Structural Information on Hyaluronic Acid Solutions As Studied by Probe Diffusion Experiments

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ABSTRACT: The fluorescence recovery after photobleaching technique (FRAP) was used to measure the diffusion coefficients (D) of fluorescein isothiocyanate (FITC)–dextrans in diluted, semidiluted, and concentrated hyaluronic acid solutions. The decrease of the diffusion coefficients as the hyaluronic acid concentration increases was consistent with the universal scaling equation $D/D_0 = \exp(-ac^{\nu})$. The diffusion experiments were carried out to obtain structural information on the transient network structure in hyaluronic acid solutions. On the basis of scaling laws, the concentration dependence of the average mesh size ξ was determined as $\xi \sim c^{-0.58\pm0.07}$. Additionally, the average ξ-values were estimated. The concentration dependence of ξ and the absolute vlaues for ξ were compared with structural information obtained from rheological experiments performed on hyaluronic acid solutions. Even though accurate results were found for the concentration dependence of ξ, there was a semiquantitative relation between the ξ-values estimated from the diffusion experiments and the rheological experiments.

Introduction

Hyaluronic acid (HA) is a high molecular weight polysaccharide. It is a linear polysaccharide consisting of repeating disaccharide units of β-D-glucuronic acid and N-acetyl-β-D-glucosamine.[1] HA occurs in many living substrata: extracellular matrix, vitreous body, synovial fluid. Nowadays, HA is a commercially available polymer isolated from several sources: umbilical cord, rooster comb, and streptococci bacteria.[2] Because of its outstanding rheological and biocompatible properties, it finds applications in human and veterinary surgery.[3] Pharmaceutical studies were performed on HA and HA derivatives (hylans) to use these polymers in controlled drug delivery systems.[4] A recent review was made by Larsen et al.[5]

From the rheological experiments we performed on HA solutions it became clear that HA chains start to overlap each other if the HA concentration exceeds 0.5 mg/mL.[6] Above this concentration the HA chains form a transient network. Theoretical considerations on the network structure of semidilute polymer solutions were made by de Gennes.[7] In his well-known "blob" model de Gennes assumes that the polymer solution can be considered as a collection of blobs which can each be characterized by a scaling length ξ, the "correlation length". ξ describes the average distance between the entanglement points of the chains and can be considered as a measure of the mesh size of the transient network.

To use HA in controlled drug delivery systems, it is fundamentally important to know how the HA network structure influences the diffusion of probes through the HA solutions. The correlation length may provide us with useful information to understand the diffusion of probes in HA solutions.

This paper deals with the diffusion of fluorescein isothiocyanate (FITC)–dextrans (with varying molecular weight) in HA solutions as a function of HA concentration. Several research studies have been published which deal with diffusion phenomena in HA solutions. It has been shown that HA solutions have only a limited effect as a barrier to the diffusion of oxygen and nitrogen.[8] In certain studies, HA had important effects on the diffusion of low molecular weight probes,[9,10] but for other authors these results were questionable.[11,12] A more recent study also shows that the diffusion rate for low molecular weight compounds is unaffected by the concentration of HA up to 0.35%.[13] Another author even reported an enhanced diffusion coefficient of glucose in HA solutions.[14] Interesting studies were performed on the sedimentation and the diffusion of proteins in HA solutions.[15,16] They found the following empirical relation:

$$f_0/f_c \sim \exp(-dc^{0.5}) \quad (1)$$

where f_0 and f_c are the friction coefficients respectively in the pure solvent and at polymer concentration c, with d being the diameter of the probe. These results were supported by the theoretical work of Ogston et al. based on the stochastic approach.[17] Another interesting study dealt with the diffusion of linear polymers in HA solutions.[18]

Our aim in this study was to perform probe diffusion experiments in HA solutions in order to calculate structural information on the transient network structure in HA solutions. On the basis of the diffusion coefficients, we calculated the concentration dependence of ξ. Moreover, we estimated the ξ-values. We also compared the results with information obtained from rheological experiments which were presented in a previous paper.[6] Since the fluorescence recovery after photobleaching (FRAP) method allows us to measure the diffusion coefficients in

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strongly concentrated polymer solutions, experiments were performed in a broad HA concentration range.

