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Direct observation of spatiotemporal dependence of anomalous diffusion in inhomogeneous fluid by sampling-volume-controlled fluorescence correlation spectroscopy

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The direct observation of a spatiotemporal behavior of anomalous diffusion in aqueous polymer [hyaluronan (HA)] solution was achieved by fluorescence correlation spectroscopy (FCS) using a modified instrument, enabling continuous change of the confocal volume of a microscope, namely, sampling-volume-controlled (SVC) FCS (SVC-FCS). Since HA chains form a mesh structure with a pore size of about 10–40 nm, the observed diffusion coefficient (D_{obs}) is markedly dependent on the diffusion distance (L). By SVC-FCS, the curve of the distance dependence of diffusion coefficient was directly obtained as a continuous profile in L =245–600 nm showing evidence of anomalous diffusion. On plotting D_{obs} against either of the sampling time (τ_{obs}) or the diffusion distance (L), D_{obs} turnover was observed near the anomalous diffusion area. The appearance of this turnover is attributed to the nonuniform mesh structure that can be observed only by a fast observation and that should be dynamically averaged by polymer motions with large τ_{obs}. This behavior is similar to that revealed in glass, colloidal systems, and gel solutions using dynamic light scattering, neutron scattering, and other techniques.
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Material transport in *homogeneous* media, where only unobstructed (normal or Euclid) diffusion is expected, can be characterized by the diffusion coefficient (D) that is related to the mean square displacement (MSD) $\langle r^2 \rangle$ of diffusing particles [1],

$$\langle r^2 \rangle = 2dDt, \tag{1}$$

where d is the space dimensionality (d=3 for three-dimensional diffusion) and the MSD is proportional to the time evolution after sufficiently longer duration than the characteristic time of the random translational motion t_c, as $t \gg t_c$. D in a homogeneous system is constant and

$$\frac{d}{dt}\langle r^2 \rangle = 2dD \tag{2}$$

is equivalent to Eq. (1).

In *inhomogeneous* media including various biological systems [2–6], however, diffusion may be anomalous; that is, the MSD after a long period is not always proportional to the time evolution and Eqs. (1) and (2) are not equivalent any more. In one particular case, MSD can be expressed as a fractional power of time not equal to 1 [7],

$$\langle r^2 \rangle = 2dDt^{\alpha} \quad (\alpha < 1), \tag{3}$$

based on the fractal theory reflecting the characteristics of inhomogeneous spaces [8,9]. As an efficient experimental approach, direct observations of distance and time dependences of diffusion coefficient (DDDC and TDDC, respectively) can help us deeply understand the complex behavior of diffusion in inhomogeneous media. Various spectroscopic techniques, which are available today, have their proper sampling time or space, with which D value is determined after the accumulation of a large number of observables such as the displacements of particles. Generally, the diffusion coefficient (D_{obs}) observed in an inhomogeneous system can be described as a function of the observation time τ_{obs} or the diffusion distance L.

$$\lim_{t \to \infty} \frac{\langle r^2 \rangle}{t} = 2dD_{obs}(\tau_{obs}) = 2dD_{obs}(L), \tag{4}$$

here, $t \gg t_c$ and $\tau_{obs} \gg t_c$, as shown in our previous studies [10,11].

Among these spectroscopic techniques, fluorescence correlation spectroscopy (FCS) [12] is one efficient method for D measurement extensively applied to various biological systems using confocal microscopes. FCS instruments have an extremely small sampling space [the confocal volume (CV) is about 0.2–1 femtoliters] and D is determined from the time correlation function that is obtained from the intensity fluctuation of emission from a small number of fluorescent molecules [13].

