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**Study on Role of Liposome Membrane on Gene Expression of
GFP in *E.coli* Cell-Free Translation System**

A dissertation submitted to

THE GRADUATE SCHOOL OF ENGINEERING SCIENCE

OSAKA UNIVERSITY

In partial fulfillment of the requirement for the degree of

DOCTOR OF PHILOSOPHY IN ENGINEERING

BY

BUI THI HUONG

MARCH, 2009

Preface

This dissertation work was conducted under the supervision of Professor Ryoichi Kuboi at the Division of Chemical Engineering, Graduate School of Engineering of Science, Osaka University from 2005 to 2009.

The objective of this thesis is to clarify the role of liposome membrane under stress condition on gene expression *in vitro* system and to clarify their mechanism on elementary steps of gene expression including transcription, translation and folding. The interest is focused on (1) possible role of biomembrane in gene expression and (2) regulation of the gene expression based on the membrane under the stress condition, and (3) deeper understanding of the gene silencing *in vivo*.

The author hopes that this research would contribute to clarifying the potential role of liposome membrane on control to enhance/inhibition of gene expression. The role of liposome membrane *in vitro* and *in vivo* opens a new possible application of the membrane-based gene regulation in the medical and industrial research.

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Summary

Liposome plays an important role in application of drug delivery and can enhance gene delivery and expression inside the cell. However, the role and function of liposome to enhance gene delivery and promote gene expression were still not yet clarified. In this study, the possible role and function of various kinds of liposome on gene expression and their mechanism were investigated and clarified at elementary step of the gene expression of Green fluorescent protein (GFP) such as transcription, translation and folding.

Chapter I: Role of Biomembrane in Gene Expression through Analysis of Elementary Steps: The effect of some kinds of liposome to enhance gene expression of a reporter protein, green fluorescent protein (GFP), was investigated through the investigation of the elementary steps of the gene expression, such as transcription, translation and folding. The result indicated that GFP product was enhanced when it was expressed in the presence of liposome prepared with the 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidyl choline (POPC) and cholesterol (Ch). The amounts of mRNA were found to be promoted and higher than those in the control sample. However, negative and positive charged liposome inhibited gene expression. The above results show that the POPC/Ch liposome could primarily affect the transcription of GFP gene among the possible steps of gene expression, resulting in the enhanced GFP expression.

Chapter II: Role of Model Biomembrane on Gene Expression ~ Negative Charged Liposome Effect on *in vitro* Gene Expression~: The role of negatively-charged liposome prepared by (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) on GFP expression was investigated. The results indicated that, in the presence of liposome, the GFP fluorescence was reduced although total product of synthesized GFP was not changed. The above results indicated that the POPC/POPG liposome could inhibit the GFP expression at a post-translational process (folding) of GFP during its gene expression. The liposome also could strongly interact and inhibit the refolding of unfolded state of GFP to its native one. In this case, surface and interior membrane fluidity of liposome was reduced significantly, implying that the liposome could interact with polypeptide GFP by electrostatic attraction and then interact strongly by hydrophobic interaction to give an inhibition of folding of expressed GFP and refolding of unfold native GFP.

Chapter III: Role of Foreign Membrane on Gene Expression ~ Effect of Cationic Liposome and Stressed Liposome on Gene Expression at Transcription and Translation level~: The effect of cationic liposome on inhibition of gene expression and its mechanism was investigated. The result indicated that 1,2-dioleoyl-3-trimethyl-ammonium-propane (DOTAP) liposome can knock out gene for inhibition of gene transcription. This result implied that liposome could strongly interact with DNA by electrostatic attraction between cationic amino groups in liposome and negative charged phosphate groups of DNA, and/or by hydrogen-bond interaction. Furthermore, cationic liposome can also knock down and interfere mRNA translation. In the presence of DOTAP liposome, mRNA could still exist although it could not be translated into protein. The results indicated that cationic amino groups of liposome can strongly interact with negative charged phosphate groups of mRNA, and neutralized mRNA, resulted in interference of mRNA and silenced its translation. DOTAP liposome can be applied as a new drug modified with specific ligand (ex: ESA (for interaction with target cell (ex cancer cell), inhibition of its growth by knock-out of gene and/or knock-down of mRNA inside the cell.

Chapter IV: Gene Expression Regulated by Membrane and Stress ~ Gene Expression *in vitro* Regulated by Stressed Liposome and Deeper Understanding of *in vivo* Gene Silence ~: The role of the liposome membrane to promote and inhibit the gene expression systems has been summarized to establish the strategy to regulate the gene expression. First, the spectra of the gene regulation by the membrane were summarized with respect to the elementary steps included in the gene expression system, such as (i) transcription, (ii) translation, and (iii) folding. A possible regulation of the gene expression has been achieved by employing some case studies, such as (1) proposal on the GFP production regulated by liposome membrane, (2) re-evaluation of the gene silence of cry protein in *Bacillus thuringiensis* cells, and (3) stress-mediated regulation of GFP gene on the liposome membrane in the cell-free translation system.

The obtained results on the strategy for the regulation of the gene expression by the stressed membrane (membrane interference) were summarized in general conclusion together with the suggestions for future works focusing on the gene silencing and/or bioprocess design.

GENERAL INTRODUCTION

A gene regulation in a biological cell becomes an important issue for the deeper understanding of the gene expression system and the application to the design of the bioprocess or gene-related drug. For example, the regulation of specific gene provides us the opportunities of the medical treatment of disease cells by suppression of target gene (Keizeimi-Esfarjani *et al*; 2000), silence a target gene (Zhang *et al*; 2006) or inactivation of specific enzyme (Kuboi *et al*; 2000). However, there have been many problems in natural gene expression in bacteria and in recombinant host cell, such as (1) low expression of protein product, (2) low stabilization and activity of released protein product, and (3) gene silencing. It is known that the regulation of gene expression could be performed by (i) DNA transcription and (ii) mRNA translation, and (iii) folding of polypeptide in post-translation during the gene expression process, that are described in **Fig.1**.

Gene expression can separately be classified into two aspects: (1) The regulation of enhancement of gene expression that plays an important role in improvement of production technology of target protein, (Klier *et al*; 1982). (2) On the contrary, inhibition of gene



Fig.1 Central dogma in gene expression

expression product that also plays very important role in expression inhibition of undesirable gene such as: (i) oncogene from cancer cell and (ii) gene producing mycotoxin from infective pathologic cells of bacteria, molds that are very dangerous to human, animal and plant. However, the control of gene expression at transcription or translation level is very complicated in their mechanism owing to the complexity of the molecular machinery (McIntosh *et al*; 2001).

Conventionally, nowadays, the DNA delivery by liposome has been accepted as one of the most promising ways for the gene expression (Taylor *et al*; 2003) when there are some problems in low efficiency of gene delivery and unstable gene expression. Beside of this, RNA interference technology supported by liposome, has recently been reported as a novel technique for silencing of a target gene as well as the inhibition of gene expression (Zhang *et al*; 2006, Fire *et al*; 2006). It is therefore supposed that the study on the role of liposome on regulation of enhance or inhibition of gene expression that are very necessary to be carried out.

A biomembrane (**Fig.2**) is commonly known to act as just a physical boundary to separate interior cytoplasm from the environment and the cellular biomolecules. The potential aspects of biomembrane itself as an active and functional interface of biological cell to make a response of the dynamic change of environmental condition (stress) have recently been revealed through the basic research on a model biomembrane. Liposome, a closed

phospholipid bilayer

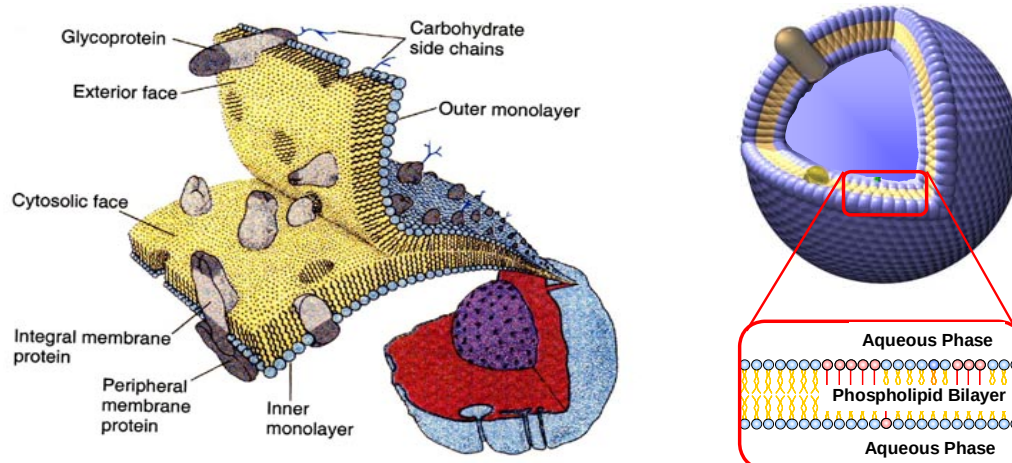


Fig.2 Structure of biomembrane (left) and model biomembrane (right)

membrane, is commonly used as a model biomembrane. The liposome has been applied for drug delivery, gene delivery and expression for the suppression of the specific gene in disease cell (Zhang *et al*; 2006, Hussein *et al*; 2008). Liposome was also applied for RNA interference (RNAi) technology as a carrier for delivery siRNAs to cell for enhancing siRNA stability *in vitro* and *in vivo* for final inhibition of target mRNA translation (Zhang *et al*; 2006). It has also been reported that the liposome can improve gene expression by increasing mRNA transcription by enhanced stability of its polyA tail (Barreau *et al*; 2006). The relation of the liposome is thus attracting many interests.

A model biomembrane, liposome (**Fig.2**), has previously been reported to induce a variety of potential functions under the variable environmental condition that are summarized in **Table 1**. Kuboi *et al*; 2000, have previously indicated that the negative charged liposome POPG and DMPG liposomes can strongly interact by electrostatic and hydrophobicity

interaction with denatured lysozyme and then inhibit its refolding, resulted in inhibition of 88% and 52% lysozyme activity.

Table 1 Summary of potential functions of liposome in enzyme technology

References	Liposome function on
Kuboi <i>et al</i> ; 1997	chaperone-like function
Umakoshi <i>et al</i> ;1998	translocation of proteins across the membrane
Yoshimoto <i>et al</i> ; 1999	membrane fusions
Tuan <i>et al</i> ; 2008	reactivation of oxidized and fragmented SOD
Ngo <i>et al</i> ; 2008	enhancement of released enzyme from Bacterial cell under heat stress condition
Kuboi <i>et al</i> ; 2000	Negative charged liposome inhibit folding of lysozyme

Table 2 Summary on application of liposome on gene technology

References	The role of liposome on gene technology
Taylor <i>et al</i> ; 2003	enhance 10 times of gene delivery and expression in eukaryote cell
Tachibana <i>et al</i> ; 2002	inhibit gene expression in <i>E.coli</i> cell free translation system
Barreau <i>et al</i> ; 2006	stability poly A tail, enhance mRNA stability and translation
Zang <i>et al</i> ; 2006	delivery siRNA to interfere target mRNA translation for treatment of target cancer cell
Safinya <i>et al</i> ; 2001	complex DNA/liposome can increase compact state of DNA, storage high-density genetic information of absorbed DNA, stabilize nucleotide for gene delivery and expression
Oberholzer <i>et al</i> ; 1995	enhancement of the PCR reaction
Nomura <i>et al</i> ; 2003	gene expression within cell-sized liposome or vesicles
Ishikawa <i>et al</i> ; 2004	expression of the cascading genetic network

From this study, possible problem should be further clarified such as whether liposome can also interact with DNA, mRNA and expressed polypeptides or not, if yes by what kind of interaction ? electrostatic, hydrophobic, hydrogen bond interaction. After their

interaction, how about their effects?: liposome can affect on DNA (gene) transcription ? mRNA translation ? folding of expressed polypeptides ?. It is expected that the conformation and activity of DNA, mRNA and some enzymes or peptides during gene expression in a biological system could also be regulated by the interaction of lipid membrane with the above macromolecules. The above subject needs to be further investigated.

Some examples of the liposome applications in gene technology were summarized in **Table 2**. The liposomes also have been emerged as the cell-like microreactor due to many prospective advantages in gene technology, such as enhancement of the PCR reaction (Oberholzer *et al*; 1995), expression of the cascading genetic network (Ishikawa *et al*; 2004) and gene expression within cell-sized lipid vesicles (Nomura *et al*; 2003), increasing mRNA transcription by enhanced stability of its polyA tail (Barreau *et al*; 2006), inhibit gene expression in *E.coli* cell free translation system (Tachibana *et al*; 2002), delivery siRNA to interfere target mRNA translation for treatment of target cancer cell (Zhang *et al*; 2006).

Model biomembrane, liposome, has been emerged with many prospective advantages in gene technology. It has also been reported that liposome can be successfully applied to enhance gene delivery and expression up to 10 times in Eukaryote cell (Taylor *et al*; 2003). The application of liposome in gene delivery and expression has been indicated that liposome has been widely applied in complexing with gene as “lipoplexes” to enhance gene delivery and expression inside the cell. Conventionally, the DNA delivery by liposome has been accepted

as one of the most promising ways for the gene expression although there are still some problems in low efficiency of gene delivery and unstable expression (Taylor *et al*; 2003, Brown; 2001).

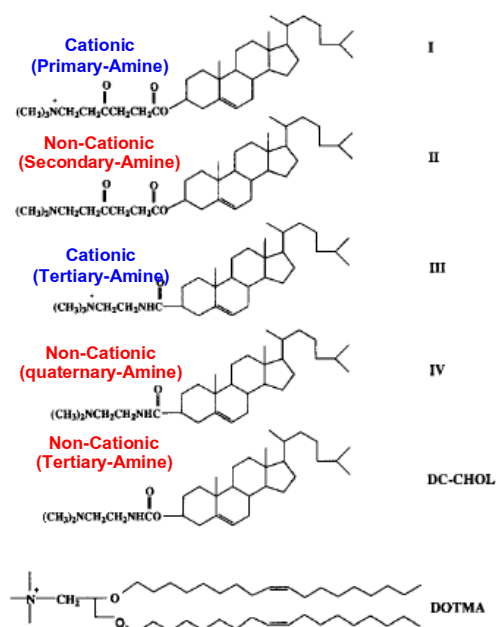


Fig.3 Structure of some cationic amphiphiles used in cationic liposomes.

Table 3 Summary of functions of some cationic amphiphiles inside the cell

Cationic amphiphiles	PKC inhibition K_i (μM)	CAT activity (mU/mg protein)	Cellular toxicity (% viable cells)
I ^b	59	5	85
II	191	143	95
III	12	4	78
IV	258	56	92
DC-Chol	> 1000	108	84
DOTMA	95	40	63

According to Song Li *et al*; (1995) the effects of Hetero-surface liposome on gene transfection efficiency are protein kinase C (PKC) inhibition, expression of chloramphenicol acetyltransferase (CAT) and cellular toxicity in Hela cell are also described in **Fig.3** and **Table 2**. Structures of some cationic amphiphiles are described in **Fig.3** including: (1) A cationic moiety containing an amino group (primary, secondary, tertiary and quaternary), (2) A spacer arm, (3) Lipid anchor to be chosen by cholesterol because of its lipid bilayer stabilizing activity and minimal toxicity to the treated cell. The effects of cationic amphiphiles inside the cell are also summarized in **Table 3**.

According to Brown *et al*, (2001), cationic liposome can complex with gene because liposome has following function: (1) Cationic lipids possess a hydrophobic group, which may either be one or two fatty acid, or alkyl moieties of 12–18 carbons in length, or a cholesteryl moiety in addition to an amine group. (2) Amine group: necessary for DNA binding moiety interacting electrostatically with DNA, condensing the large anionic molecule into small transportable units-lipoplexes. (3) Cationic lipid tends to fully neutralize the phosphate groups on DNA (**Fig.4**).

Cationic liposome can enhance gene delivery when increasing: (1) Number of amino groups, (2) distance between amine groups and hydrophobic units. DNA/cationic liposome produce condensed DNA along with tubular structures and aggregated liposomes, interact with cell membrane, followed by endocytosis and disruption of endosomes. According to

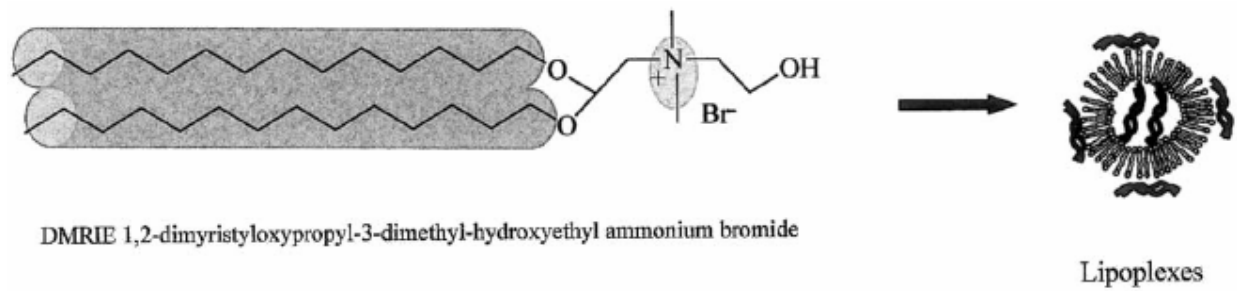


Fig.4 Lipoplexes of cationic lipid and DNA

Safinya, R, C *et al*, (2001), the cationic liposome can be used for gene delivery because complex DNA/liposome can increase compact state of DNA, storage high-density genetic information of absorbed DNA, stabilize nucleotide, and resulted in enhancement of gene delivery and expression (**Fig.5**). Furthermore, liposome is also known to be less toxic inside the cell, it was applied to enhance the gene delivery and expression in eukaryote cell as shown in **Fig.6**, (Brown *et al*, 2001; Taylor *et al*, 2003).

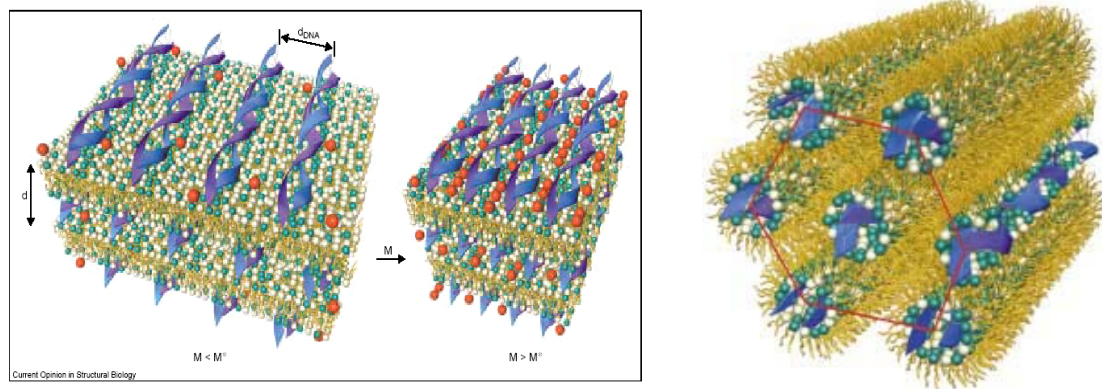


Fig.5 DNA chains adsorbed onto surface cationic lipid membrane

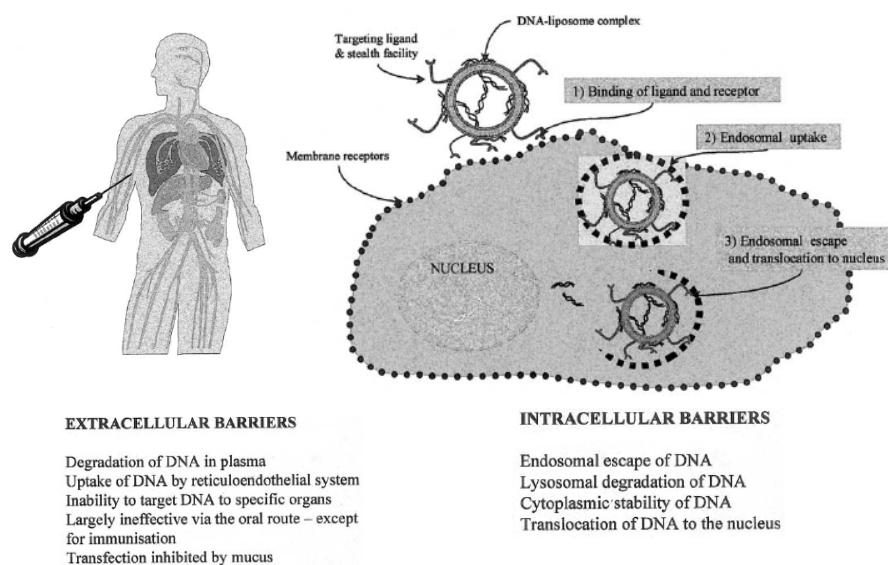


Fig.6 Various barriers to gene delivery by liposome at extracellular and Intracellular.

Such a problem in the gene regulation in the biosystems can be seen in the screening and isolation of effective microorganism for the development of the bioprocess. Bioinsecticides from *B. thuringiensis* are recognized as safety for health of human and animals and for the environment (Tabashnik *et al*; 1983). In Vietnam, the demand for bioinsecticides is increasing as people learn more about the dangers of chemical pesticides. They want to consume so-called “clean vegetables” or clean food that has no chemical pesticide residues. The Ministry of Agriculture and Rural Development as well as the Ministry of Science Technology and Environment of Vietnam supported the trial production of *B. thuringiensis* bioinsecticide to control insects in vegetables, fruit and stored grains. They realized that it was necessary to improve the production technology for the *B. thuringiensis* bioinsecticide as well as to find formulation suitable for controlling insects on vegetables, fruit and stored grains.

Bacillus thuringiensis (Bt) is a gram-positive, spore-forming bacterium that produces toxin

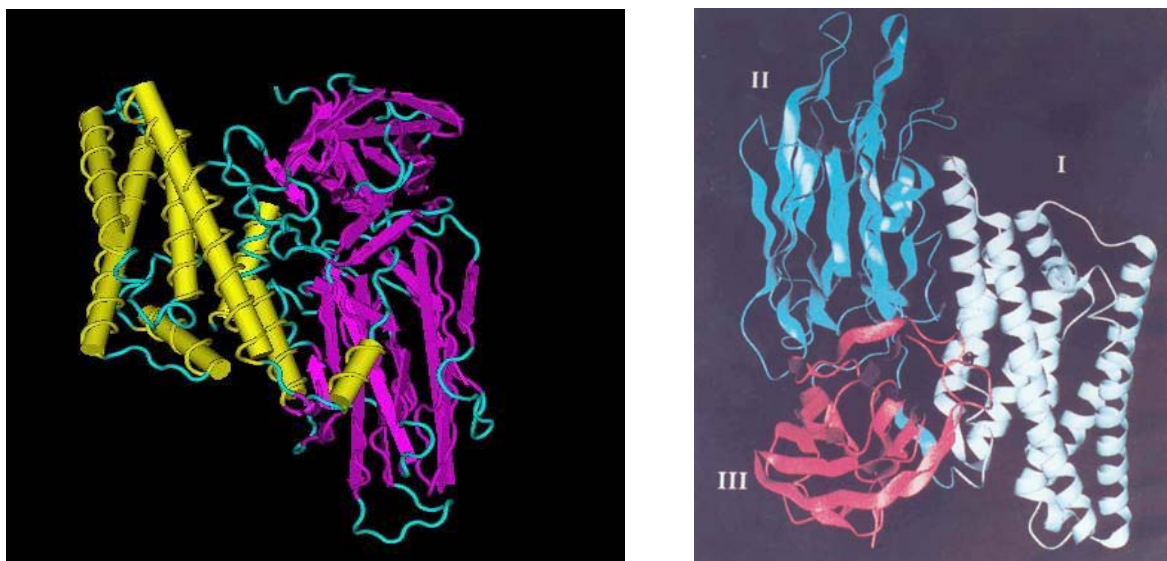


Fig.7 Structure of *cry1Ab* gene from *B.thuringiensis* encoding for toxic protein to insect

crystal protein active against Lepidopteran, Dipteran and Coleopteran insects. Bt crystal protein genes have been designated *cry*. Insecticidal activities of Cry1Ab, Cry1C proteins were showed by Asano *et al*, (1999). The *cry1* gene group encodes bipyramidal protein active mainly against lepidopteran insects. The Cry1Ab protein has insecticidal activity to *Manduca Sexta*, *Heliothis virescens*, *Pieris brassicae*, *Plodia interpunctella*, *Spodoptera lituralis*, *Plutella xylostella*. The Cry1C protein has insecticidal activity to *Spodoptera lituralis*, *Plodia interpunctella*, *Plutella xylostella*. Structure of *cry1Ab* gene encoding toxic protein to insect was described in **Fig.7**. However, some Bt strains contain “silent” crystal toxin genes which are carried on the genome but not expressed. As a result, some Bt strains that do not show high insecticidal activity may carry toxin genes that encode insecticidal protein with high specific activities, Asano *et al*, (1999). Klier *et al*, (1985) have succeeded to clone *cry* gene for expression in *E.coli* host cell for enhancement of gene expression of toxic crystal protein.

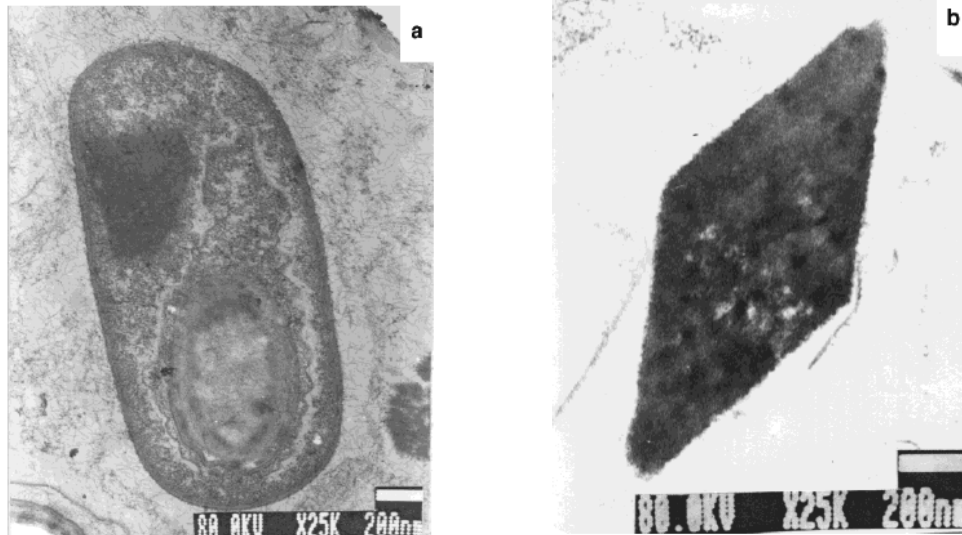
However, when one would produce and recover the Cry proteins from the *B.thuringiensis* strains, it seems that some problems were remained including: (1) low activity protein product, (2) long fermentation for producing target protein product, low release of protein product from inside to outside the cell, (3) low stabilization of released protein product, (4) low activity of protein product, and (5) unexpected gene silencing in bacterial cell as schematically shown in **Fig.8**. These problems are very necessary to be solved and contribute to enhance target protein production technology.

Liposome were successfully applied to enhance gene delivery and expression, beside of these, the liposome has been successfully applied for drug delivery, gene delivery and expression for the suppression of the specific gene in disease cells (Husseini *et al*; 2008). According to Ngo *et al*, (2008), under heat stress condition, liposome can enhance release enzyme product during fermentation and finally to enhance enzyme activity. Furthermore, according to Tuan *et al*, (2008), under oxidative stress condition, liposome has effect on reactivation of oxidized and fragmented SOD, resulted in enhancement of enzyme activity. The use of the potential functions of liposome induced under stress condition could solve the above problems remained (1)~(5). It is expected that the conventional problems could be solved by deeper understanding of the practical phenomena which were happened inside a bacterial cell during the operation.

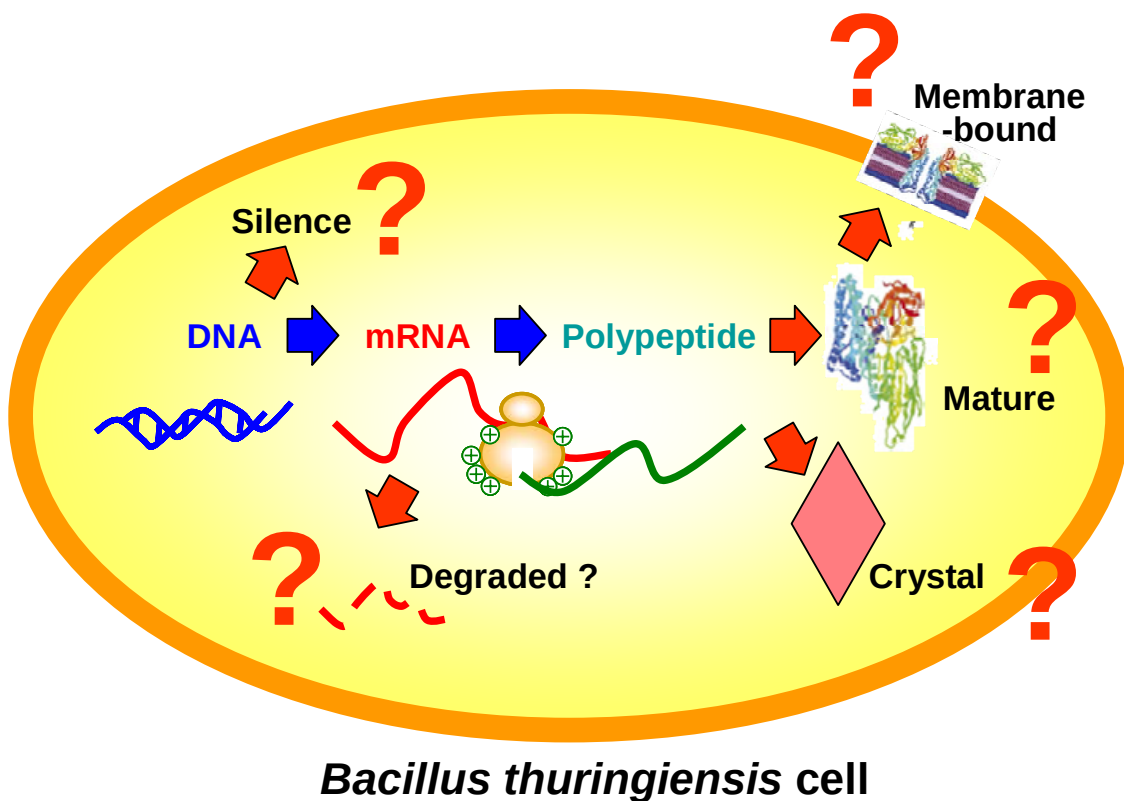
The inhibition of gene expression of toxic target gene that also plays a very important

role in prevention of toxic enzyme from infective bacteria or the growth of disease cells (for example of cancer cell) as well as in treatment of their effect in plants, animals and human. Although the inhibition of gene expression was indicated by some studies *in vitro* and *in vivo* experimental systems, their mechanisms were still not clarified yet (Tachibana *et al*; 2002). By using the liposome, Yoo *et al*, (2001) have successfully achieved the delivery E1A gene that was appreciated for its ability to produce proteins that inhibit oncogene HER-2/neu expression in both of rodent and human breast cancer cells. The human HER-2/neu oncogene over-expressed in many human cancers, and the E1A after delivery by liposome, has been shown to act as a tumor inhibitor gene by down-regulating HER-2.neu transcription.

Nowadays, target gene inhibition by siRNA, “RNAs interference” mechanism was revealed in Nobel Prize 2006, (Andrew *et al*; 2006). The human oncogene is over-expressed in many human cancers, in order to inhibit its expression and many researchers have been studying on application of liposome for drug delivery to treatment of cancer cell. According to Zhang *et al*, (2006), liposome was successfully used to encapsulate and delivery siRNA for interference of target mRNA of cancer gene HDM2 in cancer cell SKMES, resulted in 70% suppression of cancer cell growth. However, in this study, the role of liposome itself can directly interfere mRNA translation that was still not yet clarified. The role of liposome on interference of mRNA will be identified in our study.



Transmission electron microscope view of sporulating *Bacillus thuringiensis* (left) And bipyramidal crystal parasporal body (Right) (Bravo and Sobero, 2008)



Problems in cry proteins in *Bacillus thuringiensis*

- | | |
|-----------------------------------|-----------------------------------|
| # Low Activity of Protein Product | # Long Fermentation |
| # Low Release | # Low Stability of Target Product |
| # Gene Silencing | |

Fig.8 Problems in cry Protein Expression in *Bacillus thuringiensis*

Yoo *et al*, (2001) has reported to use the liposome to delivery E1A gene that was appreciate for its ability to produce proteins that can inhibit oncogene HER-2/neu expression in both rodent and human breast cancer cells. The human HER-2/neu oncogene is over-expressed in many human cancers, and the E1A after delivery by liposome, has been shown to act as a tumor inhibitor gene by down-regulating HER-2.neu transcription. The possible role of cationic liposome on mRNA interference and their mechanism will be investigated and clarified in chapter 3 from our study.

Beside of this, it has been reported that the apoptosis (cell suicide) can be triggered by production of improperly folded or incomplete proteins (Sugita *et al*; 1995, Chow *et al*; 1995), when there is a large, rapid, and overwhelming accumulation of unfolded protein, a rapid apoptosis occurs and then most cells will die. Similarly, apoptosis can be triggered by incubation with amino acid analogs or low doses of puromycin (Sugita *et al*; 1995). The inhibition of gene expression is therefore very important in this case

The gene expression inhibition of toxic gene also plays a very important role in prevention of toxic enzyme or disease cells (cancer cell) as well as in treatment their effect in plant and animal. At present, the increase of bacterial resistance to conventional antibiotics has become a major problem giving rise to an urgent need for new approaches to treat bacterial infection. In cystic fibrosis patients, pathogens like *Pseudomonas aeruginosa* show high levels of resistance and lead to progressive lung deterioration and premature death

(Hancock *et al*; 1998). To overcome antibiotic resistance, study on gene expression inhibition of toxic infective bacteria is very important and need to be carried out. Although gene expression inhibition was indicated by some studies *in vitro* and *in vivo*, their mechanisms in many cases were still not yet clarified.

According to Tachibana *et al*, (2003), cationic liposome can inhibit gene expression *in vitro* translation system. However, mechanism of cationic liposome effect on inhibition of gene expression was still not yet clarified. Beside of this, the extracellular DNA existences in environment such as in terrestrial environments, aquatic environment, water microcosms were dangerous problems to environment and human health, that need to be treated, (Zhu, B *et al*; 2006). So, degradation of extracellular DNA and silencing mRNA by liposome are also necessary to be solved. Studies on the potential role of the biomembrane on the gene expression have thus been limited until now. The knock-out of gene for inhibition of transcription and knock-down of mRNA for inhibition of translation by liposome should therefore be investigated and clarified.

