Temperature dependent FCS studies of supercooled liquids viscosity and particle size

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Abstract

Fluorescence correlation spectroscopy (FCS) in combination with the Stokes-Einstein relation (SE) can be used to obtain the values of the microscopic viscosity (nanoviscosity) of a fluid in a broad temperature range for a small sample volume of about 50 μl. The use of dry, long working distance objective was essential for avoiding temperature gradients in the sample. The nanoviscosity is equal to the macroscopic viscosity obtained from rheometric experiments when no characteristic length scale larger than the size of the fluorescent probe exists in the sample. In this work we describe experimental setups for FCS measurements in a broad temperature range. We show for selected molecular and polymeric supercooled liquids that despite the fact that in these systems a characteristic length scale can be defined, the nanoviscosity obtained from FCS is in a very good agreement with the macroscopic (rheometric) viscosity of the sample. This result corroborates the applicability of the SE relation to supercooled liquids at temperatures above 1.2 T_g. We also show that the temperature dependent size of thermoresponsive microgel particles can be determined by FCS using the designed cells and a long working distance objective.

Introduction

Optical scanning confocal microscopy in combination with Fluorescence Correlation Spectroscopy (FCS) is frequently used for studies of location and dynamics of fluorescently labeled particles on a single molecule level both *in vitro* and *in vivo*. Every year many papers are published on studies of complex systems using this method [1]. The majority of work is performed at ambient temperature using water or oil immersion objectives. In very few applications [2-5] temperature is changed in a narrow range around the ambient temperature. In principle, this should be done by placing the microscope in a box where the temperature is controlled in a limited range. This solution would allow to minimize the temperature gradients when using the water/oil immersion objectives but simultaneously would substantially limit the available temperature range. Instead, in all cases small temperature stages adapted for use with immersion objectives were used.

Another possibility to perform temperature dependent FCS (and confocal microscopy) studies is to use long working distance (LD) objectives without immersion liquid. The feasibility of using such objectives for FCS experiment was critically tested in our previous publication [6]. In this optical configuration we encounter additional problems: (i) LD objectives have much lower numerical aperture, usually NA<0.6 which results in a larger confocal volume leading to lower resolution, lower fluorescence intensity and larger diffusion times, where then bleaching can be substantial. (ii) For practical reasons one has to use a thicker window what introduces additional spherical aberration. (iii) The refractive index of the solvent (usually water) depends on temperature. The spherical aberration resulting from problems (ii) and (iii) can be compensated in a well defined range only if the LD objectives are equipped with the correction collar. These issues were extensively studied earlier [6].

Temperature dependent FCS studies which allow to measure the temperature dependence of the self- (tracer-) diffusion coefficient and the reaction rate are important for several reasons. In the case when the substrates of a chemical reaction exhibit fluorescent properties different from its products, FCS can be used to determine the chemical reaction rate. Therefore, the effect of temperature on the biochemical reactions rate can be studied, both *in vitro* and in living cells. The FCS can also be used as a tool for measurements of the size of colloidal and polymeric particles as a function of temperature using the Stokes-Einstein (SE) relation:

$$D = \frac{k_B T}{6\pi\eta R_h} \tag{1}$$

where R_h is the hydrodynamic radius of the fluorescent probe, η - the viscosity, k_B – the Boltzmann constant and T – absolute temperature. Potential materials for such studies are thermo-responsive microgel suspensions or proteins in the studies of temperature denaturation.

However, the most interesting environment to be studied by FCS are crowded and complex systems. It was shown that for colloidal systems the SE relation holds not only at infinite dilution but also at high volume fractions of the solute [7], if the long-time self diffusion coefficient, i. e. the quantity which is measured by FCS, is considered. More complex are systems with internal structure (dynamic network) formed by asymmetric (usually rod-like) particles. Results obtained in such systems probed with tracers of different size were interpreted in terms of the ratio of the probe size to the mesh size either directly through theoretical calculations of diffusion coefficient in such environment [8-10] or through the concept of nanoviscosity [11-13]. Namely, nanoviscosity (η_{FCS}) is the value of η calculated from the FCS experiment using the SE relation (Eq. 1) and represents the effective viscous drag exerted on the probe of this size in that particular environment. It was shown that for probe sizes smaller than the characteristic length scale of the internal structure η_{FCS} is systematically lower than macroscopic viscosity η_0 measured with a rheometer and that both values coincide when the probe size exceeds this length scale.