Recently, we developed a technique called sampling-volume-controlled (SVC) FCS (SVC-FCS) in which we can vary the CV in a manner shown in Fig. 1 to observe the DDDC of diffusing dye molecules directly in inhomogeneous hyaluronan (HA) solutions [14]. The diameter of an incident laser beam is continuously varied using a zooming beam expander and the illumination volume at a sample is smoothly controlled [12]. When the diameter of the laser beam is increased, the sampling space shrinks as shown in Fig. 1(b). The effectiveness of this measurement was justified by recent calculations using a simple model for FCS measurement in which $D(t)$ is introduced as a step function [15]. In this paper, the scanning region of CV radius w in SVC-FCS providing a DDDC plot was extended to 200–490 nm, and the total anomalous diffusion behavior was successfully observed after the first observation in our previous study.

We previously showed a DDDC plot for a globular protein [cytochrome c (cytc)] in aqueous HA solution [11]. A

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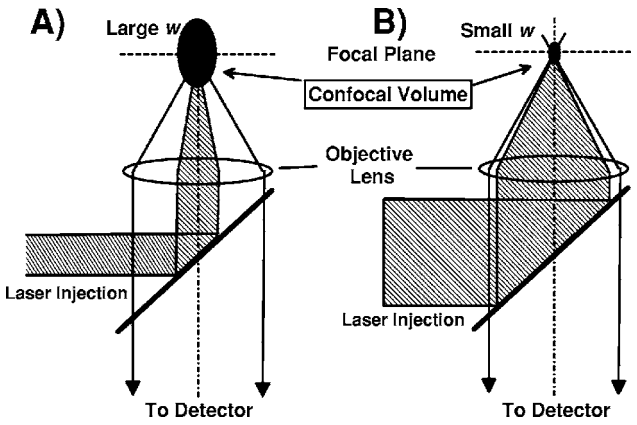

FIG. 1. Method for changing the confocal volume (CV) by changing the diameter of an injected laser beam for excitation to an inverted microscope. (a) A small laser beam makes a large CV (w). (b) A large laser beam makes a small CV (w).

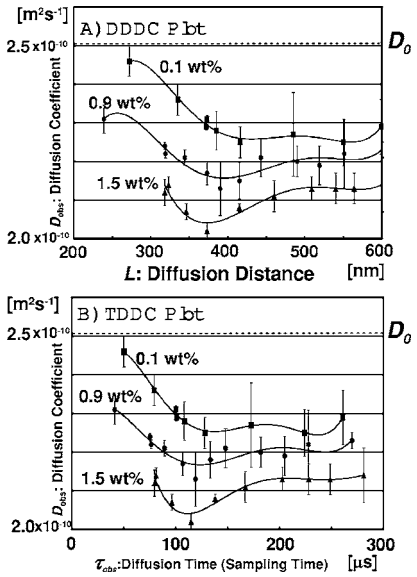

FIG. 2. Diffusion distance (L) dependence of diffusion coefficient (DDDC) plot and observation time (τ_{obs}) dependence of diffusion coefficient (TDDC) plot obtained for Alexa488 in aqueous HA solution by SVC-FCS technique. The HA concentration [C_{HA}] is equal to 0.1, 0.9, and 1.5 wt. %.

TDDC plot is also possible because τ_{obs} can be approximately expressed as

$$\tau_{obs} = L^2/6D_{obs} \tag{5}$$

near the observation point. This equation can be used for the interconversion between DDDC and TDDC plots.

Details of SVC-FCS measurement and sample preparations are described elsewhere [11]. We used Alexa 488 from Molecular Probe Inc. as the fluorescent dye with a molecular diameter of 1.4 nm with spherical shape approximation. HA with a labeled molecular weight of 300 kDa was used and dissolved in phosphate buffer solution to make the polymer matrix.

