DEVELOPMENT OF NANOPARTICLES SIZING METHOD BASED ON FLUORESCENCE POLARIZATION

Yuki ISHIZAKI[1], Terutake HAYASHI[1], Masaki MICHIHATA[1] and Yasuhiro TAKAYA[1]

1 Department of Mechanical Engineering, Osaka University, Japan
ishizaki@optim.mech.eng.osaka-u.ac.jp

Abstract:
We suggest a novel nanoparticle sizing method based on fluorescence polarization analysis. Particle size evaluation can be achieved by measuring the rotational diffusion coefficient, which is sensitive to the particle size. We develop a rotational diffusion coefficient measurement system and a fluorescent probe, which is labelled to particle. In order to verify the feasibility of the proposed method, fundamental experiments are performed. We measure the rotational diffusion coefficients of gold nanoparticles, whose diameters are 5 nm, 10 nm and 15 nm, using the developed system. The measured rotational diffusion coefficients decrease with increasing the particle size. It indicates that nanoparticle size, which is below 15 nm, can be measured with at least 5 nm resolution.

Keywords: Nanoparticle, Particle sizing, Fluorescence polarization, Brownian motion, Dynamic light scattering

1. INTRODUCTION

Metal nanoparticles, which exhibit unique optical, electrical and chemical properties, are key materials for nanotechnology. They have applications to functional nanostructure devices [1-3]. In the process of manufacturing the device, control of the particle size and monitoring of the aggregational state of particle are strongly demanded because the functionalities of the devices are determined by the particle size and their arrangements.

Conventional technique, dynamic light scattering (DLS) can evaluate particles in liquid without drying process. However analyzing the sample which contains both non-aggregated and aggregated particles is difficult owing to the particle size dependence of the signal intensity [4]. For monitoring of the aggregational state of particle, a novel technique, which can evaluate the sample contains both non-aggregated and aggregated particles, is required.

Therefore we suggest a particle sizing method based on the analysis of rotational Brownian motion. Rotational Brownian motion of nanoparticle depends on the size. We evaluate average size of particle from rotational diffusion coefficient, which is a factor representing the rotational speed of Brownian motion. Rotational diffusion coefficient can be measured by using fluorescence polarization technique. By labelling a fluorescent probe to a particle and analyzing the polarization direction of fluorescence emitted from the probe, the rotational diffusion coefficient of the particle can be measured. In this method, the particle in liquid is evaluated without drying process. In the case of the sample, which contains both non-aggregated and aggregated particles, the average size of non-aggregated particles can be evaluated accurately due to the constancy of intensity of fluorescence signal to particle size change.

In order to measure the particle size, we develop a rotational diffusion coefficient measurement system and a fluorescent probe which is labelled to particle. In this paper, we verify the feasibility of proposed method by performing fundamental experiments.

2. PARTICLE SIZNG METHOD

2.1 Relationship between particle size and rotational diffusion coefficient

We label a fluorescent probe to nanoparticle and measure the rotational diffusion coefficient of the particle. In the case of rigid spherical particle whose volume is V, rotational diffusion coefficient can be described by Debye-Stokes-Einstein equation [5]

$$D_r = \frac{k_B T}{6V\eta} = \frac{k_B T}{\pi d^3 \eta} \tag{1}$$

where k_B is the Boltzmann constant, T is temperature, η is viscosity of solvent and d is diameter of particle. Equation (1) shows that rotational diffusion coefficient is proportional to the inverse of third power of the particle diameter and thus it is very sensitive to the change of particle size.

Therefore the particle size can be calculated from Eq. (1). if rotational motion of the particle, which is labeled by the fluorescent probe, is similar to that of a spherical rigid rotor.

2.2 Rotational diffusion coefficient measurement method

In a standard coordinate system to evaluate the fluorescence polarization is shown in Fig.1. Sample which contains fluorophores is located in the origin and illuminated by excitation light which travels along z-axis. The sample emits the fluorescence in all directions. $I_{\parallel}$ and $I_{\perp}$ are parallel and perpendicular components of fluorescence intensity with respect to the polarization direction of the excitation light.

