A ‘NanoSuit’ surface shield successfully protects organisms in high vacuum: observations on living organisms in an FE-SEM

Yasuharu Takaku1,5, Hiroshi Suzuki2, Isao Ohta3, Takami Tsutsui1, Haruko Matsumoto1, Masatsugu Shimomura4,5 and Takahiko Hariyama1,5

1Department of Biology, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan
2Department of Chemistry, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan
3Laboratory for Ultrastructure Research, Research Equipment Center, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan
4Departments of Bio- and Material Photonics, Chitose Institute of Science and Technology, 758-65 Chitose, Hokkaido 066-8655, Japan
5CREST, Japan Science and Technology Agency, Hon-cho 4-1-8, Kawaguchi 332-0012, Japan

Although extremely useful for a wide range of investigations, the field emission scanning electron microscope (FE-SEM) has not allowed researchers to observe living organisms. However, we have recently reported that a simple surface modification consisting of a thin extra layer, termed ‘NanoSuit’, can keep organisms alive in the high vacuum (10⁻⁵ to 10⁻⁷ Pa) of the SEM. This paper further explores the protective properties of the NanoSuit surface-shield. We found that a NanoSuit formed with the optimum concentration of Tween 20 faithfully preserves the integrity of an organism’s surface without interfering with SEM imaging. We also found that electrostatic charging was absent as long as the organisms were alive, even if they had not been coated with electrically conducting materials. This result suggests that living organisms possess their own electrical conductors and/or rely on certain properties of the surface to inhibit charging. The NanoSuit seems to prolong the charge-free condition and increase survival time under vacuum. These findings should encourage the development of more sophisticated observation methods for studying living organisms in an FE-SEM.

1. Introduction

Soon after the first scanning electron microscopic observations were performed in 1935 using non-organic materials [1], biological observations followed, and the fine structural details of many organisms were investigated. Because the scanning electron microscope (SEM) produces images by probing the specimen with a focused electron beam, the specimen chamber requires a high vacuum to prevent electron scattering. Hence, biological samples routinely require fixation and dehydration before observation, because approximately 70–80% of all organic matter is water, which rapidly evaporates under high vacuum and consequently causes structural disruption and collapse. To stabilize biological specimens for conventional SEM observations, complex treatments are required: chemical fixation, dehydration and critical point drying [2]. Furthermore, non-conductive materials cannot be imaged in a conventional SEM because they are poor emitters of secondary electrons owing to the low atomic number of carbon. Therefore, samples usually require an ultrathin coating of electrically conducting materials [3]. Consequently, the preparation of biological samples for SEM is time-consuming and, as a rule, does not permit observations of living samples.

To avoid complex procedures and to overcome the limitations of the conventional SEM, techniques such as low-vacuum scanning electron microscopy [4] and use of...
an environmental scanning electron microscope (ESEM) were developed [5]. Low-vacuum SEMs allow semi-wet samples to be imaged without coating, but are unable to attain the resolution of conventional SEMs. ESEMs also can be used to observe specimens without coating and produce better images than low-vacuum SEMs. In particular, Stokes [6] has reported (using an ESEM) observing the movement of an adult fruitfly during imaging at a temperature of 3°C in a water vapour environment at a pressure of 4.7 × 10⁻² Pa. However, despite this innovative report, there have been no further such reports, suggesting that the ESEM technique is not reliable enough to investigate living organisms at this level of resolution. Successful observation of living specimens with a high-resolution SEM would be a significant advance for biological research.

We have reported that living organisms covered with either a layer of natural extracellular substance (ECS) or an artificial substance mimicking the ECS (e.g. 1% aqueous solution of the surfactant polyoxyethylene sorbitan monolaurate; TW20), polymerized by plasma or electron beam irradiation, rendered organisms highly tolerant to high vacuum [7,8]. The simple surface modification gives rise to a thin extra layer, which we call a ‘NanoSuit’, and allows organisms to survive for up to 2 h in the high vacuum (10⁻⁵ to 10⁻⁷ Pa) of a field emission scanning electron microscope (FE-SEM). In addition to seeing spontaneous movements of the organisms, we found that the surface fine structure of living organisms covered with a NanoSuit remained undamaged, when compared with traditionally prepared specimens. This result strongly supports our notion that the NanoSuit can preserve the ‘real-life appearance’ down to microscopic details. To examine the role of the NanoSuit further, we have investigated, in this report, live specimens protected by TW20 NanoSuit in an FE-SEM. Our method, which permits the use of a high vacuum and reveals fine structural details on live specimens at magnification up to 200,000×, is expected to be suitable for a wide range of applications in the biological sciences.