We measured D_{obs} by SVC-FCS for solutions with C_{HA}=0.1, 0.9, and 1.5 wt. % [C_{HA} is the concentration of HA] for w=200–490 nm [corresponding to L =245–600 nm where $L=(1.5)^{1/2}w$] and plotted against both L [Fig. 2(a), DDDC plot] and τ_{obs} [Fig. 2(b), TDDC plot]. The estimated mesh sizes for the 0.1, 0.9 and 1.5 wt. % HA solutions are 33, 15, and 7 nm, respectively [16]. When L decreases in the region of $L<200$ nm, D_{obs} should gradually approach D_0 (D_{obs} without HA) similarly to the previous case of cytchrome c [11] because most dye molecules have almost no opportunity to interact with polymer chains. In the shortest limit of L (the short diffusion mode), then, D_{obs} becomes a constant equal to D_0 and the time differential of MSD is constant as in Eq. (1). It is rather clear in Fig. 2 for Ha solution equal to C_{HA}=0.1 wt. % while the approaching areas for C_{HA}=0.9 and 1.5 wt. % may be out of range of the present measurement. In the longest limit at somewhere L >600 nm, D_{obs} approaches a smaller value because the diffusing particles feel a continuous friction induced by the contact with the polymer chains (the long diffusion mode). In this region, D_{obs} becomes a constant again, indicating the invariant time differential of MSD with a normal diffusion behavior. We call the mechanism of this conversion from the short diffusion mode to the long diffusion mode, which is compatible with our previous reports, the mesh-scaling effect because the relationship between L and the mesh size is the determinant.

There exists a transient region between these two extreme modes of normal diffusion, as seen in Figs. 2(a) and 2(b). In this region, D_{obs} depends on both distance and time: anomalous diffusion due to the "mesh-scaling effect." The area of this region is about one order larger than the estimated mesh size probably because the transient gradual reduction of D_{obs} is induced by the fractal character of the HA mesh structure inside a percolation cluster. The effect of the fractal structure is observed in a particular characteristic finite space [17,18], which may be one order larger than the HA mesh size. The diffusing molecules are interfered with on each rare opportunity of encountering the polymer chains in a water-rich passage inside HA solution and statistically obtained diffusion coefficients gradually decrease showing the effect of the fractal structure. The relationship between the transient behavior and the characteristic structure of the percolation cluster can be explained using "the ant in the labyrinth" model with the percolation theory [8]. According to this theory, at long times, the simple crossover from anomalous to normal diffusions occurs and D_{obs} becomes constant again, the normal diffusion that corresponds to the long diffusion mode of our case.

In this paper, however, the turnover regions (dips) in the DDDC plot are prominent showing a bottom at L =360–380 nm for 1.5 wt. %, L=380–420 nm for 0.9 wt. %, and L=400–450 nm for 0.1 wt. % before reaching the constant D_{obs} at the long diffusion mode. The plots in Fig. 2(b) are automatically derived from Fig. 2(a) using Eq. (5). In both Figs. 2(a) and 2(b), the dips appear deeper in more concentrated HA solutions. Since the positions of the dips are aligned in a markedly narrow time region in Fig. 2(b), we consider that these dips are reasonably correlated to the ef-

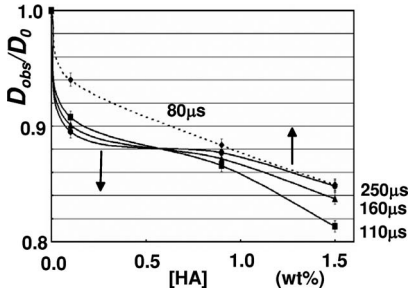

FIG. 3. Time slices of TDDC plot (D_{obs}) in Fig. 2(b) at 80, 110, 160, and 210 μs. The plot of D/D_0 vs HA concentration did not show a typical dependence on the exponential law presented by Ogston *et al.* (see Ref. [20]).

fect of the dynamic motion of HA chains that have an unknown time constant. The translational diffusion coefficient of HA molecules, D_{HA}, is a parameter that can be related to its local chain motion. D_{HA} determined by FRAP (fluorescence recovery after photobleaching) was reported as $\sim 7.9 \times 10^{-12}$ m^2 s^{-1} for 500 kDa HA [19]. Since we suppose that the magnitude of local motion of HA is similar to that of the translational diffusion, the chain can move 50 nm within 100 μs to reorganize the mesh structure completely on the same time scale.

