Form 1

2017 Report Form for Collaboration with Research Center for Biomedical Engineering

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To Chairman, Board of Directors, Research Center for Biomedical Engineering

Applicant
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Report Form for Collaboration Research

Research Theme	Effect of membrane potential on entry of cell-penetrating peptide Transportan10 into single vesicles.
Research Area	1. Biomaterials 2. Bioengineering **√3. Functional molecules** 4. Chemistry/Electrical Engineering/Mechanical Engineering/Materials Science
Research Period	From: Date/month/Year 01/06/2017 To: Date/month/Year 31/03/2018

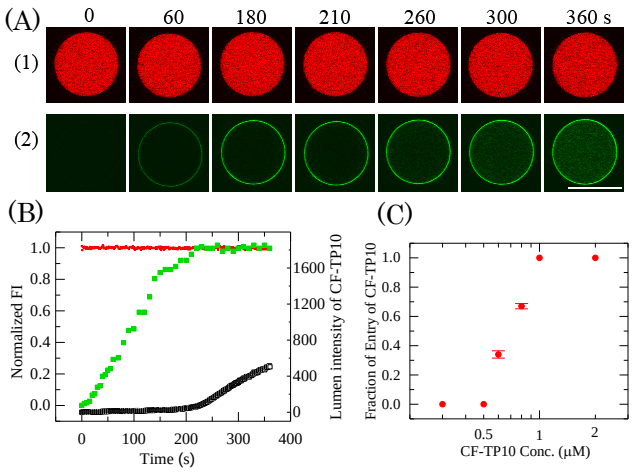
Applicant Organization			
Name	Department	Title	Role
Md. Zahidul Islam	Department of Biotechnology and Genetic Engineering, Jahangirnagar University, Ban gladesh	Lecturer	Experiments and analysis
Md. Mizanur Rahman Moghal	Integrated Bioscience Section, Graduate School of Science and Technology, Shizuoka Univ.	Ph. D course student	Experiments
Masahito Yamazaki	Research Institute of Electronics, Shizuoka Univ.	Professor	Analysis
Collaboration Partners in the Research Center	Masahito Yamazaki		

Research Results (Including Purpose, Results, Figures, etc.)

Plasma membrane or biomembrane of biological cells prevents the entry of pharmacologically active biomacromolecules (polypeptides and oligonucleotides) from outside of the cells. In contrast, cell-penetrating peptides (CPPs) such as transportan 10 (TP10) can translocate across the plasma membrane of living eukaryotic cells to enter their cytoplasms, and thus can be used for the intracellular delivery of biological cargo such as proteins and oligonucleotides. However, the elementary processes of the entry of CPPs into cells, the mechanisms underlying its translocation, and the delivery of large cargo remain unclear. On the other hand, all biological cells, irrespective of eukaryote or prokaryote, have membrane potential in their plasma membranes, which play important physiological roles. The purpose of this project is to reveal the effect of membrane potential on translocation of CF-TP10 into single giant unilamellar vesicles (GUVs) using the single GUV method.

