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INVESTIGATION OF THE CHEMICAL COMPOSITION OF ARTIFICIAL CELL SEEDS: SPHINGOSINE-DNA BOUND COMPONENTS FROM EXTRACT OF THE MEAT FROM ADULT ASCIDIANS

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ABSTRACT

Objective: To examine the components from the meat of adult ascidians (H-extract) that bound to sphingosine-DNA.
Methods: High performance liquid chromatograpy (HPLC) was used to examine the components of the H-extract fraction that bound to sphingosine-DNA.
Results: At least 15 such components were detectd in the HPLC analysis. Of these 15 components, three were nucleosides-uridine,guanocine, and thymidine. These findings showed that at least 15 distinct components, including multiple nucleosides, bound to Sph-DNA. Also, small particles were formed when uridine alone was added to Sph-DNA,
Conclusion: At least one type of artificial cell seeds comprised just three compounds;Sph,DNA, and uridine. The extract fraction that contributed to seed formation contained at least 15 components, including the nucleosides uridine, guanosine, and thymidine. Additionally, seeds for generating artificial cells could be produced by combining Sph-DNA with uridine.

Key words: Artificial cell seeds, Seeds composition, Sph-DNA-uridine.

INTRODUCTION

Artificial cells are potentially useful for the study of a wide variety of problems in the life sciences (Cavalier, Smith T, 1987, Gilbest). Until recently, most attempts to generate artificial cells have resulted in only cell-like vesicles that contain only a few cellular components, such as DNA and enzymes (Noinedux *et al*., 2011). Such vesicles can do many things that cells can do, including transcribe RNA, translate proteins, and generate ATP. Previously, I described a method for generating fully operational (self-replicating) artificial cells (Inooka, 2012). Artificial cells that can be cultivated and can produce protein were generated by combining tissue extract prepared from the meat of adult ascidians (H-extract) with lipid and DNA. However, the characteristics of H-extract that are associated with the generation of both artificial cell seeds and artificial cells themselves remain unclear. Here, H-extract was fractionated, and the fractions were used to assay the generation of artificial cells. The initial findings showed that the H-extract fraction that lacked protein and nucleic acids was associated with artificial cell generation. Next, high performance liquid chromatography (HPLC) was used to examine the components of the H-extract fraction that bound to sphingosine (Sph)-DNA during seed generation.

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At least 15 such components were detected in the HPLC analysis. Of these 15 components, three were nucleosides—uridine, guanocine, and thymidine. These findings showed that at least 15 distinct components, including multiple nucleosides, bound to Sph-DNA. Moreover, I showed that small particles were formed when uridine alone was added to Sph-DNA, and I found that these small particles were seeds that could generate artificial cells; specifically, artificial cells were generated when these small particles were incubated with white-egg. The findings indicated that at least one type of artificial cell seeds comprised just three compounds: Sph, DNA, and uridine.

Additionally, the HPLC findings presented two interesting possibilities.

- Components that bind to Sph-DNA may metabolize and generate new components
- Minor or other components that were not detected with the methods used may also have been crucial to production of the artificial cell seeds.

Such possibilities will give me a new chance to resolve current problems in the basic life sciences how seeds that are formed when Sph- DNA- uridine acquire self-replicating abilities and generate for artificial cells.

MATERIALS AND METHODS

Preparation of *Halocynthia roretzi* extract (H-extract)

H-extract was prepared from the meat of an ascidian, the edible sea squirt (*Halocynthia roretzi*). In the present study, ascidian meat and NaCl were mixed at a mass ratio of 95%:5% to generate H-extract.

Sample preparation of the fraction from H-extract that was associated with artificial cell generation

The following protocol was designed to generate samples from H-extract and to use those samples in assays for artificial cells generation. The protocol was similar to the protocol for phenol/chloroform extraction of DNA. Here, one case is described

