Fluorescence Correlation Spectroscopy Study of TAMRA Diffusion in Poly(vinyl-alcohol) and Ficoll70 Solutions.

Ariel Michelman-Ribeiro[1,2], Ferenc Horkay[1], Ralph Nossal[1], Hacène Boukari[1]
[1]Laboratory of Integrative & Medical Biophysics, National Institutes of Health, Bethesda, MD.
[2]Physics Department, Boston University, Boston, MA.

ABSTRACT

We compare fluorescence correlation spectroscopy (FCS) measurements of the fluorescent nanoparticle, TAMRA, diffusing in non-fluorescent -hence invisible- poly(vinyl-alcohol) (PVA) or Ficoll70 solutions as a function of the polymer concentration, *c*. We determine changes of the translational diffusion coefficient of TAMRA and fit the data with the universal scaling law ($D \sim \exp[-a(c/c^*)^n]$) to extract information about solvent quality. For PVA, we find $n = 0.74$, suggesting that water in this case acts as a good solvent, whereas $n = 1.02$ in Ficoll70 solutions, indicating theta-like behavior.

INTRODUCTION

A typical use of fluorescence correlation spectroscopy (FCS) is to determine the diffusion coefficient of nanoscopic particles moving in dilute solutions, yielding the same coefficient as obtained from dynamic light scattering (DLS). However, FCS has several advantages over DLS [1-5]: 1) detection specificity of fluorescence targets when the latter move in a complex, non-fluorescent, environment; 2) use of small (nanomolar) concentrations of the targeted particles and small volumes (~10-40 μl); 3) ability to study targets having a wide range of sizes, including particles as small as 1 nm; 4) applicability to both in-vivo and in-vitro conditions. Our objective is to exploit these properties to extend FCS to investigations of the behavior of macromolecules in concentrated solutions, gels, and cellular systems –a problem of interest to both basic research and applied engineering [6-11].

In this paper we describe results of analysis of FCS measurements of TAMRA (MW=430 Da), a small fluorescent probe (~ 1.6 nm), moving within non-fluorescent poly(vinyl alcohol) (PVA) polymer and Ficoll70 solutions. PVA is a neutral, water-soluble, linear polymer commonly used as a model for tissue engineering matrices [6,9-12]. Ficoll70 is a water-soluble, highly-branched sucrose-polymer that behaves as an ideal neutral sphere. It is used in perfusion experiments and research studies of the effects of crowding on, for example, the enzymatic activity or protein stability [7-8]. However, the solutions demonstrate different behaviors as the polymer concentrations are increased. Above a threshold concentration ($c^* \sim 2.5\%$ W/V) PVA chains entangle to form a flexible network that can be cross-linked to create a gel [6,12]. According to scaling theory of polymers, the PVA solution is characterized by a mesh size, x, that varies as $x \approx r_g (c/c^*)^{-n}$, where r_g, c^*, and n are, respectively, the radius of gyration of the polymer chain, the critical concentration, and the solvent quality ($n = 0.75$, good solvent; $n = 1$, theta condition) [13]. For Ficoll70 the particles appear unentangled in the concentration range studied ($\leq 30\%$ W/V), and the main characteristic lengthscale is the average spacing between the particles. In both solutions, the size of TAMRA is smaller than the characteristic

length of the solution. An interesting question is whether the structural differences between the two polymer solutions can be "seen" through the hydrodynamic behavior of the probe.

EXPERIMENTAL DETAILS

Sample Preparation:

PVA ($M_w \approx$ 85 kDa., Sigma Aldrich) was dissolved in de-ionized water at 95° C and kept at this temperature for several hours. Samples with this fresh PVA were then prepared at room temperature with concentrations ranging from 1% to 8.6% (w/v). Ficoll70 ($M_w \approx$ 70 kDa, Pharmacia Biotechnology) was added to de-ionized water at high concentration (300 mg/ml) at room temperature and left to fully dissolve overnight. Various samples with different concentrations were then prepared by dilution. The fluorescent probe, carboxytetramethylrhodamine (TAMRA) (Molecular Probes, M_w = 430 Da) was mixed at nanomolar concentration with either PVA or Ficoll70 solutions. For FCS measurements, the solutions were loaded into shallow 1-cm diameter chambers (65 μl), the bottoms of which were made from standard glass coverslips. All the experiments were performed at 24° C.