A reporter protein is often used to confirm the expression of the specific gene. Various kinds of the reporter proteins, such as β -galactosidase, β -glucuronidase, and chloramphenicol acetyltransferase, have been commonly used as the reporter proteins which show (i) easily measurement of the activity, (ii) less toxicity to host cell and (iii) detectable color or easily-stained nature. Since Shimomura, Nobel Prize winner in chemistry, 2008,

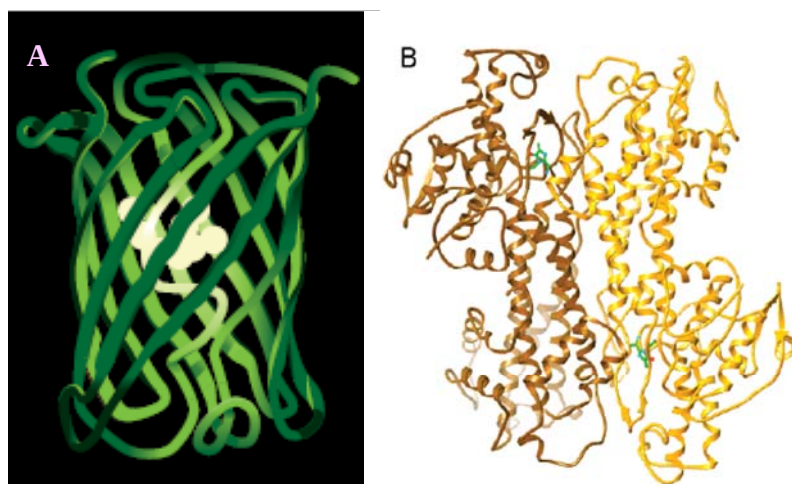


Fig.9 Overall GFP structure of (A) fold (mature GFP) and (B) unfold style (modification in Post-translation). A and B cited by Shimomura *et al*, Nobel prize in chemistry 2008 and Barondeau, P.D. *et al*, 2005; respectively.

discovered the green fluorescent protein (GFP) (**Fig.8**), the GFP structure look like a useful beer can, inside the beer can structure, the amino acids 65.66 and 67 form the chemical group reporter protein in the past decade as a guiding star for biochemists, biologists, medical scientists and other researchers. The strong green color of this protein appears under blue and ultraviolet light. It can, for example, illuminate growing cancer tumors; show the development of Alzheimer's disease in the brain or the growth of pathogenic bacteria. GFP has 238 amino acids, linked together in a long chain. This chain folds up into the specific structure that absorbs UV and blue light and green fluorescence.

The chemical processes of cells are usually regulated by proteins. There are tens thousands of different proteins, each with different functions. By connecting GFP to one of these proteins, researchers can obtain vital information. They can see which cells a particular

protein inhabits, they can follow its movements and watch its interactions with other proteins. Thanks to GFP's green light scientists can now track a single protein under the microscope. GFP is a reporter protein; its fluorescence represents for folded (mature) GFP product as well as activity of enzyme. It is rational to select the GFP as a reporter protein to investigate the gene expression *in vivo* and *in vitro*.

The final purpose of this study is to investigate the possible role of the biomembrane on the regulation of gene expression and to clarify their mechanism by using an *E.coli* cell free translation system (*in vitro*). The gene expression was investigated, focusing on the role of the membrane in elemental steps of the total GFP gene expression process, such as (i) transcription, (ii) translation and (iii) folding of the gene product (polypeptide). Based on the obtained results and previous findings, a possible role of the liposome during the gene expression was finally discussed. The framework and flow chart of the present study is schematically shown in **Fig.9** and **Fig.10**.

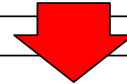
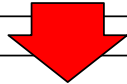
In **Chapter I**, the model experimental system to verify the role of liposome membrane during the gene expression process has been established based on the problems underlying the conventional recombinant technology. A model system was secondly established to investigate the possible effect of various kinds of liposome under stress condition for the enhancement of the gene expression and the clarification of their mechanism. *in vitro* scale gene expression of a reporter protein, GFP, was selected with and without

liposome to investigate the role of liposome membrane on gene expression of GFP in *Escherichia coli* cell-free translation system. The effect of some kinds of liposome on enhance gene expression of a reporter protein, green fluorescent protein (GFP) was investigated. The result indicated that the expression of GFP product was enhanced in the presence of liposome prepared with the 1-palmitoyl-2-oleoyl-sn- glycerophosphatidyl choline (POPC) and cholesterol (Ch). The amounts of mRNA were found to be promoted and higher than those in the control sample. However, negative and positive charged liposome inhibited gene expression. The above results show that the POPC/Ch liposome could primarily affect the transcription of GFP gene among the possible steps of gene expression resulted in enhance GFP expression. However, anionic charged liposome and cationic liposome inhibited gene expression of GFP that mechanism will be clarified in our next study in chapter II and chapter III. In **Chapter II**, a typical response of the gene expression system was performed in the presence of the model liposome, which has a similarity with the natural biomembrane, by selecting the negatively-charged lipid as a target liposome. The role of negatively-charged liposome prepared by (POPC) and 1-palmitoyl-2-oleoyl-sn-glycerophosphoglycerol (POPG) on GFP expression was investigated. The results indicated that in the presence of liposome, the GFP fluorescence was reduced although total synthesized GFP product was not changed. The above results mean that the POPC/POPG liposome could inhibit GFP expression at post-translational process of

Chapter I

Role of Biomembrane in *in vitro* Gene Expression through Analysis of Elementary Steps

- # Analysis of Elementary Step of Gene Expression (Transcription, Translation and Folding)
- # Role of Membrane at Elementary Step
- # Role of Standard Liposome and Microdomain Liposome



Chapter II

Role of Model Biomembrane on Gene Expression

~ Negative Charged Liposome on *in vitro* Gene Expression~

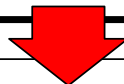
- # Negatively-Charged Liposome (POPC/POPG)
- # Role of Negatively-Charged Liposome at Post-Translation Level

Chapter III

Role of Foreign Membrane on Gene Expression

~ Effect of Cationic Liposome on Gene Expression at Transcription and Translation level~

- # Role of Cationic Liposome at Transcription Level
- # DOTAP liposome for "Knocking-Out" of Gene
- # Role of Cationic Liposome at mRNA Translation Level
- # DOTAP Liposome for "Knocking-Down" of mRNA



Chapter IV

Gene Expression Regulated by Membrane and Stress

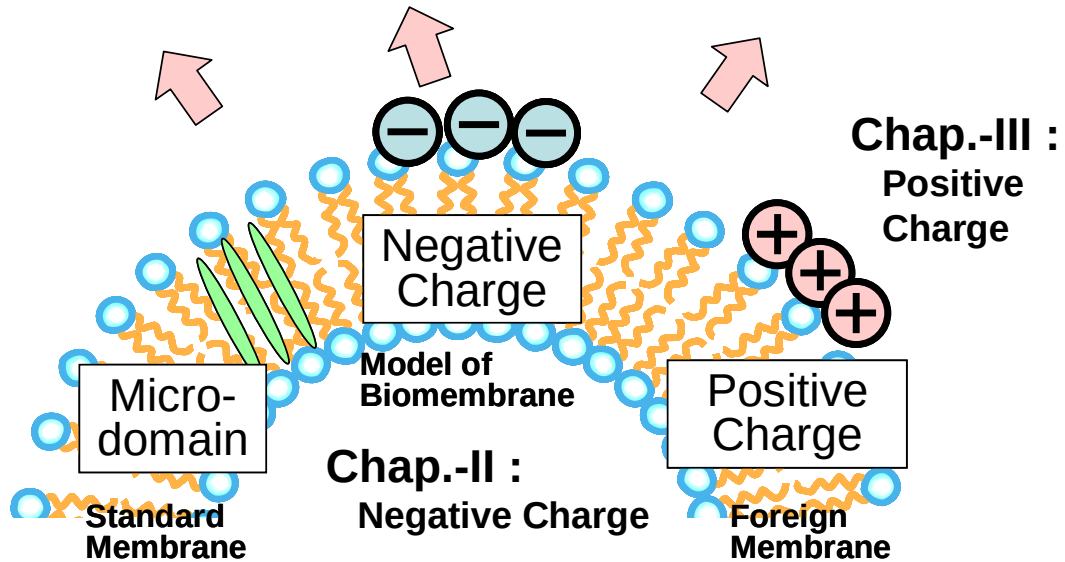
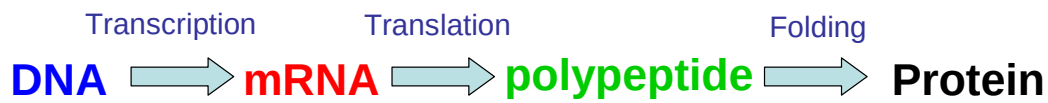
~ Gene Expression *in vitro* Regulated by Stressed Liposome and Deeper Understanding of *in vivo* Gene Silence ~

- # [Spectra of Membrane-Affected Regulation of Gene](#)
- # Enhanced Production of GFP Gene Expression
- # Membrane-Based Understanding of *cry* Gene Silence in Bt Strains
- # Role of Stressed Membrane on GFP Gene Expression (Switchable Regulation by Oxidative Stress)

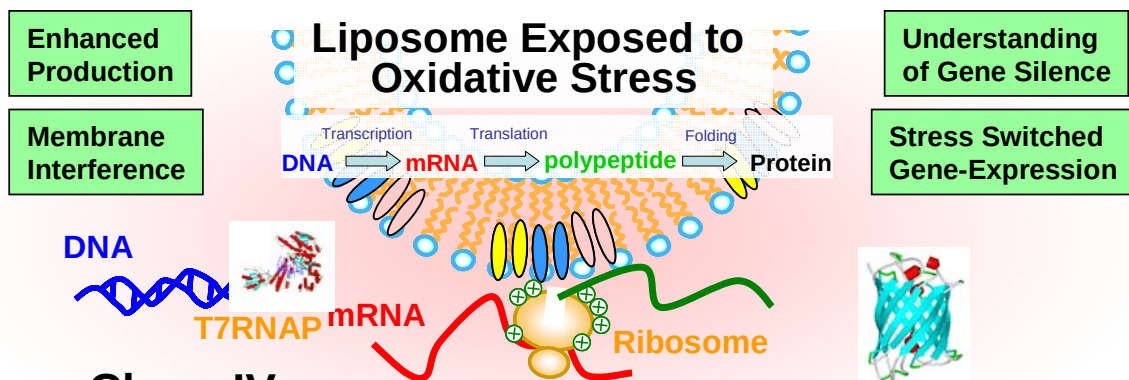
Fig.9 Flowchart of the present study

Chap.-I :

Analysis of Elementary Steps of Gene Expression



	Neutral POPC, PMPC, DPPC, etc	Microdomain POPC/Ch etc	Negative-Charge POPC/POPG	Positive-Charge		
				POPC/SA	DOTAP	DOTAP/ Oxidative Stress
DNA						
Transcription	+	++	++	++	--	~0
RNA						
Translation	+	++	~0	--	--	-
Polypeptide						
Folding	+	+	-	+/-	+/-	+/-
Protein						



Chap.-IV :

Spectra of Membrane-Affected Gene Expression and Its Application

Fig.10 Conceptual illustration of the present study

the folding of GFP during gene expression. The liposome could also interact with mature expressed GFP in cell-free system. Beside of this, liposome also could strongly interact and inhibit the refolding of unfolded state of GFP to its native one. In this case, outer and inner membrane fluidity of liposome was reduced significantly. This result implied that liposome could attract with polypeptide GFP by electrostatic attraction and then interact strongly by hydrophobic interaction, resulted in inhibition of folding of expressed GFP and refolding of unfold native GFP.

In **Chapter III**, the role of the foreign (non-natural) membrane on the gene expression has been investigated by selecting the cationic liposome. The cationic DOTAP liposome of 1,2-dioleoyl-3-trimethyl- ammonium-propane liposome can knock out gene for inhibition of gene transcription in *E.coli* cell free translation system. This result implied that liposome can interact with DNA by electrostatic attraction between cationic quaternary ammonium groups in DOTAP liposome and negative charged phosphate groups of DNA, and/or by hydrogen bond interaction between liposome and hydrogen linkage of 2 strands of DNA. Furthermore, cationic liposome can also knock down and interfere mRNA translation. In the presence of DOTAP liposome, mRNA could still exist but it could not be translated into protein. The results indicated that cationic quaternary ammonium groups of liposome can interact with negative charged phosphate groups of mRNA, and neutralized mRNA, resulting in the interference mRNA and the silence of its translation. The obtained results demonstrated

the potential roles of membrane in prospectively applicable in possible future studies such as:

(1) using neutral liposome for enhancement of the target gene expression, (2) Using cationic DOTAP liposome for treatment of extracellular free DNA that may be dangerous to environmental and human health. (3) Knock-out of target gene in human and animal by using DOTAP liposome as a high quality drug; (4) Knock-down of target mRNA and interfere its translation for inhibition of gene expression at translation level.

In **Chapter IV**, a total view of membrane-affected gene regulation has been indicated. The role of the liposome membrane to promote and inhibit the gene expression systems has been summarized to establish the strategy to regulate the gene expression based on the “Membranome”. First, the spectra of the gene regulation by the membrane were summarized with respect to the elementary steps included in the gene expression system, such as (i) transcription, (ii) translation, and (iii) folding. The gene regulation with the involvement of membrane can be applied for the following case studies.

(1) Gene regulation in E.coli cell-free translation system: The effect of microdomain liposome membrane POPC/Ch and DOTAP to enhance and inhibit gene expression of GFP that are also further applied with MBP, and GST, that prospective effect of biomembrane can be expected to regulate other gene expression in cell-free translation system and inside the cell.

(2) Understanding of gene silence in vivo: The expression of *cry* gene in *B.thuringiensis* could be regulated by the biomembrane. That problem is very important and necessary to be

resolved as well as many other target genes expression inside the cell. All *B. thuringiensis* containing *cry1Ab* gene encoding Cry1Ab protein having high insecticidal activity to target insects, except for 28S isolate. The 28S isolate harboring “silent” *cry 1Ab* was studied.

(3) *Stress-mediated gene switch in E.coli cell-free translation system*: The DOTAP liposome can inhibit 100% gene expression. However, in the presence of liposome, under oxidative stress condition, GFP expression from DNA and mRNA could be re-expressed in comparison with control samples without oxidative stress, indicating that the oxidative stress was induced by hydrogen peroxide could prevent the “silence effect” and “inhibitory effect” of the DOTAP liposome.

The results obtained in this work are summarized in general conclusion. Suggestions for future work are described as extension of the present dissertation.

Chapter I

Role of Biomembrane *in vitro* Gene Expression through Analysis of Elementary Steps

Introduction

A biomembrane is commonly known to act as just a physical boundary to separate the environment and the cellular biomolecules. The potential aspects of biomembrane itself as an active and functional interface of biological cells to make a response of the dynamic change of environmental condition (stress) have recently been revealed through the basic research on a model biomembrane. A model biomembrane, liposome, has previously been reported to induce a variety of potential functions under the variable environmental condition, such as a chaperone-like function (Kuboi *et al*;1997), translocation of proteins across the membrane (Umakoshi *et al*; 1998), and membrane fusions (Yoshimoto *et al*; 1999). It has recently been reported that the activity of fragmented superoxide dismutase (SOD) with no specific conformation and no activity is enhanced under an oxidative stress condition in the presence of liposome prepared by 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) through the enhancement of the interaction between the fragmented SOD and the liposome (Nagami *et al*; 2005; Tuan *et al*; 2008). The lipid bilayer membrane is thus known to play an important role in controlling the enzymes structure and activities under the above stress

condition. It is expected that the conformation and activity of some enzymes or peptides in a biological system could also be regulated by the interaction of lipid membrane with the above macromolecules under appropriate stress conditions.

A genotype does not always match with the phenotype although a demand for the rapid engineering of complex phenotypes in industrial bacteria gradually increased (Patnaik *et al*; 2008). Among the possible reasons of no matching, some evidences of the possible roles of liposome in “gene expression” have recently been reported both of *in vivo* and *in vitro*. For example, in the case of the design of the gene delivery system, cationic-lipid-based carriers have emerged as the most popular nonviral method to deliver a gene into a host cell efficiently. However, the liposome used as a vector has been claimed to have the enhanced effect of not only gene delivery but also the gene translation itself in eukaryote cells (Brown *et al*; 2001; Taylor; 2003). From another aspect, the liposomes have emerged as the cell-like microreactor due to many prospective advantages in gene technology, such as enhancement of the PCR reaction (Oberholzer; 1995), expression of the cascading genetic network (Ishikawa *et al*; 2004), and gene expression within cell-sized lipid vesicles (Nomura; 2003). Although the liposome-allowed gene expression inside liposome vesicles has been reported by some researchers (Ishikawa *et al*; 2004; Noireaux; 2004), the role of the liposome membrane on gene expression including transcription and translation of gene and folding of translated polypeptide, has not been fully clarified yet. As a result, almost all researchers consider that

only “elementary effect” of liposome could be a key factor of the gene-expression inside a liposome. Studies on the potential role of the biomembrane on the gene expression have thus been limited until now.

The final purpose of this chapter is to clarify the possible role of the biomembrane on the gene expression. A model system to investigate the possible role of the biomembrane was established by using the *in vitro* cell free translation system and by employing the green fluorescent protein (GFP) as a target product. The effect of the addition of liposome, a model membrane, on gene expression was investigated by using a cell-free (*in vitro*) translation system, focusing on its role in elemental steps of the total gene expression process, such as (i) transcription and (ii) translation of gene, and (iii) folding of the gene product (polypeptide). Based on the obtained results and previous findings, a possible role of the liposome during the gene expression was finally discussed.

Materials and Methods

1. Materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dimirystoyl-*sn*-glycero-3-phosphocholine (DMPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol (POPG) and 1,2-dioleoyl-3-trimethyl-ammonium-propane (DOTAP) were purchased from Avanti Polar Lipids, Inc.

(Alabaster, AL, USA). Cholesterol (Ch) was purchased from Sigma-Aldrich. The other reagents were of analytical grade. Rapid Translation system RTS 100 *E.coli* HY Kit, an *in vitro* protein synthesis system based on *E.coli* lysate, was purchased from Roche Diagnostics (Indianapolis, IN, USA). The purified green fluorescent protein (Pure GFP) was purchased from Roche Diagnostics (Indianapolis, IN, USA). Other chemicals were purchased from Wako Pure Chemical (Osaka, Japan).

2. Liposome preparation

The phospholipids used in this study are phospholipid molecules of (i) POPC, (ii) DMPC, (iii) DPPC, (iv) POPC and Ch at a ratio of (6.67/3.33), (v) POPG, and (vi) cationic lipid DOTAP were solubilized in chloroform; a phospholipid thin layer was then prepared in a round-bottom flask by evaporation at 37°C using a vacuum rotary evaporator and dried under nitrogen flow for at least 5 hours. In order to generate multilamellar vesicles, the dried phospholipid thin layer was hydrated with water (pH 7). The freeze–thaw cycle was repeated 5 times with hydrated phospholipid solution for large unilamellar vesicle formation. This vesicle solution was extruded through a polycarbonate membrane with a pore size of 100 nm to form unilamellar vesicles (liposome).

3. *in vitro* expression of GFP with and without liposome.

A rapid translation system (RTS 100 *E.coli* HY Kit), *in vitro* protein synthesis system

based on *E.coli* lysate, was used as a cell free translation system (Spirin *et al*; 1998). The expression vector, pIVEX2.3d, contained the gene for expression of the green fluorescent protein (GFP). The reaction solution (50µl) contained the following components: 12µl *E.coli* lysate, 10µl reaction mix, 12µl amino acids, 1µl methionine, 5µl reconstitution buffer, and 0.5µg of circular DNA template in 10µl of sterilized DNase and RNase free water. The reaction solution was initiated by mixing the DNA with other solutions at 30 °C for 6 – 18 hours (“Expression” operation) and was kept for 24 hours at 4 °C (“Maturation” operation). The liposome solution prepared at higher concentration was added in the reaction mixture in the replace of the above water before and after the “Expression” operation and the effect of liposome on the gene expression was evaluated based on the following methods.

4. Analysis of gene product *in vitro* gene expression

Three kinds of analysis, such as (i) GFP fluorescence, (ii) SDS-PAGE, and (iii) mRNA content, were employed to the quantification of gene product according to the previous reports (Noireaux; 2004; Tachibana *et al*; 2002). The amount of folded GFP product was evaluated by analysis of the GFP fluorescence intensity at 395 nm (excitation) and 509 nm (emission) by using the fluorescence spectrophotometer (JASCO, FP-777, Tokyo, Japan) with slit width of 5nm at excitation and emission of light path. All the GFP fluorescence spectra were analyzed after 100 times dilution of the reaction samples to water in order to avoid the

possible interference of the liposome included in the final reaction sample. It was confirmed that the fluorescence spectra of pure GFP was not affected in the above experimental condition including liposome at the finally-diluted solution and also POPC liposome itself at its final concentration has no emission spectra in the above condition for fluorescence measurement. Total amount of GFP products (unfolded and folded GFP) were also analyzed by SDS-PAGE in order to analyze the net amounts of translated GFP. A 10 μ l sample of reaction solution was dissolved with 17 μ l of SDS-PAGE sample buffer and 5 μ l of mercaptoethanol and was heated for 5 min at 95°C for denaturation. The samples with 1 to 4 μ l were applied onto a ready-made gel (Homologous 12.5, GE Healthcare, Chicago, IL, USA) set for electrophoresis system (PhastSystem, GE Healthcare, Chicago, IL, USA). The gel was finally stained with Coomassie blue dye and GFP was detected as major staining band at 27 kDa. The distribution of the protein in the stained gel was further quantified by densitometer analysis by using the SCION image software obtained at <http://www.scion.com/>, where the densitometer analysis was carried out at least three times at different points along the lane. It was confirmed that the band of GFP was not observed in the reaction solution for protein translation without plasmid DNA.

SV total RNA isolation Kit (Promega, Madison, WI, USA) was used to isolate total RNA for the quantification of mRNA contents. The initial mRNA content (4 hours expression) was herewith measured with and without plasmid DNA in the presence or

absence of POPC/Ch liposome to avoid the mRNA degradation in the reaction solution owing to the instability of mRNA. The 25 μ l solution of GFP expression was transferred to the 212.5 μ l buffer. Samples were transferred into spin column assembly and 100 μ l 95% nuclease free-ethanol was added to precipitate the proteins. After centrifugation (14,000 rpm for 1 minute) and wash by buffer, DNA was removed by 25 μ l DNase. After incubation for 15 minutes at 25 °C, 100 μ l DNase stop solution was added into spin basket. After centrifugation and wash by buffer (repeat 2 times), the RNA was finally transferred from the collection tube to elution tube by centrifugation (14,000 rpm for 2 minute). Total RNA products in elution tube under spin basket were analyzed from the absorbance at 260nm. mRNA contents in control sample were normalized from the mass-balance of total RNA contents of the solution with and without plasmid DNA, resulting that approximately 70% of total RNA was included without DNA (non-expression condition). mRNA content was calculated as the total RNA of sample containing DNA vector (including mRNA) minus to that without DNA vector (no mRNA product).

5. Statistical analysis

Results are expressed as mean $\pm$ standard derivation (SD). All experiments were performed at least in triplicate. The data distribution was analyzed, and statistical differences were evaluated using the Student's t-test. A P value of $< 0.05\%$ was considered significant.

Results and Discussion

1. Effect of liposome on gene expression in *E.coli* Cell-free translation system

The effect of the liposome addition on the gene expression was investigated in an *in vitro* protein translation system in order to investigate the possible role of the biomembrane on gene expression. The *in vitro* translation system, originated from *Escherichia coli* lysate (RTS-100, Roche), was used as a minimal *in vitro* expression machinery and the vector pIVEX2.3 was used as a template vector for expression of GFP as a reporter protein. The liposome prepared with 1-palmitoyl-2-oleoyl-sn-glycero-3- phosphocholine (POPC) and

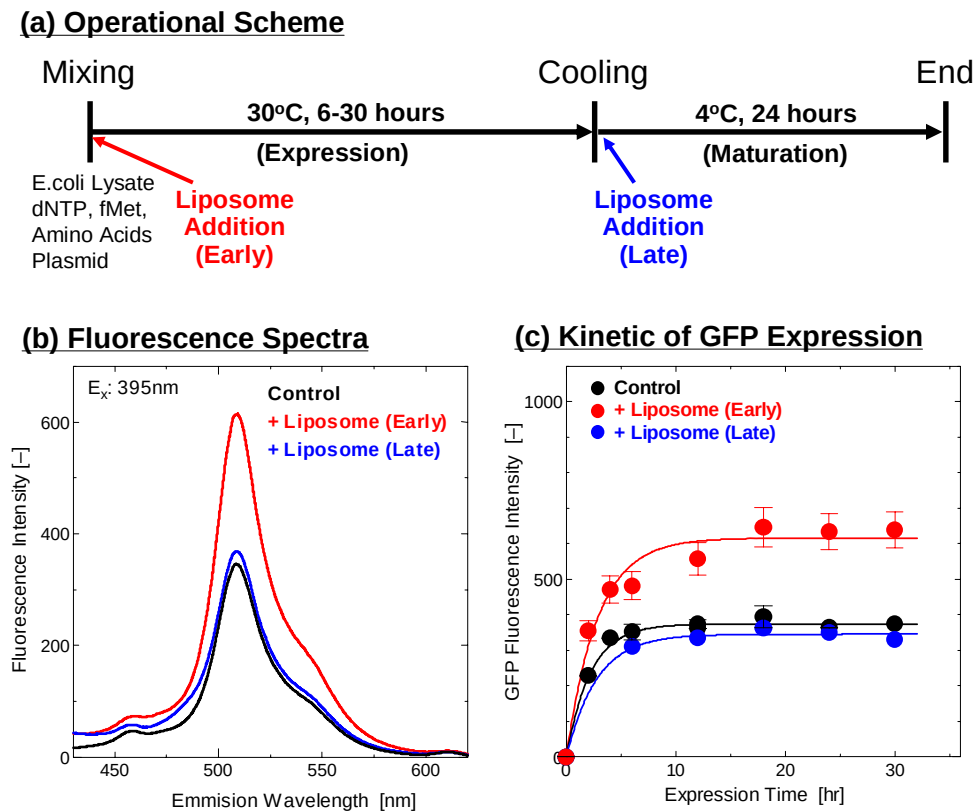


Fig.1-1 Effects of liposome addition on GFP expression in a cell-free translation system. (a) Operational scheme for GFP expression, (b) Fluorescence spectra of expressed GFP with and without liposome. (c) Time course of GFP expression with and without liposome. Liposome: Ch/POPC (33/67). The liposome was added to the solution before and after the expression operation.

cholesterol (Ch) was used as a model liposome.

Figure 1-1(a) shows the typical operational scheme of GFP expression in an *in vitro* translation system, where the transcription, translation and folding of GFP are mainly achieved in “Expression” step and the conformation of produced GFP is finally adjusted in “Maturation” step. The liposome was added at the beginning of and late in the protein expression operation (shown as “arrows” in **Fig. 1-1(a)**) in order to confirm which process of gene expression could be affected by the added liposome. As shown in **Fig.1-1(b)**, the fluorescence intensity of GFP increased in the presence of POPC/Ch liposomes (3.5 mM in lipid concentration), resulting in the GFP fluorescence with the liposome being 1.67 times greater than that in the control (without liposome). In contrast, the GFP expression was found to be similar to that of the control when the liposome was added after the gene expression operation (at the beginning of “Maturation” step). In addition, the effect of the liposome on the fluorescence intensity of the purified standard GFP was investigated in order to study the possible interaction of liposome with GFP itself, resulting in no significant change in the intensity of GFP fluorescence regardless of the variation of the lipid concentrations (data not shown). It is commonly known that the gene expression process consists of (i) transcription from DNA to mRNA, (ii) translation from mRNA to polypeptide, and (iii) folding of the polypeptide. The above results on the difference of GFP fluorescence with different timing of liposome addition and the liposome effect on the conformation of pure and matured GFP

imply that the liposome could not so significantly affect the conformation of the GFP at mature conformation and that the POPC/Ch liposome could affect both the GFP expression (above (i) and (ii)) and in post-translation pathway, GFP folding (above (iii)). **Figure 1-1(c)** shows the time course of the GFP expression. The GFP expression increased in a time-dependent manner, and the values reached a plateau at more than 6 hours of expression time in the absence of POPC/Ch liposome. In the presence of POPC/Ch liposome, the intensity of the GFP fluorescence was higher than that of the control at the initial 6 hours expression and the maximal value of GFP expression was obtained at more than 18 hours, resulting in the increase to 1.67 times higher values than that of the control sample. The above results imply that the increase of GFP expression in the presence of POPC/Ch liposome could be caused by the interaction of POPC/Ch liposome with biomolecules contributing to the GFP expression process, including (i) transcription, (ii) translation, and (iii) folding of the translated polypeptide (**Fig.1-2**).

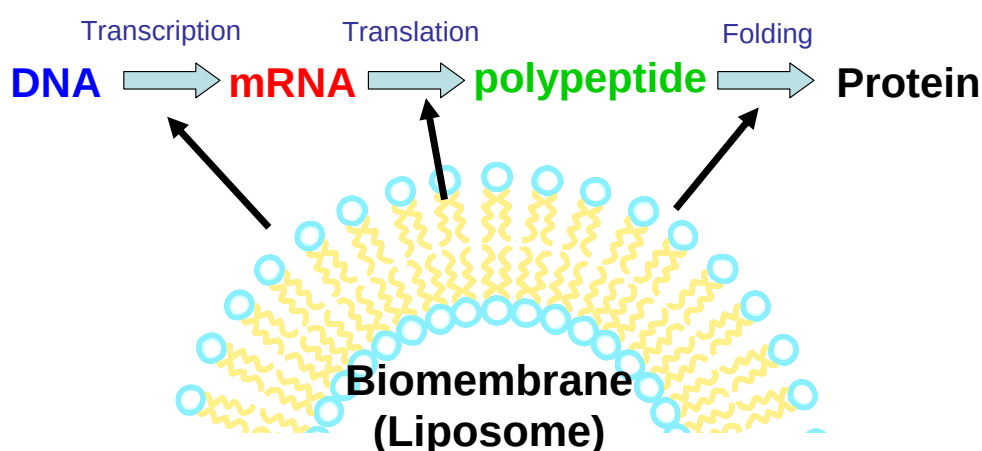


Fig.1-2 Schematic illustration of the elementary steps of gene expression process

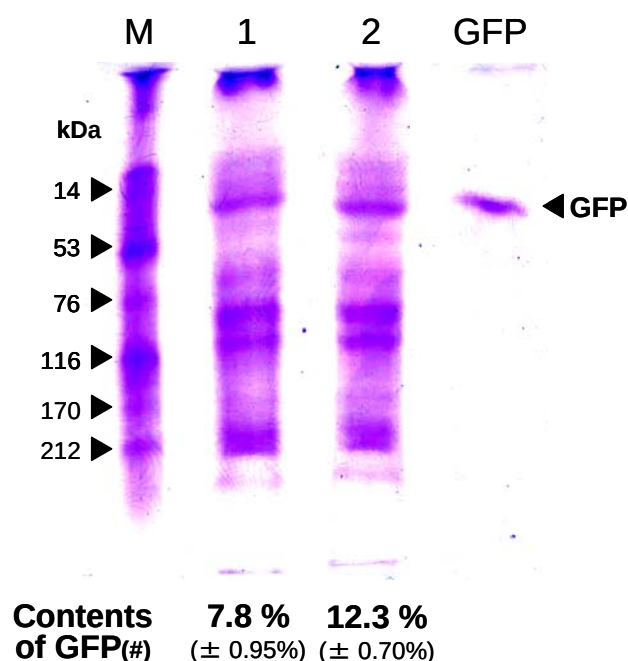


Fig.1-3 SDS-PAGE image of expressed GFP with and without liposome. The sample was analyzed by using the Phastsystem (GE Healthcare Science), and the gel was stained by the CBB method. Lane M: marker, 1: control, 2: with POPC/Ch liposome, GFP: GFP itself. GFP expression was performed at an expression time of 18 hours at 30°C. The distribution of the protein in the stained gel was further quantified by densitometer analysis, which was carried out at least three times at different points along the lane. The quantified results are shown at the bottom of each lane together with errors of quantification.

The net amounts of GFP expressed in the presence and absence of POPC/Ch liposome was further analyzed by SDS-PAGE as shown in **Fig.1-3** in order to investigate the liposome effect on the upstream steps ((i) transcription and (ii) translation steps). Densitometer analysis with the CBB stained gel indicated that the amount of GFP expressed in the presence of POPC/Ch liposome was approximately 1.58 times higher than that of the control sample (lane 1 and 2, **Fig.1-3**).

2. Role of POPC/Ch liposome at elementary steps of gene expression

In addition, the amount of mRNA was analyzed in the presence and absence of

POPC/Ch liposomes in order to investigate the liposome effect on (i) the transcription process. After the total RNAs were extracted from the reaction solution of *in vitro* translation system, the amounts of mRNA were determined from the UV spectra considering the initial concentration of the RNAs (tRNA and rRNA) in the original reaction solution. The total content of mRNA with POPC/Ch liposome was increased to 1.29 times more than that in the control, indicating that the POPC/Ch liposome could positively affect the transcription process (above (i)). The above results on the role of liposome in all the steps during the gene expression were summarized in **Fig.1-4**.

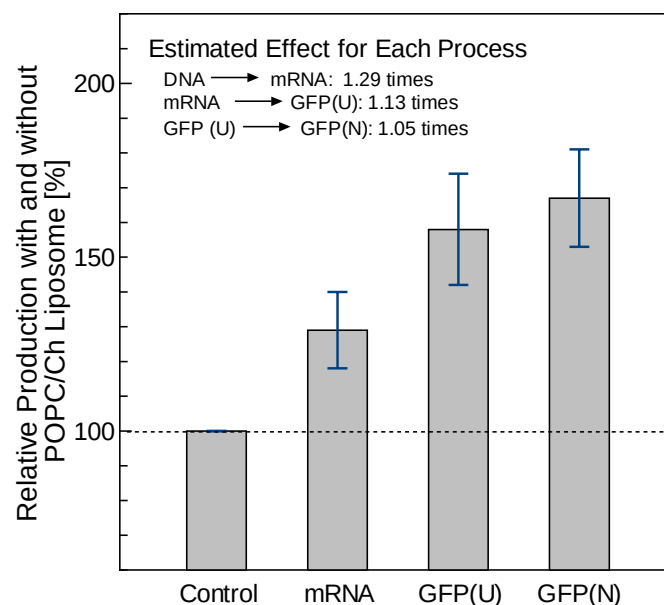


Fig.1-4 Summary of relative production of each species with and without POPC/Ch liposome. The content of each species with POPC/Ch liposome after gene expression were calculated as a relative value against that without liposome. mRNA was recovered and quantified by analysis its absorbance at A260. The amount of translated GFP was evaluated from the SDS-PAGE analysis. The amount of GFP at mature conformation was analyzed from the fluorescence measurement.