- 83 mg (wet weight) of H-extract was incubated at 65^{o}C for 30 min in a microfuge tube. Then, 400 µl of F-solution from the DNA extraction kit DNAs-ici!-F (Rizo Inc,Japan) was added to the tube and were mixed. Then, 400 µl of phenol and 400 µl of chloroform were added to the tube. The tube contents were mixed until an emulsion formed.
- The tube was centrifuged for 10 minutes at 6,714 xg
- Precipitates were kept at 4^{o}C (Sample 2)
- A pipette was used to transfer 300 µl of the upper layer of liquid (the aqueous phase) to a fresh tube.
- An equal volume (300 µl) of isopropanol was mixed with the aqueous phase in the fresh tube.
- The tube was centrifuged for 10 minutes at 15,107 xg.
- The upper fluid (Sample-1) was collected and kept at 4^{o}C.
- 1 ml of 70% ethanol was added to the tube in f).
- The tube was centrifuged at 15,107 xg for 10 minutes.
- The precipitated DNA fraction was dissolved in 45 µl of distilled water. The fraction is used as a sample-3.
- Samples 1, 2, and 3 were each dried and then dissolved in 1 ml of distilled water. These samples were used for the generation of artificial cells.

Artificial cell generation assay

Tests for generation of artificial cells were carried out as previously described [4]. Briefly, 90 µl of 10 mM Sph (Sigma, USA) and 40 µl of 1.7 µg / µl of DNA from *Escherichia coli* strain B (Sigma, USA) were mixed for about 90 sec in a glass tube; this mixture was then boiled for a few minutes. For each sample type (1, 2, and 3), 300 µl of sample was separately mixed with approximately 100 µl of the Sph-DNA mixture. It was used in each assay. A hole was drilled in the shell of an edible White Leghorn egg (from the market) and 100~200 µl of Sph-DNA-sample mixture (potential seeds) was injected into the white (albumin) of individual eggs. Each egg was then incubated for 5~7 days at 37^{o}C. Whole albumin was collected from each egg that potentially contained artificial cells, and 1~2 ml of each egg-white was transferred to 10 ml of Dulbeccos' high glucose-modified Eagle's medium containing 10% bovine serum (DMEM) in a screw-capped culture tube and incubated at 37^{o}C. The generation of artificial cells was assessed based on the presence or absence of aggregates at the bottom of each culture tube; phase contrast microscopy was used to detect aggregates.

Integrity study on seeds (Sph-DNA-sample 1 mixture)

Phase contrast microscopy was also used to assess the structural integrity of the seeds. To test whether the seeds contain DNA, the seeds were stained with ethidium bromide solution; these stained samples were smeared on glass slides and directly observed by fluorescence microscopy.

Preparation of HPLC samples

To investigate the substances adsorbed to Sph-DNA, Sph (10 mM, 450 µl) and DNA (1.7 µg / µl, 300 µl) were mixed for about 1 minute, and this mixture was then heated. Sample 1 (450 µl) was added to the mixture, and each Sph-DNA-sample 1 mixture (seeds) was divided in two tubes (each about 450 µl). Precipitates were collected. They were then suspended in water and dried. Dried materials were dissolved in 1 ml of buffer (50 mM NaH_2PO_4, pH 2.4). Also, untreated sample 1 (Sph-DNA non-adsorbed sample 1) was dissolved in buffer. Several kinds of nucleosides were prepared to estimate substances in HPLC. Subsequently, HPLC was conducted using the following protocol.

Column: Develosil RPAQUEOUS (C30 UG-5) 4.6 mm I.D.X250 mm (NOMURA CHEMICAL. CO., LTD, Japan). Flow Rate: 1.0 ml/min,

Temperature: 40^{o}C Eluent: 50 mM NaH_2PO_4 (pH 2.4)/acetonitrile(97/3), Volume of sample; 10 µl Detection: UV at 260 nm.

Artificial cells generation using uridine, and related nucleosides.

Whether artificial cells were generated with uridine instead of H-extract was tested as described above. Briefly, 90 □l of Sph (10 mM) and 40 □l of DNA (1.7□□g /□□l) were mixed; the mixture was then heated. 300 □l of uridine solution (Sigma, 0.1 M) was added into the Sph-DNA mixture. In the same manner, guanosine, cytidine, thymidine, and adenosine from Sigma (each 0.1 M) were also tested. Then, each mixture was separately injected into the white (albumin) of a separate egg. After incubation at 37^{o}C for 7 days, 1-2 ml of egg white that potentially contained artificial cells was transferred to DMEM.

