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Emergence of Living Cells by Gas-Exposed Sphingosine, DNA and Adenosine

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ABSTRACT

The preparation of self-replicating artificial cells, referred to as DNA crown cells, has been reported. Such cells could be prepared when crown cell seeds were cultured with egg white. Moreover, it was demonstrated that crown cell seeds exposed to gases could induce the emergence of living cells with agar culture. This study reports a novel in vitro method for producing living cells using gas-exposed sphingosine (Sph). Crown cell seeds consist of Sph, DNA, and adenosine and proliferated to DNA crown cells within egg white. Recently, gas-exposed Sph was used to prepare crown cell seeds instead of Sph, resulting in the in vitro emergence new living cells in seed culture. This method represents a practical and scalable alternative to previous egg-based techniques and provides a foundation for future development of synthetic cellular systems. Gas-exposed Sph was named G–Sph, and crown cell seeds prepared with G–Sph were named G–crown cell seeds. The resulting cells were named G–Sph–coli crown cells, reflecting the DNA used in the preparation of crown cell seeds.

Keywords

DNA crown cells, Sphingosine–DNA, G-crown cell seeds, G–Sph–crown cells, Gas powder (Crown bovine meat–Kaiware seeds –ex–P), Monolaurin, Gas production.

Introduction

The creation of self-replicating artificial cells is a central challenge in a synthetic biology and origin of life. In such fields, artificial cells capable of self-replication within egg white were first reported in 2012 [1]. These cells were initially constructed using sphingosine (Sph), DNA and Ascidian (Sea squirt) extract. In 2016, the active components of the Ascidian extracts were identified as the nucleosides uridine and thymidine, with adenosine found to be particularly effective in cell proliferation among nucleosides. Moreover, additional characteristics of artificial cells were described, including artificial cell seeds (hereafter referred to as Crown cell seeds) (Sph–DNA–adenosine), and their general morphology [3].

The outer membrane of artificial cells was identified as being comprised of DNA, resulting in the designation of these cells as "DNA Crown Cells" in 2016 [4].

DNA crown cells, which have been shown to develop into fully self-replicating cells after incubation in egg white, produced using DNA crown cell seeds prepared with Sph, DNA, and adenosine. Synthetic DNA crown cells can be prepared with Sph, DNA, adenosine, and monolaurin, all of which are commercially available compounds. A comprehensive procedure for generating DNA crown cells with synthetic DNA crown cells was reported in 2017 [5] and numerous DNA crown cells and associated cell strains have been produced to date [6-14]. These cells have been prepared and stored stably at 4°C for nearly a decade. Based on these findings, methods for preparing self-replicating cells (DNA crown cells) were established using a egg-based matrix.

In addition, when cultured in agar with egg white, monolaurin, salt, or microorganisms, synthesized DNA crown cells formed assemblies of diverse morphological objects [15-20]. Antibiotic-producing cells were also isolated from beer produced using co-cultures of DNA crown cells and yeast [21]. Egg white powder enclosing different DNA crown cells in conjunction with different substances has been prepared, and numerous antibiotic-producing cells have been produced using agar cultures of these powders [22-28].

In previous experiments, it was shown that gas production occurs when egg white powder enclosing DNA (bovine) crown cells combined with a blueberry extract is cultured [29]. The findings showed that four kinds of gas were produced. Subsequently, further findings showed that exposure of crown cell seeds (Sph, DNA adenosine) to these gasses resulted in the formation of living cells, which were designated as Gas (Bovine blueberry ex) crown cells [30]. This gas-mediated transformation provides a new experimental model for studying the physicochemical process that potentially underlines the origin of living cells and establishes a practical method for generating living cells from non-living DNA. Based on these findings, it was hypothesized that exposing Sph, a component of crown cell seeds, to specific gases could affect molecular interactions and promote spontaneous assembly into cell-like compartments. If successful, this would provide a fully *in vitro* controllable and reproducible method for preparing self-replicating artificial cells. This work establishes a second, more convenient, pathway for artificial cell preparation and expands the toolkit for construction of basic cellar systems. The present findings demonstrate a new method for generating self-replicating cells (designated as G–Sph–crown cells) using gas-exposed Sph (designated as G–Sph) and that new cells could be prepared with relative ease *in vitro*.

