Hydrophobically ethoxylated modified urethane (HEUR) form the flower micelles and transient network structures through the aggregation of end groups depending on the polymer concentration. The HEUR networks show the viscoelastic relaxation which is described by the Maxwell model. Although there have been many attempts to understand and predict the molecular mechanism, the full understanding remains incomplete. To understand the relaxation mechanism of associative polymers, we measured the linear viscoelasticity and the diffusibility using the fluorescence recovery after photobleaching method. With increasing the polymer concentration, the self-diffusion coefficient decreased, while the relaxation time increased, suggesting that the viscoelastic relaxation is correlated with the diffusion of the polymers. The calculated diffusion distance of the HEUR chains within the viscoelastic relaxation time is 100 times larger than the size of a HEUR chain. This significant deviation suggests that some of the HEUR chains diffuse quickly through the unimer and flower micelles at low concentrations and through the recombination process of the network strands at high concentrations.

Key Words: Associative polymer / Transient network / Viscoelasticity / Hydrophobically modified ethoxylated urethane (HEUR) / Fluorescence recovery after photobleaching (FRAP)

1. INTRODUCTION

Hydrophobically ethoxylated modified urethane (HEUR) is composed of the hydrophilic main chain and the hydrophobic end groups. HEUR forms the flower micelles and transient networks through the hydrophobic interaction depending on the concentration. It is well known that the HEUR networks exhibit single viscoelastic relaxation. Due to this mechanical simplicity, the HEUR networks have been employed as a model system of the transient network\(^1\)\(^-\)\(^8\).

Theoretically, this single relaxation behavior was explained by the classical transient network theory, developed by Green, Tobolsky\(^9\), and Yamamoto\(^10\). They assumed that the viscoelastic relation is characterized only by the lifetime of the bonds, which corresponds to the lifetime of aggregation cores. Under these assumptions, the anisotropic orientation disappears immediately after the dissociation of cores, leading to single relaxation behavior. According to the classical theory, the viscoelastic relaxation time is predicted to be the same with the lifetime of the cores and independent of the polymer concentration and the molecular mass of the chains. However, there have been experimentally reported that the relaxation time depends both on the concentration and the molecular mass\(^1\)\(^-\)\(^3\),\(^7\). Tanaka and Edwards proposed the modified model considering the recombination of the chains, to describe the deviation of the experimental results from the prediction of the classical theory\(^11\)\(^-\)\(^13\). In their model, a chain which dissociates from an aggregation core does not lose the anisotropy until when the chain recombines a different core which does not correlate to the initial core. To describe the recombinant effect, they introduced the possibility that a chain recombines a different core after the dissociation from the initial core. Their modification to some extent explained the experimental results, while the molecular origin of the recombinant possibility remains unclear.

Furthermore, they assumed that the networks are formed ideally: there is no heterogeneous structure including dangling chains, superbridge structures, and flower micelles.
However, the practical networks examined experimentally have the flower micelles, loop structures, especially in lower concentration. Also, the number of the bridge chains increases with increasing the concentration\(^2,7\). Thus, under the presence of these heterogeneous structures, the diffusion of the HEUR chains is expected to be accelerated, which should influence the viscoelastic relaxation.

To discuss the relationships between the diffusion and the viscoelastic relaxation, the measurement of the self-diffusion coefficient is essential. There are two primary methods to determine the self-diffusion coefficient; the pulsed-field gradient nuclear magnetic resonance (PFG-NMR) and the fluorescence recovery after photobleaching (FRAP). The PFG-NMR applies the magnetic gradient fields with a particular direction and generates the phase difference of the nuclear spins, which entitles the protons of the target molecules to the positional information in the applied direction. After the removal of the gradient field, the decay of the echo signals due to the self-diffusion is observed. In practical measurement, the signal intensity and the interval of the pulse are tuned. According to the obtained relationships between them, the diffusion coefficient is evaluated\(^14,15\). The PFG-NMR measurement is easy because the diffusion of the protons is detected and the chemical modification is not required. While the PFG-NMR is not good at measuring the slow diffusion including the transient networks since the diffusion time slower than the \(T_2\) relaxation time cannot be observed.

