

# 論文の内容の要旨

## 論文題目 Caging Technology for Delivery of Oligonucleotides into Cells

(細胞へのオリゴヌクレオチド送達のためのケーシング技術)

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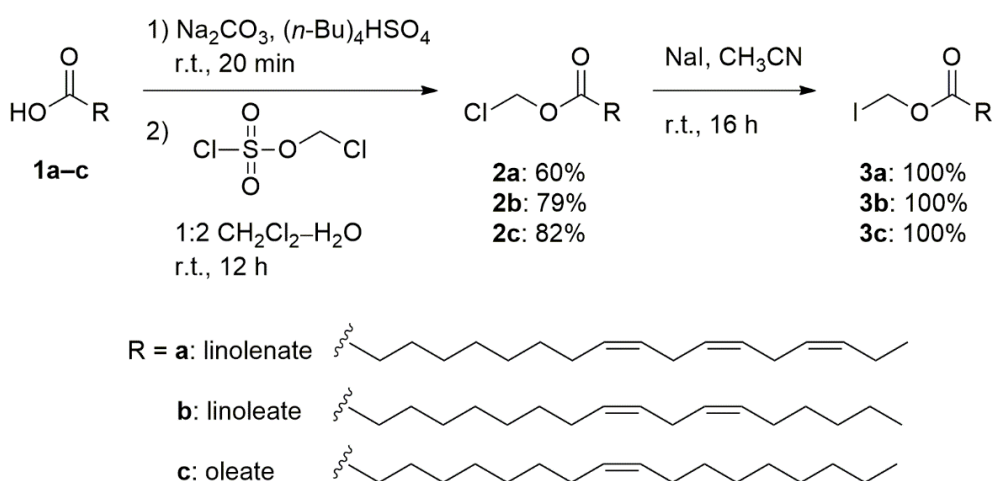
Oligonucleotides play important roles as powerful tools for controlling gene expression and their potential in using for therapeutics are obvious and massive. However, the application of oligonucleotides as therapeutic agents has been limited due to poor bioavailability and cellular penetration. The physicochemical properties of cell membranes are the barriers of the oligonucleotide delivery into the cells. In addition, the polyanionic and hydrophilic properties of oligonucleotides are the major factors that prevent the passive diffusion to the cells. The development of the efficient delivery of oligonucleotides to a specific target site is highly required for the therapeutic application.

To date, various delivery systems have been developed for the oligonucleotides delivery including viral vectors and nonviral vectors delivery systems. However, genotoxicity of viral vectors delivery systems has been investigated due to immunogenicity and possible recombination of oncogenes. On the other hand, most of nonviral vectors composed from polycationic lipids or liposomes have been well- documented of cytotoxicity. Thus, the use of neutral lipid-oligonucleotide conjugates have become an outstanding interest to improve the safe delivery of oligonucleotides and enhance their pharmacokinetic behavior of transmembrane delivery. Recent work in the area of neutral lipid-oligonucleotides conjugates has been used different types of cholesterol-conjugated oligonucleotides to elucidate the requirements for oligonucleotides delivery. However, cholesterol may be linked with cardiovascular events via intravenous administration. In this concept, polyunsaturated fatty acids are safe and naturally essential neutral lipids that play crucial roles in cell growth. Previous studies were proven that polyunsaturated fatty acids conjugates of clinical anticancer drugs have provided significant advances in improving drug's pharmacokinetics and efficacy. We propose that polyunsaturated fatty acids conjugate such as linolenic acid, linoleic acid and oleic acid will be able to deliver oligonucleotides in the cells.

In this study, we developed new method for the conjugation of polyunsaturated fatty acids to the oligonucleotides by postsynthetic modification that motivated by the previous studies showing the phosphate group of ethyl(hydroxymethyl)phosphonate can be selectively reacted with acetoxymethyl bromine. We hypothesized that our strategy in which iodomethyl alkyl ester group that more reactivity than bromine can be reacted with the phosphate group on the internucleotide linkages. Furthermore, polyunsaturated fatty acids have been applied to oligonucleotides for the first time to facilitate the oligonucleotides delivery.