RESULTS

Components that associated to generate artificial cells in H-extract

Samples 1, 2, and 3 were obtained following phenol/chloroform extraction of DNA, and then used in assays for artificial cell generation. No artificial cell generation was observed with sample 2 or sample 3. In contrast, artificial cells were generated when sample 1 was used in the assays. Specifically, mixtures of Sph-DNA and sample 1 were injected into egg white and incubated for 5~7 days at 37^{o}C. Artificial cells were observed following egg-white incubation (Fig. 1A). Sample 2 was the fraction that precipitated with phenol/chloroform and may contain most of the protein from the H-extract. Sample 3 contained the nucleic acids from the

H-extract. Therefore, sample 1 was the fraction devoid of protein and nucleic acid. The findings showed that Sph-DNA was bound to H-extract substances that were not protein or nucleic acid.

Integrity of seeds formed with Sph-DNA-sample 1

Mixing Sph with DNA from quail lymphocytes leads to the formation of fibrous assemblies (Sph-bound DNA) that become particles (Sph-DNA particles) when heated. [5] Seeds of artificial cells were obtained by adding H-extract to these Sph-DNA particles. Here, we examined whether such seeds would form when sub-fractions of H-extract (sample 1) was used instead of H-extract.

When sample 1 was added to Sph-DNA particles, large aggregates formed and could be seen with the naked eye (Fig. 1B). The aggregates seemed to be very weakly bound because they broke upon shaking. The aggregates were further examined by phase contrast microscopy. Most aggregates appeared as roughly fibrous assemblies (Fig. 1C).

To test whether the assemblies contained DNA, the aggregates were stained with ethidium bromide solution and observed by fluorescence microscopy. Russent light was evident in the fibrous assemblies (Fig. 1D). The findings demonstrated that sample 1 bound to Sph-DNA particles and formed fibrous assemblies.

HPLC study of the components of sample 1

The components of sample 1 that bound to Sph-DNA were examined by HPLC. Sph and DNA were mixed and boiled; sample 1 was added to the mixture. Then, precipitate was recovered and dissolved in 1 ml of buffer.

The chromatogram from the HPLC analysis of this precipitate is shown in Fig. 2a. The chromatogram indicated that at least 15 components from sample 1 had bound to Sph-DNA particles, and most of these components had a retention time of between 2 min and 10 min.

