Title	分子進化工学を簡略化するための‘Head-to-Head' mRNA/cDNA ディスプレイとエレクトロスプレーマイクロアレイプラットフォームの開発
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Citation	
Issue Date	2018-09
Type	Thesis or Dissertation
Text version	ETD
URL	http://hdl.handle.net/10119/15535
Rights	
Description	Supervisor:高村　禅，マテリアルサイエンス研究科，博士

Development of 'Head-to-Head' mRNA/cDNA display and electrospray microarray platform for simplifying molecular evolutionary engineering

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Background:
Directed molecular evolution is defined as the mimic of natural evolution (Darwinian theory of the survival of the fittest) at small scale in laboratory (*in vitro*) to isolate the desired specific properties from the pool of "millions-to-quadrillion" molecules. It is basically based on the principles of diversification and selection of the evolved desired phenotype. *In vitro* selection had steadily advanced from the selection of nucleic acids (DNA and RNA) aptamers to the peptide based aptamer. But to select peptide based aptamers, which plays actual role of highly functional molecules in the form of enzymes, signalling molecules etc; the peptide needs to be linked with its genotype as peptide itself cannot be amplified to get the genotypic information. The significance of directed molecular evolution has been so far well explained by the non-compartmentalized display technologies like phage display, ribosome display, mRNA display and compartmentalized display technology called *in vitro* compartmentalization (IVC). Phage display[1] is a cell-based method, where library size is directly depended on the host cell. Weak physical bonding of the ternary complex (mRNA, linkage molecule and ribosome) and large size of ribosome leads to easy dissociation and uncontrolled inter molecular interactions resulting in misleading results in ribosome display[2]. In mRNA display[3], the tether moiety, puromycin enters the P site of ribosome which results in pre-mature proteins and incorrect folding of proteins. In the past 10 years, IVC[4] have been extensively used by entrapping gene and protein in one compartment to remove non-specific interaction between the surrounding biomolecules along with protection from degradation. All the previous mentioned non-compartmentalized technologies produces low Genotype-Phenotype (GP) yield and are affinity-based selection involving multiple steps and long-time procedures leading to the loss of information. IVC are simple techniques with lot of advantages over non-compartmentalized display techniques but they lack to encapsulate the "million-to-quadrillion" library all together both in terms of time and capacity.

By studying the above rise in the molecular evolution over the time, it has encouraged to shift from affinity-based selection to function-based selection in parallel dealing with the higher number of molecules together in short time. By conducting this work, I report to create a platform by developing not only function-based *in vitro* selection with improved Genotype-Phenotype (GP) yield but also IVC-based *in vitro* selection for improved library size.

Aim:
This work is a parallel study to develop two fundamental platforms to simplify molecular evolutionary engineering. On one side, 'Head-to-Head' (H2H) mRNA/cDNA display for high efficiency of genotype-phenotype yield is established whereas on the other side, electrospray microarray platform for larger library is developed.

Experimental:

1. **'Head-to-Head' mRNA/cDNA display**

'Head-to-Head' (H2H) is an opposite link mode to unite genotype and phenotype compared to the available conventional technologies. The key technique in H2H is the formation of a covalent bond between O^6-benzylguanine (BG) and O^6-alkylguanine-DNA alkyl transferase (AGT), where mRNA is linked to a nascent AGT via a BG-linker, resulting in a "(C-terminus) protein-BG-DNA linker-mRNA (5′-terminus)" conjugate (Fig. 1). Conventional display techniques are based on the 'tail-to-tail' linking: i.e., 3′-end of the mRNA is linked with the carboxyl terminal end of the protein, thus allowing a free N-terminus of protein to be available. In these displays, polyribosomal phenomenon is theoretically thought to be occurring near the translation end point due to a halt of the first running ribosome. In H2H, the 5′-end of the mRNA (head of mRNA) was linked to the head protein moiety (i.e., the most N-terminal proximal protein, AGT) of a fusion protein via a BG-containing DNA linker, making the C-terminus of the fusion protein freely available. Thus, a head (N-terminus) to head (5′-terminus) linkage is formed. In this case, due to the stop codon (UAA), ribosomes can be expected to work in a translation cycle of initiation, elongation, and release, resulting in no ribosomes being stalled. The PCR-purified template DNA was used for cell-free *in vitro* transcription. The mRNA thus transcribed holds a stop codon for the iterative

