Reconstitution of intracellular environments *in vitro* and in artificial cells

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Toward reconstitution of living cells by artificial cells technology, it is critical process to understand the differences between mixtures of biomolecules and living cells. For the aim, we have developed procedures for preparation of an additive-free cell extract (AFCE) and for concentrating biomacromolecules in artificial cells. In this review, we introduce our recent progress to reconstitute intracellular environments *in vitro* and in artificial cells.

Key words: Liposome, cell-free protein expression, molecular crowding, cell reconstitution

Can we reconstruct living cells from a mixture of biomolecules?

All present cells are derived from their parent cells as a consequence of cell division. The only exception is the last universe common ancestor (LUCA), and thus the birth of LUCA is the start of life[1]. The origin of life must be a cell, because cells are the fundamental unit of all known lives. LUCA is assumed to be appeared more than billions years ago[2]. However, it is still mystery how the LUCA was born. It is difficult to demonstrate the event directly, because we cannot come back to the time. However, it should be possible to find conditions that a life can be born.

Rebuilding cells from biomolecules mixtures is a way to reveal critical conditions for the birth of life. Many scientists believe that life was born by encapsulation of all essential components for self-reproduction in lipids membrane (liposomes). This encapsulation process has been reproduced in laboratories by formation of cell-sized unilamellar liposomes (giant unilamellar vesicles, GUV)[3–6]. Genomic DNA of phages[7], cell extract of bacteria and eukaryote[8–11], and subsystems of cells have been encapsulated in GUVs[12,13]. It has been shown that protein expression systems work in the liposomes[9,13,14]. Membrane proteins have also been reconstituted on membrane surface of liposomes by detergent methods[15–17] and by protein expression inside liposomes[8–11,18]. Very recently, Sec insertion system for membrane proteins was reconstituted on GUVs by the cell-free protein expression method[19]. Reconstitution of cytoskeletons, including actin and tubulin system, in GUVs has directly shown their activity on change of cell morphology[10,20,21]. GUVs with cell-free protein expression system can be used as a platform for directed evolution of membrane proteins[22]. In this review, artificial cells indicate that GUV with cellular functions. These situations drove us to start the challenge to reconstitute a living cell itself by encapsulating all cellular components into artificial cells at physiological levels.

Cell extracts as a material to reconstitute the physiological environment *in vitro*

Mixtures of intracellular components can be easily prepared as cell extracts. Cell extracts is the soluble fraction after cells disruption by physical or chemical treatment like

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sonication or detergent. In the case of a well-studied model organism, *Escherichia coli*, cell extracts maintain critical biological systems including DNA replication system[23], transcription-translation (TX-TL) system[24–26], and glycolysis pathway[27,28]. Since bacteria do not have compartment or organelle, their cell extracts are very good materials for reconstitution of their intracellular environments *in vitro*. These are the reason we selected *E. coli* as a target of the challenge to reconstitute living state from biomolecules mixtures.

Concentration and additive-free: Mimicking the physiological conditions of live cells *in vitro*

Simple encapsulation of cell extracts into artificial cells is not enough to reconstitute living cells. Actually, artificial cells with typical cell extract and genome DNA did not show self-replication (data not shown). This is resulted from that typical cell extracts are seriously distinct from the intracellular condition of living cells. For examples, biomacromolecules in cell extracts were diluted by solutions with artificial chemicals. Living cells have 300 mg/mL of macromolecules inside their membrane[29], and small metabolites works as the stabilizer of fragile biomacromolecules. On the other hands, typical cell extracts are consisted with tens mg/mL of macromolecules and exogenous chemical solutions. This difference obscured the critical differences between living cells and biomolecules mixtures that should give us useful hints to reconstruct living cells. Hence, we aimed at making a cell extract that mimics intracellular environments[26,30].

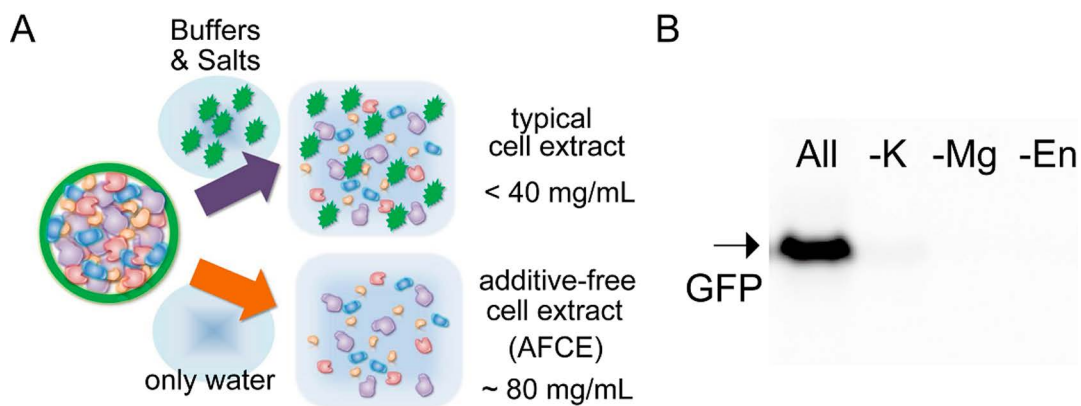## Preparation of an additive-free cell extract (AFCE)[26]