These results imply that neutral liposomes, POPC/Ch liposome, can affect both transcription and its translation of GFP gene, together with folding of GFP polypeptide, in cell

free translation system although the magnitude of the liposome effect was accelerated in upstream steps during the gene expression.

3. Previous findings on gene expression with liposome

A possible mechanism of the role of liposome on the gene expression was further investigated. It has been reported that the gene expression was efficiently achieved inside the liposome or vesicle (Nomura *et al*; 2003). According to Oberholzer *et al*, (1999) the POPC liposome has been shown to host the synthesis of a polypeptide. The enhancement of mRNA synthesis by T7 RNA polymerase occurs inside giant vesicles according to Fischer *et al* (2002). In addition, according to Nomura *et al*; (2003), the protein expression also occurs effectively inside giant vesicles constructed from POPC. The above authors reported that, after gene expression within cell-sized lipid vesicles, the level of internal rsGFP is approximately ten times higher than that in the external solution at the first stage of expression because of the “elementary effect”. According to Ishikawa *et al*; (2004), the

Table 1-1 Summary of GFP expression in a Cell-free expression system with and without liposome.

references	lipid composition	expression condition	GFP conc [μ M]
control, Yomo et al. ¹⁶	no liposome	control for 96 h at 30 °C	1.6
liposome, Yomo et al. ¹⁶	POPC/PLPC/SOPC/SLPC/Ch/DSPE-PEG 5000 = 129:67:48:24:180:14	inside liposome for 96 h at 30 °C	0.31
liposome, Tachibana et al. ¹⁷	DOPE/cationic-lipid/Ch	outside liposome for 4 h at 30 °C	0
GUV, Noireaux et al. ¹⁴	egg lecithin	inside liposome at 250C for 5 h	0.00013
control, our research	no liposome	control for 18 h at 30 °C	3.33
liposome, our research	POPC/Chol 67:33	outside liposome for 18 h at 30 °C	5.55

two-stage genetic network encapsulated in liposomes is functional and works as a transcriptional activation cascade, implying that gene expression is effectively carried out inside the liposome. As shown in such previous reports, almost all researchers have regarded the liposome effect on the gene expression or gene-relating reactions as a “elementary effect”. GFP expressions with and without liposome are summarized in **Table 1-1**. The GFP expression inside the liposome based on lipid components of POPC/PLPC/Cholesterol/DSPE-PEG 5000 was 0.31 μM , which was 5 times lower than that in the control sample according to Yomo *et al*, (2006). In contrast, almost all the GFP expression were inhibited when expressed in the presence of 0.05 mM cationic liposome based on the lipid components of DOPE/Cationic lipid/Ch (Tachibana *et al*; 2002). From these results, it is hypothesized that the effect of liposome on gene expression could be explained by the interaction of the liposome surface with the gene-expressing components such as T7 RNA polymerase, ribosome, and so on, rather than the “elementary effect”, which has been discussed previously by other researchers. In accordance with such a hypothesis, the GFP expression was enhanced just by adding the liposome to the GFP synthesis solution as shown above, where all the molecules relating to the protein synthesis exist outside the liposome membrane or on its surface. The obtained results relating to the enhanced GFP expression in the presence of POPC/Ch liposome indicate that another possible mechanism that has not been clarified yet could underlie behind the above phenomena, different from the “elementary effect”.

4. Surface characteristics of biomolecules relating to all the elementary steps

One can herewith speculate that the liposome could enhance the GFP expression through its interaction with the molecules, such as proteins and nucleotide in the protein synthesis system. A mechanism of the liposome-enhanced expression of GFP in cell-free translation system has been discussed based on the expected surface properties of the macromolecules. The basic characteristics of the macromolecules relating to the total gene expression were summarized in **Table 1-2** and **Fig.1-5**. The POPC/Ch liposome is basically

Table 1-2 Summary of characteristics of macromolecules relating to gene expression

components	hydrophobicity, ΔG				role in elemental step for gene expression		
	M_w [kDa] (ASA [nm ²])	surface	[kcal/mol] (ΔG /ASA [kcal/mol/nm ²]) ^e	hydrogen-bond stability, ρ [-] ^f	transcription	translation	folding
		charge, Z [C] (Z/ASA [C/nm ²])					
DNA	2795.1(2242)	-8470 (-3.78)	-	-	template	-	-
T7 RNA polymerase ^(b)	98.8 (482)	-2.3 (-0.005)	-561.4 (-1.16)	6.88	catalysis	-	-
mRNA	242.4 (439)	-713 (-1.62)	-	-	product	template	-
tRNA	25.8 (111)	-76 (-0.68)	-	-	-	transport/support	-
16S rRNA	521.6 (2044)	-1534 (-0.75)	-	-	-	catalysis	-
ribosomal proteins ^c	252.0 (1357)	173 (0.13)	-1673.7 (-1.23)	6.55	-	catalysis	-
polypeptide (unfolded GFP) ^d	26.7 (105)	-6.7 (-0.06)	-169.1 (-1.61)	6.74	-	product	substrate
POPC/Ch liposome	-(31 400)	0 zwitterionic (0)	-1722 (-0.05)	-	++	++	+

^a The values in brackets were calculated on the basis of the amino acid sequence of proteins. Surface net charge (Z) was calculated on the basis of the pK_a values of side residues of acidic amino acids (Glu and Asp) and basic ones (Lys, Arg, and His).^{21,22} The accessible surface area (ASA) was calculated by using VeierLite (<http://www.accelrys.com/>) with a probe molecule of 1.4 Å. ^b Calculated on the basis of PDB ID 1CEZ. ^c Calculated on the basis of PDB ID 2GYB without nucleotides. ^d Calculated on the basis of PDB ID 1EMF. ^e Calculated on the basis of the hydrophobicity scale (White, S. H.; Wimley, W. C. *Biochim. Biophys. Acta* 1998, 1376, 339). ^f Calculated on the basis of PDB data according to Fernandez, A.; Berry, R.S. *Proc. Natl. Acad. Sci. U.S.A.* 2004, 100, 2391 and Fernandez, A.; Scott, R. *Biophys. J.* 2003, 85, 1914.

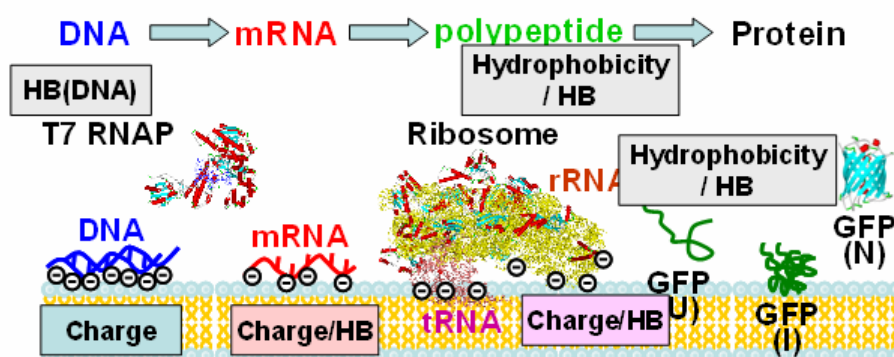


Fig.1-5 Schematic illustration of the possible biomacromolecules relating to gene expression.

prepared by using the zwitterionic lipid (POPC), where the choline-group and phosphate-group respectively show a positive and negative charge and the inert of lipid bilayer membrane provides a hydrophobic environment. The surface of T7 RNA polymerase in transcription step and GFP polypeptide in post-translation (folding) step has negative charge and that of the ribosomal complex in the translation step has a positive charge whereas the surface of polynucleotide shows the zwitterionic surface. The possible effect of liposome could further be assessed by analyzing the variation of GFP fluorescence, as an output of gene expression, in the presence of various liposomes with different surface characteristics as investigated as follows.

5. Effect of liposome types on gene expression and their possible models

The effects of liposome type, such as zwitterionic liposomes and charged ones, on GFP expression in a cell-free translation system was investigated (**Fig.1-6**). In the case of zwitterionic (neutral) liposomes, the GFP fluorescence with DPPC, DMPC and POPC were enhanced to 122%, 127% and 145%, respectively. It has been reported that the membrane fluidity of the liposome is dependent on the phase-transition temperature, and the order of the membrane fluidity of the above liposome is POPC > DMPC > DPPC, implying that the GFP expression is affected by the membrane fluidity. In the presence of POPC/Ch liposome, the synthesized GFP from the cell-free translation system gave the highest fluorescence intensity

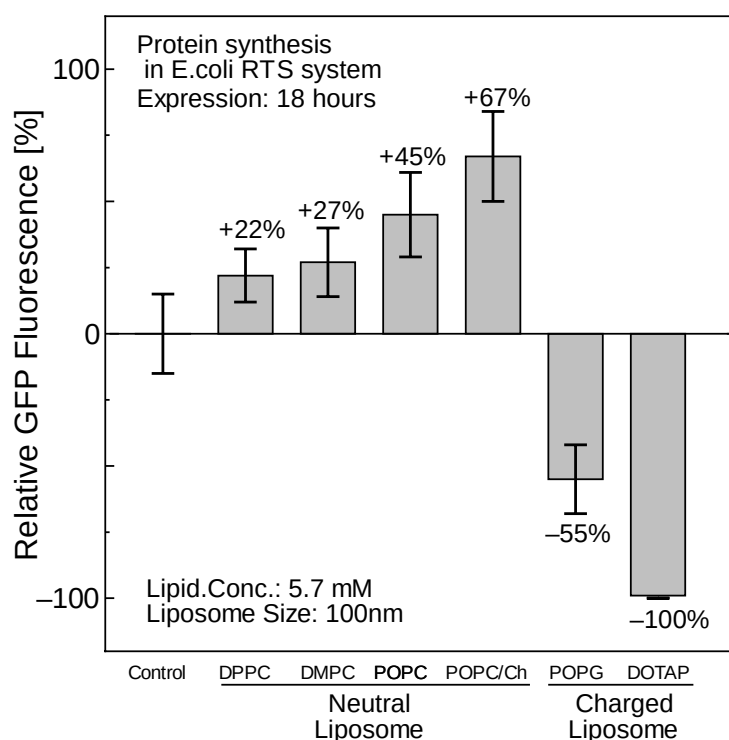


Fig.1-6 Effects of liposome types on GFP expression

(167%) in comparison with that of the other neutral liposomes. The microdomain structure of cholesterol on surface of liposome has been reported to enhance the adsorption of DNA or mRNA, resulting in the enhanced stability of their nucleotides (Safinya *et al*; 2001). In general, the ribosome is known to synthesize the polypeptide on ER membrane containing cholesterol and phospholipid (Alberts *et al*; 2002). With the lipid bilayer membrane, the polynucleotide of DNA has also been shown to interact and be stabilized there (Safinya *et al*; 2001). It has been reported that the POPC/Ch liposome possesses microdomain structures on its surface and the microdomain structure shows higher affinity with the peptide components (Taylor *et al*; 1996). The microdomain could favorably interact with protein components such

as T7 RNA polymerase, ribosome or immature polypeptide of GFP or with nucleotides, such as DNA, mRNA. On the contrary, the GFP expression was suppressed by anionic liposome POPG (45%) and cationic liposome DOTAP (0%) (**Fig.1-6**). In our series of papers, it has been reported that the liposome could act as molecular chaperone to assist the protein refolding (Kuboi *et al*; 1997), where the suitable modulation of the surface charge density of the liposome could enhance the efficiency of protein refolding (Kuboi *et al*; 1997, 2000; Umakoshi *et al*; 2007). The anionic/cationic liposomes used above have higher surface charge density, resulting in the inhibition of (iii) the folding of the translated GFP polypeptide on the surface of the liposome. The effect of the liposome membrane on the gene expression process has been extensively reported in the previous works. Tachibana *et al*, (2002) have reported that, when GFP was expressed in the presence of cationic liposome, an inhibitory effect on gene expression was observed in a cell-free translation system (*in vitro*), resulting in an almost complete inhibition of GFP expression at a cationic liposome concentration of 50 nM. As shown in **Fig.1-6**, the GFP expression detected by its fluorescence was significantly inhibited and such an inhibitory effect was also observed through the SDS-PAGE analysis with and without cationic liposome (data not shown).

The present results shown above are well corresponding with the previous findings. It was thus shown that (a) the neutral liposome membrane could affect all the steps of gene expression positively in correspondence with its membrane fluidity and microdomain

structure, (b) negatively-charged liposome could negatively affect the (iii) folding of GFP polypeptide, and (c) positively-charged liposome could negatively affect the (i) transcription or (ii) translation steps during the protein expression. Furthermore, the effect of negative charged and positive charged liposome on gene expression and their mechanism will be investigated and clarified in chapter II and chapter III, respectively.

Although the additional mechanisms of the effect of the charged liposome are now under investigation, the above findings on the liposome-affected gene expression could elucidate a possible role of biomembrane in the mismatching between genotype and phenotype inside a cell.

Summary

The possible role of liposome membrane on gene expression using GFP as a reporter gene was carried out, focusing on its role at elementary steps such as “transcription”, “translation” and “folding”. Gene expression process was analyzed by the GFP fluorescence (finally-matured product as an indicator of “total process”), SDS-PAGE (net production of GFP with polypeptide form as an indicator of “translation”), mRNA (as an indicator of the “transcription”). It was found that neutral and domain liposome enhance gene expression of GFP. The liposome was shown to enhance the (i) transcription and (ii) translation, resulting in the promotion of the gene expression, through the analysis of the fluorescence intensity and SDS-PAGE analysis of GFP and the analysis of mRNA. However, anionic charged liposome and cationic liposome inhibited the gene expression of GFP. A possible model for the “liposome membrane” affected gene expression has been discussed based on the surface characteristics of the possible biomacromolecules (such as DNA, RNAs, T7 RNA polymerase, ribosome, GFP) and the surface characteristics of the liposome. It implies that the liposome effect on the gene expression could be induced by the interaction of the molecules and the liposome. A possible role of the negatively-charged liposome based naturally-abundant lipid and the positively-charged liposome based on foreign lipids will be further investigated in chapter II and chapter III. The “elementary step analysis of gene expression” has thus been shown to be effective for its deeper understanding.

Chapter II

Role of Model Biomembrane on Gene Expression

~ Negative Charged Liposome Effect on *in vitro* Gene Expression~

Introduction

A gene regulation in a biological cell becomes an important issue for the deeper understanding of the gene expression system and the application to the design of the bioprocess or gene-related drug. For example, the regulation of specific gene provides us the opportunities of the medical treatment of disease cells by suppression of target gene (Keizeimi-Esfarjani *et al*; 2000), silence a target gene (Zhang *et al*; 2006) or inactivation of specific enzyme (Kuboi *et al*; 2000). It is known that the inhibition of gene expression could be performed by inhibition of (i) DNA transcription and (ii) mRNA translation, and (iii) folding of polypeptide in post-translation during the gene expression process (**Fig.1**). However, the control of gene expression at transcription and translation level is very complicated in their mechanism owing to the complexity of the molecular machinery (Mcintosh *et al*; 2001). Conventionally, the DNA delivery by liposome has been accepted as one of the most promising ways for the gene expression (Taylor *et al*; 2003) when there are some problems in low efficiency of gene deliver and unstable expression. RNA interference technology supported by liposome has recently been reported as a novel technique for silence of a target gene as well as the inhibition of gene expression (Zhang *et al*; Fire *et al*; 2006).

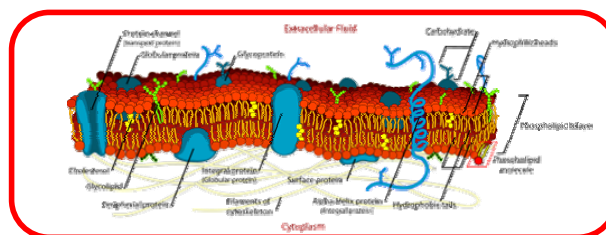
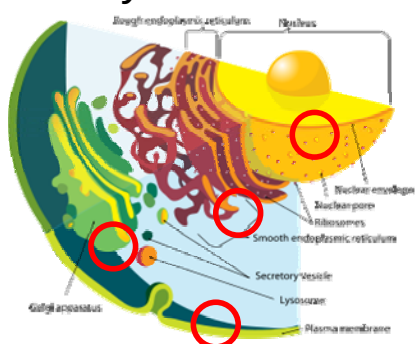
The folding of the synthesized polypeptide in post-translational process seems favorable in contrast to the above upstream steps. It has been reported that the apoptosis (cell suicide) can be triggered by production of improperly folded or incomplete proteins (Sugita *et al*; 1995). When there is a large, rapid, and overwhelming accumulation of unfolded protein, a rapid apoptosis occurs and then most cells will be died. Similarly, apoptosis can be triggered by incubation with amino acid analogs or low doses of puromycin (Sugita *et al*; 1995). The above example of protein conformation-related phenomena could be related with the possible relationship between the conformation of the synthesized polypeptide inside the cell and the response of the biological cells, including the variation of characteristic, morphology and activity of the biomembrane. If one could assume the acceptability of the reverse logic of the above phenomena, the inactivation of the synthesized polypeptide could be carried out by the regulation of the biomembrane itself.

A biomembrane is commonly known to act as just a physical boundary to separate the environment and the cellular biomolecules. The potential aspects of biomembrane itself as an active and functional interface of biological cell to make a response of the dynamic change of environmental condition (stress) have recently been revealed through the basic research on a model biomembrane. Liposome, a closed phospholipid bilayer membrane, is commonly used as a model biomembrane. The liposome has been applying for drug delivery, gene delivery and expression for the suppression of the specific gene in disease cell (Zhang *et al*; 2006,

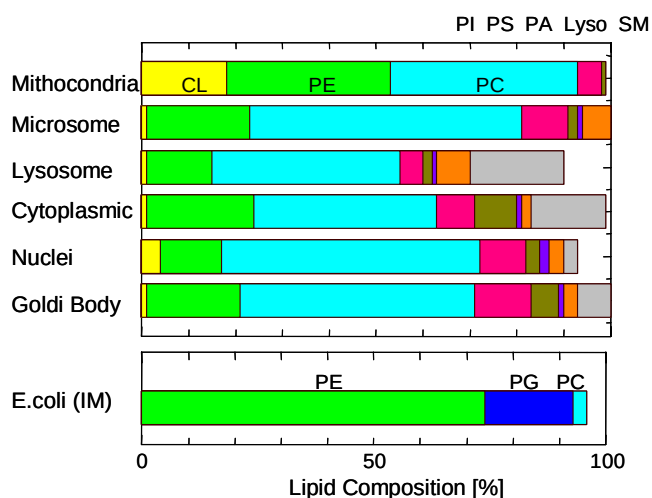
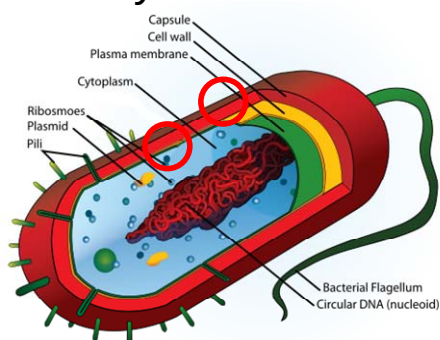
Husseini *et al*; 2008). Liposome was also applied for RNA interference (RNAi) technology as a carrier for delivery siRNAs to cell for enhancing siRNA stability *in vitro* and *in vivo* for final inhibition of target mRNA translation (Zhang *et al*; 2006). It has also been reported that the liposome can improve the gene expression by increasing mRNA transcription by enhanced stability of its polyA tail (Barreau *et al*; 2006). The relation of the liposome is thus attracting many interests.

The liposome has previously been reported to induce a variety of potential functions under the variable environmental condition (stress condition), such as a chaperone-like function (Kuboi *et al*; 1997) translocation of proteins across the membrane (Umakoshi *et al*; 1998), enhanced production and release of enzyme from bacterial cell under heat stress condition (Ngo *et al*; 2008) and membrane fusions (Yoshimoto *et al*; 1999). It has recently been reported that the activity of fragmented superoxide dismutase (SOD) with no specific conformation and no activity is enhanced under an oxidative stress condition in the presence of liposome prepared by 1-palmitoyl-2-oleoyl- sn-glycero-3-phosphocholine (POPC) through the (i) recognition of the peptide, (ii) its folding, (iii) metal chelating, and (iv) active center formation (Tuan *et al*; 2008). The lipid bilayer membrane is thus known to induce a variety of potential roles in controlling the enzymes structure and activities under the above stress condition. It is expected that the conformation and activity of some enzymes or peptides in a biological system could also be regulated by the interaction of lipid membrane with

Eukaryotic Cell



Prokaryotic Cell



**Charged / Neutral Lipids
are Important Components**

Fig.2-1 Schematic illustration of structure of biomembrane and their lipid composition

the above macromolecules under appropriate stress conditions.

The negatively-charged lipids, such as phosphatidylcholine, phosphatidylglycerol and so on, are very much abundant in the biomembrane (**Fig.2-1**). In addition to the cytoplasmic membrane, almost all the membranes of the cellular organelle consist of the negatively-charged lipid. The role of the negatively-charged lipids has been studied in various research areas. The previous study (Kuboi *et al*; 2000) indicated that negatively-charged liposome POPG and DMPG liposome can strongly interact by electrostatic and hydrophobicity interaction with denatured lysozyme and then inhibit its refolding, resulting in

the inhibition of 88% and 52% lysozyme enzyme activity. This result suggests some possibilities of negatively-charged liposome, such as (i) the inhibition of protein refolding and, also, (ii) the interference of folding of early translated polypeptide. A possible mechanism of the negatively-charged liposome on the protein refolding and the folding of early synthesized polypeptide in post-translation of gene should be investigated. As described in chapter I, the effects of liposome on gene expression were also investigated, indicating that the neutral liposome and microdomain-forming liposomes have a positive effect on gene expression of GFP in RTS system (Bui *et al*; 2008). From another study, the complex of neutral and cationic liposome has previously shown to act as an inhibitor of the gene expression in *vitro* system (Tachibana *et al*; 2005).

In this chapter, the potential role of the negatively charged lipid bilayer membrane (liposome) on GFP gene expression was further investigated, especially focusing on the role of negatively-charged liposome because most of biomembrane is composed of the negatively-charged lipids (**Fig.2-1**). By using the previous strategy based on the *in vitro* protein synthesis system, the gene expression of reporter protein, GFP, was performed in the presence of negatively-charged liposome. The variation of the gene expression of GFP was discussed, focusing on the possible role in folding of synthesized polypeptide in post-translation of gene expression.

Materials and Methods

1. Materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl -*sn*-glycero-3-phosphatidylglycerol (POPG) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Rapid Translation system RTS 100 *E.coli* HY Kit, an *in vitro* protein synthesis system based on *E.coli* lysate, was purchased from Roche Diagnostics (Indianapolis, IN, USA). Diphenylhexatriene (DPH) and tetramethyl-DPH (TMA-DPH), used as fluorescent probes of membrane fluidity analysis, were purchased from Molecular Probes Corp (MO, USA). The purified green fluorescent protein (Pure GFP) was purchased from Roche Diagnostics (Indianapolis, IN, USA). Other reagents of analytical grade were purchased from Wako Pure Chemical (Osaka, Japan).

2. Liposome Preparation

The phospholipids used in this study are phospholipids molecules of (i) POPC, (ii) POPG, at different ratios of 0:100, 95:5, 90:10, 70:30 and 100:0 were solubilized in chloroform; a phospholipid thin layer was then prepared in a round-bottom flask by evaporation at 37°C using a vacuum rotary evaporator and dried under nitrogen flow for at least 5 hours. In order to generate multilamellar vesicles, the dried phospholipid thin layer was hydrated with distilled water. The freeze–thaw cycle was repeated 5 times with hydrated phospholipid solution for large unilamellar vesicle formation. This vesicle solution was

extruded through a polycarbonate membrane with a pore size of 100 nm to form unilamellar vesicles (liposome).

3. *in vitro* expression of GFP with and without liposome

A Rapid Translation system (RTS 100 *E.coli* HY Kit), *in vitro* protein synthesis system based on *E.coli* lysate, was used as a cell free translation system. The expression vector, pIVEX2.3d, contained the gene for expression of the green fluorescent protein (GFP). The reaction solution (50µl) contained the following components: 12µl *E.coli* lysate, 10µl reaction mix, 12µl amino acids, 1µl methionine, 5µl reconstitution buffer, and 0.5µg of circular DNA template in 10µl of sterilized DNase and RNase free water. The reaction solution was initiated by mixing the DNA with other solutions at 30°C for 6 – 18 hours (“Expression” operation) and was kept for 24 hours at 4°C (“Maturation” operation). The liposome solution prepared at higher concentration was added in the reaction mixture in the replace of the above water before and after the “Expression” operation and the effect of liposome on the gene expression was evaluated based on the following methods.

4. Analysis of gene product of at post-translation level

Three kinds of analysis, such as (i) GFP fluorescence and (ii) SDS-PAGE, were employed to the quantification of gene product according to the previous reports. The amount of folded GFP product was evaluated by analysis of the GFP fluorescence intensity at 395 nm

(excitation) and 509 nm (emission) by using the fluorescence spectrophotometer (JASCO, FP-777, Tokyo, Japan) with slit width of 5nm at excitation and emission of light path. All the GFP fluorescence spectra were analyzed after 100 times dilution of the reaction samples to water in order to avoid the possible interference of the liposome included in the final reaction sample. It was confirmed that the fluorescence spectra of pure GFP was not affected in the above experimental condition including liposome at the finally-diluted solution and also POPC.POPG liposome itself at its final concentration has no emission spectra in the above condition for fluorescence measurement.

5. Analysis of gene product at translation level

Total amount of GFP products (unfolded and folded GFP) were also analyzed by SDS-PAGE in order to analyze the net amounts of translated GFP. A 10µl sample of reaction solution was dissolved with 17 µl of SDS-PAGE sample buffer and 5 µl of mercaptoethanol and was heated for 5 min at 95°C for denaturation. The samples with 1 to 4 µl were applied onto a ready-made gel (Homologous 12.5, GE Healthcare, Chicago, IL, USA) set for electrophoresis system (PhastSystem, GE Healthcare, Chicago, IL, USA). The gel was finally stained with Coomassie blue dye and GFP was detected as major staining band at 27 kDa. The distribution of the protein in the stained gel was further quantified by densitometer analysis by using the SCION image software obtained at <http://www.scion.com/>, where the densitometer analysis was carried out at least three times at different points along the lane. It was confirmed

that the band of GFP was not observed in the reaction solution for protein translation without plasmid DNA.

6. Analysis of gene product of at transcription level

Evaluation of remained mRNA products after a duration of GFP expression by using Reverse Transcription Polymerase Chain Reaction (RT-PCR). After GFP expression from mRNA in RTS system of 4 hours, at that time, about 65% GFP was expressed, total RNA was extracted by using SV total isolation Kit (Promega) according to the supplier's instruction. The primer pairs are as follows: PY504-01: 5'-ATGACTAAAGGTGAAGAAC-3' (forward) and PY504-02: 5'-CGATTCTATTAACAAGGGTATCACCT-3' (reverse) were used for amplification of specific fragment of GFP's mRNA having 371bp in length. cDNA reaction was carried out by using specific reverse mentioned primer according to protocol from PrimeScript™ RT-PCR Kit, TAKARA BIO INC with following temperature program: 65°C for 5 min, 46°C for 30 min and 95°C for 5 min, a synthesized cDNA product from 2 to 4 µg was applied for RT-PCR reaction with following temperature program: 30 min at 46°C, denature 2 min at 94°C, annealing 30s or 1 min at the primer's melting temperature, elongate 1 min 20 s or 2 min at 72°C and prolonged elongation 3 min to 10 min at 72°C. RT-PCR products were then analyzed by electrophoresis on agarose gel 1%.

7. Ultrafiltration operation

Interaction between liposome with mature synthesized GFP product in RTS system was carried out by ultra filtration, Millipore Ultra free-MC filter with a molecular mass cutoff of 50kDa was applied to identify GFP content interacted with liposome and remained in upper phase after ultra filtration. After GFP expression in RTS system of 18 hours was carried out, liposome was added, incubated at 30⁰C for 2 hours, and then solution was applied to the ultra filtration tube and centrifuge at 12.000 rpm for 20 minutes at room temperature. The upper portion (non passed solution) harboring GFP bound liposome (vesicles 100 nm in diameter), and the lower portion (passed solution) contain free GFP (27 kDa) called free GFP. Both solutions were then analyzed by fluorescence intensity of GFP of excitation at 395m, and emission at 509 nm to check GFP content remained in upper portion after ultra filtration.

8. Membrane fluidity analysis by using DPH and Tma-DPH

The change of outer and inner membrane fluidity when liposome interacted with native GFP was carried out by TMA-DPH and DPH method, respectively. DPH reaction was carried out with native GFP and native GFP denatured by guanidine hydrochloride. 1µg native GFP was denatured with 9µl guanidine 6M, inoculation at 30⁰C in 3 hours, and then mixed with 250µM liposome and 1µM DPH as final concentrations in 1ml of 0.1M Tris-HCl buffer, pH: 8 for DPH reaction. Then, the mixture was incubated for 1 hour at 25⁰C. The fluorescent polarization was measured with a spectrofluorometer (FP-777 JASCO Co. Ltd; Tokyo) to

which excitation and analyzing polarizes light (360nm), and emission intensities (430nm) both parallel ($I_{//}$) and perpendicular ($I_{\perp}$) to the excited light were recorded. The polarization of DPH was then calculated by the value of $1/P$ by using following equation:

$$P = (I_{//} - I_{\perp}) / (I_{//} + I_{\perp})$$

TMA-DPH reaction was carried out in the same steps of DPH method. The ratios of 4 μ g GFP, 36 μ l guanidine and 1mM liposome, and 2 μ M TMA-DPH were used for TMA-DPH reaction.

9. Liposome effect on inhibition of refolding of GuHCl-denatured GFP

1 μ g purified GFP was denatured by 9 μ l of GuHCl 6M, keep at 30⁰C for 3 hour. After that, POPC.POPG liposome 0.25mM was injected as final concentration in 1ml of 0.1M Tris-HCl buffer (pH 8). Its refolding was performed by 100 times dilution of the GuHCl-denatured GFP in Tris-HCl 0.1M (pH 8.0) for analyzing kinetic folding with excitation at 395nm and emission at 509 nm.

10. Statistical analysis

Results are expressed as mean $\pm$ standard derivation (SD). All experiments were performed at least in triplicate. The data distribution was analyzed, and statistical differences were evaluated using the Student's t-test. A P value of < 0.05% was considered significant.

Results and Discussion

1. Negatively charged liposome, POPC/POPG, affects the post-translational process of GFP expression

It has recently been reported that the liposome membrane itself could affect the gene expression *in vitro* protein expression system (Bui *et al*; 2008). The effect of the possible role of model membrane on the *in vitro* gene expression has been investigated. It has been indicated, in chapter I, that the GFP expression was enhanced to 167% in the presence of 5.7 mM lipid of POPC/Ch liposome in comparison with control. However, in the presence of negatively- (POPG) and positively-charged liposome (DOTAP), the GFP expression was reduced to 45% and 0%, respectively in comparison with control sample. In this chapter, the role of the negatively charged liposome was investigated by employing the same concentration of 5.7 mM lipid composed of 1-palmitoyl-2-oleoylsn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG), at the different ratios, considering the lipid composition of natural biomembrane.

The GFP expression was first achieved in the presence of POPC/POPG liposome with different lipid compositions at the constant lipid concentration of 5.7 mM. The fluorescence intensity of GFP was reduced at highest level in the presence of POPC/POPG mixed liposome, depending on the molar ratio of anionic lipid POPG as shown in **Table 2-1**. The fluorescence was reached to the saturated value of approximately 50% of control at more than 30% POPG.

**Table 2-1 Effect of surface charge densities of POPC/POPG liposome
on GFP expression**

POPC/POPG molar ratios	Surface charge density of liposome (C/nm²)	Fluorescence intensity of GFP expression (%)
100:0	0	140
95:5	- 6.9x10 ⁻²	97
90:10	- 1.4x10 ⁻¹	76
70:30	- 4.2x10 ⁻¹	40
0:100	- 1.4	45
Control	0	100

The above phenomena were also confirmed by the qualitative observation by using the microscopic observation of GFP product expressed with and without negative charged liposome. The result in **Table 2-1** indicated that, in the presence of POPC/POPG liposome at the ratios of (7:3), GFP expression showed by fluorescence intensity was 40% in comparison with control sample without liposome. It has been reported that the cytoplasmic membrane is composed of 77% zwitterionic lipids (i.e. phosphatidylcholine (PC) and phosphatidylethanolamine (PE)) and 18% anionic lipid (i.e. phosphatidylglycerol (PG)), suggesting that such a decrease of gene expression could occur inside a bacterial cell.