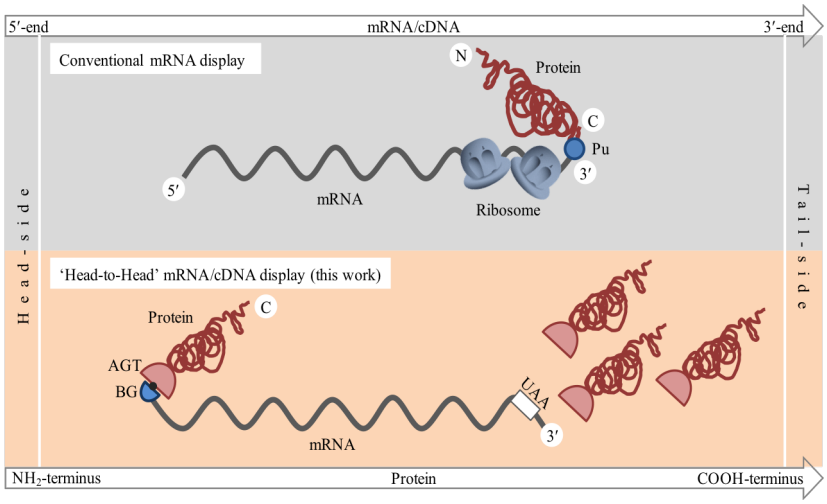

Figure 1: Schematic drawing for the comparison between conventional display techniques (ribosome display and mRNA display) and novel 'Head-to-Head' mRNA/cDNA display.

production of proteins. The 5'-terminal end of mRNA was hybridized to a complementary DNA stretch of BG-DNA linker, whereas the 3'-terminal end was hybridized with that of the 3'-protection-primer DNA. The BG-DNA linker-mRNA was subjected to fusion to obtain phenotype and genotype conjugate and GP yield was calculated. It is an information-to-function based biomolecular display method for simplified and rapid *in vitro* molecular evolution.

2. Electrospray microarray platform

Over the two decades, three main approaches have been widely used for IVC as i) Conventional methods using homogenizers and vortexing, ii) microfluidics-based approaches iii) use of single nozzle in electrospray. Each technique has its own disadvantages from polydispersity of droplet size to low-throughput generation to time consuming respectively, and thus, these approaches limit the application of IVC in the chemical and biological sciences.

Electrospray incorporating single nozzle generates monodisperse and fL-sized droplets[5] but it consumes hours-to-days for encapsulation of µl scale reaction with a limit of library size to 10^{8-9}. Hence, in this part of the research, I present a novel platform by integrating micro-hole chip with immersed electrospray for ultrahigh-throughput generation of highly monodisperse water-in-oil or agarose-in-oil compartments. The set-up of Micro-Hole Array Electrospray (µHAES) system is shown in Fig. 2 where a conductive aqueous solution is electrospray through an electrified micro-hole chip containing an array of 7x7 mm (24x16=384 holes) in an immiscible phase (mixture of oil and surfactant). A jet of water-in-oil droplets are obtained into the oil chamber when voltage of 1000 V was applied through the micro-hole array thereby generating bulk water-in-oil droplets.

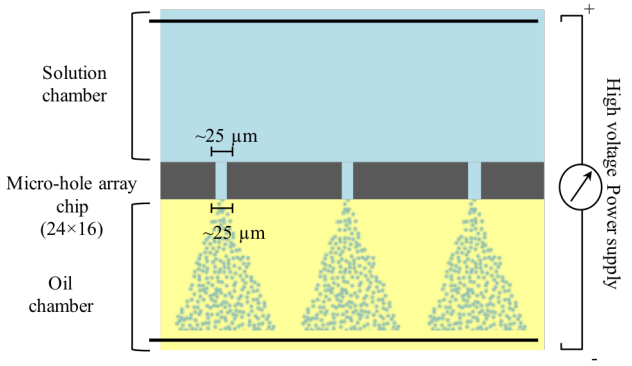

Figure 2: The concept of Micro-Hole Array Electrospray (µHAES) system.

Results and Discussion:

1. 'Head-to-Head' mRNA/cDNA display

Fortunately, the H2H linkage of BG and the nascent protein proceeds more rapidly (within 0.5–2 h) than the case for the tail-to-tail method, probably due to a lesser waiting time during the translation event. The H2H directed genotype-phenotype conjugate was analysed by PAGE (Fig. 3). Lane 3 allows for an estimation based on the amount of fluorescent FITC, that around 20–50 % of the BG-linker (contained in the forms of Protein-Linker-mRNA plus Protein-Linker) could bind to AGT under these experimental conditions, since the addition of the bottom band of the Linker only accounts for the total amount of the

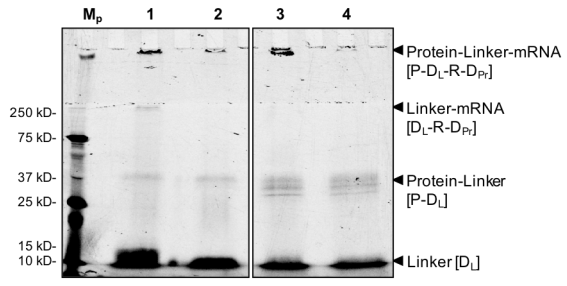

Figure 3: PAGE analysis for the formation of H2H conjugate. The fusion time was 0.5 h for Lanes 1 and 2, and 2 h for Lanes 3 and 4. Lanes 1 and 3 showed direct migration after the reaction, while Lanes 2 and 4 were nuclease-treated using RNase H and RNase ONE. M_P indicates the protein marker.