In general, buffers and salts have been added in typical

cell extract to stabilized biomacromolecules. However, their concentration is quite high (hundreds mM) and similar to the total concentration of small molecules in cells. Such high concentration of exogenous chemicals may change the environment of biomacromolecules. Thus, we developed a method to prepare an additive-free cell extract (Fig. 1A).

We considered that these chemicals as stabilizer were dispensable if the cell extract contain enough concentration of small metabolites. However, it is not easy to disrupt small cells like *E. coli* without adding solutions. Thus, addition of small amount of double distilled water (DDW, highly purified water) was inevitable. Ultrasonic treatment of 1 g wet cells in exogenous 1 mL DDW on ice gave the best result to prepare an additive-free cell extract (AFCE). Typically, 55 mg/mL proteins and 25 mg/mL nucleotides were extracted by the treatment. Protein expression was observed by adding DNA, energy recycling system, potassium, and magnesium into the AFCE (Fig. 1B). Since protein expression system requires more than 100 factors, we concluded that additive-free is no problem for preparation of functional cell extract.

Reconstitution of intracellular concentration of AFCE using evaporation at low pressure[26]

Concentration of macromolecules is another problem of typical cell extract. The crowding environment in living cells limits diffusion space of biomacromolecules, and raises the possibility of interaction among neighbors[31]. As a result, crowding affect the processes on formation of tertiary structures and association of macromolecules. Actually, presence of polymer like PEG and PVA, increases the possibility of interaction between DNA and protein, and activate efficiency of replication, cutting, and ligation of DNA. However, such additives in protein mixture sometimes trigger aqueous phase separation[32]. Thus, reconstitution of the high concentration of macromolecules without additives was

Figure 1 Preparation of an additive-free cell extract (AFCE). A: schematic representation of AFCE preparation. B: cell-free expression of GFP using AFCE. Fluorescence of GFP expressed was observed after non-boiled SDS-PAGE. -K, -Mg, and -En indicate potassium ion, magnesium ion, energy recycling system, respectively, were omitted in the mixtures of cell-free protein expression.
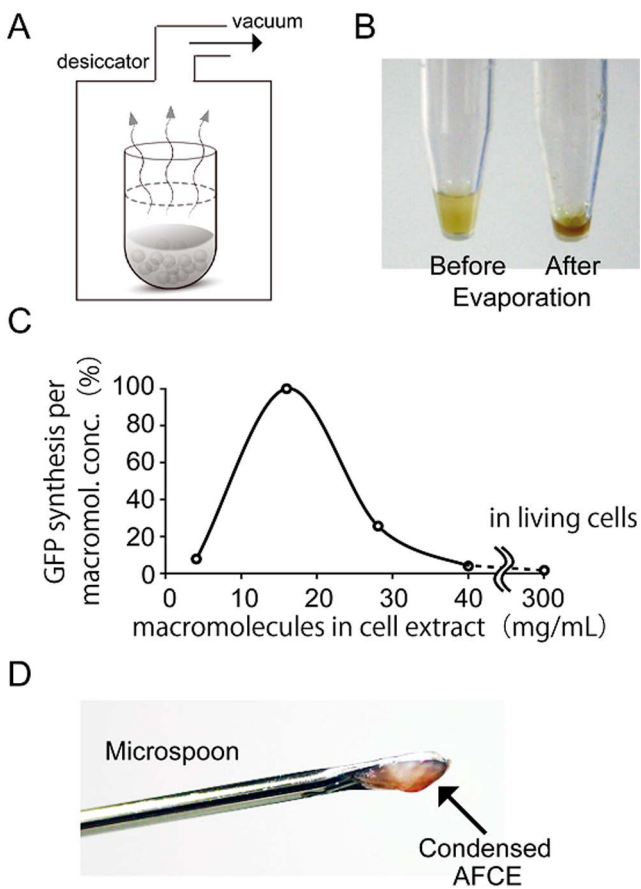
Figure 2 Evaporation under low pressure at low temperature. A: schematic illustration of the apparatus of evaporation under low pressure at low temperature. B: AFCE before and after condensation by the evaporation. C: Efficiency of protein expression using AFCE before evaporation. The efficiency is max around 15 mg/mL of macromolecules in cell extract. D: Viscosity of condensed AFCE. Micro-spoon was turned over after scooping the condensed AFCE. B&C: these figures were an edited version of Figure 1b and 1c in Seibutsubutsuri 53(5), 262–263 (2013)[33]

necessary to understand the critical difference between live cells and mixtures of biomolecules.

