

COMMUNICATION

Molecular Rotors as Reporters for Viscosity of Solutions of Collagen Like Peptides

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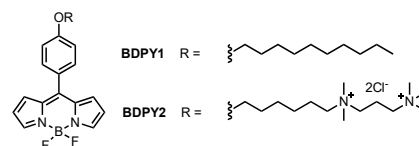
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We have studied the suitability of using a molecular rotor-based steady-state fluorometric assay for evaluating changes in both the conformation and the viscosity of collagen-like peptide solutions. Our results indicate that a positive charge incorporated on the hydrophobic tail of the BODIPY molecular rotor favours the dye specificity as a reporter for viscosity of these solutions.

Molecular rotors (MR) are fluorescent molecules that have variable emission states dependent upon different molecular conformations. MR have been used as biological sensors in applications that include monitoring local viscosities *in vitro* and *in vivo*.¹⁻³ Evaluating viscosity in solution using MR is reliant on the fact that the fluorescent lifetime and quantum yield (QY) of the fluorescent molecular rotors change as a function of viscosity. Given that intramolecular twisting and rotation of such rotors typically lead to non-radiative decay of the excited state back to the ground state; it would seem apparent that a local increase in viscosity³⁻⁸ and/or crowding⁹⁻¹¹ around these probes would affect this twisting and rotation. This leads to deactivation of the non-radiative decay pathway, which results in an increase in fluorescence QY and lifetime.^{4-6, 12-14} It has been previously demonstrated that BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) based molecular rotors are capable of probing the micro-viscosity of solutions, cellular membranes, cellular compartments and tissues within *in vitro*, *ex vivo*, and *in vivo* settings.^{3, 15, 16} The exhibited changes in fluorescence are typically characterized as changes in fluorescence lifetime (τ) as measured via Fluorescence Lifetime Imaging Microscopy (FLIM) or other specialized equipment fitted with detectors capable of Time-Correlated Single Photon Counting (TCSPC).^{4, 17-23} The

BODIPY probes being tested here have shown drastic changes in fluorescence lifetime as a function of viscosity, ranging from 0.01 Pa.s to 10 Pa.s, with the highest sensitivity being observed in the viscosity range of 0.1 Pa.s to approximately 4-5 Pa.s, while viscosities of 0.001- 0.05 Pa.s result in little to no sensitivity.¹⁹ While this approach provides quantitative viscosity measurements, it may be advantageous to use intensity-based measurements that allow for use of more easily and readily accessible equipment.^{8, 24} The advantage of use of fluorescence intensity is primarily due to the accessibility and cost of steady-state fluorimeters over their time-resolved FLIM counterparts. Using fluorescence intensities for determining conformation, and by extension viscosities in solutions of self-assembling structures, could serve as a reliable quality control tool for streamlining quality check of materials such as bioinks, often produced as aqueous solutions of short peptides.

Here, we used fluorogenic molecular rotor-based reporter molecules to quantify the viscosity of short collagen like peptides (CLP) solutions with viscosities ranging between 0.25 to ca. 100 Pa.s, considerably higher than those typically probed in the literature using BODIPY dyes. We hypothesised that our rotors would interact with the peptide structures and, therefore, will respond to the change in their supramolecular conformation, as reported previously for other environmentally sensitive molecules,^{11, 14, 25-27} thus indirectly reporting on viscosity. Two different BODIPY molecular rotors bearing a carbon chain (non-charged BDPY1 and charged BDPY2) were used in this study (see Scheme 1). To the best of our knowledge, the use of fluorescence intensities of molecular rotors to determine conformation and viscosities of collagen like peptide solutions had not yet been explored.



Scheme 1. Chemical structures of tested BODIPY's

We confirmed that the emission lifetimes and quantum yields of BDPY1 and BDPY2 (Scheme 1) strongly vary with the solution viscosity (Fig. S1). Then we investigated the interaction of BDPY1 and BDPY2 with solutions of CLP-PEG (collagen-like-peptide polyethylene glycol conjugate),²⁸ at variable

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temperature (Fig 1 and Fig S2). It is clear to see that the emission of BDPY2 changes significantly as a function of temperature of these solutions (Fig. 1A, 1B). However, spectral shape of BDPY1 may be indicative of the dye aggregation.