The mechanism of GFP expression affected by liposome POPC.POPG (7:3) was further investigated. The effect of the concentration of POPC/POPG liposome on GFP expression, showed by fluorescence intensity, was investigated as shown in **Fig.2-2**. In the presence of only 4mM of POPC.POPG liposome, GFP expression was reduced 65%, when

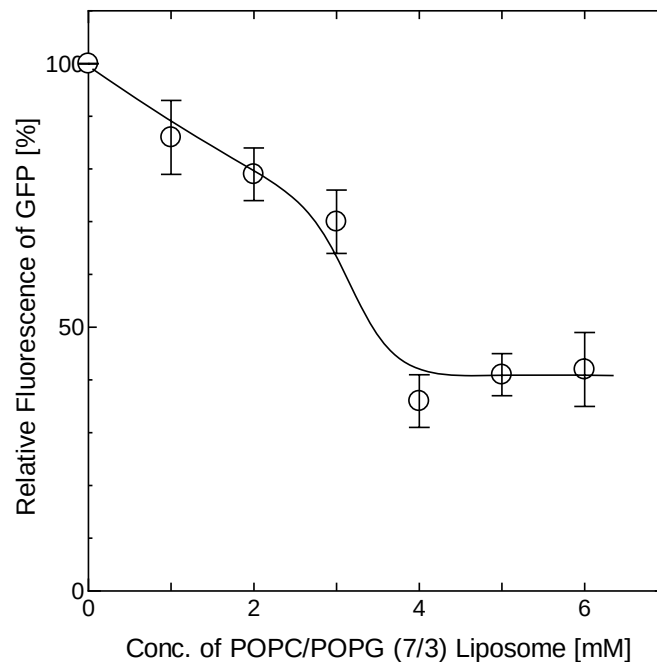


Fig.2-2 Effect of lipid concentration of POPC.POPG on GFP expression

increasing of lipid concentration of POPC/POPG (~6mM), the value of GFP fluorescence intensity was nearly same. This result indicated that 4mM of POPC/POPG liposome could affect the inhibition of GFP expression up to 65% in comparison with that of control sample without liposome. The fluorescence spectra of GFP expressed in the presence of POPC/POPG at different concentrations was shown in **Fig.2-3**. The GFP fluorescence was reduced with the increase of the POPC/POPG concentration, showing that the GFP expression process could be inhibited by the surface of the negatively-charged domain on the liposome surface. In order to assess the role of the POPC/POPG liposome, the time-chase experiment was done. When the POPC/POPG liposome was added at the beginning of the gene expression, the GFP fluorescence was 35%

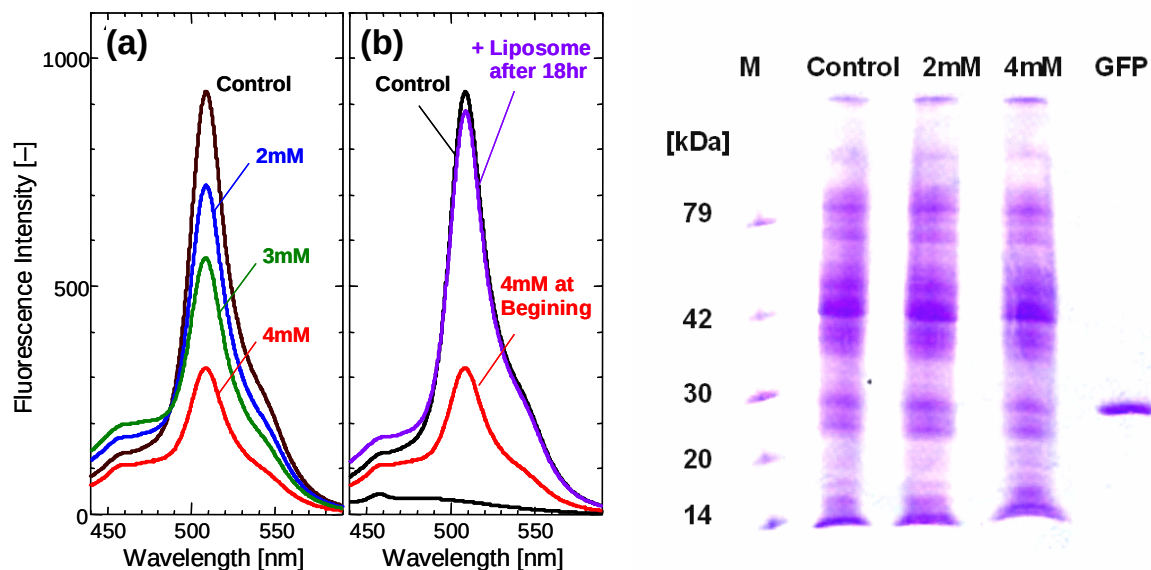


Fig.2-3 GFP fluorescence in the presence of POPC/POPG liposome. (a) Effect of concentration of POPC/POPG liposome. (b) Effect of timing of liposome addition. liposome: POPC/POPG=7:3.

Fig.2-4 CBB-stained image of SDS-PAGE of GFP expressed in the presence of POPC/POPG liposome. POPC/POPG=7:3. Lane M: marker; control: expression without liposome; 2mM and 4mM: concentration of POPC/POPG liposome of 2mM and 4mM, respectively; GFP: pure GFP.

in comparison with control sample after 18h of expression. On the contrary, the GFP fluorescence was similar with control sample when the POPC/POPG liposome was added after the GFP expression of 18 hours (**Fig.2-4**), implying that the POPC/POPG liposome did not affect the matured GFP shown by fluorescence intensity. Liposome could affect only the upstream process of gene transcription and/or translation before matured GFP structure formation.

The expressed amounts of GFP and its matured form were differently analyzed to assess the possible contribution of the POPC/POPG liposome on the GFP translation and its post-translation process (folding process).

It is known that the GFP fluorescence reflects the final product after the gene expression process, including: transcription, translation, and protein folding in post-translation. In order to investigate its effect at upstream of the gene expression process, the net amounts of GFPs expressed in the presence and absence of POPC/POPG liposome was further analyzed by SDS-PAGE as shown in **Fig.2-4**. Through the densitometer analysis with the CBB stained gel, the amount of expressed GFP products was found to be same when expressed with and without liposome at the concentration of 2-4mM. The above results on GFP fluorescence and the net amount of its expression product were summarized as a function of the POPC/POPG concentration as shown in **Fig.2-5**

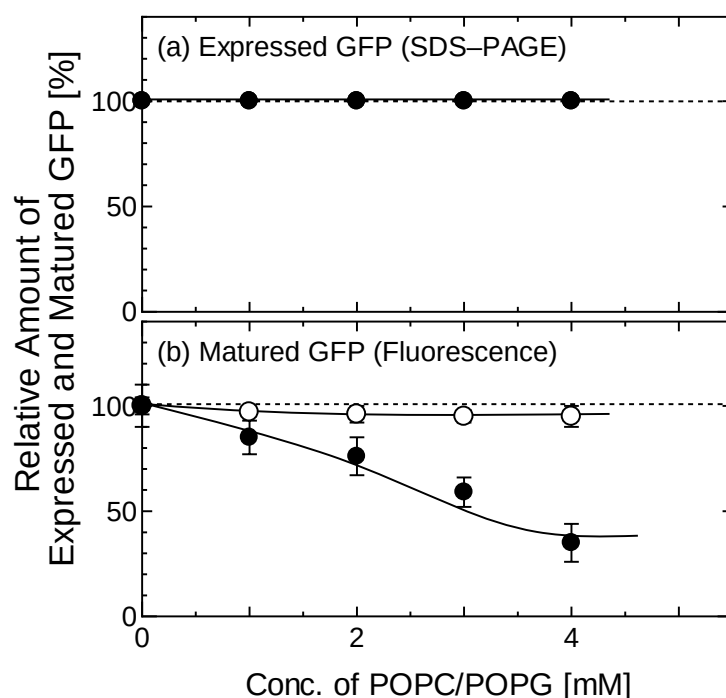


Fig.2-5 Effect of liposome POPC/POPG (7/3) concentration on: (a) GFP Synthesis and (b) Maturation of GFP conformation; Symbols: closed circle and open circle: fluorescence intensity of GFP expressed in the absence and presence of POPC/POPG liposome, respectively.

It is considered that, among the possible steps of the gene expression such as (i) transcription, (ii) translation, and (iii) folding of the polypeptide, the POPC/POPG could negatively affect the (iii) folding process though it did not significantly affect the process upstream ((i) or (ii)). This result indicated that liposome could inhibit only the folding of synthesized polypeptide GFP in the post-translation process of gene expression from *E.coli* cell free translation system. According to Kuboi *et al*, (2000), POPG and DMPG liposome can interact and change conformation of lysozyme and inhibit its refolding into native ones, reduced lysozyme activity of 88% and 52%, respectively. This result showed that POPC/POPG liposome has only negative effect on refolding of post-translation process (**Fig. 2-3** and **Fig.2-4**), it has no effect on the translation of GFP (**Fig.2-4**).

2. Effect of negatively-charged liposome on GFP expression at transcription level

In order to clarify the role of anionic liposome effect on transcription of gene expression in *E.coli* cell free system; whether anionic liposome POPC/POPG affect on gene expression at transcription or not, it is necessary to deeply understand the mechanism on the role of liposome POPC/POPG on gene expression *in vitro* translation system. mRNA content was further identified during GFP expression. After GFP expression in 3 hours, there were 65% to 75% of GFP expressed and identified by time course of GFP expression from control sample (data not shown). At the time of GFP expression for 3 hours, total RNAs in RTS reaction including tRNA (transport RNA), rRNA (ribosome RNA) and transcribed RNA

(messenger RNA) were recovered and purified from RTS system for reverse transcription reaction (cDNA reaction).

cDNA reaction was carried out based on target template mRNA (GFP's mRNA) and a specific reverse transcription primer desined based on nucleotide sequence of GFP's mRNA. The synthesized strand cDNA was then used as a template for PCR reaction by using a specific primer pairs for amplification of specific fragments of GFP's mRNA (371 bp). The RT-PCR products of specific fragment of mRNA were then analyzed on agarose gel as shown in **Fig.2-6**. When GFP expression in the presence of POPC/POPG liposome at 4mM, RT-PCR

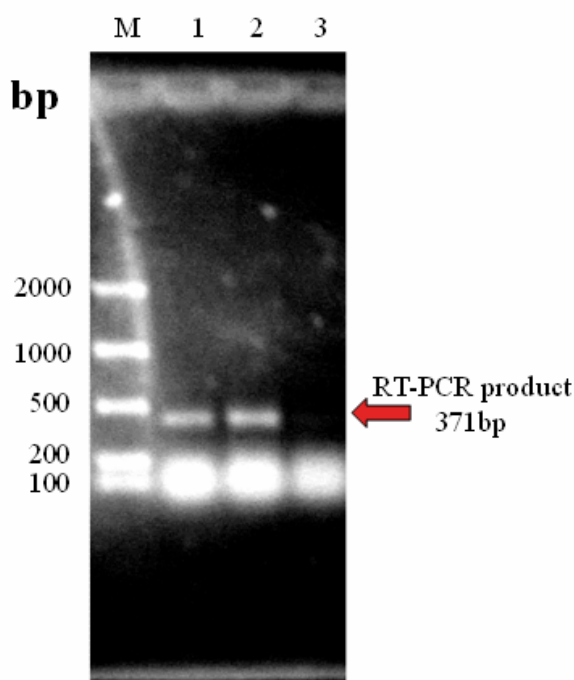


Fig.2-6. Evaluation of remained mRNA product after its translation in *E.coli* cell-free translation system in the presence of with and without anionic liposome POPC/POPG (4mM). Specific fragment of GFP's mRNA (371 bp) was amplified by RT-PCR method. Lane M: Marker (2000, 1000, 500, 200 and 100bp); Lane 1 and 2: RT-PCR product of specific fragment of remained mRNA content when it was translated in the presence of with and without liposome (4mM), respectively after 3 hours of GFP expression; Lane 3: Negative translation of control sample without GFP vector DNA.

product of specific fragment of mRNA synthesized was found to nearly same with that of control sample (**Fig.2-6**, lane 1 and 2, respectively) and no RT-PCR product was presented in the negative control sample of GFP expression without vector pIVEX plasmid containing GFP gene (no mRNA product), it has also confirmed to have no amplified specific fragments of GFP's mRNA. (**Fig.2-6**, lane 3). This result indicated that the negatively-charged liposome POPC/POPG has no effect on gene transcription, and translation, it only has effect on the inhibition of post-translation of refolding of GFP during GFP expression in *E.coli* cell-free translation system.

3. Interaction between POPC/POPG liposome with mature GFP in *E.coli* cell-free translation system

The results in **Fig.2-3** and **Fig.2-4** suggest that the POPC/POPG liposome could interact with GFP at immature or unfold form just after the GFP translation. The interaction of POPC/POPG liposome with the translated mature GFP was further investigated. After GFP expression of 18 hours, the POPC/POPG liposome was added and was kept at 30°C for 6 hours to complete the possible interaction between the liposome and GFP. After the mixture was applied to the ultrafiltration with 50 kDa molecular cutoff filter, which enables us to separate the liposome and GFP bound on it from the non bound GFP, the fluorescence spectra of both samples were analyzed as schematically shown in **Fig.2-7(a)**. After the POPC/POPG sample was added to the GFP sample expressed for 18 hours, the GFP fluorescence in the

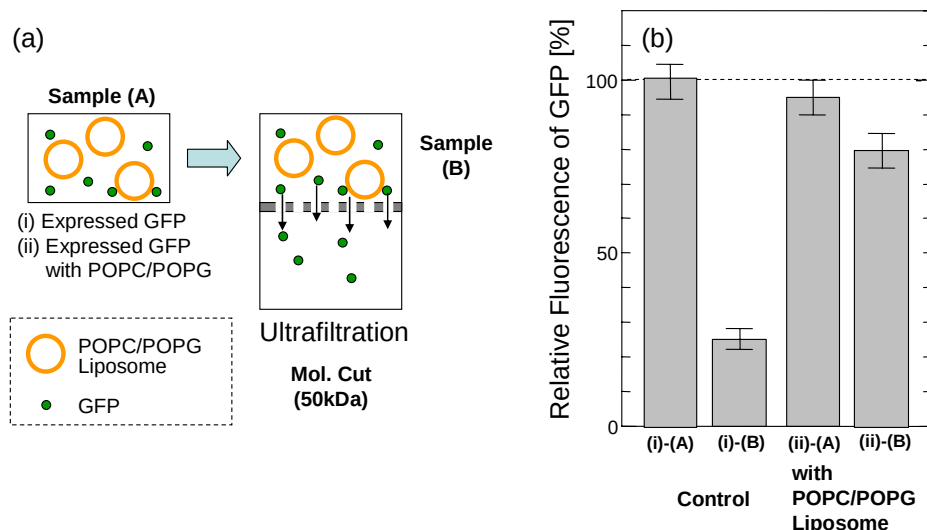


Fig.2-7 Interaction of expressed GFP with POPC/POPG liposome. Expressed GFP in control sample and expressed GFP with POPC/POPG liposome were treated by ultrafiltration (Mol.Cut:50kDa). The volume of the remained sample was adjusted to the original volume and the fluorescence was measured. POPC/POPG=7:3.

sample solution was not significantly changed (95% of the initial value, (ii)-(A)). However, after the ultrafiltration treatment, the value of GFP fluorescence intensity in the original sample solution was reduced 73% with liposome ((ii)-(B)) and 22% without liposome ((i)-(B)). It was also confirmed that the POPC/POPG liposome with 100nm was not filtrated by the ultrafiltration membrane with molecular cut of 50kDa in a preliminary experiment.

The above results show that the GFP with mature conformation could also strongly interact with the POPC/POPG liposome, so that this complex could not pass through membrane. However, the GFP conformation was not so much affected when interacting with liposome. As a result, the GFP fluorescence intensity is still stable and 51% fluorescence intensity higher than that of control sample after ultrafiltration in upper phase. The above results can be interpreted that the liposome also strongly interacted with mature GFP possibly

by electrostatic interaction of local charged surface, hydrophobicity and hydrogen bond, so that the conformation of mature GFP is still stable and not be much changed based on the analysis of fluorescence intensity of GFP after ultrafiltration.

4. Interaction between POPC/POPG liposome with immature GFP

In order to clarify the effect of liposome on folding inhibition of synthesized polypeptide GFP, the role of POPC/POPG liposome on the refolding of standard GFP was also investigated in *E.coli* cell-free translation system. Standard GFP was denatured with 5M guanidine hydrochloride (GuHCl) for 3 hours and at that time almost GFP was denatured. After the treatment of the denatured GFP at 50 times dilution, the time course of GFP folding was analyzed with and without liposome. The finally obtained values of the GFP folding was

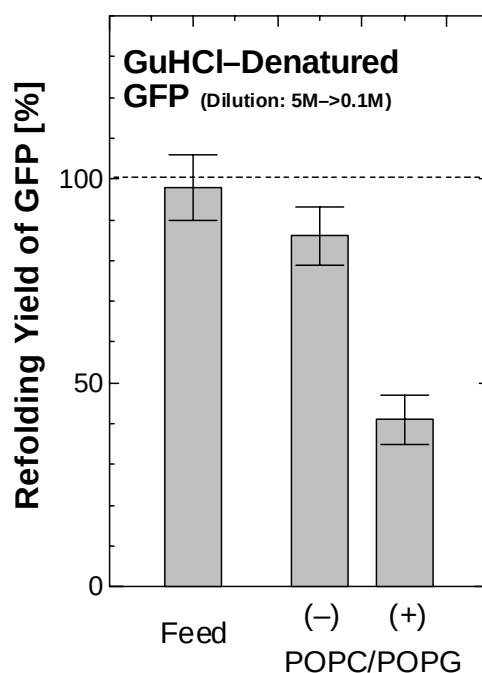


Fig.2-8 Relative percentage of refolding yields of GFP denatured by guanidine in the presence of with and without liposome.

shown in **Fig.2-8**. The refolding yield of denatured GFP was 42% with POPC/POPG liposome and 87% at control sample, so POPC/POPG liposome also strongly inhibit up to 48% refolding of standard GFP denatured by guanidine in comparison with control sample without liposome. This result one more time confirm that POPC/POPG liposomes not only inhibit folding of expressed GFP in *E.coli* cell-free translation system (**Fig.2-8**), but also inhibit the refolding of standard GFP denatured by GuHCl in *E.coli* cell-free translation system.

5. Variation of outer and inner membrane fluidity of POPC/POPG liposome when interacted with immature GFP

Among the properties of the liposome membrane, membrane fluidity is one of the most important factors to evaluate the chaperone-like function of liposomes (Kuboi *et al*; 2000; Yoshimoto *et al*; 1999, Kuboi *et al*; 1997). This is because membrane fluidity corresponds with hydrophobicity of lipid membranes and is essential for the dynamic interaction between proteins and liposomes. The membrane fluidity of POPC/POPG liposome membrane was also analyzed in the presence of with and without the standard GFP at mature and denatured GFP form. Standard GFP and GFP denatured by guanidine were mixed with POPC/POPG liposome; TMA-DPH and DPH were added to the mixture solution and incubation for 1 hour at 25⁰C for analysis of outer and interior liposome membrane fluidity, respectively. The change of membrane fluidity of liposome in the presence of with and

without POPC/POPG liposome was indicated in **Fig.2-9**. The result in **Fig.2-9(a)** indicated that, in the presence of GFP, the outer membrane fluidity of liposome (TMA-DPH) reduced to 10% and 14% in the presence of both standard GFP and guanidine-denatured GFP, respectively. Similar tendency can also be obtained in the case of inner membrane fluidity shown by DPH analysis, resulting that the inner membrane fluidity of liposome reduced 24% and 25.5% in the presence of native and GuHCl-denatured GFP, respectively (**Fig.2-9(b)**).

The above results indicated that the POPC/POPG liposome could basically recognize the surface of GFP at immature form and could inhibit the (re)folding of GFP through the strong electrostatic and hydrophobic interaction..

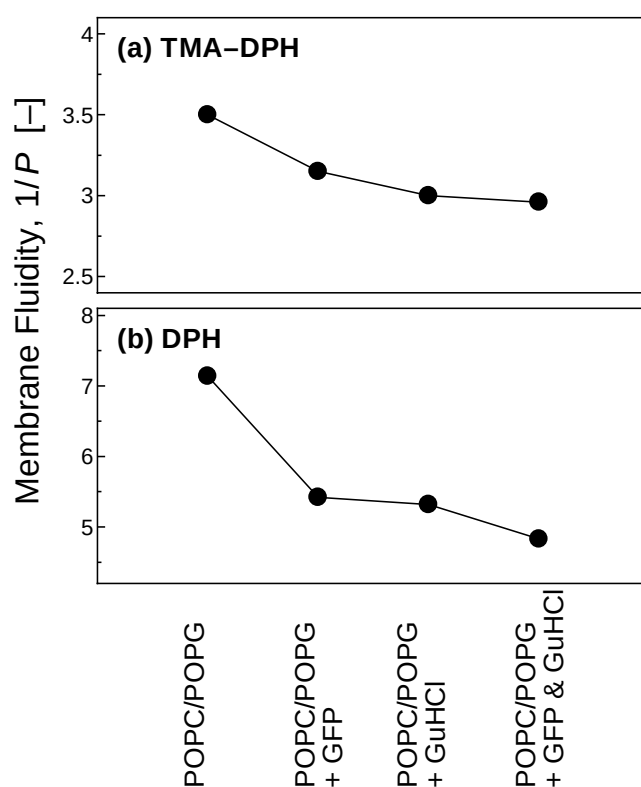


Fig.2-9 Variation of membrane fluidity of POPC/POPG liposome, measured by (a) TMA-DPH and (b) DPH, with and without native GFP and denatured one. POPC/POPG=7:3.

6. Discussion of role of negatively-charged liposome on gene expression

A mechanism of the role of negatively-charged liposome was finally discussed based on the above findings. The gene expression can be regarded as a sequential process consisting of several elemental processes, such as (i) transcription of target gene, (ii) its translation and (iii) folding of the synthesized polypeptide. It has been reported that the neutral liposome could enhance the GFP expression (Bui *et al*; 2008), where the elemental processes were considered as a result of the interaction of the liposome with the molecules, such as proteins and nucleotide in the protein synthesis system. In relation to (i) transcription, the effect of the interaction of the relating proteins with the POPC/POPG liposome could be neglected because there could exist a repulsive force between the negatively-charged liposome and negatively charged DNA and T7 RNA polymerase ($pI \sim 6.8$). The translation process is achieved by the molecular assembly of ribonucleosides (mRNA, tRNA, and rRNA) and the ribosomal proteins ($pI_{total} \sim 11.9$). This translation process could not be significantly affected because of the negatively-charged surface of the RNAs although the binding of positively-charged protein surface of complex could occur. No significant change in the amounts of synthesized GFP (**Fig.2-3** and **Fig.2-4**) supports the above assumption. The remaining folding process (iii) could be a possible key elemental process during the GFP expression in the presence of POPC/POPG. The GFP, which has acquired its conformation, is not affected (**Fig.2-2** and **Fig.2-5**) although the denatured or unfolded GFP was affected by the POPC/POPG liposome

during its (re-) folding. The decrease of the membrane fluidity measured by TMA-DPH and DPH, especially in the case of denatured GFP, clearly show the interaction of GFP on the hydrophobic interior of the POPC/POPG liposome membrane, followed by the electrostatic interaction with its negatively charged surface. It has also been reported that the charge density of the liposome could affect the protein refolding (Kuboi *et al*; 2000).

Among the properties of the liposome membrane, the membrane fluidity is one of the most important factors to evaluate the chaperone-like function of liposomes. According to Kuboi *et al*, (2000), the interaction between liposome with high surface charge density (-0.15 to -1.4C/nm^2) are used. The result indicated that negative charged liposome POPG and DMPG liposome can strongly interact by electrostatic and hydrophobicity interaction with denatured lysozyme and then inhibit its refolding, resulting in inhibition of 88% and 52% lysozyme enzyme activity. Lysozyme-liposome interaction proceeds as follows: at first step positively charged lysozyme binds to negatively charged liposomes mainly through electrostatic attraction. Then, in the second step, the conformation of change of lysozyme is induced by hydrophobicity interaction with the acyl chain region of liposomes.

The basic characteristics of the GFP, such as surface net charge and hydrophobicity, were shown along the amino acid sequence (**Fig.2-10**), together with its tertiary structure. There are some possible domains in which positively-charged amino acids are clustered (i.e. region I (domain 3 – 4) and region II (domain 6 - 9)). Among the above regions, the former

one locates in the N-terminal region, which can first be translated, and has a hydrophobic surface. The GFP, translated on the ribosomal complex, could be interacted with the POPC/POPG surface because of the electrostatic and hydrophobic interaction. GFP has reported to possess 2 cystein residues that are neighboring to two hydrophobic residues, not bounded to each other in the native structure (Fukuda *et al*; 2000). It can be supposed that 2 mentioned surface residues in polypeptide GFP are strongly interact with acyl chain region (hydrophobicity) in liposome POPC/POPG by hydrophobic interaction. Beside of this,

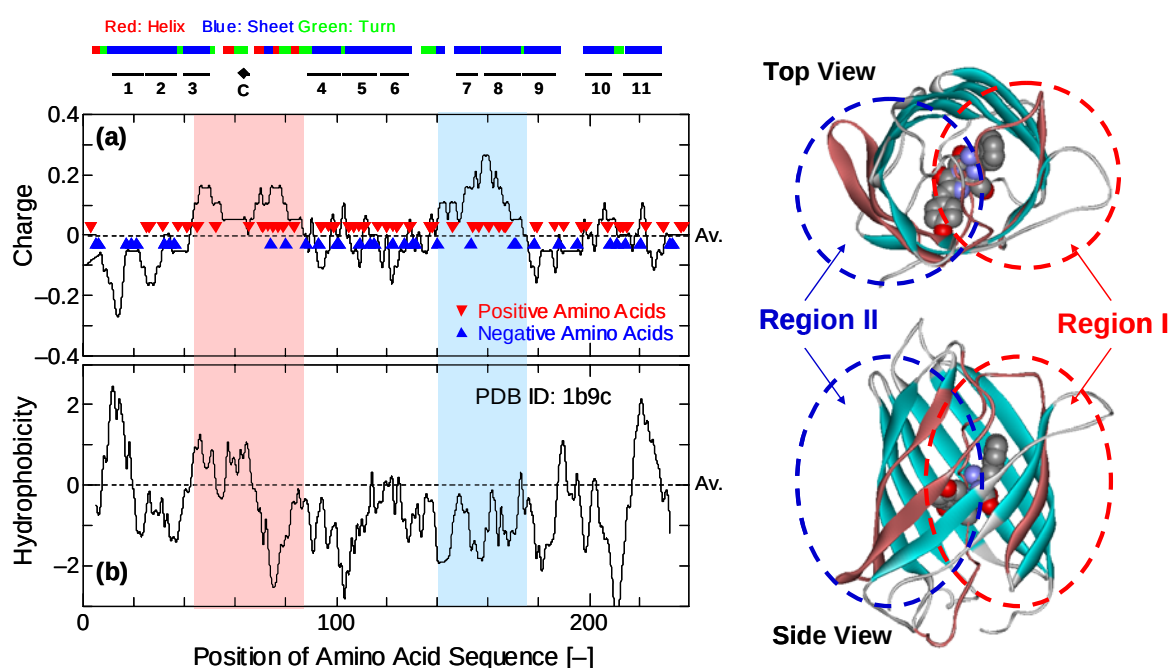


Fig.2-10 Basic characteristics of GFP at any position along amino acid sequence from N- to C-terminal. All the data were analyzed based on structure data of domain A of native GFP registered in Protein Data Bank (<http://www.rcsb.org/>, PDB ID: 1b9c). (a) shows the moving-average of the surface net charge of neighboring nine amino acids by assuming the Glu and Asp show -1 and His, Arg, and Lys show +1. (d) shows the moving-average of the surface net charge of neighboring nine amino acids by using the hydrophobicity scale reported previously (Kyte J. and Doolittle R.F., J. Mol. Biol., 157, 105 (1982)). (c) GFP conformation (domain A) displayed by viewer Lite 4.0 based on PDB Data of GFP (1b9c).

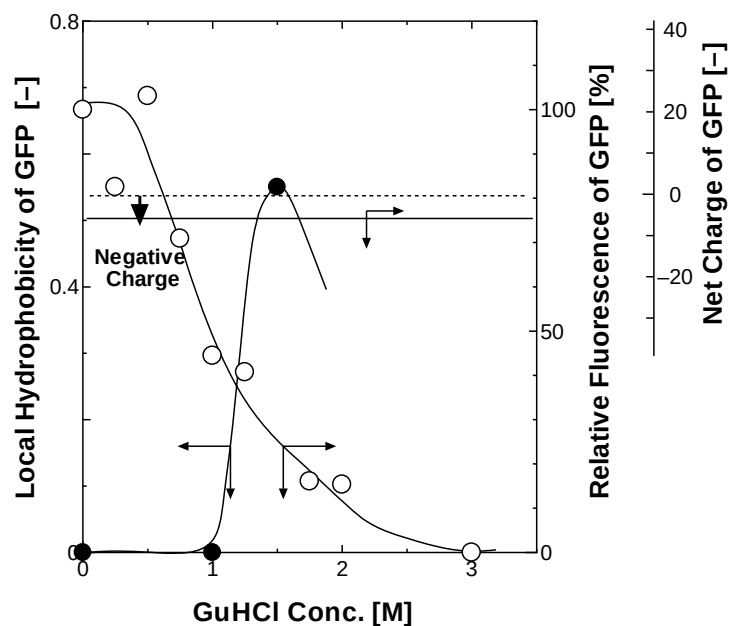


Fig.2-11 Surface characteristics of GFP at different GuHCl concentration

hydrogen bond interaction is also occurred, resulting that the synthesized GFP polypeptide could not refold into mature GFP and has no fluorescence intensity. As shown in **Fig.2-7**, the folding of the GuHCl-denatured GFP was carried out, resulting in the inhibition of the GFP refolding in the presence of POPC/POPG liposome (**Fig.2-8**). The surface characteristics of the GFP along with the GuHCl concentration were shown in **Fig.2-11**. The surface of the GFP has a slightly “negatively-charged” surface because the GFP has an isoelectric point at neutral pH region (pI=5.8). During the GFP refolding, the local hydrophobicity became maximal, giving a hydrophobic interaction between the intermediate of GFP and the liposome surface. Although the refolding process is different from the post-translational folding of the protein, the GFP could basically be folded along the similar folding pathway. From this study, the phenomena illustrate that, in the case of GFP, local negative charged density liposome is

important for GFP folding indicating by at first of electrostatic interaction, and next, interaction of hydrophobic acyl-chain group of local charged liposome with hydrophobic amino acid residue of GFP, and then hydrogen bond interaction might be included. All these mentioned interaction resulted in the inhibition of refolding of GFP and folding of synthesized GFP polypeptide in post-translation of gene (**Fig.2-4**). Negatively-charged liposome with characteristic of local charged surface, hydrophobicity and hydrogen bond that are potential for the inhibition of refolding protein and folding of newly synthesized polypeptide at post-translation level for inactive products of gene expression (**Fig.2-12**). It is thought that the inhibitory effect of the POPC/POPG liposome on the GFP folding could occur owing to the existence of the positively-charged domains in the hydrophobic domains in the polypeptide.

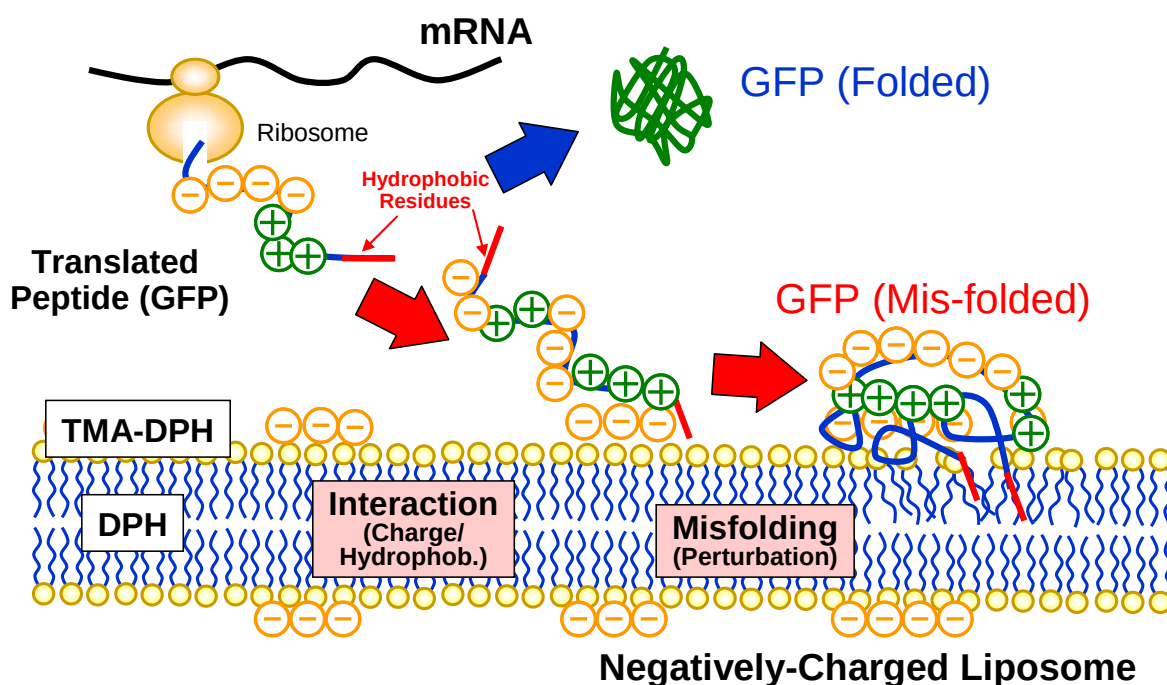


Fig.2-12 Conceptual illustration of role of negatively-charged liposome (as a model of biomembrane) in “Post-translation”

Summary

The effect of the addition of the negatively-charged POPC/POPG liposome on gene expression of GFP was carried out as a case study of the role of typical biomembrane on the gene expression. The gene expression of mature GFP product was identified by detection of fluorescence intensity after 18 hours of expression. The result of RT-PCR product of specific fragment of remained mRNA content indicated that POPC/POPG liposome has no effect on gene transcription and translation level. Furthermore, the analysis of total synthesized GFP expression with and without liposome also indicated that, the POPC/POPG liposome has no effect on gene translation. However, the POPC/POPG liposome has effect on inhibition of post-translation of refolding of GFP. In the presence of liposome at 4 mM, the GFP fluorescence (synthesized and folded amounts GFP) was reduced up to 60 to 65 % in comparison with control sample.

The total products of fold and unfold synthesized GFPs expressed in the presence and absence of POPC/POPG liposome were further analyzed by SDS-PAGE. Interestingly, total expressed GFP products were the same when the GFP was expressed with and without liposome at the concentration of 1~4mM. This result indicated that liposome only inhibit the folding of synthesized polypeptide GFP (mature protein - active protein) in post-translation but it did not inhibit the gene translation of total synthesized protein product. The interaction between liposome and mature expressed GFP was also investigated by ultrafiltration analysis.

The liposomes were found to interact strongly with mature synthesized GFP and the GFP complexed with liposome could not pass through membrane filter, account up to 72% of total GFP product before ultrafiltration.

The effect of liposome on the refolding of native standard GFP denatured by guanidine in refolding buffer was also carried out. The result showed that the liposome inhibited 55% refolding of native GFP denatured by guanidine in comparison with native GFP before denaturation. A variation of outer and inner membrane fluidity of liposome in the presence of GFP was studied, indicating that the POPC/POPG liposome strongly interacted with native standard GFP (10% reduction in surface membrane fluidity and 24% in internal membrane fluidity). The above findings on the negatively-charged liposome POPC/POPG during the GFP expression imply a possible application on “silence” of gene product, such as toxic enzyme expressed in infective toxic bacteria.

Chapter III

Role of Foreign Membrane on Gene Expression

~ Effect of Cationic Liposome on Gene Expression at Transcription and Translation levels~

Introduction

An increase of bacterial resistance to conventional antibiotics has become a major problem giving rise to an urgent need for new approaches to treat bacterial infection. In cystic fibrosis patients, pathogens like *Pseudomonas aeruginosa* show high levels of resistance and lead to progressive lung deterioration and premature death (Hancock *et al*; 1998). In order to overcome antibiotic resistance, inhibition of gene expression plays a very important role to treat infective pathogenic toxic bacteria. The extracellular DNA existence in environment is also dangerous problem to environmental and human health. Therefore, degradation of these free extracellular DNA is also necessary to be solved (Bin Zhou *et al*; 2006).

