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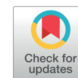
Drug delivery using polyhistidine peptide-modified liposomes that target endogenous lysosome



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ABSTRACT

Cell-penetrating peptides (CPPs) can deliver payloads into cells by forming complexes with bioactive molecules *via* covalent or non-covalent bonds. Various CPPs have been applied in CPP-modified liposomes, and their effectiveness is highly regarded in liposomal drug delivery systems (DDSs). Previously, we have reported on the polyhistidine peptide (H16 peptide: HHHHHHHHHHHHHHHH-NH_2) as a new CPP. The H16 peptide has a higher cell-penetrating capacity than well-known CPPs and delivers small molecules such as fluorescent dyes, bioactive peptides, and proteins into mammalian cells. However, it is not known whether the H16 peptide can deliver large cargos such as liposomes into cells. To assess the potential of the H16 peptide, in this study, we developed H16 peptide-modified liposomes (H16-Lipo) and evaluated their effectiveness in a liposomal DDS. The H16-Lipo was prepared by inserting a stearyl-H16 peptide into the hydrophobic region of a liposome. The H16-Lipo was internalized into human fibrosarcoma cells *via* multiple endocytosis pathways and localized to intracellular lysosomes. Based on this result, we used the H16-Lipo as a lysosome-targeting DDS. The H16-Lipo delivered alpha-galactosidase A (GLA), one of the lysosomal enzymes, to intracellular lysosomes and improved the proliferation of GLA-knockdown cells. These results suggest that the H16-Lipo is an effective drug carrier for lysosomal enzymes in a lysosome-targeting DDS. The loss of lysosomal enzymes has been known to induce metabolic disorders, called lysosomal storage diseases (LSDs). Our findings indicate that this combination of the H16 peptide and a liposome is a promising candidate as a DDS for the treatment of LSDs.

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1. Introduction

Cell-penetrating peptides (CPPs) have been extensively studied as promising delivery tools for the cellular delivery of bioactive molecules such as peptides, proteins, and nucleic acids [1]. A significant number of CPPs have been reported to date. For example, HIV-Tat (48–60) (TAT peptide: GRKKRRQRRRPPQ-NH_2) [2], and octa-arginine peptide (R8 peptide: RRRRRRRR-NH_2) [3] are major CPPs. These CPPs deliver bioactive molecules into the cytoplasm effectively by forming complexes with bioactive molecules *via* covalent or non-covalent bonds. For example, TAT peptide has delivered peptide nucleic acids into cells *via* covalent bonds [4]. However, fatty acid-modified R8 peptide has delivered siRNA into cells *via* non-covalent bonds [5]. Moreover, nano-capsule carriers such as liposomes encapsulating bioactive molecules have been also delivered into the cytoplasm using CPPs [1].

Liposomes are one of the ideal drug carriers which were discovered in the 1960s [6]. Because liposomes consist of phospholipids, which are components of the cell membrane and biological membranes, they are a highly biocompatible drug carrier. Moreover, since liposomes have both hydrophilic and hydrophobic regions, they can entrap hydrophilic and hydrophobic drugs. In addition, liposomes can be easily chemically modified on their surface. Therefore, liposomes have been noted as a promising drug delivery system in the medical field [7].

Recently, CPP-modified liposomes have been extensively investigated and developed as effective drug delivery systems

Abbreviations: CPPs, cell-penetrating peptides; DDSs, drug delivery systems; GLA, alpha-galactosidase A; LSDs, lysosomal storage diseases; MEM, minimal essential medium; FBS, fetal bovine serum; DOPC, dioleoyl phosphatidylcholine; PBS, phosphate-buffered saline; MFI, mean fluorescence intensity; CPZ, chlorpromazine; CLSM, confocal laser scanning microscopy.

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(DDSs). For example, TAT peptide-modified liposomes [8] and R8 peptide-modified liposomes [9] have been reported as typical CPP-modified liposomes. These CPP-modified liposomes are internalized into the cell *via* the endocytosis pathway [8,9]. Moreover, CPP-modified liposomes have been widely studied as DDSs. For example, R8-modified liposomes have been used to deliver nucleic acids or antitumor drugs such as taxol and paclitaxel into cells [9,10]. Therefore, liposomes modified using CPPs represent a powerful combination to achieve an effective DDS.

However, we have previously found that the polyhistidine peptide (H16 peptide: HHHHHHHHHHHHHHHH-NH_2), as a new CPP, has a higher cell penetrating ability against mammalian cells than well-known CPPs. We have demonstrated that the H16 peptide delivers small molecules such as fluorescent dyes, bioactive peptides, and proteins into mammalian cells *via* covalent bonding [11]. Furthermore, the H16 peptide showed stable cellular uptake in the presence or absence of serum, whereas the presence of serum is known to dramatically suppress the cellular uptake of well-known CPPs, such as the R8 or the TAT peptide [12,13]. Our previous findings have indicated that the H16 peptide represents a promising candidate for a DDS in the medical and biotechnological fields. However, it is not known whether the H16 peptide can deliver macromolecules such as liposomes into mammalian cells.

In the current study, we synthesized H16 peptide-modified liposomes (H16-Lipo) and evaluated this liposomal DDS. The H16-Lipo was prepared by inserting a stearyl-H16 peptide (STR-H16) into the hydrophobic region of a liposome. Cellular uptake efficiency and the uptake mechanism of the H16-Lipo were determined. In addition, we determined the intracellular distribution of the H16-Lipo and applied it to an organelle-targeting DDS. Our findings in the present study expand the capability of the H16 peptide as a component of a promising DDS and indicate that the combination of the H16 peptide and liposomes is a good strategy for a unique DDS.

