

Paper

Backscattered Electron Contrast Imaging of Scanning Electron Microscopy for Identifying Double Layered Nano-Scale Elements

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Identification of double layered thin film elements by backscattered electron (BSE) contrast imaging by scanning electron microscope (SEM) was examined. A flat type sample was formed with an inner layer composed of five different element areas (Au, Ag, Ge, Cu and Fe) 50 nm thick and a thin gold outer layer 2, 5, or 10 nm thick on a silicon substrate. Dependence of BSE intensities of the sample on the acceleration voltage of incident electrons was measured from 3 to 30 kV, and the acceleration voltage sufficient to discriminate different elements shifted from 8.8 to 10.2 kV depending on the increase of the thickness of outer gold layer. Moreover, the dependence of the substrate shape was also evaluated using Ge/Au double layered spherical nano-shells. The contribution of the nano-shell diameter to the BSE intensity was tested using different sized nano-shells. The contribution was within 10% for 1.5 times difference of the diameter of nano-shells. Results between flat thin film and the nano-shell were compared and those differences were within 6% for 10 to 20 kV of acceleration voltage, which indicates that inner elements of double layered thin film objects can be identified using BSE observation of the SEM at appropriate acceleration voltage.

1. Introduction

Scanning electron microscopy (SEM) is widely used not only for observation of surface conformation but also for element analysis. Secondary electron (SE) imaging is widely used to observe surface conformation because it reveals nanometer-order structures. For the analysis of sample surface elements, one typical way is to use energy dispersive spectroscopy (EDS). In EDS, the analysis is performed with measurements of characteristic x-rays and quantitative evaluation of components on a surface. However, the lateral resolution is 30 to 80 nm even if low acceleration voltage is used to reduce the noise from the substrate layer under the target samples [1].

Backscattered electron (BSE) is a beam

electron that is reflected from the sample by elastic scattering. Because the intensity in BSE images is strongly related to the atomic number of the specimen, BSE images can provide information about the distribution of elements in the sample [2–5]. In a previous study, we found that at least six kinds of thin film elements can be simultaneously discriminated with the contrast greater than 1.3 [6]. The lateral resolution of BSE images depended on the size of the incident electron beam as SE imaging; therefore, BSE measurement is suitable for identifying nano-scale elements such as nano-particle mixtures composed of different elements spread on the substrate.

Metal nano-particles are used as labels to

visualize biomolecules using SEM [7–9]. In a previous study, we proposed a method to use various metal nano-particles as labels and the particles were observed with high spatial resolution using field emission SEM (FE-SEM) [10]. In this method, various metal nano-particles were produced by covering polystyrene bead surfaces with metals using thermal deposition [11]. We also developed metal nano-shells, which have uniform BSE intensities in comparison with nano-particles, by removing polystyrene beads from the metal nano-particles [12]. For practical use, these nano-shells should be produced as double layers with an inner layer composed of various elements for identification of the label and a gold outer layer to immobilize biomolecules through Au-thiol bonding [13].

In this study, identification conditions of double layered elements were quantitatively evaluated. Evaluations were performed using both thin flat films formed on a silicon (Si) substrate as a model and double layered metal nano-shells as a practical example of biological labels.

2. Materials and methods

Preparation of double layered thin flat films

Element thin films were formed on a Si substrate using thermal deposition as reported in previous literature [6]. An area of the Si substrate, about 5 mm square, was subdivided into six small areas, and five kinds of metals, Au, Ag, Ge, Cu, and Fe, were deposited on these separate areas, with the thickness of 50 nm. Next, a 2-nm layer of Au was formed on the whole substrate as the second layer. After FE-SEM observations, the thickness of the Au layer was increased to 5 and 10 nm by additional deposition, and FE-SEM measurements were performed at each thickness.

