High-resolution imaging of living mammalian cells bound by nanobeads-connected antibodies in a medium using scanning electron-assisted dielectric microscopy

Tomoko Okada & Toshihiko Ogura

Nanometre-scale-resolution imaging technologies for liquid-phase specimens are indispensable tools in various scientific fields. In biology, observing untreated living cells in a medium is essential for analysing cellular functions. However, nanoparticles that bind living cells in a medium are hard to detect directly using traditional optical or electron microscopy. Therefore, we previously developed a novel scanning electron-assisted dielectric microscope (SE-ADM) capable of nanoscale observations. This method enables observation of intact cells in aqueous conditions. Here, we use this SE-ADM system to clearly observe antibody-binding nanobeads in liquid-phase. We also report the successful direct detection of streptavidin-conjugated nanobeads binding to untreated cells in a medium via a biotin-conjugated anti-CD44 antibody. Our system is capable of obtaining clear images of cellular organelles and beads on the cells at the same time. The direct observation of living cells with nanoparticles in a medium allowed by our system may contribute the development of carriers for drug delivery systems (DDS).
low radiation damage and high-contrast imaging without staining or fixation\(^{24–27}\). The spatial resolution of the SE-ADM system reached 8 nm\(^{26}\). Moreover, our system is capable of producing high-contrast images of untreated biological specimens in aqueous conditions\(^{26,27}\). Biological samples are enclosed in a liquid holder composed of tungsten (W)-coated silicon nitride (SiN) film and are not directly exposed to electron beam. Irradiated electrons are almost absorbed in a tungsten layer on the SiN thin film; thus, the negative electric-field potential arises at this position\(^{24}\). This negative potential is detected at the bottom measurement terminal through the specimen in water. The detection mechanism is based on the difference of electric dipoles of the water and specimen materials\(^{24}\). Because water has a high electric permittivity; the electric-potential induced by the irradiated electron in W-coated SiN film is propagated to the lower SiN film through the sample solution\(^{24}\). On the other hand, as the biological specimens consist of organic materials (for example amino acids and lipids) with low electric permittivity, they decrease the transmission electric signal\(^{24–27}\). Therefore, our system enables high-contrast imaging with low radiation damage.

In the previous report, we firstly showed our SE-ADM system observing the untreated living mammalian cells under aqueous condition\(^{27}\). In contrast, here, we first report that the SE-ADM system is capable of observing antibody-binding nanoparticles in liquid-phase. Moreover, we successfully observe nanobeads directly binding to mammalian cancer cells via antibodies in a medium and their intracellular structure at the same time.

**Results**

Figure 1 shows a schematic outline of the SE-ADM system for detecting culture cells with antibody-binding nanoparticles. Our SE-ADM system is based on a field-emission scanning electron microscope (FE-SEM) (Fig. 1a). Mouse cancer cells (4T1E/M3)\(^{28–30}\) are cultured in the dish holder containing medium\(^{27}\). The holder, which contains cells, is separated from the plastic culture dish and attached to an acrylic holder\(^{27}\). Cultured cancer cells in the interspace between SiN films are maintained in medium conditions under atmospheric pressure (Fig. 1b). The binding of the nanobeads onto the cells via antibodies is directly observed by our SE-ADM system under medium conditions (Fig. 1c).

Initially, we observe the streptavidin conjugated polystyrene beads under untreated liquid conditions (Fig. 2a). These beads are clearly shown to have spherical form and to be dispersed in water at 50,000 \(\times\) magnification. The beads’ diameter is detected to be approximately 100 nm in its SE-ADM image (Fig. 2a). Then, we observe the mixed solution of the streptavidin-conjugated 100–nm polystyrene beads and biotin-conjugated anti-CD44 antibody (Fig. 2b). The biotin-conjugated antibodies are bound by the streptavidin-conjugated beads. The image of the antibody-binding beads shows a rough surface with a small spinal form (Fig. 2b). The 100-nm polystyrene beads indicated by red arrows in Fig. 2a and b are magnified and shown in a pseudo-colour map for detailed analysis (Fig. 2c–f). The surfaces of spherical beads without antibodies look rather smooth (Fig. 2c,d); in contrast, the beads bound to antibodies exhibit many spines on their surfaces (Fig. 2e,f). We compare the surface structures of...
Fig. 2c and e using three-dimensional (3D) pseudo-colour maps (Fig. 2g,h), which clearly show their differences. The white arrow in Fig. 2e and h exhibits an antibody-like structure; moreover, line plots of both beads’ centres (Fig. 2d,f) clearly show that the antibody-binding beads are significantly wider in diameter than those without antibodies (Fig. 2i).