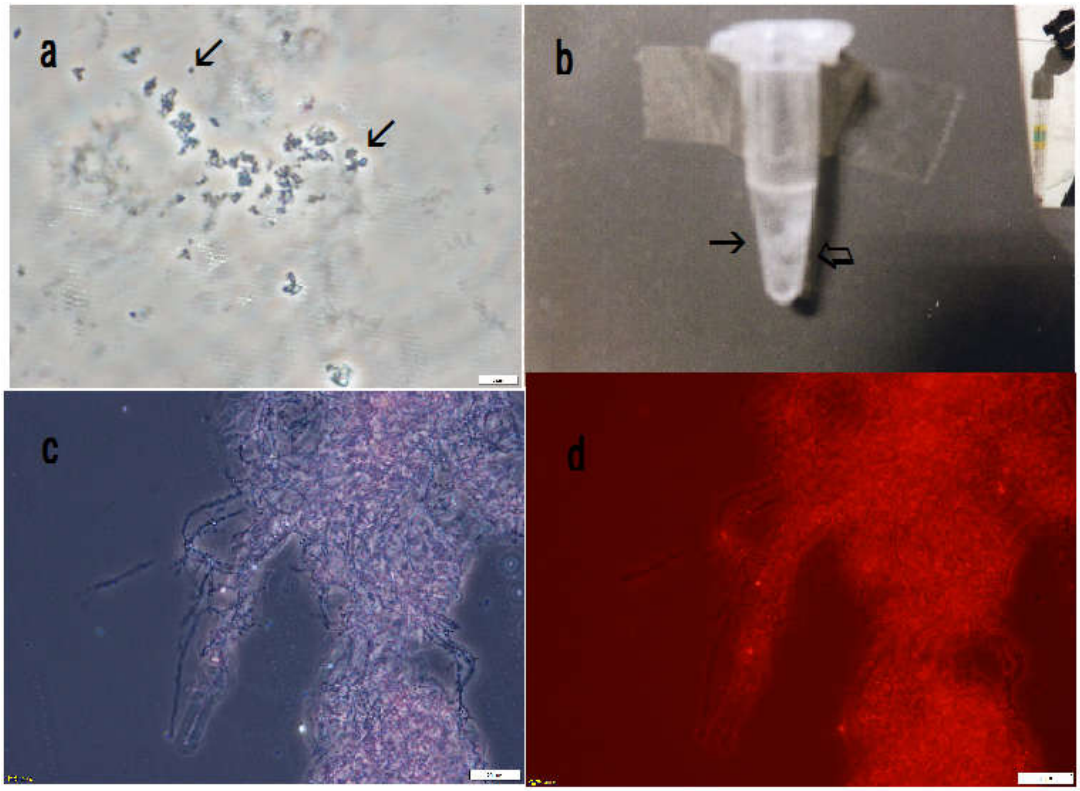

Fig.1 Inooka

Figure 1. Artificial cells that were generated with Sphingosine (Sph)-DNA-sample 1

a) The seeds (the mixture of Sph-DNA-sample1) of artificial cells were incubated in egg white at 37°C for 5 days, and 1 ml of egg white was cultivated in Dulbecco's high glucose-modified Eagle's medium containing 10% bovine serum (DMEM). Aggregates were smeared on slide glass and observed under a phase contrast microscope. Artificial cells(arrow) were observed as a dot or aggregates. (Scale bar 5 μm)

b) Sph was added to DNA. Then, sample 1 was added to the sph-DNA mixture. Aggregates (arrow) that could be observed with the naked eye had formed.

c) and d) Fluorescence microscopy of the Sph-DNA-sample 1 assembly Sph was added to DNA. After mixing, sample 1 was added to Sph-DNA mixtures. Then, the mixtures were stained with ethidium bromide solution and smeared on a slide glass. The images in c) and d) are the same field of view.

