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DEVELOPMENT OF NANO PARTICLE SIZING SYSTEM USING FLUORESCENCE POLARIZATION

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Abstract: In order to measure the sizes of nanoparticles with a wide size distribution in a solvent, we developed an optical microscopy system that allows for fluorescence polarization (FP) measurement and optical observation. This system allows the evaluation of nanoparticle sizes over a wide range, because the fluorescent signal intensity is independent of changes in the nanoparticle sizes. In this paper, we describe a fundamental experiment to verify the feasibility of using this system for different sizes of nanoparticles.

Keywords: nanoparticle, fluorescence polarization, Brownian motion, diffusion coefficient, particle sizing

1. INTRODUCTION

The integration of nanoparticles allows devices to exhibit unique electronic, photonic, and catalytic properties. It offers the prospects of both new fundamental scientific advances and useful technological applications. Many of the fundamental properties of materials (optical, electrical, mechanical, etc.) can be expressed as functions of their size, shape, composition, and structural order [1]. It is important to evaluate nanoparticle sizes and maintain a constant state (size distribution, average size, shape uniformity).

The size distribution of monodispersed nanoparticles in a solvent can be measured using dynamic light scattering. However, the scattering method is inappropriate to detect the sizes of mixture particles, which include both small monodispersed particles and large particles such as agglomerates. The signal intensity used to evaluate the particle diameter is linear to the sixth power of the particle diameter. Therefore, the scattering signal from monodispersed particles, compared to the signal from their agglomerates, is too small to detect.

The size distribution of nanoparticles with a wide size distribution can be measured using transmission electron microscopy (TEM). This requires a sample in a dried condition. However, it is difficult to evaluate both the average diameter and size distribution of nanoparticles because of the instability of their diameters in a solvent.

In order to measure the sizes of nanoparticles with a wide size distribution in a solvent, we developed an optical microscopy system that allows for fluorescence polarization (FP) measurement and optical observation. The FP method can trace the rotational diffusion constant of Brownian motion in a fluorescent molecule. When the fluorophore is used to label nanoparticles, the rotational diffusion coefficient corresponds to the size of the nanoparticles. The system makes it possible to evaluate nanoparticle sizes over a wide range, because the fluorescent signal intensity is independent of the changes in nanoparticle sizes. In this paper, we describe a fundamental experiment to verify the feasibility of using this method for different sizes of nanoparticles.

2. PRINCIPLE

2.1 Measurement of rotational diffusion coefficient

We have developed a fluorescence polarization method to measure nanoparticle sizes by measuring the rotational diffusion coefficient, D_r . When linearly polarized light is irradiated on a nanoparticle labeled with a fluorophore, a fluorescent signal maintains the polarization state of the irradiation light at first. The fluorescent signal then depolarizes as time passes. The fluorescence anisotropy, $r(t)$, depends on the angle of the particle rotation. In addition, the depolarization is quick for particles with small volumes, while the polarization state is maintained longer in particles with large volumes.

The fluorescence anisotropy, $r(t)$, is related to the rotational diffusion coefficient, D_r, of a nanoparticle. The particle size can be calculated from the rotational diffusion coefficient, D_r, on the basis of an analysis using the Debye-Stokes-Einstein (DSE) relationship [2,3], when the particle shape is approximated to that of a sphere.

$r(t)$ is defined by the following equation:

$$r(t) = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \qquad (1)$$

where the first and second subscripts refer to the orientation of the excitation and the orientation of the emission, respectively. $I_{\parallel}$ and $I_{\perp}$ are the horizontally polarized (p-polarized) and vertically polarized (s-polarized) components of fluorescence, respectively, when horizontally polarized light is irradiated.

$r(t)$ is also expressed as a function of time t as follows:

$$r(t) = \left(r_0 - r_\infty\right) e^{-t/\theta} + r_\infty \qquad i(t) \; = \frac{e^{-t/\tau}}{\tau} \tag{2}$$

where $i(t)$ is the instantaneous emission intensity, normalized to a unit-integrated value. Parameter r_∞ reflects the extent of the rotational reorientation of the fluorophore. In this case, $\bar{r}$ is obtained by integrating the intensity-weighted $r(t)$, as shown in Eq. (3).

The steady-state anisotropy, $\bar{r}$, of a fluorophore undergoing isotropic rotational diffusion is related to the fluorescence lifetime, τ, and the rotational correlation time, θ [4].

$$\bar{r} = \int_0^\infty r(t) \bullet i(t) \;\Leftrightarrow\; \frac{r_0 - r_\infty}{\bar{r} - r_\infty} \; = 1 + \sigma \tag{3}$$

where r_0 is a limiting value in the absence of rotation given by the relative orientation of the absorption and emission transition moments, and σ is the ratio of τ/θ.