Preparation of double layered nano-shells

Double layered metal nano-shells were fabricated using the same procedure as previously reported [12]. For fabrication, polystyrene beads (STADDEX SC-020-S, SC-030-S and SC-051S, nominal diameter is 196, 309 and 506 nm, respectively, JSR Co., Tokyo) were used as casts of the shells and placed on a Si substrate. Ge and Au metals were sequentially deposited on 200 and 300 nm beads 50 and 5 nm thick, respectively, as the inner and outer layers. For 500 nm beads, Au layers 55 nm thick (i.e., 50 nm inner and 5 nm outer Au layers) were deposited and used as a BSE intensity reference in FE-SEM observation. The metal-deposited nano-particles were set into UV-ozone cleaner (UV253HR, Filgen, Inc.,

Aichi) and they were UV-ozone oxidized to remove the polystyrene casts. Double layered metal nano-shells (Ge inner and Au outer layers) were then obtained, dislodged from the Si substrate with an application of gentle ultrasound using a desktop sonicator, suspended in water, and placed again on a clean Si substrate.

FE-SEM observation

Both thin flat films and fabricated nano-shells were observed with BSE detection mode using an FE-SEM (JSM 6701-F, JEOL Ltd., Tokyo) having a BSE detector (SM-74071, JEOL). Observation was carried out under the following conditions: 3 to 30 kV acceleration voltage, magnification of 80 × (films) and 8,000 × (nano-shells), 200 pA probe current, 8 mm working distance, 20 sec capture time, 1280 × 1024 pixels, and BSE detection mode. Incident electrons were vertically applied to the sample surface and BSE was monitored with its scattered angle range between 16° and 60° from the axis of the incident electron beam. The observation area was set as all elements (Au/Au, Ag/Au, Ge/Au, Cu/Au, Fe/Au, and Si/Au subdivided areas for flat films, and 200 nm Ge/Au, 300 nm Ge/Au and 500 nm Au/Au for nano-shells) were simultaneously captured in a picture. Pictures were taken with maximum contrast using the intensities of Au and Si as the brightest and darkest references, respectively. The acceleration voltage, V , was increased from 3 to 30 kV with a 1 kV step. The emission current was reset immediately before taking pictures to make the capture conditions the same.

Data analysis

Intensities of elements in BSE images were quantified using an image analysis software (Image J 1.40 g, National Institute of Health, Bethesda, MD, USA, <http://rsb.info.nih.gov/ij/>). These intensities were digitized as 8-bit gray scale values. As large an area as possible was selected for each element in each picture, and the mean value of the intensity was calculated using the software. This measurement was carried out for all elements in all pictures.

To compare intensities of elements, the intensities of Au and Si were used as references of maximum intensity, I_{max} , and minimum intensity, I_{min} , respectively. Relative intensity of an arbitrary element, \tilde{I}_Z , was calculated using I_{max} , I_{min} , and the measured intensity of the element, I_Z , as in [6]

$$\tilde{I}_Z \equiv (I_Z - I_{min}) / (I_{max} - I_{min}) \quad (1)$$

The \tilde{I}_Z was calculated for all acceleration voltages of incident electrons, V , and the relationship between \tilde{I}_Z and V was plotted.

3. Results and discussion

BSE observation of double layered thin flat films

To evaluate the contribution of the outer thin Au layer to the discrimination of inner elements by BSE measurements using FE-SEM, double layered thin film specimens were fabricated on a Si substrate and the relative intensity, \tilde{I}_Z , was calculated using Eq. (1) by setting the intensities of Au and Si as 100 and 0%. Figure 1 shows the relationships between calculated \tilde{I}_Z and V for Au outer layer thicknesses of 2 (Fig. 1a), 5 (Fig. 1b) and 10 nm (Fig. 1c). The ratio of \tilde{I}_Z to each element was then calculated to find appropriate conditions to discriminate all elements. The criteria for choosing the appropriate voltage is as follows; the ratio of \tilde{I}_Z for elements closest to each other in the atomic number should be significantly greater than 1 for every such pair. For example, of Ge, Cu and Fe, Ge has the largest atomic number while Fe has the lowest. Thus, the ratio between \tilde{I}_Z of Ge and Cu, as well as the ratio between Cu and Fe, both must be significantly larger than 1 for clear identification of all three elements when these elements used to present simultaneously. In the current case where Au, Ag, Ge, Cu and Fe need to be identified, the following ratios of \tilde{I}_Z , $\tilde{I}_Z(\text{Au})/\tilde{I}_Z(\text{Ag})$, $\tilde{I}_Z(\text{Ag})/\tilde{I}_Z(\text{Ge})$, $\tilde{I}_Z(\text{Ge})/\tilde{I}_Z(\text{Cu})$ and $\tilde{I}_Z(\text{Cu})/\tilde{I}_Z(\text{Fe})$ should all be significantly greater than the unity. For this specimen, we decided that appropriate condition of V satisfy all of the above ratio to be more than 1.3, because the sample on which second Au layer is absence satisfy this condition [6].

