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Fluorescence-based Broad Dynamic Range Viscosity Probes

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Abstract We introduce two new fluorescent viscosity probes, SYBR Green (SG) and PicoGreen (PG), that we have studied over a broad range of viscosity and in collagen solutions. In water, both dyes have low quantum yields and excited state lifetimes, while in viscous solvents or in complex with DNA both parameters dramatically (300–1000-fold) increase. We show that in log-log scale the dependence of the dyes' quantum yield vs. viscosity is linear, the slope of which is sensitive to temperature. Application of SG and PG, as a fluorescence-based broad dynamic range viscosity probes, to the life sciences is discussed.

Keywords SYBR Green I · PicoGreen · Fluorescent viscosity probe · Intramolecular quenching · Molecular rotors · Collagen sensing · Fluorescence probes

Abbreviations

SG	SYBR Green I®
PG	PicoGreen®
VG-probe	SG/PG viscosity probe

Introduction

Fluorescence approaches, based on molecular probes that are specifically designed for sensing the physicochemical characteristics of different bio-medical systems (e.g. pH, temperature, ionic strength, selected ions etc [1]) are in high demand. One of the important parameters of bio-fluids is viscosity. Viscosity is a thermodynamic characteristic of an ensemble of molecules, which depends, in particular, on the concentration of molecules, their ability to interact with each other and the solvent, and the state of molecular polymerization, i.e. fibrilization. While in general the viscosity is a macroscopic characteristic of a solution, it can be also applied to characterize a microsystem, using for this purpose small molecular probes, the inner structural dynamics of which is sensitive to the micro-viscosity, [2, 3].

The importance of viscosity registration is linked to the diagnostic of various diseases. On a macroscopic level, for example, the change in viscosity of blood plasma as well as in lymphatic fluid may be an indicator of diseases such as inflammation, cardiovascular and cerebrovascular disorders and others [4, 5]. At a microscopic state, the change in micro-viscosity can be an indication of malfunction at the cellular level, connected with disorder in diffusion processing, i.e. changes in the rate of bio-chemical reactions, transporting of molecules and proteins inside cells and the structural state of cellular membranes.

Traditional mechanical viscometers are not able to measure viscosity of microsystems and, more important, cannot measure fluid viscosity in real-time conditions. At the present time fluorescent molecular probes are most suitable tools for real-time measurements of viscosity in both micro- and macro-systems [2, 3]. There are many fluorophores, the quantum yield of which is sensitive to viscosity, but only a limited number of chromophores can actually be utilized as viscosity probes. Suitable fluorophores for sensing fluid viscosity are fluorescent molecules that exhibit inner rotation (dynamic structural isomorphism) and intensive quenching at certain conformeric states, usually based on excited state charge transfer. Consequently, in a highly viscous environment the inner dynamics of a molecule are sufficiently damped, which in turn decreases the rate of monomolecular quenching, and

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subsequently increases its quantum yield. Representatives of this subclass of fluorescent viscosity probes are known as molecular rotors [1, 6–10]. Molecular rotors belong to a group of fluorophores that form twisted intramolecular charge transfer complexes (TICT) [1].

In this paper we report the results of our study of the PicoGreen (PG) and SYBR Green I (SG) fluorophores (Fig. 1) sensitivity to viscosity. These fluorophores are well known as DNA staining dyes, employed in DNA gel visualization and in PCR [11, 12]. Recently, we have investigated the energetics and their interaction with double stranded DNA and photophysical properties of these fluorophores in the free state and in complex with DNA [13–15]. A remarkable feature of this type of fluorophore is their ability to dramatically (≈1, 000-fold) increase both the fluorescence yield and excited state lifetime upon transition from a free state (bulk water environment) to the rigid bound state, demonstrating dynamical structural isomorphism based quenching. Another interesting detail is that currently there is no data regarding charge transfer for this particular class of molecules. The quenching effect is supposedly caused by high frequency flexibility, stretching in the thiazol-quinolinium coupled system [13, 15]. Based on the results of this study, we introduce a new subclass of fluorescence-based *broad dynamic range* viscosity probes.

