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ORIGINAL PAPER

Viscosity-Dependent Diffusion of Fluorescent Particles Using Fluorescence Correlation Spectroscopy

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Abstract Fluorescent particles show the variety characteristics by the interaction with other particles and solvent. In order to investigate the relationship between the dynamic properties of fluorescent particles and solvent viscosity, particle diffusion in various solvents was evaluated using a fluorescence correlation spectroscopy. Upon analyzing the correlation functions of AF-647, Q-dot, and beads with different viscosity values, the diffusion time of all particles was observed to increase with increasing solvent viscosity, and the ratio of diffusion time to solvent viscosity, τ_D/η, showed a linear dependence on particle size. The particle diffusion coefficients calculated from the diffusion time decreased with increasing solvent viscosity. Further, the hydrodynamic radii of AF-647, Q-dot, and beads were 0.98±0.1 nm, 64.8±3.23 nm, and 89.8± 4.91 nm, respectively, revealing a linear dependence on τ_D/η, which suggests that the hydrodynamic radius of a particle strongly depends on both the physical size of the particle and solvent viscosity.

Keywords Fluorescence correlation spectroscopy · Diffusion coefficient · Quantum dot · Alexa Fluor 647 · Fluorescent beads

Introduction

The diffusion motion of particles is a fundamental feature of molecules due to their physical and chemical properties [1–3]. Analysis methods that utilize diffusion motion, such as gel chromatography, electrophoresis, and fluorescence correlation spectroscopy (FCS), are the most popular methods of quantitative analysis. Since the development of dynamic light scattering (DLS) in 1970, FCS, which measures fluorescence fluctuations after removal of a scattered excitation beam, was independently developed by Webb and Rigler in 1972 and 1974, respectively. FCS has become an advanced and accurate technique for the analysis of the dynamic information of molecules with the development of confocal microscopy and photo-detection techniques [4–9]. Currently, FCS is the most popular technique for the observation of interactions between bio-molecules, such as protein and DNA, from a very small sample in a short amount of time [10, 11]. However, commercial FCS equipment is expensive, and therefore is limited to professional organizations [12].

In order to overcome the high cost associated with using commercial equipment in the laboratory, an FCS system based on a commercial optical microscope was developed in our laboratory, and we measured the diffusion of fluorescent particles in several viscosity systems similar to that of human protein.

Theory

In a typical FCS experiment, the fluorescent signal is a function of the time–dependent fluctuation of fluorescence in a femtoliter-sized open volume of sample solution. The effective focal volume is formed by the focal point and pinhole. As shown in Fig. 1, the effective focal volume is determined by the radial length (diameter) 2 w and axial length 2 z. The valid focal zone of the FCS is very small and easily affected by the intensity of the fluorescent beam. Therefore, the minor axis, w, is calculated using the diffusion coefficient of a small standard sample.

FCS equipment detects the fluorescence from a small observation region, called the effective focal volume, using an

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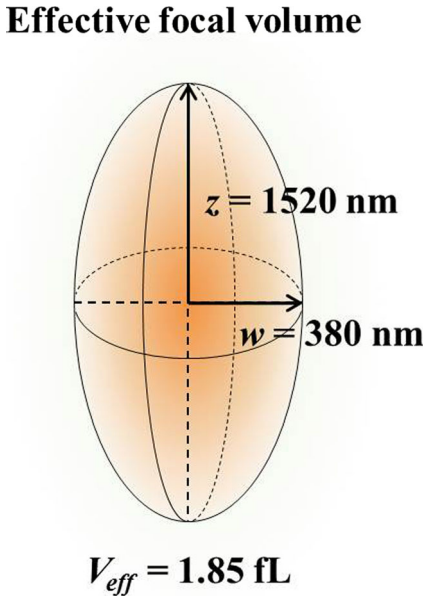

Fig. 1 Effective focal volume; the minor axis is 380 nm, the major axis is 1,520 nm, the effective focal volume is 1.85 fL, and the structure factor is 4

objective lens. When the fluorescent particles in Brownian motion enter and exit the effective focal volume illuminated by the laser, fluorescence fluctuation is produced and is dependent on the number, size, and speed of the particles. If the autocorrelation function of the time series signal for the fluorescence intensity is measured, quantitative information about the particles can be obtained.