On the other hand, in the FRAP measurement, molecules with fluorescent probes in a limited area are exposed to intense light and photobleached. The self-diffusion coefficient is evaluated according to the time-development of the intensity recovery due to the self-diffusion of the molecules. The FRAP method requires chemical modification by the fluorescent probe. Although one should consider the effect of the modification, the FRAP method can detect the wide-ranged diffusion coefficient regardless of whether the sample is solid or liquid.

In this study, we synthesized the HEUR modified with a fluorescein isothiocyanate (F-HEUR) and performed the FRAP and linear viscoelastic measurements. Here, the polymer concentration ranged from 1.8 to 6.0 wt%, which covers from the sparse networks with the heterogeneous structure to the dense networks with relatively high bridge chain content. In this paper, we propose a general method to analyze the FRAP recovery data and to determine the average diffusion coefficients for systems with dynamical heterogeneity due to the heterogeneous structure of HEUR depending on the concentration. By comparing the diffusion coefficient with the linear viscoelastic behavior, we discuss the molecular mechanism of the relaxation.

2. MATERIALS AND METHODS

2.1 Synthesis of F-HEUR

As a telechelic associative polymer, ADEKA NOL GT-700 (ADEKA), which was kindly supplied from ADEKA, was employed. The chemical structure is shown in Fig. 1 (a). The molecular mass, \(M_w\), was \(3.5 \times 10^4\) g mol\(^{-1}\) and the polydispersity index, \(M_w/M_n\), was 3.7. 10.09 g of the HEUR was dissolved into 300 mL of \(N, N\)-dimethylformamide, which was preliminarily dried using the molecular sieve 4A. After stirring at 60 °C for 20 minutes, 105.4 mg of sodium hydride was added mildly and the solution was stirred for 15 minutes. 105.3 mg of FITC (\(M_w = 389.38\) g mol\(^{-1}\)) was added into the resultant solution which was reacted for 24 hours under stirring. In order to remove unreacted elements, the mixture was dialedyzed by dimethyl sulfoxide and milliQ water for three days, respectively. After the dialysis, the F-HEUR was obtained by the freeze drying. The schematic illustration of the reaction is shown in Fig. 1 (b).

2.2 Gel Permeation Chromatography (GPC)

The F-HEUR sample was characterized with size-exclusion chromatography utilizing a column/pump system (DP-8320 GPC EcoSEC, Tosoh) equipped with a refractive index monitor (RI-8011). The elution solvent was THF. Commercially available monodisperse polystyrene samples (Tosoh) were utilized as the solution standards. The concentration of the F-HEUR was 0.50 wt%.

2.3 Dynamic Viscoelastic Measurement

Dynamic viscoelasticity was measured using a stress-controlled rheometer MCR 302 (Anton Paar). The
measurements were carried out at 10-35 °C with a cone-plate fixture with 25- and 40-mm diameters. Cone angles were 4.0° and 4.011°, respectively. The angular frequency sweep tests were carried out from 0.1 to 100 rad s⁻¹. The oscillatory shear strain amplitudes for all the tests were within the range of linear viscoelasticity.

2.4 Fluorescence recovery after photobleaching (FRAP)

The self-diffusion coefficients of the F-HEUR were measured by the FRAP method using a laser scanning confocal microscopy (Nikon C2 Plus, Tokyo, Japan) at room temperature. A circular range with a diameter of 100 μm was selected as a bleaching area. Before the bleaching, the fluorescence intensity of the target area was measured for 30 s. After the observation, the laser (the wavelength: 488 nm) was utilized for the bleaching. The duration of the bleach was set at 1 s. Then, the detection of fluorescence recovery was observed for 10 min.