We also plotted the time slices of the TDDC plot in Fig. 2(b) at $\tau_{obs}=80$, 110, 160, and 250 μs, indicating the relative reduction in $D_{obs}(=D_{obs}/D_0)$ on HA addition, as shown in Fig. 3. For an invariant permanent mesh, these plots should follow the concentration law proposed by Ogston *et al.* providing a fitting equation as [20]

$$\frac{D_{obs}}{D_0} = \exp(-aC_{HA}{}^{0.5}). \tag{6}$$

The parameter a is related to the mesh size ξ in the cubic mesh approximation, and the diameter of the diffusing molecules b is expressed as

$$\xi = (b/a)C_{HA}{}^{-0.5}. \tag{7}$$

In our previous study using cytc as a diffusing molecule, this dependence was clearly observed in both FCS and pulsed field gradient (PFG) NMR experiments [11]. However, these equations cannot be applied to the results displayed in Fig. 3, where the parameter a cannot be obtained as a constant. Therefore, the results in Figs. 2(a) and 2(b) cannot be explained only by the existence of the permanent mesh structure.

To explain the appearance of the dips in Figs. 2(a) and 2(b), we suggest the following model picture taking polymer dynamics into consideration for the present Alexa488-HA system. We assume that the HA mesh has a characteristic time constant t_{re} to reorganize its structure by the translational motion or fluctuation of the polymer chain. In other words, t_{re} is the lifetime of one local structure that forms a pore space including the dye molecules. From the experimental curve in Fig. 2(b), we estimate t_{re} to be located in 130–160 μs.