The normalized auto-correlation function, $G(\tau)$, of the fluorescence fluctuation is calculated from the fluorescence intensity $I(t)$ as

$$G(\tau) = 1 + \frac{< \delta I(t) \cdot \delta I(t+\tau) >}{< I(t) >^2} \quad (1)$$

where $< >$ is the time average of the time series signals. The autocorrelation function of the fluorescence intensity fluctuation is given by the time averaged production of fluorescence fluctuation at time t and after a delay time τ, over a large number of measurements.

The autocorrelation function for translational diffusion can be also calculated as

$$G(\tau) = 1 + \frac{1}{N}\left(\frac{1}{1+\tau/\tau_D}\right)\left(\frac{1}{1+(1/s)^2(\tau/\tau_D)}\right)^{1/2} \quad (2)$$

The autocorrelation function of the diffusion motion for a particle involves the translational diffusion time τ_D, structure parameter $s=z/w$, and the number of particles N existing at the same time in the effective focal volume [13]. The effective focal volume is represented by $V_{eff}=\pi^{3/2}w^2z^2$.

Information about the diffusion coefficient can be obtained using the autocorrelation function of the fluctuation obtained in the equilibrium state. When the particles are in solution, the diffusion coefficient for the translational diffusion, D, can be expressed using the Stokes-Einstein equation [14]

$$D = \frac{k_B T}{6\pi\eta r} \quad (3)$$

where D is diffusion coefficient, and r is the hydrodynamic radius of the particle. The hydrodynamic radius, or Stokes radius, of a solute is the radius of a hard sphere that diffuses at the same rate as that solute. k_B is Boltzmann's constant, T is the absolute temperature, and η is the viscosity of the solvent [15]. The translational diffusion time τ_D is given by the expression

$$\tau_D = \frac{< w >^2}{4D} \quad (4)$$

The parameters of N, τ_D, and w/z can be estimated using least square fitting of the experimental data to Eq.(2), and w and z can be determined by Eq. (4) through the diffusion coefficient D.

Experiment

Sample Preparation

The particles used in this experiment were fluorescent beads and quantum dots. Figure 2a, b and c show the structures and sizes of Alexa Fluor 647 (AF 647, Invitrogen, A20006), quantum dots (Q-dot 655, Invitrogen, Q21321MP), and fluorescent beads (Bead, Invitrogen, 830866), respectively.

AF-647 is a standard sample with a known diffusion coefficient and a molecular weight and size of 1,300 g/M and 0.84±0.1 nm, respectively [7]. The Q-dot with a diameter of 18±0.2 nm was composed of a CdTe core, a ZnS outer shell, and a carboxylate surface and can be used to investigate or quantify a specific protein through chemical reaction with a solution containing tissue, cells, or protein. The carboxylate groups can form covalent bonds with amine radicals, which are present in most proteins, such as immunoglobulin, bovine serum albumin (BSA), and avidin. The diameter of the beads was 24± 0.3 nm, and the fluorescent seed in the center was coated with carboxylate groups after treatment with sulfate.

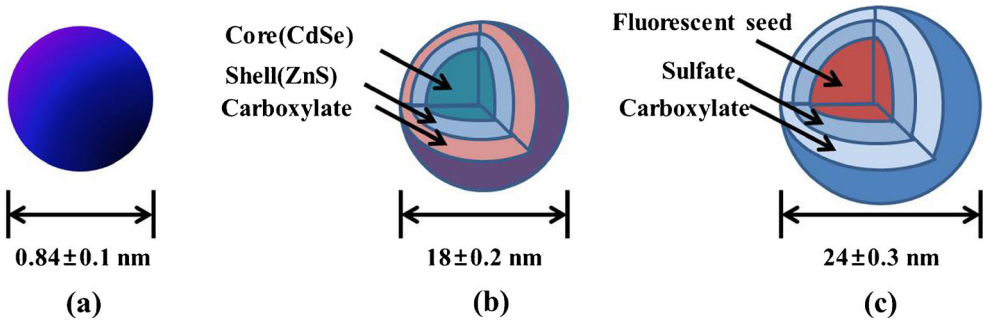

Fig. 2 Structures of the analyzed fluorescent particles: **a** Alexa Fluor 647, **b** quantum dot, and **c** fluorescent beads