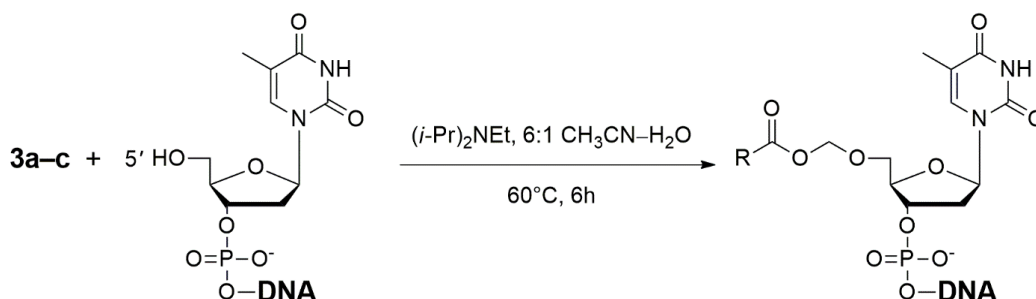
## Results and Discussion

The synthesis of iodomethyl alkyl ester of polyunsaturated fatty acids were synthesized through the two-step transformations (**Fig. 1**). Firstly, the reaction of chlorination at carboxyl group of polyunsaturated fatty acids was employed to form chloromethyl alkyl ester group. Under this condition, chloromethyl chlorosulfate was used as the reagent to obtain the desired chloromethyl alkyl ester of polyunsaturated fatty acids (**2a-c**). Secondly, chloromethyl alkyl ester group were subjected into iodomethyl alkyl ester group. Sodium iodide was treated as the reagent to obtain iodomethyl alkyl ester of polyunsaturated fatty acids (**3a-c**).



**Fig. 1** Synthesis of iodomethyl alkyl ester of polyunsaturated fatty acids

Conjugated polyunsaturated fatty acids with oligonucleotides was synthesized through postsynthetic modification of oligonucleotide by using iodomethyl alkyl ester of polyunsaturated fatty acids. Poly(dT)sequence that is complementary to the poly(A) tails of mRNA was applied as the surrogate for oligonucleotides (**Fig. 2**) DIPEA was added as a catalyst. The reaction mixture was incubated  $60^\circ\text{C}$  for 6 hours. Purification of reaction mixture of the linolenic acid conjugated a poly(dT)sequence was subjected to HPLC analysis. The production of the conjugates were confirmed by MALDI-TOF MS.