FCS Apparatus:

The experimental setup has been described elsewhere [5]. We used a 543 nm HeNe laser (JDS Uniphase) to illuminate the sample through a 60X objective (NA=1.2, water) in an Olympus IX70 inverted microscope. The 27-μW incident beam was expanded and focused onto a small spot of radius r_0 (< 1 micron). The emitted fluorescent light was collected by the same objective and focused onto an optical fiber with a core diameter of either 10 or 25 microns. This small diameter ensured the confocal detection necessary for delimiting small volumes of interest. For signal detection, two avalanche photodiodes (PerkinElmer, EG&G, Vandreuil, Canada) were used in cross-correlation mode to reduce the effects of spurious detector afterpulsing on the correlation function, which is important at short time scales (<10 μs.). The pulses of the photodiodes were processed by a digital correlator (Brookhaven Instrument, Holtsville, NY, USA), yielding the time-correlation functions.

FCS Theory:

Fluorescence Correlation Spectroscopy utilizes the fluctuations in emission from fluorescent particles moving through a system. These fluctuations are typically caused by changes in the number of fluorescent particles in a small-illuminated volume or changes in the emission quantum yield of the particles. The illuminated volume is made small (~femptoliter) by confocal setup or by two-photon emission [1,3-4]. The detected intensity, *I(t)*, of the fluorescent particles in the sample volume at time, *t* , is time-correlated to generate a correlation function defined as:

$$F(t) = 1 + \frac{< dI(t) dI(t+t) >}{< I(t) >^2} \qquad (1)$$

where $dI(t) = I(t) - < I(t) >$ denotes the deviation of the measured intensity from the average intensity, *<I(t)>*. For the ideal case of freely diffusing monodisperse and uniformly bright fluorescent particles, the correlation function can be written as [2]:

$$F(t) = 1 + \frac{1}{N}\frac{1}{\left(1+\frac{t}{t_d}\right)}\frac{1}{\left(1+p\frac{t}{t_d}\right)^{1/2}}, \qquad (2)$$

when the excitation beam is a 3-D Gaussian beam, with an intensity profile given by: $W(r,z) = Ae^{-2\left(r/r_0\right)^2} e^{-2\left(z/z_0\right)^2}$. Here r_0 and z_0 characterize the width of the focused beam and the length along the optical axis defined by the size of the pinhole, respectively, and t_d is a characteristic time for a particle to diffuse along the lateral width (r_0) of the focused incident beam, viz., $t_d = r_0^2/4D$, where D is the translational diffusion coefficient of the particle. In Eq.2 N denotes the average number of particles in the excitation volume and $p = \left(r_0/z_0\right)^2$.

RESULTS AND DISCUSSION

In Fig.1 we show normalized correlation functions, $[F(t)-1]/[F(0)-1]$, of TAMRA in water and in several PVA and Ficoll70 solutions prepared at various concentrations. Each correlation function was collected over a 30 to 45 min period. Note the systematic shift of the curves with increasing concentration, indicating slower movement of the TAMRA. The data were readily fit with the expression in Eq.2 (see solid lines in the figure), showing that each of the solutions can be satisfactorily described by a single characteristic time, τ_d. That is, no anomalous diffusion process is observed.

In Fig.2a we plot the diffusion coefficient, D, of TAMRA (scaled by the diffusion time of TAMRA in water, D_o) as a function of the PVA concentration of the various solutions. As expected, the curve shows a systematic and monotonic decrease of diffusion coefficient with increasing polymer concentration; no measurable effect was observed when the threshold concentration was crossed ($c^* \sim 2.5$ % W/V). Similarly, as shown in Fig.2b, the diffusion coefficient decreases monotonically with an increase in Ficoll70 concentration, although with a slower dependence on polymer concentration.