c) Phase contrast microscope image without filters, Scale bar 20 μm

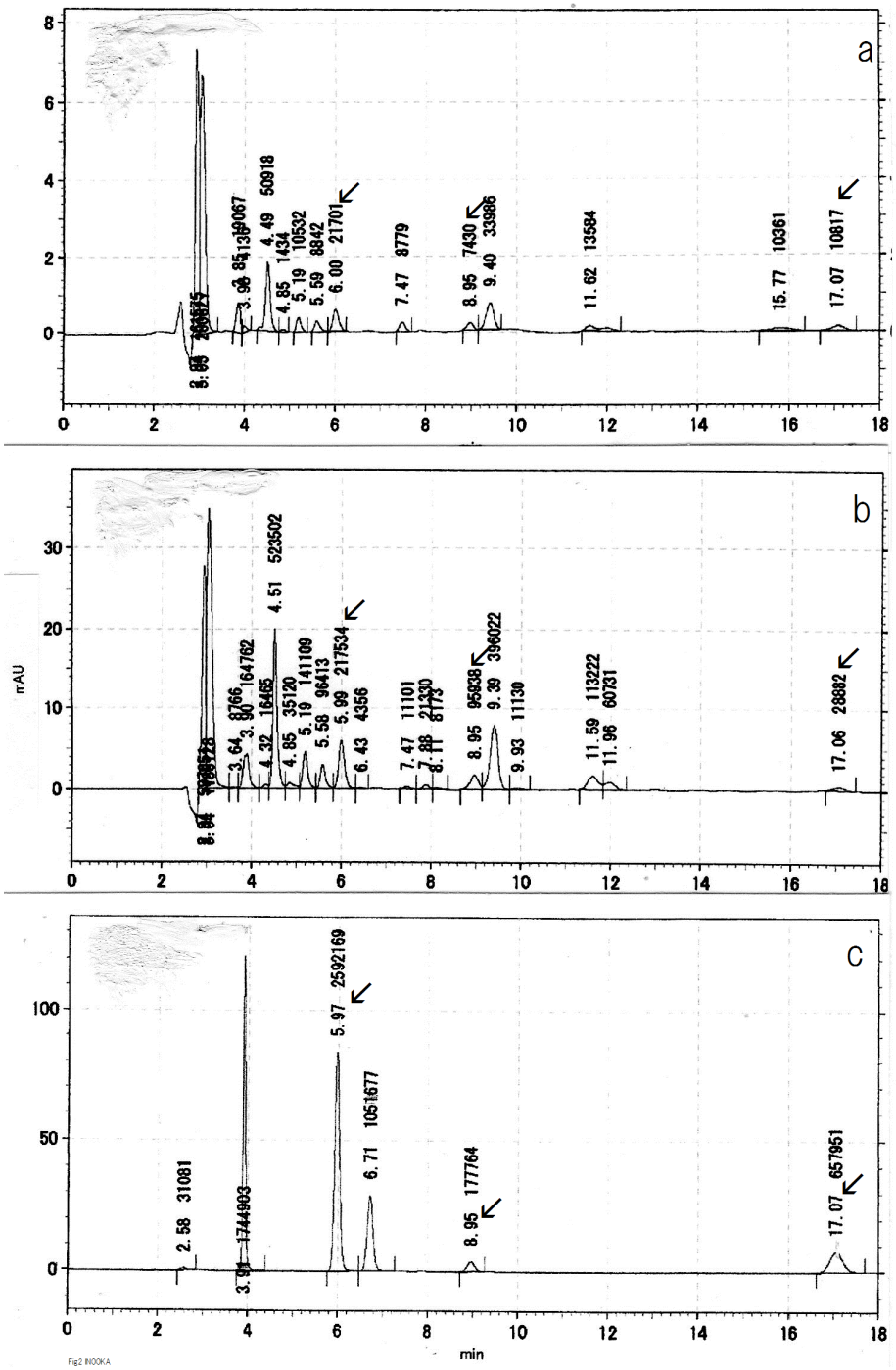

Figure 2 High performance liquid chromatography (HPLC) chromatograms of the components from sample 1 that bound to Sph-DNA Sph and DNA were mixed. After boiling, sample 1 was added to the Sph-DNA mixture. Precipitates were recovered and dissolved in buffer

a) The chromatogram of the adsorbed substances of sample 1
Vertical scale is absorbance at 260 nm, and the chapter on the vertical scale represents retention time (minutes). At least 15 components are represented in the vertical scale. Uridine (6.00 in retention time), guanosine (8.95 in retention time) and thymidine (17.07 in retention time), respectively, were represented with arrow.

b) The chromatogram of the non-adsorbed substances from sample 1(as a control).
Vertical scale is absorbance at 260 nm, and the chapter on the vertical scale is retention time (minutes). At least 20 components are represented in the vertical scale. Uridine (5.99 in retention time), guanosine (8.95 in retention time) and thymidine (17.07 in retention time),respectively, were represented with arrow.

c) The chromatogram of purified nucleosides.
Uridine (5.97 in retention time), guanosine (8.95 in retention time,) and thymidine (17.07 in retention time), respectively,