Supercooled liquids do not behave like simple liquids in terms of dynamical properties. Their dynamics is heterogeneous [14-17], related time correlation functions have a stretched exponential Kohlrausch-Williams-Watts form and their viscosity is changing by about 13 orders of magnitude in a narrow temperature range between T_g and T_m. A characteristic correlation range is discussed in relation to the dynamic heterogeneity [14-17]. In the temperature range from T_g to 1.2 T_g translational diffusion in these liquids strongly decouples from their macroscopic viscosity [18-22]. Also structural heterogeneities (clusters) were reported in supercooled liquids with a characteristic correlation length reaching 100 nm [23-28].

Important question arises related to all these features: Do the supercooled liquids behave like simple or like complex liquids in terms of viscosity? Is the nano-viscosity measured locally by means of FCS equal to the macroscopic (rheological) viscosity like in simple liquids, or does it depend on the a characteristic length scale of the system and the size of the fluorescent probe, like in complex liquids?

In order to answer these questions we measured the nano-viscosity η_{FCS} by means of FCS and compared it to the macroscopic viscosity obtained from rheological measurements for one polymeric and two molecular supercooled liquids. Since the η_{FCS} was obtained from FCS self diffusion coefficients using the Stokes-Einstein (SE) relation, we have limited our studies to temperatures above 1.2 T_g, where the SE relation was shown to hold.

In FCS diffusion time τ of the dye or fluorescently labeled probe through the confocal volume of the microscope is measured and can be transformed into viscosity data using SE relation. For smallest available dye molecules the accessible to FCS range of diffusion times (~20 μs to 100 s) corresponds to viscosity values from ~1 cP to ~10^7 cP. This range can be further extended by several orders of magnitude towards higher viscosities (lower diffusion coefficients) when the method of single particle tracking (SPT) is used [29].

In this work first a home-made FCS cell in combination with a LD objective for use in the temperature range of 10 – 100°C is described. It was demonstrated that spherical aberration due to changing refractive index of the medium can be compensated by means of the correction collar [6]. The first temperature dependent measurement was performed using the home-made cell on a suspension of thermoresponsive microgel particles. The same setup was used for studies of viscosity of two glass forming liquids in the temperature range from room temperature to ~80°C, finding a good agreement with the available literature data.

Extension of the temperature range required a new design of the cell. Modified Linkam THMS600 temperature stage (with a temperature range from -196 to +200°C) was used to measure the viscosity of ortho-terphenyl (OTP) doped with N,N′-Bis(2,5-di-tert-butylphenyl)-3,4,9,10-perylenedicarboximide in the temperature range from 15 to 200°C covering four orders of magnitude of viscosity in a single run and using only 50 μl of the sample. No other single technique could provide such data. Moreover, for the first time OTP viscosity was measured in the temperature range 145 - 195°C.

The results obtained in this study proved the applicability of FCS to measurements of the temperature dependence of either particle size or medium macroscopic viscosity. Small sample quantity and a broad range of measured viscosity make it a very promising tool for measuring viscosity of many extremely viscous materials.

2. Materials and methods

2.1 Materials

The copolymer poly(ethylene-co-propylene) of molecular weight of 6000 Da (PEP6) was kindly provided by Dr. Lutz Willner and Dr. Jürgen Allgaier and was synthesized according to a procedure described elsewhere [30]. The glass transition temperature of PEP6 amounted to T_g = –62°C. The fluorescent dye Rubrene (5,6,11,12-tetraphenylnaphthacene) was purchased from Sigma Aldrich. This dye in cyclohexane at room temperature has a strong absorption in the range of 480-500 nm and fluorescence in the range of 500-620 nm [31].

A homogeneous 1 nM solution of Rubrene in PEP6 was prepared by dissolving first the appropriate amounts of the copolymer and dye in toluene and evaporating toluene from the mixture in a vacuum oven.

Ortho-terphenyl (OTP) from Aldrich was distilled under vacuum.

N,N′-Bis(2,5-di-tert-butylphenyl)-3,4,9,10-perylenedicarboximide was purchased from Sigma.

The dye was added to OTP in the following way: a stock solution of the dye in toluene was prepared at about 1 mM concentration. Then the target dye solution in toluene was prepared reaching ~10 nM concentration in two steps. To transfer the dye to the 30 μl sample of OTP, 30 μl of the toluene solution was mixed with the OTP sample directly in the measuring cell (4 mm inner diameter). Vigorous mechanical mixing at 60°C for about 1 hour provided good homogenization of the sample. The measuring cell was left under vacuum at 60°C for 2 days to remove toluene.