Materials and Methods

In summary of methods, the procedure begin with the preparation of G-Sph. G–Sph was produced by co-culturing with cells under the culture conditions established for producing gas powder. Gas powder is identical to antibiotic crown cell-powder, only the name has been changed and the method for preparing the antibiotic crown cells powder has been described previously.

G–crown cell seeds were prepared in a mixture consisting of G–Sph, DNA, and adenosine. The living cells emerge when these cell seeds were cultured.

Materials

Sph (Tokyo Kasei, Japan), DNA (from beef), *E. coli* DNA (Sigma-Aldrich, USA), adenosine (Sigma-Aldrich; Wako, Japan), and monolaurin (Tokyo Kasei). Monolaurin solutions were prepared to a final concentration of 0.1 M in distilled water. Agar plates were prepared using agar medium (SMA) (AS ONE, Japan). Tissue culture plates (35 mm) were obtained from (IWAKI**,** Japan), and Kaiware seeds were obtained from a local market.

Methods

To prepare gas powder requires the preparation of DNA crown cells, followed by egg white powder enclosed DNA crown cells and partner.

Preparation of DNA crown cells [5,22]

Step 1 A total of 180 µL of Sph (10 mM) and 90 µL of Bovine meat DNA (1.7 µg/µL) was combined, and the mixture was heated and cooled twice.

Step 2. A–M solution (100 µL) was added and the mixture was incubated at 37°C for 15 min.

Step 3. A total of 30 µL of monolaurin solution was added, and the mixture was incubated at 37°C for another 5 min.

Step 4 Approximately 0.3 mL of the suspension was then injected into egg white and incubated for 7 days at 37°C. The egg white was recovered and used as DNA (Bovine meat) crown cells.

Preparation of Kaiware seed extracts for use as a partner

Kaiware seeds (about 50 grains) were ground using a mortar and pestle and suspended in 3 mL of distilled water.

Preparation of gas powder (Egg white powder)

1. First, 3 mL of Kaiware seed extract was mixed with 3 mL of egg white.
2. The mixture was incubated for 5 h at 37°C.
3. Then, approximately 25 mL of fresh egg white was added to the mixture.
4. The fluid was poured into two Petri dishes and dried for 1–2 days at 37°C.
5. Dried materials were collected and powder was prepared using a mortar and pestle.
6. The powder was named Gas powder (Crown–Bovine–meat–Kaiware seeds–ex–P).

This powder was stored at room temperature until use.

Procedures and apparatus to prepare G–Sph

A small amount of Gas powder (40–50 mg; Crown Bovine–meat kaiware–ex. P) was added to two agar plates and incubated for 2 days at 37°C. Then, approximately 1.5 mL of 0.1 M monolaurin solution was dispensed onto each plate.

1. Two dishes were placed in the plate which the powder was produced and monolaurin solution was added (Figure 1).
2. Then, 500 µL of Sph was dispensed onto each four dishes, which were then placed in a bag, and the plates were then incubated for 2 days at 37°C.
3. After incubation, the dishes were removed and objects on the surface were collected after the addition of 0.5 mL of distilled water to each dish. The solution (G–Sph) was then stored in a freezer at approximately 4°C.

Preparation of G–Sph crown cell seeds

1. First, 90 µL of DNA (*E. coli*) was treated by heating and cooling twice.
2. Then, 100 µL of adenosine was added to the DNA and the mixtures were incubated at 37°C for 15 min.
3. G–Sph (180 µL) was then added to the DNA–adenosine mixtures and incubated at 37°C for 15 min. The resulting material was designated as G–Sph–crown cell seeds.

Cultivation of G–Sph–crown cell seeds

1. First, 200 µL of G–Sph–crown cell seeds was poured to new agar plate and incubated at 37°C for 18 h.
2. Each plate was then incubated at 37°C for 1, 2 and 3 days (primary culture).

Some of the objects that were grown in the primary culture were cultivated (secondary culture) using fresh agar medium. The plate was then incubated at 37°C for 1 and 2 days to obtain the secondary

culture. The various resulting structures were designated as G–Sph–crown (*E. coli*) cells, specifically, G (Bovine–Kaiware)–Sph–Crown (*E. coli*) cells, with the designation reflecting that these structures were generated though the interaction of gas powder (Bovine–Kaiware seeds ex.P) and crown (*E. coli*) cell seeds derived using *E. coli* DNA.

Figure 1: Shows a photograph of a dish with the lid removed that had been placed on an agar plate at 2 days after spreading the gas–powder.