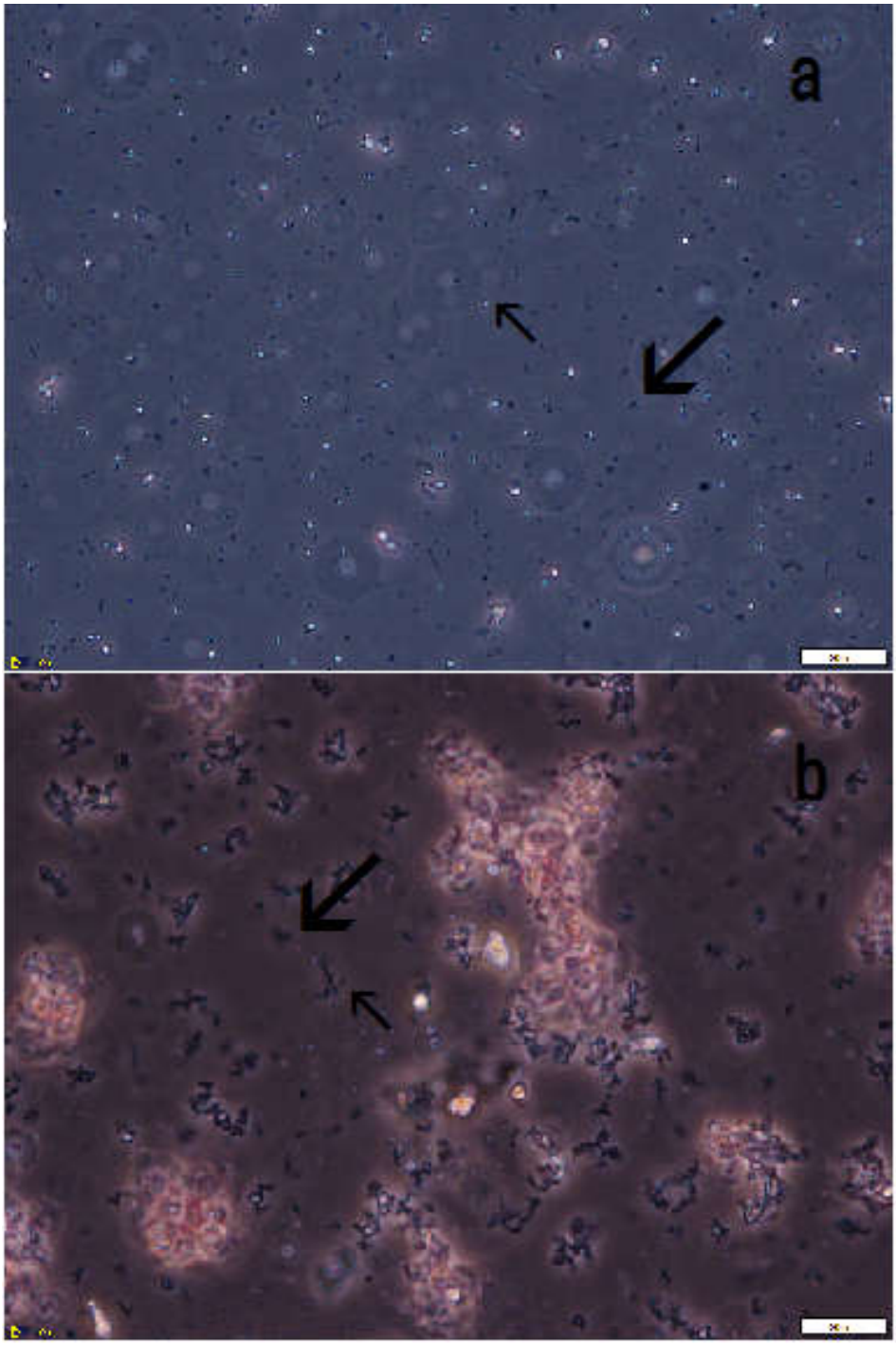

Fig3 Inooka

Figure 3 Structural integrity of seeds and artificial cells that were formed in the Sph-DNA-uridine mixture

a) Seeds: Sph was added to DNA. After mixing, uridine was added to the sph-DNA mixture. A drop was smeared on a slide glass, and was observed by phase contrast microscopy. Many particles, all almost identical in size, were observed as dots (arrow). Scale bar 20 μm.

b) The seeds in a) were incubated in egg white for 5 days, and 1 ml of egg white was then cultivated in D-MEM for 2 days. These artificial cells were smeared onto a glass slide. These cells are observed as dots (arrow) or aggregates (arrow), respectively. Scale bar, 20 μm.

The chromatogram of the precipitate from non-adsorbed sample 1 is shown as a control in Fig. 2b. Non-adsorbed sample 1 contained at least 20 components, and most of these components had a retention time between 2 min and 10 min. Though there was a difference in the number of components between adsorbed sample 1 and non-adsorbed sample 1, the HPLC profiles were nearly identical for these two sample types.

Additionally, several components of the sample were tentatively identified based on retention times and comparisons with standard compounds (Fig. 2C). Both the adsorbed sample and the non-adsorbed samples contained uridine (6.00 in the adsorbed sample and 5.99 in the non-adsorbed sample), guanosine (8.95 in the adsorbed sample and 8.95 in the non-adsorbed sample), and thymidine (17.07 in the adsorbed sample and 17.06 in the non-adsorbed sample), notably, adenosine and cytidine were not detected in this HPLC analysis of sample 1. The findings indicated that some nucleosides may be associated with the formation of artificial cell seeds.

Generation of artificial cells using individual types of nucleosides

Next, each type of nucleoside detected in sample 1 was used as isolated, purified nucleoside in the artificial cell generation assay to determine whether artificial cells could be generated with nucleosides instead of sample 1. Uridine, which was detected in sample 1, led to the generation of many artificial cells, but neither guanosine nor thymidine led to abundant generation of artificial cells. Sph and DNA were mixed, and heated to generate Sph-DNA particles. Uridine solution was added to the Sph-DNA particles. The mixture was incubated in egg white, and the egg whites were then incubated with D-MEM. Artificial cells that were generated with Sph-DNA-uridine are shown in Fig. 3b. Interestingly, adenosine, which was not detected in sample 1, could be used to generate artificial cells, while cytidine was weak in this assay. The findings demonstrated that 1) various components were adsorbed to Sph-DNA particles, 2) one of these components was uridine, and 3) uridine could interact with Sph-DNA particles and in the absence of sample 1 to generate artificial cells.