Materials and Methods

The PicoGreen (PG) and SYBR Green (SG) dyes were purchased from Invitrogen. The concentrations of PG and SG in solution were determined from optical absorption spectra using the following extinction coefficients: 75,000 M^{-1} cm^{-1} and 70,000 M^{-1} cm^{-1}, respectively [13, 16, 17]. The structure of PicoGreen and SYBR Green dyes is shown in Fig. 1. The IUPAC (International Union of Pure and Applied Chemistry) name of PG is (2-(n-bis-(3-dimethylaminopropyl)-amino)-4-(2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene)-1-phenyl-quinolinium), the molecular mass= 552.5 g·mol^{-1} and SG is N',N'-dimethyl-N-[4-[(E)-(3-methyl-1,3-benzothiazol-2-ylidene)methyl]-1-phenylquinolin-1-ium-2-yl]-N-propylpropane-1,3-diamine. The mass of SG is 509.73 g·mol^{-1}.

Glycerol (anhydrous "super glycerol"), ethylene glycol and collagen, type I solution from rat tail were purchased from Sigma-Aldrich, USA. The concentration of a stock solution of collagen was ~4 mg/ml. Purity of collagen is >95 %, SDS-PAGE shows two doublets at apparent molecular weights of 115 and 130 kDa, and at 215 and 235 kDa, which is the typical band pattern for type I collagen. In this study we used serial dilutions of collagen (from 2.2 mg/ml to 0.06 mg/ml) using 20 mM Na-acetate buffer, pH 3 as a solvent.

Fluorescence spectra of the dyes were measured using the Fluoromax-4 fluorometer (Horiba, USA), equipped with the software controlled Peltier temperature controlling system, F-3004. A 1×1 cm path-length Suprasil quartz cuvette (Hellma, Plainview, NY) was used for fluorescent measurements. The PG and SG dyes were excited at 485 nm and the fluorescence monitored over the wavelength range 490 to 800 nm. In all experiments, the concentration of the dyes has been kept constant at 1 μM.

In this study we have used mixtures of ethylene glycol (low viscosity solvent) and anhydrous glycerol (high viscosity solvent) to change the viscosity of the dye's solution in a broad range without considerable change in the solvent polarity (the dielectric constant). At 20 °C ethylene glycol (polar protic solvent) has dielectric constant (ε) of 37.7, viscosity (η) – 13.5 cP, glycerol (polar protic solvent) has ε =42.5 and η=945 cP. Viscosity for the mixtures were calculated using the following equation [18]:

$$\ln(\eta_{mix}) = \sum_i w_i \times \ln(\eta_i), \quad (1)$$

where η_{mix} is the viscosity of the mixture, η_i is the viscosity of each component, and w_i is the weighting factor ($0 < w < 1$) of each component.