2. Materials and methods

(a) Experimental organisms
Fourth-instar larvae of the mosquito *Culex pipiens molestus* (approx. 6 mm in body length), collected from puddles and maintained in the same water they had come from, were used. In order to exclude any effects of the water, they were transferred to distilled water at 24 ± 1°C 2 days prior to the experiment, with distilled water changes every 12 h. The larvae were rinsed thoroughly in distilled water 1 h before experiments began.

Adult specimens of the beetle *Lilioceris merdigera* were collected from Hamamatsu City, Japan. The animals were maintained in the laboratory at 20 ± 1°C and fed daily. Individuals chosen for the experiment were rinsed thoroughly in distilled water 1 h prior to experimentation.

Specimens of the amphipod *Talitrus saltator* were collected on a sandy beach in southern Tuscany (42°46’ N, 11°6’ E; Albegna, Grosseto, Italy), transported to the laboratory in plastic boxes containing wet sand and maintained in an aquarium containing sand moistened with artificial seawater. They were fed weekly with dry fish food placed on blotting paper. The experimental animals were rinsed thoroughly in distilled water 1 h before experiments began.

(b) Microscopy
For FE-SEM, we used a Hitachi S-4800 operated at acceleration voltages of 1.0 kV. The vacuum level of the observation chamber was 10⁻⁵ to 10⁻⁷ Pa. The detector for secondary electron was a mixture of signal with upper and lower detectors. Other details in conditions were as follows: working distance, 8 mm; aperture size, φ 100 μm; scan speed, each beam is 10–15 frames per second. To record the dynamic movements of animals, imaging data from the SEM were directly transferred to a Hi-band digital-formatted video recorder (Pioneer, DVR-DTP95). Transmission electron microscopy (TEM) observations were carried out using a JEM-1220 (JEOL) at an acceleration voltage of 120 kV.

(c) Preparation of Tween 20 solutions and sample preparation for the FE-SEM to observe the living specimens
The amphiphilic compound polysorbitan monolaurate (Twee 20; Wako Pure Chemical Industries) was used for all the experiments to mimic natural ECS [7,8]. To form the NanoSuit, organisms were dipped for 1 min into different concentrations of Tween 20 (0.01–50%) dissolved in distilled water and blotted briefly thereafter on dry filter paper to remove excess solution, and then irradiated by plasma or electron beam of SEM [7]. For plasma irradiation, the metal-emitter from a standard ion-sputtering device (JFC-1100, JEOL) was removed, so that the plasma ions produced within the chamber were based on the remaining rainified air-derived gas molecules. Specimens were irradiated with plasma inside this device for 3 min at a vacuum level of approximately 1.0 Pa and 1.0 kV DC (8.0 mA) at room temperature. For electron beam irradiation, specimens were treated with electron beam inside SEM at a vacuum level of approximately 10⁻⁵ Pa and 1.0 kV DC (7.0 μA) at room temperature. The samples were not exposed to any traditional treatments such as chemical fixations, dehydration or ultrathin coating with electrically conducting materials. In figure 2d–f, after formation of 1% (v/v) Twee 20 NanoSuit, the specimen was covered with an additional thin layer of gold with an ion-sputtering device (JFC-1100, JEOL) for 5 min at a vacuum level of approximately 1.0 Pa and 1.0 kV DC (8.0 mA) at room temperature. Twenty samples were used for each experiment.

(d) Preparation for standard scanning and transmission electron microscopy
For standard SEM observation, animals were prefixed with 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and post-fixed in 1% OsO₄ in the same buffer. The specimens were then dehydrated, freeze dried (FD300, JEOL) and ultrar thin coated with OsO₄ (PMC-5000, Meiwa). For TEM to observe the surface fine structure of the samples, specimens were prefixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4), and then post-fixed in 1% OsO₄ in the same buffer. The dehydrated specimens were embedded in an epon–araldite mixture. Ultrathin vertical sections (approx. 70 nm) of the surface were stained with 2% uranyl acetate followed by 0.4% lead citrate for 5 min each.