Cationic liposome, 1,2-dioleoyl-3-trimethyl-ammonium-propane (DOTAP), has several advantages as an attractive non-viral vector, such as lipofection of cationic/neutral/domain liposome, where DNA plasmid can be effectively delivered and expressed in mammalian cell, *Eukaryote* cell (Taylor *et al*; 2003). The cationic liposome has been successfully applied in drug delivery, gene delivery and expression. Recently, the liposome has been widely applied as a vector to complex with gene as Lipoplexes for the enhancement of gene delivery and expression inside the cell. The human oncogene is

over-expressed in many human cancers in order to inhibit its expression; many researchers have been studying on the application of liposome for drug delivery. A treatment of the cancer cell using the liposome (Zhang *et al*; 2006, Yoo *et al*; 2001) has successfully been achieved to delivery the E1A gene that was appropriate for its ability to produce proteins that inhibit oncogene HER-2/neu expression in both rodent and human breast cancer cells. The human HER-2/neu oncogene is over-expressed in many human cancers and the E1A, after delivery by liposome, has been shown to act as a tumor inhibitor gene by down-regulating HER-2 neu transcription.

An inhibition of toxic gene expression plays a very important role in the prevention of toxic enzyme or disease cells (cancer cell) as well as in treatment of their effect in plant and animal or disease cells, such as cancer cell (Zhang *et al*; 2006). Although the inhibition of the gene expression was indicated by some studies *in vitro* and *in vivo*, their mechanisms in many cases have still not been clarified yet (Tachibana *et al*; 2002). Nowadays, the target gene inhibition by siRNA, “RNAs interference” mechanism, was revealed by Fire and Mellow (Nobel Prize in 2006), where a short antisense RNA can link to target mRNA strand and stringers degradation of mRNA by base-pairing (Zhang *et al*; 2006). The cationic liposome was successfully applied for siRNA delivery for the inhibition of target gene expression by interference of target mRNA translation of cancer gene HDM2, resulting in the inhibition of 70% the growth of cancer cell SKMES. However, the role of liposome was utilized as just a

factor for the siRNA delivery for degradation of target mRNA based on the strategy of “RNA interference”. The effect of the liposome itself on the mRNA translation has not been clarified yet. Tachibana *et al*, (2002) indicated that the cationic liposome could inhibit the GFP expression although the mechanism was still not clarified yet. It is important to study the role of the lipid membrane itself on the gene expression for the deeper understanding of the gene expression inside a cell and to apply it for the gene regulation process.

In the previous chapters, the role of neutral liposome, microdomain-forming liposome and negatively-charged liposome on the gene expression of GFP was investigated as the main membrane components of the biomembrane, focusing on the role of biomembrane in the elementary steps of the gene expression process. A possible regulation of the above gene expression should be considered based on the “membrane-affected phenomena” in order to extend the above findings for the design of the bioprocess or the deeper understanding the unsolved problems on the gene expression. There are some possibilities such as (i) use of the foreign membrane to regulate the biomembrane and (ii) stress exposurement to bacterial cells for the regulation of the biomembrane characteristics as described in the general introduction. In this chapter, the role of the cationic liposome on the gene expression of the GFP, a reporter protein, was investigated, focusing on the elementary steps of the gene expression process as applied in the previous chapters. First, a possible use of the cationic liposome was investigated for the inhibition of gene transcription and transcription levels, followed by

expression with “knock-out of gene” and “knock-down of mRNA” concept by using *E.coli* cell free translation system with GFP as a reporter gene. Finally, a mechanism for the regulation of the gene expression based on the “Membrane interference” was discussed. This study may have broad utility for possible future studies such as: (1) treatment of extracellular free DNA that may be dangerous to environmental and human health; (2) Inhibition of infective toxic bacteria; and (3) “knock-out” of oncogene (cancer - disease gene) in cancer tumor of human and animal.

Materials and Methods

1. Materials

1,2-Dioleoyl-3-trimethyl-ammonium-propane (DOTAP) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Rapid Translation system RTS 100 *E.coli* HY Kit, an *in vitro* protein synthesis system based on *E.coli* lysate, was purchased from Roche Diagnostics (Indianapolis, IN, USA). The purified green fluorescent protein (Pure GFP) was purchased from Roche Diagnostics (Indianapolis, IN, USA). Other chemicals were purchased from Wako Pure Chemical (Osaka, Japan).

2. Liposome preparation

Liposome was prepared from cationic lipid 1,2-dioleoyl-3-trimethyl-

ammonium-propane (DOTAP) that was initially dried from chloroform; in layer was then prepared in a round-bottom flask by evaporation at 37°C using a vacuum rotary evaporator and dried under nitrogen flow for at least 5 hours. In order to generate multilamellar vesicles, the dried phospholipids thin layer was hydrated with water (pH 7). The freeze–thaw cycle was repeated 5 times with hydrated phospholipids solution for large unilamellar vesicle formation. This vesicle solution was extruded through a polycarbonate membrane with a pore size of 100 nm to form unilamellar vesicles (liposome).

3. mRNA recovery for GFP expression

A transcription reaction was carried out from expression vector GFP in transcription Kit (Promega), circle GFP vector was opened by restriction *Apal* I enzyme for getting line DNA fragment used for transcription reaction. mRNA product was recovered by SV total RNA isolation Kit (Promega, Madison, WI, USA) that was described in our previous study.

The initial mRNA content (4 hours expression) was herewith measured with and without plasmid DNA in the presence or absence of DOTAP liposome to avoid the mRNA degradation in the reaction solution owing to the instability of mRNA. The 25µl solution of GFP expression was transferred to the 212.5µl buffer. Samples were transferred into spin column assembly and 100µl 95% nuclease free-ethanol was added to precipitate the proteins. After centrifugation (14.000 rpm for 1 minute) and wash by buffer, DNA was removed by 25µl DNase. After incubation for 15 minutes at 25°C, 100µl DNase stop solution was added

into spin basket. After centrifugation and wash by buffer (repeat 2 times), the RNA was finally transferred from the collection tube to elution tube by centrifugation (14.000 rpm for 2 minutes). Total mRNA products in elution tube under spin basket were analyzed from the absorbance at 260nm and electrophoresis on agarose gel 1%.

4. *in vitro* expression of GFP with and without liposome

A Rapid Translation system (RTS 100 *E.coli* HY Kit), *in vitro* protein synthesis system based on *E.coli* lysate, was used as a cell free translation system. The expression vector, pIVEX, contained the gene for expression of GFP. The reaction solution (50µl) contained the following components: 12µl *E.coli* lysate, 10µl reaction mix, 12µl amino acids, 1µl methionine, 5µl reconstitution buffer, and 13 µg of purified mRNA. The liposome solution prepared at higher concentration was added in the reaction mixture in the replace of the above water before and after the “Expression” operation and the effect of liposome on the gene, expression was evaluated based on the following methods.

5. Analysis of gene product

The amount of synthesized and folded GFP product was evaluated by analysis of the GFP fluorescence intensity at 395 nm (excitation) and 509 nm (emission) by using the fluorescence spectrophotometer (JASCO, FP-777, Tokyo, Japan) with slit width of 5nm at excitation and emission of light path. All the GFP fluorescence spectra were analyzed after

100 times dilution of the reaction samples to water in order to avoid the possible interference of the liposome included in the final reaction sample. It was confirmed that the fluorescence spectra of pure GFP was not affected in the above experimental condition including liposome at the finally-diluted solution and also POPC liposome itself at its final concentration has no emission spectra in the above condition for fluorescence measurement.

6. SDS-PAGE analysis

Total amount of GFP products (unfolded and folded GFP) translated from mRNA were also analyzed by SDS-PAGE in order to analyze the net amounts of synthesized GFP. A 10 μ l sample of reaction solution was dissolved with 17 μ l of SDS-PAGE sample buffer and 5 μ l of mercaptoethanol and was heated for 5 min at 95°C for denaturation. The samples with 1 to 4 μ l were applied onto a ready-made gel (Homologous 12.5, GE Healthcare, and Chicago, IL, USA) set for electrophoresis system (PhastSystem, GE Healthcare, and Chicago, IL, USA). The gel was finally stained with Coomassie blue dye and GFP was detected as major staining band at 27 kDa. The distribution of the protein in the stained gel was further quantified by densitometer analysis by using the SCION image software obtained at <http://www.scion.com/>, where the densitometer analysis was carried out at least three times at different points along the lane. It was confirmed that the band of GFP was not observed in the reaction solution for protein translation without plasmid DNA.

7. Semi-quantitative mRNA product expressed in *E.coli* cell-free translation system

mRNA content in *E.coli*-cell free translation system also quantitative after 3h of GFP expression, at that time, 70% of GFP was synthesized. The mRNA content was semi-quantitatively determined to be the total RNA content of the sample containing the DNA vector (including mRNA) minus that without the DNA vector (no mRNA product) and then analyzed by A260, 1 unit of absorbance equal to 40 µg RNA.

8. Evaluation of remained mRNA products after duration of gene transcription, mRNA translation by using Reverse Transcription Polymerase Chain Reaction (RT-PCR)

After GFP expression from mRNA in RTS system of 4 hours, at that time, about 65% GFP was expressed, total RNA was extracted by using SV total isolation Kit (promega) according to the supplier's instruction. The primer pairs are as follows: PY504-01: 5'-ATGACTAAAGGTGAAGAAC-3' (forward) and PY504-02: 5'-CGATTCTATTAACAAGGGTATCACCT-3' (reverse) were used for amplification of specific fragment of GFP's mRNA having 371bp in length. cDNA reaction was carried out by using specific reverse mentioned primer according to protocol from PrimeScriptTM RT-PCR Kit, TAKARA BIO INC with following temperature program: 65°C for 5 min, 46°C for 30 min and 95°C for 5 min, a synthesized cDNA product from 2 to 4 µg was applied for RT-PCR reaction with following temperature program: 30 min at 46°C, denature 2 min at 94°C, annealing 30s or 1 min at the primer's melting temperature, elongate 1 min 20 s or 2 min at 72°C and prolonged elongation

3 min to 10 min at 72°C. RT-PCR products were then analyzed by electrophoresis on agarose gel 1%.

Identification of the stabilization of mRNA outside RTS system with/without liposome: mRNA was complexed with different DOTAP liposome concentrations that were inoculated with the same incubation condition in *E.coli* cell free system at 30°C for 6h. The mixtures mRNA/liposome with and without treatment of SDS 5% were then analyzed by electrophoresis on agarose gel 1%.

9. Statistical analysis

Results are expressed as mean $\pm$ standard derivation (SD). All experiments were performed at least in triplicate. The data distribution was analyzed, and statistical differences were evaluated using the Student's t-test. A P value of < 0.05 percentage was considered significant.

Results and Discussion

1. Effect of cationic liposome DOTAP on GFP expression

GFP expression in the *E.coli*-cell free translation system was evaluated by using the vector plasmid pIVEX2.3-GFP as a template for gene expression of GFP in the presence of various concentrations of DOTAP liposome. **Figure 3-1** shows the effect of the DOTAP concentration on the GFP expression shown by fluorescence intensity. When the liposome concentrations in the standard RTS-100 reaction mixture was increased, the GFP fluorescence, which is equivalent to the amounts of synthesized and folded GFP protein, was reduced in comparison with that of control sample without liposome (**Fig.3-1**). This results suggest that the addition of liposome DOTAP in *E.coli* cell-free system is effective for the inhibition of gene expression of mature and folded GFP, resulting that at 4mM DOTAP liposome, GFP

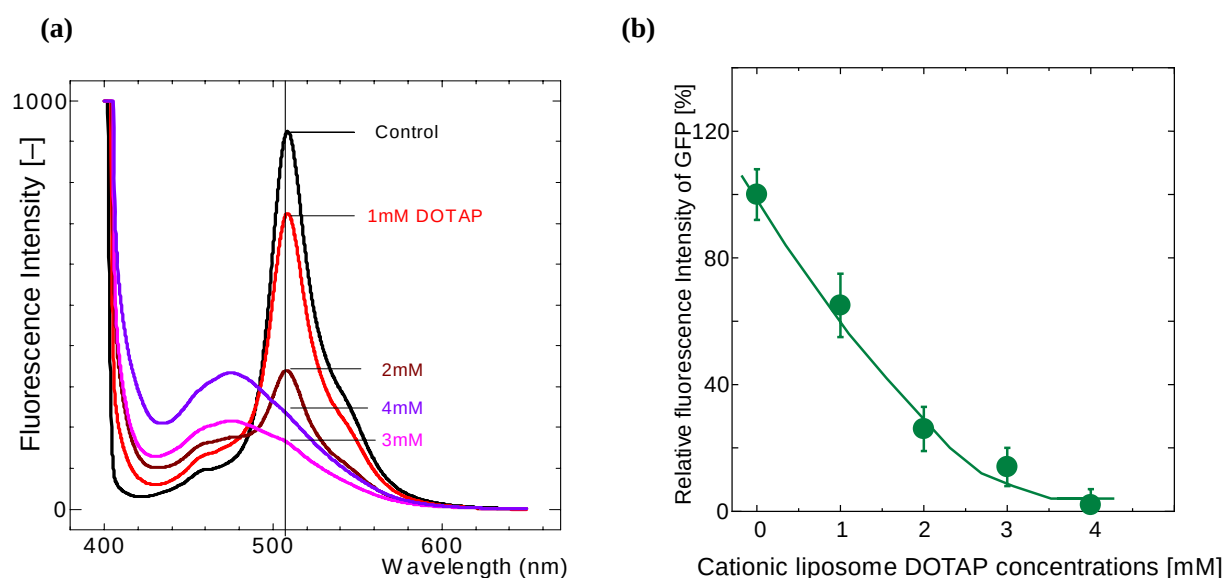


Fig.3-1 Effects of cationic liposome DOTAP addition on GFP expression in a Cell-free translation system. (a) Fluorescence spectra of expressed GFP with and without liposome. (b) Relative percentage of GFP products expressed with and without DOTAP liposome.

expression was 100% inhibited. A microscopic observation of GFP expression was also carried out to determine whether cationic liposome DOTAP inhibit GFP expression shown by fluorescence intensity. Specific GFP fluorescence was emitted in control sample although it was not emitted when GFP expression was performed in the presence of DOTAP liposome at 4mM (data not shown). From this measurement, it became clear that the expressed and folded GFP as well as proper GFP protein folding (showed by GFP fluorescence) was almost inhibited by cationic liposome DOTAP.

However, the fluorescence of GFP indicated by fluorescence measurement and microscopic observation may show the contents of only folded and mature GFP. It is thought that the fluorescence intensity corresponding to the GFP production was reduced in the presence of the DOTAP liposome not only owing to the decrease of the folded GFP but also to the decrease of the total synthesized GFP product. **Figure 3-2** shows the results of the SDS-PAGE analysis of the protein mixture that was synthesized in the presence and absence of DOTAP liposome. The amount of GFP synthesis was also estimated by densitometric analysis of the SDS-PAGE gel stained by Coomassie Brilliant Blue R-250 (CBB). It was found that the band for the GFP was not observed in the presence of DOTAP liposome, corresponding with the results obtained from the fluorescence data. The result in **Fig.3-2** also indicated that the cationic liposome DOTAP at 4mM could inhibit 100% GFP expression of total polypeptide in *E.coli* cell-free translation system (lane 2) in comparison with that of

control sample without liposome (lane 3).

The effect of various sizes of liposome having diameters of 50 nm, 100nm, 200nm, 400nm on GFP expression was investigated in order to investigate the other possible factor of the DOTAP liposomes. The result of fluorescence intensity analysis showed that the liposome with various sizes gave nearly the same effect on the inhibition of GFP expression in the presence of 4mM liposome, resulting that the 100% inhibition of the GFP expression was similarly observed (data not shown). This result implies that the gene expression was inhibited by cationic lipid DOTAP, depending on the concentration of lipid and not on the size of liposomes.

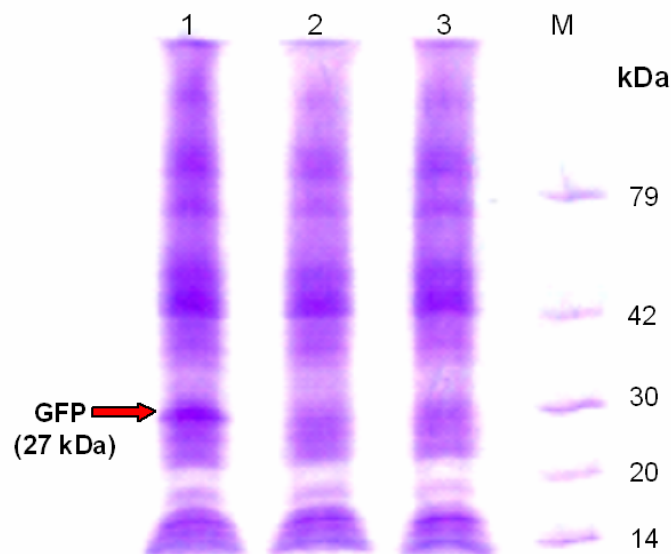


Fig. 3-2 SDS-PAGE image of GFP when expressed of with and without DOTAP liposome. The sample was analyzed by using the PhastSystem (GE Healthcare Science), and the gel was stained by the CBB method, lane 1: control; lane 2: GFP expression in the presence of 4 mM DOTAP liposome, lane 3: negative control (GFP expression without DNA and mRNA), lane M: marker with molecular weights of 79, 42, 30, 20, 14 kDa. GFP expression was performed at an expression time of 6h at 30°C. The distribution of the protein in the stained gel was further quantified by densitometer analysis, which was carried out at least three times at different points along the lane.

2. Role of cationic liposome DOTAP on GFP expression at transcription level

It is necessary to clarify whether the cationic DOTAP liposome could inhibit the gene expression originated from the inhibition of gene transcription or gene translation in order to deeply understand the mechanism on the role of liposome DOTAP during the gene expression in *in vitro* translation system. The mRNA content was measured after the GFP expression of 3 hours, at that time 60-75% of GFP was expressed at the control sample. Total RNAs in RTS reaction including tRNA (transport RNA), rRNA (ribosome RNA) and transcribed RNA (messenger RNA) were recovered and purified from RTS system for reverse transcription reaction (cDNA reaction). cDNA reaction was carried out based on the target template mRNA (GFP's mRNA) and a specific reverse transcription primer designed based on nucleotide sequence of GFP's mRNA. The synthesized strand cDNA was then used as a template for PCR reaction by using a specific primer pairs for amplification of specific fragments of GFP's mRNA (371 bp). RT-PCR products of specific fragment of mRNA were then analyzed on agarose gel as shown in **Fig.3-3**. In the case of GFP expression in the presence of 4mM DOTAP liposome, no RT-PCR product was observed, showing the no production of mRNA (**Fig.3-3**, lane 2) in comparison with control sample without liposome (lane 3). The result at lane 1 in **Fig.3-3** was negative control sample, where it was confirmed to have no amplified specific fragments of GFP's mRNA without vector pIVEX plasmid

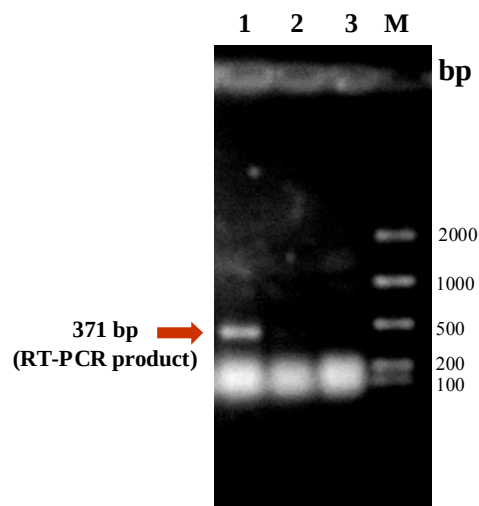


Fig.3-3 Electrophoresis on agarose gel of RT-PCR product of specific fragments of GFP-mRNA with the length of 371 bp. Lane 1: RT-PCR product of specific fragment of mRNA from control sample of GFP expression without DOTAP liposome; lane 2: GFP expression in the presence of DOTAP 4mM; lane 3: negative control, GFP expression without GFP vector DNA; lane M: marker with molecular weight of 2000, 1000, 500, 200, 100 bp.

containing GFP gene (no mRNA product). On the contrary, the specific fragment of mRNA was recovered from the control sample of GFP expression containing GFP gene in the absence of DOTAP liposome, resulting that mRNA with the length of 371bp was amplified and presented in the recovered sample (lane 3). The above results clearly show that the cationic DOTAP liposome inhibited the gene transcription in *E.coli* cell free translation system.

3. Role of DOTAP liposome on inhibition of gene transcription by knocking-out of gene

In order to clarify the mechanism on the inhibitory effect of gene transcription by cationic liposome DOTAP more in details, the stability of expression vector GFP was studied with and without the liposome. GFP expression vector was mixed with liposome DOTAP at

1-4 mM in total volume of sterilized water equal to that of *E.coli* cell free translation reaction for GFP expression, incubation was carried out at 30°C for 6 hours. GFP vector before and after incubation was analyzed by agarose gel electrophoresis as shown in **Fig.3-4**. The result indicated that the cationic liposome DOTAP gradually degraded GFP vector in comparison with control sample, depending on the DOTAP concentration below 3mM (**Fig.3-4**, lane 1, 2, 3, and 4). At 4mM of DOTAP liposome, no band for vector GFP was observed and the content of the vector can be estimated as almost 0 % in comparison with that of control sample (**Fig.3-4**, lane 1 and 5). It was thus found that the cationic liposome DOTAP at higher concentration level knocked out (or degraded) the gene to inhibit its transcription.

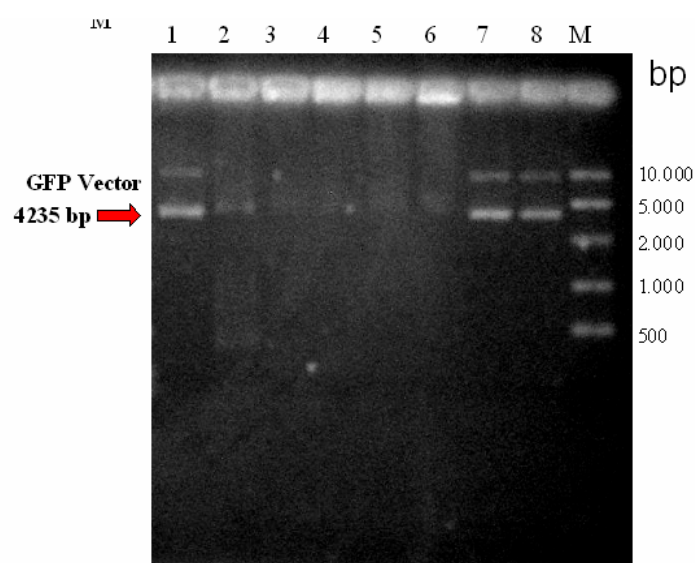


Fig.3-4 Electrophoresis on agarose gel of GFP vector DNA and complex liposome/vector DNA of GFP after inoculation in the same condition with GFP expression in *E.coli* cell-free translation system at 30°C for 6h, showed from lane 1 to lane 7. Lane 1: DNA+ 5% SDS, from lane 2 to 5: DNA+ DOTAP of 1, 2, 3, 4 mM, respectively; lane 6: DNA+ 4mM DOTAP + 5% SDS; lane 7, 8: GFP vector after 6h and 0h of inoculation. Lane M: marker with molecular weight of: 10.000, 5000, 2000, 1000, 500 bp.

There are some possible mechanisms on the “knock-out effect” of DNA by DOTAP

liposome. According to Zhang *et al*, (2006), a positively-charged liposome could interact with strongly negatively-charged RNA or DNA by 2 types of different forces acting in the system: (1) electrostatic interaction between anionic phosphate groups of polynucleotide (DNA, RNA) surface and the cationic of quaternary ammonium groups of polar heads of DOTAP liposome, and (2) hydrophobic interaction of the side residues of the polynucleotide with the liposomal membrane of lipid bilayer. The tight interaction of cationic liposome and negative charged DNA gene could result that the DNA was degraded by possible mechanism based on some interactions: at 1st step, strong electrostatic interaction between positive charge of quaternary ammonium groups of liposome and negative charge from phosphodiester bond that linked nucleotides in DNA strand; at 2nd step, the destabilization of hydrogen bonds of double strands DNA affected by above strong electrostatic interaction on phosphodiester bond resulting in the unstabilization of single strand DNA (ssDNA) and, at the 3rd step, the hydrolysis of double strands DNA(dsDNA) on the surface of liposome. It has been reported that the hydrolytic activity can be induced on the surface of the molecular assemblies such as the reverse micellar system (Walde, 2006). Cationic liposome can interact with DNA by electrostatic interaction. This strong interaction could make phosphodiester bond is not stable and could break down in DNA structure. As an alternative explanation, the gene of GFP and others became highly compact conformational state when it was interact with liposome (Safinya *et al*; 2001), so that the T7 RNA polymerase (T7RNAP), a transcription enzyme,

could not bind with T7 promotor located in GFP gene for transcription into mRNA.

Polynucleotide, such as DNA and RNA, is known to form complex with cationic liposome. When negatively-charged DNA chains were adsorbed onto the surface of the cationic/neutral/domain liposome, the structure of the polynucleotide was changed to compact state, so that the liposome could support to storage high-density genetic information and stabilize nucleotide of adsorbed DNA (Safinya *et al*; 2001) and also enhanced the gene delivery and expression inside the cell. The cationic lipids possess a hydrophobic group, which may be either one or two fatty acids, or alkyl moieties of 12–18 carbons in length in addition to an amine group. The amine group is necessary for its binding with DNA moiety through the electrostatic interaction (Brown *et al*; 2001), resulting in the condensation of the large anionic molecule into small transportable unit's lipoplexes. Because of the complex formation, the cationic lipid tends to fully neutralize the phosphate group on DNA. DNA/cationic liposome complex condensed DNA along with tubular structures and aggregated on the surface of liposome, resulting in the interaction with cell membrane to induce the endocytosis and disruption of endosomes to give the enhanced gene delivery to inside the cell. Nowadays, cationic/neutral/domain liposome was successfully in use for drug delivery for treatment of target cell (Zhang *et al*; 2006, Sugahara *et al*; 2002, Fukuda *et al*, 2006). It is expected that the above phenomena could be applied to various fields.

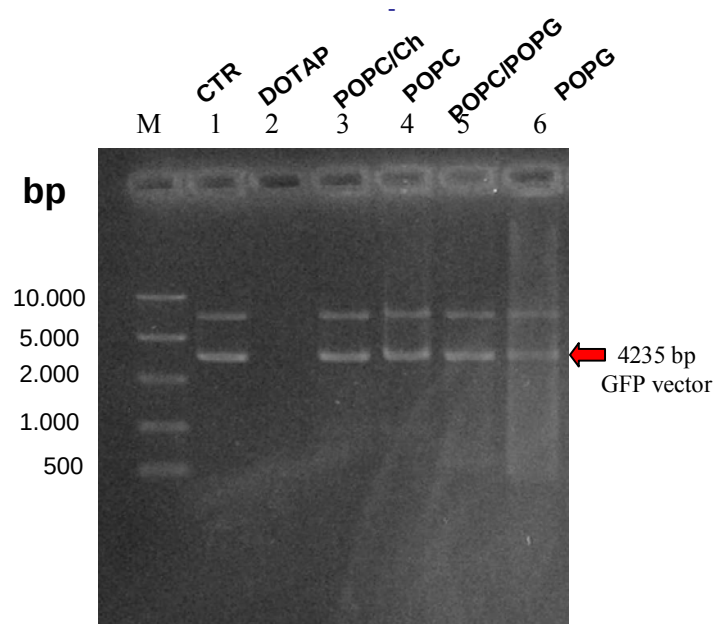


Fig.3-5 DNA vector with some kinds of liposome at 4mM with the same ratios and volume used in *E.coli* cell-free translation system for GFP expression. (H₂O was replaced for components solution of of translation system), after inoculation of 6h at 30⁰C: Lane M: marker with molecular weight of: 10.000, 5.000, 2.000, 1.000, 500 bp; lane 1: control sample: vector GFP, from lane 2 to 6: complex of DNA with following liposome including: DOTAP, POPC.Ch (7:3), POPC, POPC.POPG and POPG, respectively.

4. Stabilization of DNA vector in complex with various kinds of liposome

The stabilization effect of DNA was further investigated in the presence of some kinds of liposomes including cationic liposome DOTAP, microdomain-forming and neutral liposome POPC/Ch, neutral liposome POPC, negative charged liposome POPC/POPG (7/3) and POPG. The results in **Fig.3-5** indicated that the DOTAP liposome as an additive to the cell free translation system could degrade the DNA vector at the ratios of liposome/DNA: 0.0516 μ M DOTAP/0.1 μ g DNA vector, that ratios was also used inside *E.coli* cell-free translation system for the inhibition of 100% of GFP expression. On the contrary, DNA is still stable when complexed with other liposomes, such as POPC/Ch, POPC, POPC/POPG, POPG,

(Fig.3-5. lane 3, 4, 5, 6, respectively). The ratios of positive and negative charges between DOTAP liposome and DNA vector that was calculated based on the ratios of liposome and DNA are 0.0516 μM DOTAP and $0.03935 \times 10^{-6} \mu\text{M}$ DNA vector, equal to (+) 3.108×10^{16} (C) and (-) 0.01825×10^{16} (C), and the charge ratios of liposome and DNA are 170 and 1, respectively. We supposed that DNA vector is often presented at supercoiled style with overlap structure, so that DNA can absorb on surface of liposome, but only a part of negative charge DNA surface can be easily neutralized by liposome, other parts of DNA surface can be stored in DNA supercoiled and overlap structure, so they are difficult to be neutralized by liposome. So that, transcription of DNA can be inhibited by DOTAP liposome when the number of cationic charge of liposome is 170 times higher than negative charge of DNA, in theory. However, that ratio is very important to get the “knock-out effect” of gene by DOTAP liposome to inhibit gene expression at the transcription level.

This result once more confirms that the electrostatic interaction between liposome and DNA could play a very important role in the stabilization of gene as well as its ability for transcription. DNA can be existed stably when complexed with neutral liposome POPC, microdomain-forming liposome POPC/Ch, partially negatively-charged liposome POPC/POPG (7:3). However, DNA can be almost degraded when it was complexed with cationic liposome at the ratios of charged density of liposome/DNA of 170 and 1.

5. Role of cationic liposome DOTAP on gene expression at mRNA translation level

A possible role of the DOTAP liposome at mRNA translation level was furthermore investigated in the *E. coli* cell-free system in order to clarify whether the cationic liposome inhibit only gene transcription and how about the liposome effect on the gene translation. The recovery of the mRNA was carried out for the translation reaction in the presence of DOTAP liposome, the role of DOTAP liposome on mRNA translation was clarified by initiating the GFP expression reaction by adding the recovered mRNA.

In three hours of GFP expression from DNA, 70% GFP was shown to be expressed in control sample as described in previous section. At that time, mRNA content in *E.coli* cell-free translation system was also recovered together with total RNA of tRNA, rRNA for semi-quantitative of mRNA. The calculation indicated that there was about 13 µg mRNA could be transcribed from DNA vector from a 50 µl of total RTS reaction after 3 hours of expression. The recovered and purified mRNA (13 µg) was also used for the template to replace the DNA vector for GFP translation from mRNA in *E.coli* cell-free translation system.

(1) Recovery of mRNA for GFP expression.

At first, a circle plasmid DNA, encoding GFP, (**Fig.3-6**) was opened into line DNA by using the *Apal* I restriction enzyme for requirement in carrying out gene transcription and knock-out of AmpR gene for recovery only GFP gene. The plasmid DNA after treatment with *Apal* I can be separated into three DNA fragments of 426bp, 1245bp and 2563bp in **Fig.3-6(a)**.

enzyme though the GFP gene will still be located in the DNA fragment of 2563 bp that can be

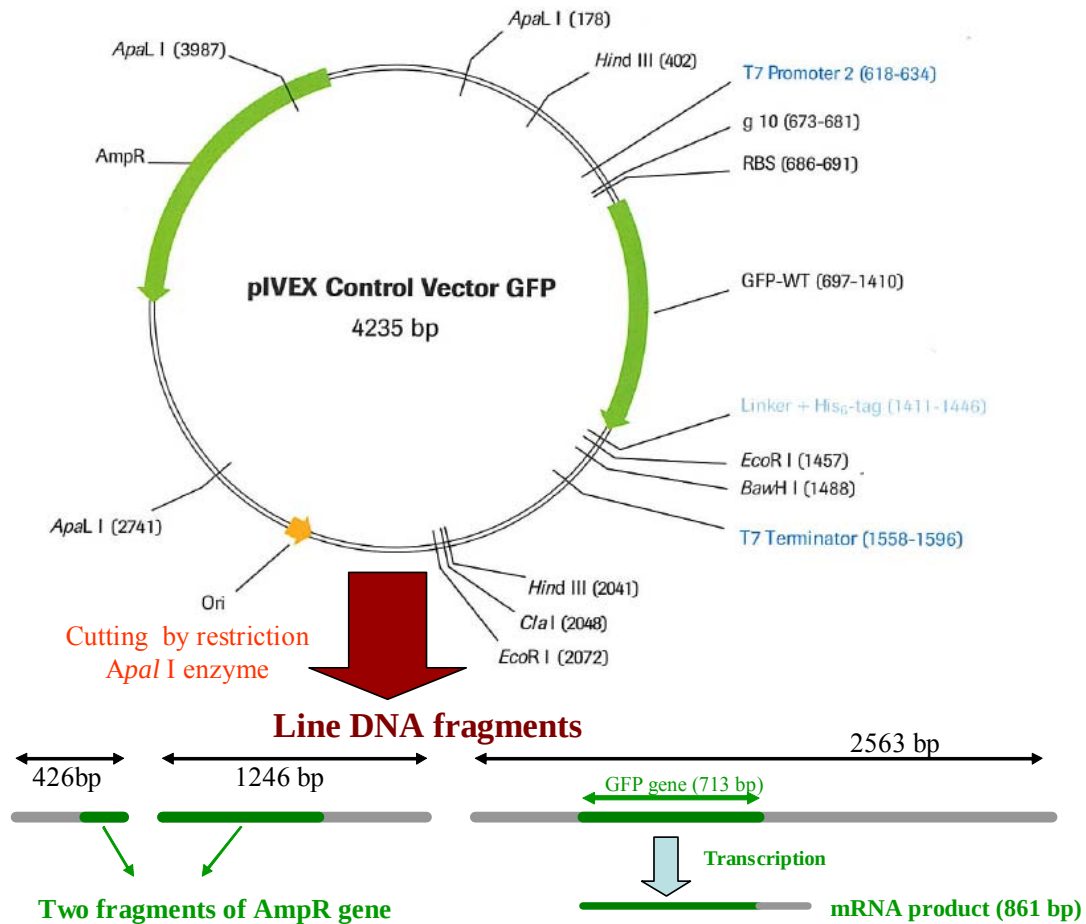


Fig.3-6(a). Opening circle GFP vector by restriction enzyme *ApaI* for creation of 3 line DNA fragments including: 2563 bp (habouring GFP gene), 1246bp and 426 bp. AmpR gene was cleaved by *ApaI* restriction enzyme and could not be transcribed and translated.

