We investigated secondary ion mass spectrometry (SIMS) for cellular imaging with high spatial resolution. Cellular level imaging would require nanoscale processing techniques for removal of contamination from cell surfaces and exposing the inner matter of cells. In this study, we applied gas cluster ion beams to the etching of biological samples. Molecular ions were detected with high-intensity from cholesterol samples etched with Ar cluster ions. Additionally, the intensity of secondary molecular ions with gas cluster ion irradiation was compared to that with Au$^{3+}$ or C$_{60}^+$ beam irradiation and we demonstrated the advantage of using gas cluster ions as etching beams for biological samples with low damage. Finally, we performed etching of animal cells with Ar cluster ions and SIMS analysis of the etched cells. We demonstrated that the cell surfaces were etched with low damage by using gas cluster ion beam.

Keywords: sputtering, gas cluster, mass spectrometry, animal cells, cholesterol

1. INTRODUCTION

Imaging mass spectrometry techniques are becoming increasingly valuable tools for visualization of the spatial distribution of biomolecules in tissues and cells. Matrix assisted laser desorption ionization (MALDI) is a soft ionization technique that has been applied for imaging of biomolecules up to 100 kDa, for instance, proteins in a rat whole body [1]. MALDI is generally applied for the imaging of tissues, because its spatial resolution is more than 10 $\mu$m. On the other hand, SIMS is applied to cellular imaging, for instance imaging of elemental disposition in animal cells with a spatial resolution of 500 nm [2]. SIMS analysis is generally used for imaging elements, because of the low sensitivities of molecular ions.

We have developed a method for SIMS imaging of biomolecules with high spatial resolution by using high-energy heavy ions as projectiles. Desorption of large molecular ions from organic compounds was discovered by Macfarlane and coworkers in 1974 [3] using fission fragments from a $^{252}$Cf source. The process of biomolecular ion emission has been explained by electronic excitations and by the pressure pulse model [4]. It has been known that secondary ion yields from biomolecular samples increase by using a primary ion beam in the MeV energy range; we performed imaging mass spectrometry of animal cells by using MeV ion beams [5].

Careful sample preparation is very important for cellular level imaging. Contamination must be removed from the sample surface and the inner matter of the cells must be exposed, because secondary ions are emitted from the sample surface at a depth of several nm. However, it is difficult to process the structure and retain the chemical composition of cells at the nano scale by using conventional methods, such as cutting by microtome and fixating with alcohol. Therefore, for imaging mass spectrometry of cells, innovative methods of sample processing are required.

In order to fabricate biological samples at the nanoscale, we applied gas cluster ion beams to these samples. When a biological sample is irradiated with atomic ion beams, the molecular ion yield decreases rapidly with increasing dose, because irradiation damage is accumulated on the sample surface. Therefore, the maximum dose in SIMS measurements is determined by irradiation damage. This dose, called ‘the static limit’, is generally about $1 \times 10^{12}$ to $1 \times 10^{13}$ ions/cm$^2$. On the other hand, it was reported that the accumulated damage is suppressed using cluster ions for irradiation at a dose above the static limit, because the velocity of incident ions is very low and the
sputtering rate is very large. We have investigated the effect of incident cluster size on irradiation damage and molecular ion yields. It was shown that the large gas cluster ions are well suited for nanoscale processing with low damage [6–8]. In this study, we demonstrate the advantages of using gas cluster ions as etching beams for biological samples.

2. EXPERIMENTAL

2.1 Sample preparation

Cholesterol (supplied by Nacalai Tesque, Kyoto, Japan) thin films were prepared by spin coating 10 µl ethanol solution (0.01 mg/µl) onto a clean Si wafer. The films were irradiated with either 10 keV Ar monomer or cluster ion beam at dose of 1×10¹³ ions/cm².