A 1 μM aqueous sample solution was made from a 1/1,000 dilution of samples (solubility at 25 °C) in distilled water [7]. Sucrose aqueous solutions of different concentrations (0, 20, 30, 40, 50 wt.%) were made by mixing 999 μL sucrose solution and 1 μL of 1 μM aqueous sample solution.

Optical Setup of FCS

Figure 3 shows the schematics of the prototype FCS system. The optical system of the FCS apparatus was designed for single-photon excitation, and the auto-correlation function was calculated using software.

A He-Ne laser (Thorlab, 5 mW beam diameter of 0.8 mm) beam was expanded approximately 12 times to 9 mm by passage through a beam expander. After reflection by a dichroic mirror (SEMROCK FF545/650-Di02), the expanded beam passed through the objective lens (NA=1.2), immersion water, and cover glass (thickness=0.17 mm), in succession, and then formed an effective focal volume in the sample solution. The optical system was composed of several optical components and a commercial fluorescent microscope system (Olympus IX71) (the shaded-box in Fig. 3). A portion of the fluorescent light emitted from the particles in the focal volume passed successively through the objective lens and dichroic mirror and was focused on the core of the optical fiber (core diameter=62.5 μm) [14]. Passing through the optical fiber, the fluorescence emission was converted into a TTL signal by the photo detector of a single-photon detection module (id Quantiqe, id100-MMF).

A correlator converted the light signals, transformed from a single light quantum count module, to a correlation function using a NI PCI-6602 board and LabVIEW software. Using LabVIEW, the pulse signal, caused by light entering the detector, was detected for about 45 s. The algorithm in LabVIEW followed the multi-tau method [16–18].