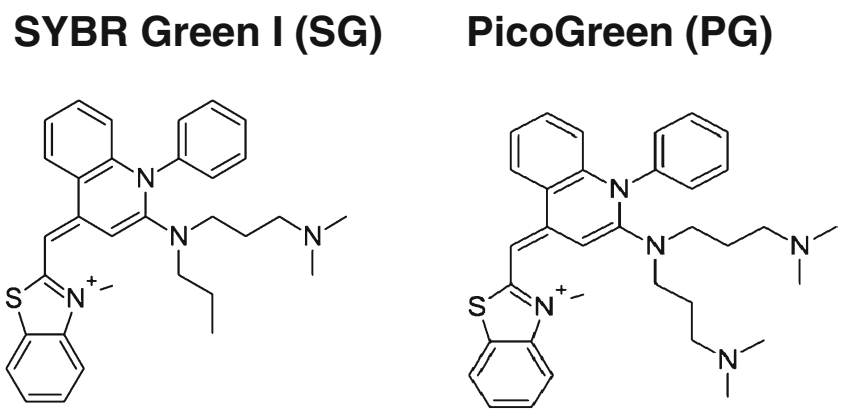

Fig. 1 Molecular structure of SYBR Green I and PicoGreen

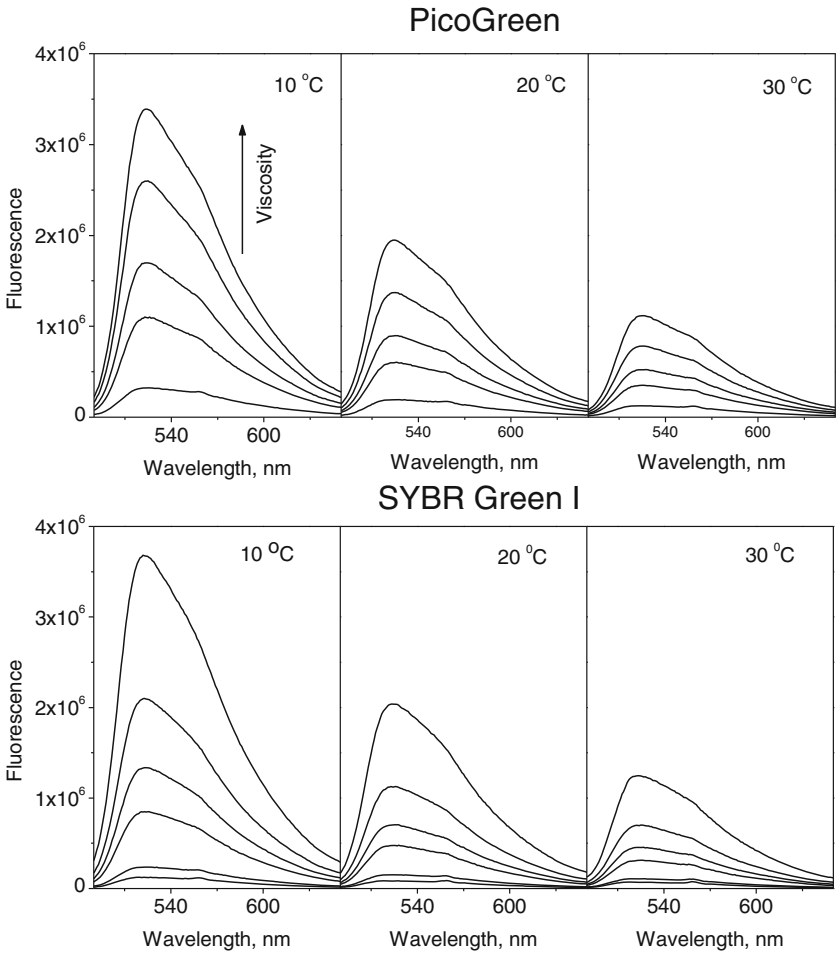

Fig. 2 Fluorescence spectra of PicoGreen (PG) and SYBR Green I (SG) in solutions of different viscosity (Ethylene glycol/Glycerol mixtures) and at different temperatures (10, 20 and 30 °C). Concentration of the dyes was 1 μM. Excitation was at 473 nm

Results and Discussion

Characterization of PG and SG Fluorophores Sensitivity to Viscosity

Figure 2 shows the fluorescence spectra of PG and SG that have been recorded at different solvent viscosities and temperatures, from 10 to 30 °C. An increase in viscosity leads to a large increase in fluorescence intensity of the dyes (Fig. 2) and, consequently, demonstrates their high propensity for viscosity sensing.

The change in fluorescence intensity is almost the same for both PG and SG, and in the studied range of solvent viscosity (from 13 to 900 cP), at 20 °C, can be fitted with the linear function (Fig. 3 left). The chemical structure of the PG and SG dyes is similar except for the dimethylaminopropyl groups (Fig. 1). These groups are far away from the dye fluorescent core and thus are thought to have a relatively small influence on chromophore electronic states. It should be noted that the fluorescence intensity (F) and quantum yield (Q_o) of the dyes is proportional to the excited state lifetime (τ) of the chromophore, i.e. $F \propto Q_o \propto \tau$, in a broad range of solvent conditions

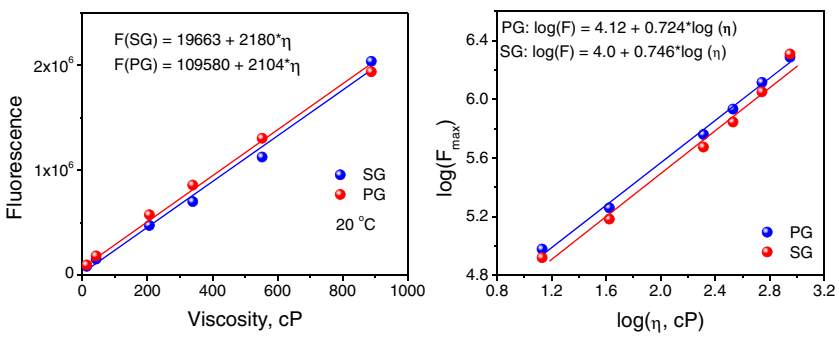

Fig. 3 (*Left*) The dependence of fluorescence intensity of the PG and SG dyes upon viscosity of ethylene glycol/glycerol mixtures at 20 °C. (*Right*) figures shows the data in log–log scale