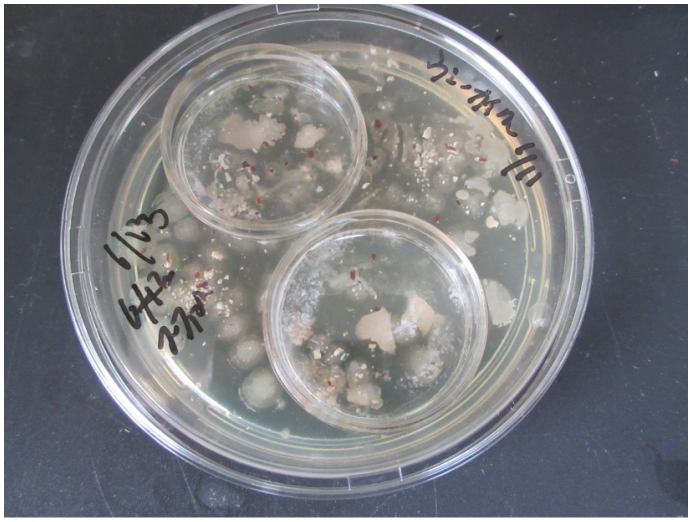

Figure 2: Photograph of a dish with cultures at 2 day after the addition of monolaurin. Brownish objects in the gas-generation system are observed, suggesting that the cultured gas powder interacted in some way with the monolaurin.

Gas-generation system

Gasses were produced after incubating gas–powder for two days following monolaurin addition. Since the gas-generating system comprises cells that are generated through the interaction between the gas produced by the gas powder and the sphingosine, numerous different systems can be established by changing the gas powder, and numerous kinds of G–Sph can potentially be prepared.

General observations

Objects on the plates could be observed directly with the naked eye and under an optical microscope.

Results and Discussion

Figure 1 shows a photograph of a dish with the lid removed that had been placed on an agar plate at 2 days after spreading the gas–powder.

Figure 2 shows a photograph of a dish with cultures at 2 days after the addition of monolaurin. Brownish objects in the gas generation system were observed, suggesting that the cultured gas powder interacted in some way with the monolaurin.

Figure 3 shows several relatively large colony-like objects on the agar medium after culturing the crown cell seeds which were prepared using G–Sph. The image shows that the gas–crown cell seeds were living, i.e., that multiple living cells were generated from non-living gas–crown cell seeds spread onto a agar dish.

Figures 4 and 5 show microscopic images (No. 1 and No. 2, respectively) after 1 day of primary culture.

Figure 6 shows the microscopic appearance of objects after 2 days of culture.

Figure 7 shows the microscopic appearance of objects after 3 days of culture.

Figure 8 shows a photograph of a second-generation culture obtained by culturing a portion of the material shown in Figure 3. The image shows that cells in Figure 3 could be serially cultivated.

Figure 9 shows the microscopic appearance of objects (No. 1) after 1 day of secondary culture.

Figure 10 shows the microscopic appearance of objects (No.2) after 1 day of secondary culture.

Figure 11 shows the microscopic appearance of objects (No. 1) after 4 days of secondary culture.

Figure12 shows the microscopic appearance of objects (No. 2) after 4 days of secondary culture.

Figure 13 shows the microscopic appearance of objects (No. 3) in a secondary culture.

Figure 14 shows the microscopic appearance of objects (No. 4) in a secondary culture.

Figure 15 shows the microscopic appearance of objects (No. 5) in a secondary culture.

Figure 16 shows a photograph of a plate on which some of the cells from the gas-generation system were cultured. Microorganism-like objects were observed, suggesting that the cell-like objects in the gas-generation system were viable.

Figure 17 shows a microscopic image of the growing objects shown in Figure 16.

The results of this study demonstrated that gas-exposed Sph can serves as a robust substrate for the formation of self-replicating artificial cells. It was previously demonstrated that antibiotic-producing cells were generated through the combination of DNA crown cells and partners [21-28]. Importantly, the generation process was found to be common across all combinations tested. Therefore, the use of gas powder prepared from the combination of DNA crown cells (bovine meat) and Kaiware seeds extract in this study is not particularly unique. As noted previously, numerous

antibiotic-producing cells were produced during the culture period following the addition of monolaurin. However, the underlying reason for such a phenomenon and the processes involved during the course of cultivation remain unclear.