BMPC (1,1'-di(p-methoxyphenyl)cyclohexane) was synthesized according to procedure described in [32].

Alexa488 was obtained as a “waste” after reaction of staining a protein sample with Alexa Fluor® 488 NHS ester.

The 70/30 (by weight) OTP/BMPC mixture containing about 1nM of Alexa488 was prepared by dissolving proper amounts of the components in toluene and then removing toluene by evaporation in a vacuum oven for several days.

The sample of PNIPAM – acrylic acid co-polymer (PNIPAM-co-AAc) microgels was synthesized and kindly provided by dr. Dzina Kleshchanok (at that time from RWTH Aachen).

2.2 Methods

The home made temperature FCS cell

The home made temperature FCS cell was built at the Forschungszentrum Jülich. The schematic diagram of the cell is shown in Fig.1. The temperature was maintained by a Lakeshore controller connected to the heating ring and a temperature sensor. At lower temperatures water cooling was provided. The cell was fixed to a 160 × 110 mm adapter fitting to most of microscope tables.

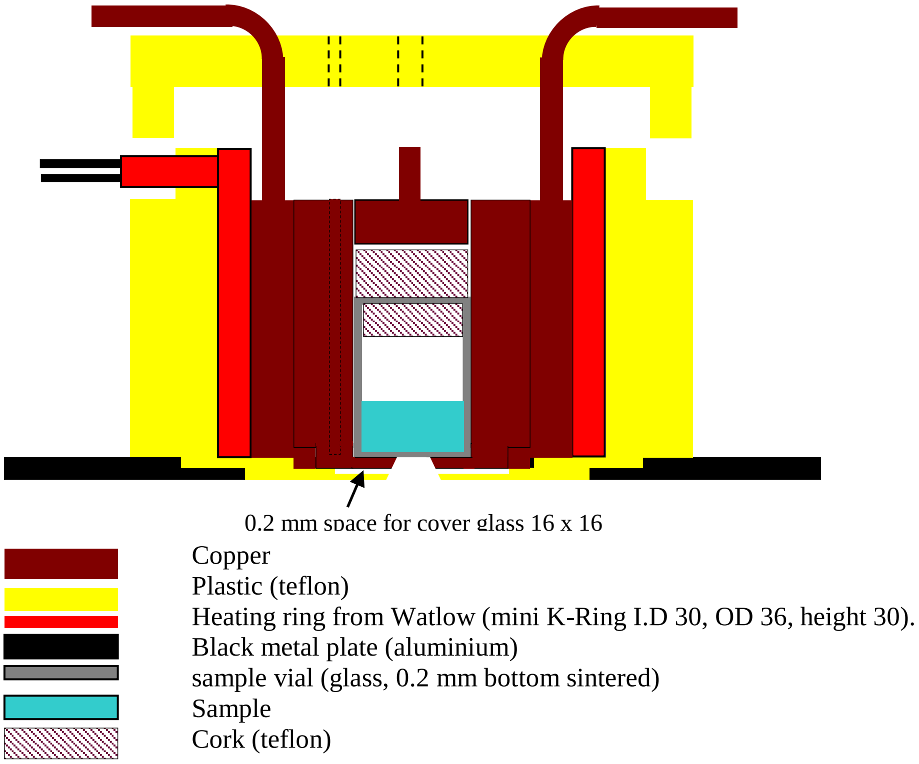

Figure 1: Schematic diagram of the high temperature FCS cell.

In the case when immersion objective is to be used, the bottom glass window and the Teflon base should be removed and a different adapter for the cell should be used. This would allow for lowering the cell all the way down, enabling the immersion objective to access the bottom of the cell.

The customized Linkam THMS600 temperature stage

A customized cell based on the standard Linkam THMS600 temperature stage was designed to fit the inverted microscope geometry and to accommodate a small cylindrical glass container for the sample. The heating block was inverted and lowered towards the bottom window such that LD objectives with working distance of 3-4 mm could reach the sample container placed in a cylindrical 6.2 mm diameter hole in the heating block. In order to minimize the optical distortions due to the presence of the bottom window of the stage, it was replaced with a cover glass placed ~0.5 mm from the heating block. Standard Linkam temperature controller and nitrogen pump were used to stabilize the temperature in the cell. The temperature range is -196 to +200°C.