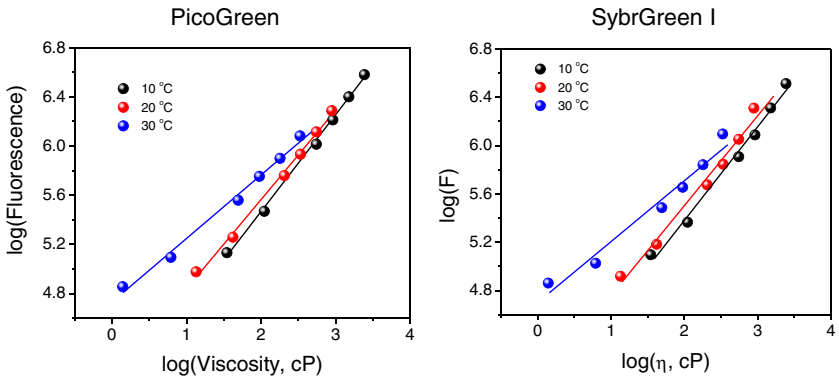

Fig. 4 The dependence of fluorescence intensity of the PG (*left*) and SG (*right*) dyes upon viscosity of ethylene glycol/glycerol mixtures at three temperatures: 10, 20 and 30 °C

and states (bound to DNA and free in solution) [13], which is a result of the dynamic nature of the quenching/enhancement effect. Another notable feature of the dyes is their inner structural isomorphism. As it has been shown [13, 15], the origin of both PG and SG fluorescence quenching/enhancement is intra-molecular dynamics that results in perturbation of the thiazol-quinolinium coupled system. The unimolecular quenching constant, k_q, which characterizes intra-molecular quenching effect, depends on the viscosity, which invariably reduces the rates of the internal dynamics of the PG molecule. In this case the relationship between the quantum yield and viscosity can be expressed by the following equation based on the Förster-Hoffman model [19]:

$$Q_o = Z \times k_r^{-1} \times \eta^{\alpha}, \tag{2}$$

where Z and α are constants, and k_r is a radiative rate constant. For data analysis Eq. (2) is often used in the logarithmic form. Taking into account that the fluorescence intensity is proportional to the dye quantum yield, the relation between intensity (F) and viscosity (η) can be written in the following logarithmic form:

$$\log(F) = \Re + \alpha \times \log(\eta), \tag{3}$$

where ℜ is a constant determined by the physical properties of a dye, including its radiative rate constant and solvent properties; the α parameter is a constant that depends on inner properties of the chromophore molecule (in particular, the ability to intra-molecular rotation and isomerization) and inter-molecular interactions with a solvent.

The dependences of fluorescence signatures of PG and SG upon viscosity, plotted in the double logarithmic scale, are shown in Fig. 3 right. The parameters obtained for the dyes by fitting the data to Eq. (1), are, as expected, very similar: the α parameter for PG is 0.72, for SG $\alpha = 0.75$. The α parameter is an important characteristic of probes sensitivity to the viscosity of an environment. It gives a slope of the viscosity-sensitive function, i.e.

$$\alpha = \delta\log(F)/\delta\log(\eta), \tag{4}$$

Noting that for the dyes F α Q_o [14], Eq. (4) can be rewritten in the following form, containing the fundamental parameter, the quantum yield of a chromophore:

$$\alpha = \delta\log(Q_o)/\delta\log(\eta) \tag{5}$$

Temperature Dependence of PG and SG Sensitivity to Viscosity

Figure 4 shows the evolution of the dyes log(fluorescence) vs. log(viscosity) functions with a temperature. The slope of the functions decreases with the temperature rise. At 10 °C the

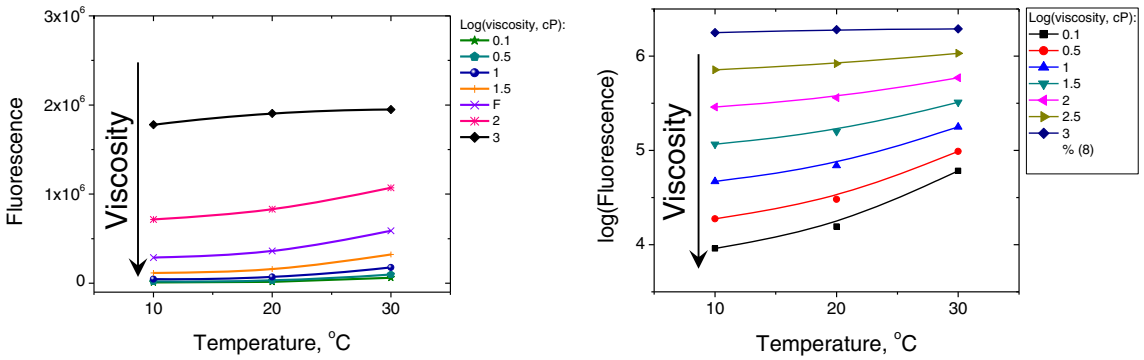

Fig. 5 The dependence of PG fluorescence (*left*) and logarithm of fluorescence intensity (*right*) upon the temperature at different solution viscosity

