Living Organisms under an Electron Microscope: the NanoSuit®

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Scanning electron microscopy (SEM) has made remarkable progress and has become an essential tool for observing biological materials at microscopic level. However, various complex procedures have precluded observation of living organisms to date. Here, a new method coined the “NanoSuit®” is presented by which living organisms can be observed by an SEM. A simple surface modification to extracellular substances (ECS) of the certain multicellular organisms by electron beams or plasmas can coat of thin polymer membrane made of ECS. The “NanoSuit®” acts as a barrier to the passage of gases and liquids and thus protects the organism. Based on this discovery, we invented the coating method by the “biomimetic ECS” composed of biocompatible substance for the organism which lack the natural ECS. The new “NanoSuit®” methods will be useful for numerous applications, particularly in the life sciences.

Keywords: NanoSuit®, Nano-polymer, Electron microscope, Living organisms, Thin membrane, Morphology, Biomimetics

1. Introduction

About 20 years after the first scanning electron microscopic observation was performed using non-organic materials [1], biological observations began and the fine structure of several organisms was investigated, therefore, many paradigm shifts in biology occurred, especially the findings of viruses and organelles. However, it is necessary to evacuate the specimen chamber in order to prevent scattering by molecules in the air, because an electron microscope uses a beam of electrons to illuminate the specimen. Because approximately 80% of all living organisms are water, the biological samples have been routinely required sacrifice and dehydration before observation, otherwise they are rapidly evaporated under high vacuum, and consequently leads to disruption and collapse of structure. To preserve and stabilize biological structure for SEM observations, complex treatments are required; chemical fixation, careful drying procedures, and coating by electrically conducting materials. Consequently, many and time-consuming procedures were required to observe biological samples. In addition to the traditional treatment, researchers have tried to modify SEM procedures to allow lower vacuum levels, as in low-vacuum scanning electron microscopy or by use of an environmental scanning electron microscope (ESEM) [2-5]. All such methods, however, resulted in inferior resolution. Therefore, no living samples were observed by SEM except few reports [6,7].

In order to observe the living and/or wet biological samples in an SEM, we invented a new method to cover the surface of the samples with a thin extra layer. We coined the “NanoSuit®” which could prevent passing gases and liquid. The simple surface modification by electron beams or plasmas can equip the “NanoSuit®” on biological samples and hence can keep them alive/wet under the high vacuum conditions [8-12]. In this paper, we summarize the role of the NanoSuit® for an SEM observations.

2. Biomimetics technologies
A biofilm can be said to be a familiar example of the slime that can be gathered in the kitchen sink and a plaque of teeth. Biofilms comprise any syntrophic consortium of bacteria in which cells stick to each other. In the medical field, the formation of a biofilm by microorganism, such as *Staphylococcus aureus* in the catheter is a major problem. This is because bacteria wrapped in a biofilm acquire resistance to antibiotics and immunity in the catheter. Among ecosystems, biofilms are also observed everywhere and play an important role in maintaining the ecosystem. Bacteria possesses a cell wall on the outside of the cell membrane, and a biofilm is formed on the outside.

On the other hand, many of eukaryotes release secretions, like the biofilm, to the outside of cells to protect individual cells. We, human beings also secrete outside the skin to enhance the moisturizing effect. Extremely abundant exocrine secretions are found in the larvae of Dipteran (Fig. 1).

![Fig. 1. Larvae of *Drosophila melanogaster*.](image1)

The larvae produces secretions outside the body and the extracellular substances of secretions maintains its external environment. Thus many organisms are able to withstand environmental changes by those secreted biofilms and/or ECS. Organisms might have acquired those drought tolerance in evolution.

### 3. Drought tolerance in high vacuo

In order to find the nature of tolerance of living organisms to *high vacuo*, we introduced several living organism of various taxa directly into an SEM to see whether they could survived under high vacuum conditions. We found that the larvae of the fruit fly *Drosophila melanogaster* (Oregon-R) were tolerated in *high vacuo*, when they were irradiated by electron beam immediately in an SEM (Fig. 2). Although the larvae possesses a soft cuticle, they continued to move actively around 60 min in high vacuum condition.

![Fig. 2. Hydra (A), Planarian (B), and Mosquito larvae (C) were dehydrated but not the larvae of *Drosophila melanogaster* (D).](image2)

These results led to the hypothesis that electron-beam or plasma irradiation enhanced cross-linking within the ECS to form a durable polymer on the surface and that this polymer increased resistance to vacuum conditions (Fig. 3). This finding could shed light on the new observation technique for various living/wet organic samples with a high resolution SEM.

![Fig. 3. The living *Drosophila* larva which was exposed with electron-beam or plasma irradiation (C, D), it can survive in high vacuum condition (A). TEM images are shown of vertical sections through the surface of the animal. The layer between the arrowheads in (B) indicates the limits of the newly formed outer membrane.](image3)

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### 4. NanoSuit® method for the organism with low ECS

To investigate the chemical properties of the ECS of *Drosophila* larvae, we carried out FTIR analysis. This revealed that the ECS contained amphiphilic molecules. From a biomimetic point of view, solutions including nontoxic amphiphilic molecules were then tested in an attempt to mimic
the ECS layer. To test the barrier properties of the NanoSuit® made by this solution, the surfaces of several different animals previously unable to survive SEM exposure were provided exogenous materials by immersing them in nontoxic amphiphilic solution before electron or plasma irradiation. When live larvae of mosquito were observed under the SEM without any additional treatment, they quickly shrank and ceased to move. Larvae treated with a nontoxic amphiphilic solution but not irradiated in the SEM showed the same collapsed structure when observed 30 min later.