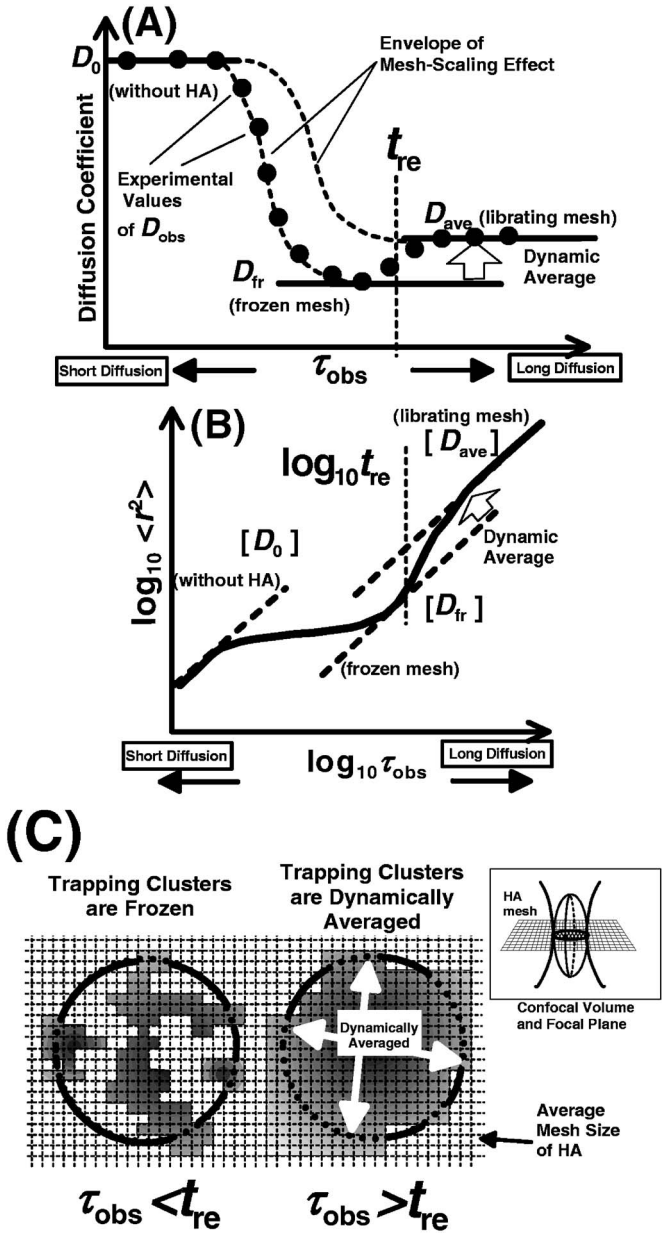

FIG. 4. (a) Figure explaining TDDC plot (D_{obs} vs τ_{obs}) in Fig. 2(b) based on transitions among three normal diffusion coefficients (D_0, D_{fr}, and D_{ave}). The transition between D_{fr} and D_{ave} is supposed to be induced by the librating translational motions of the HA polymer chain that reorganize the mesh structure with the time constant t_{re}. The line shape of the transition area is due to the mesh-scaling effect. (b) Dual logarithmic plot of MSD vs observation τ_{obs} directly derived from (a). (c) The effect of trapping clusters, that are frozen on $\tau_{obs} < t_{re}$ and dynamically averaged on $\tau_{obs} > t_{re}$, in SVC-FCS measurement: The figure indicates the horizontal projection of confocal volume (CV) as shown in the inset. The aria on the boundary surface of CV, where molecules can come in and go out, is narrow when trapping clusters are frozen on $\tau_{obs} < t_{re}$.

Using this model picture, we suppose that the diffusion coefficient observed near the dips has two typical values before and after the dynamic averaging of the mesh structure induced by the polymer motion, i.e., D_{fr}, related to frozen mesh structures, and D_{ave}, an average value obtained for a

librating mesh. A model figure is shown in Fig. 4: The plots of D_{obs} against τ_{obs} and corresponding $\log_{10}\langle r^2\rangle$ against $\log_{10}\tau_{obs}$ are shown in Figs. 4(a) and 4(b), respectively. Figure 4(b) is more familiar for researchers engaged in other experiment such as neutron or light scattering. For $\tau_{obs} < t_{re}$, the polymer structure is frozen in one geometry sometimes exhibiting inhomogeniety accompanied by a wide mesh size distribution. In such a situation, D_{obs} is approximated using D_{fr} corresponding to the permanent mesh structure. On the other hand, for $\tau_{obs} > t_{re}$, D_{obs} approaches D_{ave} because the material transport in a rapidly librating mesh can be approximated as the material transport in continuous media with a particular time-averaged friction. Experimentally observed diffusion coefficients are indicated by black circles in Fig. 4(a) and the rough profile is in good agreement with that shown in Fig. 2(b).

Since the variation in D_{obs} is small ($<30\%$), we have not expressed our results of the previous works as a logarithmic plot of MSD, which is familiar to the physicists in a wide variety of research fields. Normal diffusion is expressed as a straight line with slope equal to 1. The transient behavior in Fig. 4(a) is expressed as a single curve (linking three straight lines) in Fig. 4(b) that converges to normal diffusion in the short and long diffusion modes. Similar curves have been obtained for various experiments in glass, lattice, polymer solution, and colloidal solution. The turnover of MSD was sometimes observed, showing that trappings occur on this time scale [21–23].

In this HA system, we tentatively propose the existence of a trapping effect in a nonuniform mesh structure of a polymer chain. In a frozen mesh, there is a variety of pore spaces of various effective sizes. In a place where polymer chains are clouded, the probability of the existence of diffusing molecules becomes extremely small. On the other hand, the diffusing molecules tend to stay in large pore spaces, which act as percolation clusters, for a long period. As the result, the distribution map of pore spaces are divided into two regions in a sharp contrast; one is the region occupied by clusters of large pore spaces where diffusing molecules can smoothly transport and the rest space is an area where diffusing molecules can hardly travel. The cluster of large pores acts as a trap of diffusing molecules (trapping cluster) as shown in Fig. 4(c). Since the position of the turnover corresponds to the 10–15 meshes in average (L=300–500 nm), the size of this trapping cluster seems to be about the same order of CV radius w. The intersecting area with the trapping clusters is restricted on the CV boundary surface, and then, the frequency of fluorescence fluctuation is somewhat reduced to decrease D_{obs}.