Experimental Section

Sample Preparation. HA, isolated by extraction from rooster combs, was supplied by Diosynth (Holland). The water content was about 5%. The protein content was lower than 0.5%, and the concentration of sulfated (glycosamino)glycan was lower than 1%. The molecular weight was determined by high-performance gel permeation chromatography. By means of the universal calibration method, M_n, M_w, and M_z were respectively determined as 390 000, 680 000, and 960 000.[19] The HA solutions were prepared by adding the solvent (containing the fluorescent probe) slowly to the HA (to avoid the formation of clumps). The solutions were stirred overnight at 4 °C. After stirring, we waited at least 1 day before performing the diffusion experiments. The composition of the solvent was 0.067 M phosphate buffer (pH 7.3) and 0.179 M NaCl. The NaCl, NaH_2PO_4, and $Na_2HPO_4 \cdot 2H_2O$ used were of an analytical grade (Merck).

Fluorescein (Sigma). The fluorescein concentration in the examined solutions was 0.004 mg/mL.

Albumin Bovine–Fluorescein Isothiocyanate (FITC-BSA; Sigma). The molecular weight of the albumin was 67 000. FITC-BSA contained 11.2 mol of fluorescein isothiocyanate per 1 mol of albumin. The final FITC-BSA concentration in the solutions was 0.30 mg/mL.

Fluorescein isothiocyanate–dextrans. (FITC-dextran; Sigma) of several weight-average molecular weights were used. They were 71 200, 147 800, and 487 000 containing respectively 0.005, 0.005, and 0.007 mol of FITC per 1 mol of glucose. The polydispersity index was lower than 1.35 ($M_w/M_n < 1.35$). The final FITC-dextran concentration in the HA solutions was 0.25 mg/mL.

Coumarin-labeled polystyrene latex microparticles (Fluoresbrite Plain Microspheres, Polysciences, Inc.) were used with a diameter (weight average) of 45 ± 3 nm, as measured by Polysciences, Inc., using a transmission electron microscope and a centrifugal particle size analyzer. The final concentration of the spheres in the solution was 4.20 mg/mL.

Diffusion Measurements. The diffusion coefficients of the probes were measured using the fluorescence recovery after photobleaching method.[20,21] In this technique, the tracer diffusion coefficient of the fluorescent probe is measured by bleaching the fluorescent molecules (using an intense laser beam) moving in the focus area of the laser beam. Immediately after the bleaching process, a highly attenuated laser beam measures the recovery of the fluorescence in the bleached area due to the diffusion of the fluorescent probe from the surrounding nonbleached areas into the bleached area. The probe diffusion coefficient D can be derived from the characteristic diffusion time T_D. We calculated T_D using the formula of Soumpasis.[22] The experimental setup of the FRAP instrument used is represented in detail elsewhere.[23]

As diffusion is dependent on temperature, the measurements were performed after equilibrating the temperature of the samples using a heated microscope table (25 ± 1 °C). Since a focused laser beam can also cause an increase in temperature of the samples, we tried to calculate the heating effect of the laser beam (see the appendix).

Results and Discussion

The diffusion of tracer molecules through polymer solutions is a complex matter, as several factors have an influence on it. Basically, the factors of influence can be classified into two groups. First, the diffusion can be influenced by chemical interactions between tracer molecules or between polymer chains and tracer molecules. Second, diffusion is influenced by a steric hindrance which depends on the size and the shape of the diffusing tracer molecules.[24] Moreover, this steric hindrance is determined by the network structure of the polymer solutions. As we are specifically interested in the influence of the HA network structure on the probe diffusion, we used FITC-dextrans as tracer molecules. The diffusion of FITC-dextrans is mainly determined by the steric hindrance which dominates the chemical interactions. Figure 1 represents the fluorescence recovery profile of FRAP experiments on HA solutions using FITC-dextran and -BSA as probes. While there is a complete fluorescence recovery in the case of FITC-dextran, there exists only a partial fluorescence recovery in the HA–BSA experiment. The partial fluorescence recovery indicates the well-known chemical interactions between proteins and HA chains.[25] The complete recovery of the fluorescence signal in the FITC-dextran experiment is an indication of the absence of considerable chemical interactions between the FITC-dextran molecules and the HA chains. Other arguments for the use of FITC-dextran are the commercial availability of several molecular weights, the excitation possibility at 488 nm (an argon laser was used as the light source), and the stability of FITC-dextrans in aqueous solvent (so that no FITC is liberated in the solutions).