Viscosity measurements

Viscosity measurements of the PEP6 polymer doped with 1 nM of the fluorescent dye rubrene were performed using the commercial rheometer Physica MCR 501 (Anton Paar) in the temperature range of 0-70°C in steps of 5°C at shear rates of 0.01-1s^{-1}, at 3 points per decade [33]. Viscosities of OTP/BMPC mixtures were measured using Contraves LS40 rheometer. The low shear rate range was chosen in order to obtain the zero shear viscosity, defined as:

$$\eta_0 = \lim_{\omega \to 0} \frac{G''}{\omega}. \tag{2}$$

where G'' is the dynamic loss modulus and ω the angular frequency.

The temperature scan was done both ways: by warming up and cooling down the sample. Before starting a new measurement the sample was equilibrated for 10 minutes at the new temperature.

FCS measurements

The FCS measurements were performed using a ConfoCor 2 instrument (Zeiss) with a long working distance objective (Olympus LUCPLFLN 40×/0.6, working distance 3-4.2 mm, equipped with a correction collar for compensation of the window thickness in the range of 0-2.0 mm). The measured samples were contained either in the home-built temperature cell, described above, or in the customized Linkam stage. In both cases the temperature was stabilized within ±0.05°C or better.

The FCS correlation functions (CFs) were analyzed using the usual analytical form which was derived [34] under the assumption that the confocal volume has a Gaussian shape of dimensions $\sigma_x = \sigma_y = \sigma$ and σ_z and $SP = \sigma_z / \sigma$:

$$G(t) - 1 = \frac{1}{N}\left(1 + t/\tau\right)^{-1}\left(1 + SP^{-2}t/\tau\right)^{-0.5}\left(1 + \frac{T_t}{1 - T_t}e^{-t/\tau_t}\right), \tag{3}$$

where [6]

$$\sigma^2 = D\tau, \tag{4}$$

D is the particle translational self diffusion coefficient, T_t and τ_t are the amplitude and characteristic decay time of the triplet contribution and N is the average number of particles in the confocal volume.

During each measurement series the position of the confocal volume was maintained at 50 μm above the bottom. After each temperature change the correction ring was adjusted to reach the maximum amplitude of the correlation function [6].

Absolute viscosity measurements by FCS

To estimate the viscosity η of an unknown solvent from FCS measurements, the diffusion time τ_{ref} of the same fluorescent probe must be measured in a reference solvent and in the studied solvent. As the solvents will usually have different refractive indices, a careful correction collar adjustment is required. In our case the compensation range of the correction collar of the Olympus LD objective was sufficient for any combination of solvents, however if an immersion objective is to be used, its range of compensation might often be too narrow for a pair of solvents with distinctly different refractive indices.

Combining the SE (Eq. 1) with Eq. 4, one arrives at

$$\eta = \frac{\tau k_B T}{6\pi R_h \sigma^2}\,. \tag{5}$$

As for a given probe R_h = const and also the σ value is maintained constant by adjusting the correction collar, the solvent viscosity can be calculated as

$$\eta(T) = \eta_{ref}(T_{ref})\frac{\tau T}{\tau_{ref} T_{ref}}\,, \tag{6}$$

where T_{ref} is the temperature at which the reference measurement was carried out and η_{ref} is the viscosity of the reference solvent at this temperature. In the following, η in Eq. 6 will be referred to as η_{FCS}.

In principle, if at certain conditions (certain temperature) the probe is known to fulfill the SE relation in the studied solvent, an FCS measurement performed in those conditions can also play the role of a reference point.

3. Results and discussion

3.1. PCS / FCS study of PNIPAM copolymer

The choice of PNIPAM – acrylic acid copolymer as a material for microgel particles allowed for a better control of their thermoresponsive properties. The dissociable groups made the system sensitive to the pH value of the solution and substantially reduced the response time. At the temperature of 32°C the microgel particles underwent a transition from the swollen state to the collapsed state. The study was performed at pH = 4, where the effect was most pronounced. We have applied the standard photon correlation spectroscopy (PCS) method to measure the T-variation of R_h using a conventional ALV PCS setup. Almost twofold reduction of R_h was found between the room temperature and the temperature of 40°C. The value of $R_{40^\circ C}$ obtained from PCS amounted to 56.5 nm. Likewise, FCS correlation functions were measured in the temperature range of 22-40°C and fitted with Eq. 3. For comparison with the hydrodynamic radii obtained from PCS, the ratios of the hydrodynamic radius at given temperature to the radius at 40°C were calculated according to:

$$\frac{R_{FCS}(T)}{R_{FCS}(40^\circ\,\mathrm{C})} = \frac{\tau_{FCS}(T)}{\tau_{FCS}(40^\circ\,\mathrm{C})}\frac{T}{(273+40)}\frac{\eta(40^\circ\,\mathrm{C})}{\eta(T)} \tag{7}$$

