Analyses of Size and Morphology of Nanoparticles by Atomic Force Microscope <*G1-9-182*>

（原子間力顕微鏡によるナノ粒子のサイズ及び形態解析法〈*G1-9-182*〉）

Atomic Force Microscopy (AFM) is a technique to analyze the size, morphology, and surface shape of nanoparticles with the images captured by detecting the atomic force between a sample surface and a tiny probe tip with a curvature radius in the order of nanometers (Fig. 1) mounted on a cantilever. It can be performed in air or liquid. Mechanical attributes of nanoparticles such as stiffness can also be determined as well. AFM has been used for the characterization of pharmaceuticals based on nanotechnology.

1. Equipment and operating principle

1.1. AFM system

The AFM system consists of a semiconductor laser, an AFM head (the component part of the instrument that a cantilever is mounted on), a cantilever with a probe tip, a sample stage, and a split photodiode, etc. and is equipped with an optical microscope and a CCD camera to properly align the laser beam irradiated at the cantilever (Fig. 1). The AFM system is placed on a vibration-isolation table to prevent any vibration that may affect the measurement.

1.2. AFM operating principle

The operating principle of AFM is generally described as follows (Fig. 1).

1) A semiconductor laser is irradiated to the back of the cantilever and the reflected laser beam is constantly monitored at the split photodiode.
2) As the cantilever approaches the vicinity of a sample surface, the cantilever deflects in response to the bending moment generated by the intersurface force (attractive or repulsive). The deflection is measured as upward or downward displacement of the laser detection position at the split photodiode.
3) For the deflection of the cantilever to remain constant, while the distance in the z-axis direction between the cantilever and the sample surface is controlled by a piezoelectric drive attached to the sample stage or AFM head, the cantilever is scanned in the directions of *x* and *y* directions of the sample.

Based on the operating principle as 1) ~3) above, an AFM image is captured with the height information recorded per pixel. In actual image acquisition, the nanoparticles to be measured are fixed onto a flat solid substrate and the height of the particles is measured from the substrate surface. In measuring the size of nanoparticles, assuming that the object nanoparticles are spherical, the height measured by AFM is equivalent to the particle size. By further using a standard sample for calibration, the height in the z-axis direction in AFM images can be highly accurate and precise. On the other hand, information on the lateral (*x*, *y*) dimension obtained with AFM images needs to consider the difficulty of calibration and the influence of the geometry of the probe tip.

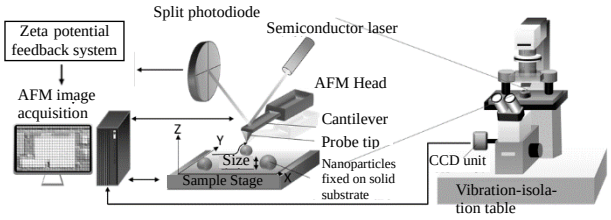

Fig. 1. Schematic diagram of a typical atomic force microscope system and PC for image acquisition[1)]

1.3. Other equipment

Acoustic enclosure: An acoustic wall box may be used to house the AFM system, to protect from vibration that may be caused by external sound.

UV cleaning system: This is used to clean the cantilever.

Temperature control system: This is used when samples need to be measured at a constant temperature.

2. Measurement

Measurement of the size of nanoparticles using AFM is generally performed in the following procedure.

2.1. Preparation of samples

Samples are prepared by dispersing the object nanoparticles in a suitable solvent at an appropriate concentration. The solvent and concentration are selected so as to maintain the stable dispersion of the nanoparticles.

2.2. Preparation of substrate for fixing nanoparticles

Fixing the sample to be observed onto a solid substrate is essential for acquiring AFM images. To select an appropriate substrate based on physicochemical properties of the sample to be observed is an important element in studying for the optimal conditions such as the number of the observed particles and the morphology.