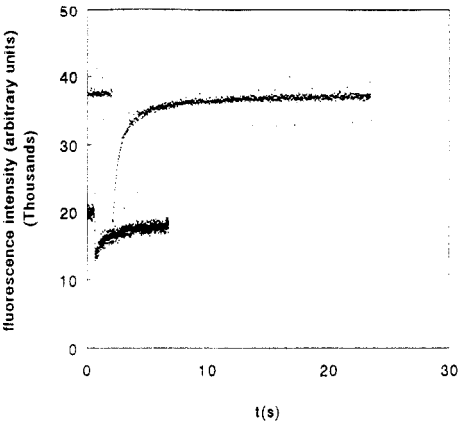

Figure 1. Typical fluorescence recovery profile of FRAP experiments using FITC-BSA (lower curve) and FITC-dextran (upper curve) as diffusing probes.

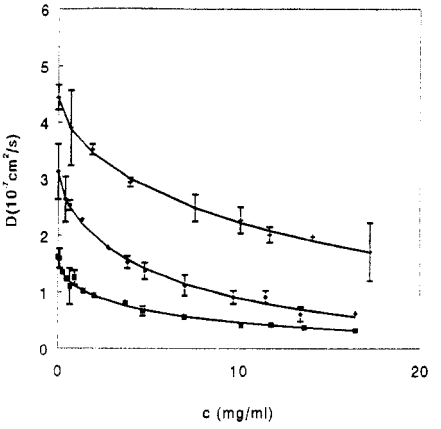

Figure 2. Diffusion coefficients (D) of the FITC-dextrans as a function of HA concentration (c). The molecular weights are 71 200 (+), 148 000 (♦), and 487 000 (■). The standard deviation is not indicated when the bar is smaller than the marker size.

Figure 2 represents the diffusion coefficients of the FITC-dextrans as a function of HA concentration. The data shown are the results of a large number of FRAP experiments performed on the HA solutions: for each HA concentration, four samples were applied on the FRAP instrument, to avoid systematic errors on D due to the application. Moreover, on each of the applied samples, four FRAP experiments were performed on different places in the sample (to avoid systematic errors due to microheterogeneities in the sample).

During the last decades, intense studies have been performed to provide theoretical concepts dealing with the diffusion of probes in polymer solutions.[17,26,27] In-

Table 1. Obtained Parameters by Fitting the Experimental Data of Figure 2 to $D = D_0 \exp(-ac^{\nu})$[a]

	M_w	$D_0 \pm \sigma$	$a \pm \sigma$	$\nu \pm \sigma$
FITC-dextran	71 200	4.46 ± 0.06	0.170 ± 0.014	0.607 ± 0.029
FITC-dextran	148 000	3.14 ± 0.06	0.293 ± 0.023	0.629 ± 0.032
FITC-dextran	487 000	1.66 ± 0.05	0.400 ± 0.040	0.500 ± 0.040

[a] D in 10^{-7} cm^2/s; c in mg/mL.

teresting work was performed by Langevin and Rondelez,[28] who considered the sedimentation of probes in semidilute polymer solutions. They made use of a scaling law for the friction coefficients proposed by de Gennes:

$$f_0/f_c = \psi(d/\xi) \tag{2}$$

It was expected that, for extremely small molecules ($d/\xi \to 0$), $\psi(d/\xi) \cong 1$. For extremely large molecules ($d/\xi \to \infty$), $\psi(d/\xi) \sim \eta_0/\eta_M$, where η_0 and η_M are respectively the viscosity of the solvent and the macroscopic viscosity of the polymer solution. Furthermore, de Gennes assumed that the movement of a probe through the network can be considered as a process which involves an activation energy. The activation energy is determined by the elastic free energy that is coupled with the expansion of the mesh ($\xi \to d$) when a probe molecule passes through it. He found the following relation:

$$D = D_0 \exp[-\beta(d/\xi)^{\delta}] \tag{3}$$

In eq 3, β approaches 1.[28] As ξ can be expressed as a function of the polymer concentration[7]

$$\xi \sim c^{-\nu} \tag{4}$$

eq 3 can be written as

$$D = D_0 \exp(-\beta' d^{\delta} c^{\delta\nu}) \tag{5}$$

Although many theories[17,26-28] show a linear dependence on d in eq 5 ($\delta = 1$), experimental δ-values do not always agree with it.[29] As we only used three kinds of FITC-dextran molecules, we were not able to determine the δ-value accurately. Assuming $\delta = 1$, eq 5 may be written as

$$D = D_0 \exp(-\beta' d c^{\nu}) \tag{6}$$

Equation 6 agrees with the well-known empirical equation of Phillies[29,30] usually written in the following way:

$$D = D_0 \exp(-ac^{\nu}) \tag{7}$$

Equation 7 can be considered as a generalized expression of Ogston's diffusion model.[17]

We tried to fit the data represented in Figure 2 to eq 7. In the fitting procedure (nonweighted nonlinear least-squares method) three parameters were simultaneously fitted: D_0, a, and ν. As there was also an experimental error on D_0, we preferred to consider D_0 as a free parameter in the fitting procedure. The lines in Figure 2 show that the data points are well-fitted to eq 7. This was confirmed by the random scatter of experimental points around the fitted curve. In Table 1 we have summarized the values of the parameters D_0, a, and ν.

As we stated in eq 4, the ν-values of Table 1 show the concentration dependence of ξ: it indicates how the average distance between the entanglement points of the transient network decreases as the HA concentration increases. As ν is independent of the size of the diffusing probe, it should be expected that the difference between the ν-values mentioned in Table 1 is not significant. Although there exists a good agreement between the ν-values obtained by using FITC-dextrans 71 200 and 148 000 as probes, there is a considerable difference with the ν-value obtained in the case of FITC-dextran 487 000. The reason is unclear. On the basis of the ν-values of Table 1, we calculated the mean value for ν as 0.579 ± 0.069. This mean value is consistent with the ν-value obtained from our rheological experiments which were reported in detail in a previous study.[6] From those experiments we calculated ν as 0.589 ± 0.020. In that rheological study we measured the plateau modulus of the entangled HA solutions which made it possible to estimate the concentration dependence of ξ. Interpreting the ν-values from the diffusion study, it is important to emphasize that we assumed $\delta = 1$ in eq 5.

In de Gennes' theory,[7] the following expression exists for ξ:

$$\xi = R_G(c/c^*)^{\nu} \tag{8}$$

In eq 8, R_G and c^* are respectively the radius of gyration of the polymer molecules and the critical concentration for overlapping. Additionally, it can be shown[7] that R_G and c^* scale as follows:

$$c^* \sim N^{(1-3\upsilon)} \tag{9}$$

$$R_G \sim N^{\upsilon} \tag{10}$$

N being the polymerization degree of the polymer and υ being the dependence of R_G on the polymerization degree as defined by Flory.[31] Since ξ must be independent of the degree of polymerization (a basic assumption in de Gennes' theory), it follows that ξ must scale as $\sim N^0$. Therefore, using eqs 8-10, we can show that ν has to fulfill the following condition:

$$\nu = -\upsilon/(1 - 3\upsilon) \tag{11}$$

From eq 11, and considering $\nu = 0.579$, the υ-value can be calculated: $\upsilon = 0.785$. As the HA molecules are very stretched due to their polyelectrolyte properties, the calculated υ-value is greater than the typical υ-value ($0.5 < \upsilon < 0.6$) for neutral flexible polymers and indicates the semiflexible characteristic of the chains.

