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学 位 論 文 題 名

# Mechanism-based development of peptide-modified nanoparticles for intracellular delivery of nucleic acids

（機能性核酸の細胞内動態制御を指向した機構論に基づいた
ペプチド修飾ナノ粒子の開発）

## 学位論文内容の要旨

The studies presented in this thesis showed that modification of the novel vector called multifunctional envelope-type nano device "MEND" particles with a high density of R8 peptide resulted in an efficient carrier capable of cytosolic delivery of plasmid DNA and siRNA to cells in culture. Further modification of R8-MEND with a novel peptide derivative of the INF7 fusogenic peptide resulted in enhancement of the gene expression of R8-MEND in the presence of PEG coating and *in vivo*.

**1. Chapter 1**

Chapter 1 of the thesis included a brief introduction about gene therapy and MEND system and its applications. This was followed by the aims of the current study and thesis outline.

**2. Chapter 2**

Chapter 2 comprises a literature review about therapeutic nucleic acids and their delivery systems.

**3. Chapter 3**

In chapter 3 a thorough comparison between the effects of MEND surface modification with octaarginine (R8) versus octalysine (K8) regarding cellular uptake, endosomal escape, and efficiency in cytosolic delivery of the encapsulated cargo was carried out. Results of the study were as follows:

K8 and R8 linked to stearyl moiety were used to modify MEND composed of DOPE (Dioleoyl phosphatidyl ethanolamine) / Phosphatidic acid (PA) in 7:2 molar ratio. K8-MEND and R8-MEND had comparable diameters equal~160 nm and zeta potential ~40 mV. No significant difference ($p>0.05$) was found between the measured intracellular fluorescence of rhodamine-DOPE-labeled K8-MEND and R8-MEND using flow cytometry at the four concentrations of MEND tested in NIH 3T3 cells. The rate of uptake of both MENDs was also similar ($p>0.05$) between 15 min and 3 hrs. Amiloride, a selective macropinocytosis inhibitor strongly inhibited the uptake of both K8-modified liposomes (K8-Lip) and R8-modified liposomes (R8-Lip); while chlorpromazine, a clathrin-mediated endocytosis inhibitor, had no effect on their uptake. These results showed that K8-MEND is taken up in via macropinocytosis as efficiently as R8-MEND in agreement with the previously published data on the mechanism of uptake of R8-MEND.[1] The results also emphasized the importance of the high positive-charge density on the surface of MEND in the stimulation of macropinocytosis.

Confocal laser scanning microscopy (CLSM) of live cells showed that the extent of R8-MEND escape from endocytic vesicles was higher than that of K8-MEND—~62% compared to only ~26% for K8-MEND. In the presence of bafilomycin A1, a selective vacuolar proton pump inhibitor, the extent of K8-MEND and R8-MEND escape was comparable. This result was confirmed by the transfection activities of K8-MEND and R8-MEND encapsulating plasmid DNA encoding a luciferase reporter gene in the presence of the membrane-disrupting agent, chloroquine or bafilomycin A1. Chloroquine enhanced K8-MEND gene

expression approximately 4-fold, while it had only a negligible effect on that of R8-MEND. The endosome acidification inhibitor resulted in ~90% inhibition of R8-MEND gene expression, while it enhanced that of K8- MEND by ~315%. These results supported the confocal microscopy observations.

Then the contribution of fusion between K8-MEND or R8-MEND and the endosomal membrane during escape was tested using spectral imaging of double labeled FRET liposomes modified with K8 (K8-Lip) or R8 (R8-Lip). Both K8-Lip and R8-Lip were shown to fuse to endosomes during escape. The acidification inhibitor, bafilomycin A1, failed to inhibit fusion of K8-Lip. In contrast, R8-Lip fusion was partially inhibited in the presence of bafilomycin A1. In another *in vitro* experiment, K8-Lip could fuse to liposomes composed of negatively charged lipid components, simulating those present in the endosomal membrane, only at neutral pH while R8-Lip could fuse at both acidic and neutral pH conditions. These results collectively indicated that R8-MEND fusion to endosome takes place at both neutral conditions (just after uptake) and at acidic conditions (upon maturation of endosome), unlike K8-MEND which can escape only just after uptake.

Finally, modification of MEND with R8 was shown to enhance the encapsulated luciferase gene expression ~320-fold compared with unmodified MEND and 17-fold compared with K8-MEND. Similarly, when silencing effect of MEND encapsulating anti-luciferase siRNA was checked in HeLa cells stably expressing luciferase R8-MEND equivalent to 0.1 μg of siRNA resulted in around two times the silencing effect resulting from K8-MEND.

To conclude, modification of MEND with R8 resulted in efficient uptake of MEND and high endosomal escape of the encapsulated nucleic acids. Efficient endosomal escape took place through fusion to endocytic vesicles under neutral and acidic conditions resulting in enhanced delivery of the functional nucleic acids to the cytosol and/or the nucleus of cells in cultures.

**4. Chapter 4**

In chapter 4, R8-MEND was optimized for *in vivo* applications. Testing R8-MEND *in vivo* showed that it results in low gene expression levels in the organs tested. PEGylation of R8-MEND resulted in severe inhibition to its endosomal escape. A novel stearylated derivative of the well known peptide INF7 [2] was designed and optimized for surface modification of R8-MEND. When included to the MEND lipid coat, the stearylated derivative (STR-INF7) was more potent than the parent INF7 peptide in improving the gene transfection activity of R8-MEND.

