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In Vitro Cellular Gene Delivery Employing a Novel Composite Material of Single-Walled Carbon Nanotubes Associated With Designed Peptides With Pegylation

Takahisa Ohta [1], Yasuhiko Hashida [1], Yuriko Higuchi [2], Fumiyoshi Yamashita [2], Mitsuru Hashida [1, 2, *]

[1] *Institute for Integrated Cell-Material Sciences (iCeMS), Kyoto University, Yoshida Ushinomiya-cho, Sakyo-ku, Kyoto 606-8501, Japan*
[2] *Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Yoshida Shimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan*

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ABSTRACT

Single-walled carbon nanotubes (SWCNTs) attract great interest in biomedical fields including application for drug delivery system. In this study, we developed a novel gene delivery system employing SWCNTs associated with polycationic and amphiphilic H-(-Lys-Trp-Lys-Gly-)$_7$-OH [$(KWKG)_7$] peptides having pegylation. SWCNTs wrapped with $(KWKG)_7$ formed a complex with plasmid DNA (pDNA) in aqueous solution based on polyionic interaction but later underwent aggregation. On the other hand, a complex of pDNA and SWCNT-$(KWKG)_7$ modified with polyethylene glycol (PEG) chains of 12 units [SWCNT-$(KWKG)_7$-$(PEG)_{12}$] afforded good dispersion stability for 24 h even in a cell culture medium. The *in vitro* cellular uptake of SWCNT-$(KWKG)_7$-$(PEG)_{12}$/pDNA complex prepared with fluorescence-labeled pDNA was evaluated with fluorescent microscopic observation and flow cytometry. The uptake by A549 human lung adenocarcinoma epithelial cells increased along with the extent of pegylation, suggesting the importance of dispersion stability in addition to the cationic charge which facilitates ionic cellular interaction. The expression of pDNA encoding the monomeric Kusabira-Orange 2 fluorescent protein in the form of the SWCNT-$(KWKG)_7$-$(PEG)_{12}$/pDNA complex demonstrated remarkable enhancement of transfection depending also on the extent of pegylation and the *N*/*P* ratio. The potential of the SWCNT composite wrapped with polycationic and amphiphilic $(KWKG)_7$ with pegylation as a carrier for gene delivery was demonstrated.

Introduction

Single-walled carbon nanotubes (SWCNTs) have a cylindrical structure with a diameter of nanometer size, a length from several tens to hundreds of nanometers, and a broad effective surface for the modification of functional molecules.[1,2] Moreover, SWCNTs dispersed in aqueous solution have been shown to be taken up into cells via penetration through the cell membrane.[3,4] In addition, dispersed SWCNTs show low cytotoxicity, whereas the non-dispersed SWCNTs and their aggregates are highly toxic to cells.[5,6] Because of these characteristics, SWCNTs have been investigated as potential drug delivery system carriers. However, because it is extremely difficult to disperse SWCNTs in aqueous media due to their hydrophobicity, the application of SWCNTs to biomedical fields has been limited. Therefore, various dispersing agents have been developed for the physical modification and surface coating of SWCNTs, such as polyethyleneimine,[7] polyethylene glycol (PEG) modified (PEGylated) phospholipid,[8] DNA,[9] and so on. These agents have amphiphilic properties that allow them to interact with hydrophobic SWCNTs to form a complex and solubilize SWCNTs with hydrophilic moieties. In our previous studies, we developed a novel composite of SWCNTs with amphiphilic designed peptides and demonstrated that their chemical and physicochemical characteristics were suitable for biomedical applications.[10] We demonstrated, for example, the utility of an SWCNT-peptide composite for photothermal therapy in cancer treatment with infrared laser irradiation based on its improved dispersion stability in aqueous water.[11]

One of the most promising applications for the SWCNT-peptide composite would be as a drug or gene delivery carrier. In a previous series of investigations, we developed various carriers for gene

* *Correspondence to:* Mitsuru Hashida (Telephone: +81-75-753-4525; Fax: +81-75-753-4575).
E-mail address: hashidam@pharm.kyoto-u.ac.jp (M. Hashida).

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delivery, such as emulsions,[12] liposomes,[13] and bubble liposomes,[14] all of which had a spherical structure. In contrast, SWCNTs have a cylindrical structure and their surface can be modified with ligands for active targeting and delivery.[15,16] In addition, the possibility of assembling multiple functions on SWCNTs would seem to further encourage application of the SWCNT-peptide composite in gene delivery.[17,18]

In general, it is difficult to transfer bare genes into cells due to the electrostatic repulsion between the genes and cell surface as well as their instability. In order to achieve penetration of the cell membrane by genes, various mechanical, physicochemical, and biological methods have been proposed, such as electroporation,[19] the use of local suction,[20] hydrodynamics administration,[21] application of cationic carriers,[22] and virus vectors.[23] Among these methods, gene delivery systems using cationic carriers would seem to be especially promising due to the simplicity of their cellular uptake and avoidance of degradation. However, cationic carriers sometime have the problem of aggregation under physiological condition. In addition, not only complex formation but also detachment from cationic carriers, that is, endosomal escape, should be considered in order to avoid degradation and effectively express the target genes.

