Graduate School of Advanced Science and Engineering
Waseda University

博士論文概要
Doctoral Thesis Synopsis

論文題目
Thesis Theme
Design of Lipophilized Molecule or Self-assembled Peptide Nanotube for Cellular Uptake

申請者
(Applicant Name)

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<th>Siyoong</th>
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Department of Life Science and Medical Bioscience,
Research on Environmental Biotechnology

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Bioactive molecules that work inside the cell have to be internalized through the cell membrane. In this study, two different methods were tested for enhancement of cellular uptake. The first method involved the lipophilization, whereas the second method utilized self-assembled peptide nano-tubes as nano-carriers to enhance cellular uptake.

A general introduction to these studies is provided in Chapter 1. Certain medications are administrated as chemically modified versions called prodrugs, which are in an inactive state at the time of injection. The prodrugs must undergo transformation into the active state by enzymatic or chemical reactions within the body. Lipophilization, which is one of the prodrug strategies, controls the hydrophilic/hydrophobic (lipophilic) balance of the therapeutic molecule, and has been typically used to enhance cell membrane permeability and increase stability. This strategy helps the drug penetrate the hydrophobic phospholipid bilayer of the cell membrane. Once inside the cell, diverse functional groups can be enzymatically cleaved.

Alternatively, carriers may be used to envelope the drugs to facilitate mass transport through the cell membrane without directly modifying the drug. Typical drug delivery carriers have been designed using micelles, liposomes, dendrimers and nanoparticles. These carriers have unique properties, depending on their source materials. The design of carriers has evolved to support advanced functions such as enhancement of cell membrane permeability, site-specific delivery, and control of the drug release rate from the carrier. Further considerations include the biostability, biocompatibility, non-toxicity, and non-immunogenicity of the carrier.

Chapter 2 focuses on the development of a DNA labeling agent modified by lipophilization. This new compound was designed not only to increase cell membrane permeability, but also to bypass the antimetabolic pathway of 5-ethynyl-2’-deoxyuridine (EdU). Based on the EdU, chemical phosphorylation was performed on the 5’-hydroxyl group. Chemical monophosphorylation was anticipated to enhance the efficiency of the substrate for DNA polymerization, by its circumvention of the enzymatic monophosphorylation step, which requires greater time and energy. However, phosphorylated compounds are generally ineffective at penetrating cell membranes due to negative charges on the phosphate groups. Therefore, cell membrane permeability was enhanced by masking the negative charges using a bis-pivaloyloxymethyl (bis(POM)) group, which is quite stable in buffer and plasma, and cleavable inside various cell types. Progressive cleavage of the bis(POM) protecting groups gradually generates EdU monophosphate and prevents excessive concentration of antimetabolites. In this study, phosphorylated EdU (PEdU) was compared with EdU in terms of bioactivity, cytotoxicity and sensitivity in vitro. In addition, its labeling ability in vivo was also evaluated.

EdU was synthesized from 5-iodo-2’-deoxyuridine (IdU) by introduction of trimethylsilyl acetylene and elimination of trimethylsilane. Chlorobis(POM) phosphate was synthesized from trimethyl phosphate. All methyl groups were substituted with POM groups, and one of them was hydrolyzed and reacted with oxalyl chloride. Finally, PEdU was obtained by reaction of the chlorobis(POM) phosphate with the EdU.

The cytotoxicity of PEdU was compared with EdU using mouse embryonic fibroblasts (3T3) and human cervical cancer cells (HeLa cell line). After 24 hours of incubation with the compounds, only EdU initiated cell death in both 3T3 and HeLa cells at 100 µM. After 48 hours, EdU induced cytotoxicity in both cell lines at a 10 µM concentration, and many of the cells were dead after incubation with a 100 µM concentration of
EdU. In contrast, PEdU did not reduce cell viability in either line, even at 100 µM. After 72 hours, the viability of both cell lines decreased rapidly when treated with 10 or 100 µM concentrations of EdU. However, PEdU did not induce cell death in the 3T3 cell line prior to the 72-hour timepoint, or prior to the 48-hour timepoint for the HeLa cell line, even at 100 µM. PEdU induced cytotoxicity at 100 µM after 72 hours only in the HeLa cell line, which is more sensitive to chemicals than the 3T3 cell line.

The cellular DNA labeling ability of PEdU was evaluated using a fluorescent azide (Alexa Fluor 488). Various concentrations (0.1-100 µM) of EdU or PEdU were incubated with 3T3 and HeLa cells for 24 and 72 hours. After a click reaction with Alexa Fluor 488, the incorporated DNA was observed by fluorescence microscopy. After 24 hours, the fluorescence intensity of PedU-treated cells was at similar level to that of EdU-treated ones. After 72 hours, PEdU showed higher fluorescence intensity than EdU, since many cells had been damaged by the EdU. Thus, PEdU was less toxic than EdU, and more effective at DNA labeling.

DNA labeling by PEdU was evaluated in vivo using 3-week-old mice, which were injected intraperitoneally with various concentrations of PEdU. After 24 and 48 hours, their tissues were harvested, sectioned, and stained with Alexa Fluor 488 and DAPI. Alexa Fluor 488 staining enabled visualization of newly synthesized DNA after PEdU injection, whereas DAPI staining enabled visualization of total DNA. PEdU was successfully incorporated into animal DNA during replication and marked the location of cell division.

In conclusion, PEdU was designed as an efficient DNA labeling agent, and was synthesized by chemical phosphorylation on the 5' hydroxyl group of EdU. PedU also exhibits low toxicity due to progressive elimination of the bis(POM) protecting groups. PEdU bypassed the toxic pathway of EdU and showed a remarkable reduction in cytotoxicity compared to unmodified EdU. Nevertheless, PEdU was incorporated as efficiently as EdU during metabolic DNA labeling. Accordingly, PEdU can be employed as a less toxic DNA labeling agent for studies requiring subsequent cell survival over a long period of time or those utilizing sensitive cell lines. This study indicates that chemical modifications of nucleoside analogues and prodrugs may be employed for enhancement of their biological activities.