To properly interpret the present diffusion data one needs to go beyond the Stokes - Einstein model, which assumes a homogeneous polymer-solvent medium. Because of its size, TAMRA probes an inhomogeneous polymer-solvent medium, having features on the lengthscale, x, estimated to be between 2 and 10 nm [12]. Several models have been suggested to take into account the interplay between the mesh size and the probe size and its effect on probe diffusion in a polymer solution. Notably, de Gennes et al. argue (see [14] and reference therein) that $D/D_o = 1$ for $d/x \ll 1$, (d being the probe size), $D/D_o = h_o/h$ for $d/x \gg 1$ (h_o and h being the viscosities of the solvent and the polymer-solvent solutions, respectively), and, in the intermediate range $d/x \sim 1$, $D/D_o = y(d/x)$, where Y is a scaling function which, according to Langevin and Rondelez, is $D/D_o = \exp(-d/x)$ [14] (Note that this expression originally was derived for sedimentation coefficients [14].) As has been pointedly emphasized by Phillies as well as other authors, this prediction, which is based on the reptation model,

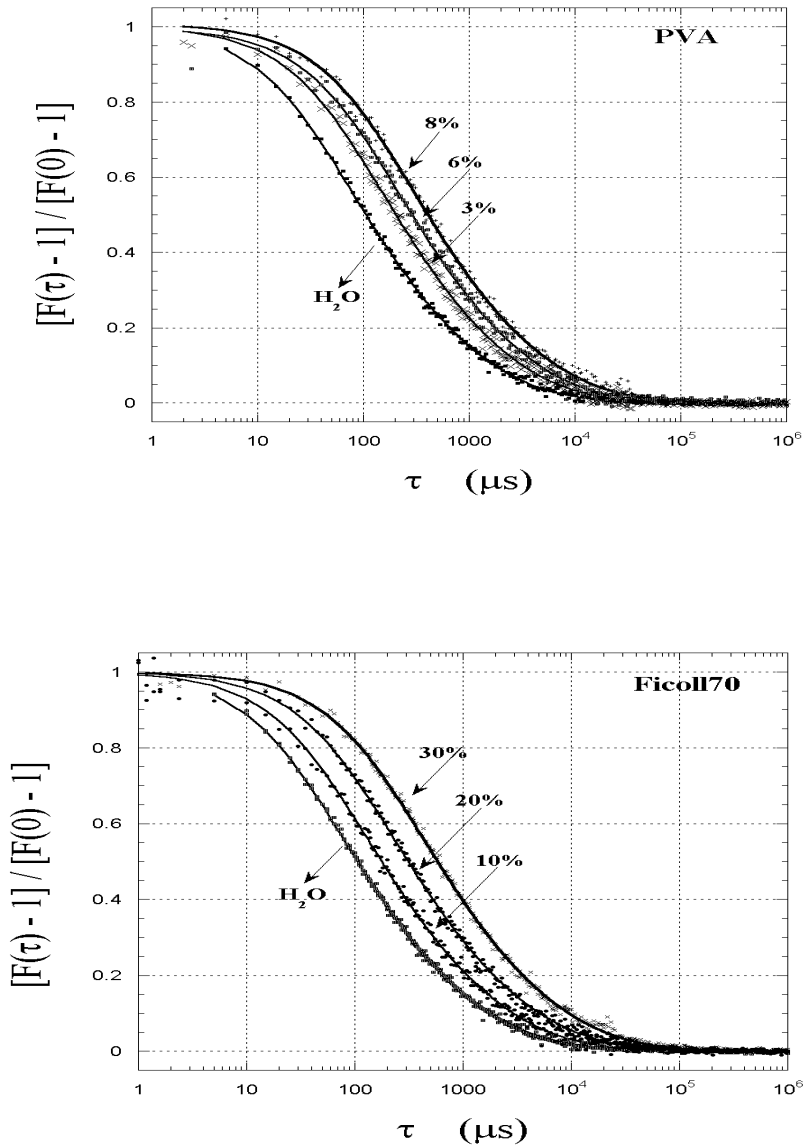
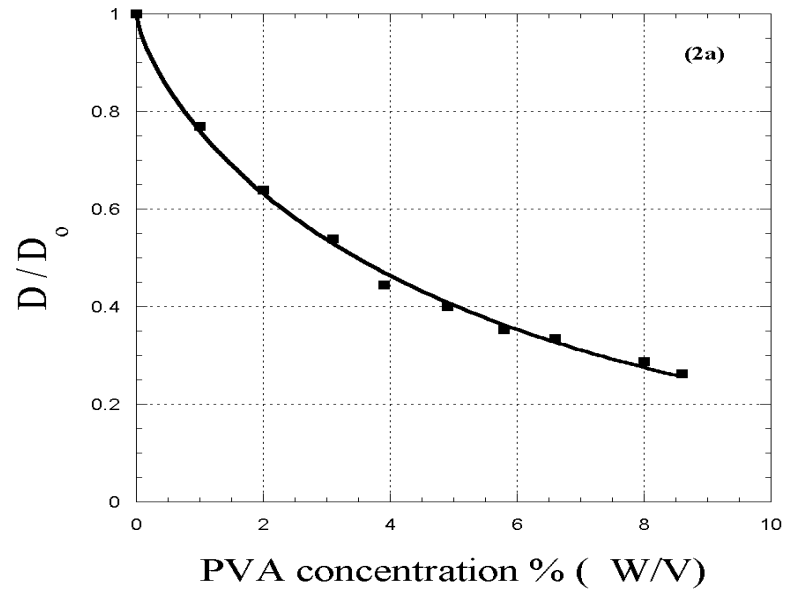