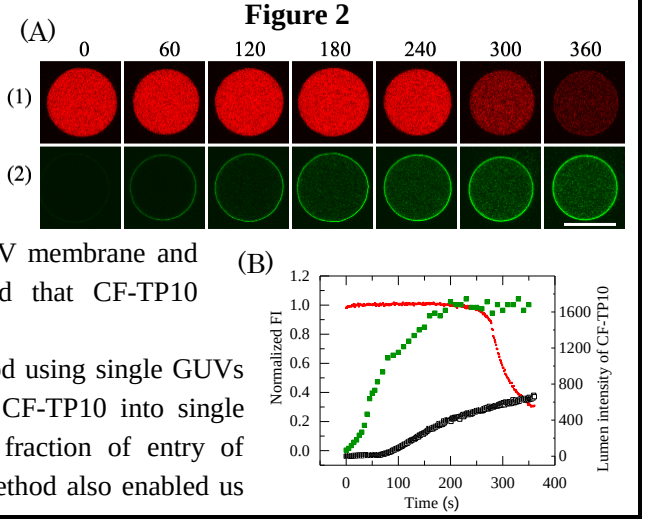
First, we have developed a new method for the continuous, quantitative detection of the entry of CPPs into GUVs, where we investigate the interaction of fluorescent probe-labeled CPPs with single GUVs containing large unilamellar vesicles (LUVs) with a diameter of 100 nm and water-soluble fluorescent probes in their lumens using confocal laser scanning microscopy (CLSM). Using this method, we investigated the interaction of carboxyfluorescein (CF)-labeled TP10 (CF-TP10) with single GUVs comprised of dioleoylphosphatidyl-glycerol (DOPG) and dioleoylphosphatidylcholine (DOPC) containing LUVs of the same membrane and Alexa Fluor 647 hydrazide (AF647) in their lumens. At low concentrations of CF-TP10 (such as 1.0 μM CF-TP10), first the fluorescence intensity (FI) of the GUV membrane due to the binding of CF-TP10 increased with time (green square in Fig. 1B), and then after some lag time the FI of the GUV lumen due to the binding of CF-TP10 with the LUVs increased continuously with time (Fig. 1A (2) and black square in Fig. 1B), whereas FI of the GUV lumen due to AF647 did not change (Fig. 1A and red circle in Fig. 1B), indicating no pore formation in the GUV membrane. As a measure of the rate of entry of CPPs into GUV lumen, we can use the fraction of GUVs which CPPs entered before a specific time t with respect to total examined GUVs, $P_{entry}(t)$, because the entry of CPPs occurred stochastically. Here we can determine the values of $P_{entry}(t)$ based on the FI of the GUV lumen due to CF-TP10 at a specific time t. The values of the I_{lumen} (6 min) for all examined GUVs (n = 20) interacting with CF-TP10 were larger than 50, indicating that the fraction of GUVs which CPPs entered before 6 min without pore formation among all examined GUVs, P_{entry} (6 min), was 1.0 for 1.0 μM CF-TP10. Figure 1C (red circle) shows the dependence of P_{entry} (6 min) on CF-TP10 concentration; at and less than 0.50 μM CF-TP10, P_{entry} (6 min) = 0, and above 0.50 μM, P_{entry} (6 min) increased with an increase in CF-TP10 concentration, indicating that the rate of entry of CF-TP10 into GUV lumen increased with CF-TP10 concentration.

Figure 1



At higher concentrations of CF-TP10 (such as 2.0 μM CF-TP10), after the FI of the GUV lumen due to CF-TP10 (black square in Fig. 2B) increased significantly, leakage of AF647 started (Fig. 2A and red circle in Fig. 2B). These results indicate that CF-TP10 entered the GUV lumen by translocating across the GUV membrane and then bound to the LUVs before pore formation and that CF-TP10 concentration in the lumen increased with time.

Figure 2



The above results clearly indicate that the new method using single GUVs containing LUVs can detect continuously the entry of CF-TP10 into single GUVs without pore formation and the values of the fraction of entry of CF-TP10 increased with CF-TP10 concentration. This method also enabled us

to follow the time course of CF-TP10 concentration in the GUV lumen continuously and quantitatively. Moreover, in this method, the I_{lumen} greatly increased after entry of CF-TP10 into GUV lumen, and therefore other experimental methods such as flow cytometry can detect the entry of CF-TP10. We can reasonably expect that this method can be applied to the interaction of other CPPs with single GUVs.

Next, to elucidate the mechanism of the translocation of TP10 across lipid bilayers, we investigated the effect of the membrane potential on the entry of CF-TP10 into single DOPG/DOPC-GUVs using the single GUV method. Various membrane potential were applied to GUV membranes using the K^+ concentration gradient, which was confirmed by the potential sensitive-fluorescence probe. The rate of entry of CF-TP10 into the GUV lumen before leakage of AF647, i.e., pore formation, increased with an increase in membrane potential. There are several elementary processes involved in the entry of CF-TP10 into GUV lumen. It is thus important to elucidate which elementary process is affected greatly by the membrane potential. We have to investigate more this point next year.