Chapter 3 describes the synthesis and use of self-assembled peptide nanotubes as carrier molecules. Amphiphilic peptides were synthesized by liquid phase condensation for the addition of a hydrophobic helix and by living radical polymerization for the addition of a hydrophilic chain. The hydrophobic helix was first synthesized by repetition of deprotection and condensation of the amino acids leucine (Leu) and aminoisobutyric acid (Aib) until a helix of the desired length (12 to 16 amino acids) was formed. Subsequently, sarcosine-N-carboxyanhydride (Sar-NCA) was synthesized as a monomer and then polymerized into a hydrophilic polymer chain by living radical polymerization. Lastly, the terminus of the hydrophilic chain was capped by glycolic acid.

The lengths of both the hydrophobic and hydrophilic chains play an important role in determining the shape of the nanostructure after self-assembly. First, in order to examine the effect of the number of amino acids in the hydrophobic helix on the shape of the nanostructure, 12 and 16 amino acid compounds were compared. Our results indicate that tube and sphere shapes result from the self-assembly of 12 and 16 amino acid compounds, respectively. In contrast, a well-organized structure was not formed when the hydrophilic sarcosine
chain in the amphiphilic peptide was shorter than 18 or longer than 34. Only compounds that contain hydrophilic sarcosine chain lengths between 20 and 28 formed well-organized nanotubes.

Conditions during self-assembling affected the resulting shape of the nanotube. At 70°C, amphiphilic peptides were assembled into sheet shapes and aggregated over heating time. At 80°C and 90°C, they formed nanotube shapes, which interestingly elongated over heating time. A positively charged amphiphilic peptide was synthesized without neutralization by glycolic acid capping. Zeta potential of self-assembled peptide nanotubes responded to the ratio of the positively charged amphiphilic peptide added, but the shape was unaffected. The positive charge on the surface of nanotubes is a very important factor for cellular uptake, since stable attachment of the carriers to the negatively charged cell membrane is required.

Cytotoxicity of the self-assembled peptide nanotubes was investigated by evaluating the viability of HeLa cells. None of the prepared nanotubes had a significant effect on HeLa cell viability at different concentrations (10, 50, and 250 µg/mL). Cellular uptake was investigated using fluorescently labeled nanotubes and HeLa cells. The length of peptide nanotubes within 400 nm did not seriously affect cellular internalization. Even though the length of nanotube was 900nm without charge, it was internalized more than nano-sphere. Positively charged peptide nanotubes that contain positively charged amphiphilic peptide were more readily internalized than neutral peptide nanotubes, even after 1 hour of incubation. Nanotubes could encapsulate certain molecule and deliver into cell effectively, even though the molecule was cell membrane impermeant.

In conclusion, amphiphilic peptides with both hydrophobic and hydrophilic motifs were synthesized, and modification of synthesis conditions enabled control of the final length and electric charge of each motif. The length of each motif affected the final shape of the nanostructure after self-assembly, forming a well-organized, uniform nanotube at a given ratio of hydrophobic to hydrophilic motifs. The length of the nanotubes could be controlled by adjusting the temperature and length of time for the self-assembly. Positively charged nanotubes and fluorescent nanotubes were produced by including positively charged amphiphilic peptides and fluorescent peptides, respectively, in the self-assembly. Length of peptide nanotube affected cellular uptake, and positively charged peptide nanotube showed vigorous cellular uptake and it could encapsulate certain molecule which is cell membrane impermeant, and deliver into cell effectively. The flexible design of nanotubes can help to optimize properties for effective cellular uptake.

Chapter 4 summarizes the two designs that were performed to achieve vigorous cellular uptake. The DNA labeling agent, EdU, was modified by lipophilization with a monophosphate group to create PEdU. Compared to the EdU, PEdU showed significantly reduced cytotoxicity and higher DNA labeling efficiency. Likewise, the self-assembled peptide nanotubes of varying lengths, charges, and fluorescence were successfully prepared and capable of efficient cellular uptake. Both the designs—lipophilization and self-assembled peptide nanotubes—can be employed for studies concerning uptake.
### List of research achievements for application of doctorate (Dr. of Engineering), Waseda University

**氏名** (SEO SIYOONG)  
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(As of December, 2015)

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<th>題名、発表・発行掲載誌名、発表・発行年月、連名者 (申請者含む) (theme, journal name, date &amp; year of publication, name of authors inc. yourself)</th>
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<td>2. Phosphorylated 5-ethynyl-2’-deoxyuridine for advanced DNA labeling, RIKEN Summer School, Kobe, Japan, Sep. 2013, Siyoong Seo, Kazumitsu Onizuka, Chieko Nishioka, Eiki Takahashi, Satoshi Tsuneda, Hiroshi Abe, Yoshihiro Ito</td>
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**Presentation**

1. Phosphorylated 5-ethynyl-2’-deoxyuridine for advanced DNA labeling, RIKEN Summer School, Kobe, Japan, Sep. 2013, Siyoong Seo, Kazumitsu Onizuka, Chieko Nishioka, Eiki Takahashi, Satoshi Tsuneda, Hiroshi Abe, Yoshihiro Ito


5. Phosphorylated 5-ethynyl-2’-deoxyuridine for advanced DNA labeling, E-MRS, Lille, France, May 2015, Siyoong Seo, Kazumitsu Onizuka, Chieko Nishioka, Eiki Takahashi, Satoshi Tsuneda, Hiroshi Abe, Yoshihiro Ito