured 24 h after transfection and in vitro cytotoxicity was de-

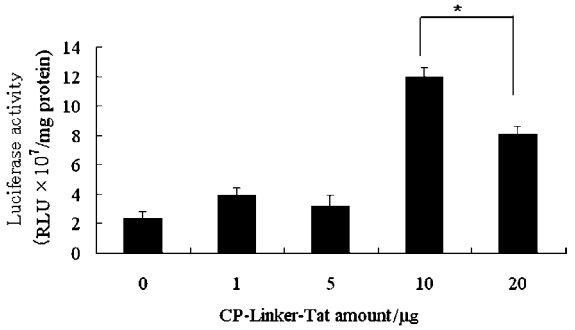

Figure 2. pDNA transfection efficiency of CP–Tat. CP–Tat and pCMV–Luc (1 μg) complexes were transfected into COS7 cells (2×10^5 cells) using serum-free Opti-MEM I medium. Luciferase activity was measured 24 h after transfection. Data represent mean ± S.E ($n = 3$). $^*P < 0.1$.

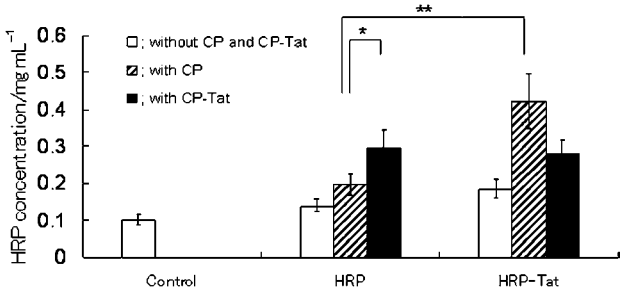

Figure 3. Cellular uptake efficiency of HRP and HRP–Tat with CP and CP–Tat. HRP (100 μg) and HRP–Tat (100 μg) were introduced into COS7 cells with or without CP (600 μg) and CP–Tat (600 μg) by incubating for 4 h in serum-free medium. HRP concentrations were determined using a TMB Microwell Peroxidase Substrate System. Data represent the mean ± S.D ($n = 3$). $^*P < 0.1$ $^{**}P < 0.02$.

termined based on survival rates measured by MTT assay. CP–Tat elicited remarkable cytoplasmic luciferase activity compared to the control (Figure 2)[10] and showed 100% survival (data not shown). The best DNA expression efficiency in COS7 cells was obtained when the weight ratio of plasmid DNA to compound **4** was 1:10.

CP self-aggregates in water to form nanoparticles and transports encapsulated proteins. CP–Tat was expected to be a vehicle that carried proteins into cells. Cellular uptake efficiency of horseradish peroxidase (HRP) with CP and CP–Tat **4** was also examined (Figure 3). HRP–Tat was synthesized as follows. Compound **1** (15 mg, 10 μmol) was treated with TFA and the resulting Cys(Acm)–peptide was allowed to react with $AgNO_3$ (45 μmol) in aqueous acetic acid at 4 °C for 1 h. Dithiothreitol (138 mg, 0.89 mmol) was added and left to stand under N_2 gas overnight. The reaction mixture was evaporated and the residue was applied to a column of Sephadex G-25 using 0.1 M acetic acid as a solvent to afford the SH-peptide **5**. Compound **5** and 3-(2-pyridyldithio)propionyl–HRP[11] (10 mg) were assembled in 0.1 M acetic acid then purified by Sephadex G-25 and dialysis followed by lyophilization to afford HRP–Tat (Figure 4).[12] HRP

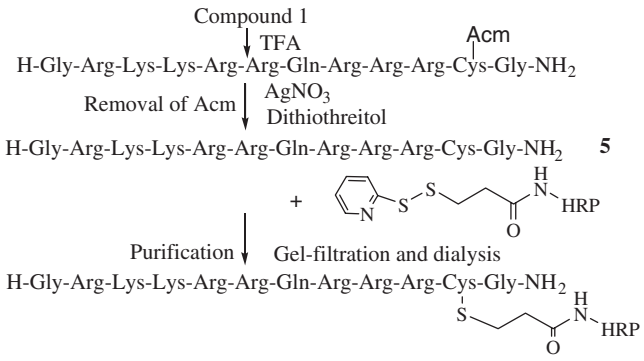

Figure 4. Synthetic scheme of HRP–Tat.

(100 μg) and HRP–Tat (100 μg) introduced into COS7 cells with or without CP (600 μg) and **4** (600 μg) by incubating for 4 h in serum-free medium. The HRP concentration was determined with a TMB (3,3′5,5′-tetramethylbenzidine) Microwell Peroxidase Substrate System (KPL, Gaithersburg, USA). Based on this assay, HRP with compound **4** penetrated into the cells with high efficiency. Tat-modified HRP with CP showed the highest penetration, but in this case it was doubtful whether CP contributed to cell penetration. On the other hand, the combination of CP–Tat and HRP–Tat was not highly efficient due to the repulsion of Tat conjugated with each molecule not to form a complex.

In summary, CP–Tat conjugate was synthesized, and its efficient properties as a vector for DNA and protein were shown. Based on these results for CP–Tat conjugate, new developments in drug delivery are anticipated.

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