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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論文題目	Development of ‘Head-to-Head’ mRNA/cDNA display and electrospray microarray platform for simplifying molecular evolutionary engineering		
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論文の内容の要旨

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

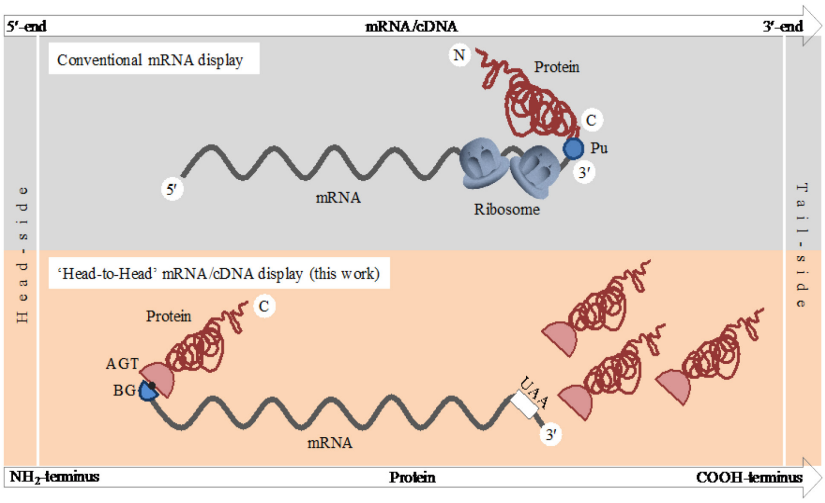

Figure 1: Schematic drawing for the comparison between conventional display techniques (ribosome display and mRNA display) and novel 'Head-to-Head'

iterative 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.

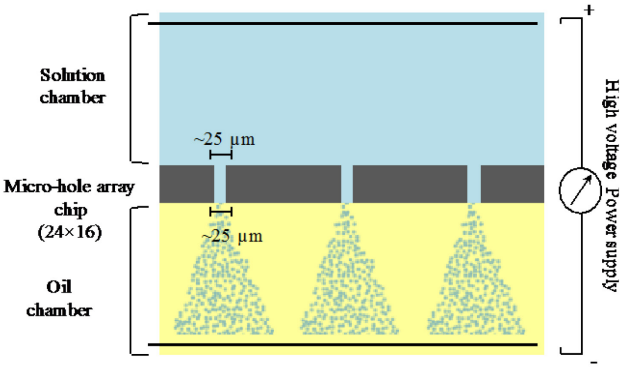

Figure 2: The concept of Micro-Hole Array Electrospray

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

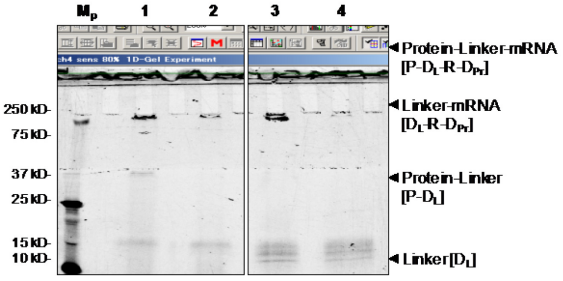

Figure 3: PAGE analysis for the formation of H2H conjugate. The fusion time was 0.5 h for Lanes 1 and 2, and 2 h for Lanes 3 and 4. Lanes 1 and 3 showed direct migration after the reaction, while Lanes 2 and 4 were nuclease-treated using RNase H and RNase ONE. M_P indicates the protein marker.

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.