Figure 2 shows the alteration of rotational motion of fluorophore and the polarization direction of fluorescence emitted from the fluorophore over time on the x-y plane. Fluorophore has absorption and emission moment and is excited by the light, whose polarization direction is parallel to the absorption moment. After fluorophores are excited, $I_{\parallel}$ decreases and $I_{\perp}$ increases over time owing to the rotational motion. The fluorescence polarization can be described by

$$r(t) = \frac{I_{\parallel}(t) - I_{\perp}(t)}{I_{\parallel}(t) + 2I_{\perp}(t)} \tag{2}$$

Assuming that the sample is spherical rigid rotor, $r(t)$ is

described by

$$r(t) = r_0 \exp\left(-\frac{t}{\theta}\right) \tag{3}$$

where r_0 is an initial anisotropy, θ is a rotational correlation time. If rotational motion of sample is hindered, $r(t)$ is described by Eq. (4) [6].

$$r(t) = (r_0 - r_\infty)\exp\left(-\frac{t}{\theta}\right) + r_\infty \tag{4}$$

where r_∞ is an anisotropy when t is ∞. The relationship between θ and D_r is described by following equation.

$$\theta = \frac{1}{6D_r} \tag{5}$$

In order to evaluate a rotational diffusion coefficient of sample from fluorescence anisotropy, we adopt frequency-domain method [7]. Sample is excited by sinusoidally-modulated excitation light and then the fluorescence emitted from the sample is also modulated. The fluorescence lifetime is calculated from the phase difference between the fluorescence and the excitation light (φ) by Eq. (6) [8].

$$\tau = \frac{\tan\phi}{\omega} \tag{6}$$

where ω is modulation angular frequency. The schematic of each polarized component of fluorescence signal is illustrated in Fig.3. The amplitudes ratio of parallel and perpendicular components shows the fluorescence anisotropy of the sample. Rotational diffusion coefficient D_r can be calculated from τ, r_0, r_∞ and the amplitude ratio of two components Y_{AC} ($= A_\parallel / A_\perp$) according to Eq. (7) [7]. By assuming that rotational motion is not hindered ($r_\infty = 0$) and using Eq. (5), Eq. (7) is transformed to Eq. (8). Equation (8) is the main equation in our study to calculate D_r from experimental data and when we calculate D_r, r_0 in Eq. (8) is fixed to 0.4, which is the value when absorption and emission moments of fluorophore correspond.

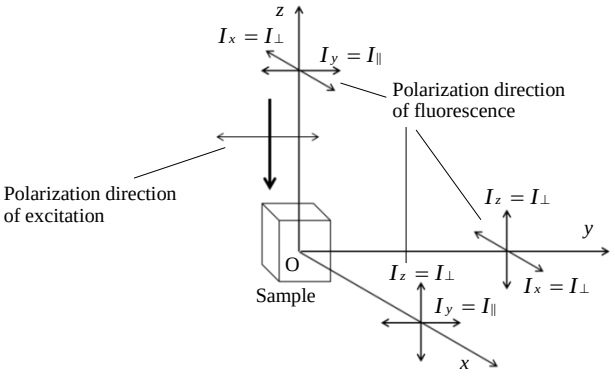

Fig.1 : Standard coordinate system

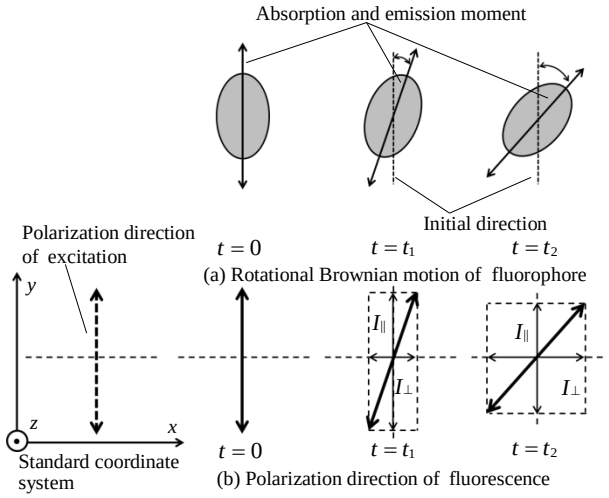

Fig.2 : Relationship between rotational Brownian motion of fluorophore and polarization direction of fluorescence ($0 < t_1 < t_2$)

2.3 Structure of fluorescent probe

In order to measure the rotational diffusion coefficient of the nanoparticle, we have to label a fluorescent probe to the particle. However fluorophore, which is directly labeled to metal nanoparticle, is quenching due to surface energy transfer (SET) from a fluorophore to a metal nanoparticle. The energy transfer efficiency is described by Eq. (9) [9].

$$\Phi_{EnT} = \frac{1}{1 + (l/l_0)^4} \tag{9}$$

where l is a distance between fluorophore and nanoparticle, l_0 is called Forster distance and equal the distance at which energy transfer efficiency is 50 %.