Figure 2 shows the relationships between the ratio of \tilde{I}_Z among each element and V for Au outer layer thicknesses of 2 (Fig. 2a), 5 (Fig. 2b) and 10 nm (Fig. 2c) calculated with results in Fig.1. The appropriate conditions can be found from Fig.2 as (i) intersection point of Ag/Ge with Ge/Cu and (ii) high values of V , which is 28 kV for 2 nm, more than 26 kV for 5 nm, and more than 27 kV for 10 nm. The objective of this study is discrimination of metal nano-shells conjugated with biomolecules such as mRNAs or proteins in a cell, and practical use of high V , i.e. case (ii), is not suitable for this purpose because of serious damages of biomolecules. Therefore, we decided the appropriate condition as case (i) in this study.

In Fig.2, the solid lines indicate the appropriate V decided in the above case (i) and the dotted lines indicate the appropriate V when there

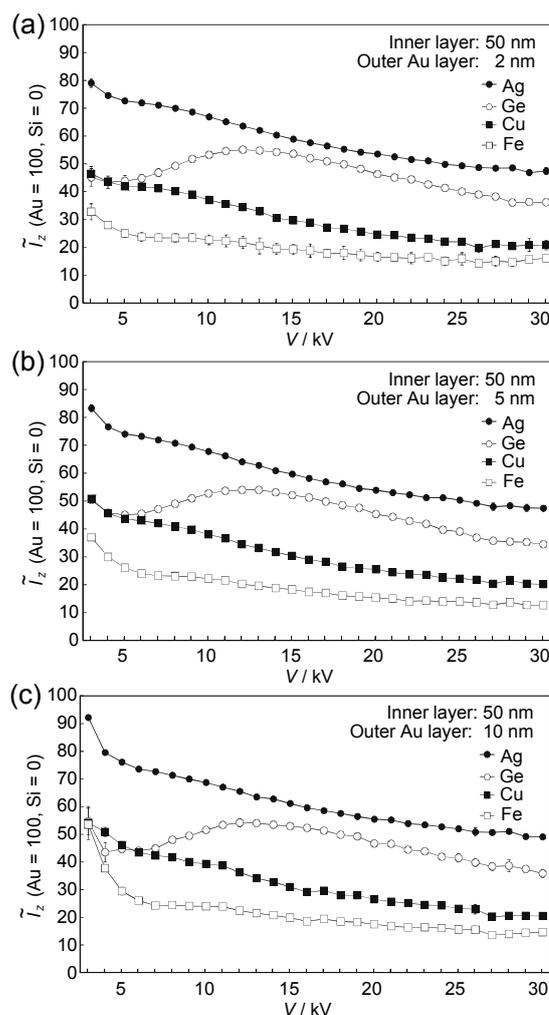


Figure 1. Relationship between \tilde{I}_Z and V for 2 nm (a), 5 nm (b), and 10 nm (c) of outer Au layer.

is no outer Au layer [6]. The thickness increase of the outer Au layer indicates that the V appropriate to discriminate inner layer elements shifted to a higher value. Values of V appropriate for each thickness of the outer Au layer were 8.8 kV for 0 nm [6], 9.0 kV for 2 nm, 9.4 kV for 5 nm, and 10.2 kV for 10 nm. The increase of appropriate V was proportional to the thickness of the outer Au layer with its gradient of 0.143 kV/nm ($R^2 > 0.99$). Though the thickness of the outer Au layer increased, the ratio of \tilde{I}_Z at the appropriate V did not decrease between 1.32 and 1.34, which indicated that we can identify inner elements of double layered thin flat films using BSE observation of FE-SEM by selecting an appropriate V .