To make sure a stable baseline for measuring the height, the substrate surface roughness needs to be sufficiently flat compared to the particles to be measured. The surface roughness (arithmetic mean roughness, which is the average of the absolute values of deviations from the center line for bumps and dips of the surface) is desirable not more than 5% of the size of the particles to be measured. It is also important that the physical properties of the substrate surface are relatively uniform for fixing nanoparticles easily.

In general, the surfaces of nanoparticles in a stable dispersion state are either positively or negatively charged, and the fixation of the nanoparticles onto a solid substrate is often

made by electrostatic interaction. Negatively charged polystyrene standard nanoparticles, for example, can be easily fixed onto a positively charged solid substrate surface. A number of studies are required for selecting an appropriate solid substrate, particularly when the interactions are complex such that surface forces between a particles and a substrate depend on van der Waals or hydrophobic interactions, or such that due to the interaction with a substrate deformation or disintegration occur on the soft particles to be fixed. A high-quality mica (muscovite mica), gold (111) -vapor fixed mica and single crystal silicon are commercially available representative substrates for AFM measurement. These substrates have atomically flat surfaces and can be surface-treated for controlling the electronic charge state of the substrate surface. For instance, to fix negatively charged nanoparticles, 0.3 vol % of 3-aminopropyltriethoxysilane (APTES) aqueous solution can be used to treat the surface for a positive charge. Relatively flat cover glass with surface roughness of approximately not more than 5 nm is commercially available, which may be used as a substrate for particles with the size of approximately not less than 100 nm. It is desirable to acquire AFM images of the substrate in advance to get the information on the surface roughness of the substrate to be used.

2.3. Nanoparticle fixation on a solid substrate

Apply a liquid sample of nanoparticles onto a suitable substrate and incubate for a sufficient time to allow the particles fixed onto a substrate. When AFM images are acquired in air, after the incubation, rinse the substrate with ultrapure water to remove excess components such as salts and dry.

2.4. Acquisition of AFM images

2.4.1 Selection of measurement mode

Nanoparticles are fixed to a substrate by weak intermolecular interactions such as electrostatic interaction and van der Waals interaction. It is therefore important to minimize the force applied to the lateral dimension by an AFM measurement mode. One of the measurement modes to meet this requirement is the intermittent contact mode (also called dynamic mode, tapping mode, dynamic force mode, or amplitude-modulated mode), which is available for most commercially available AFM equipment. In recent years, however, the nonresonant mode (force curve mapping) that does not vibrate a cantilever may be used in observing a particularly soft sample or in the measurement of mechanical properties (e.g., stiffness).

In the intermittent contact mode, the cantilever is oscillated up and down at a frequency close to the resonance frequency by a small piezoelectric element fit in the cantilever holder. The amplitude of the oscillation is very sensitive to the distance between the probe tip and the sample, and the amplitude quickly becomes small upon the probe tip contacting the sample surface by dissipating the kinetic energy of the cantilever toward the sample. With the distance between the probe tip and the sample being feed-back controlled to keep the oscillation amplitude constant, the particle surface in the sample is scanned by oscillating the cantilever up and down constantly, resulting in an advantage of almost nil force toward the lateral dimension. For this reason, this is a valid measurement mode also for those samples that move easily, have uneven surface, are soft or with adsorption to the surface. Size measurement of nanoparticles can be made in air or in liquid environments by the intermittent contact mode. How to acquire images by the intermittent contact mode is described in the following.

2.4.2. Selection of cantilever

The characteristics and geometry of the cantilever and the probe tip attached to its end are important factors that determine the sensitivity and resolution of AFM. Points to consider are given in the following.

Images obtained by AFM include factors derived from both the probe tip shape and the shape of sample particles. In other words, the shape of the probe tip has no effect in measuring the height of a particle, while it has effect in displaying the shape in the *x* and *y* directions of the nanoparticle. Therefore, caution is required to deal with the information on the sizes of nanoparticles in the *x* and *y* directions. To minimize artifacts derived from the probe tip shape, use of a probe tip having the tip radius of not more than 10 nm is recommended.