However, larvae covered with nontoxic amphiphilic solution and observed by SEM ab initio retained their morphology and exhibited active movements for 30 min.

5. NanoSuit® method for the life science

Although the nontoxic amphiphilic based NanoSuit® has a property of surface shield effect and protected some organisms in an SEM, it was unable to protect isolated tissues excised from intact organisms. To overcome this limitation, we have modified the technique and developed a new solution; surface shield enhancer (SSE) solution, which enables FE-SEM observations on wet tissues. The samples by NanoSuit® method for “wet fixed specimens” and by the conventional method for the dried fixed pathological samples were compared. The samples prepared with conventional method (Fig. 4 A, B) were completely different from the specimens treated with SSE solution (Fig. 4 C, D).

6. An example of design and implementation of a biomimetic gloves for cancer patients

Many patients undergoing cancer medication therapy sometimes suffer from several disorders, one of which is the disappearance of fingerprints. Because fingerprints are one of the important forms of skin that governs the function of grasping objects, those patients fail to grab things, therefore, various restrictions are caused in their daily life. They are seeking to be able to spend their daily life as smoothly as possible by themselves. It is urgent to improve their quality of life.

To solve this problem, we used the idea of “Biomimetics”. Legs of geckos and insects have very high adhesiveness. Its’ adhesion is due to the accumulation of Van der Waals forces caused by the fact of dense hairs called SETA (bristle) of which tips are micrometre size. We looked for the similar cloth to SETA using NanoSuit® method. Inspired by the function and structure of SETA, we developed new gloves using the NANOFRONT® cloth which possesses the similar ultra-structure as SETA (Fig. 5).

7. Discussion

In order to observe living organisms by an SEM in high vacuum, there are two major problems to be solved: how to produce a sufficiently thin barrier of gas and/or liquid through which to observe surface fine structure; and how to increase the electric conductivity of the surface of the animal, without any toxicity or damage to the living/wet organism. We found that after modification of material on the surface of organisms through exposure to an electron beam or plasma ionization, i.e. conditions known to enhance polymer formation, and the treated organisms possess the potential to tolerate high vacuum environments without any electrical charges. The advance that we have made is to
preserve life in high vacuum long enough to observe the active movements of living specimens at good resolution. In addition to the discovery described above, other organisms which lack ECS were successfully protected with a NanoSuit® composed of nontoxic amphiphilic molecules. Those results revealed that the natural ECS and the artificial compounds play the same role to compose the “NanoSuit®” to protect the life of whole organism in a harsh environment in an SEM.

By contrast, excised tissues do not have such a protective cover and the amphiphilic solution is harmful for naked cell membranes, so that an alternative barrier was needed. We used a surface shield enhancer solution with glycerine as a main component.

The structures observed by the “NanoSuit®” method showed apparent differences from those obtained by the conventional method. This ongoing progress in electron microscopy is starting to have a significant impact on our understanding of the subcellular world. The several NanoSuit® method will allow more sophisticated observation to reveal many real structures from the subcellular size materials to the whole organism as living state.

8. Conclusion

Here we showed that a simple surface modification can render living/wet organisms strongly tolerant to high vacuum condition coat-like nontoxic amphiphilic substances to an electron beam or plasma ionization, i.e., conditions known to enhance polymer formation.

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References
1. M. Knoll, Z. Tech. Phys., 16 (1935) 467.
2. G.-D. Danilatos, J. Microsc., 162 (1991) 39.
3. A. Mohan, N. Khanna, J. Hwu, and D. C. Joy, J. Scan Microsc., 20 (1998) 436.
4. W. O. C. Symondson and I. B. Williams, Entomol. Exp. Appl., 85 (2003) 75.
5. D. J. Stokes, “Science, Technology and Education of Microscopy an Overview”, (2003) 564.
6. R. F. Pease, T. L. Hayes, A. S. Camp, and N. M. Amer, Science, 154 (1966) 185.
7. A. Sokoloff, T. L. Hayes, R. F. W. Pease, and M. Ackermann, Science, 157 (1967) 443.
8. Y. Takaku, H. Suzuki, I. Ohta, D. Ishi, Y. Muranaka, M. Shimomura, and T. Hariyama, Proc. Natl. Acad. Sci. USA, 110 (2013) 7631.
9. I. Ohta, Y. Takaku, H. Suzuki, D. Ishii, Y. Muranaka, M. Shimomura, and T. Hariyama, Microscopy, 63 (2014) 295.
10. Y. Takaku, H. Suzuki, I. Ohta, T. Tsutsi, H. Matsumoto, M. Shimomura, and T. Hariyama, Proc. R. Soc. B, 282 (2014) 2857.
11. Y. Takaku, H. Suzuki, H. Kawasaki, I. Ohta, D. Ishii, S. Hirakawa, T. Tsutsui, H. Matsumoto, S. Takehara, C. Nakane, K. Sakaida, C. Suzuki, Y. Muranaka, H. Kikuchi, H. Konno, M. Shimomura, and T. Hariyama, R. Soc. Open Sci., 4 (2017) 160887.
12. S. Takehara, Y. Takaku, H. Suzuki, I. Ohta, M. Shimomura, and T. Hariyama, Sci. Rep., 8 (2018) 1685.