On the other hand under a dynamically averaged condition, trapping clusters move, are newly formed and disappear from time to time due to both of the local fluctuations and the translational diffusion of polymer chains. The clusters are occasionally connected to each other to make a larger percolation cluster; the molecules can reach any places if they wait longer than the reorganization time t_{re} ($\tau_{obs} > t_{re}$). The molecules can pass almost everywhere on the CV boundary surface within τ_{obs}. At present, we suppose that this trapping mechanism, which disappears in $\tau_{obs} > t_{re}$, makes D_{fr} smaller than D_{ave}.

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[1] P. A. Netz and T. Dorfmüller, J. Chem. Phys. **107**, 9221 (1997).
[2] T. V. Ratto and M. L. Longo, Langmuir **19**, 1788 (2003).
[3] M. Wachsmuth, W. Waldeck, and J. Langowski, J. Mol. Biol. **298**, 677 (2000).
[4] M. Weiss, H. Hashimoto, and T. Nilsson, Biophys. J. **84**, 4043 (2003).
[5] P. Schwille, Cell Biochem. Biophys. **34**, 383 (2001).
[6] T. J. Feder *et al.*, Biophys. J. **70**, 2767 (1996).
[7] M. J. Saxton, Biophys. J. **66**, 394 (1994).
[8] A. Aharony and D. Stauffer, Phys. Rev. Lett. **52**, 2368 (1984).
[9] D. Stauffer and A. Aharony, *Introduction to Percolation Theory* (Taylor & Francis, London, 1994).
[10] A. Masuda, K. Ushida, H. Koshino, K. Yamashita, and T. Kluge, J. Am. Chem. Soc. **123**, 11468 (2001).
[11] A. Masuda, K. Ushida, G. Nishimura, M. Kinjo, M. Tamura, H. Koshino, K. Yamashita, and T. Kluge, J. Chem. Phys. **121**, 10787 (2004).
[12] *Fluorescence Correlation Spectroscopy: Theory and Applications*, edited by R. Rigler and E. S. Elson (Springer, Berlin, 2001).
[13] W. W. Webb, Q. Rev. Biophys. **9**, 49 (1976).
[14] A. Masuda, K. Ushida, and T. Okamoto, Biophys. J. **88**, 3584 (2005).
[15] K. Seki, A. Masuda, K. Ushida, and M. Tachiya, J. Phys. Chem. A **109**, 2421 (2005).
[16] These mesh sizes were calculated using Eq. (6) derived from the HA concentration dependence of D_{obs} as in Ref. [11].
[17] S. Havlin, *Molecular Diffusion and Reactions* (Wiley, Chichester, 1989).
[18] Y. Suzuki and I. Nishio, Phys. Rev. B **45**, 4614 (1992).
[19] P. Gribbon, B. C. Heng, and T. E. Hardingham, Biophys. J. **77**, 2210 (1999).
[20] A. G. Ogston, B. N. Preston, J. D. Wells, and J. M. Snowden, Proc. R. Soc. London, Ser. A **333**, 297 (1973).
[21] L.-S. Luo and G. D. J. Phillies, J. Chem. Phys. **105**, 598 (1996).
[22] T. Odagaki and Y. Hiwatari, Phys. Rev. A **41**, 929 (1990).
[23] T. Odagaki, J. Matsui, and Y. Hiwatari, Phys. Rev. E **49**, 3150 (1994).