STR-INF7 modification resulted in improvement of the endosomal escape of R8-MEND and PEGylated R8-MEND in the absence of the fusogenic lipid DOPE. Spectral imaging of double labeled liposomes showed that STR-INF7 doesn't mediate fusion between the liposomes and the endosomal membrane. Non-fusogenic R8-Lip could rupture negatively charged liposomes entrapping calcein and could release calcein at neutral pH and acidic pH. In contrast, the unmodified non-fusogenic R8-Lip failed to release calcein at either condition. These results showed that STR-INF7 mediates endosomal escape through rupture of the endosomal membrane at both neutral and acidic conditions of the endocytic vesicles.

STR-INF7 enhanced gene expression of PEGylated R8-MEND in a STR-INF7-concentration-dependent manner. The enhancement in gene expression was up to 72 folds in absence of DOPE and 22 folds in presence of DOPE over the peptide-unmodified R8-MEND. Surface modification of PEGylated R8-MEND with STR-INF7 didn't affect the long-circulation properties of the PEGylated MEND. In addition, STR-INF7 modified R8-MEND was non-toxic to cells in cultures. Finally, upon i.v. injection to mice, STR-INF7-modified R8-MEND resulted in gene expression levels in liver 240-folds higher than unmodified R8-MEND.The enhancement was 116-fold and 6-fold in spleen and lung respectively compared to that of unmodified R8-MEND. These results showed that STR-INF7 is an efficient device that improves endosomal escape of MEND both *in vitro* and *in vivo*.

To conclude, modification of R8-MEND with the novel compound STR-INF7 resulted in a competent nonviral vectors that could transfect cells in cultures and *in vivo*. The new STR-INF7 surface modification was non-toxic and functioned well in PEGylated R8-MEND through a mechanism independent on fusion between MEND coat and the endosomal membrane.

## 5. Chapter 5

Chapter 5 summarized the results presented in the thesis and discussed possible extensions for this research and future research projects.

## 6. References


1. Khalil, I.A., K. Kogure, S. Futaki, and H. Harashima, *J Biol Chem.* **281**(6): p. 3544-3551(2006)
2. Plank, C., B. Oberhauser, K. Mechtler, C. Koch, and E. Wagner, *J Biol Chem.* **269**(17): p. 12918-12924(1994)

# 学位論文審査の要旨

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# Mechanism-based development of peptide-modified nanoparticles for intracellular delivery of nucleic acids

## (機能性核酸の細胞内動態制御を指向した機構論に基づいた ペプチド修飾ナノ粒子の開発)

革新的な人工遺伝子デリバリーシステムを開発するためには、遺伝子およびキャリアーの細胞内動態の制御が不可欠である。本論文は、多機能性エンベロープ型ナノ構造体（以下、MENDと略）の細胞内動態の中で主要な律速段階であるエンドソーム脱出過程に着目し、オクタアルギニン(R8)のエンドソーム脱出機構の解明を行なった。さらに、新たなエンドソーム脱出素子としてINF7の機能評価を行ない、*in vivo*遺伝子送達システムへの応用の可能性を示した。

R8は、細胞膜透過性ペプチドとして優れた細胞送達性を示すことが明らかになっている。R8で表面修飾した多機能性エンベロープ型ナノ構造体(MENDと略)は強力な遺伝子導入力を示し、優れたエンドソーム脱出能が報告されている。本論文では、R8のエンドソーム脱出機構をオクタリジン(K8)と比較することにより解析した。その結果、R8とK8は共にマクロピノサイトーシスを誘起して同程度の効率で細胞内へ侵入することが明らかになった。エンドソーム脱出機構を両者で比較したところ、R8の方がK8よりも効率的に脱出することが明らかとなった。両者の脱出機構を解明するため、エンドソームの酸性化を阻止するバフィロマイシン存在下で比較したところ、R8は感受性を示し、K8は非感受性であった。In vitroの実験系で、FRETの原理を用いて膜融合能を両者で比較したところ、K8-リポソームは中性条件下でのみ膜融合を示し、R8-リポソームは中性でも酸性でも膜融合を示した。この結果は、細胞系の実験結果と良く対応し、R8-リポソームはマクロピノサイトーシスで取込まれた後、エンドソーム内が酸性になっても効率的に脱出できるのに対して、K8-リポソームは酸性になると脱出できないことが明らかとなった。本研究成果は、細胞膜透過性ペプチドの細胞内動態特性における新たなメカニズムの発見であり、この領域で高く評価されている。

さらに、本論文では、新たなエンドソーム脱出素子としてインフルエンザ由来のINF7に着目した。インフルエンザはエンベロープ型ウイルスで、エンドサイトーシスで内在化した後、膜融合によりエンドソームから脱出することが知られている。そこで、INF7に

着目し、ステアリル化したINF7をMENDの膜に導入すると、遺伝子発現活性は飛躍的に上昇した。さらに、ポリエチレングリコール(PEG)で修飾したMENDにおいてもその効果は減弱することはなかった。そこで、INF7によるエンドソーム脱出過程のメカニズムを解明するために、spectraledge法を用いて、膜融合の可能性を検証した。その結果、予想とは異なり、INF7は膜融合では無いメカニズムでエンドソーム脱出を促進していることがあきらかとなった。そこで、in vivoでの機能評価を行なったところ、静脈内投与において肝臓と脾臓で高い遺伝子発現の促進がみられた。

以上、著者は、多機能性エンベロープ型ナノ構造体(MEND)の細胞内動態の主要な律速段階であるエンドソーム脱出課程に着目し、R8の脱出機構の解明とINF7による膜融合に依存しない脱出機構の発見に成功した。本研究は、遺伝子治療や再生医療など21世紀にその実現が期待されている革新的医療の実現に大きく貢献するものと確信する。

よって、著者は、北海道大学博士（生命科学）の学位を授与される資格あるものと認める。