Here the T-variation of the water viscosity was taken into account. The obtained from FCS temperature dependent hydrodynamic radius is plotted in Fig. 2 together with the temperature variation of R_h, as obtained by light scattering. Obviously the two methods give exactly the same information regarding the size variation of PNIPAM-co-AAc microgels with varying temperature.

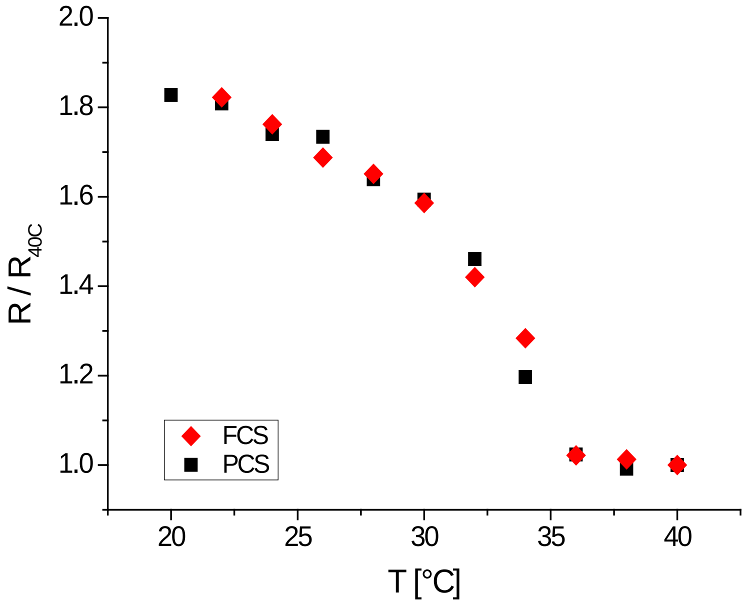

Fig. 2 Comparison of the temperature variation of the normalized hydrodynamic radii of a PNIPAM-co-AAc microgels at pH 4.0 obtained using the FCS and PCS techniques at the concentration of 0.03 mg/ml.

3.2 PEP6 doped with Rubrene

The FCS correlation functions were measured for Rubrene in PEP6 in the temperature range of 25 – 95°C. The analytical correlation function (Eq. 3) with a single diffusion process and the triplet contribution fitted well the shape of the experimental CFs. With decreasing temperature the diffusion time becomes longer due to the increase of viscosity of the polymer. From the FCS correlation time τ nanoviscosity η_{FCS} defined in Eq. 5 was calculated from Eq. 6.

The values of η_{FCS} together with the macroscopic viscosities η_0 measured in a rheological experiment versus temperature are plotted in Fig. 3. As it can be seen, the agreement is very good. This means that the slowing down of the rubrene motion is solely due to the increase of the polymer viscosity while lowering temperature. Thus, η_{FCS} can be used as a measure of the macroscopic viscosity.

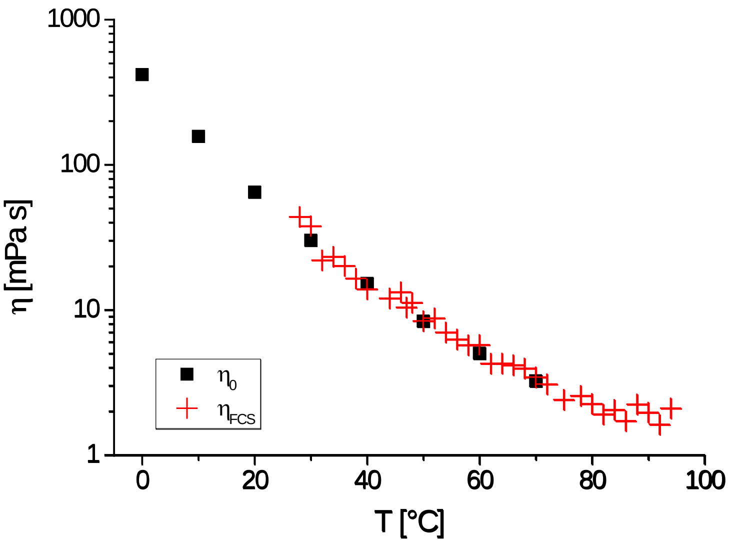

Fig. 3. Comparison of η_{FCS} (red crosses) with macroscopic viscosity η_0 (black squares) of PEP6 measured in the temperature range of 25 – 95°C and 0 – 70°C, respectively.