Measurements of anisotropy decay can reveal a multiplicity of rotational correlation times reflecting heterogeneity in the size, shape, and internal motions of the fluorophore-nanoparticle conjugate. Determinations of rotational correlation times are performed either in the time or in the frequency domain. The rotational diffusion coefficient can be precisely calculated from the fluorescence anisotropy in the frequency domain. Using an excitation pulse as a forcing function, Eq. (3) is transformed into a decay process in which the fluorescence lifetime, τ, is no longer linked to the correlation time.

The determination of rotational correlation times is achieved by measuring three parameters: Φ, Y_{AC}, and Y_{DC}. In order to define Φ, Y_{AC}, and Y_{DC}, we measure the sinusoidal waveforms of both $I_\parallel$ and $I_\perp$ in the frequency domain. A series of $I_\parallel$ and $I_\perp$ are measured while adjusting the relative phase between the excitation light and the detector gain, when the excitation light and detector gain are modulated using the same radial frequency.

After the acquisition of the sinusoidal signals of $I_\parallel$ and $I_\perp$, each data set is processed to yield frequency-dependent amplitudes and the phase shift between the excitation and emission light. The resulting polarized emission components are modulated at the same frequency but phase shifted with phase decay Φ relative to each other [4].

$I_{\parallel AC}$ and $I_{\perp AC}$ are the amplitudes of $I_\parallel$ and $I_\perp$ in the frequency domain, respectively. $I_{\parallel DC}$ and $I_{\perp DC}$ are the average values of $I_\parallel$ and $I_\perp$, respectively. These signals are characterized by the ratio:

$$Y_{AC} = I_{\parallel AC} / I_{\perp AC}\,,\; Y_{DC} = I_{\parallel DC} / I_{\perp DC} \tag{4}$$

Furthermore, the fluorescence lifetime of the emission light can be measured in the frequency domain. The fluorescence lifetime, τ, is determined by both a series of data sets for the excited light intensity, $i(\omega)$, and the total light intensity of the emission light, $I(\omega)$.

The three parameters, Φ, Y_{AC}, and Y_{DC}, are then related to parameters $\bar{r}$, r_0, r_∞, and σ by

$$\Phi = \tan^{-1} \frac{3\sigma\left(r_0 - r_\infty\right)}{2\sqrt{\begin{matrix}\left(1-r_0\right)\left(1+2r_0\right)\left[\left(1-r_0\right)+\left(1-r_\infty\right)\sigma\right] \\ \bullet\left[\left(1+2r_0\right)+\left(1+2r_\infty\right)\sigma\right]\end{matrix}}} \tag{5}$$

$$Y_{AC} = \sqrt{\frac{\left(1+2r_0\right)^2\left(\omega\tau\right)^2 + \left[\left(1+2r_0\right)+\left(1+2r_\infty\right)\sigma\right]^2}{\left(1-r_0\right)^2\left(\omega\tau\right)^2 + \left[\left(1-r_0\right)+\left(1-r_\infty\right)\sigma\right]^2}} \tag{6}$$

$$Y_{DC} = \frac{\left(1+2r_0\right)+\left(1+2r_\infty\right)\sigma}{\left(1-r_0\right)+\left(1-r_\infty\right)\sigma} \tag{7}$$

$$\bar{r} = \frac{Y_{DC} - 1}{Y_{DC} + 2} \tag{8}$$

The rotational correlation time, θ is given by

$$\theta = \left[\frac{Y_{AC}\begin{bmatrix}\left(Y_{AC}^2 - 2Y_{DC}\right)\left(Y_{DC}+2\right)\sqrt{1+\tan\Phi^2} \\ +Y_{AC}\left(Y_{DC}-4\right)\end{bmatrix}\tan\Phi \bullet \omega\tau}{\left(Y_{AC}^2 - 4\right)\left(Y_{AC}^2 - Y_{DC}^2\right) + \left(Y_{AC}^2 - 2Y_{DC}\right)^2 \tan\Phi^2} - 1 \right]^{-1} * \tau \tag{9}$$

In order to define the rotational correlation time, θ, the fluorescence life time, τ, is required. The fluorescence lifetime, τ, is determined by the phase decay δ and the modulation m of fluorescence, which is excited with light whose intensity, i(t), varies sinusoidally with time [5]:

$$i(t) = a + b\sin\omega t \tag{10}$$

where ω is the angular velocity of the modulated excitation light [5]. As a consequence of the finite duration of the excited state, the modulated fluorescence emission is delayed in phase by angle δ, relative to excitation. In addition, a decrease in the modulation of the fluorescence occurs. The intensity of the fluorescence is given by

$$I(t) = A + B\sin\left(\omega t - \delta\right) \tag{11}$$

We acquire the phase decay, δ, between the sinusoidal curve of the excitation light and the emitted fluorescence without the emission polarizer in the frequency domain.