Figure 1: Normalized FCS correlation functions of TAMRA probe PVA (top) and Ficoll70 (bottom) solutions. Each curve is labeled with the polymer concentration (PVA or Ficoll70) in weight percentage per unit volume. The solid lines are the fits of Eq.2 to the data.

ignores various hydrodynamic effects (see review [15] and references therein). However, whatever the model, it appears the observed decrease in the diffusion coefficient can be readily fit with a stretched exponential. Clearly, the expression by Rondelez and Langevin provides explicitly meaningful physical parameters and takes into account the dependence on the probe size. If we use the expression relating x to the concentration, viz., $\xi = r_g (c/c^*)^n$ [13], we end up with the following expression:

$$D/D_o = \exp[-d/r_g (c/c^*)^n]. \qquad (3)$$



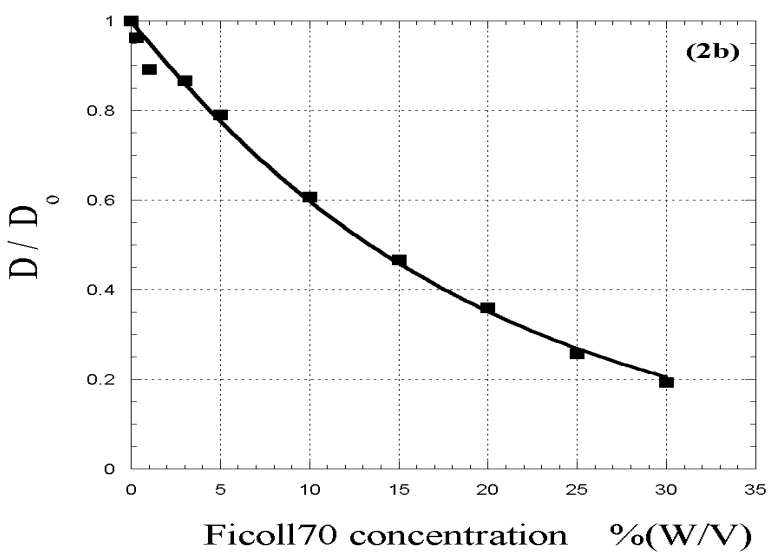


Fig.2: The diffusion coefficient of TAMRA, scaled by the corresponding value in water, is plotted as a function of the concentration of either PVA (top) or Ficoll70 (bottom). The solid lines are fits to Eq.3 (see text).