Next, we directly observe the binding of nanobeads to cancer cells via anti-CD44 antibodies in a medium condition. CD44 is known as a hyaluronic acid (HA) receptor and a prominent marker of malignancy in several types of cancers31–34. Therefore, an understanding of the binding mechanism of CD44 to cancer cells may aid in the design of effective DDS therapeutic techniques for cancer patients35. 4T1E/M3 cells are stained first with the biotin-conjugated anti-CD44 antibodies and then with streptavidin-conjugated rhodamine, then observed via optical fluorescent microscopy (Fig. 3). Phase-contrast (Fig. 3a) and fluorescence (Fig. 3b) images clearly show that the CD44 protein is widely localized on cellular membranes.
CD44 is a complex transmembrane glycoprotein initially identified as a receptor for HA and a lymphocyte-homing receptor, which is involved in many processes, including cellular adhesion, angiogenesis, inflammation and tumour development. Much evidence suggests that CD44 is a prominent marker of several types of cancer-cell malignancy, including invasion and metastasis, and may be an important target for lymphocyte-homing receptor, which is involved in many processes, including cellular adhesion, angiogenesis, inflammation and tumour development. Much evidence suggests that CD44 is a prominent marker of several types of cancer-cell malignancy, including invasion and metastasis, and may be an important target for lymphocyte-homing receptor. We now directly observe the streptavidin-conjugated nanobeads bound to the biotin-conjugated anti-CD44 antibody on the cell surface using the SE-ADM system. Mouse cancer cells (4T1E/M3) are incubated with biotin-conjugated anti-CD44 antibody for 30 min; then, they are stained with 100-nm streptavidin-conjugated beads. Through this treatment, the 100-nm beads conjugated with anti-CD44 antibodies are bound to the CD44 protein on the cell membrane. A low-magnification image of the cells taken by the SE-ADM system clearly shows the intracellular structure. To detect the 100-nm beads, we image the central region of the cell at a magnification of 20,000× (Fig. 4d–f). The black spherical particles are found to be dispersed on the cell membranes when they are bound to anti-CD44 antibodies (Fig. 4d), whereas few beads are detected on the cells stained by 100-nm beads alone (Fig. 4e) and almost no beads are detected on the untreated cells (Fig. 4f). The average number of 100-nm beads/field is 29.5 with anti-CD44 antibodies, 3.75 without antibodies and 1.25 in the control, in four scanned images at each condition of 20,000× (Fig. 4g).

The CD44-binding 100-nm beads on the living cells are further analysed at various cell positions and high magnification (Fig. 5). Figure 5a shows a 3,000× magnification of the SE-ADM image of the nuclear region of the cell. The large spherical black object at the bottom of Fig. 5a is a typical mammalian nucleus. For detailed observation, the centre of the nucleus region (red square) is scanned at 10,000× magnification (Fig. 5b); many small black dots are detected on cell membrane above the nucleus. This suggests that these dots correspond to the 100-nm beads bound to CD44 proteins, which can be clearly observed at a magnification of 30,000× (Fig. 5c). Similar spherical beads are shown in other cell regions (Fig. 5d–f). Figure 5d shows another living cell imaged by SE-ADM system at 3,000× magnification, which shows clear intracellular structures. Figure 5d shows the border area of the nucleus and cytoplasm; a section of this image (red square at the bottom) is shown at 20,000× magnification in Fig. 5e. Another cytoplasmic region in Fig. 5d (the red square at the top left) is shown at 40,000× magnification in Fig. 5f. Both images (Fig. 5c and f) clearly show many 100-nm beads dispersed over the whole area of the cell membrane. Figure 5g and h show colour maps of enlarged images of the 100-nm beads indicated by the red arrows in Fig. 5f. Figure 5i shows a 3D colour map of Fig. 5h. The white arrows in Fig. 5g–i indicate protrusions from the bead’s surface, which are suspected to be the anti-CD44 antibody.

