Novel Distance Dependence of Diffusion Constants in Hyaluronan Aqueous Solution Resulting from Its Characteristic Nano-Microstructure

Akiko Masuda, Kiminori Ushida,* Hiroyuki Koshino, Koichi Yamashita, and Thomas Kluge†

Contribution from the RIKEN (The Institute of Physical and Chemical Research), Wako, Saitama 351-0198, Japan

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Abstract: Material transports in hyaluronan (HA) aqueous solution were investigated applying two different techniques, i.e., pulsed field gradient NMR (PFG-NMR) and photochemical quenching, to the measurement of diffusion constants to show a sharp contrast resulting from the difference of the spectroscopic observation time while the same probe molecules were commonly used in two experiments. The value from PFG-NMR reflects the relatively long transport along which the majority of the molecules are retarded by the mesh structure of HA solution. In such inhomogeneous fluids, the observable diffusion constant should generally depend on the observation time and, i.e., the averaged distance of diffusion. Quantitative discussion, which compares the obtained characteristic distance of diffusion with the pore size, clarifies the role of the nano-microstructure of HA solution forming small pores surrounded by the polymer chain networks.

Introduction

The extracellular substrate in connective tissue contains various kinds of high-molecular-weight polysaccharides.[1,2] Among them, hyaluronan (HA) is a linear polysaccharide with repeating disaccharide units of β-D-glucuronic acid and *N*-acetyl-β-D-glucosamine.[3−6] HA is soluble in water at neutral pH and behaves as a weakly charged polyelectrolyte due to the carboxylate group on each disaccharide unit. The characteristic properties of HA are that it forms an infinite mesh in solution the size of which is of the order of 15 nm and that it shows extremely high macroscopic viscoelasity even in dilute amounts of HA.[7−12] Various rheological experiments have clearly shown that HA chains start to overlap mutually if the concentration exceeds critical overlap concentration depending on the molecular weight (MW) of HA: e.g., 1.0 mg mL^{-1} for MW $\sim$ 390 kDa to form a transient network.[13,14] The mesh structure of HA plays a mechanical role as space filler and it also has permeability for small molecules such as urea, sugars, and proteins. Therefore, from biological, physiological, and medical points of view, the mass transport in HA solutions has attracted much attention because it can be regarded as a model phenomenon for various material transports commonly found in animal and human bodies. Moreover, the effectiveness of physiologically important molecules, such as drugs, hormones, and enzymes, should be controlled by both their mobility and that of substrates in this matrix which may be affected by the concentration (C_{HA}) and the molecular weight (MW) of HA and, also, the practical migration pathways which are seriously interfered with by the sugar mesh.

To clarify the mass transport in HA solutions with microstructures of the order of tens of nanometers, we employed two different methods of determining the diffusion constants (D) in the polymer solution: (1) direct observation by the pulsed field gradient (PFG)-NMR method and (2) estimation from the rate constants of photochemical bimolecular reactions (PCBR). For HA solutions, which should be considered as an inhomogeneous system on a microscopic scale, this comparative analysis should be valuable because observable diffusion constants result from an ensemble average of various transport steps depending on the observation time (t_{obs}) for the applied techniques. Since a characteristic distance of diffusion is given by $(2Dt_{obs})^{1/2}$ for PFG-NMR, the measurements under our experimental conditions (vide infra) provide the information of 1−43 μm diffusion along

* To whom correspondence should be addressed: (phone) +81-48-467-7963, (fax) +81-48-462-4668, (e-mail) kushida@postman.riken.go.jp.

† Present address: RMH Polymers GmbH & Co. KG, Hallesche Strasse 27, D-06258 Schkopau, Germany.

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Table 1. The Observed Diffusion Constants for Various Concentrations of HA and Viscosities of HA Solutions[c]