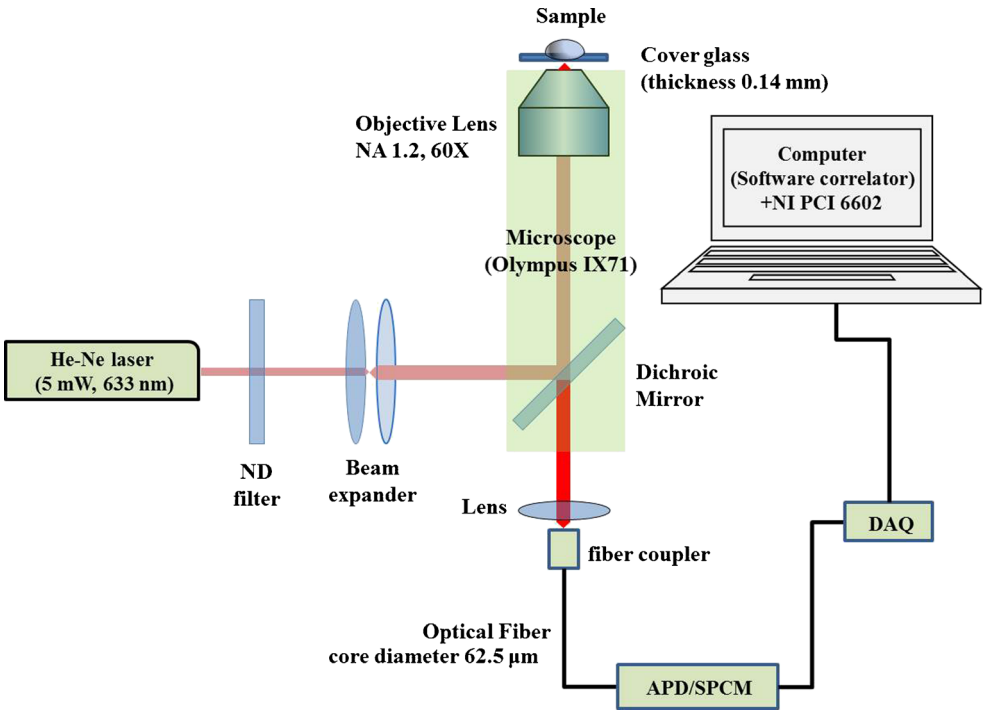

Fig. 3 Schematics of the experiment

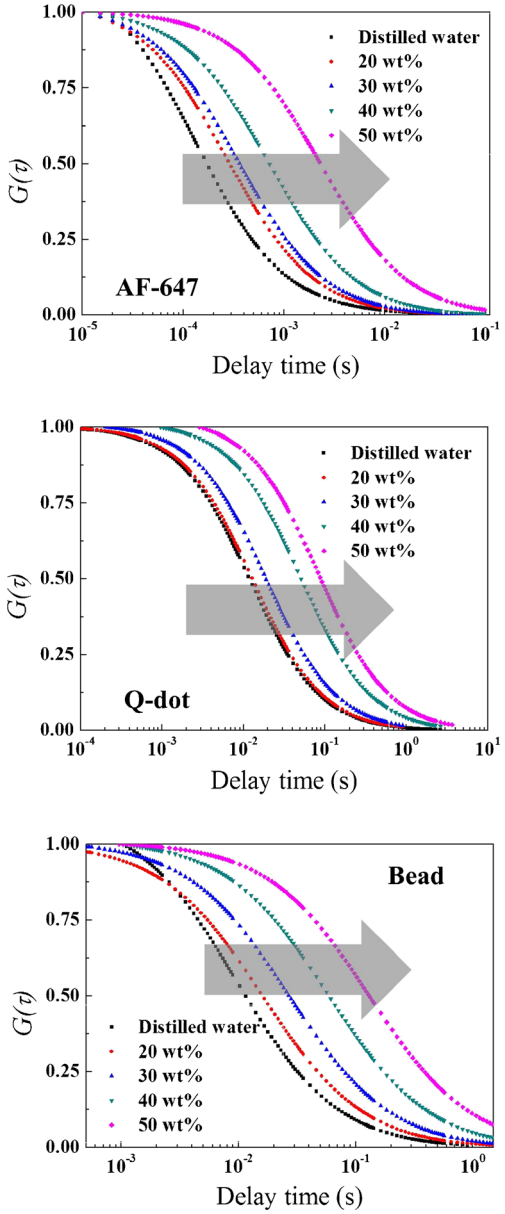

Fig. 4 Correlation functions of samples with viscosity variation: **a** Alexa Fluor 647, **b** quantum dot, and **c** fluorescent beads

Results and Discussion

Sample solutions were prepared by dissolving AF-647, Q-dot, and beads in various sucrose solvents (0 to 50 wt.%), and the solvent concentration was converted into a viscosity using data offered in Refs. [19, 20]. To ensure homogeneity of the fluorescent particles, the mixed aqueous solutions were allowed to rest for 2 hours. Viscosity indicates the thickness of a liquid, and as the viscosity of a material increases, the resistance to flow in cases. Generally, the viscosities of human blood and serum are 3–4 cP and 5.1 cP, respectively [21]. Therefore, in this study, solvents with various viscosities, including those equal to human blood and serums, were prepared. Using the known diffusion coefficient of Alexa Fluor 647 (280 $\mu m^2/s$ in distilled water) [13], the minor axis of the effective focal volume of our FCS system was calculated as approximately 380 nm. Additionally, the diffusion coefficient of spherical particles with known size was obtained by Eq. (3), and the half-width of the effective focal volume for the FCS system was obtained using the diffusion time and diffusion coefficient [1–5].