In the rheological study we also estimated the absolute values of ξ as a function of HA concentration.[6] In these calculations we made use of an "equivalent network model"[32] to estimate ξ. As we made many assumptions in these calculations, it was intriguing to compare these ξ-values with values obtained by another independent technique. Making the following considerations, ξ could be estimated from the diffusion experiments. From eqs 6 and 7, we see that

$$a = \beta' d \tag{12}$$

Using eqs 3, 4, and 6, ξ can be expressed in the following way:

$$\xi = c^{-\nu}/\beta' \tag{13}$$

Combining eqs 12 and 13 provides the following expression for ξ:

$$\xi = (d/a)c^{-\nu} \tag{14}$$

For calculating ξ from eq 14 it is necessary to know the size of the FITC-dextrans. The diameters of the FITC-dextrans were calculated from the measured D_0-values using the Stokes-Einstein relation making the following assumptions:

(a) The geometry of the FITC-dextrans can be approximated as spherical.

(b) The FITC-dextran concentration in the solution is low enough to avoid intermolecular interactions between

Table 2. ξ-Values Calculated on the Basis of the Diffusion Experiments and the Rheological Experiments

HA concn (mg/mL)	$\xi \pm \sigma^a$ (nm)	$\xi \pm \sigma^b$ (nm)	$\xi \pm \sigma^c$ (nm)	$\xi \pm \sigma^d$ (nm)
0.5	99 ± 9	83 ± 10	107 ± 11	
3	33 ± 3	27 ± 3	44 ± 5	
6	22 ± 2	17 ± 2	31 ± 4	
8	18 ± 2	14 ± 2	27 ± 4	
10	16 ± 2	13 ± 2	24 ± 3	37 ± 2
14	13 ± 2	10 ± 1	20 ± 3	28 ± 2
17	12 ± 1	9 ± 1	18 ± 3	
19				26 ± 2
24				22 ± 2
28				20 ± 2

[a] Probe: FITC-dextran 71 200. [b] Probe: FITC-dextran 148 000. [c] Probe: FITC-dextran 487 000. [d] Results from the rheological measurements.

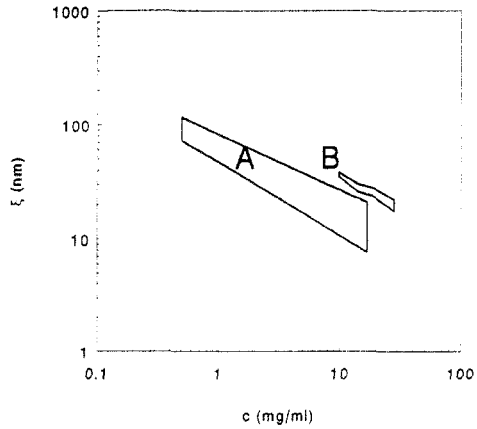

Figure 3. Average mesh size (ξ) as a function of HA concentration (c). The letters A and B indicate the ξ-values calculated respectively from the diffusion and the rheological experiments.

the FITC-dextran molecules so that the measured D_0-value approximates the diffusion coefficient for an infinite diluted solution.

The diameters of the FITC-dextran molecules with average molecular weights (M_w) 71 200, 148 000, and 487 000 were respectively 11.0, 15.7, and 30.3 nm. We also found that $d \sim M_w^{0.527\pm0.018}$ which is consistent with scaling laws relating the size of a polymer chain to its molecular weight.[31]

The calculated ξ-values, both from the diffusion experiments and from the rheological experiments, are represented in Table 2 and Figure 3. The indicated standard deviations of ξ are calculated, taking into account the errors in the individual parameters of eq 14. It is important to emphasize that, in practice, ξ will show some distribution. Moreover, as the network in the HA solution is a transient network, each mesh has a finite lifetime and, due to the permanent movement of the chains, the size and the shape of each mesh will continuously change within its lifetime. Roughly speaking, from Table 2 and Figure 3 there seems to be a "semiquantitative" agreement between the ξ-values calculated from the diffusion experiments and the rheological measurements. Comparing the ξ-values from the diffusion measurements with the ξ-values from the rheological experiments, it is important to take into account the following:

(a) Due to instrumental difficulties keeping the temperature in the FRAP apparatus at a constant 37 °C, we had to perform the FRAP experiments at 25 °C. However, the rheological experiments were done at 37 °C because we were interested in the rheological behavior in physiological conditions. As the polymer chains become more extended at a higher temperature, ξ is expected to decrease