The only exogenous component of the AFCE is DDW, which can be removed by evaporation. Thus AFCE was evaporated under low pressure at low temperature (below 20°C) to avoid aggregation or degradation of proteins that occur at high temperature (Fig. 2A). After 3.5 h evaporation, macromolecule concentration of AFCE reached near ~300 mg/mL (Fig. 2B). However, the concentrated AFCE did not show efficient protein expression. We should note that the disability of AFCE was not resulted from aggregation or degradation, because dilution of the AFCE by DDW restored the activity. Moreover, AFCE before evaporation showed less efficient levels of protein expression at high concentration of macromolecules, especially more than 30 mg/mL (Fig. 2C). Similar dysfunction at high concentra-

tion was observed when typical cell extracts with buffers and salts were examined. Adding over 7% PVA gave the same dysfunction. These results indicate that TX-TL system of biomolecules mixtures at high concentration could not work dissimilar to that in living cells.

Another remarkable feature of highly condensed AFCE was high viscosity. The condensed AFCE was like gels and remained even if the tubes were turned over. Figure 2D shows the result of AFCE scooped by spoon. The condensed AFCE did not drop from turned over spoon because of the high viscosity.

Reconstitution of intracellular condition in artificial cells using semi-permeable property of lipid membranes[30]

Lipids membranes are one of the most important components of life. In addition to the role as boundaries between living systems and environment, lipids membrane affect chemical reactions inside artificial cells[33,34]. Although we tried to encapsulate such condensed AFCE into GUVs, it was found to be very difficult due to the high viscosity. Therefore, we developed a method to condense macromolecules inside artificial cells using hypertonic conditions. The method depends on the semi-permeable property of lipids membrane. Small and non-charged molecules like water can go through the membrane by osmotic treatment[35]. Consequently, the volume of artificial cells decreases until the osmotic pressure of inner media is equal to that of outer media. On the other hand, charged or relatively large molecules (>100 Da) remain inside[35]. Taken together, large or charged macromolecules in artificial cells were condensed inside liposomes by hypertonic treatment (Fig. 3A). Simply, the condensation factor after the hypertonic treatment depends on the initial ratio of osmotic pressure between outer media and inner media. Macromolecule concentration inside the artificial cells showed 4-fold increase after 6 minutes of hypertonic treatment. This method enables us to reconstruct artificial cells with physiological macromolecule concentration (300 mg/mL). We termed this artificial cell as life-mimicking artificial cells (L-MACs).

Characters of artificial cells with physiological concentration of biomacromolecules[30]

To understand characteristic features of L-MACs, we prepared GUVs containing a single component macromolecule, BSA, at physiological macromolecule concentration (BSA GUVs) by the same method. Comparison between L-MACs and BSA GUVs identified two different characters. First, condensation ratio was different between them (Fig. 3B). BSA inside GUVs was linearly condensed in response to the external osmotic pressure, and the concentration of BSA reached over 400 mg/mL. On the other hands, AFCE inside GUVs were linearly condensed up to around 260 mg/mL of