To clarify this point, gas production was investigated during cultivation. The findings showed that four kinds of gas were produced [29]: dimethyl sulfide (DMS), 5-(2-methylpropyl) nonane, 1-3-dichlorobenzene, and dodecane. The findings showed that living cells were generated from gas-exposed non-living crown cell seeds [30]. It was hypothesized that exposing sphingosine to specific gasses could alter its molecular interactions and promote the spontaneous assembly of cell-like compartments. Therefore, the present experiments examined whether living cells emerge when G–Sph were used as a components of crown cell seeds instead of Sph.

The DNA–adenosine–G–Sph mixtures were poured in agar plate and incubated for 1 day at 37°C. Many colony like objects were observed (Figure 3).

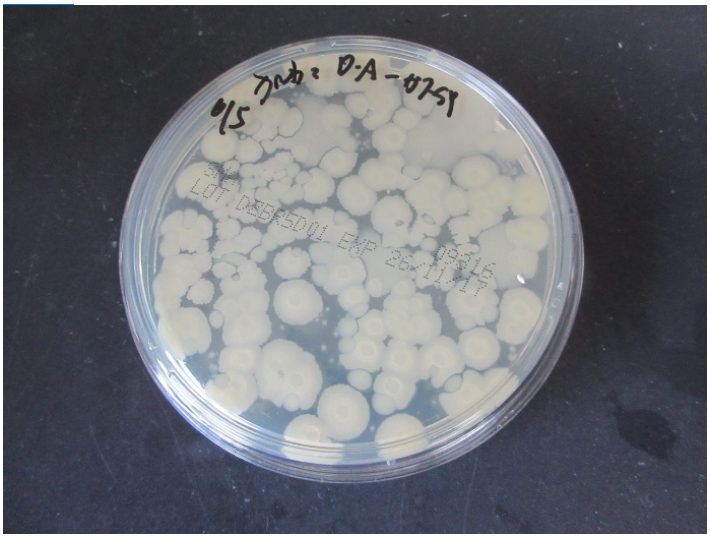

Figure 3: Relatively large colony-like objects on the agar medium obtained after culturing the crown cell seeds prepared using G–Sph (primary culture).

The findings imply that living cells could be prepared from non-living materials (G–Sph crown cell seeds). The observed cells were named G–Sph–*E. coli* crown cells, specifically, Gas (bovine Kaiware ex) crown (*E. coli*) cells. Thus, this would provide a controllable, fully in vitro, and reproducible method for producing living cells. The microscopic appearance of the objects cultivated in the agar plates (is shown in Figures 4-7 and 9-15). Most of these objects exhibited diverse morphologies, including elongated, connected, cottony-like, bar-like, and branched structures. In addition, most of these objects contained colorless structures, possibly representing the origin of the proliferative assemblies. However, experiments have not yet been conducted to clarify the nature or function of these internal components.

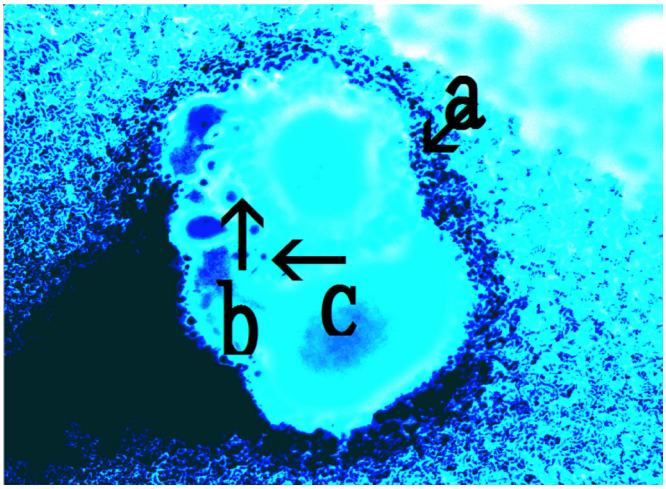

Figure 4: Microscopic image (No. 1) after 1 day of primary culture. A large object (Figure 4a) containing small objects with colorless regions (Figure 4b) is shown. Approximate size: 2–3 μm.

