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Self-assemblies of enzymatically degradable amphiphilic oligopeptides as nonviral gene carrier

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ABSTRACT

Novel biodegradable oligopeptide-type gene carriers composed of cationic residues (KRRRKRKRRRKRKRRC) and oligo leucine segments were developed. The amphiphilic carrier was found to form micelle-like assemblies in aqueous solutions, when the oligo leucine is 12 amino acids length (Pep-L12). NMR, CMC, and GPC analysis revealed their hydrophobic/cationic core/shell morphology. Hydrophobic interaction between leucines is thought to be the major driving force behind formations of assemblies. The transient expression of luciferase introduced to COS-1 cells using Pep-L12 below the CMC is as low as that by the control cationic peptides without leucine residue (Pep-L0), while improved transgene expression was observed in the case of Pep-L12 above CMC. The self-assembly raised the apparent molecular weight and gene transfection ability without loosening their low cytotoxicity. These results indicate that the amphiphilic oligopeptides are very promising materials as highly efficient and less toxic gene carriers.

1. Introduction

Polymeric gene carriers are now extensively studied due to their high abilities to deliver and protect pDNA, but the polyplexes formed between the carriers and pDNAs are sometimes too highly compacted to be recognized by transcription factors in nucleus. Recently, the destabilization of the polyplexes by conjugating hydrophilic or hydrophobic segments to polymeric carriers has been reported [1–3]. However, excess modification of side chains results in the low resistance to DNase at the same time.

Effect of the carrier molecular weights (Mws) has been also being studied [4–8]. Recently, high potential of low Mw polymeric carriers was attracting great attention. Kunath et al. reported that low Mw PEI (5 kDa) was much less toxic than high Mw PEI (48 kDa), and reporter gene expression of 5 kDa PEI was 3.7-fold higher than 48 kDa PEI in various cell lines [6]. Breuning et al. compared PEIs with the Mw of 1–9 kDa and showed that the highest reporter gene expression was obtained at 5.6 kDa, with low cytotoxicity. Schaffer et al. reported higher gene expression for low Mw PLL (19 and 36 residues) than high Mw PLL (180 residues) because of effective *in vitro* transcription and easy pDNA release [4]. Taken together, low cytotoxicity and high DNA releasing ability of low Mw carries were important key features for the high potential gene carriers. On the other hand, low Mw carriers are pointed out to reduce cellular uptake [9] and decrease stability of polyplexes at the same time. Thus, a new type "low Mw carriers", which have low cytotoxicity, high cellular uptake, and adequate polyplex stability, would be more useful gene carriers.

In the present study, oligopeptide-type carriers were selected in order to reduce cytotoxicity and to induce the intracellularly digestible feature. Since the chemical chain elongation of cationic oligopeptide would increase the cytotoxicity, we tried to raise the apparent Mw of oligopeptide-type carriers by their self-assembly. Amphiphilic oligopeptides having cationic and hydrophobic sequences were then designed. Hydrophobic interactions between oligo leucine sequences make carriers form assemblies and increase the apparent Mw. Cationic sequences for interacting with pDNA include cleavable sequences (Arg-X-Lys/Arg-Arg (R-X-K/R-R)) by intracellular proprotein convertase, furin [10,11]. We have previously found that carriers including this cleavable sequences are enough cationic to form polyplexes with pDNA, and these polyplexes became destabilized if carriers were cleaved by furin [12]. Increased apparent Mw is expected to increase cellular uptake of the polyplex and the enhanced stability can be destabilized by furin cleavage resulting in the pDNA release in intracellular environments.

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2. Experimental

2.1. Amphiphilic oligopeptides

We synthesized four oligopeptides by Fmoc-based solid phase method using 9050 plus PepSynthesizer (Applied Biosystems, CA, USA) and purified in the usual way. They are composed of cationic KRRRKRKRRRKRKRRC and hydrophobic oligo leucine segment with different lengths.

Oligopeptide solutions were analyzed by GPC (Shimadzu Corporation, Kyoto, Japan) which fitted with a combination of two columns of TSK gel G6000PWXL (21.5 mm I.D. × 300 mm length, Tosoh Corporation, Tokyo, Japan) and TSK gel G3000PWXL, RID-10A Refractive index detector, and SPD-M10A UV–VIS detector. Elution was carried out with 1/15 M phosphate buffer (pH 7.5) at 0.3 mL/min.

2.2. Critical micelle concentration (CMC) measurements

CMCs of oligopeptides in aqueous solution were measured on a RF5300PC (Shimadzu Corporation, Kyoto, Japan) using pyrene (Nacalai Tesque, Inc., Kyoto, Japan) as a hydrophobic region probe [13]. Five μL of pyrene solution in acetone at a concentration of 6×10^{-5} M was transferred into a vial and evaporated. Five hundred μL of oligopeptide solutions which ranging from 5.0×10^{-4}–1.5 g/L were added dropwise to make the pyrene concentration of 6.0×10^{-7} M, incubated at 65 °C for 3 h, and cooled down to the room temperature. Pyrene excitation spectra were measured with the slit widths of 5 and 1.5 nm for excitation and emission at an emission wavelength of 380 nm.