2.5 Pulsed Field Gradient-¹H Nuclear Magnetic Resonance (PFG-¹H NMR) Measurements

In order to measure the self-diffusion coefficient of HEUR in methanol, the PFG-¹H NMR measurements were carried out. The apparatus used was ECA-500 manufactured by JEOL Co., Ltd. The probe was 5 mm HX/FG probe. The measurement temperature was 30 °C. The HEUR solutions were poured into an inner tube with an outside diameter of 3 mm. The inner tube was set inside the outer tube containing deuterated water as a locking solvent. Conventional ¹H single-pulse measurements were performed to confirm chemical shifts, shim adjustment, and the value of receiver gain. 90° pulse measurement was then carried out to obtain an accurate 90° pulse width. PFG-¹H NMR measurements were carried out using the obtained parameters. PFG-¹H NMR was performed with a pulse sequence called BPP-STE-LED. The diffusion time, Δ, was 200 ms, the PFG intensity varied from 3 mT/m to 900 mT/m, and the PFG irradiation time was adjusted through the sample. We utilized the methanol just in the NMR measurement; we did not use it in the FRAP and viscoelastic measurements.

3. RESULTS AND DISCUSSION

3.1 Characterization of F-HEUR

To characterize the synthesized HEUR, we performed the GPC measurements. Figure 2 shows the GPC spectrum for the original HEUR and F-HEUR solutions. In both the original HEUR and F-HEUR, three pronounced peaks at 1.4×10⁴, 2.8×10⁴, and 4.4×10⁴ g mol⁻¹ were observed. The F-HEUR showed a little peak shift to the larger molar mass region, which is attributed to the change in the addition of the FITC. This good correspondence of the GPC spectrum before and after the chemical modification indicates that the scission of main chains by the unexpected radical attack is negligible and that the chain length of the F-HEUR is almost similar with that of the original one. It should be noted that the excess amount of fluorescence modification induces the scission of the main chains. There are some possibilities that the scission of the main chains occurs, which is below the detection limit of the GPC. We believe that these small chains were eliminated by dialysis in dimethyl sulfoxide to some extent.

To evaluate the network formation of the F-HEUR, we performed the dynamic viscoelastic measurements. Figure 3 (A) shows the frequency dependences of the F-HEUR and HEUR solutions with concentration of 2.0 wt%. Gray and black symbols represent the data of the F-HEUR and HEUR, respectively. Filled and open symbols are the storage and loss moduli, respectively. Both spectra well agree with the prediction of the Maxwellian model as

\[ G' = G_0 \frac{\alpha^2 \tau^2}{1 + \alpha^2 \tau^2} \]  \hspace{1cm} (1)

\[ G'' = G_0 \frac{\alpha \tau}{1 + \alpha^2 \tau^2} \]  \hspace{1cm} (2)

where \( G_0 \) is the relaxation strength, \( \tau \) is the viscoelastic relaxation time. This agreement suggests that the F-HEUR forms the transient networks even after the modification of the FITC, while the \( G_0 \) was smaller and \( \tau \) was shorter compared with the original one. The reduction of the \( G_0 \) for the F-HEUR means that the decrease of the effective network density and the decrease of the \( \tau \) implies the faster time scale of the
recombination of the hydrophobic core. Both phenomena are related to each other and due to the modification with the FITC.

Figure 3 (B) shows the composite curve of the F-HEUR solution, measured at 10-35 °C. The time-temperature superposition principle is employed, where a reference temperature is 25 °C. The composite curves are constructed by shifting \( G' \) and \( G'' \) data at different temperatures horizontally and vertically, to superimpose the low-frequency data. \( a_T \) and \( b_T \), respectively denote the horizontal and vertical shift factors. In Fig. 3 (C), the natural logarithm of the shift factor, \( \ln a_T \), is plotted against \( T^{-1} \). Values of \( \ln a_T \) of the F-HEUR (circle) and the original HEUR (triangle) well correspond to each other. This agreement indicates that the activation energy for the main relaxation mode mainly related to the recombination of the hydrophobic core even in the F-HEUR is identical with that in original HEUR, suggesting that the stability of the hydrophobic core is not so affected by the FITC labeling.