	C_{HA}/wt %	η_0/mPa s	D(water)/m^2 s^{-1}	D(Ru(bpy)$_3^{2+}$)/m^2 s^{-1}	D(Cyt)/m^2 s^{-1}
D_2O	0	1.096[a]	$(1.90 \pm 0.02) \times 10^{-9}$	$(3.75 \pm 0.04) \times 10^{-10}$	$(1.1 \pm 0.1) \times 10^{-10}$
HA30	0.18	*b*			$(9.8 \pm 0.2) \times 10^{-11}$
	0.35	*b*			$(9.3 \pm 0.2) \times 10^{-11}$
	0.5	*b*	$(1.84 \pm 0.03) \times 10^{-9}$	$(3.53 \pm 0.05) \times 10^{-10}$	$(8.1 \pm 0.1) \times 10^{-11}$
	0.9	94			$(7.8 \pm 0.1) \times 10^{-11}$
	1.5	570	$(1.85 \pm 0.07) \times 10^{-9}$	$(3.19 \pm 0.05) \times 10^{-10}$	$(7.3 \pm 0.1) \times 10^{-11}$
	2.0	2000	$(1.88 \pm 0.03) \times 10^{-9}$	$(2.96 \pm 0.08) \times 10^{-10}$	$(7.3 \pm 0.1) \times 10^{-11}$
HA100	0.5	560	$(1.83 \pm 0.07) \times 10^{-9}$	$(3.64 \pm 0.05) \times 10^{-10}$	$(8.3 \pm 0.2) \times 10^{-11}$
	1.5	32000	$(1.81 \pm 0.01) \times 10^{-9}$	$(3.19 \pm 0.01) \times 10^{-10}$	$(8.9 \pm 0.2) \times 10^{-11}$

[a] This value was quoted from ref 18. [b] These values could not be determined because they were below the measurable range of our viscometer. [c] All the experimental values are the average of 3−4 observations.

which most of the particles should be perturbed by the mesh and show relatively small D values. On the other hand, based on an isotropic approximation that the mean distance of diffusion can be expressed as $(6Dt_{obs})^{1/2}$, PCBR can be related with 16−34 nm diffusion with which a large population of particles travels only inside the rooms between the mesh, and the observable D should be very close to that for the aqueous solution without HA. In this report, we present our successful results of this comparative study for migration using the same set of probe molecules in common, Ru(bpy)$_3^{2+}$ (bpy = 2,2′-bipyridine) and cytochrome *c* (Cyt).

Results and Discussion

In PFG-NMR experiments, the diffusional attenuation of the echo amplitude is generally given by

$$\frac{I(G)}{I(0)} = \exp\left[-\gamma^2 G^2 \delta^2 D\left(\Delta - \frac{\delta}{3}\right)\right] \qquad (1)$$

where $I(G)/I(0)$, γ, G, δ, and Δ are the ratio of echo amplitudes in the presence and the absence of applied field gradient (FG), the gyromagnetic ratio, the magnitude of FG, the duration of each FG pulse, and the interval between the two FG pulses (corresponding to the diffusion time), respectively.[15] In the present study, a series of echoes were measured systematically by varying Δ with constant FG (max. 0.91 T m^{-1}) using a stimulated echo (STE) sequence. (See Experimental Section.) In all cases, a plot of $\ln(I(G)/I(0))$ against $\gamma^2G^2\delta^2(\Delta-\delta/3)$ gave a straight line with a slope of $-D$ as shown in Figure 1. Systematic variation of δ also gave linear plots similar to Figure 1 for water and Ru(bpy)$_3^{2+}$. In the examination of the δ dependence for Cyt, however, sufficient accuracy for small D was not obtained probably because of some instrumental problems. Consequently, we could not find any evidence of restricted diffusion promoted by the HA mesh. We also took several points of G, and no contradiction among D values obtained for various G, δ, and Δ was found throughout this study.

The self-diffusion constants were obtained at 298 K for water, Ru(bpy)$_3^{2+}$, and Cyt in D_2O as $(1.90 \pm 0.02) \times 10^{-9}$, $(3.75 \pm 0.04) \times 10^{-10}$, and $(1.10 \pm 0.05) \times 10^{-10}$ m^2 s^{-1}, respectively. These values are consistent with those estimated from literature values[16,17] with a correction based on the Stokes−Einstein equation.[18] The observed D values for various concentrations

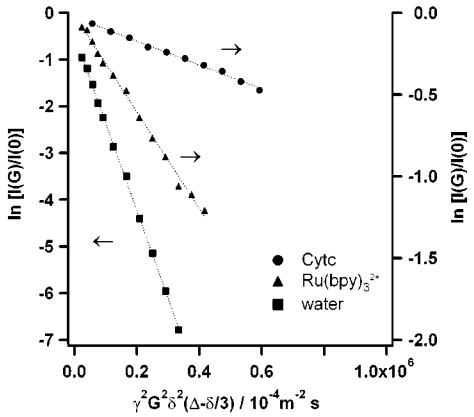

Figure 1. Semilogarithmic STE attenuation plot against $\gamma^2G^2\delta^2(\Delta-\delta/3)$ for water, Ru(bpy)$_3^{2+}$, and Cytc in the 2% aqueous solution of HA30 obtained from the systematic variation of Δ (see eq 1): ●, Cytc (G = 0.91 T m^{-1}, δ = 1 ms); ▲, Ru(bpy)$_3^{2+}$ (G = 0.34 T m^{-1}, δ = 1 ms); ■, water (G = 0.34 T m^{-1}, δ = 1 ms). The slope of the linear plot corresponds to $-D$ for each sample.