3. Results and discussion
To observe living organisms in an FE-SEM, it is necessary to cover them with a barrier to reduce gas and liquid loss under high vacuum. Such a barrier should not be toxic or damage the living organisms and should form a layer thin enough to preserve the surface fine structure while maintaining electrical conductivity. We have previously found that, with the assistance of a polymerized thin film
`NanoSuit`, organisms can tolerate high-vacuum environments [7,8], presumably because the NanoSuit acts as a barrier to gas and liquid release from the organism: a property that we call the `surface shield effect`. This unexpected finding has allowed us to observe living specimens under a high-resolution SEM.

**Figure 1.** Comparison of mosquito larvae treated with different concentrations of Tween 20 (TW20) and observed for 30 min in the SEM. The small white squares in a, e, i, m, q indicate the position of images shown at high magnification in b–d, f–h, j–l, n–p, r–t. Each image comes from a different treated larva. The thin TW20 NanoSuit in the specimens shown in a–d and h was not sufficient to protect them from dehydration and/or resulted in electrostatic charging (b,c,d,h). The thick TW20 NanoSuit (m,q) prevented imaging of the surface fine structure (n–p, r–t). Scale bars, 500 μm (a,e,i,m,q), 500 nm (b–d, f–h, j–l, n–p, r–t).
To investigate the ‘surface shield effect’ morphologically and to understand how the NanoSuit allows visualization of fine structures with good resolution in the SEM, we prepared NanoSuits of varying thickness and compared the SEM images with cross sections of the NanoSuit in TEM images (figures 1 and 2). Figure 1 shows results of individual mosquito larvae treated with different concentrations of TW20. The images indicate that a low concentration of TW20 (0.01%) is not sufficient to protect surface structures (figure 1b–d), and specimens dehydrate rapidly (figure 1a). Higher concentrations of TW20 (0.1 and 1.0%) yield thin NanoSuits, which prevent dehydration (figure 1e,i) and preserve the underlying structure (figure 1f–h,j–l). Even higher concentrations of TW20 (10 and 50%) result in thick NanoSuits and prevent imaging of the surface fine structure (figure 1n–p,r–t).

Figure 2 compares SEM and TEM images for larvae covered with a 1% TW20 NanoSuit. The TEM image shows that the surface of the specimen was covered with a NanoSuit less than 200 nm thick (figure 2c). The alternating pattern of furrows and ridges detected by TEM under the NanoSuit appears to closely correspond to the SEM image (figure 2h; see also figure 1f–h,j–l). These results suggested that the electron beam is able to penetrate the NanoSuit and reveal the surface fine structure under it.

Specimens treated with 0.1% TW20 had thin NanoSuits, which provided sufficient protection for SEM imaging (figure 1f–h) but could not be properly fixed for TEM imaging. Larvae treated with 10% and 50% TW20 solutions had rather ‘thick’ NanoSuits (approx. 1–5 μm), which prevented imaging of the underlying fine structure (figure 2g–i; see also figure 1n–p,r–t). Consistent with this interpretation, covering the surface of 1% TW20 NanoSuit-protected specimen with a thin layer of gold, which interfered with the electron beam penetration, also drastically changed the high magnification image. The alternating pattern of furrows and ridges was no longer visible in SEM (figure 2e), although in TEM images the ridges were still clearly present (figure 2f).

Table 1 summarizes the imaging results on mosquito larvae using different concentrations of TW20. NanoSuits prepared with 0.01% TW20 are too thin to protect specimens.
from dehydration and hence show partially disrupted structures in SEM. Nanosuits prepared with 0.1% TW20 prevent dehydration, but some samples show electrostatic charging, which prevents good imaging (figure 1h). NanoSuits prepared with 1% TW20 prevent dehydration while still being thin enough to permit successful imaging of underlying structures. Higher concentrations of TW20 (10% and 50%) led to formation of thick NanoSuits, which prevent imaging of underlying fine structure.