Figure 4 shows the experimentally obtained correlation functions of AF-647, Q-dot, and beads with several solvent viscosities. The FCS signal was counted for 45 s using a PCI-6602 (National Instrument) instrument, and the measurements were performed 20 times in a viscosity range of 1 cP–12 cP. To correct for solvent evaporation over time, the values on the vertical axis were normalized. In all samples, $G(\tau)$ shifted to longer delay times with increasing solvent viscosity. To determine the effective focal volume of our FCS system, the fluctuation in fluorescence intensity emitted from the AF-647 aqueous solution was measured, and the autocorrelation function was calculated using the software correlator in LabVIEW [22]. The diffusion times of AF-647, Q-dot, and beads solutions were obtained by least-square fit using Eq. (2).

Figure 5 shows the diffusion times of AF-647, Q-dot, and beads with viscosity values obtained by analyzing the correlation functions in Fig. 4. The diffusion times of fluorescence particles are proportional to solution viscosity as predicted by Eqs. (3) and (4). As shown in Fig. 5, as solvent viscosity increased from 1 cP to 12 cP, the diffusion times of AF-647, Q-dot, and beads increased from 1.3 to 2.5 ms, 11.3 to 122.9 ms, and 10.8 to 162.7 ms, respectively. Also, the slopes, τ_D/η, indicating the ratio of diffusion time to solvent viscosity for AF-647, Q-dot, and beads were 0.18, 10.03, and 14.18 ms/cP, respectively. These slopes show a linear dependence on particle size. This result suggests that larger particles are more sensitive to solvent viscosity.

Figure 6 presents the diffusion coefficients of AF-647, Q-dot, and beads with viscosities calculated from the results in