**Discussion**

Recently, the super-resolution fluorescence microscopies reached to the resolution which is higher than 50 nm. However, this method needs to use the fluorescence dye or fluorescence beads. On the other hand, our SE-ADM system enables to observe the beads and/or specimens without fluorescence dye. Moreover, the spatial resolution of our SE-ADM system reached 8 nm measured by 25–75% rising edge of IgM protein particle. High-resolution scanning TEM and cryo-TEM enabled observation of the high-contrast imaging of the biological specimens in water and/or in ice. Our results presented here demonstrate that our SE-ADM system (Fig. 1) clearly observed 100-nm polystyrene beads bound to antibodies in a liquid phase (Fig. 2b), without staining or metal coating. The density of polystyrene beads is 1.03 g/ml, which is very close to the liquid density. Therefore, observing the beads structure with very clear contrast using a traditional liquid-sample holder for SEM is difficult because the irradiated electron beam is scattered and absorbed by both the liquid and polystyrene beads with antibodies. Our system enabled clear detection of the antibody-binding beads, which showed wider diameters than those without antibodies (Fig. 2c–i). Using our SE-ADM technique, we previously reported the direct observation of intact mammalian cancer cells and changes of their intracellular structure under medium conditions. In DDS, polymeric nanoparticles are commonly used as the drug carriers. Therefore, our system would be useful for analysing the mechanism by which drugs are delivered by observing drug carrier particles binding to the living cells in medium.

**Figure 3.** Optical phase-contrast and fluorescence observation images of cells stained with biotin-conjugated anti-CD44 antibodies and streptavidin-conjugated rhodamine. (a) Optical phase-contrast image of antibody-stained cultured cells obtained with an optical microscope at 400× magnification. (b) Fluorescence image of anti-CD44 immunostained cells obtained from an optical microscope with a fluorescence filter at 400× magnification. Anti-CD44 antibodies are localized on the cell membranes. The scale bar represents 10 μm in (a).
DDSs. CD44 protein exhibits high expression on the cell membranes of mouse cancer 4T1E/M3 cells (Fig. 3). In this study, we successfully detected streptavidin-conjugated 100-nm nanobeads bound to the CD44 protein on the cell membrane via a biotin-conjugated anti-CD44 antibody (Figs 4 and 5). These SE-ADM images showed several 100-nm beads dispersed over the entire cell area (Figs 4d and 5c,f). Notably, our system could simultaneously observe 100-nm beads and intracellular structure without metal staining (Fig. 5a–f). Conventional super-resolution microscopes, including optical microscope and X-ray microscopes are difficult to detect both the undamaged structures of polystyrene beads and intracellular structure without staining in the medium condition. By contrast, our system can clearly observe both structures without metal staining or fixation. Future application of our SE-ADM system to the DDS or correlative work with lipid stain to show specificity would contribute the establishment of useful DDS.

At present, the spatial resolution of our SE-ADM system remains unsatisfactory for more detailed structural analysis of membrane proteins bound by nanobeads. For a more precise analysis of such protein structures, the spatial resolution may be better than 3 nm. To approach this, we are currently constructing an SE-ADM system based on a SiN film thinner than 10 nm and a fine electron beam of approximately 1-nm diameter using super high-resolution FE-SEM.