Rat fibroblast cells (3T3-L1, supplied by DS Pharma Biomedical Co., Osaka, Japan) were seeded and cultured with Dulbecco’s Eagle’s minimum essential medium (MEM) (Nacalai Tesque), fetal bovine serum (HyClone, Logan, Utah, U.S.A) and gentamicin (Nacalai Tesque) on 10 mm × 10 mm indium tin oxide (ITO) glass substrates. To avoid surface charging in secondary ion measurements, electrically conducting substrates are required. The ITO glass substrates were sterilized by flaming prior to cell seeding. Chromosome on the cell required. The ITO glass substrates were sterilized by flaming prior to cell seeding. Chromosome on the cell

2.2 Methods

To measure the mass spectra of cholesterol samples, a 6 MeV Cu⁴⁺ ion beam provided by Kyoto University’s 1.7 MV tandem accelerator was chopped to a width of 50 ns with 10 kHz. The pulsed ion beam was incident on the sample after being collimated to a diameter of 1 mm. The beam current of the primary ions was 70 pA at a diameter of 1 mm. The secondary ions were accelerated at 6 kV and detected by a microchannel plate (MCP) detector after passing through a fieldfree drift region. The timings of primary ion pulsing and secondary ion detection were used as start and stop signals for the time-of-flight (TOF) measurement, respectively.

For analysis of the cell surface etched with Ar cluster ions, a TRIFT IV (ULVAC-PHI, Japan) instrument was used. It provides a 30 keV Au⁺ beam focused to a spot size of about 1 µm diameter. The secondary ions were extracted through a 3 kV electric field into a TOF mass analyzer equipped with three hemispherical electric sectors. To increase detection sensitivity, secondary ions were accelerated with a voltage of 8 kV just in front of the MCP detector. The mass resolution, m/Δm, was about 4400 at an m/z ratio of 27 in this setup.

A method for generating large gas cluster ion beams was developed at Kyoto University. The experimental details of the gas cluster ion formation and ionization techniques were described elsewhere [9–11]. Neutral clusters are ionized with electrons ejected from a hot filament and accelerated toward the target samples with a voltage of up to 10 kV. Magnets installed between the ionizing and target chambers remove small cluster and monomer ions included in the cluster ion beams. For monomer ion beam irradiation, the magnets are detached. An Ar gas cluster ion beam consists of a number of Ar atoms, ranging from 100 to 10000 atoms, and mean size was about 2000 atoms. The Ar cluster ion beam was focused to 0.5 mm by an einzel lens and raster-scanned over a 1.5 mm × 1.5 mm square area by deflectors. The cluster ion beam current was measured with a wire (0.15 mm diameter) installed in front of the target. The working pressures in the source, ionization and target chambers were, respectively, 1, 1×10⁻⁷ and 1×10⁻⁴ Pa.

3. RESULTS AND DISCUSSION

3.1 Etching of cholesterol by Ar gas cluster irradiation

In order to evaluate the effect of cluster ion irradiation on biological molecules, the SIMS analyses of the cholesterol films etched with Ar cluster ions or monomer ions were performed. Fig. 1 (a, b) shows the positive ion spectra for a cholesterol thin film acquired with 6 MeV Cu⁴⁺ primary ions at a dose of 2.8×10⁵ ions/cm² for the m/z ranges 0–450 and 350–400, respectively. The intensities of secondary ions were normalized with primary ion dose. The continuous line indicates the spectrum for the unirradiated cholesterol thin film. The dash and dot lines indicate the spectrum for the sample after irradiation with 10 kV Ar monomer and Ar cluster ions, respectively, at a dose of 1×10¹³ ions/cm². It is extremely important to mention that the samples were irradiated with Ar monomer or cluster ions at doses above the ‘static limit’. The molecular ions, [Ch-H₂O+H]+ (m/z 369) and [Ch-H]+ (m/z 385) were detected with low intensity from the sample etched with Ar monomer ions. (Fig.1). In contrast, molecular ions were detected with high-intensity from the sample etched with Ar cluster ions.