Identification condition of double layered metal nano-shells

Next, we examined whether the results of flat thin films apply to nano-scale objects. For this

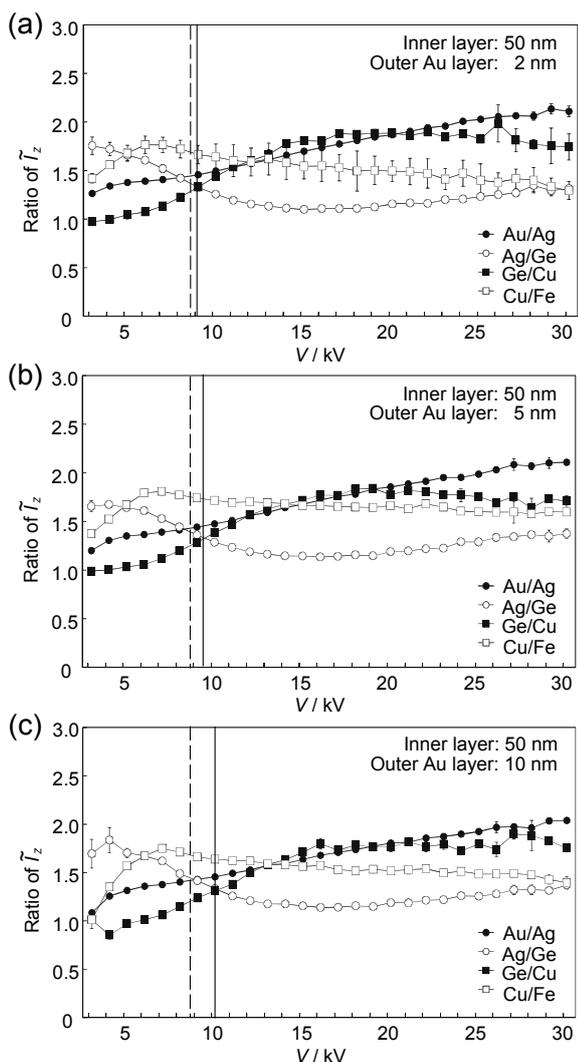


Figure 2. Relationship between the ratio of \tilde{I}_Z among each element and V for 2 nm (a), 5 nm (b), and 10 nm (c) of outer Au layer. Solid lines indicate the appropriate condition of discrimination for each case and dotted lines indicate that for the absence of outer Au.

evaluation, Ge was chosen as a model element and Ge/Au double layered nano-shells were fabricated at thicknesses of 50 nm for Ge as the inner layer and 5 nm for Au as the outer layer. An Au nano-shell 55 nm thick and 500 nm in diameter was also fabricated and used as a maximum intensity reference, I_{max} , in BSE images to calculate \tilde{I}_Z of Ge/Au nano-shells using Eq. (1). Two different diameters of Ge/Au nano-shells (200 and 300 nm) were fabricated to evaluate the contribution of the size effect for BSE intensity. These two different sized nano-shells were mixed with 500 nm Au nano-shells, and the mixture was dropped on a Si substrate.

Figures 3 a to d show both SE (Fig. 3a) and BSE (Figs. 3b–d) images of the nano-shell mixture. As indicated in Fig. 3a, five types of nano-shells are observed in these pictures; (i) 500

nm Au nano-shell, outer layer is apparent, (ii) 300 nm Ge/Au nano-shell, outer layer is apparent, (iii) 300 nm Ge/Au nano-shell, inner layer is apparent, (iv) 200 nm Ge/Au nano-shell, outer layer is apparent, and (v) 200 nm Ge/Au nano-shell, inner layer is apparent, respectively. Orientations of the nano-shells can be identified in the SE image as the absence (i, ii, and iv) or presence (iii and v) of an aperture of the nano-shell.

The \tilde{I}_Z of Ge/Au nano-shells whose inner layer is apparent in the picture (i.e. ii and iv) was calculated for each V and was compared with the result of flat film. Figure 3e shows a summary of the relationships between \tilde{I}_Z and V for 200-nm nano-shells, 300-nm nano-shells, and flat film.