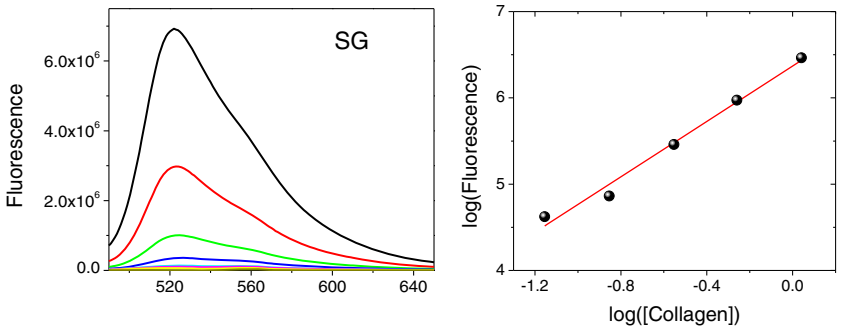

Fig. 6 (*Left*) Fluorescence spectra of SYBR Green I (SG) at different concentrations of collagen, from 2.2 mg/ml to 0.06 mg/ml. (*Right*) Dependence of SG fluorescence intensity at 520 nm (maximum of fluorescence spectrum) upon the concentration of collagen in solution. Concentration of SG was 6 μM. Buffer: 20 mM Na-acetate buffer, pH 3

slope is about 0.8 decreasing to about 0.5 at 30 °C. The observed temperature dependent change in the α parameter manifests the influence of heat on the thermodynamic properties of the system, including intra-molecular motions and coupling of a chromophore with polar protic solvent molecules. A remarkable feature of the temperature-dependent evolution of the viscosity functions (Fig. 4) is that they are divergent at low viscosity but converge at high viscosity (>1,000 cP). Convergence of the functions suggests that in viscous solutions, the influence of the temperature on the dynamics of the chromophore intra-molecular motion (rotation) diminishes possibly due to the crowding effect, i.e. proximal inter-molecular interactions, which effectively dampen inner dynamics.

The change in temperature causes a change in the solution viscosity, i.e. these two macroscopic parameters are coupled thermodynamically. To separate and evaluate their particular influences on spectral parameters of the probes we have used the data shown in Fig. 4 and their analytical expressions. Based on this we have calculated the dependence of PG fluorescence upon temperature at constant viscosities, form 10 to 1,000 cP. These functions are shown in Fig. 5. The interesting aspect of the temperature-dependent change in fluorescence is its non-linear character at low viscosity and a gradual decrease in the steepness followed by vanishing of the temperature dependence, at high viscosity. These changes in quantum yield cannot be explained by the simple influence of temperature on the internal dynamics of the thiazol-quinolinium coupled system, which typically induces quenching of fluorescence. We subsequently suggest that the positive temperature effect might well be caused by specific intermolecular interactions that decrease the rate of quenching.

Application of the PG and SG Probes for Analysis of Collagen Viscosity

Type I collagen (from rat tail) is a protein that forms a triple helical structure. Multiple molecules form collagen fibrils. In certain conditions multiple fibrils form collagen fibers. At acidic pH, collagen is soluble, but is characterized by high solution viscosity, which linearly depends upon the protein concentration [20]. Subsequently to show the usefulness of the probes to the life-science applications, we have studied the collagen protein, a highly studied one in the life sciences today.