Stable excitation oscillation of a cantilever is an important factor in imaging sample surfaces by the intermittent contact mode, and the use of a cantilever with large stiffness (a high spring constant) is desirable to overcome adhesive forces between the probe tip and sample particles (e.g., capillary, van der Waals, and electrostatic forces). On the other hand, since particles may be deformed by the force upon contact with the cantilever, it is thus desirable to use a cantilever with small stiffness (a low spring constant) than that of the particles to be measured. A cantilever with a high resonance frequency may shorten the measurement time due to better scanning sensitivity, but caution is required for the damage to the particles to be measured due to the large stiffness (spring constant) in general. In addition, a cantilever with different stiffness may have to be used depending on observation in air or in liquid. The cantilever to be used should be determined in consideration of these points and should be optimized as required.

2.4.3. Acquiring AFM images

A prepared sample is placed on the sample stage, and an AFM image is acquired. The AFM image has information on the *x*-*y* plane coordinates and the vertical z coordinates. The number of the data points in the *x*-*y* plane, or the pixel number, must be considered, when acquiring and analyzing the image. For example, a 10 μm $\times$ 10 μm image with 200 pixels on a side acquired gives the size per pixel of 50 nm $\times$ 50 nm.

With this setting condition, particles smaller than 50 nm are not discriminable. The scan size should therefore be set for the size of the particles to be measured. Generally, it is desirable to set a scan size to be 10 pixels or more per particle in the measurement. In the analysis of the average particle size and particle size distribution by AFM, assuring a random sampling of representative particles plays an important role. Generally, it is recommended to measure the size of at least 100 or so nanoparticles and to acquire images from different fields to avoid artificiality in measurements due to a single field of view. In the event of the image quality becoming suddenly deteriorated during the acquisition, the cantilever should be cleaned or replaced as is often the cause that the cantilever gets dirt or worn.

An AFM image should be acquired under the same operation conditions using a substrate without the test nanoparticles fixed. This is to make sure that artifacts or foreign matters that may be misconstrued as the target nanoparticles are not contaminated from the operating process or the substrate itself.

3. Image analysis and size (height) measurement of nanoparticles

Acquired AFM images are analyzed after correcting the tilt of height on the images derived from the placement of the sample or the thermal drift of the equipment, using the software provided by the AFM equipment manufacturer (software by other developers for AFM image analysis can be also used as well). Essential operating procedure for data analysis in the size measurement of nanoparticles is described hereunder.

3.1 Size measurement by cross-sectional shape analysis

A cross-sectional shape profile in a vertical direction along a line drawn across any part of an image is obtained using the cross-sectional shape analysis tool of the software, thereby making measurement of distance in horizontal and vertical directions possible. With the cross-sectional shape profile, it is possible to know the agglomerating property of nanoparticles, as well as the height. The information can be obtained on the appropriateness as to correcting the tilt of the substrate around the nanoparticles. Cross-sectional shape analysis is performed with respect to individual nanoparticles in the image to measure the height. The benchmark for the height measurement is the lowest point of all the data, the point where geometry of the particle begins to rise in the scanning direction, or the benchmark is set by the operator's discretion. The series of measurement should be performed under the same conditions regardless of the benchmark chosen. To avoid influence of artifacts derived from the sample preparation, obvious foreign particles and large agglomerates that are not individually distinguishable should be excluded in calculating the average particle size.

3.2. Size measurement by automated particle analysis

Software can be used to automatically identify particles and enable the batch mode measurement of the particle size in a short time. Particles are identified based on the threshold of height set by users. That is, particles with a height equal to or higher than the set threshold value are included in the analysis while those with a height lower than the set threshold value are excluded. In addition, obvious foreign particles or large agglomerates of indistinguishable particles are picked up by the software and can be excluded from the analysis. After the above operations, the maximum height of individual particles relative to the height of the substrate as the benchmark is automatically measured. In the automated particle analysis, caution is required of artificial influence in the results unless the tilt of the image to be analyzed is appropriately corrected. When performing automated particle analysis, the output of the results should be checked against the results obtained by the cross-sectional shape analysis to confirm the validity of the results. The average height of nanoparticles by the automated analysis software tends to be greater than the average height by the cross-sectional shape analysis. In addition, some software analyzes particle size based on the area occupied by individual particles in images. In this case, the particle size is analyzed as an area-equivalent diameter.