**Figure 4.** SE-ADM images of nanobeads binding to cancer cells via anti-CD44 antibodies. (a) An image of the streptavidin-conjugated 100-nm beads binding to cells via biotin-conjugated anti-CD44 antibodies in medium using the SE-ADM system at an electron beam-acceleration of 6 kV, 5,000× magnification and −32 V bias. (b) An image of the streptavidin-conjugated 100-nm beads binding cells without anti-CD44 antibodies using SE-ADM system at an electron beam-acceleration of 10 kV and 3,000× magnification. (c) An image of unstained cancer cells at electron beam-acceleration of 8 kV and 5,000× magnification. (d–f) Expanded images of the red boxes in (a–c) with 20,000× magnification. In (d), many clear black spherical particles are dispersed over the whole area. In (e), few spherical beads are observed. In (f), almost no spherical beads are observed. (g) The average number of nano-beads/field with or without anti-CD44 antibody to the 4T1E/M3 cells. The average number of nano-beads/field is 29.5 with antibodies, 3.75 without antibodies and 1.25 in the control, in four scanned images of each condition at 20,000× magnification (an image size of 5.8 μm × 4.8 μm). Values are means ± SD; ***p < 0.0001. The scale bars represent 1 μm in (a–c), 500 nm in (d–f).
Figure 5. High-resolution images of anti-CD44 antibodies binding 100-nm beads on the membranes of cancer cells. (a) A dielectric image of the nucleus region of a cancer cell stained with antibody-binding 100-nm beads at an electron beam acceleration of 6 kV, 3,000 × magnifications and −32 V bias. (b) An expanded image of the red boxed area in (a) at 10,000 × magnification. (c) High-resolution image of the red boxed area in (b) at 30,000 × magnification. Clear black spherical particles are dispersed over the whole area. (d) Another image of the cancer cells stained with antibody-binding 100-nm beads at an electron beam acceleration of 6 kV, 3,000 × magnification and −32 V bias. (e) An expanded image of the red boxed area at the bottom centre in (d) at 20,000 × magnification. (f) High-resolution image of the red boxed area in (d) at the top left at 40,000 × magnification. This area also shows 100-nm beads of clear black spheres. (g,h) Pseudo-colour maps of enlarged images of the bead binding areas indicated by red arrows in (f). (i) 3D colour map of (h). The white arrows suggest the anti-CD44 antibody. The scale bars are 2 μm in (a) and (d), 1 μm in (b), 500 nm in (e), 200 nm in (c) and (f), 50 nm in (g) and (i).
Conclusion
In conclusion, we have reported the successful direct observation of non-fluorescence 100-nm polystyrene beads binding to antibodies in aqueous condition by our SE-ADM system. Moreover, we have performed the clear detection of streptavidin-conjugated nanobeads binding to untreated cell membranes in a liquid medium via biotin-conjugated antibodies using our system. These cells were placed between two SiN films in a liquid holder and detected using our newly developed SE-ADM system. Our system was capable of simultaneously imaging both structures of the cellular organelles and antibody-binding 100-nm polystyrene beads. Therefore, our SE-ADM system would contribute the analysis of the mechanism by which drugs are delivered to cells. Our method can also be applied to various liquid samples across a broad range of scientific fields, including nanotubes, organic materials and ceramics.

Methods
100-nm-beads and CD44-antibody preparation. Polystyrene-matrix particles having 100-nm diameter conjugated with streptavidin in PBS (phosphate buffered saline) solution were obtained from Micromod Partikeltechnologie GmbH (Rostock, Germany). The beads density was 1.03 g/ml. The 100-nm beads alone in liquid solution (2μl) were added and sealed in the liquid-sample holder as a control sample.

Biotin-conjugated rat anti-mouse CD44 antibodies (catalog #: 553132) were obtained from BD Bioscience. The biotin anti-CD44 antibodies (1μl) were mixed with the streptavidin-conjugated 100-nm beads (1μl) and attached to the surfaces of the beads via biotin-streptavidin interaction. Then, this mixture solution was introduced to the liquid-sample holder.