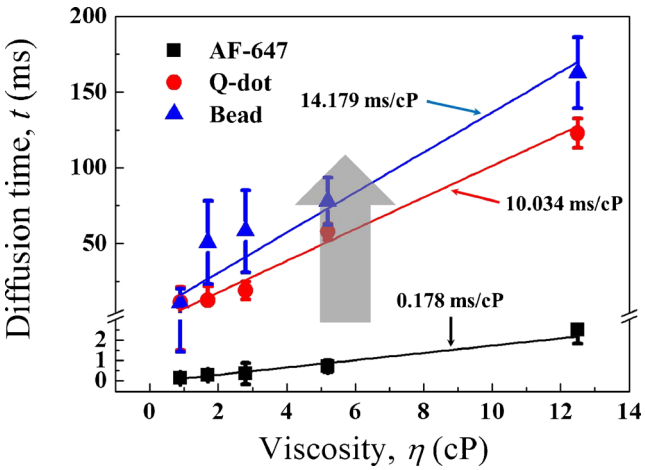

Fig. 5 Diffusion times of fluorescent particles with viscosity variation

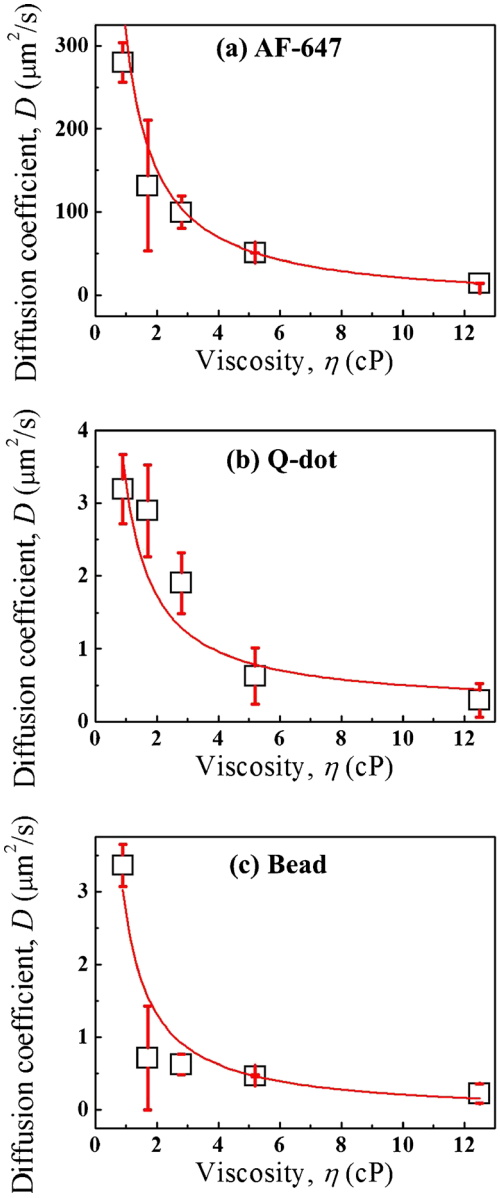

Fig. 6 Diffusion coefficients of fluorescent particles with viscosity variation: **a** Alexa Fluor 647, **b** quantum dot, and **c** fluorescent beads

Fig. 5. As shown in Fig. 6, the diffusion coefficients of the particles decrease with increasing solvent viscosity as predicted by Eq. (3). In the case of AF-647, as solvent viscosity increased from 1 cP to 12 cP, the diffusion coefficient decreased from 280 $\mu m^2/s$ to 145.2 $\mu m^2/s$, while the coefficients of Q-dot and beads decreased from 3.2 $\mu m^2/s$ to 0.3 $\mu m^2/s$ and from 3.7 $\mu m^2/s$ to 0.2 $\mu m^2/s$, respectively.

The hydrodynamic radii of AF-647, Q-dot, and beads were calculated via Eq. (3) using the results in Fig. 6, and the values were found to be 0.98±0.1 nm, 64.8±3.23 nm, and 89.8±4.91 nm, respectively. The hydrodynamic radius of particles in solution is closely related to the actual size of those particles and the solvent viscosity [23, 24]. Figure 7 shows the hydrodynamic radius of

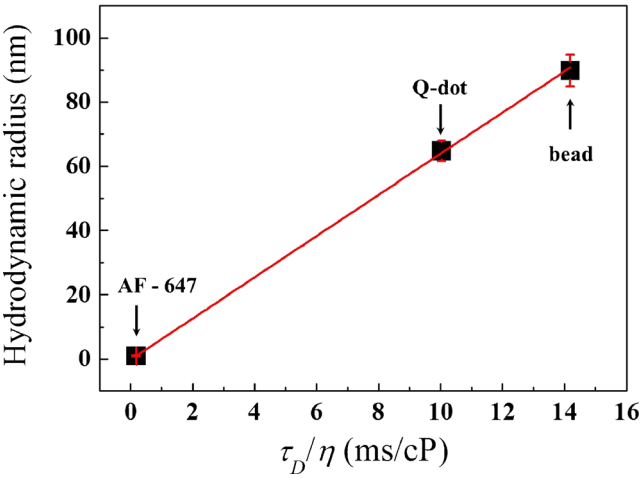

Fig. 7 Solvent viscosity and diffusion time ratio as related to particle hydrodynamic radius

a particle with the ratio of diffusion time to solvent viscosity, τ_D/η. As shown in Fig. 7, the hydrodynamic radius reveals a linear dependence on τ_D/η. This illustrates that the hydrodynamic radius of a particle depends on both particle size and solvent viscosity.

Conclusions

We investigated the dynamic properties of fluorescent particles of various sizes and in various solvent viscosities. In order to perform the study, an FCS system, based on a commercial optical microscope was developed, and the diffusion coefficients of AF-647, Q-dot, and beads in various solvents with viscosities similar to human blood were determined.

By analyzing the correlation functions of AF-647, Q-dot, and beads with solvent viscosity, the diffusion time of all particles was shown to increase with solvent viscosity. The diffusion coefficients of the particles as a function of viscosity were calculated from the diffusion time, and the diffusion coefficients decreased with increasing solvent viscosity.