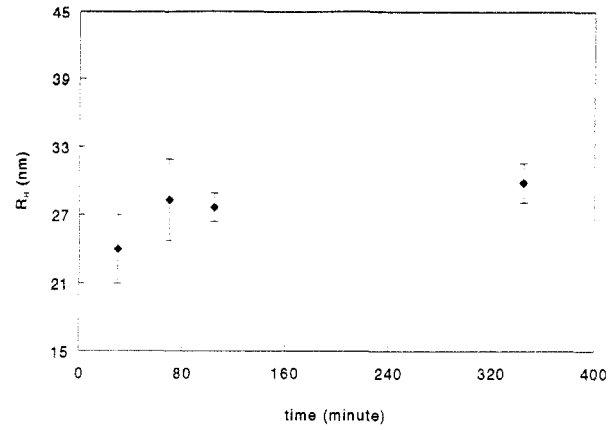

Figure 4. Hydrodynamic radius of the microspheres (R_H) as a function of time (t) after the preparation of the solutions.

as the temperature increases due to a stronger overlap. The temperature dependence of ξ is rather complex and may depend on the polymer concentration and on the glass transition temperature of the polymer solution.[33]

(b) For the ξ-calculations, both from the diffusion experiments and from the rheological measurements, we made assumptions so that the calculated ξ-values are actually estimates for ξ.

In our previous study[6] we performed intensive experimental work on the zero shear rate viscosity behavior of the HA solutions. As non-Stokes-Einsteinian effects in polymer solutions have been observed earlier,[34,35] we were interested in which way the measured D-profiles deviate from the D-profile expected from the η_0-values of the HA solutions. In this framework we also studied the diffusion behavior of polystyrene microparticles in the HA solutions. Although we did not observe chemical interactions between the spheres and the HA chains, the FRAP recovery curves showed a capricious profile, indicating an aggregation of the spheres due to the high salt concentration in the solvent. This made it impossible to fit the curves. Another indication for the aggregation was the decrease of the diffusion coefficient as a function of time. Figure 4 illustrates how the hydrodynamic radius of the spheres increases during longer time periods between the preparation of the solution and the FRAP measurement. The aggregation was retarded by adding Triton X-100 (0.1%) to the HA solutions. Reproducible measurements and the absence of aggregation phenomena were obtained when we performed the FRAP measurements between 1 and 2 h after adding the microparticles (treated with ultrason) to the HA solutions. Figure 5 shows the relative D-values (D/D_0) of the FITC-dextrans and the microparticles (d = 42 nm) as a function of the HA concentration. Fitting the measured diffusion coefficients of the microparticles to eq 7 resulted in the following values for the parameters a and ν: $a = 0.643 \pm 0.303$; $\nu = 0.597 \pm 0.212$. As these values showed a large standard deviation, we did not take them into consideration for calculating structural information. We also performed experiments with still larger spheres (d = 140 nm), but as the aggregation tendency was so strong we did not succeed in obtaining reliable measurements.

Figure 5 clearly shows that the probe diffusion in the HA solutions did not go as predicted based on the macroscopic zero shear rate viscosity of the HA solutions. In all the experiments, the probe molecules moved more quickly through the HA network than predicted. For the FITC-dextrans, failures of the Stokes-Einstein equation decrease as the size of the FITC-dextrans increases.

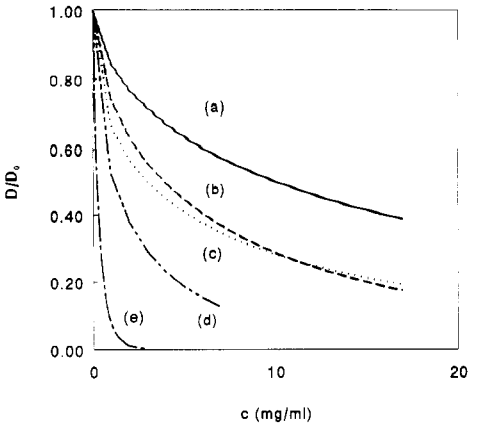

Figure 5. Relative diffusion coefficients (D/D_0) of the FITC–dextrans and microparticles as a function of HA concentration (c) calculated from the experimental data presented in Figure 3: FITC–dextran 71 200 (a), FITC–dextran 148 000 (b), FITC–dextran 487 000 (c), polystyrene microparticles (d), and the Stokes–Einstein prediction (e).