3.3. Analysis of nanoparticles having shapes other than true sphere

When evaluating particle size, if particles are deformed upon fixing the particles to a substrate or non-spheric particles are to be analyzed, it is important to consider that parameters other than the height are additionally analyzed using particle analysis software. Where particles become deformed upon fixation to a substrate, for example, the volume-equivalent diameter may be used as a size evaluation parameter (Fig. 2A), assuming that the volume is constant before and after fixation to the substrate. In addition, it is also possible to obtain information on the deformed shape of particles under analysis based on the area-equivalent diameter, or the height/area-equivalent diameter ratio (Fig. 2A). When the particle under analysis is elliptically shaped, it is possible to measure the length of the major and minor axes, assuming that the particle corresponds to an ellipse, and is further possible to evaluate the shape based on the oblateness of the particle derived from the minor axis/major axis ratio (Fig. 2B). In the analysis of non-sphere particles with additional information on the lateral (x y) dimension, evaluate the tip shape of the cantilever using a calibration grating to pay attention because of the significant influence of the tip curvature.

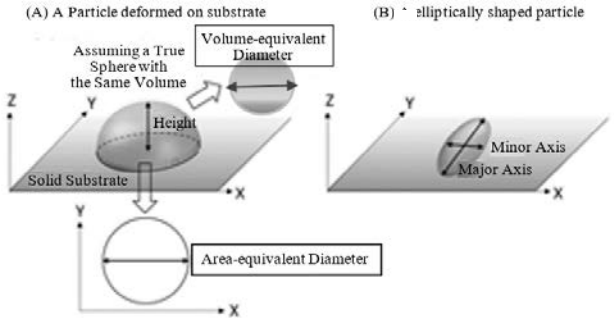

Fig. 2. Geometry evaluation of a particle deformed on a substrate (A) and an elliptically shaped particle (B) [1)]

3.4. Reporting size data

The size (height) distribution and the average with the standard deviation of nanoparticles measured should be reported. The information on the method used to fix the particles, the cantilever, the measurement mode, whether the measurement was made in air or in liquid, the number of the nanoparticles measured, and the analysis method for size should be described, as these factors involved in the measurement have impact on the results of size measurement of nanoparticles.

4. Verifying AFM performance

In AFM, the *z*-position of the cantilever is controlled for the distance by the expansion and contraction of the piezoelectric element. The expansion and contraction have properties such as non-linearity and hysteresis to the applied voltage. The *z*-height is determined based on the voltage applied to the piezoelectric element in conventional AFM. However, due to the properties mentioned above, 'height correction' based on a calibration curve prepared by measuring actual samples with certified height should be required. For example, selecting a calibration grating with a step height close to the height of nanoparticles to be measured, the average of step heights measured in at least three different locations using a sharp probe tip should be compared to the certified step height of the calibration grating used.

If the average value measured is significantly different from the certified value, it is necessary to consider the recalibration of the *z*-displacement of the piezo drive device by the manufacturer or others.

Some of recent AFM equipment is, on the other hand, equipped with a length-measuring sensor attached to the piezoelectric element and can precisely measure the degree of expansion and contraction, thereby allowing constant measurement of *z*-height. In other words, there exists the equipment with a control method that correct the height or the displacement.

References

1) Sakai-Kato, K, *et al*., Pharmaceutical and Medical Device Regulatory Science, 50, 634-640 (2019).
2) ASTM International E2859-11: 2017 Standard Guide for Size Measurement of Nanoparticles using Atomic Force Microscopy.