It should be noted that the linear relationship between \( \ln a_T \) and \( T^{-1} \) was observed as shown in Fig. 3 (C), which corresponds to the Arrhenius behavior described as

\[
\ln a_T = \frac{E_a}{R} \left( \frac{1}{T} - \frac{1}{T_f} \right)
\]

Here, \( E_a \) and \( R \) represent the activation energy and the gas constant, respectively. Using Eq. (3), the activation energy is evaluated to be \( E_a = 54 \text{ kJ mol}^{-1} \).

### 3.2 Concentration dependence of linear viscoelastic properties for F-HEUR

Figure 4 (A) shows the concentration-dependence of the relaxation strength for the F-HEUR and original HEUR solutions. The relaxation strengths for the F-HEUR and HEUR increased with increasing the concentration. In Fig. 4 (A), the dashed line is the prediction of the affine network model\(^{17-22} \), where it is assumed that the HEUR networks have the ideal structure composed of the bridge chains. At the low concentration region, the experimental results are lower than the affine network prediction, suggesting the presence of the flower micelles, dangling chains, and super bridge chains. On the other hand, at the high concentration region, the experimental values asymptotically approach to the prediction value, suggesting that the number of the bridge chains increases, and that a resultant network is close to the ideal one without heterogeneous structures.

Figure 4 (B) shows the relaxation time against the polymer concentration. The relaxation time increased with increasing the concentration as well. These experimental results are consistent with the previous data\(^{2,7} \). This increment reflects two possibilities. One is the fact that the surrounding chains and networks retard the dynamics of the HEUR chains and networks. In high concentration region, the increment of
the number of chains increases the local viscosity, resulting in the retardation of the relaxation time. The other one is due to the relaxation accompanied with chain scissions. In the system including superbridge strands with \( n \) precursor chains, when one of the ends in a superbridge strand breaks, the strand lose the energy \( kT \), leading to the viscoelastic relaxation. The frequency for effective breakup increases with increasing the length of the superbridge strand, which accelerates the viscoelastic relaxation, because the number of the breakable points increases\(^{23}\). In the high concentration region, the reduction of the size of the network strands would reduce the frequency for effective breakup, resulting in the increment of the relaxation time.

It should be noted that the relaxation time and strength of the F-HEUR decreased compared with those of the original HEUR, reflecting the reduction of the hydrophobic interaction. However, the concentration-dependence of these parameters can be overlapped with those of the original one only by vertical shifts, suggesting that the viscoelastic behaviors of HEUR and F-HEUR solutions differ due to the difference in the effective concentrations to form the network structures. These results and the correspondence of the activation energy, and the Maxwellian viscoelastic behavior inferred that the aggregation structures of the F-HEUR and HEUR are different, but the mechanism of the viscoelastic relaxation is identical.

### 3.3 Analysis of FRAP recovery data and estimated diffusion coefficient of F-HEUR

Figure 5 (A) shows the time-development of the fluorescent intensity for the 3.0 wt% F-HEUR solution in the FRAP measurement. The fluorescent intensity was reduced step-wise at \( t = 0 \) by the laser irradiation. The intensity recovered with time and reached the plateau around 100 s. This fluorescent intensity, \( F \), in the recovery process is fitted with the Kohlrausch-Williams-Watts (KWW) type equation expressed as

\[
F = A - B \exp\left(\left(-\frac{t}{\tau_{\text{KWW}}}\right)^\beta\right)
\]

\( A \) is the intensity at the plateau region, \( B \) is the reduction in the intensity, and \( \tau_{\text{KWW}} \) is the characteristic time. It should be noted that the fluorescence photobleaching recovery process for single component diffusing species is well described by a two-dimensional diffusion equation\(^{24}\). As described in Appendix 1, the functional form of time-dependent fluorescent intensity depends on initial photo-bleaching patterns. In the present case, shallow beaching with a Gaussian beam, the KWW function can approximately fit the time dependence of the intensity recovery.