The hydrodynamic radius revealed a linear dependence on τ_D/η, indicating that the hydrodynamic radius of a particle is strongly dependent on both the physical particle size and solvent viscosity. These results can be used for investigations of interactions between bio-molecules, such as protein and DNA.

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References

1. Cooper A (2004) Biophysical chemistry. Royal Society of Chemistry, Scotland
2. Kim SH, Shim T, Kim D (2010) Particles size measurement of silole nano-clusters by fluorescence correlation spectroscopy. J Korean Phys Soc 56:1264–1268
3. Schwille P, Haupts U, Maiti S, Webb WW (1999) Molecular dynamics in living cells observed by fluorescence correlation spectroscopy. Biophys J 77:2251–2265
4. Magde D, Elson E, Webb WW (1972) Thermodynamic fluctuation in a reacting system-measurement by fluorescence correlation spectroscopy. Phys Rev Lett 29:705–708
5. Ehrenberg M, Rigler R (1974) Rotational brownian motion and fluorescence intensify fluctuations. Chem Phys 4:390–401
6. Mertz J (2010) Introduction to Optical Microscopy. Robert and Company Publishers, Colorado
7. A. Einstein (1905) Über die von der molekularkinetischen Theorie der Wärme geforderte Bewegung von in ruhen- den Flüssigkeiten suspendierten Teilchen. *Ann.d.Phys.* 17, 549–560
8. Hodgdon JA, Stillinger FH (1993) Stokes-Einstein violation in glass-forming liquids. Phys Rev E 48:207–213
9. Jacobson K, Sheets ED, Simson R (1995) Revisiting the fluid mosaic model of membranes. Science 268:1441–1442
10. Sengupta D, Balaji J, Maiti S (2002) Measuring diffusion in cell membranes by fluorescence correlation spectroscopy. Meth 27:374–387
11. Krichevsky O, Bonnet G (2002) Fluorescence correlation spectroscopy: the technique and its applications. Rep Prog Phys 65:251–297
12. Renz M, Langowski J (2008) Dynamics of the CapG actin-binding protein in the cell nucleus studied by FRAP and FCS. Chromosome Res 16:424–437
13. Lide DR (1994) CRC Handbook of Chemistry and Physics. CRC Press, Cleveland
14. Pawley JB (2006) Hand book of Biological Confocal Microscopy 3rd ed. Springer, New York
15. Lakowicz JR (2006) Principles of Fluorescence Spectroscopy, 3rd edn. Springer Science + Business Media, New York
16. Magatti D, Ferri F (2001) Fast multi-tau real-time software correlator for dynamic light scattering. Appl Opt 40:4011–4021
17. Magatti D, Ferri F (2003) 25 ns software correlator for photon and fluorescence correlation spectroscopy. Rev Sci Instrum 74:1135–1144
18. Culbertson MJ, Burden DL (2007) A distributed algorithm for multi-tau autocorrelation. Rev Sci Instrum 78:044102
19. Bourne M (2002) Food texture and viscosity :concept and measurement. Academic, California
20. Mathlouthi M, Reiser P (1995) Sucrose properties and applications. BLACKIE A&P, London
21. Sakai H, Takeoka S, Sungick P, Kose T, Nishide H, Izumi Y, Yoshizu A, Kobayashi K, Tsuchida E (1997) Subscutaneous microvascular responses to hemodilution with a red cell substitute consisting of polyethyleneglycol-modified cesicles encapsulating hemoglobin. Bioconjug Chem 40:66–78
22. Rigler R, Mets U, Widengren J, Kask P (1993) Fluorescence correlation spectroscopy with high count rate and low background: analysis of translation diffusion. Eur Biophys J 22:169–175
23. Schmidt M, Burchard W (1981) Translational diffusion and hydrodynamic radius of unperturbed flexible chains. Macromolecules 1: 210–211
24. Daly E, Saunders BR (2000) Temperature-dependent electrophoretic mobility and hydrodynamic radius measurements of poly(N-isopropylacrylamide) microgel particles: structural insights. Chem Phys 2:3187–3193