of HA for HA30 (MW = 426k) and HA100 (MW = 1280k)[19] are listed in Table 1. We ignored the effect of the exclusion volume of HA (0.65 cm^3/g from ref 19) because it gave only ∼1−1.5% deviation at the maximum concentration. The values of D were independent of MW of HA. Typical $(2Dt_{obs})^{1/2}$ values were 6−43, 3−18, and 1−4 μm for water, Ru(bpy)$_3^{2+}$, and Cytc, respectively. The 1−43 μm diffusion processes observed by PFG-NMR were not significantly affected by the polymer chain lengths of 1 and 3.5 μm for HA30 and HA100, respectively.

The D/D_0 ratio in HA30 solution was plotted against C_{HA} in Figure 2A, where D_0 is defined as the diffusion constant in the absence of HA. Almost no effect of HA was found on the diffusion of water molecules of 0.3 nm diameter. This result indicates that water molecules diffuse relatively freely through HA networks at least for several tens of μm. The diffusion of Ru(bpy)$_3^{2+}$ with a diameter of 1.0 nm was slightly retarded in proportion to C_{HA}. This result suggests that an increased HA concentration leads to a decrease in the effective volume available for diffusion. Based on the estimated value of D/D_0 and the viscosity of D_2O, Ru(bpy)$_3^{2+}$ molecules appear to feel a microviscosity of 1.4 mPas in 2 wt % HA solution. For Cytc, D/D_0 decreases rapidly with increasing C_{HA} in contrast to Ru(bpy)$_3^{2+}$ even in the very dilute region (<0.3 wt %). The large difference in the C_{HA} dependence cannot be caused only by the effective volume for diffusion and the molecular size of Cytc. According to the diffusion model proposed by Ogston et al.,[19]

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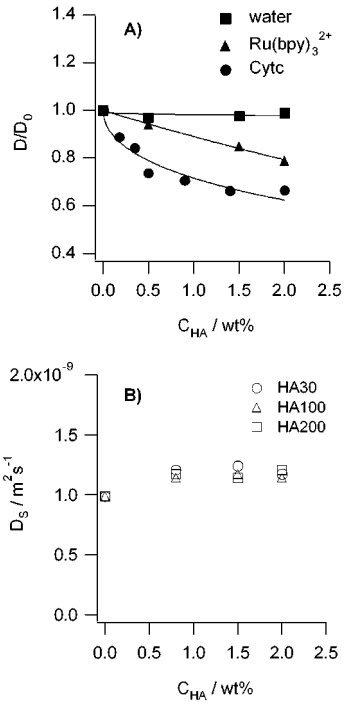

Figure 2. (A) The ratio of the diffusion constants in the presence and absence of HA30 plotted against the HA concentration. (B) The sum of the diffusion constants of $Ru(bpy)_3^{2+}$ and Cytc depending on concentration and molecular weight of HA.

the C_{HA} dependence of the D/D_0 can be expressed empirically by the exponential law,

$$D/D_0 = \exp(-aC_{HA}^{0.5}) \quad (2)$$

where a is a constant that is related to the size of the mesh and the probe molecule. This exponential scaling was supported by the theoretical work based on the stochastic approach for diffusional migration in rod systems with an obstruction[20,21] and also by the experimental works using sedimentation measurements.[22−25] The application of this model gave a fitting curve for Cytc with $a = 0.105$ as shown in Figure 2A. If we naively assume a simple mesh model, the average mesh size ξ is expressed as

$$\xi = (b/a)C_{HA}^{-0.5} \quad (3)$$

where b is the diameter of Cytc and we obtain $\xi = 9$ nm at 14 mg/mL of HA. A FRAP study[26] gave $\xi = 10-20$ nm at the same HA concentration depending on the kind of probe molecules and an ESR spin probe work[27] gave $\xi = 11$ nm. Our present results provide slightly smaller values in the same order probably because of the difference in the observation time of each method. The observed C_{HA} dependence suggests that the direct retardation of diffusion occurs by the entangling of HA chains and that the spherical space among the HA chains should gradually shrink with an increase of C_{HA}. It is a very important fact that the diffusivity of protein is reduced by HA even in dilute amounts because activities of proteins can be related to their mobility in some cases.