In Fig. 5 (A), the dashed line represents the fitting result using Eq. (4), where \( A = 3.6 \times 10^3 \), \( B = 3.3 \times 10^2 \), \( \tau_{\text{KWW}} = 13 \) s, and \( \beta = 0.61 \). As mentioned in the Appendix 1, the case of \( \beta = 0.6 \) approximately corresponds to the functional form deduced from the diffusion equation. Therefore, the case of \( \beta < 0.6 \) suggests that there exists distribution in the diffusion time. The concentration \( C_{\text{HEUR}} \) dependence of \( \beta \) is shown in Fig. 5 (B). It is seen that \( \beta \) value decreases from around 0.6 to 0.4 with increasing \( C_{\text{HEUR}} \), which is also observed in the previous study by Rao et al.\(^{25}\) This result suggests that the heterogeneity increases with \( C_{\text{HEUR}} \). It is known that the first- and second-order average relaxation times, \( <\tau_{\text{KWW}}^2>^{11}\) and
By considering that the Eq. (4) with $\beta = 0.6$ are the standard curve without distribution, the average of the diffusion time in Eq. (4) is given by

$$\langle \tau_D \rangle^{[1]} = \frac{\tau^{(1/\beta)}_{\text{KWW}} 0.6 \tau_{\text{KWW}}}{\beta \tau^{(1/\beta)}_{\text{KWW}}} \gamma$$  \hspace{1cm} (6a)

$$\langle \tau_D \rangle^{[2]} = \frac{\tau^{(2/\beta)}_{\text{KWW}} \tau^{(1/\beta)}_{\text{KWW}}}{\tau^{(2/\beta)}_{\text{KWW}}} \gamma$$  \hspace{1cm} (6b)

Here we use $\gamma = 1.5$, which is the factor to convert $\tau_{\text{KWW}}$ to $\tau_D$ (cf. Fig. S1), and determine two kinds of diffusion time. According to the definition of the diffusion time, the two-dimensional lateral self-diffusion coefficient is calculated by the following equation,

$$D = \frac{w^2}{4\langle \tau_D \rangle^{[n]}} \hspace{1cm} (n = 1 \text{ or } 2)$$  \hspace{1cm} (7)

where $w$ is the radius of the bleached area ($w = 50 \mu$m). Figure 5 (C) show the values of $D$ estimated from the second-order average time plotted against the concentration. Noted that $D$ can be estimated from the first order average time as well, which is shown in Fig. S2 of Appendix 2. Although the absolute values are different, the tendency of both parameters is similar. Thus, from here we discuss based on the second-order average time.

The dashed line in Fig. 5 (C) represents the value of $D$ for the HEUR methanol solution, estimated by the PFG-NMR measurement. In methanol, the hydrophobic interaction is suppressed, and the HEUR are dissolved as a unimer. The obtained $D$ for the F-HEUR is slightly lower than that of the HEUR-methanol solution, suggesting that the association retards the diffusion of the HEUR chains. This result quantitatively corresponds to the prediction of the sticky Rouse model. $D$ decreased with increasing the concentration, suggesting that the dynamics of the HEUR chains are restricted by the local viscosity and/or the increment of the number of large micelles.

It should be noted that the values of $D$ we obtained were larger than those previously reported by Rao et al.\textsuperscript{25} Rao et al. measured the self-diffusion coefficients for the HEUR polymers with the PFG-NMR method. They obtained the self-diffusion coefficients ranging from $10^{-14} - 10^{-12}$ m$^2$s$^{-1}$ with the concentrations below 1 wt%. This is attributed to the differences in the chemical structures. Our polymers have the urethane bonds and the fluorescent molecules in the main chains. These two structures reduce the hydrophilicity of the main chains and the aggregation strength of the end groups, which was already discussed in the previous section. A reduction of the aggregation interaction by
the fluorescent-modification was also reported by Suzuki et al.\textsuperscript{27} Thus, we cannot compare the absolute values with the conventional data, but we believe that the comparison of the FRAP and viscoelastic data using the same sample is permitted.