We fit the data in Fig.2a and Fig.2b with Eq.3, where the fitting parameters are n and exponential prefactor. For PVA the fit yields $n = 0.74$ (TAMRA), which is very close to the theoretical value for a good solvent ($n = 0.75$). Further, taking $r_g \approx 4$ nm and $c^* \approx 2.5\%$

we estimate $d \approx 2.1$ nm, which is comparable to the size of TAMRA that we measure by FCS (~1.6 nm). In contrast, the data of TAMRA in Ficoll70 solutions are well fit with a single, pure exponential ($n = 1.02$), which suggests a theta-like behavior for the Ficoll70 solution. Of course, one needs to clarify whether this assertion (theta-solvent) is consistent with the rigorous definition of a theta-solvent, or rather results from an empirical fit having no particular physical meaning. Certainly, one cannot interpret the observed changes in probe diffusion in this case as resulting from (bulk) viscosity changes: over the concentration range of the Ficoll70 solution, the viscosity increases by more than 20 fold (see Fig.2 in [11]), whereas the decrease in the diffusion of TAMRA is about 80% (see present Fig.2b). This significant difference is often attributed to the phenomenon of microviscosity in a molecularly heterogeneous solution.[7,16] However, should one expect the increase in the microviscosity to be exponential with increase of Ficoll70 concentration?

In summary, we successfully applied FCS to measure the diffusion coefficient of a fluorescent probe, TAMRA, in both PVA and Ficoll70 solutions. Despite the smallness of the probe with respect to the concentration-dependent mesh size of the polymer solution, it appears the probe experiences significant slowing down with increasing polymer concentration. The changes in the apparent diffusion coefficient of the probe cannot be accounted for merely by changes in the bulk viscosity of the solution.

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REFERENCES

[1] W. W. Webb, *Appl. Optics* **40**, 3969 (2001)
[2] S. R. Aragon and R. Pecora, *J. Chem. Phys.* **64**, 1791 (1976).
[3] R. Rigler and E. S. Elson, *Fluorescence Correlation Spectroscopy: Theory and Applications''*, Springer Series in Chemical Physics, Springer-Verlag; New York 2001.
[4] Y. Chen, J. D. Müller, K. M. Berland, E. Gratton, *Methods* **19**, 234 (1999).
[5] H. Boukari, R. Nossal, and D. L. Sackett, *Biochemistry* **42**, 1292 (2003).
[6] A. Michelman-Ribeiro, H. Boukari, R. Nossal, and F. Horkay, *Macromolecules* **37**, 10212 (2004).
[7] N. A. Busch, T. Kim, and V. A. Bloomfield, *Macromolecules* **33**, 5932 (2000).
[8] A. H. Elcock, *PNAS* **100**, 2340 (2003).
[9] A.S. Hoffman, *Advanced Drug Delivery Review* **43**, 3 (2002).
[10] C. R. Nuttelman, S. M. Henry, K. S. Anseth, *Biomaterials* **23**, 3617 (2002).
[11] R. H. Schmedlen, K. S. Masters, J. L. West, *Biomaterials* **23**, 4325 (2002).
[12] F. Horkay, A. M. Hecht, E. Geissler, *Macromolecules* **1994**, *27*, 1795.
[13] P. G. de Gennes, *Scaling Concepts in Polymer Physics*; Cornell University Press; Ithaca, NY, 1979.
[14] D. Langevin and F. Rondelez, *Polymer* 19, 875 (1978).
[15] G. D. J. Phillies, J. Non-Cryst. Solids **131-133**, 612 (1991).
[16] K. S. Stojikovic, A. M. Berezhkovkii, V. Y. Zitserman, S. M. Bezrukov, *J. Chem. Phys.* **119**, 6973 (2003).