Biochemical Engineering Journal 46 (2009) 154–160

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Negatively charged liposome as a potent inhibitor of post-translation during *in vitro* synthesis of green fluorescent protein

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ARTICLE INFO

Article history:
Received 13 December 2008
Received in revised form 27 March 2009
Accepted 1 May 2009

Keywords:
Liposome
Green fluorescent protein (GFP)
Protein folding
Hydrophobicity

ABSTRACT

The effect of negatively charged liposome on *in vitro* synthesis of a reporter protein, green fluorescent protein (GFP), was investigated using a cell-free translation system. GFP was expressed with and without negatively charged liposome prepared with 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylchorine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG), resulting in the GFP fluorescence being reduced to approximately 60% in the presence of POPC/POPG at more than 30% POPG, depending on its concentration. However, the amount of synthesized GFP products, as analyzed by SDS-PAGE, did not change with and without the POPC/POPG liposome. The results of the ultrafiltration operation indicate that the liposome interacts not only with synthesized polypeptide GFP but also with folded synthesized GFP (mature GFP). Liposome also inhibited refolding of unfolded GFP to its native state. The above results show that the POPC/POPG could inhibit the folding of GFP in the post-translational process of the gene expression product.

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1. Introduction

Determining the details of gene regulation in a biological cell is important to obtaining a deeper understanding of the gene expression system and its application to the design of bioprocesses or gene-related drugs. For example, the regulation of a specific gene provides us with opportunities for medical treatment of pathogenous cells by suppression of the target gene [1], silencing a target gene [2], or inactivating a specific enzyme [3,26–28]. It is known that the inhibition of gene expression can be carried out by inhibition of (i) DNA transcription and (ii) mRNA translation, and (iii) the folding of polypeptides in post-translation during the gene expression process. However, controlling gene expression at the transcription or translation level is quite complicated due to the complexity of the molecular machinery [4,5]. Conventionally, DNA delivery by liposome has been accepted as one of the most promising means of achieving gene expression [6,7] when there are some problems with a low efficiency of gene delivery and unstable expression. RNA interference technology supported by liposome has recently been reported as a novel technique for silencing of a target gene as well as inhibiting gene expression [2,8].

Folding of the synthesized polypeptide in a post-translational process seems favorable in contrast to the above upstream steps [23]. It has been reported that apoptosis (cell suicide) can be triggered by production of improperly folded or incomplete proteins [9,10,29]: When there is a large, rapid, and overwhelming accumulation of unfolded protein, rapid apoptosis occurs and most cells will die. Similarly, apoptosis can be triggered by incubation with amino acid analogs or low doses of puromycin [9]. The above example of protein conformation-related phenomena could be related to the possible relationship between the conformation of the synthesized polypeptide inside the cell and the response of the biological cells, including variations in the characteristics, 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 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 the biomembrane itself as an active and functional interface of biological cells responding to dynamic changes in environmental conditions (stress) have recently been revealed through basic study of a model biomembrane. Liposome, a closed phospholipid bilayer membrane, is commonly used as a model biomembrane and has been applied to drug delivery, gene delivery, and expression for the suppression of the specific genes in disease cells [2,11]. Liposome has also been applied to RNA interference (RNAi) technology as a carrier for delivery of siRNAs to cells to enhance siRNA stability *in vitro* and *in vivo* for final inhibition of target mRNA

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doi:10.1016/j.bej.2009.05.002

translation [2]. It has also been reported that the liposome can improve gene expression through increases in mRNA transcription caused by enhanced stability of its polyA tail [12]. The use of liposome is thus attracting a great deal of interest from researchers.

Liposome has previously been reported to induce a variety of potential functions under variable environmental conditions; these include a chaperone-like function [13], translocation of proteins across the membrane [14], release of enzyme product from bacterial cells under heat stress conditions [15], and membrane fusion [16]. 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 enhancement of the interaction between the fragmented SOD and the liposome [17–19]. It has recently been reported that the liposome itself can affect in vitro gene expression by selecting the green fluorescent protein (GFP) as a reporter protein [20]. The lipid bilayer membrane is thus known to play a variety of potential roles in controlling enzyme structures 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.