2. Materials and methods

2.1. Peptide synthesis and purification

The stearyl-polyhistideine peptide (STR-H16) was synthesized by Biologica (Shanghai, China) with stearic acid ($C_{17}H_{35}COOH$) labeling at the N-terminus and an amide group at the C-terminus.

The STR-H16 was purified to homogeneity using a Waters purification system (Waters, Milford, USA) and a reversed-phase COSMOSIL 5C_{18}-MS-II column (10 mm × 250 mm) (Nacalai Tesque, Kyoto, Japan). The column was run for 30 min at 1 mL/min with a linear gradient from 10 to 60% (v/v) acetonitrile in water containing 0.1% (v/v) trifluoroacetic acid. The molecular masses were confirmed using matrix assisted laser desorption/ionization time-of-flight mass spectrometry (Auto FlexII Bruker Daltonics, Billerica, USA).

2.2. Cell culture

HT1080 human fibrosarcoma cells were cultured in Eagle's minimal essential medium (MEM) containing 10% fetal bovine serum (FBS) (v/v), 100 μg/mL streptomycin, 100 units/mL penicillin, and 250 ng/mL amphotericin B. Cells were maintained at 37 °C in a humidified 5.0% CO_2 incubator and a subculture was performed every 3–4 days.

2.3. Preparation of the polyhistidine-modified liposomes

Liposomes were prepared through the lipid film hydration method. Lipid films were formed on the bottom of a glass tube by evaporating a chloroform solution containing 500 nmol dioleoyl phosphatidylcholine (DOPC) and 50 nmol cholesterol. After evaporation, 100 μL of 10 mM fluorescein in phosphate-buffered saline (PBS) (pH 7.4) was added to the lipid film, and mixed vigorously for 15 s to hydrate the lipids. The hydrated lipids were sonicated for 1 min in a bath-type sonicator (440 W, AS ONE, Osaka, Japan) and incubated for 30 min at room temperature in the dark to form liposome encapsulating fluorescein. Next, the liposome solution was subjected to five freeze-thaw cycles, and 400 μL of 10 mM fluorescein in PBS was added. Finally, to form liposomes of a predetermined diameter, the liposome solution was extruded 19 times through a Mini-Extruder (Avanti Polar Lipids, Alabaster, AL, USA) equipped with a poly carbonate membrane filter (100 nm pore size). Presized liposomes containing fluorescein and free fluorescein were separated using illustraTM MicroSpinTM S-300 H R Columns (GE Healthcare UK Ltd, England) for 2 min at 800 × *g*. Flow-through was collected and 1 mM liposome-encapsulated fluorescein was prepared.

Polyhistidine-modified liposomes, i.e. H16-Lipo, were prepared by incorporating STR-H16 into the surface of the liposomes prepared above. The liposomes were incubated with various concentrations (0–30 mol % of the total lipid) of STR-H16 for 1 h at room temperature in the dark.

2.4. Flow cytometric analysis

Flow cytometric analysis was performed as described previously [11]. Briefly, the HT1080 cells were incubated in media containing of the H16-Lipo (final concentration of DOPC: 100 μM) for predetermined periods at 37 °C and 5.0% CO_2. The cellular uptake of the H16-Lipo was evaluated through flow cytometric analysis, using the mean fluorescence intensity (MFI). Approximately 10^4 cells were collected per specimen with three repeats.

2.5. Endocytosis inhibition assay

To determine the endocytosis pathway involved in the cell uptake of H16-Lipo, chlorpromazine (CPZ), nystatin and amiloride were used as inhibitors against clathrin-mediated endocytosis, caveolae-mediated endocytosis and micropinocytosis, respectively. The HT1080 cells were precultured onto 6-well plates at a density of 5.0×10^5 cells/well in a final volume of 1 mL and incubated for 24 h at 37 °C and 5.0% CO_2. After pre-culture, the cells were pretreated using 50 μM of CPZ (30 min), 50 μM of nystatin (1 h), or 100 μM of amiloride (2 h). Then, the cells were washed twice using PBS and incubated with the H16-Lipo (final concentration of DOPC: 100 μM) for 3 h at 37 °C. The inhibitory effect of each inhibitor on the cellular uptake of the H16-Lipo was determined through flow cytometric analysis.

2.6. Confocal laser scanning microscopy (CLSM)

The cultured HT1080 cells were seeded onto a multi-well glass bottom dish (Matsunami Ind., Osaka, Japan) at a density of 2.0×10^4 cells/well in a final volume of 100 μL and incubated for 24 h at 37 °C and 5.0% CO_2. After complete adhesion, the cells were incubated with the H16-Lipo (final concentration of DOPC: 100 μM) for 24 h at 37 °C and 5.0% CO_2. After washing twice using PBS, fresh media containing 500 nM of LysoTracker Red (Life Technologies, CA, USA) was added to the cells and they were incubated for 2 h. Hoechst 33342 nuclear staining dye (Dojindo, Kumamoto, Japan) was also added to cells for 1 h. The intracellular distribution of the H16-Lipo was observed and fluorescence images were acquired using the Fluo View (Olympus, Tokyo, Japan).

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