To avoid a quenching due to SET, we develop the fluorescent probe which consists of fluorophore and double strand-DNA. Double-strand DNA takes a rigid structure and thus has a role of spacer to keep distance between a fluorophore and a metal particle. By reaction of metal

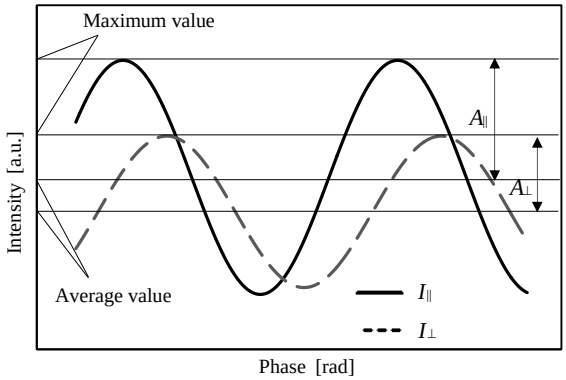

Fig.3 : Schematic of modulated fluorescence signal

$$\theta = \frac{Y_{AC}^{\,2}(1-r_\infty)^2 - (1+2r_\infty)^2}{(1+2r_0)(1+2r_\infty) - Y_{AC}^{\,2}(1-r_0)(1-r_\infty) + \sqrt{\begin{array}{l} Y_{AC}^{\,2}[9(r_0-r_\infty)^2 + \{2 + r_\infty(2+5r_\infty) + r_0[2-4r_\infty(4+r_\infty)] + r_0^{\,2}[5-4r_\infty(1-2r_\infty)] \\ - Y_{AC}^{\,2}(1-r_0)^2(1-r_\infty)^2\}(\omega\tau)^2] - (1+2r_0)^2(1+2r_\infty)^2(\omega\tau)^2 \end{array}}} \cdot \tau \tag{7}$$

$$D_r = \frac{1 + 2r_0 - Y_{AC}^{\,2}(1-r_0) + \sqrt{Y_{AC}^{\,2}[9r_0^{\,2} + \{2 + 2r_0 + 5r_0^{\,2} - Y_{AC}^{\,2}(1-r_0)^2\}(\omega\tau)^2] - (1+2r_0)^2(\omega\tau)^2}}{6(Y_{AC}^{\,2} - 1)\tau} \tag{8}$$

nanoparticle with 3'thiol modified and 5'fluorophore modified DNA, the probe attaches to the surface of the metal particle as shown in Fig.4. We use 23 bases DNA. Given the lengths of particle-thiol and DNA-fluorophore linker (≈1.8 nm) [10], the distance between a fluorophore and a metal particle is about 9.6 nm and can be longer than 9 nm, which is the Forster distance found by Yun in the case of the energy transfer from organic fluorophore to gold nanoparticle [9]. Therefore the energy transfer efficiency is below 50 % and we can acquire enough fluorescence intensity to measure.

3. EXPERIMENTAL SETUP

The experimental setup is shown in Fig.5. Argon laser with 488 nm of wavelength is used as the excitation light. The excitation light is modulated into sinusoidal waveform by passing through Acoust-Optic Modulator (AOM). After the polarization direction of the excitation light is adjusted to y-axis by the half wave plate and the polarizer, the excitation light is focused into the sample through the objective lens. The fluorescence emitted from sample passes along z-axis through dichroic mirror and emission filter, while the reflected excitation light from the sample is blocked by these two components. The beam displacer is used to divide the fluorescence signal into parallel and perpendicular components. The image intensifier enhances the fluorescence signal and the signal is detected by the cooled CCD camera. The temperature of the sample is controlled by temperature controller with feedback.