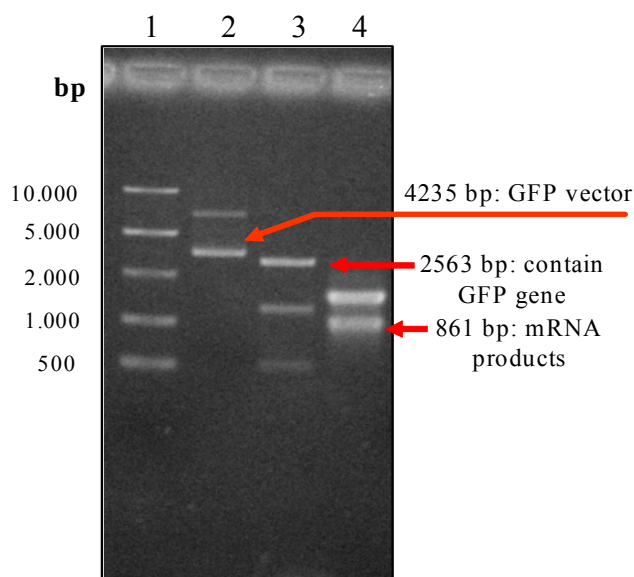


Fig.3-6 (b). Electrophoresis on agarose gel of circle GFP vector before and after opening by restriction enzyme *ApaI*. lane 1: marker: (10.000, 5000, 2000, 1000, 500 bp); lane 2: circle GFP vector: 4235 bp; lane 3: opened line DNA fragments of 2563, 1246 and 426 bp, after cutting circle GFP vector; lane 3: mRNA product: 861 bp including mRNA-GFP 713 bp.

AmpR gene located in vector GFP could be cleaved after the treatment with *Apal* I restriction transcribed into cell free translation system. Circle DNA, DNA line fragments, and mRNA products were then analyzed by electrophoresis on agarose gel in order to confirm the quality of purified products (**Fig.3-6(b)**). The result in **Fig.3-6(b)** shows that all the products were purified and indicated by specific band products with their specific molecular weights as mentioned above. There is no sub-product of opened line DNA fragment that show high quality enough to investigate further experiments (**Fig.3-6(b)**, lane 2). 100% circle DNA vector was successfully cut by *Apal* I enzyme at specific sites and line DNA fragments was recovered as shown in lane 2 and 3 (**Fig.3-6(b)**). AmpR gene was also 100% cleaved and it was confirmed that it could not be transcribed into mRNA. After the transcription reaction from line DNA fragment, mRNA was also recovered and purified by RNA isolation kit described in the previous study (Bui *et al*; 2008) and was then analyzed by the absorbance (A260) to check its quality after purification. The result indicated that the ratios of absorbance of A260/A280 were increased up to 1.98 (data not shown). This result shows that the mRNA was recovered and purified without the presence of DNA and it has enough quality for further experiments of GFP translation from mRNA (**Fig.3-6**, lane 4).

(2) Cationic liposome inhibit mRNA translation

The purified mRNA was used to replace the DNA vector to carry out the GFP expression at mRNA translation level. The GFP expression from mRNA was used as model

system to investigate the mechanism of the liposome effect on the translation step of the gene expression *in vitro* experimental system. The time course of mRNA translation to GFP production was carried out and the GFP expression was measured by fluorescence intensity, indicating that the mRNA translation was maximal at the 6 hours translation (data not shown). In the following, the mRNA translation was carried out for 6 hours in the presence and absence of liposome, including negative charged liposome (POPG), microdomain-forming liposome (POPC/Ch), and neutral liposome (POPC) at the lipid concentration of 4mM. The fluorescence intensity of GFP product reduced to 80% with POPG and enhanced to 125% with POPC/Ch and 115% with POPC in comparison with control sample (data not shown). However, in the presence of cationic liposome DOTAP, mRNA translation was totally inhibited, resulting in the 0% GFP production. This result indicated that, although the effect of negative charged and neutral liposome is not so significant in mRNA translation step, the cationic liposome DOTAP could significantly affect the mRNA translation.

A possible role of the concentration of cationic liposome on mRNA translation was investigated through the investigation of the GFP fluorescence. DOTAP liposome at the concentrations of 1, 2 and 3 mM was added to the standard RTS-100 reaction mixture at 0h of starting translation and the GFP production was performed for 6 hours after the addition of the mRNA. As shown in **Fig.3-7**, the effect of the concentration of DOTAP on the GFP fluorescence spectra was investigated. The relative fluorescence of GFP was reduced with the

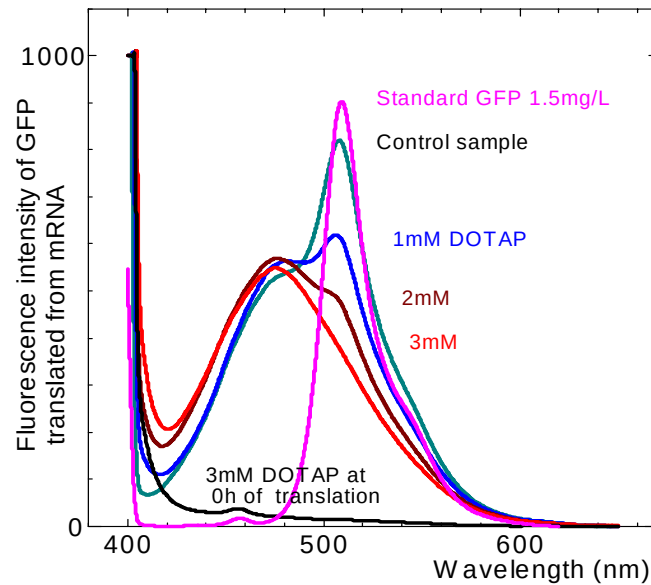


Fig.3-7 Effect of cationic liposome DOTAP concentrations on GFP expression from mRNA showed by fluorescence intensity in an *E.coli* cell-free translation system.

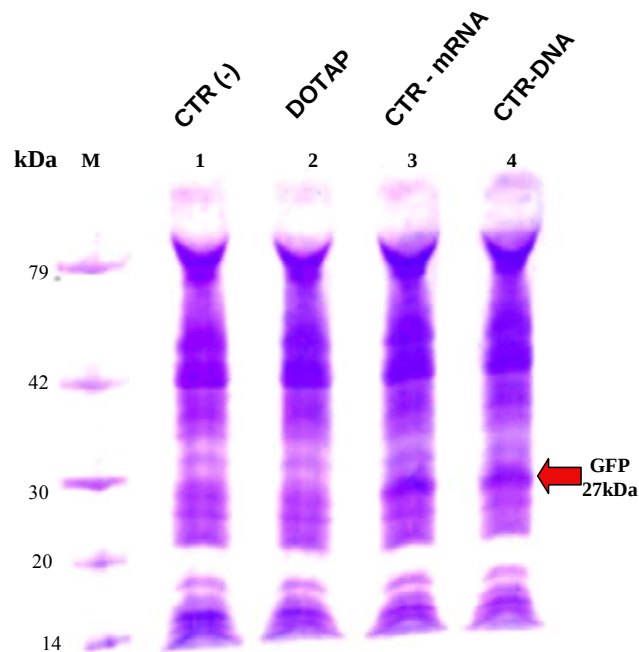


Fig.3-8 Expression of GFP from mRNA in the presence of with/without liposome. Protein product was analyzed by SDS-PAGE and stained by CBB method. Lane M: marker with molecular weight of 79, 42, 30, 20, 14 kDa. Lane 1: Negative control, GFP expression without mRNA and DNA. Lane 2: GFP expression from mRNA with DOTAP liposome; Lane 3, 4: GFP expression from mRNA and GFP-DNA vector, respectively.

increase of the concentration of DOTAP. The obtained sample was also observed by using the fluorescence microscope, resulting that the GFP fluorescence, observed in the control, was not observed in the presence of cationic liposome DOTAP at 3mM. The GFP fluorescence, indicated by fluorescence spectra and microscopic observation, show the content of only folded protein (mature GFP).

Totally-synthesized GFP was further analyzed by using SDS-PAGE. The analyzed results of GFP expression were shown as an image of CBB-stained gel. The result also indicated that the addition of the DOTAP liposome was totally inhibited the mRNA translation for GFP at 3mM (**Fig.3-8**, lane 2) in comparison with that of control sample without liposome (**Fig.3-8**, lane 3).

The above results in **Fig.3-7** and **Fig.3-8** suggest that the addition of the DOTAP liposome could be used as an effective inhibitor of “mRNA translation step” of mature GFP using *E.coli* cell free system. Although the effect of the size of the DOTAP liposome (i.e. 50nm, 100nm, and 200nm) was investigated at 3mM, the variation of the liposome size gave nearly the same effect on inhibition of GFP expression (data not shown). This result also implies that the gene expression at mRNA translation level was inhibited by DOTAP liposome, depending on the characteristic of cationic charged surface of liposome, not by the size of liposomes.

6. Role of cationic liposome on inhibition of mRNA translation as mRNAi model: Liposome can knock down mRNA for silencing its translation.

A mechanism of inhibitory role of liposome during the mRNA translation was also clarified. The time course of mRNA translation, analyzed from GFP fluorescence, indicated that mRNA could be translated into GFP during 6 hours and was suppressed at more than 6 hours of translation (data not shown). This result indicated that the lifetime of mRNA for GFP translation is very short, only around at 6 hours, and this time is nearly same as the time for GFP expression from DNA at 6 hours (about 90% GFP). The lifetime of the mRNA could also be a key factor to inhibit the GFP translation process.

The identification of the remained mRNA product after 4 hours of translation was carried out in the presence of with/without cationic liposome (DOTAP) in order to clarify whether cationic liposome can knock down mRNA (mRNA still exists but it could not be translated) or knock out mRNA (mRNA was degraded and it could not be translated), Nguyen *et al*, (2008). Total RNAs were recovered for RT-PCR reaction. The contents of remained mRNA, after 4 hours of translation, were identified by specific fragments of mRNA having 371 bp in length that were cloned and amplified based on specific primer pairs and RT-PCR method. RT-PCR products were analyzed by electrophoresis on agarose gel 1% and then staining with ethidium bromide 0.5% as shown in **Fig.3-9**. The result in **Fig.3-9** indicated that mRNA translation without liposome DOTAP from control sample (lane 2) was reduced and accounted about 30% of that in experiment sample of mRNA translation in the presence of

3mM DOTAP liposome (lane 3). As mentioned above, after 4 hours of translation, 65% GFP was translated, the result also indicated that mRNA content was also reduced about 70% in comparison with that of experiment sample containing liposome. Furthermore, the most important result in **Fig.3-9** already confirmed that, in the presence of cationic liposome DOTAP, mRNA could still exist after 4 hours of translation but it could not be translated into GFP in *E.coli* cell free system. This result indicated that the cationic liposome DOTAP “interfered” mRNA for silencing and “inhibit” its translation in spite of no degradation of mRNA.

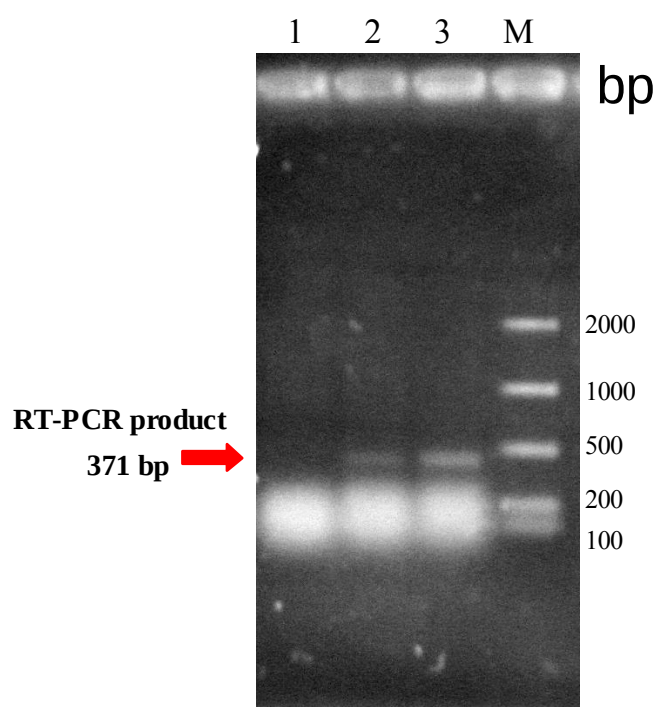


Fig.3-9 Electrophoresis on agarose gel of RT-PCR product of specific fragments of GFP-mRNA (371 bp). Lane 1: negative control, GFP translation in the absence of mRNA and DNA; Lane 2 and 3: specific product of remained mRNA product after its translation of 4h in the presence of without and with DOTAP liposome at 3mM, respectively; lane M: marker with molecular weight of 2000, 1000, 500, 200, 200 bp.

A possible degradation of mRNA in the presence of cationic liposome was further studied to verify the assumption described in the previous section. The liposome and mRNA were mixed at the same ratios used for mRNA translation reaction in *E.coli* cell-free translation system and then, the prepared complex was incubated at 30°C for 6 hours in the same condition of mRNA translation in RTS system. The mixtures of mRNA/liposome were then analyzed by electrophoresis on agarose gel 1% (**Fig.3-10**). During electrophoresis, the

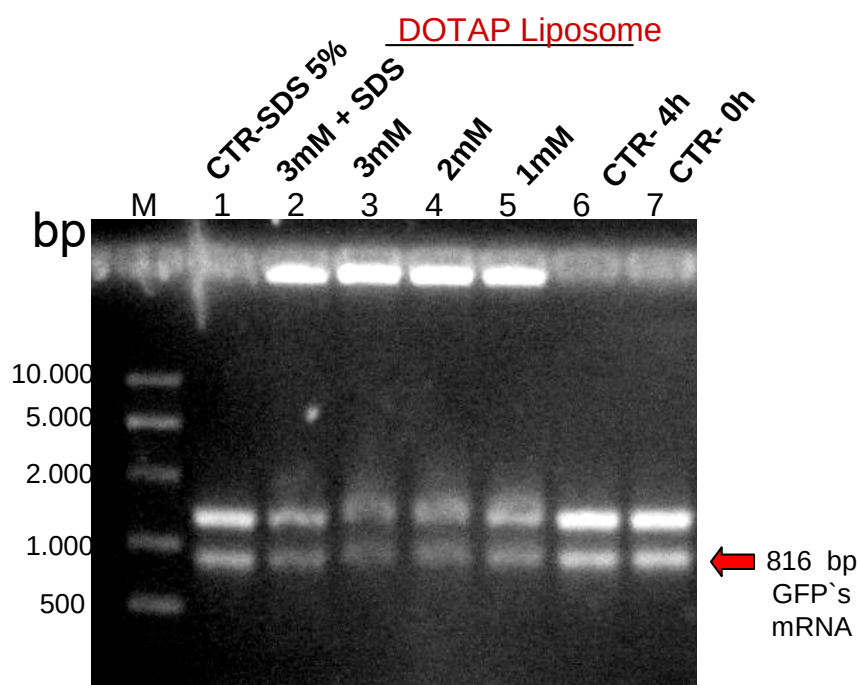


Fig.3-10 Electrophoresis on agarose gel of mRNA and complex liposome/mRNA with the same ratios and volumes used in RTS reaction (H₂O was replaced for RTS solution), after inoculation at 30°C for 6h from lane 1 to lane 6. Lane 1: mRNA + SDS 5%, lane 2: complex of mRNA + DOTAP (3mM) + SDS 5%; lane 3, 4, 5: mRNA + DOTAP of 3mM, 2mM, 1mM; respectively; lane 6: mRNA; lane 7: mRNA at 0h, lane M: maker (10.000, 5000, 2000, 1000, 500 bp).

neutralized mRNA molecules were still stable and located inside the wells of the sample application and could not move on agarose gel (**Fig.3-10**, lane 2, 3, 4, 5) in comparison with

control sample (**Fig.3-10**, lane 6, 7). This result confirmed that the negatively charged phosphate groups on surface of mRNA have been neutralized by cationic quaternary ammonium groups of DOTAP liposome, neutralized mRNA can not move from Anot to Catot during electrophoresis on agarose gel, beside of this, the structure of the mRNA could also be changed and its function of translation was “silenced” by DOTAP liposome.

The liposome was disrupted by treatment with SDS 5% before analysis of this liposome/mRNA complex by electrophoresis on agarose gel in order to confirm whether mRNA entrapped inside liposome or just absorbed on its surface in complex liposome/mRNA (lane 1, **Fig.3-10**). The result indicated that, after breaking-down the liposome in complex of mRNA/liposome, mRNA is still present in nearly same content with that of no degradation of liposome (**Fig.3-10**, lane 1 and 2). However, the mRNA was not also significantly affected by 5% SDS in comparison with control sample (**Fig.3-10**, lane 1 and 6). This result further clarified that mRNA was not entrapped inside liposome and they only absorbed on the surface of liposome in complex of mRNA/liposome. The cationic liposome, DOTAP, can strongly attract with negative charged mRNA by electrostatic interaction between anionic phosphate groups of mRNA and the cationic of quaternary ammonium groups of polar heads of DOTAP liposome, and then neutralize the mRNA surface to induce the conformational change of the secondary structure of mRNA, resulting in silencing mRNA translation (liposome interfere mRNA translation).

7. Stabilization of mRNA in complex with various kinds of liposome

The effect of possible degradation mRNA by cationic DOTAP liposome was further investigated focusing on the stabilization of mRNA in comparison with neutral, domain, and negative charged liposome. The results were indicated in **Fig.3-11**.

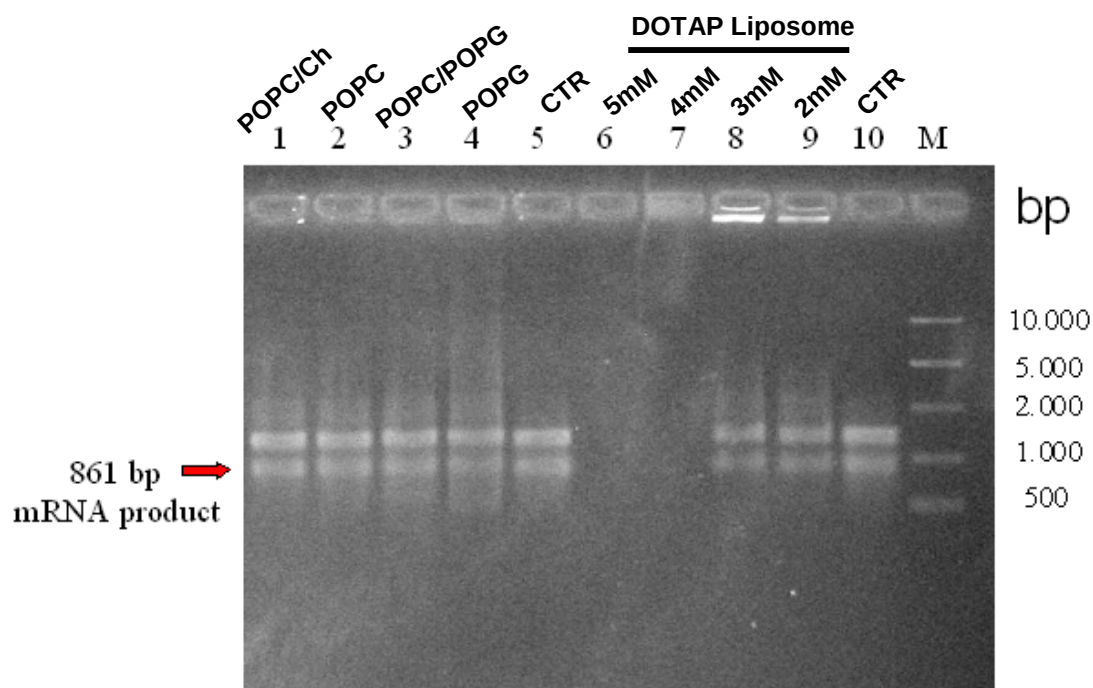


Fig.3-11 Complex of liposome with mRNA with the same ratios and volumes used in RTS reaction (H_2O was replaced for RTS solution) after 6h of inoculation at $30^{\circ}C$. Lane 1, 2, 3, 4, mRNA complex with liposome including POPC.Ch, POPC, POPC.POPG, and POPG, respectively. Lane 5: control sample (1). Lane 6, 7, 8, 9: mRNA ($2.6 \mu g$) with DOTAP liposome at 5, 4, 3, 2, 1 mM, respectively. Lane 10: control sample (2).

The cationic 4mM DOTAP liposome can degraded mRNA at the equal ratios of $0.0516 \mu M$ DOTAP lipid and $8.88 \times 10^{-6} \mu M$ mRNA, the charge ratios are 6.74 and 1, respectively. However, the addition of the other types of liposomes with the same concentration, including POPC/Ch, POPC, POPC/POPG, and POPG, did not degrade mRNA. DOTAP liposome at 3mM with mentioned ratios used in RTS reaction did not degraded

mRNA. In this case, mRNA is still existed although it cannot be translated into protein because of its silent effect by DOTAP liposome.

Cationic liposome DOTAP can therefore interfere mRNA and silence its translation. The ratios of positive and negative charges between DOTAP liposome and DNA vector that was caculated based on the ratios of liposome/DNA of 0.0358 μ M DOTAP and 8.88 $\times 10^{-6}$ μ M mRNA, equal to (+) 2.33 $\times 10^{16}$ (C) and (-) 0.46058 $\times 10^{16}$ (C), respectively. So charge ratios of positive DOTAP liposome and mRNA were 5 and 1, respectively. We supposed that mRNA is often presented at supercoine style with agregate constructure, so that mRNA can absorb on surface of liposome, but only a part of negative charge mRNA surface can be easily neutralized by liposome, some other parts of mRNA surface can be stored in supercoine style with agregate constructure, so they are difficult to be neutralized by liposome. So that, translation of mRNA can be inhibited by DOTAP liposome when the number of cationic charge of liposome is 5 times higher than negative charge of mRNA, in theory. However, that ratio is very important to get the “knocking-down effect” of mRNA by DOTAP liposome to inhibit gene expression at the translation level, “silencing” mRNA translation (mRNAi by cationic DOTAP liposome). It is seminal with the case of DNA (**Fig.3-5**), this results also indicated that mRNA can be existed stably when it make a complex with neutral liposome POPC, microdomain-forming liposome POPC/Ch, partialy negatively-charged liposome POPC/POPG (7:3). However, it can be almost degraded when complexed with cationic

liposome DOTAP at the ratios of charged density of liposome/mRNA is 5/1.

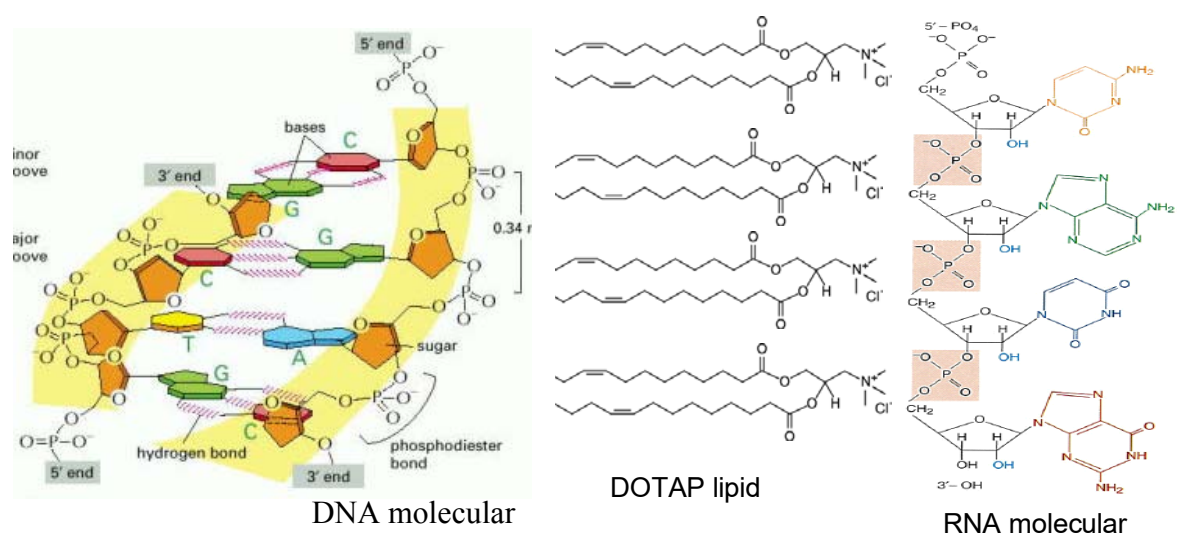


Fig.3-12 Specific interactions formed between the cationic trimethylammonium side chains of DOTAP liposome and anionic DNA and mRNA.

The chemical structure of RNA in **Fig.3-12** shows that, RNA structure contains the phosphodiester chemical linkage between nucleotides in RNA contains the sugar ribose that has an additional -OH group. RNA also contains the base uracil, which differs from thymine in DNA, by the absence of a -CH₃ group (Albert *et al*; 2002). Phosphate groups in RNA and DNA have negative charge, so that the RNA easily interacts with positively charged components. The present results clarified that the cationic DOTAP liposome could interact with negatively charged mRNA by electrostatic attraction between the positively charged quaternary ammonium groups of DOTAP and the negatively charged mRNA phosphate groups and tends to fully neutralize mRNA (Esposito *et al*; 1997). As shown in **Fig.3-12**, the charged complementarity of the nanoparticles and mRNA would bind with a high affinity

(McIntosh *et al*; 2001) as many protein involved in gene regulation dedicate the majority of DNA-bound amino acid side chains to neutralize the phosphate backbone (Darby *et al*; 1993). It is possible that the change of the secondary structure of mRNA to increase the compact state of mRNA on the surface of cationic membrane (Brown *et al*; 2001), so that, after neutralization, mRNA could not be translated in to protein in *E.coli* cell-free translation system. This is the reason for the mRNA interference (mRNAi) induced by liposome membrane itself.

The roles of lipid membranes on mRNA translation were also summarized from previous studies and our study, indicated in **Table 3-1**. A possible role of the cationic liposome during the gene expression was discussed in the following. DNA is known to consist of double helix structure of 2 strands linked each other by hydrogen bond. On the contrary, the RNA normally consists of a single strand, of which the nucleotides are linked together covalently by phosphodiester bonds through the 3'-hydroxyl (-OH) group of one sugar and the 5'-phosphate (P) of the next (Alberts *et al*; 2002). According to Barreau C *et al*, (2006), in the presence of AUUUA (instability element), a poly(A) tail in mRNA of *Eukaryote* cell was instable and, without liposome, mRNA was degraded after 4 hours of expression. However, in the presence of liposome (DMRIE-C lipofectant), mRNA product is very durable after 4 hours of transcription (both of with Cap and no Cap), showing that the liposome enhanced the stability of reporter mRNAs introduced into HeLa cells. However, it is also dependent on the presence

or absence of an AUUUA instability element, a poly (A) tail (**Table 3-1**).

Table 3-1 Summary of role of liposome on mRNA translation and its mechanism

references	materials	mechanism	result	key role
Fire, Z.A; Nobel Prize. ⁵	siRNA	specific base-pairing to target mRNA	knock-out target mRNA	interfere mRNA translation
Zhang et al. ⁶	R8-EggPC/Ch/PEG -PE/DOTAP for delivery siRNA	specific base-pairing to target mRNA	knock-out target mRNA, inhibit 70% cancer cell growth	
Our research	DOTAP	neutralize anionic phosphat groups of mRNA	knock-down, inhibit 100% mRNA translation of GFP	
Tachibana et al. ²⁷	DOPE/cationic- lipid/Ch	inhibit gene expression	inhibit 100% GFP expression	not showed
Barreau et al. ¹⁰	CDMRIE-C liposofectant	stability polyA tail, AUUUA element	enhance mRNA stability	support mRNA translation
Zohra et al. ¹⁷	DOTAP-apatite	stability of polyA tail, right cap orientation	enhance 100 times RACA-mRNA translation	

According to Kushner *et al*, (2002), mRNA can be only translated when it has secondary structure, and lifetime is not durable. The conformation of mRNA could play a key role in its stability for mRNA translation. According to Zohra *et al*, (2007), the translation inefficiency of template mRNA has reported to be caused by its quick degradation and short half life of the expressed protein as the prime reasons for this low expression. However, in the presence of DOTAP-apatite, the stability of polyA tail and support right cap orientation on mRNA, led to the enhanced stability and higher expression kinetics and to a notable enhancement of 100 times translation of anti-reverse capped mRNA (RACA-mRNA) inside HeLa cell (**Table 3-1**).

Fire *et al*, (2006) revealed a new mechanism for gene regulation based on RNA interference and Zhang *et al*, (2006) have described a novel approach for siRNA cellular delivery using siRNA encapsulated into liposome additionally bearing arginine octamer (R8) molecules attached to their outer surface (R8-liposomes). The R8-liposomal human double minute gene 2 (HDM2)-siRNA demonstrated a significant stability against the mRNA degradation in the blood serum, and higher transfection efficiency into lung tumor cell lines. The mechanism of action of R8-liposome-encapsulated siRNA is associated with the RNAi-mediated degradation of the target mRNA. siRNA in R8-liposomes effectively inhibited the targeted cancer gene HDM2 and significantly reduced of 70% the proliferation of SKMES cancer cells, (Zhang *et al*; 2006).

Based on the results described in this chapter, new original points were indicated, where mRNAs are still existed in the presence of cationic liposome but they could not be translated. The cationic liposome therefore interfered and silenced the mRNA translation (knock-down of mRNA). The most important mechanism was also indicated that the negatively-charged phosphate groups on surface of mRNA have been neutralized by cationic quaternary ammonium groups of DOTAP liposome by electrostatic attraction. At that time, the secondary structure of mRNA was changed, finally, mRNA was shown to still exist and be stable although it could not work and translate into protein in *E.coli* cell free translation system. The cationic quaternary ammonium groups in DOTAP liposome is necessary for

binding moiety interacting electrostatically with the anionic phosphate groups on mRNA, condensing the large anionic molecule of the phosphate groups into small unit's lipoplexes. This result implies that the cationic liposome electrostatically attract mRNA and interfere mRNA, resulting in silencing its translation owing to the conformational change of the mRNA on liposome.

Summary

The role of the foreign membrane and stressed membrane on the gene expression were investigated to establish the strategy of the gene regulation based on the membrane described in chapter I and II.

Cationic liposome DOTAP was found to inhibit the gene expression of GFP in *E.coli* cell-free translation system. The role of liposome on the inhibition of gene expression was studied, resulting that the DOTAP liposome can inhibit the gene expression at both of transcription and translation levels. The mechanism was also investigated, resulting that the DOTAP liposome can knock out the gene for inhibition of its transcription through the degradation of the DNA molecule. It is considered that strong electrostatic interaction of cationic quaternary ammonium groups of DOTAP liposome with negatively charged phosphate groups on surface of DNA. The strong electrostatic interaction could negatively affect on hydrogen bond linkage of 2 strands of DNA, and phosphodiester bonds in a single strand of DNA, (**Fig.3-4** and **Fig.3-5**). Furthermore, hydrogen bond interaction between DNA and DOTAP liposome can be easily formed. DNA was, therefore, degraded in the presence of DOTAP liposome.

DOTAP liposome can also inhibit the mRNA translation; it can also knock down and interfere mRNA translation (mRNAi by liposome). Cationic DOTAP liposome can neutralize negative charged mRNA by electrostatic interaction between cationic quaternary ammonium

groups in liposome with negative charged phosphate groups of mRNA resulted in interference mRNA and silencing its translation (**Fig.3-10** and **Fig.3-11**). In the presence of DOTAP liposome, mRNA could still exist although it could not be translated into protein. The above results implies that the cationic quaternary ammonium groups of DOTAP liposome can strongly interact with negative charged phosphate groups of mRNA, and neutralized mRNA, DOTAP liposome is cited as a prospective drug for inhibition of gene transcription and translation.

It was thus found that the gene expression could also be silenced by using the foreign membrane, DOTAP, through the “knocking-out of gene” and “knocking-down of mRNA” mechanism.

Chapter IV

Gene Expression Regulated by Membrane and Stress

~ Gene Expression *in vitro* Regulated by Stressed Liposome and Deeper Understanding of *in vivo* Gene Silence ~

Introduction

Stress exposurement of the bacterial cells has reported to induce a variety of potential functions because of the stress-response dynamics of the cell itself. Such a stress response function of the biological cells has generally been known to be regulated under the control of the stress factors (Tsuchido *et al.*, 1994, Murofushi *et al.*, 1998). However, it has been gradually known that not only the gene and proteins do not always govern the gene expression but also, possibly, by the lipid membrane itself *in vivo* research. A possible role of the membrane itself has recently been reported such as (i) molecular chaperone-like function to refold the partly-denatured protein (Kuboi *et al.*, 1997, Yoshimoto and Kuboi, 1999), (ii) heat induced translocation of proteins (Umakoshi *et al.*, 1998), (iii) heat-induced fusion of the liposome membranes (Felix *et al.*, 2003). According to Tuan *et al.*, (2008), under oxidative stress condition, SOD was oxidized and reduced its activity although, however, in the presence of liposome, its activity can be reactivated ((iv) LIPOzyme functions). A possible role of the stressed membrane on the gene expression has also been reported by Marc D *et al.*, (1997), where the lipid peroxidation produces a variety of compounds with profound biological activity that mediate signal transduction leading to effectively activate *PR-1* gene expression in Tobacco cell. According to Ngo *et al.*; (2005, 2008) under stress condition of

heat and oxidative stress, chitosanase enzyme produced from *S. griceus* was also enhanced in the presence of liposome through the stress-mediated interaction of liposome membrane and cell membrane. The role of the membrane under stresses condition could play an important role in the gene regulation process *in vitro* and *in vivo*. It is expected that the gene expression also be regulated in the presence of liposome exposed under the stress condition.

In the previous chapters, the role of various liposomes, such as natural-based liposomes (neutral, microdomain-forming and negatively charged liposomes) and artificial-based foreign liposomes (cationic liposomes) *in vitro* gene expression was investigated, focusing on the role of biomembrane in the elementary steps of the gene expression process. In this chapter, the strategy of the “membrane” based regulation of the gene expression has been established by summarizing the spectra of the liposome-affected gene expression of a reporter protein, GFP. The stress effect is not avoidable when one considers the gene regulation in the actual biosystem because the system is always exposed to the variable stress environment for its survival. The combination of the stress exposurement with the above membrane effect was discussed for the efficient regulation of the gene expression. The possible regulation of the gene expression has been studied by selecting GFP expressed in *E.coli* cell-free translation system and the silent expression of *cry* gene *in vivo* in natural isolated *B.thuringiensis* strains as case studies.