BG linker input. This experiment indicates the generation of the H2H construct-holding phenotype-genotype conjugate at a yield of around 20% in a 2-h translation reaction. Among the advantages of H2H, the generation of multi-copied proteins is the most promising and was proven to be possible owing to the restored stop codon, which had been intentionally removed in the conventional mRNA display. Another advantage is obviously having a free C-terminus of the protein, which can be used for modifications such as C-terminal methylation, α-amidation.

2. Electrospray microarray platform

The water-in-oil droplets generated by novel µHAES system was analysed and graph was plotted (Fig. 4). µHAES system not only reduces the time of encapsulating cell-free reaction (10 µl) in 1fL IVC to just 5 minutes but can also increase library size to $10^{11\text{-}12}$ compared to 55 hours and $10^{8\text{-}9}$ library size of single nozzle electrospray therefore, establishing an ultrahigh-through w/o droplet generation platform in less time with high library size.

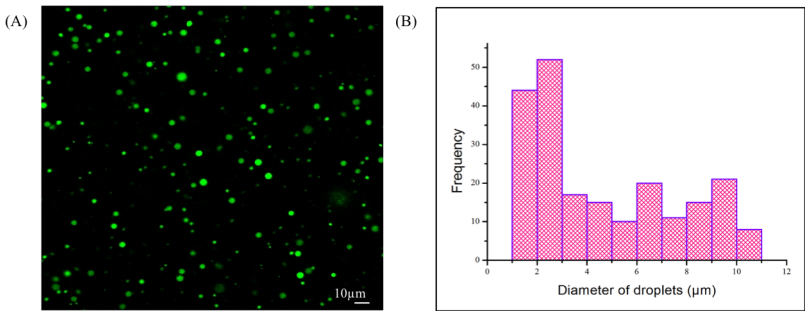

Figure 4: (A) Fluorescent image of agarose-in-oil droplets produced by µHAES. (B) Graphical representation of size distribution of the droplets. From the graph, average size was found to be 1-3 µm with a range of 1-11 µm.

Conclusion:

A novel genotype-phenotype (GP) linking method termed 'Head-to-Head' (H2H) linking was devised. The H2H construct, that is, 5′-end of mRNA bound to a BG-DNA linker, which in turn binds to the nascent protein AGT, completing the whole construct (protein-linker-mRNA conjugate), was generated in a substantial amount (an estimated yield of above 20 %). As less time is required for H2H, it protects the initial information and is a rapid system for fusion allowing free C-terminal modifications. This H2H construct can be used to find allosteric binding site based peptide aptamer to work as function-based selection. Micro-Hole Array Electrospray (µHAES) platform uses ~384 holes to produce uniform droplets in less time (5 minutes) with increased library size ($10^{11\text{-}12}$). It can be used to produce ultralow Ni-NTA beads and commercialized in the near future, application.

Hence, the two parallel fundamental techniques are successfully developed as 'Head-to-Head' (H2H) mRNA/cDNA display for high efficiency of genotype-phenotype yield and electrospray microarray platform for larger library size in the need to simplify molecular evolutionary engineering.

References:

1. Smith, G.P. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* 228, 1315 (1985).
2. Mattheakis, L.C., Bhatt, R.R. & Dower, W.J. An in vitro polysome display system for identifying ligands from very large peptide libraries. *Proceedings of the National Academy of Sciences of the United States of America* 91, 9022-9026 (1994).
3. Roberts, R.W. & Szostak, J.W. RNA-peptide fusions for the in vitro selection of peptides and proteins. *Proceedings of the National Academy of Sciences* 94, 12297 (1997).
4. Tawfik, D.S. & Griffiths, A.D. Man-made cell-like compartments for molecular evolution. *Nature Biotechnology* 16, 652 (1998).
5. Sharma, B., Takamura, Y., Shimoda, T. & Biyani, M. A bulk sub-femtoliter in vitro compartmentalization system using super-fine electrosprays. *Scientific Reports* 6, 26257 (2016).

Keywords: 'Head-to-Head' (H2H) covalent linkage, Multi-copied proteins, C-terminal modifications, Micro-Hole Array Electrospray (µHAES) system, Library size.