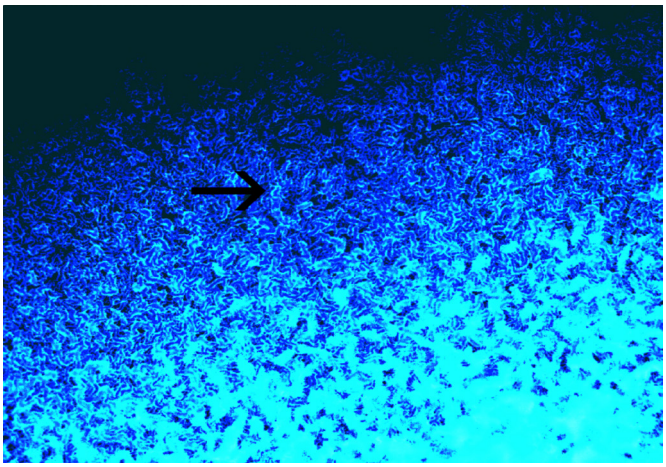

Figure 5: Microscopic image of objects after 2 days of culture. Objects like edge are observed around the membrane. Approximate size, 2–3 μm (Figure 6b).

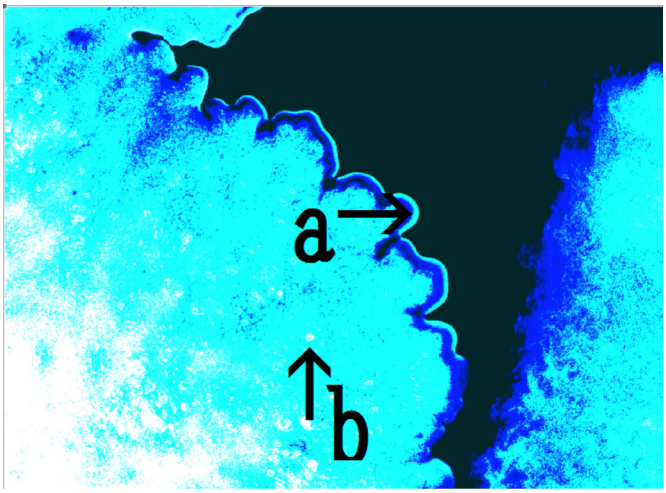

Figure 6: Microscopic image of objects after 2 days of culture. Objects like edge are observed around the membrane. Approximate size, 2–3 μm (Figure 6b).

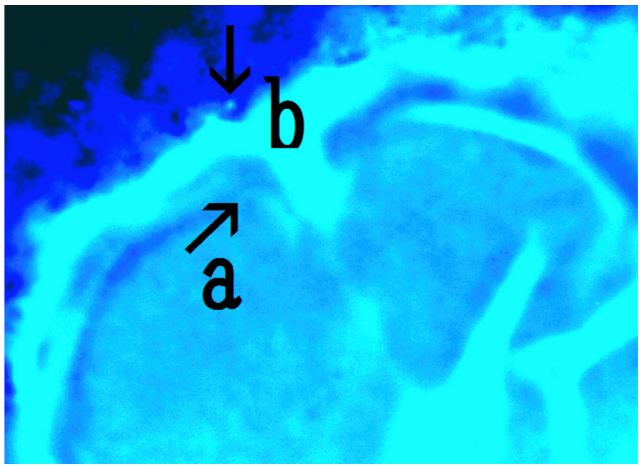

Figure 7: Microscopic image of objects after 3 days of culture. Bar-like objects are shown (Figure 7a). Approximate size, 2–3 μm (Figure 7b).

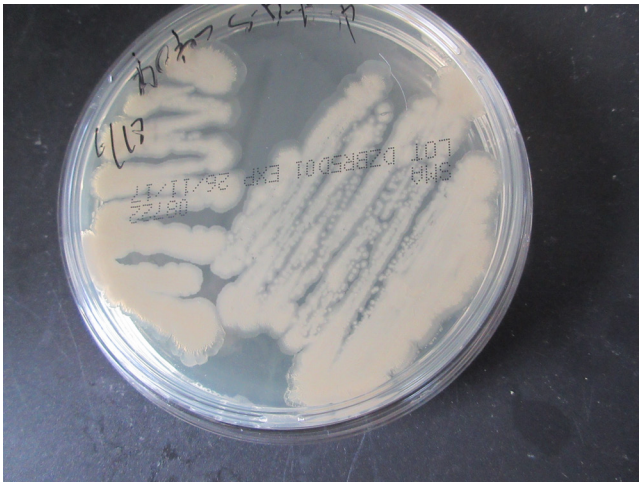

Figure 8: Photograph of second-generation culture obtained by culturing a portion of material shown in Figure 3.