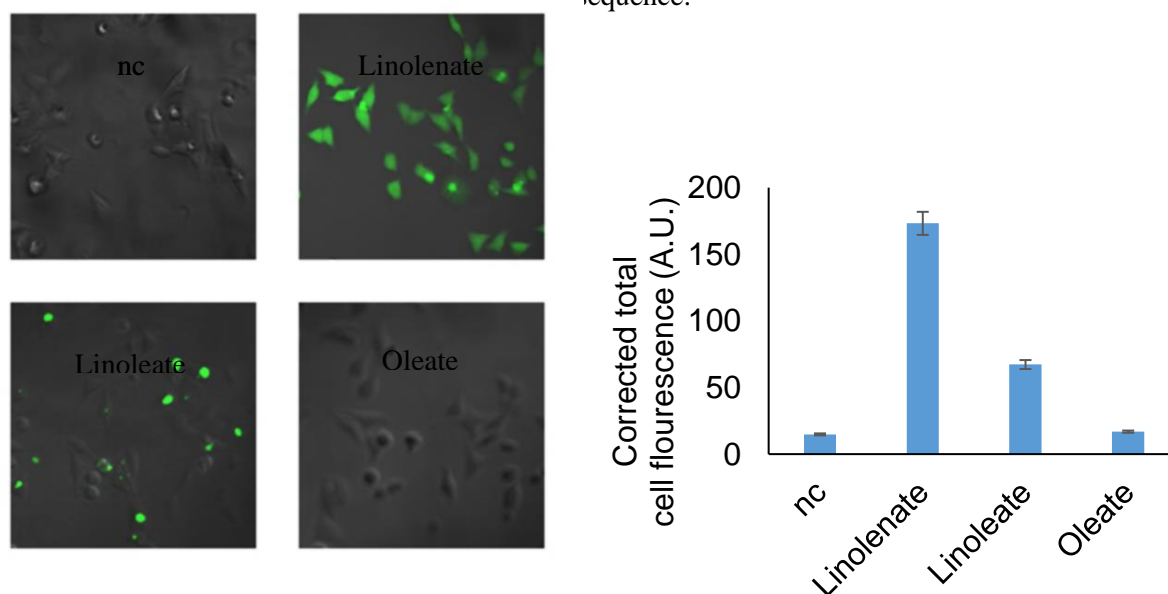


**Fig. 2** Conjugation of DNA with polyunsaturated fatty acids. R = linolenate (a), linoleate (b), and oleate (c).

MALDI-TOF MS suggested that a polyunsaturated fatty acid was attached to DNA strands. At first, we suspected that the conjugation site of oligonucleotides with polyunsaturated fatty acids can react at the phosphate group of the oligonucleotides. However, we observed only 1 conjugation of

polyunsaturated fatty acids with poly(dT)sequences and mixed-sequence of DNA. To elucidate the reaction site of the conjugated unsaturated fatty acids with DNA, the 5'phosphorylation poly(dT)sequence (20-mer) were synthesized. The conjugation of linolenic acid with 5'phosphorylation poly(dT)sequence were proceeded same as the conditions for the conjugation of unsaturated fatty acids with 5'OH poly(dT)sequence. The conjugation of linolenic acid was failed to conjugate with 5'phosphorylation poly(dT)sequence. No desired products obtained from the reaction. Polyunsaturated fatty acids preferably attached to OH- group of deoxyribose on the oligonucleotides.

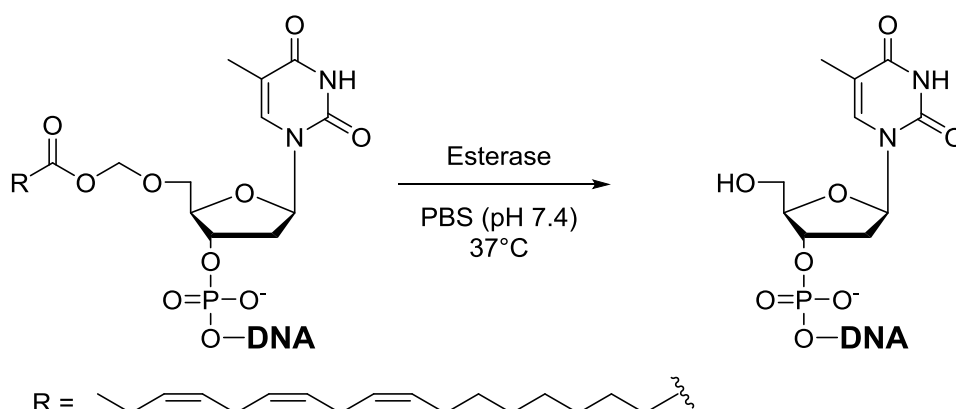
Investigation the cellular uptake properties of the polyunsaturated fatty acids conjugated oligonucleotides. Linolenate, linoleate and oleate conjugated fluorescence poly(dT)sequences were individually incubated with HeLa cells without any transfecting reagent at 37°C for 2 h. The sample solutions containing 2  $\mu$ M probes were incubated for 2 h with cultured HeLa cells and fluorescence positive cells were then detected for the non-modified probe and polyunsaturated fatty acids conjugated oligonucleotides under a confocal microscope. Surprisingly, the maximum intensity was observed for linolenate conjugated fluorescence poly(dT)sequence, which exhibited 94.2% of cellular uptake intensity (Fig. 3). Linoleate conjugated fluorescence poly(dT)sequence showed slightly cellular uptake intensity. Oleate conjugated fluorescence poly(dT)sequence did not show the cellular uptake intensity. The intensity of the fluorescence in the cells increased as the number of double bonds on the polyunsaturated fatty acids. The results suggest that linolenate conjugated fluorescence poly(dT)sequence exhibited the outstanding of the cellular uptake intensity through 1 conjugation of sequence.