3.4 The relationship between diffusion and viscoelastic relaxation of F-HEUR

To discuss the correlation between the diffusion and viscoelastic relaxation, the diffusion distance, $L$, within the viscoelastic relaxation time, is introduced. $L$ is expressed as

$$L = (r D)^{\frac{1}{2}}$$

(8)

where $r$ is the viscoelastic relaxation time and $D$ is the diffusion coefficient estimated from the FRAP data. Figure 6 shows the polymer concentration-dependence of $L$. $L$ is almost constant ($\sim 10^{-6}$ m) regardless of the concentration. The value of $L$ is on the order of 1 μm, which is 100 times larger than the precursor chain size shown as the dashed line. One of the presumable reasons for this deviation is that the viscoelastic relaxation cannot be determined only by the equilibrium of the HEUR chains after the pulling out step as assumed in the classical theory of the transient network. It is straightforward to understand that the network strands are composed of some precursor chains due to the presence of the loop structures. Hence, we need to employ network strands of length, $\xi$, instead of single precursor chains. $\xi$ is considered to be the length between crosslinks, which is assumed to be homogeneously distributed in space; it is given by the following equation.

$$\xi = \frac{v}{3} = \left(\frac{k_B T}{\sigma}ight)^{\frac{1}{2}}$$

(9)

Figure 6 also depicts $\xi$ as a function of polymer concentration. $L$ is about 10-100 times longer than $\xi$ for all the measured regions, which means that the significant difference between the diffusion distance and the strand length remains even in the high concentration, where the networks are formed containing few heterogeneities.

These deviations between $L$ and $\xi$ is attributed to the dynamic heterogeneity of the transient networks. In the low concentration region, the networks are composed not only of super bridge structures but also of unimers and flower micelles, according to the concentration dependence of the relaxation strength. In such sparse networks, the network component, which dominates the viscoelasticity, diffuses slowly, while the unimers and flower micelles diffuse rapidly. The viscoelastic parameters are sensitive to the network component. On the other hand, the FRAP measurement possibly detects only the diffusion of the fast components, i.e., unimers and flower micelles, resulting in a significant difference between $L$ and $\xi$ in the low concentration region.

However, in the high concentration region, where the networks are composed of the ideally connected chains with few unimers and flower micelles, it is unclear which component other than the network one diffuses rapidly. As shown in Fig. 5 (A), the fluorescent intensity did not return to the initial value at the long-time limit. Here, the recovery component could correspond to the rapid diffusing species, which includes unimers and flower micelles, and unrecovered component could be the molecules incorporated firmly in the network structure. However, even in the high concentration region where the nearly ideal network structure was concluded from the modulus data shown in Fig. 4 (A), the FRAP recovery ratio corresponding to the rapid diffusing component is still higher than 50%. This means that some of the molecules incorporated in the network structure can diffuse rapidly through the recombination of the crosslinking cores, whose recombination rate is much higher than the viscoelastic relaxation time.

Based on these results, we think that the viscoelastic relaxation of the HEUR networks is not primarily determined by the dissociation of the aggregation cores and the diffusion of the HEUR chains. The diffusion process is dominated by fast diffusing component such as unimers and flower micelles in the low concentration region, while some of the HEUR molecules which take part in the network structure could also diffuse faster through the recombination of the aggregation cores of the HEUR chains in the high concentration region. Notably, the viscoelastic relaxation is not completed even if...
the fast component HEUR molecules diffuse the distance of the network strands. In other words, in order to relax the viscoelasticity, the accumulation of the many diffusion steps might be necessary, which leads to the loss of the orientation anisotropy of the effective network strands.