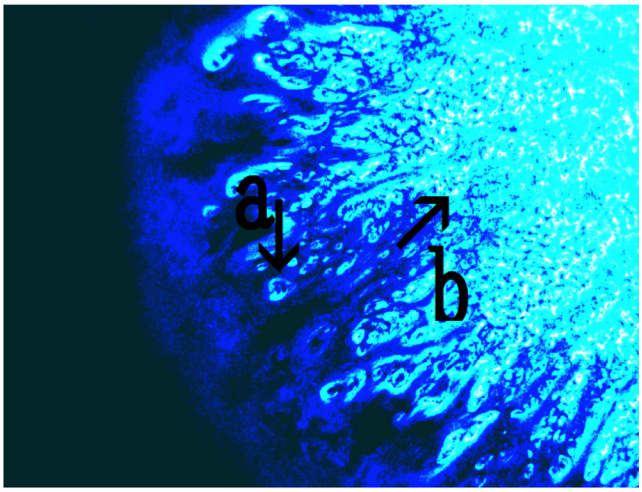

Figure 9: Microscopic image of objects (No. 1) after 1 day of secondary culture. Elongated or round objects are observed (Figure 9a). Approximate size 7–8 μm (Figure 9b).

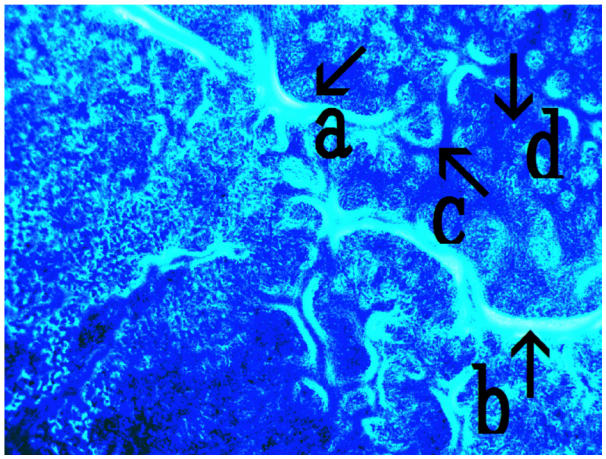

Figure 10: Microscopic image of objects (No. 2) after 1 day of secondary culture.
Bar-like objects of various sizes are shown (Figures. 10a and 10c). Colored objects are also shown (Figure 10b). Approximate size, 2–3 μm (Figure 10d).

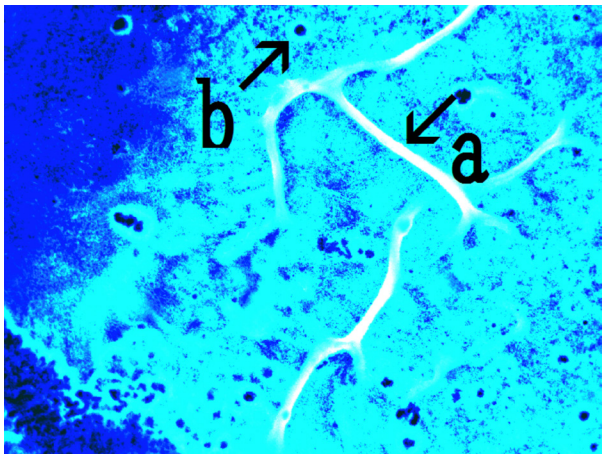

Figure 11: Microscopic image of objects (No. 1) after 4 days of secondary culture. Bar-like objects (Figure 11a) and round objects (Figure 11b) are shown. Approximate size (Figure 11b), 60 μm.

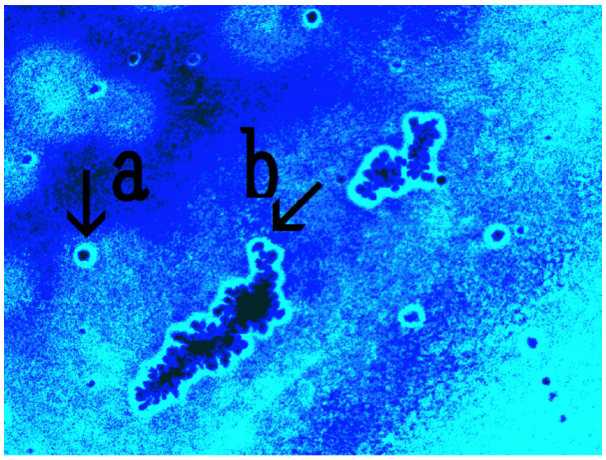

Figure 12: Microscopic image of objects (No. 2) after 4 days of secondary culture. Thorn-like objects (Figure 12b) and round objects are shown (Figure 12a). Approximate size of objects in Figure 12a, 100μm.