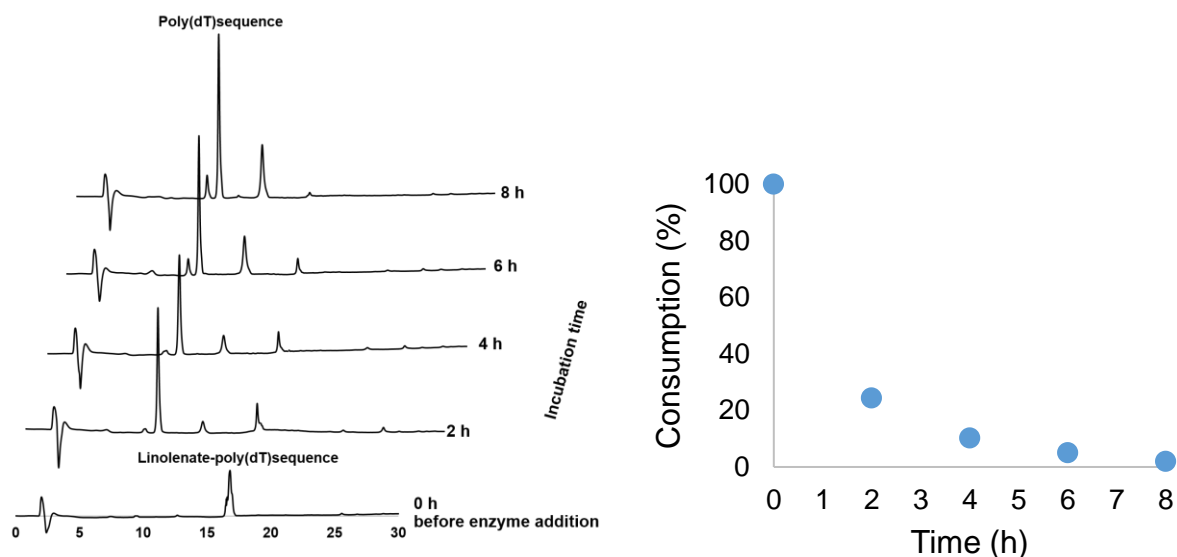
**Fig. 3** Cellular uptake experiments of polyunsaturated fatty acid-conjugate fluorescence poly(dT)sequence, Negative control (nc): unconjugated fluorescence poly(dT)sequences. Bar: 50  $\mu$ m. Statistical analysis of the cellular uptake efficiency by counting fluorescent cells (200 cells each) from imaging data.

Evaluation the deprotection of the linolenic acids on the oligonucleotides was conducted via esterase activities (Fig.4). Linolenate conjugated poly(dT)sequence was treated with esterase from porcine liver, and the reaction was monitored by HPLC. Time course HPLC chromatogram are shown in Fig.5. A peak corresponding to linolenate conjugated poly(dT)sequence was observed in the HPLC chromatogram before the addition of the enzyme. After incubation with the enzyme for 2 hours, the intensity of the peak corresponding to linolenate conjugated poly(dT)sequence extremely decreased.

The reaction proceeded until linolenate conjugated poly(dT) sequences had been finally converted to the deprotected product at 8 hours.



**Fig.5** Esterase activities of the linolenic acid on oligonucleotides



**Fig.6** Deprotection of 5'-linolenate-protected poly(dT) with an esterase. Reverse-phase HPLC profiles showing the time course for the deprotection reaction.

## Conclusions

We have designed and conjugated linolenic acid, linoleic acid, oleic acid to poly(dT) sequence via post-synthesis DNA modification. The reaction site of the polyunsaturated fatty acids attached to 5'-OH group of deoxyribose on oligonucleotides. Linolenic acid conjugated fluorescence poly(dT) sequence showed the outstanding cellular uptake in HeLa cells, as well as slightly cellular uptake was observed in linoleic acid conjugated fluorescence poly(dT) sequence. No fluorescence signals have been detected with unmodified probe and oleic acid conjugated fluorescence poly(dT) sequence. Besides, linolenic acid conjugated poly(dT) sequence was readily cleaved by porcine liver esterase. These results showed that the linolenic acid conjugated poly(dT) sequence described in this study could be used as oligonucleotides delivery owing to its natural and safe essential substances.