Figure 6 shows the sensitivity of SG to the viscosity of collagen solution. The concentration of collagen has been

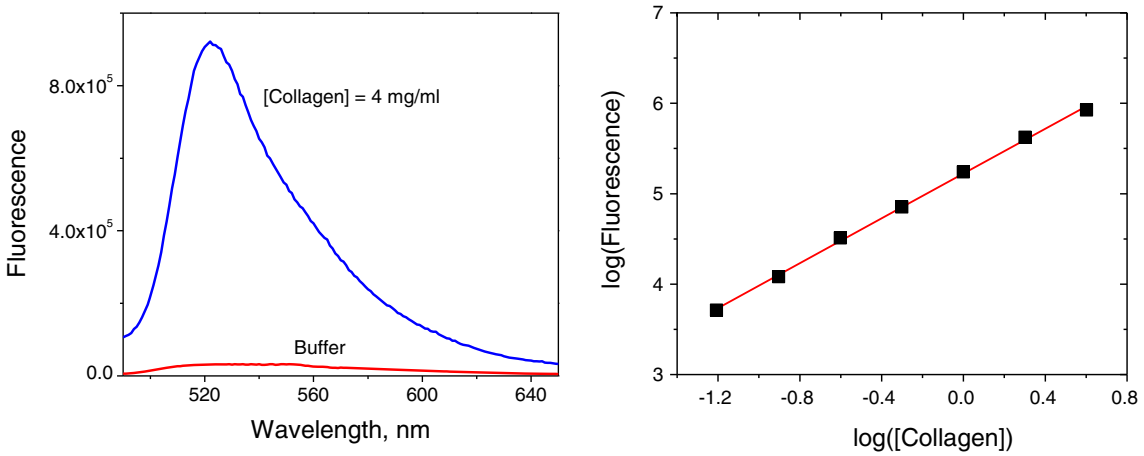

Fig. 7 (*Left*) Fluorescence spectra of PG ([PG] =0.35 uM) in collagen solution (4 mg/ml) and in the buffer. (*Right*) Dependence of PG emission intensity (ΔF = F(PG in collagen) – F(PG in buffer)) upon collagen concentration in solution; 25 °C, 20 mM Na-acetate buffer, pH 3. Excitation at 470 nm

decreased stepwise from 2.2 mg/ml to 0.06 mg/ml, i.e. totally ≈ 40-fold. The decrease in collagen concentration dramatically causes a reduction in SG fluorescence, Fig. 6 left, without any changes in shape or spectral position. Assuming that the viscosity of solution is proportional to the concentration of collagen, i.e. $\eta \propto$ [collagen], one can analyze the data by plotting them in a double log-scale. In this case, the dependence of the probe fluorescence will be a linear function of the collagen concentration/viscosity, the slope of which reveals the α parameter. As it can be seen from Fig. 6, right, the observed dependence of the log(F) vs. log([collagen]) is linear (Fig. 6 right), characterized by the slope, $\alpha = 1.6$.

We have also measured PG sensitivity to the concentration of collagen in solution (Fig. 7). The data, plotted in the log-log scale (Fig. 7 right), also shows linear fluorescence vs viscosity behavior. The slope of the dependence is $\alpha = 1.3$. The obtained α parameter is about the same as for SG. It should be noted that in this experiment we have reduced the concentration of the probe (PG) about 20-fold, as compared to SG (6 μM), to study any possible specific binding of the probe to the protein [8]. Such a decrease in concentration can significantly diminish binding of the dye to collagen and subsequently significantly change the sensitivity (the α parameter) of a probe to the protein solution viscosity. Accordingly our results suggest that both PG and SG report only on the concentration/viscosity of collagen in solution.

Conclusions

Both PG and SG are highly sensitive to viscosity in the demonstrated model system, i.e. in ethylene glycol/glycerol mixtures. The dependences of PG and SG fluorescence upon the viscosity of solution, plotted in a double log-scale, are linear and identical. The slope, $\alpha = \delta\log(Q_o)/\delta\log(\eta)$, of these functions is ≈0.7. This suggests that the main structural component of the fluorophores, responsible for viscosity sensing, is the thiazol-quinolinium coupled system. The VG-probes show an unusual positive dependence of their fluorescence (F) upon the temperature (T) at constant viscosity, i.e. fluorescence enhancement. The functions $\log(F) = f(T, \eta_i = \text{constant})$ are divergent at low viscosity, but do converge at an elevated viscosity.

VG-probes show a broad dynamic range of sensitivity to the concentration/viscosity of collagen, i.e. a fibril solution, demonstrating a linear dependence of their fluorescence in the broad range of collagen concentrations, from mg/ml to μg/ml. The VG-probe's α parameter for a collagen fibril solution is 1.3–1.6, which is about twice as large as for the model ethylene glycol/glycerin system. The results of this study, and considering the fact that in fully damped state (in complex with DNA) the quantum yield can be ≈1,000-fold larger than in a low viscosity water solution [13, 15], suggests that the VG-probes have a large propensity for viscosity sensing in various fluids, and have significant potential as broad dynamical range viscosity probes for the life sciences [21].

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