It should be noted that the photophysical properties of BODIPY rotors were previously shown to be temperature-independent, *i.e.* their fluorescence depends only on viscosity,¹³ while the temperature affects their properties indirectly, through the effect of temperature on solution viscosity. While, both BDPY1 and BDPY2 have similar fluorogenic cores and viscosity sensitivity (Fig. S1), BDPY2 with its charge bearing tail is considerably more hydrophilic. As such it might be less prone to aggregation, see Fig. S2, and more suited for the analysis of the aqueous based collagen like peptide solutions, similar to what was previously shown in biological tissue analysis.³ Isothermal titration calorimetry measurements for the association of BDPY2 to the CLP structure rendered an association constant $\approx 1 \times 10^5 \text{ M}^{-1}$ (see Fig. S3).

Fig. 1A and 1B display emission spectra obtained for BDPY2 in the presence and absence of 0.1% w/w CLP-PEG over the temperature range of 10°C – 70°C. In both cases there appears to be a consistent and drastic decrease in the fluorescence intensity of the rotor as a function of increasing temperature. The slight increase in initial BDPY2 fluorescence intensity observed in the sample containing 0.1% w/w CLP-PEG as compared to BDPY2 water only should be assigned to the increased solution viscosity due to the presence of CLP-PEG. The subsequent decrease in intensity as a function of temperature observed in both samples may be related to temperature induced changes in viscosity,^{13, 29} dielectric constant of the solution²⁹, or extent of hydrogen bonding.³⁰ To ensure that the incorporation of the BODIPY rotors did not disrupt the triple helical structure of CLP-PEG molecules, CD melting curves were recorded for 0.1% w/w CLP-PEG (Fig. 1C and 1D) in the

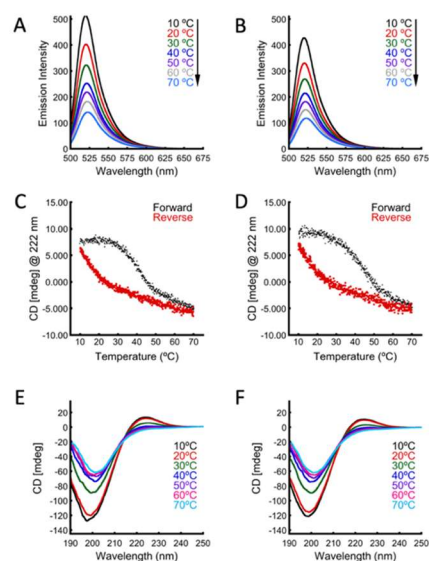


Figure 1. Temperature dependence of fluorescence and circular dichroism signals of solutions comprising BDPY2 and CLP-PEG. (A) Fluorescence spectra of a mixture of 10 μM BDPY2 and 0.1% w/w CLP-PEG and (B) 10 μM BDPY2 in water. Excitation wavelength of 480 nm. (C) CD signal at 222 nm of a mixture of 10 μM BDPY2 and

0.1% w/w CLP-PEG and (D) CD signal at 222 nm of 0.1% w/w CLP-PEG measured as a function of temperature. Forward scan (10°C – 70°C) Reverse scan (70°C – 10°C). (E) CD spectra of a mixture of 10 μM BDPY2 and 0.1% w/w CLP-PEG and (F) CD spectra of 0.1% w/w CLP-PEG at various temperatures.

presence and absence of 10 μM BDPY2. As can be seen in Fig. 1C and 1D, the addition of the charged rotor (BDPY2) did not influence the melting curve of the CLP-PEG or inhibit its reassembly upon cooling, nor did it affect the conformation as comparable CD spectra for CLP-PEG were obtained in its presence (Fig. 1E) and absence (Fig. 1F). Note that similar trends were also observed in the melting curves of the type I collagen solutions (Fig. S4) however, as expected, there was no reassembly of the helical structure observed upon heating above the melting/denaturation temperature of the type I collagen solution in the presence or absence of the BODIPY rotors.

To further examine the interaction of BDPY2 with CLP-PEG, we prepared solutions with concentrations of CLP-PEG ranging from 0% to 10% w/w and 10 μM BDPY2. These solutions ranged in viscosity from below 0.25 Pa.s (limit of detection of the employed system) to 92 Pa.s, as measured by bulk rheometry (Fig. 2A). As seen in Figs. 2B and 2C, when we measured the fluorescence spectrum of BDPY2 in these solutions we find that as the concentration of CLP-PEG increases there is a concomitant increase in fluorescence intensity and a slight red shift in the emission maxima. Further, as shown in Fig. 2D, the fluorescence emission intensity of BDPY2 decreases as temperatures increase, irrespective of the starting CLP-PEG concentration. For BDPY1 we observed minor, non-significant, changes in the emission intensity as a function of the CLP-PEG content (Fig. S5). Together with temperature dependent data presented in Fig S2, this data suggests partial aggregation of BDPY1 and its lack of sensitivity to the presence of CPL, hence, the studies with this dye were discontinued.