In a previous study [12], the etching depths of leucine were measured using Ar monomer or cluster ions. The etching depth of the sample irradiated with Ar monomer ions was 0.3 µm. In contrast, the etching depth of the sample irradiated with Ar cluster ions was 1.5 µm. Likewise, in this study, the etching depth of the cholesterol samples irradiated with Ar monomer ions was about 5 much less than that of the sample irradiated with Ar cluster ions. These results indicate the advantages of
In a previous study, the yield of cholesterol molecular ions was about $10^{-5}$ [14]. The intensity of molecular ions with gas cluster ions were detected with 90% of the beams were used as projectiles at the same dose, the intensity observed with Ar cluster ions decreased to 20% after etching with Au$_3$ cluster ions compared to other cluster ions, Au$_3$ indicating the advantages of using gas cluster ions as etching beams for biological samples. Additionally, we discuss the advantages of using gas cluster ions as etching beams for biological samples.

In a previous study [13], the intensities of molecular signals for cholesterol films etched by Ar monomer ions and Ar cluster ions respectively using gas cluster ions as etching beams for biological samples. The intensity of molecular ions decreased to 70%. However, the molecular ions were detected with 90% of the intensity observed with Ar cluster ion irradiation at the same dose. Ar cluster ions collide to samples with low speed because the size of Ar cluster is larger than other clusters. Therefore samples are etched and secondary ions are emitted with low damage. These comparisons clearly demonstrate the advantages of using gas cluster ions as etching beams for biological samples.

We performed SIMS imaging by using high-energy ions and a processing technique for biological samples with gas cluster ions. In this experiment, the yield ($Y$) of cholesterol molecular ions, namely the count of secondary ions per primary ion dose, was about $10^{-2}$ (Fig. 1). In a previous study, the yield of cholesterol molecular ions with 13 keV Ar$_{700}$ incidence was measured and was about $10^5$ [14]. The intensity of molecular ions with gas cluster ion irradiation was lower than that measured with MeV energy primary ion beams. This result indicated the advantages of using MeV ions as projectiles for imaging mass spectrometry with high spatial resolution, because secondary ions could be detected from samples even if a small area is irradiated with ion beams. However, MeV energy ion irradiation can damage the samples and the molecular ions are hardly detected at a dose above the static limit. In a previous study, the molecular ion signals for the peptide sample were exponentially attenuated under prolonged bombardment by 6 MeV Cu$_{13}^+$ primary ion beams [5]. In contrast, the secondary molecular ions were detected with high intensity from the samples etched with gas cluster ion beam. For these reasons, we applied cluster ion beams to processing techniques of biological samples and MeV energy ion beams to imaging mass spectrometry [14].

3.2 Evaluation of the effect of cluster ion irradiation of animal cells

In order to observe the advantages of gas cluster ions as etching beams for animal cells, we analyzed animal cells irradiated with Ar cluster ions. Fig. 2 shows a diagram of the process of this experiment. Fig. 3 (a, b) shows the positive ion spectra for the cells obtained with TRIFT-IV using 30 kV Au$_{13}^+$ primary ions. The spectra for the unetched and etched areas are presented in Fig. 3 (a) and (b), respectively. The intensities of the secondary ions from the unirradiated area (Fig. 3 (a)) were very low. In contrast, Fig. 3 (b) demonstrates the advantage of using gas cluster ions as etching beams for animal cells. Firstly, the In$^+$ signal at m/z 115 was detected with high-intensity. This result indicates that cells in a certain small area is irradiated with ion beams. The 3T3-L1 cells were exposed. Secondly, The [EtBr-Br]$^+$ and EtBr fragment ions signal at m/z 314 and 284 was detected with high-intensity. This result indicates that the cell nucleoli were exposed on the sample surface. Finally, the obtained signals at m/z 377 and 638 may be derived from molecules such as fatty acid methyl, a sterol and diacylglycerol that are the components of the surface and...
ion beam could be used as a technique for preparing biological samples for imaging mass spectrometry.

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