It should be noted that this deviation between \( L \) and \( \xi \) can be estimated from the activation energy. The activation energy estimated from the temperature dependence of the linear viscoelasticity is a sum of the activation energy of water viscosity and the association energy. The viscosity activation energy is known to be 15 kJ/mol. Thus, the association energy \( (E_{\text{association}}) \) can be calculated to be 39 kJ/mol. This value would give a ratio of the diffusion coefficients between chains with and without associative ends to be \( \exp\left(\frac{E_{\text{association}}}{RT}\right) = 10^{-6.8} \), leading to a ratio of diffusion distance \( \sim D^{1/2} \sim 10^{-3.4} \). This value roughly agrees with our experimental value.

Now, the diffusion coefficient estimated by the FRAP measurement was close to that of the unimer state. This agreement indicates that the ratio of the diffusion distance between the network and diffusive components is reasonable.

### 4. CONCLUSION

We investigated the linear viscoelastic relaxation through the FRAP measurements of the fluorescent-labeled HEUR aqueous solution. The results have been summarized as follows: (1) an increase in the polymer concentration led to a decrease in the value of diffusion coefficient, and an increase in the viscoelastic relaxation time; (2) the estimated diffusion distance during the viscoelastic relaxation time was approximately 10-100 times of the mesh size regardless of the HEUR concentrations. These results indicated that the viscoelastic relaxation was not primarily dominated by the detachment of the chains from hydrophobic cores. Regarding the discrepancy between diffusion distance and the mesh size, we considered that the observed diffusing species were unimers and flower micelles at low concentrations. In contrast, at high concentrations we thought that some of HEUR molecules involved in network structure could diffuse fast through the recombination of the network strands.

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### APPENDIX 1: ANALYTICAL METHOD OF FRAP RECOVERY DATA

The fluorescence photobleaching recovery process for single component diffusing species is well described by a two-dimensional diffusion equation\(^{24}\). Firstly we review the solutions of the diffusion equation with some different initial bleaching profiles to be used for the analysis of our FRAP data.

The first case is that the bleaching laser intensity profile, \( I(r) \), is given by a Gaussian function with the half-width \( w \) at \( e^{-2} \) height,

\[
I(r) = I(0) \exp\left( -\frac{2r^2}{w^2} \right)
\]

Here the center of the Gaussian beam is taken as the origin at \( r = 0 \), so that the \( I(0) \) is the maximum intensity of the beam. The second case is the uniform circular profile for \( I(r) \) with radius \( w \), which is represented by,

\[
I(r) = \begin{cases} I(0) & r \leq w \\ 0 & r > w \end{cases}
\]

After photobleaching of fluorescent molecules uniformly dispersed in a membrane by a laser beam of \( I(r) \), the concentration profile of the bleached chromophore, \( C(r, t) \), at time zero \( (t = 0) \) is given by

\[
C(r, 0) = C_0 \exp\left( -K\frac{(r)^2}{100} \right)
\]

Here the parameter \( K \) represents the amount of bleaching determined by both the time interval of bleaching and the laser power. The time evolution of the concentration profile \( C_K(r, t) \) is obtained by solving the two-dimensional diffusion equation (here the subscript \( K \) is added to note that this is dependent on \( K \)), and the fluorescence intensity \( F_K(t) \) observed at time \( t \geq 0 \) is generally given by

\[
F_K(t) = A \int I(r) C_K(r, t) \, d^2r
\]

where \( A \) is the constant related to the quantum efficiencies of light absorption, emission, detection, and the attenuation factor of the beam during observation of recovery. The fluorescence recovery curves are often converted to the fractional form \( f_K(t) \) defined by

\[
f_K(t) = \frac{[F_K(t) - F_K(0)]}{[F_K(\infty) - F_K(0)]}
\]

In the case of shallow and deep bleaching by a Gaussian
beam \((K < 1, \text{ and } K > 4, \text{ respectively})\) given by Eq (10), the following equations for \(f_K(t)\) are derived.

\[
f_K(t) = \begin{cases} 
1 - \frac{1}{1 + t/\tau_D} & \text{for } K < 1 \\
\frac{\Gamma(K^{-1}) - 1}{K - 1} & \text{for } K \geq 4 \& t/\tau_D \geq 4
\end{cases}
\](15)