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 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

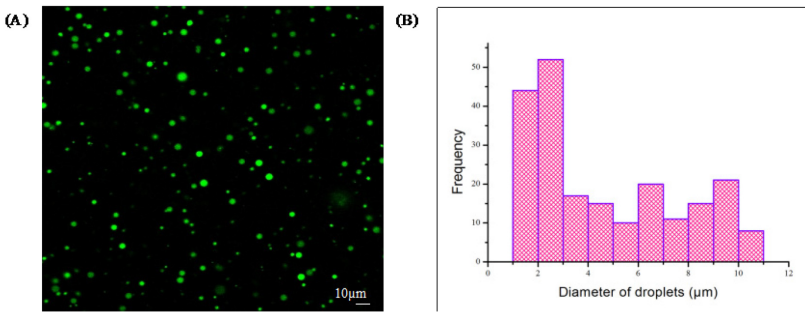

Figure 4: (A) Fluorescent image of agarose-in-oil droplets produced by µHAES. (B) Graphical representation of size distribution of the droplets. From the graph, average size was found to be 1-3 µm with a range of 1-11 µm.

55 hours and 10^{8-9} library size of single nozzle electrospray therefore, establishing an ultrahigh-through w/o droplet generation platform in less time with high library size.

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^{11-12}). 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.

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

論文審査の結果の要旨

本研究は、進化分子工学法をより簡便に実施するための、新規 mRNA/cDNA ディスプレイ法と micro hole array electrospray を用いた IVC 作成法の開発に関するものである。

進化分子工学は、あたかもダーウインの進化論のように、分子の構造にランダムな変異を起こさせ、目的の分子に近い性質を持った分子群を選別して増やす(増幅)過程を繰り返すことで、望みの性質の分子を開発していく手法である。酵素や DNA/ペプチドアプタマーの開発に用いられている。その増幅過程において DNA は PCR 等で同じ構造の分子を簡単に増やすことができるが、たんぱくやペプチドはできない。従って、たんぱくやペプチドの進化分子工学法では、性質を調べるためのタンパク・ペプチド分子（表現型）とその設計図である DNA か RNA 分子（遺伝子型）を、何等かの形で紐づけて取り扱うのが一般的である。紐づけには様々な方法が確立されているが、例えば RNA ディスプレイ法では収率が悪く（＜１％）操作が煩雑で時間がかかり（～数日/Round）、in-vitro compartmentalization (IVC)法では均一な液滴の作成に時間がかかりすぎる等の欠点があり、進化分子工学的手法の普及の大きな障害となっていた。本研究は、より簡便に進化分子工学法を実施するために、O^6-benzylguanin(BG)と O^6-alkylguanine-DNA alkyl transferase(AGT)を用いた簡便高収率の新規遺伝子型-表現型（GP）リンク法を開発し、また micro hole array electrospray による均一な IVC の高速生成法を確立したものである。

第 1 章では、進化分子工学法の現状と問題点について述べ、本研究の目的を明示している。

第 2 章では、BG を有する DNA リンカを新たに設計し、先頭に AGT モチーフをもつ mRNA ライブラリと結合させ、無細胞たんぱく合成を行うことで、合成中に mRNA（遺伝子型）とたんぱく（表現型）が頭部と頭部で結合する新規 GP リンク作成を試みている。各種のmRNA 保護方法を検討・開発し、最終的に GP リンクを確立し、基本動作の確認に至っている。GP リンクの収率は、従来法では 1%以下程度であったが、20~50%に向上し、また作業時間も、1 世代あたり数日かかっていたものが、数時間へと短縮された。

第 3 章では、本研究室で開発中の深沈型エレクトロスプレーによる IVC 作成法が、BG-AGT リンク法に適応できることを確かめている。

第 4 章では、平板に開けた 384 個の micro hole array electrospray により、直径数μm の均一な IVC の高速生成に成功している。これにより $10^{11\text{-}12}$ 規模の均一な IVC を取り扱い可能となった。IVC 法による分子進化工学法では機能ベースのセレクションが容易になりさらに適応範囲が広がると考えられる。

以上、本論文は、BG-AGT 結合を用いた、遺伝子型-表現型の高収率な新規リンク法の開発と、micro hole array electrospray の開発により、進化分子工学的手法の基礎プロセスの大幅な簡便化を達成したものであり、学術的に貢献するところが大きい。よって博士（マテリアルサイエンス）の学位論文として十分価値あるものと認めた。