The atomic interactions between BDPY2 and CLP were studied using classical molecular dynamics in an aqueous environment at different temperatures. Our results show that BDPY2 interacted closely with CLP throughout the course of the simulation (for greater than 60% of the simulation time). This

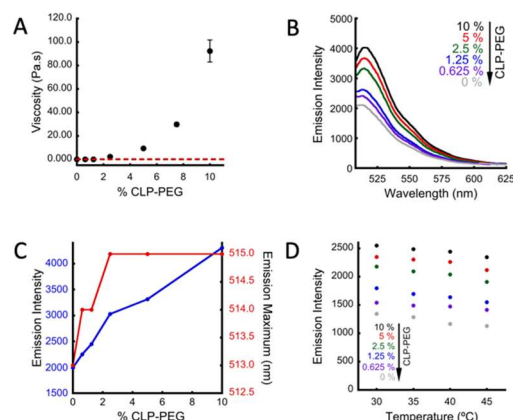


Figure 2. BDPY2 fluorescence data in solutions of various concentrations of CLP-PEG. (A) Viscosity of CLP-PEG solutions as determined via rheometric measurements. Red dashed line denotes a viscosity of 0.25 Pa.s, the lowest viscosity accurately measured under the employed geometry. (B) Fluorescence spectra of 10 μM BDPY2 recorded in solutions with increasing concentrations of

CLP-PEG. (C) Changes in fluorescence intensity (blue) and emission maximum (red) as a function of increasing CLP-PEG concentration. (D) Emission intensity at maximum of BDPY2 in various concentrations of CLP-PEG at temperatures between 30°C and 45°C.

data indicates that the BODIPY rotors bind to CLP instead of diffusing freely in solution.

As shown in Fig. 3, the contact frequency of BDPY2 with CLP decreased as a function of increasing temperature, highlighting that the dye remains attached to CLP at lower temperatures and in viscous environments throughout the 500 ns simulation. The three-dimensional volumetric contact maps (Fig. 3B) of BDPY2 in the presence of CLP at 10°C, 30°C, 50°C, and 70°C illustrate that the contacts of the probe to the peptide are governed by the generation of electrostatic interactions between the polar head groups of the CLP and the charged tail of BDPY2 at lower temperatures, whereas at higher temperatures, lower viscosities, the dye tends to diffuse freely in the solvent. Additionally, to better highlight the nature of the contacts of BDPY1 and BDPY2 with the CLP at 10°C, 30°C, 50°C, and 70°C; Fig. S6 breaks down the simulated contact frequency with each amino acid residue of the CLP peptide. Overall, despite the neutral BDPY1 showing a similar contact behavior to BDPY2 (see Fig. S7), experimentally BDPY1 showed lower hydrophilicity compared to BDPY2 and insufficient fluorescence response to CLP.

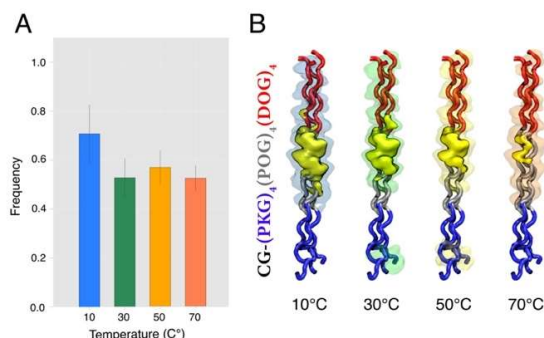


Figure 3. Binding of BDPY2 based molecular rotor molecules to CLP. (A) Contact frequency ratio between BDPY2 to CLP over a 500ns simulation. (B) Three-dimensional contact map of BDPY2 with CLP at different temperatures. Yellow surfaces represent where BDPY2 remains for greater than 40% of the simulation time, while transparent surfaces (blue, green, orange, or red) represent where BDPY2 stays >60% of the simulation time.

To better understand the effect of viscosity, temperature, and conformation, we set out to further compare the characteristics of 10 μ M BDPY2 in aqueous solutions consisting of 0% and 5% w/w CLP-PEG, an intermediate concentration. Fluorescence emission data for BDPY1 and 5% w/w CLP-PEG did not show any trend to link the dye emission with viscosity, Fig S5. For BDPY2, as seen in Fig. 4A and B, the absorption and emission spectra of the dye are similar in both the 0% and 5% CLP-PEG solutions, with absorption maxima centered at 495 nm and emission maxima centered around 517 nm at 25°C. In monitoring the steady-state fluorescence emission spectrum of both the solutions at temperatures ranging from 10°C–70°C we recorded that the emission of BDPY2 decreases and becomes slightly red shifted with increasing temperature for both the 0% CLP-PEG (Fig. 4C and E) and 5% CLP-PEG (Fig. 4D and F) solutions. Further, there is a large jump in the emission intensity

of BDPY2 in 5% w/w CLP-PEG between 10°C and 20°C, which corresponds to a significant change in viscosity of the CLP-PEG solution as measured in Fig. 4G.