3.3. OTP/BMPC mixture doped with Alexa488

Another glass forming system for which the viscosity η_{FCS} was measured by means of FCS was a mixture of two glass forming liquids OTP/BMPC doped with Alexa488. In this mixture the crystallization is completely suppressed. In Fig. 4 we show the experimental CFs of the mixture as a function of temperature in the range of 20-55°C. All CFs were fitted with Eq. 3 and as one can see, the fits are reasonably good over the whole temperature range.

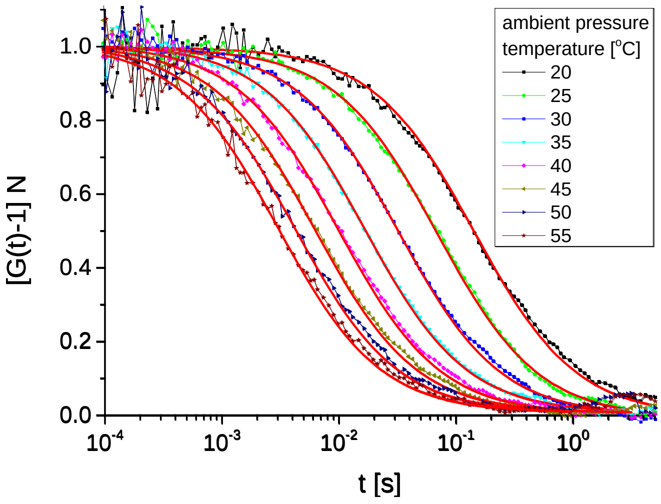

Fig. 4. Normalized FCS autocorrelation functions obtained from measurements done at temperatures from 20 to 55°C for Alexa488 in BMPC-OTP mixtures. Red lines represent the fits of Eq. 3 to the data.

The values of η_{FCS} obtained from the FCS correlation times using Eq. 6 are plotted versus temperature in Fig. 5 and compared to the macroscopic viscosity η_0 obtained from rheological measurements.

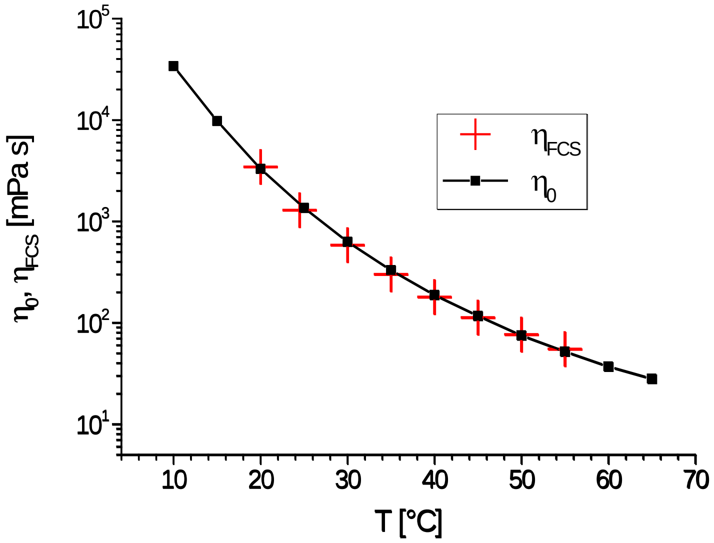

Fig. 5: Comparison of η_{FCS} (red crosses) with macroscopic viscosity η_0 (black squares) of the OTP/BMPC mixture measured in the temperature range 20–55°C and 10–65°C, respectively.

As in the case of PEP-6 doped with rubrene, cf. the previous section, also here we found a good agreement between the temperature variation of η_{FCS} and the macroscopic viscosity η_0.