We have previously reported [3] that negatively charged liposome POPG and DMPG liposome can strongly interact by electrostatic and hydrophobic interactions with denatured lysozyme and then inhibit its refolding, resulting in 88% and 52% inhibition, respectively, of lysozyme enzyme activity. It cannot be determined from these results alone whether negatively charged liposome can also inhibit refolding of other proteins or affect folding of early translated polypeptide. Therefore, the effects of negatively charged liposome on refolding of proteins and the folding of early synthesized polypeptides in post-translation of a gene and its mechanisms should be investigated and clarified. Furthermore, we have recently investigated the effects of liposome on gene expression, and our results indicated that neutral liposome as well as microdomain-dorming liposome, have a positive effect on gene expression of GFP in the RTS system [20]. In another study, a complex of neutral and cationic liposome also showed an inhibition of gene transcription and translation in an *in vitro* system [30]. Furthermore, we have previously reported in Langmuir our investigation of the role of liposome in gene expression of GFP in a cell-free translation system. Our results indicated that microdomain-forming liposome POPC/cholesterol can enhance GFP expression. In contrast, negatively and positively charged liposome can inhibit GFP expression. The positively charged liposome (DOTAP liposome), which is often used as a vector to carry the DNA/RNA, has previously reported to show the negative effect on the gene transcription and translation steps, resulted in the inhibition of GFP expression [31]. However, the mechanisms of negatively and positively charged liposome on GFP expression have not been fully investigated [20]. Therefore, in the present study, the potential role of the negatively charged lipid bilayer membrane (liposome) on GFP gene expression was further investigated by employing an *E. coli* cell-free translation system consisting of minimal components for protein synthesis and a model biomembrane, liposome, with special attention being paid to negatively charged liposome. By using a previous strategy based on an *in vitro* protein synthesis system [20], the gene expression of reporter protein GFP was carried out in the presence of negatively charged liposome. The variation of the gene expression of GFP was discussed, focusing on the possible role of folding of synthesized polypeptide in post-translation gene expression.

2. Materials and methods

2.1. 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). The 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 fluorescence probes for 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).

2.2. Liposome preparation

The phospholipids used in this study were phospholipid molecules of (i) POPC and (ii) POPG at different ratios of 0:100, 95:5, 90:10, 70:30, and 100:0 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 was dried under nitrogen flow for at least 5 h. In order to generate multilamellar vesicles, the dried phospholipid thin layer was hydrated with distilled water. The freeze–thaw cycle was repeated five 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).

2.3. In vitro expression of GFP with and without liposome

The Rapid Translation System (RTS 100 *E. coli* HY Kit), an *in vitro* protein synthesis system based on *E. coli* lysate, was used as a cell-free translation system [24]. The expression vector, pIVEX2.3d, contained the gene for expression of the green fluorescent protein (GFP). The reaction solution (50 μl) contained the following components: 12 μl *E. coli* lysate, 10 μl reaction mix, 12 μl amino acids, 1 μl methionine, 5 μl reconstitution buffer, and 0.5 μg of circular DNA template in 10 μl 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 h ("Expression" operation) and was kept for 24 h at 4 °C ("Maturation" operation). The liposome solution prepared at higher concentrations was added to the reaction mixture in place of the above water before and after the "Expression" operation, and the effect of liposome on gene expression was evaluated based on the following methods.

2.4. Analysis of gene product

2.4.1. Analysis of gene product at protein level

Two kinds of analysis, (i) GFP fluorescence and (ii) SDS-PAGE, were employed to quantify the gene product according to previous reports [20]. The amount of folded GFP product was evaluated by analysis of the GFP fluorescence intensity at 395 nm (excitation) and 509 nm (emission) using the fluorescence spectrophotometer (JASCO, FP-777, Tokyo, Japan) with a slit width of 5 nm at the excitation and emission light paths. All the GFP fluorescence spectra were analyzed after a 100 times dilution of the reaction samples with water in order to avoid the possible interference of the liposome included in the final reaction sample. It was confirmed that the fluorescence spectrum of pure GFP was not affected under the above experimental conditions, which included liposome in the finally diluted solution; in addition, POPC/POPG liposome itself at its final concentration had no emission spectra under the above conditions

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