Here \(\tau_D\) is the characteristic diffusion time which is related to the diffusion coefficient \(D\) as \(\tau_D = \nu^2 / 4D\), \(\nu = (1 + 2 t/\tau_D)^{-1}\), and \(\Gamma(\nu)\) is the gamma function. In the case of a uniform circle profile given by Eq. (11), it is also known that \(f_K(t)\) is given by the following equation\(^{28}\).

\[
f_K(t) = \exp\left(-2\tau_D/t\right)[I_0(2\tau_D/t) + I_1(2\tau_D/t)]
\](17)

Here \(I_0\) and \(I_1\) are the modified Bessel functions. It is noted that Equations (15) and (17) are not dependent on \(K\), but Equation (10) is dependent on \(K\).

Figure 5 compares these functions given by Eqs. (15), (16), and (17), plotted against \(t/\tau_D\) in the case that \(\tau_D = 100\) s. In the calculation of Eq. (16), two cases of \(K = 4\) and 20 are demonstrated. It is seen that the recovery curves given by Eqs. (15) (uniform circular shape) and (17) (shallow bleaching case) are almost overlapped while those given by Eq. (16) (deep bleaching case) deviate toward the longer time side: for the larger \(K\) values, the deviation becomes larger. In order to easily reproduce these recovery curves for the analysis of FRAP data, we fit these theoretical functions by the following Kohlrausch-Williams-Watts (KWW) type equation having extra parameters: intensity factor \(S\) and relaxation time modification factor \(\gamma\).

\[
f_K(t) = S\left[1 - \exp\left(-\left(t/\tau_D\right)^\beta\right)\right]
\](18)

The \(\beta\), \(\gamma\), and \(S\) values are summarized in Fig. 5. In the theoretical curves the complete recoveries \((f_K(t) \to 1)\) are much slower compared with the KWW function, so that the intensity modification factor \(S = 0.96\) was necessary to reproduce the recovery curves in a limited time region approximately but this factor is not so important for the determination of \(D\). Concerning the \(\beta\) parameter, similar values \(0.6 - 0.66\) were found to trace the theoretical curves, meaning that the shapes of the recovery curves are nearly universal among those four conditions. This indicates that the KWW function with \(\beta = 0.6 \sim 0.66\) can be a standard curve to fit the FRAP data. Rather the difference in the \(\gamma\) values especially for the deep bleaching case (Eq. (16)) is more serious to evaluate the \(\tau_D\) and \(D\) values. However, we think that our experimental bleaching condition was weak enough that the Equation (16) is not necessarily used. (The \(K\) values estimated from the fluorescence intensities just before and just after bleaching were on the order of \(10^{-1}\))\(^{24}\) Therefore, we use Eq. (18) with \(\beta = 0.6\) and \(\gamma = 1.50\) is used as a standard curve to fit the FRAP recovery data. However, when a heterogeneous structure exists, the diffusion of molecules may have a distribution. By considering such case, the way of using Eq. (18) is extended by regarding \(\beta\) as a parameter to represent the distribution in \(D\). In the next section we will fit the FRAP data by Eq. (18) with \(\beta\) and \(\tau_D\) as floating parameters and determine \(D\) values.

**APPENDIX 2: DIFFUSION COEFFICIENTS ESTIMATED FROM THE FIRST- AND SECOND-ORDER RELAXATION TIMES**

![Fig. S1](image1.png) Fractional fluorescence recovery \(f_K(t)\) vs. \(t/\tau_D\) given by Equations (15), (16), and (17) with \(\tau_D = 100\) s. The solid curves represent the KWW fit results. The fit parameters are shown in the figure.

![Fig. S2](image2.png) Concentration dependence of the diffusion coefficients calculated from the first- and second-order relaxation times for the F-HEUR solution (Circle). A dashed line represents the \(D\) value for 1 wt% HEUR methanol solution determined by the PFG-NMR measurement.
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