Materials and Methods

1. Materials

1,2-Dioleoyl-3-trimethyl-ammonium-propane (DOTAP) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Rapid Translation system RTS 100 *E.coli* HY Kit, an *in vitro* protein synthesis system based on *E.coli* lysate, was purchased from Roche Diagnostics (Indianapolis, IN, USA). The purified GFP was purchased from Roche Diagnostics (Indianapolis, IN, USA). Other chemicals were purchased from Wako Pure Chemical (Osaka, Japan).

2. Liposome preparation

Cationic lipids of 1,2-dioleoyl-3-trimethyl- ammonium-propane (DOTAP) was solubilized in chloroform; a thin layer was then prepared in a round-bottom flask by evaporation at 37°C using a vacuum rotary evaporator and dried under nitrogen flow for at least 5 hours. In order to generate multilamellar vesicles, the dried phospholipids thin layer was hydrated with water (pH 7). The freeze–thaw cycle was repeated 5 times for large unilamellar vesicle formation. This vesicle solution was extruded through a polycarbonate membrane with a pore size of 100 nm to form unilamellar vesicles (liposome).

3. *In vitro* expression of GFP with and without liposome

A Rapid Translation system (RTS 100 *E.coli* HY Kit), *in vitro* protein synthesis

system based on *E.coli* lysate, was used as a cell free translation system. The expression vector, pIVEX, contained the gene for expression of GFP. The reaction solution (50 μ l) contained the following components: 12 μ l *E.coli* lysate, 10 μ l reaction mix, 12 μ l amino acids, 1 μ l methionine, 5 μ l reconstitution buffer, and 13 μ g of purified mRNA. The liposome solution prepared at higher concentration was added in the reaction mixture in the replace of the above water before and after the “Expression” operation and the effect of liposome on the gene expression was evaluated based on the following methods. The hydrogen peroxide was added in the gene expression system at the final concentration of 1-10mM in order to investigate the role of the oxidative stress on the GFP expression.

4. Analysis of gene product

The amount of synthesized and folded GFP product was evaluated by analysis of the GFP fluorescence intensity at 395 nm (excitation) and 509 nm (emission) by using the fluorescence spectrophotometer (JASCO, FP-777, Tokyo, Japan) with slit width of 5nm at excitation and emission of light path. All the GFP fluorescence spectra were analyzed after 100 times dilution of the reaction samples to water in order to avoid the possible interference of the liposome included in the final reaction sample. It was confirmed that the fluorescence spectra of pure GFP was not affected in the above experimental condition including liposome at the finally-diluted solution and also POPC liposome itself at its final concentration has no emission spectra in the above condition for fluorescence measurement.

5. Isolation of *Bacillus thuringiensis* (*B.thuringiensis*) and identification their existence of specific cry gene

B. thuringiensis was first isolated from the environment in Vietnam. 56 leaf samples, 55 soil samples and 26 paddy humus samples were collected from many provinces in Northern Vietnam for isolation of *B.thuringiensis*. The isolated *B.thuringiensis* cells were characterized in relation to its toxicity on various bacterial species as shown below. Toxicity of *B.thuringiensis* against *P. xylostella*, *S. exiqua*, *S. litura* were assayed by using fresh cabbage leaf discs treated with parasporal crystal toxins of *B.thuringiensis* at the concentration of $3\text{-}5 \times 10^7$ spores of *B.thuringiensis*/ml. Similarly in the case of the above toxicity test, *H. armigera* were assayed by using cotton leaves. *C. cephalonica* was assayed by mixing spore-protein crystal toxins powder with rice, the application dose was 10^9 spore of *B.thuringiensis* per kg rice. Flagellar (H) antigen serotypes of the isolates were identified by the slide agglutination test. Flagellated bacterial suspensions were prepared by culturing bacteria in L-shaped tubes containing 4 ml nutrient broth at 37°C for 3 to 4 hours with gentle shaking. The slide agglutination test (Ohba and Aizawa., 1978) was performed by mixing one drop of flagellated bacterial suspension on a glass slide with one drop of 20 to 50-fold dilution of H antiserum. Specific H agglutination was determined 1-3 minutes after mixing.

6. Polymerase Chain Reaction (PCR)

Oligonucleotide primer pairs of TY6 (5'-GGTCGTGGCTATATCCTTCGTGT

CACAGC-3') and TY14 (5'-GAATTGCTTTCATAGGCTCCGTC-3'); TYIC (5'-CAACCTCTATTTGGTGCAGGTTC-3') and TYIUNI (5'-ATCACTGAGTC GCTTCGCATGTTTGA CTTTCTC-3'), were respectively used for the amplification of the specific DNA fragments of *cry1Ab*, *cry1C* genes by PCR. The PCR components of 25µl of total volume included: 13,6µl of H₂O, 2,5µl of dNTP, 2,5µl of buffer, 2µl of primer, 0,4µl of Tag-polymerase enzyme, 2µl of DNA template. The PCR conditions were shown as follows: denaturation at 94⁰C for 2 min, followed by 30 amplification cycles (94⁰C, 1min; 52⁰C, 2min; 72⁰C, 1,5 min) and extension at 72⁰C for 5 min. The content of 5µl of PCR products were analyzed by electrophoresis on 1% agarose gel.

7. Cloning and sequencing specific fragments of *cry1Ab* and *cry1C* gene

The PCR products of DNA fragments from isolates of *B.thuringiensis*. var *kurstaki* H15, *B.thuringiensis*. var *aizawai* H1 were ligated in to PCRTM 2.1 vector and transferred in to *E. coli* DH5α. The transformed *E. coli* DH5α were screened by colony hybridization. These recombinant plasmids were isolated and purified from transformed *E. coli* DH5α. It was then examined carefully by cutting of restrict enzyme *EcoRI*. Sequences of DNA fragments were determined by the dideoxy chain termination method with ³²PdATP on Autosequencer ALF-Express, they were compared with reference genes from Gene bank data base and were translated in to protein fragments by PC/Gene program.

8. Statistical analysis

Results are expressed as mean $\pm$ standard deviation (SD). All experiments were performed at least in triplicate. The data distribution was analyzed, and statistical differences were evaluated using the Student's t-test. A P value of $< 0.05\%$ was considered significant.

Overview of strategy on stressed membrane-based regulation of gene expression

In chapters I, II, and III, it has been shown that the biomembranes could also affect the gene expression system by employing both the liposome as model biomembrane and the cell free translation system as model gene expression systems. The spectra of the biomembrane roles in the gene expression process have been summarized in Graphical **Table 4-1**. The effect of the typical liposomes which consist of the lipid components observed in the biomembrane, such as neutral, microdomain-displaying, and negatively-charged liposomes were described in Chapter I and II and they were found to positively affect in the transcription step. There are some differences: the translation and folding of the GFP were further enhanced in microdomain-forming liposome and the folding of GFP was inhibitory affected by the negatively charged liposome. In this chapter III, the unusual membranes, such as cationic liposome (DOTAP), was selected as the target membrane to regulate the gene expression. The DOTAP liposome is herewith known to be utilized as the vector of DNA and mRNA in the DDS and in the RNAi technologies.

Table 4-1 Spectra of membrane role on Cell-free translation system

	Neutral POPC, PMPC, DPPC	Microdomain POPC/Ch etc	Negative Charge POPC/POPG	Cationic Liposome		
				POPC/SA	DOTAP	DOTAP/ Oxidative stress
DNA						
Transcription	+	++	+	+	--	+
mRNA						
Translation	+	++	0	--	--	+
Polypeptide						
Folding	+	+	--	+/-	+/-	+/-
Protein						

The addition of DOTAP liposome was found to “knock out” (degrade) the DNA for inhibition of gene expression at transcription level and “knock down” (silence) the mRNA for inhibition of gene expression at the translation level. The gene expression was found to be regulated by using the above foreign membrane.

The total views of the membrane role on the gene expression tell us that the gene expression could not always be explained by single reason. For example, the GFP expression was not observed by the fluorescence in DOTAP liposome and POPC/POPG liposome. There is a difference in the type of the liposome role; the former affected the transcription and translation and the latter affected the folding. This comparison shows that one needs “membranome” approach in addition to the conventional “genome” and “proteome” approaches when one discusses the gene expression. For example, the DOTAP liposome itself, often used in DDS and RNAi, has gene-regulating functions such as “knocking down” and

“knocking out” of the gene. If one could encounter the unexpected suppression of the DNA and mRNA in the host cell, it is reasonable to consider the possible effect of the liposome membrane used as a vector. A gene silence in the bacterial cells could also be considerable as another example of the membrane effect. In the case of *B.thuringiensis*, it has been shown that, among the several strains, there are some strains not to induce the insecticidal activity. There is no explanation from the viewpoint of genome. Inside a bacterial cell, the DNA is known to exist neighboring to the inner side of the cytoplasmic membrane. It can be possible that the difference in the membrane surface could affect the “active” and “inactive” state of the target gene inside a cell (this subject will be discussed in the following section).

In a bacterial cell, its stress-response behaviors could also be key factors to maintain the cellular homeostasis and for the cells to survive in the given stress environment. There could be some examples of the induction of the stress-response functions of the bacterial cell. It has been reported that the heat stress exposurement to the *Escherichia coli* cells could induce the potential functions of themselves, such as (i) enhancement of production of heat shock proteins (Umakoshi *et al.*, 1996) and (ii) translocation of the cytoplasmic β -galactosidase across the cytoplasmic membrane (Umakoshi *et al.*, 1998). The production of the β -galactosidase has also been enhanced by the addition of the membrane components, fatty acids, under the heat stress condition. It has recently been shown that the heat treatment of the *Streptomyces griseus* cells in the presence of POPC liposome could enhance the

production and release of the chitosanase (Ngo *et al.*, 2005) through the interaction between the liposome membrane and cytoplasmic membrane under the heat stress condition (Ngo *et al.*, 2008). It has also been shown that the oxidative stress, by the addition of hydrogen peroxide, in the presence of the POPC liposome could also enhance the release of the chitosanase and the combination of the oxidative/heat stress with the liposome could enhance both of the production and release of the chitosanase from the *S. griceus* cells (Ngo *et al.*, 2009). All the examples shows that the above potential functions could be induced by the stress-response dynamics of the membrane. The use of such stress-response dynamics of the membrane could enhance the membrane-related phenomena in the biological cell.

The control of the gene expression could be performed by the membrane under the stress condition by considering the molecular mechanism of the elementary steps of the gene expression by employing the case studies, such as (i) discussion on the model production process of GFP in cell-free translation system based on the spectra of the membrane-related gene regulation, (ii) membranome-based understanding of the silence effect of the *cry* gene in *B.thuringiensis* cells, and (iii) oxidative stress-induced regulation of gene silencing and activation by using the DOTAP liposome in *E.coli* cell-free translation system.

Results and Discussion

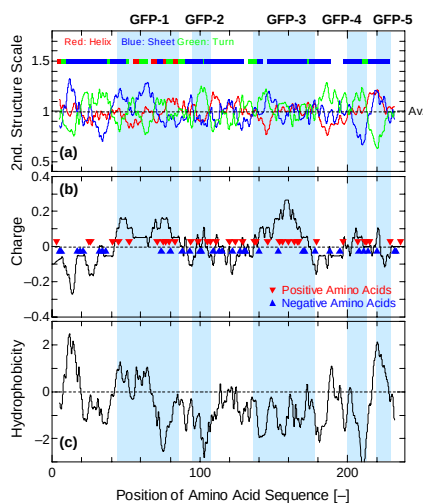
1. Comparison of basic characteristics of various proteins

An extension of the membrane-based gene expression has been first investigated. Possible effects of protein types on the gene expression have been discussed through the analysis of the basic characteristics based on their amino acid sequences by employing some target proteins, such as GFP, maltose-binding protein (MBP), glutathione S-transferase (GST), Chitosanase, Cry1C and Cry1Ab proteins. The basic characteristics of the above proteins were shown in **Table 4-2**. The pI value can be regarded as the electrostatic nature of the protein. Secretion protein, chitosanase, has a pI at relatively alkali pH, showing that it has a positive charge to be bound to the negatively-charged cytoplasmic membrane. On the other hand, cry1a, cry1c, and MBP show the pI at acidic pH region. The GFP and GST has a pI at pH around 6, showing the slightly negative charge. The hydrophobicity was calculated from the averaged value of hydrophobicity of the amino acids. Among the proteins shown here, the hydrophobicity of GFP and Cry1C was higher than that of other proteins. The local characteristics of the proteins can be shown in **Fig.4-1**. As an example, the positively charged domain of each protein has been shown as a “blue” rectangles. As described in chapter II, the folding of protein as a post-translational process is well related to the charged density buried in the hydrophobic region. Such charged domains can be seen especially in MBP and Cry1C protein at higher probability, showing that they could induce the misfolding after their

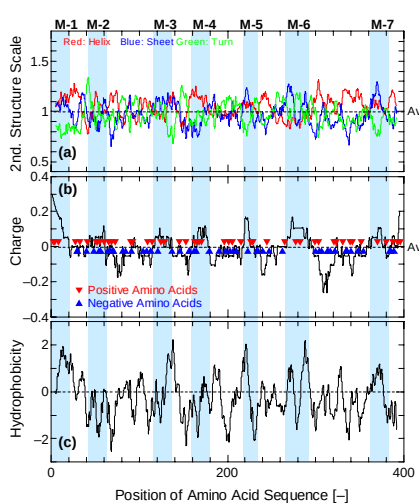
Table 4-2 Comparison of Basic Characteristics of Various Proteins

	GFP	MBP	GST	Chitosanase		Cry1C	Cry1Ab
				Signal Peptide	Main		
Amino Acid Residue [-]	236	396	232	53	306	646	1145
Molecular Weight [kDa]	27.0	43.4	27.0	5.3	33.5	73.2	130.6
pI [-]	5.8	5.5	6.1	13.0	7.89	4.78	5.02
Hydrophobicity [-]	0.99	-0.24	-0.39	0.11	-0.35	1.01	-0.42

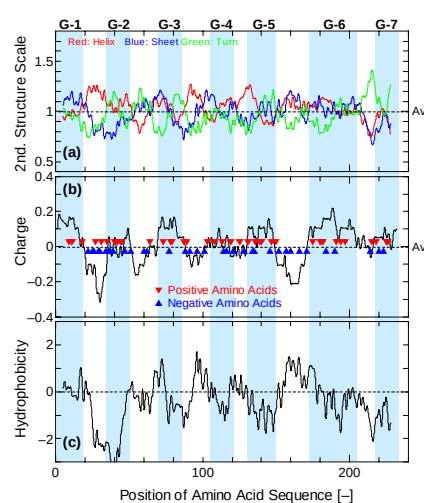
(i) GFP



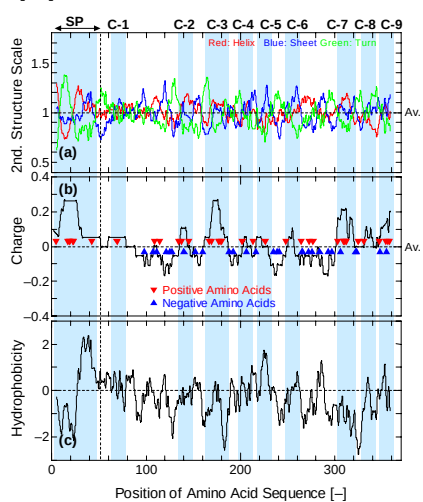
(ii) MBP



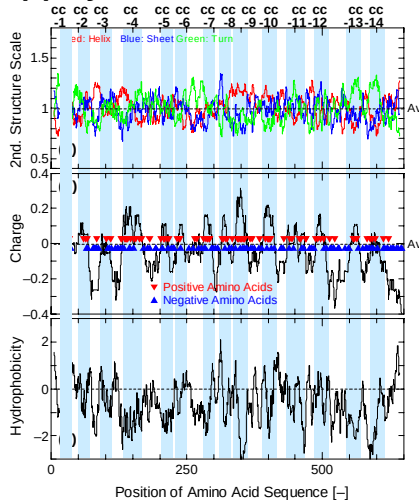
(iii) GST



(iv) Chitosanase (with Signal Peptide)



(v) cry 1c



(vi) cry 1a

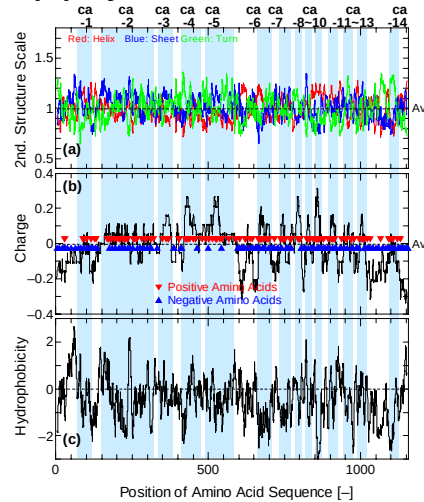


Fig.4-1 Analytical plot of characteristics of various proteins

translation step. The effect of the microdomain-forming membrane could be obtained in the proteins with higher (local) hydrophobicity as described in chapter I, implying that the GFP, MBP and Cry1C proteins could be possible to be enhanced at least until the translation level. In relation to the gene silence described in chapter III, the complex formation of the polynucleotides (DNA and mRNA) could be a key factor, implying that the size of the gene product could be related with it. Although further investigation is needed, it is expected that the large size protein has a possibility to be affected by the cationic membrane because of the stability of the mRNA.

As an example, the effects of the addition of the POPC/Ch liposomes on the gene expression of other types of proteins, such as maltose binding protein (MBP) and glutathione S-transferase (GST), was also investigated at the SDS-PAGE analysis (Data not showed). The GST expression was slightly enhanced observed in the presence of POPC/Ch liposome although the MBP expression was 10% increases similarly in the case of GST was 4% increase, Table 4-3. This also supports the effect of POPC/Ch liposome on the enhanced gene expression. Our results indicated that the POPC/Ch liposome could not only increase the folding of GFP with an immature conformation in their post-translation (GFP fluorescence intensity), but could also enhance their transcription and translation from gene to polypeptide ((i) and (ii) by approximately 58% in the *in vitro* system (**Fig.1-3**). As shown in **Table 4-2** and **Fig.4-1**, the basic characteristics of MBP and GST were also summarized.

Table 4-3 Densitometer analysis of SDS-PAGE image of expressed MBP and GST in the presence of with and without POPC/Ch liposome

POPC/Ch	Contents of proteins	Relative contents
	MBP	MBP
(-)	12.19% $\pm$ 0.81%	100%
(+)	13.35% $\pm$ 0.026	110%
	GST	GST
(-)	6.84 $\pm$ 0.30%	100%
(+)	7.11 $\pm$ 0.74%	104%

Note: (-): Control, (+): with 4mM POPC/Ch liposome.

The GFP has a relatively hydrophobic surface as compared with MBP and GST (**Table 4-2**). Although the positively charged domains can be seen in the several domains (blue colored regions), the positively charged domains are not overlapped in the hydrophobic domains (except for the M1 and M6 in MBP and G-1 in GST) (**Fig.4-1**).

As a second example, the possibility that the inhibition of the gene expression by the cationic DOTAP liposome was investigated in the case of GFP gene in *E.coli* cell-free translation system. It was also examined whether this inhibitory effect could be observed with other genes products, including maltose-binding protein (MBP) and glutathione S-transferase (GST) genes. Two vectors harboring MBP and GST genes were applied for the expression in *E.coli* cell-free system under the incubation for 6 hours at 30°C with and without DOTAP liposome. The effect of DOTAP liposome on inhibition of gene expression of MBP and GST was showed in **Fig.4-2**.

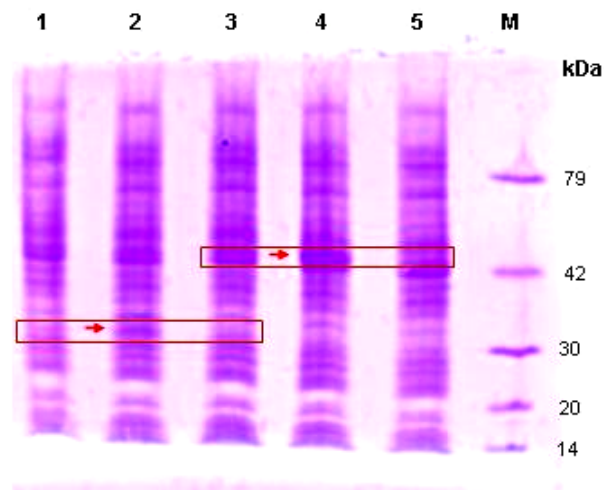


Fig.4-2 Effect of DOTAP liposome on gene expression of some kind proteins in an *E.coli* cell-free translation system. SDS-PAGE image of MBP and GST: lane 1: GST expression in the presence of DOTAP liposome, Lane 2: GST expression in control sample without liposome, lane 3: negative control sample: gene expression without DNA vector, lane 4, 5: MBP expression with and without DOTAP liposome; lane M: Marker with molecular weight of 79, 42, 30, 20, 14 kDa.

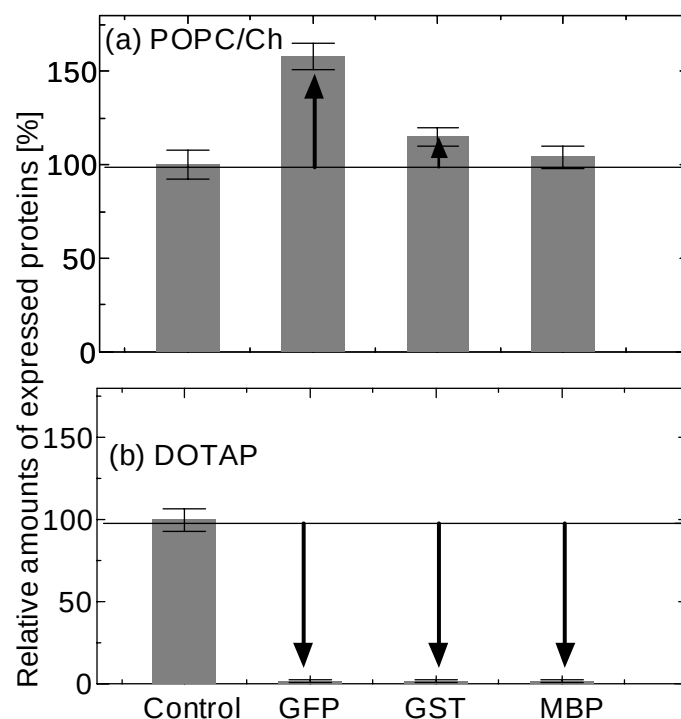


Fig.4-3 Summary the effect of POPC/Ch and DOTAP liposomes on gene expression of some proteins

The result in SDS-PAGE analysis of total protein product in **Fig.4-2** shows that, in

the presence of DOTAP liposome, the expressions of both MBP and GST were also inhibited in comparison with control sample without liposome and negative control sample without DNA. It was thus found that the cationic liposome DOTAP at 4mM inhibited 100% of gene expression of either GFP or MBP and GST in *E.coli* cell-free translation system.

The effect of (a) microdomain liposome membrane POPC/Ch and (b) DOTAP liposomes on the expression of GFP, MBP and GST was summarized in **Fig.4-3**, indicating that the gene expression of the proteins could be regulated by the liposome surface. Although further investigations are needed, a possible extension of the membrane-based regulation of the other types of genes have thus been shown as examples.

2. Enhanced production of GFP gene expression considering the role of membrane

A possible regulation of the GFP expression was discussed considering the role of the membrane surface. There are two possible regulations, such as (i) enhancement of gene expression (ii) inhibitory of gene expression by “knocking down gene” or “knocking out” mRNA).

In the former case, the use of the microdomain-like liposome (i.e. POPC/Ch) is effective for the production of the GFP expression because the liposome could enhance all the elementary steps of the gene expression (**Fig.1-5**). There are two kinds of approaches to increase the enhanced effect of the microdomain liposome: (a) the increase of the recognition functions of liposome itself through micro-domain of cholesterol on liposome; (b) the

employment of the sequential expression of the target gene; (c) the stabilization of essential components for gene expression such as DNA, mRNA, transcriptional protein, rRNA, tRNA... by liposome for enhance gene expression.

It has been reported that the biomacromolecules could be recognized by the liposome, depending on the physicochemical properties and its dynamic nature, in a series of studies using aqueous two-phase system, immobilized liposome chromatography, and membrane chip (sensor) system (Kuboi *et al.*, 2001, Kuboi and Umakoshi, 2006, Shimanouchi, 2005). The recent studies show that the biomolecules with less stable hydrogen bonds could be stabilized on the surface of the liposome (Fernandez *et al.*, 2003, Lee 2004, Yoshimoto, 2005, Shimanouchi 2006). According to their findings, the microdomain on the liposome surface could be related with the hydrogen bond stabilizing effect of the membrane itself against the HB-unstable proteins, suggesting that the biomolecules relating to the gene expression could be recognized by the above liposomes. It has been reported that the POPC/SM/Ch liposome has maximal in its effect (Shimanouchi 2006). The use of the liposome could enhance the GFP expression in the *E.coli* cell-free translation system.

A sequential expression of GFP gene can also be effective for the promotion of the gene expression process. Through this thesis, it was found that the (i) transcription of DNA, (ii) translation of mRNA and (iii) folding of the polypeptide could be affected by the liposome, depending on the difference of the interaction between the elementary step

machinery and membrane surface (**Fig.1-6**). It is naturally accepted that the product of the elementary step reaction could interact with liposome membrane if that reaction occur on the membrane surface, the product could be stocked there. When one could perform the elementary step on the designed liposome, the efficiency of the reaction could furthermore be enhanced.

The use of the DOTAP liposome could be effective for the inhibitory expression of (“knock-down” or “knock-out”) of the gene. In chapter III, the DOTAP liposome was found to induce the degradation of the DNA (**Fig.3-4, Fig.3-5**) and also the silencing of the mRNA (**Fig.3-10, Fig.3-11**) at the critical concentration. The present results imply that the DOTAP liposome can be used for the down-regulation of the gene expression. If the DOTAP liposome could be introduced into the gene expression system, the DOTAP liposome could induce the DNA degradation, resulting in the “knocking-out” of the gene. At the same time, the mRNA could be silenced by the formation of the complex with the DOTAP liposome. All the reactions would be stopped by the above condition. The activation of the silenced mRNA could be needed by the signal inside a cell (i.e. oxidative stress, heat stress, and so on.). The possible activation of the DOTAP silencing gene will be described in the final section of this chapter. There is a problem in the gene degradation and mRNA silencing using DOTAP liposome. The DOTAP liposome has been shown to inhibit the expression of several genes such as GFP, MBP and GST (**Fig.4-2**). That results open a prospective effect of DOTAP

liposome that can be applied as a new drug modified with specific ligand for example of ESA (*Eucheuma serra* agglutinin) for specific interaction and binding with its target cancer cell (Sugahara *et al*; 2002, Fukuda *et al*; 2006), and then DOTAP liposome can inhibit the growth of the target cell by knock-out of gene and/or knock-down of mRNA inside the cell.

3. Membrane-based understanding of silence of *cry* gene in *B.thuringiensis* strains

According to the discussion described in the previous sections shown by **Table 4-1**, **Table 4-2**, and **Fig.4-1**, the Cry proteins have some possibilities to be affected by the membrane; *cry1Ab* at transcriptional or translational steps in introducing DOTAP liposome and *cry1c* gene at the post translational steps in natural membrane system. Based on such a consideration, the gene silence effect of such *cry* genes has been discussed.

Natural gene expression inside the cell was investigated in *B.thuringiensis* which can produce crystal toxin protein expressed from *cry* gene. The identifications of *cry1Ab* and *cry1C* gene that can express crystal toxic protein to insects from eleven isolated *B.thuringiensis* strains in Vietnam were indicated that, 10 *B.thuringiensis* var. *kurstaki* isolates harbored *cry1Ab* gene and the isolate of *B.thuringiensis* var. *aizawai* H1 harbored *cry1C* gene. All of them have insecticidal activities except a strain in spite of harboring *cry1Ab* gene but it has no insecticidal activity.

All of them were used for bioinsecticide production technology that was used by farmers for treatment of vegetables. However, some problems are still remained and

necessary to solve including: (1) toxic protein products are still low; (2) until 48h of fermentation, when starvation of nutrition in the medium was occurred, toxic crystal proteins expressed from cry gene start to be released; (3) only from 70%-80% toxic proteins can be released from the cell; (4) Pre-toxic crystal protein products are still low and easily to be broken under high pH, protease, in fermentation environment, or UV light of environment (5) their insecticidal activity are still lower than that from chemical insecticide. Klier *et al*, (1982) have successfully cloned the *cryI* gene and expressed its gene in *E.coli* cell for expression of higher production of Cry protein. It is therefore investigated whether liposome membrane under with and without stress condition could control gene expression in *B.thuringiensis* or *E.coli* host cell or not. If yes, the prospective role and mechanism of liposome effect on gene expression should be identified and discussed.

It is expected that whether liposome under stress condition can: (1) improve gene expression of cry gene, (2) enhance release of expressed toxic protein in *B.thuringiensis* during continuous fermentation with nutrition addition, (3) enhance stabilization of pre-toxic crystal protein. All the possibility was suggested to be possibly related with the biomembrane or stressed biomembrane during the experimental operation.

(1) Isolation and identification of *B. thuringiensis* strains in Vietnam

The following samples were collected for the isolation of cry gene-constitutive *B.thuringiensis* strains; 56 leaf samples, 55 soil samples and 26 paddy humus samples from

many provinces in Northern Vietnam. Total of 36 *B.thuringiensis* were isolated and characterized. The microscopic observation showed that 11 isolates having bipyramidal shape crystals, 10 isolates having square shape crystals, 6 isolates of flat rectangular and square crystals, 2 isolates of spherical shape crystals and other having small inclusions.

The insecticidal activities of the spore-crystal mixture of isolates were tested against *Lepidopteran* insects including: *Corrcyra cephalonica*, *Plutella xylostella*, *Spodoptera exiqua*, *Spodoptera litura* and *Helicoverpa armigera*.

Table 4-4 Bioassay of insecticidal activities of *B.thuringiensis* isolates having bipyramidal shape crystals against some *lepidopteran* insects

<i>B.thuringiensis</i> strains	Gene (PCR product)		Insecticidal activities [%]			
	<i>cry1Ab</i>	<i>cry1C</i>	<i>P. xylostella</i>	<i>S. exiqua</i>	<i>S. litura</i>	<i>C. cephalonica</i>
H1	No	strong	90-100 %	90-100 %	80-90 %	100 %
47S	Weak	No	90-100 %	90-100 %	80-90 %	100 %
50S	Weak	No	90-100 %	90-100 %	80-90 %	100 %
H22	Strong	No	90-100 %	90-100 %	80-90 %	100 %
H15	Strong	No	90-100 %	90-100 %	80-90 %	100 %
29S	Weak	No	90-100 %	90-100 %	80-90 %	100 %
T58	Weak	No	90-100 %	90-100 %	80-90 %	100 %
H8	Strong	No	90-100 %	90-100 %	80-90 %	100 %
28S	Strong	No	50%	60%	50%	100%
H3	Weak	No	90-100 %	90-100 %	80-90 %	100 %
H13	Strong	No	90-100 %	90-100 %	80-90 %	100 %

The results indicated that there were eleven isolates having bipyramidal shape crystals with highly toxic to *Lepidopteran* insects, the results was showed in **Table 4-4**. All of eleven isolates having bipyramidal shape crystals gave 100% mortality to *C. cephalonica*, 10/11 of isolates gave 90-100% mortality to *P.xylostella* and *S. exiqua*, 80-90% mortality to *S.*

litura. There was one isolate having lower insecticidal activities, it gave 50% mortality to *P. xylostella* and *S. litura*, 60% mortality to *S. exiqua*. All of the above eleven isolates having lower insecticidal activities to *H. armigera*, there was one isolate gave 70% mortality; 2, 5, 2 and 1 isolates gave 60%, 50%, 40% and 30% mortality, respectively. The serological determination of 11 mentioned isolates having bipyramidal shape crystals was carried out, indicating that 10 isolates belong to the serovar *kurstaki* (H 3abc) and one belongs to the serovar *aizawai* (H7). SDS-PAGE of parasporal inclusions of eleven above isolates was carried out, ten *B.thuringiensis*. var *kurstaki* isolates showed two core fragments of 130 kDa and 70 kDa and several other minor proteins; isolate of *B.thuringiensis*. var *aizawai* H1 consisted four mainly protein core fragments of 130 kDa, 81kDa, 70 kDa and 55kDa.

(2) Identification of the existence of *cry1Ab* and *cry1C* gene from *B.thuringiensis* isolates

The identifications of *cry1Ab* and *cry1C* genes from eleven above *B.thuringiensis* isolates were based on PCR. Oligonucleotit primer pairs of TY6 and TY14, TYIC and TYIUNI were used to isolate two specific DNA fragments of *cry1Ab* and *cry1C* genes, respectively. PCR products were separated by agarose gel electrophoresis in **Fig.4-4, Fig.4-5**.

The result in **Fig. 4-5** showed that *B.thuringiensis*. var *kurstaki* A1 and ten isolates of *B.thuringiensis*. var *kurstaki* produced only one PCR product of about 236bp, *B.thuringiensis* var *aizawai* M1 and isolate of *B.thuringiensis* var *aizawai* H1 had no PCR products. Ten isolates of *B.thuringiensis* var. *kurstaki* and *B.thuringiensis*. var *kurstaki* A1 harbored *cry1Ab*

gene, *B.thuringiensis*. var *aizawai* M1 and isolate of *B.thuringiensis*. var *aizawai* H1 did not harbor *cry1Ab* gene.



Fig.4-5 Agarose gel (1%) electrophoresis analysis of PCR products obtained by using primers TY6 and TY14 for cloning *cry1Ab* gene. Lane 1, 2: *B.thuringiensis*. var *aizawai* M1 (standard strain) and isolate of *B.thuringiensis*. var *aizawai* H1, respectively; Lane 3: *B.thuringiensis*. var *kurstaki* A1 (standard strain), Lane M: Marker, Lane 4-13: isolates of *B.thuringiensis*. var *kurstaki* including: 47S, 50S, H22, H15, 29S, T58, 28S, H8, H3, H13.