2.3. Polyplex formation with pDNA

pCMV-Luc and pT7-Luc (Promega corporation, WI, USA) were amplified to sufficient quantities by standard molecular biology techniques, and purified with a QIAGEN-tip 500 (QIAGEN K.K., Tokyo, Japan). Oligopeptide solutions were mixed with pDNA solutions at a given charge ratio which is the ratio of the number of cationic groups of oligopeptide to that of anionic group of pDNA (*C*/*A* ratio). The solutions were incubated for 30 min at 37 °C to allow the polyplex formation and analyzed on 0.8 wt% agarose gel in Tris–borate EDTA buffer at 100V for 30 min. pDNA was visualized by staining with 0.5 μg/mL ethidium bromide (EtBr, Sigma chemicals, St Louis, MO, USA).

2.4. In vitro *transfection*

COS-1 cells were grown in DMEM (Nissui, Tokyo, Japan) containing 10% fetal bovine serum (FBS) (Sigma chemicals, USA) at 37 °C under a 5% CO_2 atmosphere. COS-1 cells were seeded in 96 well culture plates at a density of 1×10^4 in 100 μL DMEM containing 10% FBS per well. After 24 h incubation, cells were washed with PBS, and 40 μL DMEM was added. Ten μL of polyplex solutions containing 100 ng pCMV-Luc at the concentration above or below CMC of Pep-L12 were poured gently to the wells. Fifty μL of 200 μM chloroquine solution was added (final concentration is 100 μM) and incubated for 5 h. Cells were washed with PBS and cultured for 43 h with DMEM containing 10% FBS at 37 °C in a 5% humidified CO_2 environment. The cells were washed with PBS, treated with the lysis buffer containing 1% Triton-X100, and incubated for 30 min at 37 °C. Cell lysate was diluted into luciferase assay solution containing 470 μM luciferin. The relative light units (RLU) of expressed luciferase were measured using ATP-300 Lumicounter (Advantec Toyo Kaisya, Ltd., Tokyo, Japan). Luciferase solutions at a known concentration were used for calibration. The protein concentration was determined by DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as a standard. The obtained luciferase expression (ng luciferase) was divided by total protein content of cell lysates and expressed as ng luciferase/mg protein.

Table 1
Sequences of amphiphilic oligopeptides.

Oligopeptide	Sequences and cleavage sites	Amino acid composition (K/R/L/C)
Pep-L0	H_2N-K-R-R-R-K-R*-K-R-R*-R-K-R*-K-R-R*-C-$CONH_2$	10/5/0/1
Pep-L4	H_2N-$(L)_4$-K-R-R-R-K-R*-K-R-R*-R-K-R*-K-R-R*-C-$CONH_2$	10/5/4/1
Pep-L8	H_2N-$(L)_8$-K-R-R-R-K-R*-K-R-R*-R-K-R*-K-R-R*-C-$CONH_2$	10/5/8/1
Pep-L12	H_2N-$(L)_{12}$-K-R-R-R-K-R*-K-R-R*-R-K-R*-K-R-R*-C-$CONH_2$	10/5/12/1

* represents the cleavage site of furin.

2.5. Cell-free assay system for luciferase expression

Fifteen μL of polyplexes ($C/A = 10$) were mixed with 12.8 μL of rabbit reticulocyte lysate mixtures (T_NT Coupled Reticulocyte Lysate Systems; Promega, WI, USA) and incubated with shake at rate of 300 rpm/min for 90 min at 30 °C. After transcription/translation assay according to the manufacture's protocol, luciferase activities were measured by the same method described in the above section.

3. Results and discussion

3.1. Self-assembly of amphiphilic carriers

Sequences and abbreviation of synthesized amphiphilic oligopeptides were shown in Table 1. GPC chart for each amphiphilic oligopeptide in phosphate buffer is shown in Fig. 1. Only Pep-L12 exhibited two peaks, while the other oligopeptides showed peak which is at the similar elution time to the second peak of Pep-L12. The first peak of Pep-L12 is considered to be attributed to the self-assembly of the Pep-L12 with the apparent higher Mw and the second peak corresponds to the unimer as low Mw as the other oligopeptides, Pep-L0, Pep-L4, and Pep-L8. These results indicated that only Pep-L12 forms micelle-like assemblies in aqueous solution.

Micelle-like assemblies can be confirmed by comparing ^{1}H NMR spectra in good solvents and water [14,15]. Protons in the core structure composed of the insoluble fractions do not provide sufficient NMR signals. Thus, self-assembly of oligopeptides was analyzed in DMSO and water. Leucine contents (X_{Leu}) in water and in DMSO were measured using the signal intensity at 0.8 ppm (CH_3 in leucine) and at 1.6 ppm (β-CH_2 and γ-CH in leucine, β, γ, and δ-CH_2 in lysine, β and γ-CH_2 in arginine, and SH in cysteine). The

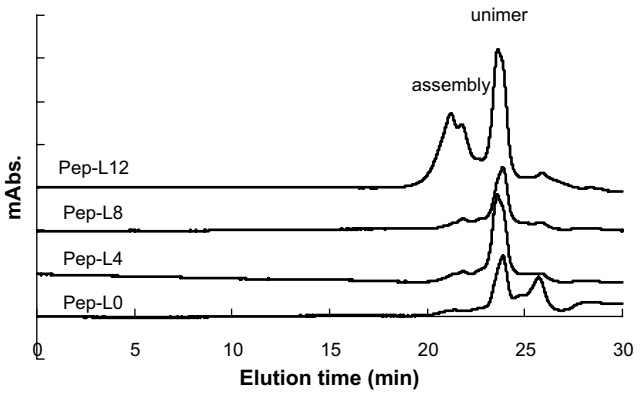

Fig. 1. GPC charts of Pep-LX ($X = 0$, 4, 8 and 12).