The diffusion experiments with the microparticles confirm this trend which may indicate the validity of the asserted limiting case in the diffusion theory of Langevin and Rondelez:[28]

$$\lim_{d/\xi \to \infty} D/D_0 = \eta_0/\eta$$

Finally, we examined the diffusion of FITC–dextrans and fluorescent microspheres in diluted, semidiluted, and concentrated HA solutions. We analyzed the diffusion experiments in order to get structural information on the HA solutions and compared the results with the information obtained from rheological experiments performed on the HA solutions. It is our general conclusion that the probe diffusion in HA solutions provides us with information on the microscopic solution structure which agrees quite well with the information obtained from the viscoelastic properties of the HA solutions. These results may support our fundamental and pharmaceutical research on HA and HA derivatives.

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Appendix: Influence of the Laser Beam on the Temperature of the Sample

An effort to get information on a possible temperature increase of the sample was first made by Lopez et al.[21] They used fluorescein as a pH indicator to measure the temperature-induced pH change of a Tris buffer solution around its pK. Under their experimental conditions, they measured a temperature increase of less than 0.4 °C. As it was experimentally impossible in our FRAP equipment to measure the temperature change while the laser beam acts upon the sample (too small of a sample volume and too short of an action time), we tried to calculate it, simulating the experimental conditions. In the calculations we assumed that the heated volume of the sample could be considered as a cube with a side of 10 μm. From the applied volume of 2 μL, we calculated the height of the liquid layer as 10 μm. The intensity of the laser beam when it entered the sample was $\cong$1.0 mW. From the spectrophotometrical measurements we performed on the FITC–dextran solutions, we calculated that only 1% of the light entering the FRAP sample was absorbed. Furthermore, we assumed that all the absorbed light was converted into heat (fluorescence quantum efficiency = 0).

The theoretical calculation of the temperature distribution $T(x,y,z,t)$ can be made by solving the heat diffusion equation:

$$\lambda \nabla^2 \mathbf{T} = \lambda\left(\frac{\partial^2 \mathbf{T}}{\partial x^2} + \frac{\partial^2 \mathbf{T}}{\partial y^2} + \frac{\partial^2 \mathbf{T}}{\partial z^2}\right) = c_v \frac{\partial \mathbf{T}}{\partial t} - p \qquad \text{(A1)}$$

where λ denotes the thermal conductivity, c_v the thermal capacity per unit volume, and p the power intensity per unit volume. For water we have $\lambda = 0.50\ \mathrm{W \cdot m^{-1} \cdot K^{-1}}$ and $c_v = 4.19\ \mathrm{MJ \cdot m^{-3} \cdot K^{-1}}$. We have assumed that the thermal transient phenomenon we are dealing with in this paper occurs in a very short time. So the liquid does not move by natural convection. Hence, heat transfer is only possible by conduction expressed by eq A1. Although eq A1 looks cumbersome at first sight, a semianalytical solution can be found because some realistic approximations will simplify the problem considerably. Remembering that the thermal transient is very short, the heat diffusion will be limited to a small volume surrounding the heat source (i.e., the focal point of the laser beam). Hence, with respect to this small volume, the liquid can be considered as the infinite space. Under these conditions, eq A1 has an analytical solution known as Green's functions:[36]

$$G(x,y,z,t) = \frac{1}{8c_v\left(\pi\left(\frac{\lambda}{c_v}\right)t\right)^{3/2}} \exp\left(\frac{x^2+y^2+z^2}{4\frac{\lambda}{c_v}t}\right) \qquad \text{(A2)}$$