A second important advantage of the NanoSuit is that it maintains specimens ‘alive’ in the high vacuum of the SEM [7,8]. As a consequence, electrostatic charging induced by the electron beam did not occur. Figure 3 shows an example. On the leg of the beetle L. medigera fine ‘hairy’ structures, whose fibrillar nanoscale elements are thought to work as an adhesion device [9], are visible on the foot (figure 3a). These structures are clearly visible in specimens covered with a NanoSuit (figure 3b–d). The forked ends of the hairy projections showed hollow depressions in the centre and remained completely free of electrostatic charging (figure 3e). Traditionally, biological samples are coated with ultrathin electrical conductors such as gold, palladium, platinum or osmium, to prevent

<table>
<thead>
<tr>
<th>TW20 (%)</th>
<th>electrostatic charge</th>
<th>fine structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>+</td>
<td>partially disrupted</td>
</tr>
<tr>
<td>0.1</td>
<td>+/-</td>
<td>good</td>
</tr>
<tr>
<td>1</td>
<td>none</td>
<td>excellent</td>
</tr>
<tr>
<td>10</td>
<td>none</td>
<td>not visible</td>
</tr>
<tr>
<td>50</td>
<td>none</td>
<td>not visible</td>
</tr>
</tbody>
</table>
the accumulation of electrostatic charges at the surface [3]. However, the living beetles in this study were observed with good resolution and no electrostatic charging for up to 120 min under continuous observation (cf. electronic supplementary material, movie S1). Even after the cessation of active movement during the SEM observation, when the beetle was returned to the normal condition in the air, the animal recovered the movements in a few minutes, indicating the specimen was still alive in the SEM. When the observation time was extended to 180 min, the tip of the hairy projections started to show electrostatic charging, although the fine structures still maintained their original shapes. Animals observed continuously for 180 min did not survive. In contrast, living beetles not treated with the TW20 solution remained charge-free for only about 30 min and did not survive after observation in the SEM.

After electrostatic charging had commenced, treatment with TW20 solution was not able to halt or reverse it (data not shown). Furthermore, dead specimens, which were directly treated with TW20 solution and allowed to form a NanoSuit in the SEM, also showed an accumulation of electrostatic charge on the surface (figure 3f,g; electronic supplementary material, movie S2). Based on these results, it appears that living organisms possess electrical conductors and/or are endowed with properties on their surface which inhibit electrostatic charging. The NanoSuit seems to be responsible for prolonging survival in vacuum and the charge-free condition.

To further explore the role of the NanoSuit, we checked the damage to the specimens caused by the electron beam. In a conventional SEM preparation, electron-beam-induced deposits sometimes appear in the observation field. The deposit is easy to detect when the magnification of the observation is shifted from high to low (figure 4a,b; cf. electronic supplementary material, movie S3). This deposit is thought to represent non-volatile substances such as hydrocarbons remaining behind after the release of gaseous molecules from the specimen [10]. In this study, the surface of living T. saltator covered by a NanoSuit showed faint deposits (figure 4c,d; cf. electronic supplementary material, movie S4), whereas specimens washed with distilled water (not coated by a NanoSuit) quickly started to exhibit deposits (data not shown), as did fixed samples. In summary, covering the surface of living animals with a NanoSuit apparently prevented deposits from forming.

4. Conclusion

We report here that live samples protected with a NanoSuit can be observed with good resolution and no electrostatic charging in the SEM. This suggests that some living organisms, in particular arthropods, have the potential to tolerate high-vacuum environments and possess their own electrical conductors and/or rely on certain properties on their surfaces to inhibit electrostatic charging. The NanoSuit prolongs the charge-free condition and increases survival time under vacuum. Because the NanoSuit maintains the integrity of the organism’s surface without interfering with high-resolution imaging in the SEM, it should prove useful in future biological work. Furthermore, the method is simpler and less time-consuming than conventional SEM procedures and greatly facilitates the imaging of biological samples hitherto considered unsuitable for the high-vacuum conditions of the conventional SEM.

Acknowledgements. We thank Research Supervisor Yasuhiro Horiike (Fellow Emeritus, National Institute for Materials Science) for allowing us to carry out research with live specimens in an FE-SEM.

References