$$\tan\delta = \omega\tau \tag{12}$$

2.2. Particle sizing using sphere model

For Brownian particles reorienting in a liquid, the Debye model defines an exponential decay for the single-particle orientational time correlation function, C, and rotational correlation time, θ:

$$C = \exp(-t/\theta) \tag{13}$$

$$\theta = \frac{1}{6D_r} \tag{14}$$

where D_r is the rotational diffusion coefficient. D_r is coupled with the shear viscosity, η, at temperature T using the Debye-Stokes-Einstein (DSE) relationship [2,3].

$$D_r = \frac{k_B T}{V_H \eta} \tag{15}$$

where k_B is the Boltzmann constant. The hydrodynamic volume, V_H, is related to the particle volume, v, through a factor that depends on the shape of the reorienting particle and the boundary conditions. Particle volume v can be calculated by measuring the rotational diffusion coefficient, D_r, when the relation between the hydrodynamic volume, V_H, and the particle volume, v, is formulated. For example, $V_H = 6v$ for a sphere with stick boundary conditions. The DSE relationship is known to be effective to the molecular length scales for low-viscosity liquids.

Consequently, the hydrodynamic volume, V_H, of a fluorophore can be determined using Φ, Y_{AC}, Y_{DC}, ω, and τ on the basis of Eq. (14), Eq. (15), and Eq. (12). When sphere approximation is applied to a fluorophore-nanoparticle conjugate, the diameter can be calculated from the hydrodynamic volume, V_H. This reflects the excited state lifetime and intrinsic rotational diffusion properties of the fluorophore and the modulation frequency.

In the proposed method, the fluorescence particle, which works as the probe for particle sizing, is connected to the nanoparticle to be measured. The rotational diffusion time for the fluorescence particle is caluculated by using stick boundary condition for the sphere particle. When the nanoparticle is connected to the probe, the rotational diffusion time for the probe are change corresponding to their volume. In order to evaluate the particle size which connects to the probe, we investigate the relation between the rotational diffusion time and particle size for the fluorescence nanaparticle which is connected to the standard nano partcile. We can evaluate the particle size by comparing the result of the standard nanoparticle whose size is determined.

3. EXPERIMENTAL SETUP

We developed a rotational diffusion coefficient measurement system using a fluorescence polarization method, as shown in Fig. 1. An Ar^+ laser (wavelength: 488 nm) is the polarized light source, and it is coupled to an acousto-optic modulator (AOM). Before coupling to the AOM, the polarization direction is oriented using a half wave plate (1/2 WP) in order to improve the diffraction efficiency of the AOM. A high-speed light amplitude modulation as fast as 80 MHz can be achieved in the unit, which consists of the AOM, lens (L), and iris.

The polarization direction of the input signal is then oriented by a half wave plate (1/2 WP) just as with the polarization direction of $I_{\parallel}$. The incident light is illuminated on the sample via a half mirror (HM) and objective lens. The sample is excited using linearly polarized light. The emission is relayed through the beam displacer, which divides the fluorescence polarization signal oriented either parallel, $I_{\parallel}$, or perpendicular, $I_{\perp}$, to the excitation beam polarizer. The fluorescence signals are finally relayed to the image intensifier. The image of the orthogonal components of the fluorescence signal is enhanced on the image intensifier and then relayed to a CCD. Images of both the horizontally polarized, $I_{\parallel}$, and the vertically polarized components, $I_{\perp}$, are analyzed to acquire the fluorescence anisotropy. The modulation of the gain of the image intensifier corresponds to the modulation of the input signal amplitude. The phase decay, d, is the phase difference between the incident light and the gain of the image intensifier. Acquisition proceeds with a series of phase shifts to acquire a first-order photo bleaching compensation. A data series is processed to yield frequency-dependent amplitudes, along with the phase shift between the excitation light and emitted lights with the two orthogonal polarization directions. The polarized emission components are modulated at the same frequency but phase shifted relative to each other.

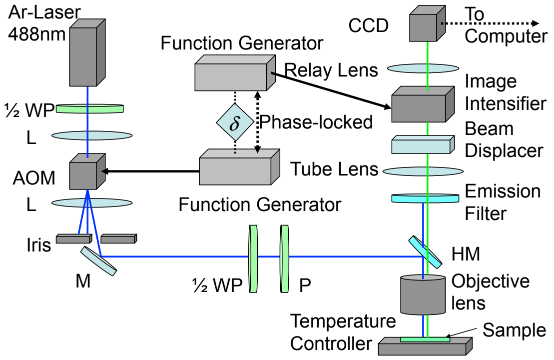

Fig.1 Experimental seteup

4. EXPERIMENTAL RESULTS

4.1 Rotational correlation time for fluorophore

In order to achieve the particle size measurement, the precise measurement of the rotational diffusion coefficient is required, which corresponds to the rotational correlation time, as shown in Eq. (14). We perform fundamental experiments to verify the feasibility of precise measurement of the rotational correlation time for the fluorophore without labeling gold nanoparticles.