The intensity of fluorescence signal is evaluated by analyzing the brightness of the fluorescent spot in the image captured by the CCD camera. Acquiring the sinusoidally-modulated fluorescence signal is achieved by shifting the phase of the trigger signal of the image intensifier. Because the period of trigger signal is synchronized with the period of modulation signal of AOM, the phase of enhanced fluorescence signal is shifted and by repeating the phase shift, sinusoidally-modulated fluorescence signal is acquired. The phase of the enhanced signal is shifted in step of 20° from 0° to 720° (2 periods). The phase difference between the fluorescence and the excitation light φ and amplitude ratio Y_{AC} are obtained from the acquired data and τ and D_r are calculated from Eq. (6) and Eq. (8).

4. EXPERIMENT RESULTS

4.1 Measurement of rotational diffusion coefficients of standard sample

For measurement performance test of the developed system, rotational diffusion coefficient of standard sample is measured and the values are compared to theoretical values.

A kind of fluorophore, Alexa Fluor 488 (Invitrogen) is used as standard sample and is dispersed in 4 solvents of different viscosities, which are water, glycerin30%, glycerin50% and glycerin60%. Assuming that the shape of Alexa Fluor 488 is spherical, theoretical values are calculated from Eq. (1). The diameter of Alexa Fluor 488 measured by DSL is about 1.1nm and thus we use this value for calculation of theoretical values.

The fluorescence lifetime τ, amplitude ratio Y_{AC} and viscosity of each solvent are shown in Table 1. The temperature of solvents is maintained at 20 ℃ and the modulation frequency is set at 60 MHz, and values in Table 1 is average of 3 times experiment. The rotational diffusion coefficient which is calculated from values in Table 1 and the theoretical curve is shown in Fig.6. The error bars in the graphs show the maximum and minimum measurement values.

The rotational diffusion coefficients are inversely proportional to viscosities of solvents as with the theoretical values and they can be measured with low deviation in the developed system. Measurement deviations, which is represented by the length of error bar, increase with the decreasing the solvent viscosity. We presume that it is caused by the change of the solvent viscosity which results from the temperature fluctuation because rotational diffusion coefficient is sensitive to small variations in the viscosity in low viscosity area.

4.2 Measurement of rotational diffusion coefficients of gold nanoparticles

We measure rotational diffusion coefficients of gold nanoparticle samples using the developed fluorescent probe. The diameters of particles are 5 nm, 10 nm, 15 nm and 0 nm (only fluorescent probe). The fluorescent probe consists of Alexa Fluor 488 and 23 bases double strand-DNA. The

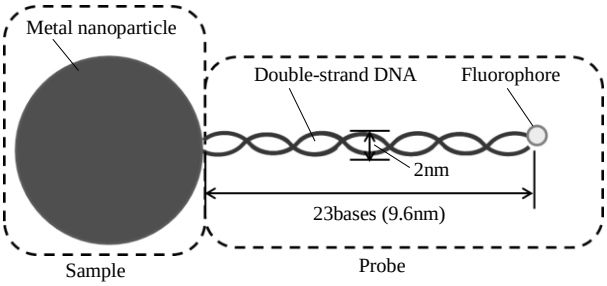

Fig.4 : Schematic of fluorescent probe

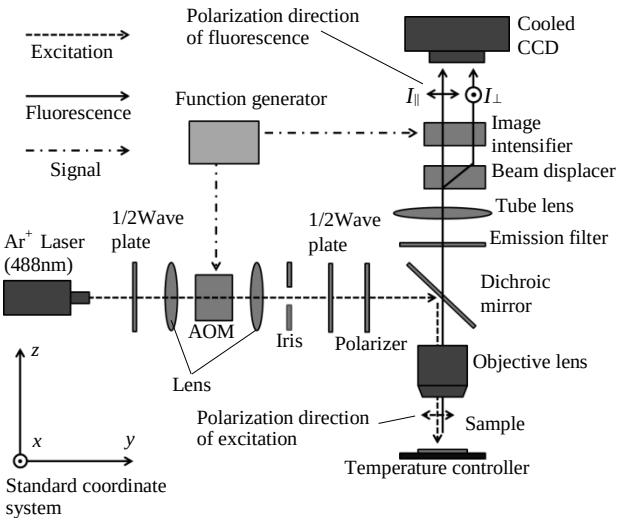

Fig.5 : Schematic of experimental set-up

probe is added to the gold nanoparticle solution (British BioCell International) and labelled to the particle. The solvent of sample is replaced to pure water.