Equation A2 is the solution of eq A1 provided that the power density is an impulse function, i.e., an infinite power density in just one point and only at $t = 0$. To get an easy physical interpretation of eq A2, notice that eq A2 as a function of x (or y and z) is nothing more than a Gaussian probability distribution with a root-mean-square deviation growing proportional to $t^{1/2}$. In our case, a uniform power p is dissipated in a cubic volume with side a (=10 μm) during a finite time t. The temperature distribution can then be found by superposition. Indeed, considering an elementary volume $dx'\,dy'\,dz'$, during an elementary short time step dt' one gets an impulse given by

$$p\,dx'\,dy'\,dz'\,dt' \qquad \text{(A3)}$$

which leads to a temperature distribution:

$$G(x-x',y-y',z-z',t-t')\,p\,dx'\,dy'\,dz'\,dt' \qquad \text{(A4)}$$

In order to get the final temperature distribution, one has to add up the whole cubic volume and also with respect to time:

$$\mathbf{T}(x,y,z,t) = \int_0^t dt'\,p \int\int\int G(x-x',y-y',z-z',t-t')\,dx'\,dy'\,dz' \qquad \text{(A5)}$$

The simplification made by the fact that the volume is approximately a cube may be a rather crude approximation for the focal point of the incoming light beam. As we are only interested in an estimation of the temperature rise, the real shape of the heat source is not so critical as long as the total power ($=pa^3$) remains the same. For a cube, the integration with respect to x', y', and z' can be carried out analytically.[36] Only the integration with respect to time has to be done numerically in order to obtain the temperature. Figure 6 shows the calculated temperature changes for a FRAP experiment with a bleaching time of 15 ms. The lines show the temperature changes at different places in the sample (respectively in the central point of

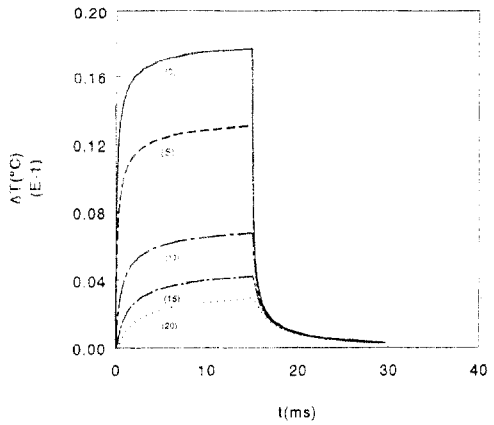

Figure 6. Temperature increase profiles during and after a bleaching pulse of 15 ms. The lines show the temperature change at different places: in the central point of the heated spot (0) and at 5 μm (5), 10 μm (10), 15 μm (15), and 20 μm (20) from the central point.

the heated spot and at 5, 10, 15, and 20 μm from this center).

On the basis of our calculations we conclude the following:

During the bleaching period (t < 15 ms) only a weak temperature increase occurs which drops sharply from the center of the bleached area to the surroundings of the sample.

After the bleaching period, the temperature decreases extremely quickly as a function of time due to the sharp temperature fall. Figure 6 clearly shows that after 30 ms the temperature increase nearly disappears. As for a FRAP experiment with a bleaching time of 15 ms, where the total measuring time is about 300 ms, we can state that no heating effect of the laser beam influences the measurement. More evidence supporting this statement is the fact that the probe moves from the surroundings in the bleached area (where there is a smaller temperature increase) to the center and that the diffusion, for the most part, happens after the bleaching period, when there is also a smaller temperature increase.

To verify the absolute value of the diffusion coefficients measured by the FRAP apparatus, we measured the diffusion coefficients of small molecules (fluorescein in water, 22 ± 1 °C) and large spheres (polystyrene microparticles in water, 25 ± 1 °C). For fluorescein the measured D-value was (51.28 ± 1.49) × 10^{-7} cm^2/s which closely corresponds to reported values in published papers.[37] Polystyrene microparticles tend to aggregate during storage, so the spheres were treated with ultrason before performing the FRAP experiments. The measured diffusion coefficient was (1.16 ± 0.08) × 10^{-7} cm^2/s. Assuming the validity of the Stokes–Einstein relation, it was possible to calculate the diameter of the spheres: 42 ± 2 nm. This was also consistent with the size measured by the producer. This consistency also indicates the absence of a significant temperature increase during the experiment; as the viscosity of water depends strongly on the temperature, much lower diffusion coefficients would be measured if the temperature of the samples increased significantly.

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