Table 2. The Obtained C_{HA} and MW Dependence of k_q[a]

	$k_q/M^{-1}\ s^{-1}$		
C_{HA}/wt %	HA30	HA100	HA200
0		$(2.9 \pm 0.1) \times 10^8$	
0.8	$(3.5 \pm 0.1) \times 10^8$	$(3.3 \pm 0.1) \times 10^8$	$(3.4 \pm 0.1) \times 10^8$
1.5	$(3.6 \pm 0.2) \times 10^8$	$(3.4 \pm 0.1) \times 10^8$	$(3.3 \pm 0.1) \times 10^8$
2.0	$(3.4 \pm 0.1) \times 10^8$	$(3.3 \pm 0.1) \times 10^8$	$(3.5 \pm 0.2) \times 10^8$

[a] All the experimental values are the average of 3−4 observations.

Details of the PCBR method have been reported in previous publications.[11,12] In this study, we investigated the fluorescence quenching of $Ru(bpy)_3^{2+}$ by Cytc, the main process of which is believed to be the intermolecular electron transfer from $Ru(bpy)_3^{2+}$ to the heme unit of Cytc. The quenching rate constant k_q was obtained from the fluorescence decay curves by using ordinary Stern−Volmer-type analysis which showed normal linear dependence in all cases. The obtained C_{HA} and MW dependencies of k_q are listed in Table 2. The k_q value of around $3 \times 10^8\ M^{-1}\ s^{-1}$ showed that this reaction was a diffusion-controlled one under all conditions. For a diffusion-controlled reaction, k_q can be expressed in terms of the distance between the metal centers (taken equal to the sum of the radii of $Ru(bpy)_3^{2+}$ and Cytc) and the sum of diffusion constants (D_S) of $Ru(bpy)_3^{2+}$ and Cytc.[28] Using ionic radii, 0.5[16] and 1.7[29] nm for $Ru(bpy)_3^{2+}$ and Cytc, and the viscosity of water, D of each solute can be estimated by the Stokes−Einstein relationship as 4.9×10^{-10} and $1.4 \times 10^{-10}\ m^2\ s^{-1}$ for $Ru(bpy)_3^{2+}$ and Cytc, respectively. Then the calculated value of k_q in water is $1.8 \times 10^8\ M^{-1}\ s^{-1}$ by considering that Cytc carries a net positive charge of approximately +9 at neutral pH.[30] On the other hand, the observed k_q in buffer solution is $2.9 \times 10^8\ M^{-1}\ s^{-1}$, showing an acceptable value within the experimental error. For HA solution, the sum of the diffusion constants, D_S, is estimated from the observed k_q values. The D_S is plotted against C_{HA} as shown in Figure 2B. No dependence of D_S on the MW of HA was observed in the investigated C_{HA} region and the D_S in HA solution is similar to that in buffer solution. The lifetime of the excited $Ru(bpy)_3^{2+}$ was 300−400 ns in the presence of Cytc and the $(6Dt_{obs})^{1/2}$ value corresponded to 16−34 nm. Macroviscosity of HA solution also did not affect the diffusion of Cytc and $Ru(bpy)_3^{2+}$, i.e., microviscosity in the HA mesh is almost equal to the viscosity of water under this condition. Although we only obtained the sum value, the results indicated that both of the D values of Cytc and $Ru(bpy)_3^{2+}$ appeared to be unchanged on the addition of HA to the solution.

Our present comparative analysis successfully demonstrates a sharp contrast between (two) observable D values in two alternative methods due to their dependence on the typical t_{obs} for each detection. PCBR, which was unchanged on HA addition to water, should reflect the movements over a short distance (short traveling period) where the majority of solutes appear to have no opportunity to interact with sugar networks. This consideration proved the existence of "rooms" of water between the sugar chains the size of which is between 16−34 nm and

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1−3.5 μm. This dimension[31] is in good agreement with those (∼15 nm in diameter with a spherical approximation) estimated earlier by electron microscopy[10] light scattering experiments,[8] ESR measurements,[27] FRAP techniques,[26] and our previous work.[11,12] However, even Cytc with a diameter of about 3.4 nm, which fills a large part of the single-pore space, did not appear to be interfered with by the HA mesh because all of our PCBR results showed a single exponential decay of homogeneous quenching. This indicates another specific characteristic of HA solution where HA chains are constantly moving in the same time scale as the Cytc transport. Due to this dynamical effect, the effective pore size for the diffusion process may be significantly larger than that defined from the structural analysis.