3.4. OTP doped with N,N′-Bis(2,5-di-tert-butylphenyl)-3,4,9,10-perylenedicarboximide

The success of the home-made temperature cell was a motivation for designing a new stage with extended temperature range. The viscosity of pure OTP in a broad temperature range of 15 – 200 °C was measured using FCS in the customized Linkam THMS600 temperature stage. The results obtained for this sample are especially interesting because there is no viscosity data for OTP in the temperature range between 145 and 195°C. Low temperature data was obtained by Cukierman [35], whereas high temperature data was provided by McLaughlin [36]. Using FCS to measure η_{FCS} we were able to bridge this gap. The data is shown in Fig. 6.

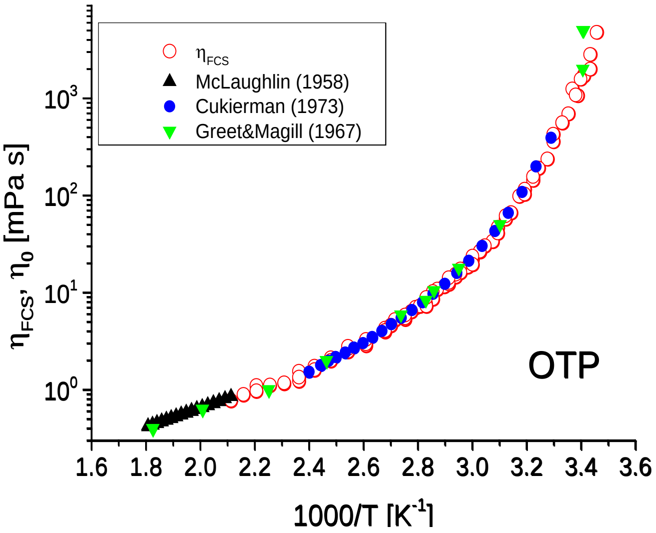

Fig. 6. Plot of the viscosity of OTP vs. the inverse temperature. Shown is the high temperature data by McLaughlin [36], ranging from about 195°C to about 280°C, the mid *T* data from Cukierman [35], ranging from 145°C to 30°C. Own FCS data as red empty circles.

The lower temperature limit of 15°C was set by the condition $T > 1.2\ T_g$ in order to avoid the temperature range where the translational diffusion might decouple from the viscosity of the system (see Introduction). The data in Fig. 6 clearly show an excellent agreement of our FCS results with the viscosity data reported in literature.

A small extension of our data towards lower temperatures is possible by means of FCS (down to ~0°C). Further supercooling would drastically increase the tracer's diffusion time making acquisition of correlation function too long to be practical. In this situation another technique could be adopted, namely single particle tracking (SPT). Provided that localization of fluorescent tracer is possible with an accuracy of 10-20 nm [37], several more orders of magnitude in viscosity could be measured.

Conclusions

Two different temperature cells were designed to conduct FCS measurements using the LD objective. It was found that such measurements were possible in a broad range of temperatures and relatively easy to carry out, although they require careful adjustment of the optics and optimum temperature insulation. The translational self-diffusion coefficients of fluorescent probes measured in these experiments were used to calculate the nanoviscosity of three studied samples of supercooled liquids. In all cases the values of nanoviscosity η_{FCS} were found to be in a very good agreement with macroscopic viscosity η_0 obtained from rheologic measurements, even though the probe size was of the order of 1 nm. As in our study $T > 1.2\ T_g$, we proved that nanoviscosity obtained from FCS measurements is compatible with the rheological literature data in this temperature range. Moreover, for the first time experimental OTP viscosity data were obtained in the range 145 – 195 °C. Good sensitivity of FCS and observed perfect agreement of η_{FCS} with η_0 above 1.2 T_g make it a promising tool for studying the temperature-dependent nanoviscosity of supercooled liquids.

The design of both temperature cells proved to be good and their performance with the LD objective was very good in the range form +15 to +200°. The Linkam customized stage potentially can be used down to -196°C with appropriate samples. A temperature dependence of the hydrodynamic radius of thermoresponsive PNIPAM based microgels was measured, showing a clear transition from the collapsed state to the swollen state at about 32°C, in a good agreement with PCS data.

Acknowledgments

A.P. J.G. and A.P. acknowledge partial financial support by the grant no. 2013/09/B/ST3/01678 of the Polish National Science Centre. Dr. Dzina Kleshchanok is gratefully acknowledged for providing the PNIPAM-based microgel samples.

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