Integrity of seeds formed with Sph-DNA-uridine

To investigate the structural integrity of the seeds that were generated in the Sph-DNA-uridine mixtures, Sph was mixed with DNA. After heating this mixture, uridine was added to the Sph-DNA mixture. The structural integrity of these seeds was assessed by phase contrast microscopy. As shown in Fig. 3a, individual seeds were observed as dots, and most seeds appeared as roughly spherical particles (approximately 50 nm in diameter).

DISCUSSION

The mechanisms and elements required for the generation of artificial cells were unclear; elements essential for artificial cell generation may exist in H-extract. Here, it was demonstrated that the components in H-extract fractions that lacked both protein and nucleic acids could be used to successfully generate seeds for the production of artificial cells. The components in the H-extract fraction that lacked both protein and nucleic acids could bind to Sph-DNA mixture and form aggregates with the Sph-DNA particles. HPLC was then used to analyze components recovered from the aggregates. As a result, at least 15 components were found to bind to Sph-DNA particles. Of the 15 components, three were tentatively identified as uridine, guanosine, and thymidine. These findings indicated that some types of nucleosides may be associated with the formation of artificial cell seeds.

Based on these findings, further experiments were performed to test whether individual types of nucleosides, including undetected nucleosides, could be used to generate artificial cells. Uridine could be used to generate artificial cells. In contrast, guanosine and thymidine seemed to be weak with regard to the generation of artificial cells. However, currently, it is not clear whether both nucleosides are associated with artificial cell generation. To clarify this, more studies must be conducted and more details must be determined. Also, it may be important that adenosine, which was not detected in sample 1, was effective for generating artificial cells. Further studies of this finding will be presented in the near future.

Next, seeds that were formed with Sph-DNA-uridine were observed using phase contrast microscopy. Most of these seeds appeared as roughly spherical particles (approximately 50 nm in diameter). It is very interesting and important that homogenous seeds were formed with the use of uridine. This finding indicated that the complex fibrous assembles formed by the adsorption of various kinds of components; in contrast, the binding of a single component, uridine, resulted in formation of simple, homogenous seeds. Moreover, it was demonstrated that the artificial cell seeds that were formed from Sph-DNA-uridine mixtures could be used to generate artificial cells. This finding indicated that one type of artificial cell seed comprised Sph, DNA, and uridine. Thus, I characterized the chemical composition of one type of artificial cell seed and described its structural integrity.

A final problem in this series of studies remains; specifically, how did seeds that were formed with Sph-DNA-uridine acquire the ability to self-replicate and generate progeny artificial cells. A study of the interaction between seeds and egg white, especially of the behavior of nucleosides, will be very important for understanding the mechanisms by which seeds differentiate into self-replicating, artificial cells.

Conclusion

At least 15 such components of the H-extract fraction that bound to sphingosine-DNA. Were detected in the HPLC analysis. Of these 15 components, there were nucleosides-uridine, guanocine, and thymidine. Moreover, small particles were formed when uridine alone was added to Sph-DNA, and it was demonstrated that these small particles were seeds that could generate artificial cells.

REFERENCES

Cavalier-Smith, T. 1987. The origin of cells: A symbiosis between genes, catalysis, and membranes. Cold Spring

Harbor Symposia on Quantitative Biology L11 pp 805-824.

Gilbert, I. W. 1986. Origin of the RNA world. Nature 319: p 618

Inooka, S. 2012. Preparation and cultivation of artificial cells. App. Cell Bio. 26: pp13-18

Krihara, K., Tamur, M., Shohda, K., Toyota, T., Suzuki, K. and Sugawara, T. 2011. Self-reproduction supermolecular giant vesicles combined with the amplification of encapsulated DNA. *Nat. Chem.,* 3: pp775-581.

Noivedux, V., Maeda, Y.T. and Labchaber, A. 2011. Development of an artificial cell from self-organization to computation and self-reproduction. *Pro. Natl. Acad. Sci.,* USA 108: pp 3473-3480.

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