A

B

C
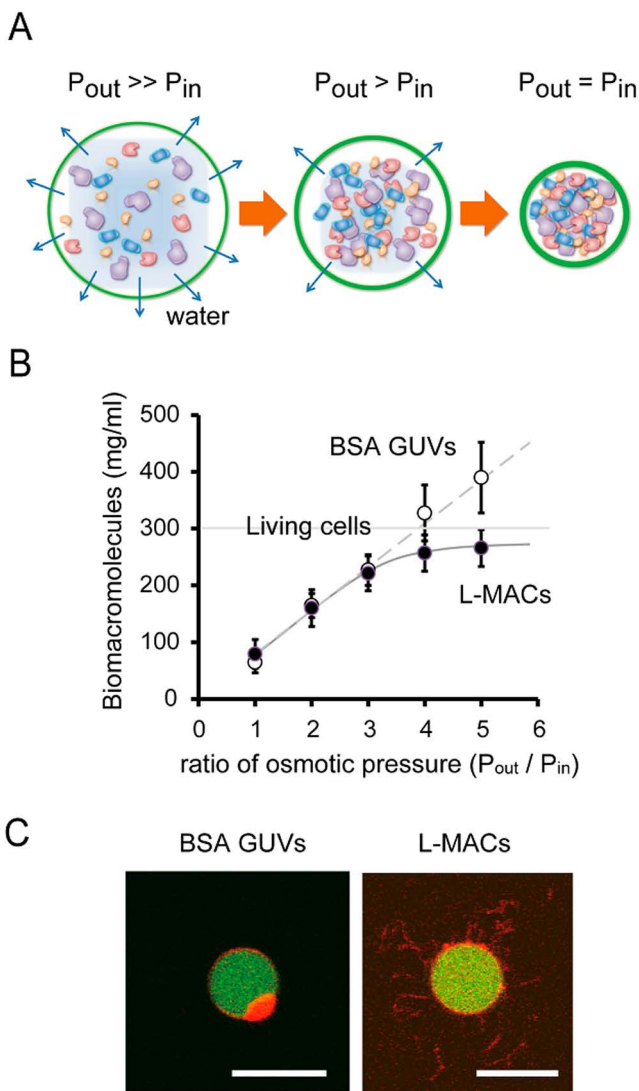
Figure 3 Condensation of macromolecules inside artificial cells. A: schematic illustration of concentrating macromolecules inside artificial cells using hypertonic condition. B: macromolecule concentrations inside BSA GUVs and L-MACs after 1h hypertonic treatment. C: typical shape deformations of BSA GUVs and L-MACs. Lipids were labeled with rhodamine (red). Green color was from GFP fluorescent. Scale bars indicate 10 μm.

macromolecules, but it reached plateau at the concentration. This difference might be resulted from the difference of viscosity between them, because 500 mg/mL of BSA were fluid and was easily pipette, unlike AFCE. Our recent results suggest that diffusion of macromolecules in the L-MACs was extremely slower than that in cells, and slower than that in BSA liposomes (in preparation of manuscripts). Recently, other group found that metabolism-depletion ceases diffusion of large molecules[36]. Since L-MACs belong a kind of metabolism-depleted cells, providing homeostatic metabolism to L-MACs may increase the diffusion rate of macromolecules inside.
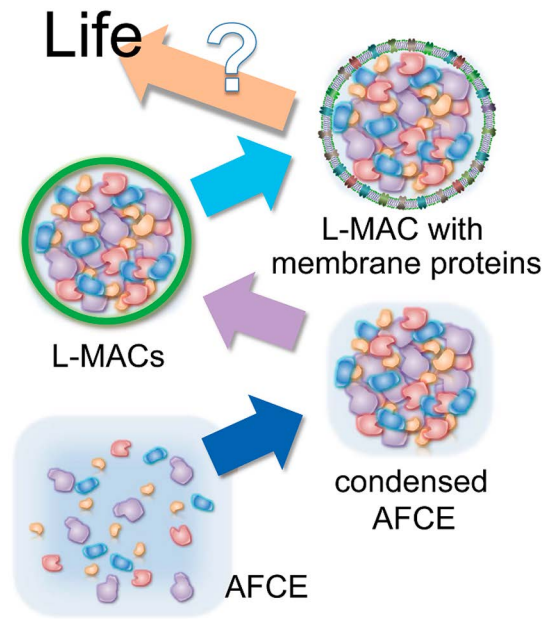
Figure 4 Future perspective of the study. L-MACs do not have membrane proteins. Introducing membrane proteins into L-MACs will reveal the critical difference between living matters and non-living matters.

Second was the morphology change (Fig. 3C). GUVs under hypertonic conditions deform their shapes to compensate the increase in membrane surface area per volume ratio[37]. BSA liposomes showed budding (production of small vesicles around the liposomal membrane), which is commonly observed in deformation process without external forces[37,38]. On the other hands, L-MACs showed tubulation (small tubules were formed around the membrane surface). Tubing deformation needs a larger driving force, ex. by actin or other materials[20]. Thus, AFCE strongly affect the liposomal morphologies, and the mechanism was still elusive.

Future Perspective

Through reconstitution of physiological environments *in vitro* (bulk solutions) and in artificial cells, we found two major disabilities of the condensed AFCE: "low protein expression" and "high viscosity". These disabilities inhibit to convert L-MACs into living cells. The results strongly indicate that simple encapsulation of all essential components inside artificial cells is not enough to produce a life. We cannot deny that our results are from some uncared artifact of our experimental condition. However, our recent results raised a question, "Was LUCA born by simple encapsulation of all essential components for self-reproduction in lipids membrane?"

Membrane proteins dominate a half of surface of biomembrane components, and are essential for homeostasis of living cells. However, L-MACs lack membrane proteins. Do introducing membrane proteins into L-MACs restore the

two major disabilities of the condensed AFCE (Fig. 4)? Through the bottom-up construction of membrane proteins in L-MACs, we believe that we will find the bridge to reconstruct life from biomolecules mixtures.

Acknowledgement

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