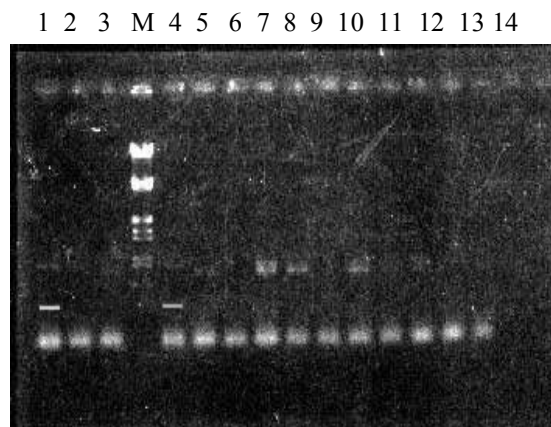


Fig.4-6 Agarose gel (1%) electrophoresis analysis of PCR products obtained by using primers TYIC and TYIUNI for cloning specific fragment of *cry1C* gene. Lane 1: *B.thuringiensis*. var *aizawai* M1 (standard strain), Lane 2,3: *B.thuringiensis*. var *kurstaki* A1, T1; lane M: Marker, Lane 4: isolate of *B.thuringiensis*. var *aizawai* H1, Lane 5-14: isolates of *B.thuringiensis*. var *kurstaki* including: 47S, 50S, H22, H15, 29S, T58, H8, H3, 28S, H13.

The result in **Fig.4-6** shows that *B.thuringiensis*. var *aizawai* M1 and isolate of *B.thuringiensis*. var *aizawai* H1 produced PCR products of about 285bp, *B.thuringiensis*. var *kurstaki* A1 and ten isolates of *B.thuringiensis*. var *kurstaki* had no PCR products. This result indicated that *B.thuringiensis*. var *aizawai* M1 and isolate of *B.thuringiensis*. var *aizawai* H1 harbored *cry1C* gene, *B.thuringiensis*. var *kurstaki* A1 and ten isolates of *B.thuringiensis*. var *kurstaki* did not harbor *cry1C* gene.

(3) Cloning and sequencing specific fragments of *cry1Ab* and *cry1C* gene from obtained strains isolated in Vietnam

In order to exactly identify the *B.thuringiensis* isolates containing *cry1Ab* as well as *cry1C* genes, PCR products of *B.thuringiensis* isolates were cloned and sequenced, they were compared with reference specific DNA fragments of *cry 1Ab* and *cry 1C* genes that were published in gene database.

The PCR products of DNA fragments from isolates of *B.thuringiensis. var kurstaki* H15 and *B.thuringiensis. var aizawai* H1 were ligated in to PCRTM 2.1 vector and transferred in to *E. coli* DH5 α . The transformed *E. coli* DH5 α were screened by colony hybridization for recovery a large content of plasmids containing PCR products. The results of sequencing analysis showed that cloned DNA fragments from *B.thuringiensis. var kurstaki* H15 and *B.thuringiensis. var aizawai* H1 had 236bp and 285bp in length respectively. They had a high level of similarity to specific DNA fragments of *cry1Ab* and *cry1C* genes respectively, the cloned DNA fragment from *B.thuringiensis. var kurstaki* H15 was homology 98, 73% in comparison with specific DNA fragment of *cry1Ab* gene published by Geiser *et al*, the cloned DNA fragment from *B.thuringiensis. var aizawai* H1 was homology 99,3% in comparison with specific DNA fragment of *cry1C* gene published by Sanchis V *et al*, in International Gene Bank Database. This results one again affirmed that 10 isolates of *B.thuringiensis. var kurstaki* harbored *cry1Ab* gene but no *cry1C* gene, isolate of *B.thuringiensis. var aizawai* harbored *cry1C* gene but no *cry1Ab* gene. Specific DNA fragments of *cry1Ab* and *cry1C*

genes from isolates in Vietnam could be translated into protein fragments of 78 and 95 amino acids, respectively by PC/gene program.

(4) Summary of the *B.thuringiensis* isolation and gene screening and their problems

B.thuringiensis isolates in Vietnam have insecticidal activities to *lepidopteran* insects such as *C. cephalonica*, *P. xylostella*, *S. exiqua*, *S. litura* they have lower insecticidal activities to *H. armigera*. They were used for bioinsecticide production technology. Isolate of *B.thuringiensis*. var *kurstaki* 28S in spite of harboring *cry1Ab* gene but it has no insecticidal activities to *P. xylostella* and *S. litura*.

However, some problems are still remained, such as (1) low productivity, (2) long term operation (Cry protein is not produced until from 48h to 72h of fermentation, when starvation of nutrition in the medium (stress condition) was occurred, (3) less secretion (only 70%-80%), (4) less stability of pre-toxic crystal protein under high pH or with protease , and (5) less insecticidal activity in contrast to chemical insecticide. In addition to the above-mentioned problems on Cry protein production process, the (6) existence of “Silent” gene in different strains with same gene sequences could also be an important problem in the isolation steps. It has previously reported that the stress could enhance the intracellular proteins, such as heat shock proteins, together with the variation of the characteristics of the biomembrane although the HSP is known to be initiated by the heat stress signaling of sigma factor (Umakoshi *et al*; 1998). It has recently reported that the production of the secretion

protein, chitosanase, was enhanced under the heat stress condition in the presence of the liposome (Ngo *et al.*, 2005, 2008). The variation of the characteristics of the biomembrane under the stress condition, generated during the isolation and process operation, could be related to the above-mentioned problems that is one unexpected. Furthermore, as described in chapter I, neutral liposome POPC and domain liposome POPC/Cholesterol can enhance the gene expression in cell-free translation system of 145% and 167%, respectively. The liposome can therefore give a prospective application for enhance gene expression inside the cell. Based on these evidences shown above, it is supposed that, inside the cell, all the above problems remained in natural gene expression inside the cell (for example of *cry* gene expression in *B. thuringiensis*), such as: (1) low productivity, (2) less secretion of target toxin proteins, (3) less stability of pre-toxic crystal protein, and (4) less activity of target toxic protein. All these problems could be successfully solved by investigating the stress response of the bacterial cells focusing on a possible role of the biomembrane, modeled as liposome. It is expected that liposome with or without stress can be prospectively applied to improve both of quality and quantity of target protein products expressed from gene in natural cells such as *cry* gene expression in natural *B.thuringiensis* cells, or target gene expression in recombinant hot *E.coli* cells.

4. Role of stressed membrane on GFP expression: effect of cationic DOTAP liposome under stress condition on GFP expression

Oxidative stress, known as the effect of reactive oxygen species (ROS) on the biomolecules, has been widely studied. H_2O_2 has also been found to play important roles as second messenger in the initiation and amplification of signaling at the antigen receptor (Reth *et al.*, 2002). A several excellent reviews about the functions of H_2O_2 as secondary messenger have already appeared (Reth *et al.*, 2002, Bae *et al.*, 1997, Mahadev *et al.*, 2001, Rhee *et al.*, 2000, Gamaley *et al.*, 1999, Finkel *et al.*, 2001, Droge *et al.*, 2002). It was also reported that the production rate of $\cdot\text{O}_2^-$, H_2O_2 were linearly related to the number of the active respiration chains that reached to the maximal values during exponential growth and significantly decreased at the stationary phase (Flecha *et al.*, 1995).

For example, it has been reported that under redox condition, heat shock protein (Hsp33), a redox-regulated molecular chaperone, which is activated by disulfide bond formation and subsequent dimerization, works as efficient chaperone that binds to unfolded protein intermediates and maintains them in folding competent conformation. Reduction of Hsp33 is necessary but not sufficient for substrate protein release.

The substrate dissociation from Hsp33 is linked to the presence of the DnaK/DnaJ/GrpE foldase system, and then supports the refolding of the substrate protein. Upon the substrate release, the reduced Hsp33 dimers dissociate into inactive monomers. This regulated substrate transfer ultimately links substrate release and Hsp33 inactivation to the

presence of available DnaK/DnaJ/GrpE, and, therefore, to the return of cells to non-stress condition (Hoffmann *et al.*, 2004).

The combination of the above oxidative stress and the liposome membrane could induce the unexpected functions, possibly through the stress-response dynamics of the membrane itself, in the previous investigation *in vitro* and *in vivo*. Recently, it has been reported that, although the addition of the hydrogen peroxide could inactivate and fragment the superoxide dismutase (SOD), the liposome could recruit the oxidized and fragmented SOD peptide to induce the original SOD-like activity (Tuan *et al.*, 2008). In the *in vivo* investigation, the addition of the liposome into the cultivation media for *Streptomyces griseus* cells has shown to induce the enhanced effect of the chitosanase production and its release from the cell, (Ngo *et al.*, 2005, 2008).

It is expected that, similarly in the case of the previous findings on the oxidative stress condition, the silencing effect of the foreign liposome membrane, cationic DOTAP liposome, could be regulated. The effect of oxidative stress condition on the cationic liposome-induced GFP expression was investigated in this section for the purpose of the possible regulation of the DOTAP-silenced expression system. The hydrogen peroxide was selected as a stressor because it is a metabolite and abundant inside a cell. As described in the previous section, two kinds of the process have been selected as target reactions, such as (a) total expression process of GFP (initiated from DNA) and (b) translation and folding steps

(initiated from mRNA), in order to investigate the role of the stressed liposome on the elementary steps of the gene expression process. **Fig. 4-6** and **Fig.4-7**, respectively, show that the GFP expression from DNA and mRNA were inhibited in the presence of DOTAP liposome and were re-expressed under oxidative stress condition . As also shown in the previous sections, the inhibitory effect of the DOTAP liposome was observed, where the GFP expressed from DNA and mRNA disappeared at the DOTAP concentration of 4mM and 3mM, respectively. However, in the presence of H₂O₂ (4.6mM) addition at 0h of GFP expression from DNA and mRNA in *E.coli* cell-free translation system, after 6 hours of expression, GFP expression can be re-expressed 50% and 30%, respectively, in comparison with that of control sample without H₂O₂.

In parallel experiment, the GFP expression from DNA and mRNA was performed in the presence of 4.6 mM H₂O₂ and without DOTAP liposome, indicating that the GFP expression was enhanced to 6% and 8 %, respectively, that are not significant in comparison with control sample of GFP expression without H₂O₂ and liposome (data not shown). It was thus found that the oxidative stress induced by hydrogen peroxide could prevent the “silent effect” of mRNA expression and “inhibitory effect” of gene expression of the DOTAP liposome.

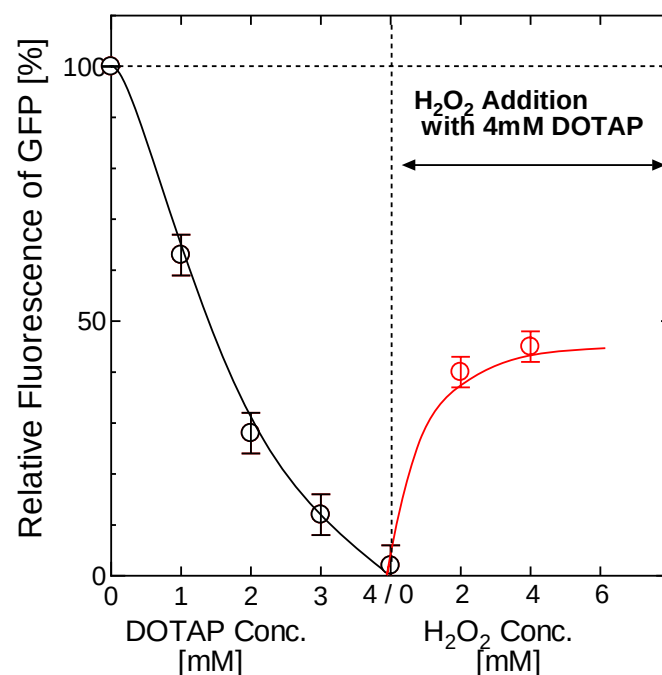


Fig.4-6 Effect of DOTAP liposome on GFP expression from DNA with H₂O₂.

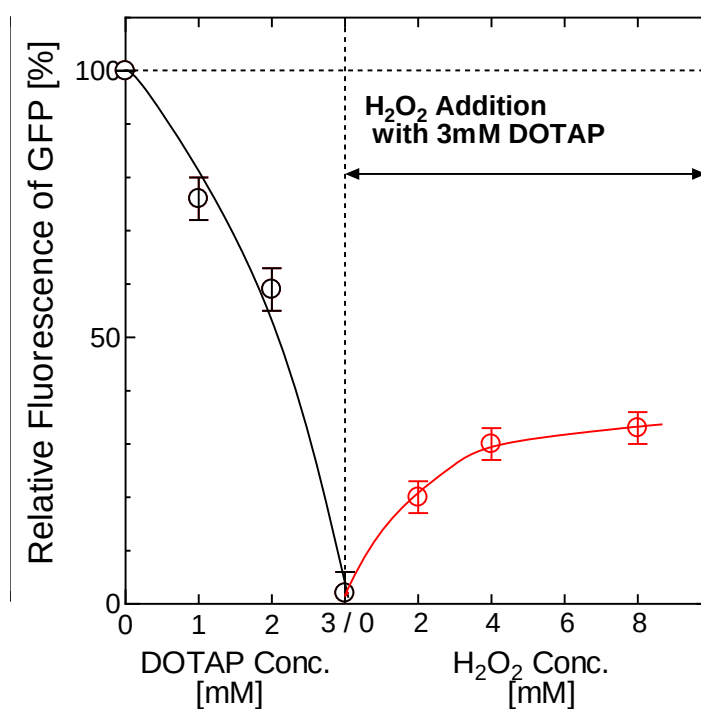


Fig.4-7 Effect of DOTAP liposome on GFP expression from mRNA with H₂O₂.

According to Tuan *et al*, (2008), oxidized POPC liposome strongly interacts with the oxidized SOD under oxidative stress condition and then reactivate its activity. Further more, according to Marc D *et al*, (1997), the lipid peroxidation produces a variety of compounds with profound biological activity that mediate the signal transduction leading to effectively activate *PR-1* gene expression in Tobacco cell. The oxidized DOTAP liposome reduced its inhibition activity of GFP expression from both DNA and mRNA. In another word, the effect of the DOTAP liposome was suppressed under oxidative stress although it normally inhibited the gene expression by “knock-out” of DNA gene or “knock-down” of mRNA. Such effect can also be observed in both cases of the “DNA”-initiated and “mRNA”-initiated expression of the GFP gene. This comparison of both cases tells us that the oxidative stress effect could be related to the “Translation step” and “Post-translation step” but not to the “Transcription step”. There are two possibilities of the less-silence effect of the oxidative stress, such as the (i) chemical conversions (oxidization) of the DOTAP surface, (ii) the strong interaction of mRNA into the oxidized or release the interaction (translation step), and (iii) its effect on the GFP itself (post-translational step).

In the presence of H₂O₂, DOTAP liposome was easily oxidized because it has two alkyl chains with unsaturated C-C bond. It is possible that the hydroxyl group could form inside a hydrophobic environment of the DOTAP liposome. In relation to the possibility (iii), it could not be possible because the oxidative stress negatively affect the gene expression of

GFP without DOTAP, resulting in the opposite effect by the oxidative stress. It has also been reported that the GFP has relatively stable structure, with β -barrel conformation, and it can be tolerant to the hydrogen peroxide at relatively higher concentration level (10 mM) without UV irradiation (Alnuami *et al.*, 2008). The possibility (ii) is the most probable among the above subjects although its could also be related with the oxidation of the DOTAP (i). It has been reported that the stability of the hydrogen bond of the main chain of the poly peptide could be a key factor to regulate its stability, where the hydrogen bonds can be stabilized in the hydrophobic and non-polar environment (Fernandez *et al.*, 2003). It has also been reported that the oxidized liposome could recruit the peptide with lower stability of the hydrogen bonds through the immobilized liposome chromatography and liposome dielectric dispersion analysis (Yoshimoto, 2004, Shimanouchi 2005). According to Tuan *et al.*, (2008), under oxidative stress condition, SOD was oxidized and reduced its activity, however, in the presence of liposome, its activity can be reactivated.

Based on their finding, the hydroxyl group formed inside a liposome membrane is thought to be unstable because it has no opponent group of the hydrogen bond in the possibly hydrophobic environment. After the mRNA was adsorbed on the DOTAP surface through the electrostatic interaction, it is possible that the hydrogen bond of the side residues of the polynucleotide could be stabilized in the membrane itself, probably give a switch of the translational machinery on. However, it should also be considered that the release of the

mRNA interacted on the DOTAP liposome through the increased fluidity of the membrane surface induced by the oxidation. The oxidized DOTAP liposome (oxidized DOTAP fragments) has cationic charge surface lower than that of control sample and less interaction with DNA or mRNA. The oxidative stress therefore reduces the ability of liposome on of knock-out of gene and knock-down of mRNA.

Although further investigation is needed for these phenomena, it was thus shown that the oxidative stress could act as a “switch” or “activation” of the “silencing” mRNA translation and “inhibitory” gene expression at least by DOTAP liposome.

Summary

A total view of the membrane-affected gene regulation has been shown as a graphical table. The significance of the concept of the membrane-based regulation of gene, also utilizing its stress-response dynamics, was finally shown by employing some case studies *in vitro* and *in vivo*. Different liposome membranes can affect on the gene expression at different levels of positive or negative effect that show the significance of the membranome concept in addition to the conventional genome and proteome approaches.

The above findings have been applied to (1) controlled expression (such as enhance or reduce gene expression) of not only GFP but also other proteins of GST and MBP in cell-free translation system. These results can open original points for possible applications of

liposome in regulation of gene expression in natural cell (for example *B.thuringiensis* cell) or recombinant host cells (for example *E.coli* cell). (2) The silent or inhibitory effect of GFP expression, which were induced by membrane, can be solved by using oxidative stress condition, implying that the “silent” *cry* gene in *B.thuringiensis* strains can be related to the above phenomena. Therefore, smart gene regulation (silencing and activation) of GFP expression using DOTAP/oxidative stress could open a possible application to improve and switch on silent gene expression inside the cell. The established strategy could further be utilized for the gene regulation *in vivo* by monitoring the stressed state of the biomembrane and for the development of the “cell-like” reactor based on the liposome-immobilizing matrix.

(3) It is expected that liposome with stress can open a prospective application to solve some remained above problems that occurred during gene expression from natural bacteria cells or recombinant host cell such as : (i) enhance productivity, (ii) enhance release protein product from the cell and (iii) increase activity of protein product, (iv) activate gene expression.

General Conclusion

The possible effect of various kinds of liposome under stress condition on gene expression and their mechanism were carried out. *E.coli* cell-free translation system represented for *E.coli* host cell choosing for gene expression of GFP as a reporter protein expression with and without liposome under stress condition.

In **Chapter I**, the effect of various kinds of liposomes on the gene expression was investigated, focusing on its role at elementary steps such as “transcription”, “translation” and “folding”. Gene expression process was analyzed by the GFP fluorescence (finally-matured product as an indicator of “total process”). It is concluded that the neutral and micro-domain liposome enhance the gene expression of GFP showed by fluorescence intensity and SDS-PAGE analysis. The mechanism also indicated that liposome was showed to enhance the gene transcription and translation, resulting in the promotion of gene expression. It is possible that the liposome containing microdomain of cholesterol on liposome surface could play very important role in stabilization of gene-expressing components such as DNA, or T7RNA polymerase, ribosome, when they interact with liposome, resulting in the enhancement of transcription.

The liposome was shown to enhance the (i) transcription and (ii) translation to promote the gene expression, through the analysis of the fluorescence intensity and

SDS-PAGE analysis of GFP and the analysis of mRNA. However, the anionic charged liposome and cationic liposome inhibited the gene expression of GFP. A possible model for the “liposome membrane” affected gene expression has been discussed based on the surface characteristics of the possible biomacromolecules (such as DNA, RNAs, T7 RNA polymerase, ribosome, GFP) and the surface characteristics of the liposome. It implies that the liposome effect on the gene expression could be induced by the interaction of the molecules and the liposome. A possible roles of the negatively-charged liposome based naturally-abundant lipid and the positively-charged liposome based on foreign lipids will be further investigated in chapter II and chapter III.

In **Chapter II**, the effect of negatively-charged liposome, as a typical biomembrane, on *in vitro* synthesis of a reporter protein GFP, was investigated by using the cell-free translation system. GFP was expressed with and without negatively-charged liposome prepared by the 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG), the effect of the addition of the negative charged POPC/POPG liposome on the gene expression of GFP was carried out. The gene expression of mature GFP product was identified by detection of fluorescence intensity after 18 hours of expression. In the presence of liposome at 4 mM, the GFP fluorescence (synthesized and folded amounts GFP) was reduced up to 60% in comparison with control sample. However, liposome only inhibits folding of synthesized polypeptide GFP (mature

protein - active protein) in post-translation but it did not inhibit gene translation of total synthesized protein product. The interaction between liposome and mature expressed GFP was also investigated by ultrafiltration analysis. The result indicated that the liposome also interact strongly with mature synthesized GFP that bind with liposome and could not pass through membrane filter, and still account up to 72% of total GFP product before ultra filtration, that is 51% higher than that from control sample without liposome of 21%.

The effect of liposome on the refolding of standard GFP denatured by guanidine in refolding buffer was also carried out. The result showed that the liposome inhibited 55% refolding of native GFP denatured by guanidine in comparison with native GFP before denaturation. A changing of outer and inner membrane fluidity of liposome in the presence of GFP was also investigated. The result indicated that the POPC/POPG liposome strongly interact with native standard GFP, resulting in the reduction of 10% outer membrane fluidity and 24% inner membrane fluidity. The above findings on the negatively-charged liposome POPC/POPG during the GFP expression imply the future study on “silence” of gene product, such as toxic enzyme expressed in infective toxic bacteria. Negative charged liposome is potential for application in inhibition of gene at post-translation level, folding.

In **Chapter III**, the role of cationic liposome, as a foreign membrane, on the gene expression of GFP was also investigated. The result indicated that the cationic liposome DOTAP can inhibit gene expression of both of GFP in *E.coli* cell-free translation system. The

role of liposome on inhibition of gene expression was clarified that DOTAP liposome can inhibit gene expression at both of transcription and translation levels. The mechanism was also clarified and indicated that DOTAP liposome can knock out gene for inhibition of gene transcription in cell free translation system for GFP. This result implied that cationic DOTAP liposome can neutralize negative charged DNA. DOTAP liposome could strongly interact with DNA by electrostatic attraction between cationic quaternary ammonium groups in liposome with negative charged phosphate groups of DNA and negatively affect on hydrogen bond linkage of two strands of DNA. The ratios of positive and negative charges between DOTAP liposome and DNA vector that were calculated as (+) $3.108 \times 10^{16}(\text{C})$ and (-) $0.01821 \times 10^{16}(\text{C})$, respectively, equal to the ratio of 170 and 1, respectively. That ratio is very important to knock out gene and inhibit gene transcription following by gene expression in *E.coli* cell-free translation system.

Furthermore, DOTAP liposome can also inhibit the mRNA translation, depending on the ratios of positive and negative charges between DOTAP liposome and mRNA that was calculated based on the ratios of liposome and DNA were (+) $2.33 \times 10^6 (\text{C})$ and (-) $0.46058 \times 10^{16}(\text{C})$, equal to the ratio of 5 and 1, respectively. At this ratio, DOTAP liposome can neutralize mRNA, resulting in silencing mRNA translation. At that time, mRNA can still exist but it can not be translated. DOTAP liposome is cited as a prospective drug for the inhibition of gene transcription and translation. It can also knock down and interfere mRNA

translation (mRNAi by liposome). Negative charged mRNA was neutralized by DOTAP. In the presence of DOTAP liposome, mRNA could still exist although it could not be translated into protein. The results implied that the cationic quaternary ammonium groups of DOTAP liposome can strongly interact with negatively-charged phosphate groups of mRNA, and neutralized mRNA, resulted in interference of mRNA and silencing its translation.

Cationic liposome can open a prospective drug in future. DOTAP liposome can be used not only in enhancing gene delivery by using complex cationic/neutral/domain liposome, but also in possible application for a new drug modified with specific ligands for treatment and interference of target cell and target DNA, mRNA inside the cell.

In **Chapter IV**, the role of the liposome membrane to promote and inhibit the gene expression systems has been summarized to establish the strategy to regulate the gene expression based on the “Membranome”. First, the spectra of the gene regulation by the membrane were summarized with respect to the elementary steps included in the gene expression system, such as (i) transcription, (ii) translation, and (iii) folding. A possible regulation of the gene expression has been achieved by employing some case studies, such as (1) the comparison of basic characteristics of proteins for possible regulation of gene expression, (2) proposal of the GFP production based on the liposome membrane, (3) understanding of the gene silence in *B.thuringiensis* cells, and (4) stress-regulation of GFP gene in the cell-free translation system.

First, the basic characteristics of the proteins, such as GFP, MBP, GST, chitosanase, and cry proteins, were analyzed and compared with each other in order to apply for the “membrane-based” regulation of their gene described in chapter’s I-III and in the beginning of the chapter IV. The basic strategy was applied for the (i) enhanced production of MBP by POPC/Ch liposome and (ii) gene silence of MBP and GST by the cationic DOTAP liposome in cell-free translation system. A possible extension of the present strategy was thus shown by using the above case studies. A proposal for the enhanced production of GFP in the cell-free translational system has also been as an example of the extension.

The gene silence *in vivo* has also been clarified based on the present model on the membrane-affected regulation of gene. Natural gene expression in bacteria cell was carried out by using *B.thuringiensis* isolates for encoding Cry protein. These Bt strains were characterized for application in Bioinsecticide production technology. However, some problems were still remained including: (1) low activity protein product, (2) long fermentation for producing target protein product, low release of protein product from inside to outside the cell, (3) low stabilization of released protein product, (4) low activity of protein product, (5) gene silencing was happened in Bacterial cell. Those problems are very necessary to resolve and contribute to enhance target protein production technology. From our above successful results in Chapter (I, II, III), prospective model application can be achieved. It is expected that the liposome under stress condition can: (1) improve gene expression, (2)

enhance release of expressed protein in *B.thuringiensis* during continuous fermentation with nutrition addition, (3) enhance stabilization of pre-toxic crystal protein. In order to investigate the possible effect of various kinds of liposome under stress condition on enhance gene expression and clarify their mechanism. *E.coli* cell-free translation system was therefore chosen for gene expression of GFP as a reporter protein in the presence of with and without liposome under stress condition. Prospective model for application of liposome with/without stress condition can be indicated by following: the effect of oxidative stress condition can activate the gene expression that could be inhibited by liposome. Gene expression can be inhibited under the presence of cationic liposome (DOTAP). However, in the presence of H₂O₂ with DOTAP liposome, gene expression can be re-expressed about 50% from DNA and 30% from mRNA that could be normally inhibited 100% by cationic liposome DOTAP.

The obtained result plays an important role of possible application case of stress condition for activate gene expression inside the cell, for example of activation of *cry* gene in *B.thuringiensis* or target gene in *E.coli* host cell. In the presence of micro domain liposome POPC/Ch indicated in enhance gene expression in *E.coli* cell-free translation system (Chapter I), that result is prospective for application to enhance gene expression (for example *cry* gene) in Bacteria of *B.thuringiensis* or target gene in *E.coli* host cell. Positive charged and negative charged liposome: POPC/POPG and DOTAP liposome can be prospectively applied for inhibit target gene expression inside the cell, such as (1) toxic gene expressed in toxic bacteria

to human and animals (*E.coli*, *Salmonella*), (2) toxic gene in molds producing mycotoxin (*Aspergillus flavus*, *Aspergillus paraciticus* that can strongly produce aflatoxin, ochratoxin), (3) disease gene in human, such as oncogene in cancer cell in tumor.

Suggestion for Future Works

1. Application of liposome for enhances gene expression and recovery synthesized protein product in Bacteria cells

It is concluded that neutral and domain liposome enhances gene expression of GFP. That liposome was showed on enhancement of gene transcription resulted in promotion of gene expression. This result indicated that, neutral and domain liposome play very important role in enhancing gene expression inside *E.coli* cell as well as other Bacteria cell. Mixing complex of neutral and domain liposome and cationic liposome at suitable ratios can be applied for enhancing gene delivery and expression not only in Cell-free translation system but also inside the cell, for example of *E.coli* cell that is useful host Bacteria cell for expression of target gene. Cationic liposome can enhance attraction between complex of liposome/DNA and anionic cell membrane for enhance gene delivery from out side to inside the cell. Neutral liposome can support for stabilization of DNA, mRNA, RNA polymerase, transcriptional protein,... for enhancing gene transcription and expression. In the presence of neutral and domain liposome inside the cell, liposome will affect on: (1) enhance gene; (2) under stress condition, liposome can be used for controlling gene expression in Bacteria cell or *E.coli* host cell; (3) interaction between liposome membrane and Bacteria cell membrane under stress condition will enhance release of synthesized protein product and stabilization of its enzyme activity. Liposome with stress can therefore open a prospective application to

solve some remained above problems that occurred during gene expression from natural bacteria cells or recombinant host cell such as : (i) enhance productivity, (ii) enhance release protein product from the cell and (iii) increase activity of protein product.

2. Anionic liposome can be applied for gene inhibition at post-translation level

The folding of the synthesized polypeptide in post-translational process seems favorable in contrast to the above upstream steps. It has been reported that the apoptosis (cell suicide) can be triggered by production of improperly folded or incomplete proteins (Chow *et al*; 1995, Hussein *et al*; 2008): When there is a large, rapid, and overwhelming accumulation of unfolded protein, a rapid apoptosis occurred and then most cells will be died. Similarly, apoptosis can be triggered by incubation with amino acid analogs or low doses of puromycin (Sugita *et al*; 1995). The above example of protein conformation-related phenomena could be related with the possible relationship between the conformation of the synthesized polypeptide inside the cell and the response of the biological cells, including the variation of characteristic, morphology and activity of the biomembrane.

Anionic liposome can be applied for inhibition of folding of polypeptide at post-translation of gene, as well as inhibition of gene expression at post-translation level. We can apply anionic liposome for inhibition of activity of toxic enzyme synthesized inside the cell. Over expressed unfolding enzyme will result in apoptosis of the cell. Negative charged liposome is potential of for inhibition toxic cell growth.

3. Application of cationic liposome for inhibition of gene expression

Cationic liposome can inhibit gene expression at transcription and translation levels, This prospective effect of DOTAP liposome open an important future for application of liposome on: (1) Treatment of extra cellular free DNA that may be dangerous to environment and human health. (2) Inhibition of infective toxic bacteria such as *E.coli*, *Salmonella* that often located in foods, fruits, vegetable. (3) Toxic gene in molds producing mycotoxin such as *Aspergillus flavus*, *Aspergillus paraciticus*, that can strongly produce aflatoxin, ochratoxin, that molds often located in foods, fruits, agro-produce such as peanuts, maize. (4) knock-out of oncogene or knock-down of mRNA (cancer - disease gene) in cancer cell in tumor of human and animal. (5) DOTAP liposome can also be possible applied as a new drug modified with specific ligand, (ex: ESA for interaction with target cancer cell)) for inhibition of its growth by knock-out of gene and/or knock-down of mRNA inside the cell, enhance drug delivery into target cells.

4. Activation of gene expression by using stress

The silence or inhibitory effect of GFP expression that were created by membrane, can be solved by using oxidative stress condition. From this result, the “silent” *cry* gene from our natural *B.thuringiensis* strains can be deeper understand. Silencing and activation of GFP expression by using DOTAP/oxidative stress opening a possible application of oxidative stress condition to improve and switch silent gene expression inside the cell.

Nomenclature

(1/P) Membrane fluidity of liposome

$I_{//}$	Fluorescence intensity of parallel to the excited light	[-]
$I_{\perp}$	Fluorescence intensity of perpendicular	[-]
ODx	Optical density at x nm	[-]
kDa:	Kilodalton	[-]
bp:	Base pairs	[-]
Ax:	Absorbance at Ax nm	[-]

List of Abbreviations

RTS	Rapid translation system
RNAi	RNA interference
GFP	Green Fluorescent Protein
MBP	Maltose-binding protein
GST	Glutathione S-transferase
GroEL/GroES	= Molecular chaperone system in <i>E.coli</i>
OD	= Optical density
U.V	= Ultra violet
TMA-DPH	trimethylammonio-diphenylhexatriene
POPG	1-palmitoyl-2-oleoyl-sn- glycero-3-[phospho-rac-(1-glycerol)]
DOTAP	1,2-Dioleoyl-3-trimethyl-ammonium-propane
POPC	= 1-Palmitoyl-2-oleoyl-3-phosphocholine
Chol	Cholesterol
RT-PCR	Reverse Transcription Polymerase Chain Reaction
mRNA	Messenger RNA
tRNA	Transfer RNA
rRNA	Ribosome RNA
GuHCl	Guanidinium hydrochloride

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List of Publications

[Original Papers]

1. Huong Thi Bui, Hiroshi Umakoshi, Kien Xuan Ngo, Masato Nishida, Toshinori Shimanouchi, and Ryoichi Kuboi: Liposome can Control Gene Expression in Cell-Free Translation System, *Langmuir*, **24**, 10537-10542 (2008)
2. Huong Thi Bui, Khang Duy Dinh, Chau Thuy Nguyen: Cloning and Sequencing Specific DNA Fragment of cry1Ab Gene from Some *Bacillus thuringiensis* var. *kurstaki* strains isolated in Vietnam. *J. Genetics Application*, **4**, 1-7 (2003). ISSN: 0866-8566.
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4. Huong Thi Bui, Hiroshi Umakoshi, Keishi Suga, Tomoyuki Tanabe, Toshinori Shimanouchi, and Ryoichi Kuboi: Cationic Liposome Inhibit Gene Expression in *E.coli* Cell-Free Translation System; *Membrane (Maku)*, accepted, **34**, 2009
5. Huong Thi Bui, Hiroshi Umakoshi, Keishi Suga, Masato Nishida, Toshinori Shimanouchi, and Ryoichi Kuboi: Negative-Charged Liposome Is Potent Inhibitor of Post-Translation During *in vitro* Synthesis of Green Fluorescent Protein, *Biochem. Eng. J.*, submitted.

[Related Papers]

1. Kien Xuan Ngo, Hiroshi Umakoshi, Toshinori Shimanouchi, Huong Thi Bui and Ryoichi Kuboi: Active Roles of liposomes and Heat Stress for Enhanced Release of Chitosanase from *Streptomyces griseus*, *J. Biosci. Bioeng.*, 106(6), 602-605 (2008)

2. Huong Thi Vu, Huong Thi Bui, Nguyen Thuy Chau, Cloning and sequencing MetA gene, a methionine biosynthetic gene encoding Homoserin acetyltransferase enzyme from isolates of *Corynebacterium glutamicum* in Vietnam. *J. Biotechnology*, in press
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[International Conferences]

1. H.T.Bui, D.N.Huyen, D.D.Khang, N.T.Chau: Some biochemical and biomolecular characteristic of *Bacillus thuringiensis* strains isolated in Vietnam, *Proc. of 5th Pacific Rim Conference on the Biotechnology of Bacillus thuringiensis and its Environmental Impact*, 17-21 November, p. 129-135, Hanoi, Vietnam (2003)
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