The fluorescence lifetime τ and amplitude ratio Y_{AC} of each sample are shown in Table 2. The temperature of solvents is maintained at 20 ℃ and the modulation frequency is set at 60 MHz, and values in Table 2 is average of 3 times experiment. The rotational diffusion coefficient which is calculated from values in Table 2 is shown in Fig.7. The error bars in the graph show the maximum and minimum measurement values.

The rotational diffusion coefficients decrease with increasing the particle diameter, but are not proportional to the inverse of third power of the particle diameter in accordance with Eq. (1). We presume that it is caused by the fact that the shape of the probe-labelled particle is not spherical, and thus nanoparticle diameter can't be calculated from Eq. (1) directly.

However, the decrease of rotational speed owing to the enlarging of particle size by 5 nm can be detected as the decrease of rotational diffusion coefficients. Therefore, nanoparticle size which is below 15 nm can be measured with at least 5nm resolution.

5. CONCLUSION

In order to verify the feasibility of proposed method, fundamental experiments are performed. Rotational diffusion coefficient of the standard sample is measured to investigate the accuracy of the fluorescence polarization measurement. As a result, rotational diffusion coefficient can be measured with low deviation in the developed system.

We measure rotational diffusion coefficients of gold nanoparticles whose diameters are 5 nm, 10 nm and 15 nm using the developed fluorescent probe. The rotational diffusion coefficients decrease with increasing the particle size, and thus the decrease of rotational speed owing to the enlarging of particle size can be detected. This result indicates that nanoparticle whose diameter is below 15 nm can be measured with at least 5 nm resolution in proposed method.

ACKNOWLEDGEMENT

This research was partly supported by KAKENHI (Grant-in-Aid for Exploratory Research 24656088).

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Table 1 : Viscosity of solvents, measured lifetime τ and amplitude ratio Y_{AC}

Solvent	Viscosity [mPa・s]	τ [ns]	Y_{AC}
Water	1.002	4.1	1.051
Glycerin30% [wt%]	2.50	3.8	1.127
Glycerin50% [wt%]	6.00	3.6	1.345
Glycerin60% [wt%]	10.8	3.1	1.630

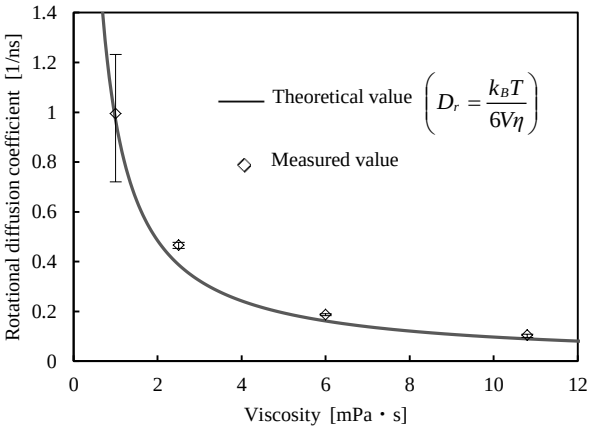

Fig.6 : Rotational diffusion coefficient versus viscosity of solvent

Table 2 : Measured lifetime τ and amplitude ratio Y_{AC}

Diameter of gold nanoparticle [nm]	τ [ns]	Y_{AC}
0 (only fluorescent probe)	2.0	1.225
5	1.1	1.406
10	1.0	1.481
15	0.9	1.710

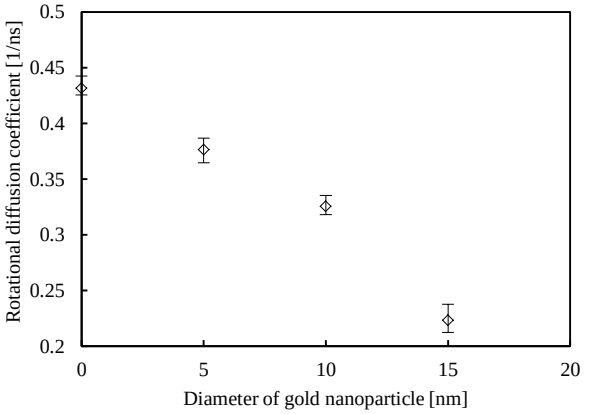

Fig.7 : Rotational diffusion coefficient versus diameter of gold nanoparticle