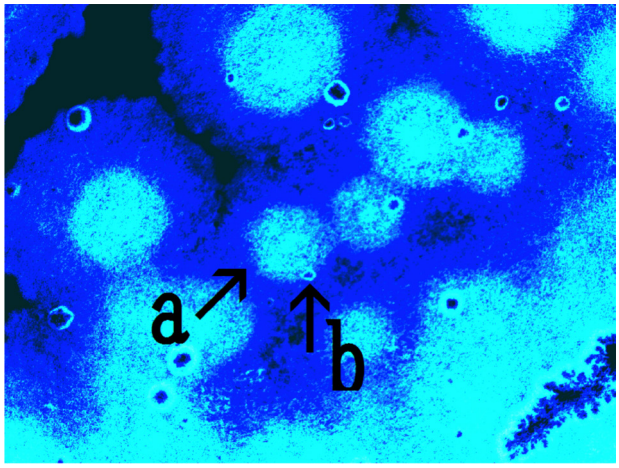

Figure 13: Microscopic image of objects (No. 3) in secondary culture. Cottony-like objects of various sizes (Figure 13a) and round objects are shown (Figure 13b). Approximate size of Figure 13b, 50 µm

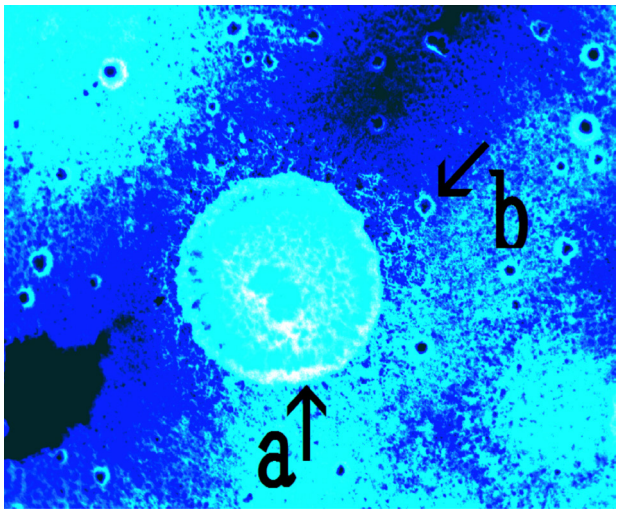

Figure 14: Microscopic image of objects (No. 4) in secondary culture. Large, colorless, Cottony-like objects are shown (Figure 14a). Round objects are also observed (Figure 14b). Approximate size, 60 µm (Figure 14b).

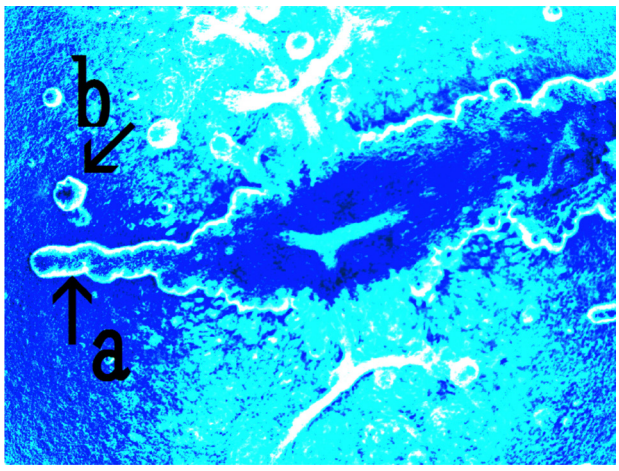

Figure 15: Microscopic image of objects (No. 5) in secondary culture. Numerous colorless bar-like objects (Figure 15a) and round colorless objects (Figure 15b) are shown. Approximate size, 100 µm (Figure 15b).