First, the fluorescence anisotropy as a function of the time, *t*, is evaluated using the developed system. Three solutions are prepared for measuring the rotational correlation time of a fluorophore by mixing water with glycerin at 30 wt%, 50 wt%, and 60 wt%. The viscosity varies, with 2.5 mPa·s, 6.0 mPa·s, and 10.8 mPa·s at 293 K, respectively. We also use water as a solution, which has a viscosity of 1.0 mPa·s at 293 K. The fluorophore is Alexa Fluore 488 (Invitorogen Corp.), which has the same size as fluorescein. The fluorescence lifetime, τ, varies, with values of 4.1 ns in water, 3.8 ns in 30 wt% glycerin, 3.6 ns in 50 wt% glycerin, and 3.1 ns in 60 wt% glycerin.

The theoretical value of the rotational correlation time, which depends on the particle size, can be calculated from Eq. (14) and Eq. (15), when we apply the sphere shape approximation for the fluorophore, as follows:

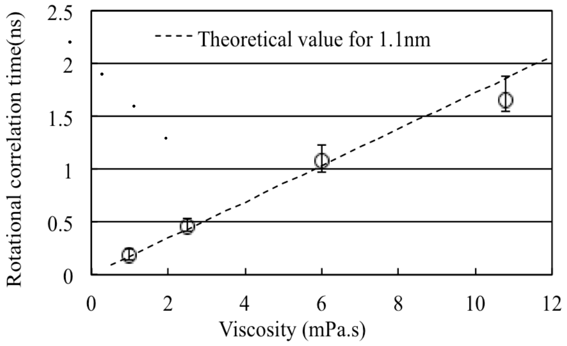

Fig. 2 Rotational correlation time of fluorophore.

$$\theta = \frac{6\pi d^3 \eta}{k_B T} \qquad (16)$$

The theoretical value is plotted according to the solution temperature, T (293 K), and nanoparticle diameter, d (1.1 nm). According to Eq. (16), the rotational correlation time of the fluorophore agrees well with the value for a nanoparticle with a diameter of 1.1 nm under the nonslip boundary condition. This value is close to that of the fluorescein, whose size is estimated to be 1.0 nm [6]. The size difference is considered to be an effect of hydration on the fluorophore in the solution. From the above results, the rotational correlation time can be precisely measured using the developed system.

4.2 Rotational correlation time for non-spherical particle

In order to evaluate the diameter of a gold nanoparticle labeled with a fluorophore, the rotational correlation time of the fluorophore with gold nanoparticles that are smaller than 10 nm is investigated. The rotational correlation time of nanoparticles in solution was validated using the fluorophore with gold nanoparticles with diameters of 5.9 nm and 8.2 nm. The sizes of the gold nanoparticles were measured using TEM.

The rotational correlation time for the 5.9-nm gold nanoparticle is 1.2 ± 0.1 ns, and that for the 8.2-nm nanoparticle is 2.0 ± 0.1 ns. The rotational correlation time for the fluorophore is 0.2 ± 0.1 ns. All of the samples are dispersed in water, and the fluorescence lifetime, τ, is 4.1 ns. The viscosity at 293 K is 1.0 mPa·s. The size validation for the gold nanoparticles smaller than 10 nm shows that the change in the rotational correlation time from 1.2 ns to 4.1 ns corresponds to an increase in the nanoparticle size. The change in the rotational correlation time for the fluorophore can be used to estimate the size of a gold nanoparticle smaller than 10 nm.

However, the sphere approximation model cannot be applied to the fluorophore with gold nanoparticles. Therefore, we need to develop a non-spherical model for the rotational motion of the fluorophore with gold nanoparticles.

5. CONCLUSION

We developed a nanoparticle sizing system using a fluorescence polarization method. This system can precisely determine the rotational correlation time of nanoparticles with a fluorophore. Based on the results, we can define the size of a nanoparticle based on the DSE relation when the particle shape is approximated by a sphere.

We also investigated the rotational correlation time of a fluorophore with gold nanoparticles that were smaller than 10 nm. A change in the rotational correlation time from 1.2 ns to 4.1 ns could be detected using the developed system. This indicates that the measurement results for the rotational correlation time of a fluorophore-labeled gold particle can be used to estimate the size of gold nanoparticles smaller than 10 nm.

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