In our studies, we have developed the H-(-Lys-Trp-Lys-Gly-)$_7$-OH [(KWKG)$_7$] peptide as a poly-cationic and amphiphilic dispersing agent suitable for wrapping the surface of SWCNTs.[24] The SWCNT-(KWKG)$_7$ composite shows stable dispersion in aqueous solution due to the protonated amino groups of lysine residue, which would also facilitate complex formation with plasmid DNA (pDNA) and cell surface interaction as a first step of cellular uptake. In addition, modification of the lysine residue of SWCNT-(KWKG)$_7$ with PEG remarkably improved the dispersion stability under physiological conditions. The obtained SWCNT-(KWKG)$_7$-(PEG)$_{12}$ is expected to form a complex with the intended gene while maintaining high dispersion stability.

The present experiments were designed to evaluate the utility of SWCNT-(KWKG)$_7$ and the SWCNT-(KWKG)$_7$-(PEG)$_{12}$ composite in gene delivery from various viewpoints. We first analyzed the dispersion stability of their pDNA complexes in cell culture medium, and then analyzed the dispersion stability by zeta potential measurement, agarose gel electrophoresis, and fluorescence spectrum measurement in ethidium bromide (EtBr) solution. The cellular uptake of the SWCNT-(KWKG)$_7$-(PEG)$_{12}$/pDNA complex was evaluated by fluorescent microscopic observation and flow cytometry (FACS) using fluorescence-labeled pDNA. Finally, the gene expression by the SWCNT-(KWKG)$_7$-(PEG)$_{12}$/pDNA complex was examined using monomeric Kusabira-Orange 2 (mKO2) fluorescent protein-coding pDNA.[25]

Materials and Methods

Materials

Purified SWCNTs (HiPco[26]; lot no. P0343) were purchased from Carbon Nanotechnologies (Houston, TX). The (KWKG)$_7$ peptide shown in Figure 1 was synthesized with more than 90% purity by Invitrogen (Carlsbad, CA). Succinimidyl-[(N-methyl)-dodecaethyleneglycol] ester [methyl-(PEG)$_{12}$-NHS ester, MS(PEG)$_{12}$] was obtained from Thermo Fisher Scientific (Rockford, IL). Label IT Plasmid Delivery Control was purchased from Mirus Bio (Madison, WI). FuGENE HD transfection reagent was obtained from Promega (Madison, WI). VECTASHIELD with 4',6-diamidino-2-phenylindole (DAPI) was purchased from Vector Laboratories (Burlingame, CA). Opti-MEM® was obtained from Invitrogen. Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui Pharmaceutical (Tokyo, Japan) and fetal bovine serum was

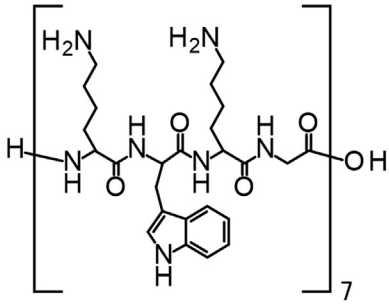

Figure 1. Chemical structure of (KWKG)$_7$ peptide.

obtained from MP Biochemicals (Irvine, CA). Other chemicals were purchased from Wako Chemicals (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan).

Preparation of the SWCNT Dispersion Solution

The SWCNT-(KWKG)$_7$ composite was prepared in 5 mL D_2O solution containing 2 mg SWCNTs and 10 mg (KWKG)$_7$ peptide in a glass tube by sonicating for 1 h with an ultrasonic disruptor UD-201 (TOMY Digital Biology, Tokyo, Japan) in an iced water bath.[10,11] D_2O solution was employed to prepare density difference between dispersed and non-dispersed SWCNT D_2O. After sonication, the dispersion solution was centrifuged at 40,000 rpm for 2.5 h with a Himac CP65β ultracentrifuge and a model P40ST rotor (Hitachi, Tokyo, Japan), and the supernatant of the SWCNT-(KWKG)$_7$ dispersion was dialyzed using a 100 kD dialysis membrane tube (Spectrum Laboratories, Inc., Rancho Dominguez, CA) against distilled water for 24 h to remove free unbounded (KWKG)$_7$ peptide.

Quantification of the SWCNT-(KWKG)$_7$ Dispersion

The concentration of SWCNTs in the SWCNT-(KWKG)$_7$ dispersion was determined by absorbance spectrum measurement at 808 nm with $A_{1mg/mL} = 40.3$[8] using a UV spectrophotometer (UV-1600; Shimadzu, Kyoto, Japan). The concentration of (KWKG)$_7$ peptide was quantified at 280 nm with $A_{280nm} = 5500\ M^{-1}cm^{-1}$ as an extinction coefficient of tryptophan residue.[27]

PEG Modification of the SWCNT-(KWKG)$_7$

PEG modification of the amino groups of the lysine residue of SWCNTs-(KWKG)$_7$ was performed by covalent amide bond formation between the amino groups and carboxyl groups of MS(PEG)$_{12}$ pegylation reagent. SWCNT-(KWKG)$_7$ dispersion in 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 8.0) was added along with various volumes of 2.5 mM MS(PEG)$_{12}$ to set the molar ratio between 10%, 20%, 30%, 40%, and 50% against total amino groups. The reaction was carried out for 2 h, and the reaction solution was run with 3 rounds of centrifugal filtration using an Amicon Ultra-0.5 centrifugal filter 100 kD device (Merck Millipore, Ltd., Darmstadt, Germany) to exchange the buffer and remove free unconjugated MS(PEG)$_{12}$. The extent of PEG modification of the amino groups was determined as reported in our previous study by using fluorescein isothiocyanate (FITC)-(PEG)$_{12}$-NHS ester and calculations from the absorbance spectrum and molar extinction coefficient of FITC.[24] In this study, the extent of PEG modification is expressed based on the results of this experiment under the same reaction conditions.