Experimental Section

Reagents and Solutions. Three synthetic HA samples labeled molecular weights 300000 (HA30), 1000000 (HA100), and 2000000 (HA200) were obtained from Denki Kagaku Kogyo Co., Ltd. Relative molecular weight data (including polydispersity) of the HA samples were obtained by gel filtration chromatography (GFC) at 30 °C as described in the previous paper.[11] $Ru(bpy)_3Cl_2 \cdot 6H_2O$ (Aldrich) and Horse Heart Cytochrome *c* type VI (Sigma) were used as received. All the solutes and HAs were dissolved in 50 mM HEPES buffer (total ionic strength is adjusted to 0.1 M by addition of NaCl, pH 7.0) and D_2O was used for NMR measurements instead of H_2O. The preparation procedure for the HA solutions was similar to those reported in the previous works.[11,12] No evidence for serious static interactions between solutes ($Ru(bpy)_3^{2+}$ and Cytc) onto HA was found by emission, optical absorption, and NMR spectroscopy in the present experimental condition.

Apparatus and Measurements. ^1H PFG-NMR experiments were performed by using a JEOL JNM-A400 Spectrometer (400 MHz, 9.39 T) with field gradient unit (FG Power ∼1.13 T m^{-1} (max)). Relatively large D (∼10^{-9} m^2 s^{-1}) is satisfactorily obtained by a conventional spin−echo pulse sequence (90°−τ−180°−τ−aquisition) with FG.[15] For large molecules such as proteins which show relatively slow diffusion ($D \sim 10^{-10}$ m^2 s^{-1}), stimulated echo (STE) sequence (90°−τ−90°−t−90°−τ−aquisition)[32,33] is suitable for measurements of D values. In the present study, we selected STE with careful consideration on the magnitudes of T_1, T_2, and D of each system. The absolute magnitude of the field gradient was calibrated with the value of the diffusion constant of HDO at 25 °C, 1.9×10^{-9} m^2/s, reported by Mills and Longsworth.[34,35]

Fluorescence decay curves were measured by using a conventional single-photon counting apparatus (Horiba NAES 1100). A hydrogen-filled flash lamp having about 4 ns pulse duration (full width at half-maximum) was used as the excitation source. The samples were excited at 480 nm with use of a monochromator. The emission of the excited $Ru(bpy)_3^{2+}$ was detected by a photomultiplier through a 570 nm cutoff filter. The fluorescence decay profiles were analyzed as a single-exponential decay with a nonlinear least-squares iterative deconvolution method. For a diffusion-controlled reaction, k_q can be expressed as $k_q = 4\pi PRD_S N_A$, where R is the distance between the metal centers (taken equal to the sum of the radii of $Ru(bpy)_3^{2+}$ and Cytc), N_A is Avogadro's number, and D_S is the sum of diffusion constants of Cytc and $Ru(bpy)_3^{2+}$. P is a correction factor for the electric charge effect of the solutes and is expressed as

$$P = \frac{Z_{cytc} Z_{rubpy} e^2}{4\pi R k_B T} \left[\frac{1}{\exp(Z_{cytc} Z_{rubpy} e^2 / 4\pi R k_B T) - 1} \right] \qquad (4)$$

where Z_{cytc} and Z_{rubpy} are the ionic charges of Cytc and $Ru(bpy)_3^{2+}$, e is the unit charge, ϵ is the dielectric constant of the solvent, and k_B is the Boltzmann constant.

Viscosity measurements were carried out by using a model DV-III cone/plate viscometer (Brookfield Engineering Labs., Inc.) with a CPE-51 cone. By using this setup, the shear rates can be varied between 0 and 768 s^{-1}. The measurable viscosity range was 25.6−512000 mPa s.

Conclusions

The distance dependence of the diffusion constant in HA solution was successfully obtained in two extreme cases above and below the pore dimension of HA solutions. This novel result proves that HA solution has two properties as a result of the nano-microstructures: (1) fast diffusion over a short distance, which does not reduce the reaction rate among adjacent (<∼10 nm) molecules, and (2) slow diffusion over a long distance and extremely high macroviscosity, which suppress the escape of precious materials from animal organisms. One of the superior features of HA as a biomaterial can be described by these two physicochemically contradicting behaviors found in one fluid solution.

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