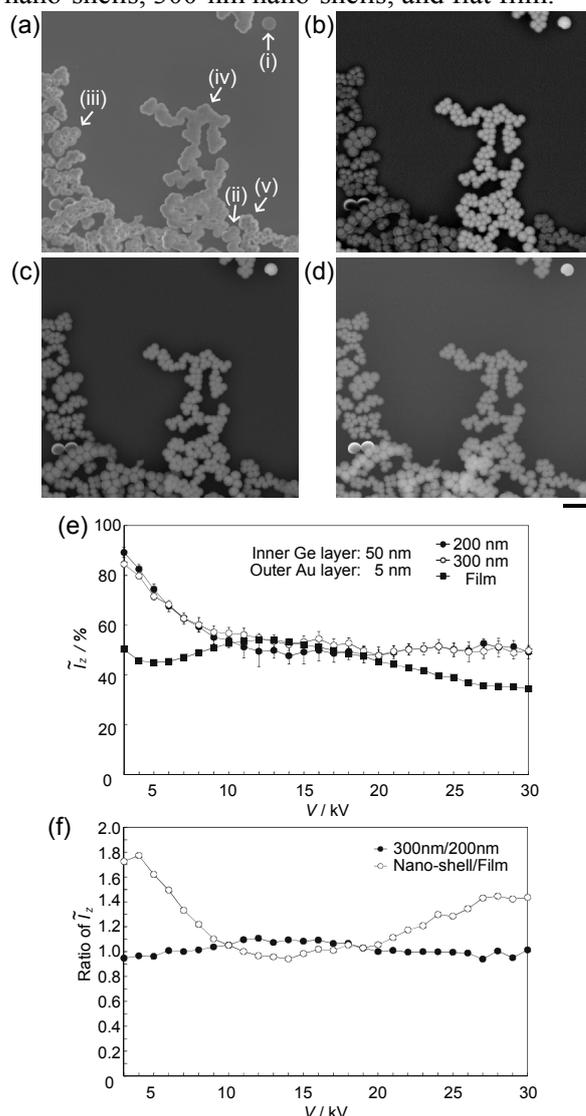


Figure 3. Observations of Ge/Au double layered nano-shells. (a) An SE image of five types of nano-shells (see body text). (b–d) BSE images of nano-shells at 5 kV (b), 10 kV (c) and 20 kV (d) of V . Bar, 1 μm . (e) Relationships between \tilde{I}_Z and V for 200 nm Ge/Au nano-shell, 300 nm Ge/Au nano-shell and Ge/Au thin flat film. (f) Relationships between ratios of \tilde{I}_Z among 200- and 300-nm nano-shells (black) and nano-shell and film (white), and V .

The ratio of \tilde{I}_Z between the 300-nm and 200-nm nano-shells were calculated (Fig.3f, black points), and was between 0.94 and 1.10 for the same V , which indicated that the contribution of the nano-shell diameter to the BSE intensity is within 10% for 1.5 times difference of the diameter.

In a comparison of the BSE intensities of nano-shells with that of flat film, the ratio of \tilde{I}_Z between an average of 200- and 300-nm nano-shells and the film for the same V was also calculated (Fig.3f, white points) and was 0.94 to 1.05 for V from 10 to 20 kV. This indicated that results of thin flat films are applicable to the nano-shell evaluations within an error of 6% in this range of V . The large difference of \tilde{I}_Z between nano-shell and flat film for low V could be caused by the contribution of element composition of quite surface region for BSE intensity, especially void of the nano-shell which is absent from the film specimen. The difference of \tilde{I}_Z for high V could be also caused by complicated contribution of Si bulk substrate to the BSE intensity.

4. Conclusion

The relationship between \tilde{I}_Z and V was studied for double layered thin film-like elements with outer layers made of thin Au surfaces that can immobilize biomolecules. Though the thickness of the outer Au layer increased, the ratio of \tilde{I}_Z among various elements at appropriate V did not decrease for flat film specimens. The difference of \tilde{I}_Z between nano-shells and flat film for the same V was within 6% for V between 10 and 20 kV. On the basis of these results, we concluded that we could identify the inner elements of double layered thin film-like specimens using BSE observation of

FE-SEM with the selection of appropriate V .

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