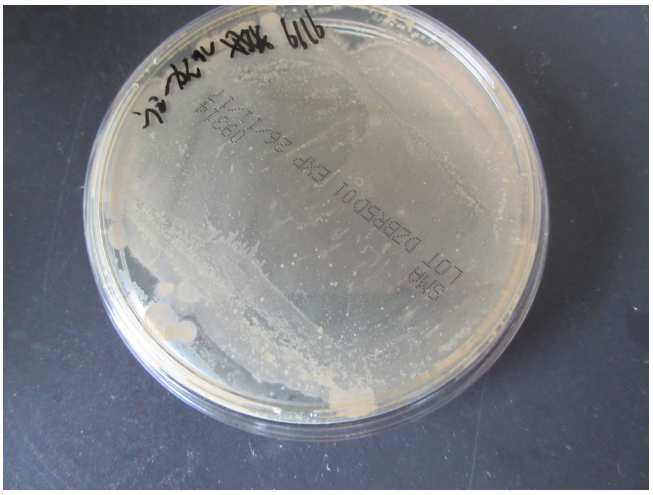

Figure 16: Photograph of a plate on which a portion of cells from the gas-generation system were cultured. Microorganism-like objects are shown.

The findings suggest that gas treatment appears to modify intermolecular interactions, promoting the assembly of dynamic membrane structures capable of autonomous division. This behavior resembles that observed in previous egg-based systems and may represent an overlooked pathway in the transition from non-living to living systems, with gasses possibly acting as a trigger for structural reorganization within DNA crown cell seeds and potentially conferring seeds with a self-replicating ability. Such seeds grew in agar medium and subsequently generated G–Sph–crown cells.

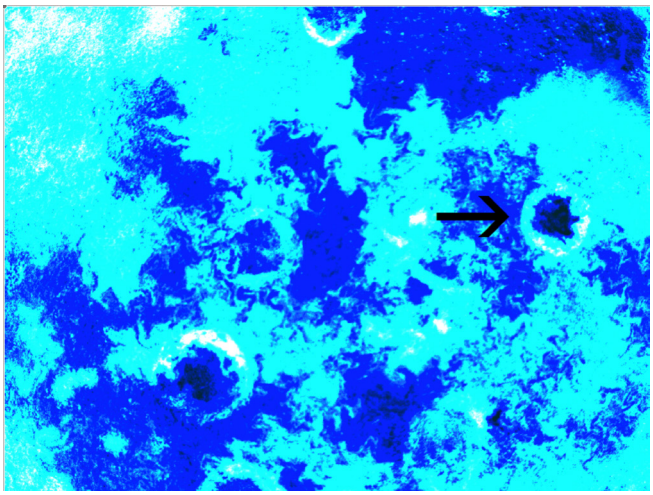

Figure 17: Microscopic image of objects observed growing in Figure 16. Round, colorless objects are shown (Figure 17←). Approximate size, 150 µm (Figure 17←).

A major advantage of the present method is its independence from biological tissues, allowing experiments to be conducted entirely *in vitro*. This improves reproducibility, reduces contamination risk, and facilitates the control of environmental parameters.

Furthermore, the scalability of the gas-exposure process suggests the potential for large-scale production of artificial cells.

The comparison with the egg-based method highlights the complementary nature of the two approaches, while egg-derived matrices provide biologically rich environments. The Sph–gas method offers a minimal and tunable system that is suitable for mechanistic studies. Further studies will involve the preparation of numerous kinds of G–Sph–crown cells using this method and should focus on identifying the specific chemical modifications induced by gas exposure. Importantly, the various fundamental results obtained to date on Gas–Sph crown cells, including the production of DNA crown cells [6-10], antibiotic-producing crown cells [21-28], crown cells Nema [31-34], gas–crown cells [30] have the potential to contribute substantially to other fields. Given the limited space available, only three of these potential applications are mentioned here.

First, countless unidentified microorganisms exist on Earth. Many contribute greatly to human wellbeing; yet how they emerge and grow remains largely unknown.

Second, microorganisms provide a wide range of fermentation products that are beneficial to humans, but many effective strains cannot be isolated from the natural environment.

Third, numerous diseases caused by cellular abnormalities, including cancer, remain difficult to treat.

Taken together, this study has the potential to address some of these challenges by providing insights that may advance our understanding of microbial origins and, consequently, improve our ability to isolate and culture beneficial microorganisms, and contribute to restoration of abnormal cellular states. Thus, these cells may support advances in scientifically and industrially fields without limitation and may contribute substantially to human welfare.

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