List of Publications Related to the Collaboration Research

(1) Md. Mizanur Rahman Moghal, Md. Zahidul Islam, Sabrina Sharmin, Victor Levadnyy, Md. Moniruzzaman, and Masahito Yamazaki, Continuous detection of entry of cell-penetrating peptide transportan 10 into single vesicles, *Chem. Phys. Lipids*, 212, 120-129, 2018.

(2) Md. Zahidul Islam, Sabrina Sharmin, Md. Moniruzzaman, and Masahito Yamazaki
Elementary Processes for the Entry of Cell-Penetrating Peptides into Lipid Bilayer Vesicles and Bacterial Cells, *Appl. Microbiol. Biotechnol. in press*, 2018

List of Presentations (Conference, Meeting, etc)

(1) Md. Mizanur Moghal, Md. Zahidul Islam, Sabrina Sharmin, Masahito Yamazaki, Effect of Membrane Potential on the Translocation of Cell-Penetrating Peptide Transportan 10 (TP10) across Lipid Bilayers, The 55th Annual Meeting of Biophysical Society of Japan, 19–21 Sept., 2017, Kumamoto Univ., Kumamoto

List of Awards

Research plan for the next year (from April 1, 2018 to March 31, 2019), if the collaboration research is continued. Prior consent from the collaboration partner in the Research Center is necessary.

As described in the above section, in the last year's project, we found that the rate of entry of CF-TP10 into the GUV lumen before pore formation increased with an increase in membrane potential applied to the GUV membrane. However, there are several elementary processes involved in the entry of CF-TP10 into GUV lumen. It is thus important to elucidate which elementary process is affected greatly by the membrane potential. For this purpose, we examine the effect of membrane potential on the rate constants of binding of CF-TP10 to GUV membrane from aqueous solution and that of unbinding of CF-TP10 from GUV membrane to aqueous solution using the method developed by us (1). Briefly, we investigate the interaction of CF-TP10 with single DOPG/DOPC-GUVs (not containing vesicles) under the membrane potential using confocal laser scanning microscopy measure, and analyze the time course of the FI of the GUV membrane due to CF-TP10 to obtain these rate constants. Then we compare the effect of membrane potential on the rate constants, and determine which process is greatly affected by the membrane potential.

So far we investigated the negative membrane potential. To increase our understanding of the effect of membrane potential on the entry of CF-TP10, we also want to investigate the effect of the positive membrane potential on the entry of CF-TP10 into GUV lumen and also on the rate constant of each elementary process. On

the basis of the results of the effects of both negative membrane potential and positive one on the entry of CF-TP10 and its elementary processes, we want to improve the mechanism of the entry of CF-TP10 proposed by us recently (2).

Next, to generalize the concept of the effect of the membrane potential on the entry of CPPs, we investigate the effect of membrane potential on the entry of other CPPs such as CF-R_9 (3) and Rh-LfcinB (4-9) (4) and its elementary processes.

The results of the effect of membrane potential on the entry of CPPs and the mechanism of the translocation of CPPs across the membranes are very helpful to develop novel vectors for the delivery of drugs, chemicals, oligonucleotides, and proteins into cells for curing various diseases.

<References>

(1) Islam MZ, Ariyama H, Alam JM, Yamazaki M (2014) Entry of cell-penetrating peptide transportan 10 into a single vesicle by translocating across lipid membrane and its induced pores. Biochemistry 53:386-396.

(2) Islam MZ, Sharmin S, Levadnyy V, Shibly SUA, Yamazaki M (2017) Effects of Mechanical Properties of Lipid Bilayers on Entry of Cell-Penetrating Peptides into Single Vesicles. Langmuir 33:2433-2443.

(3) Sharmin S, Islam MZ, Karal MAS, Shibly SUA, Dohra H, Yamazaki M (2016) Effects of lipid composition on the entry of cell-penetrating peptide oligoarginine into single vesicles. Biochemistry55:4154-4165.

(4) Moniruzzaman M, Islam MZ, Sharmin S, Dohra H, Yamazaki M (2017) Entry of a Six-Residue Antimicrobial Peptide Derived from Lactoferricin B into Single Vesicles and *Escherichia coli* Cells without Damaging their Membranes. Biochemistry 56:4419-4431.