Upon closer examination of the steady-state fluorescence anisotropy of BDPY2 in water and 5% w/w CLP-PEG solutions (Fig. 4H) we found that the fluorescence anisotropy of BDPY2 was higher in the viscous CLP-PEG solution. There was a decrease in anisotropy as the temperature of the solutions increased and the CLP-PEG conformation is lost, which resulted in a reduced viscosity. This data provides further evidence that the viscosity of the solutions are correlated with the rotation/relaxation of BDPY2, probably due to the interactions of BDPY2 with the helical structure of the assembled CLP-PEG.

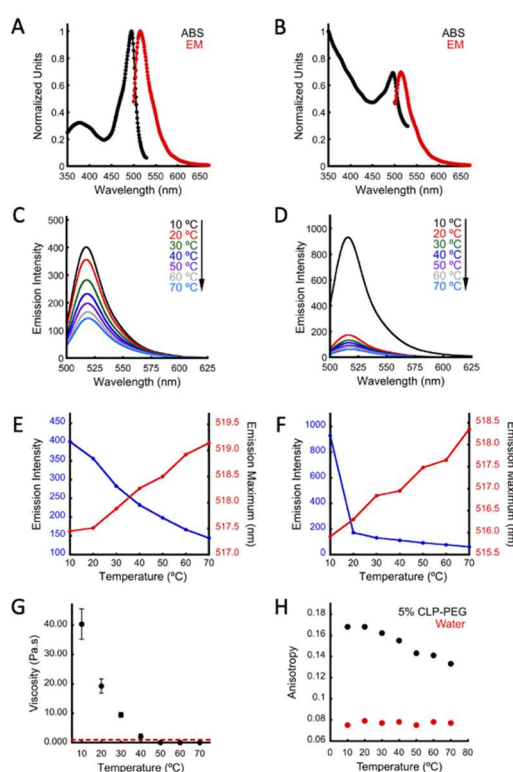


Figure 4. Comparison of spectroscopic data for BDPY2 in water and in 5% w/w CLP-PEG. (A) Normalized absorption and emission spectra of BDPY2 in water. (B) Normalized absorption and emission spectra of BDPY2 in 5% w/w CLP-PEG. (C) Steady-state emission spectra of BDPY2 in water at temperatures between 10°C - 70°C. (D) Steady-state emission spectra of BDPY2 in 5% w/w CLP-PEG at temperatures between 10°C - 70°C. (E) Change in emission intensity and maximum of BDPY2 in water at temperatures between 10°C - 70°C. (F) Change in emission intensity and maximum of BDPY2 in 5% w/w CLP-PEG at temperatures between 10°C - 70°C. (G) Viscosity of a 5% w/w CLP-PEG solution at temperatures between 10°C - 70°C as determined via rheometric measurements. Red dashed line denotes a viscosity of 0.25 Pa.s, the lowest viscosity accurately measured under the employed geometry. (H) Steady-state fluorescence anisotropy of BDPY2 in water (red) and 5% CLP-PEG (black) at temperatures between 10°C - 70°C.

Molecular analyses for the density of atomic contacts between BDPY2 and CLP show the “head-group” of the dye sitting closest to CLP structure at 10°C (see Fig. 8A). This interaction weakens at higher temperatures (see Fig. 8B-C and D). Our results indicate the binding of BDPY2 to CLP favors a stable head planar conformation of BDPY2. The modelling data suggests that the conformation of the BDPY2 upon binding to CLP increases the energy barrier for the transition to a butterfly

conformation (core-bended structure), which increases the viscosity sensitivity for the dye.³¹

In summary, we propose that the fluorescence intensity of BDPY2 rotor is strongly correlated to the viscosity of aqueous CLP solutions, in the range between 0.25 to ca 100 Pa.s, as measured by standard rheometry. Our results suggest that under well controlled conditions, one could use the emissive properties of the BDPY2 dye to develop a rapid quality control test whereby emission intensities could be directly correlated with viscosity. Such a simple quality control system will considerably simplify the standardization of peptide-based gels and bioinks.

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Conflicts of interest

There are no conflicts to declare.

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