4T1E/M3 cell culture and sample preparation. Mouse breast cancer cells (4T1E/M3) were established as previously described28–30. Cells were cultured in a high-glucose RPMI-1640 medium containing 10% fetal calf serum (FCS) and 20 mM HEPES at 37 °C under 5% CO2. After adding the culture medium (described above; 1.5 ml/dish) to the culture dish attached under the SiN-Al holder, cells (4 × 10⁴; 20 μl /dish) were seeded and cultured at 37 °C under 5% CO2. The medium was changed after 2–3 days, and the cells formed a sub-confluent or complete confluent monolayer on the SiN membrane in the holder after 4–5 days.

Immunolabelling. The cells seeded in the dish holder were stained with biotin-conjugated anti-mouse-CD44 antibodies (BD Bioscience, 1/50, 50 μl) diluted by the 1:1 mixture of PBS and medium for 30 min at 4 °C, washed twice with the mixture solution and stained with streptavidin-conjugated polystyrene particles of 100-nm diameter (Micromod Partikeltechnologie GmbH, 1/30, 50 μl) for 30 min at 4 °C. After washing twice, the holder was observed by the SE-ADM system.

Tungsten deposition on the upper SiN film. A 50-nm-thick SiN film supported by a 0.4 × 0.4 mm window in a Si frame (4 × 4 mm, 0.38-mm-thick; Silson Ltd., Northampton, UK) was coated with tungsten using a magnetron sputtering device (Model MSP-30T, Vacuum Device Inc., Japan), as previously described24.

Liquid-sample holder and culture-dish holder. The liquid-sample holder was formed as previously described24. Briefly, the liquid-sample holder comprised an upper Al holder and lower acrylic resin portion that maintained the sample solution at atmospheric pressure between the SiN films27. The upper W-coated SiN film was attached to the Al holder with double-sided tape, and the W-layer on SiN film was connected to the Al holder by silver conductive ink. A hand-made Al holder with a Si frame was attached under a 35-mm culture dish square holder via biotin-streptavidin interaction. Then, this mixture solution was introduced to the liquid-sample holder.

4T1E/M3 mouse breast cancer cells were cultured in the holder dish and stained with biotin-conjugated anti-mouse-CD44 antibodies and streptavidin-conjugated 100-nm polystyrene beads as described above. Next, the Al holder containing cells was separated from the plastic culture dish, attached upside down to another SiN film on an acrylic plate and sealed27. The Al holder received a voltage bias of approximately −32 V (Fig. 1a).

High-resolution SE-ADM system and FE-SEM setup. The FE-SEM (JSM-7000F, JEOL, Tokyo, Japan)-based high-resolution SE-ADM imaging system is shown in Fig. 1a. The liquid-sample holder was mounted onto the SEM stage, and the detector terminal was connected to a pre-amplifier under the holder26,27. The electrical signal from the pre-amplifier was fed into the AD converter after low-pass filtering, as has been previously described27. The LPF and electron beam-scan signals were logged by a PC through an AD converter.

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Image processing. SE-ADM signal data from the AD converter were transferred to a personal computer (Intel Core i7, 2.8 GHz, Windows 7), and high-resolution SE-ADM images were processed from the LPF signal and scanning signal using the image-processing toolbox of MATLAB R2007b (Math Works Inc., Natick, MA,
USA). Original SE-ADM images were filtered using a two-dimensional (2D) Gaussian filter (GF) with a kernel size of $7 \times 7$ pixels and a radius of $1.2 \sigma$. Background subtraction was achieved by subtracting SE-ADM images from the filtered images using a broad GF ($400 \times 400$ pixels, $200 \sigma$).

**Statistical analysis.** Differences in number of nano-beads with antibody and without antibody conditions were analysed using one-way ANOVA followed by Bonferroni’s multiple comparisons test. GraphPad Prism (Version 4; GraphPad Softwere, San Diego, CA USA) was used for statistical analysis.

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Author Contributions
T. Okada and T. Ogura performed and designed the research. T. Ogura designed and developed the SE-ADM system and culture-dish holder. All authors contributed to observing the experimental data, discussing the experimental results and writing the manuscript.

Additional Information
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