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Dynamics of Bacteriophage R17 Probed with a Long-Lifetime Ru(II) Metal-Ligand Complex

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Abstract The metal-ligand complex, [Ru(2,2′-bipyridine)$_2$(4,4′-dicarboxy-2,2′-bipyridine)]$^{2+}$ (RuBDc), was used as a spectroscopic probe for studying macromolecular dynamics. RuBDc is a very photostable probe that possesses favorable photophysical properties including long lifetime, high quantum yield, large Stokes' shift, and highly polarized emission. To further show the usefulness of this luminophore for probing macromolecular dynamics, we examined the intensity and anisotropy decays of RuBDc when conjugated to R17 bacteriophage using frequency-domain fluorometry with a blue light-emitting diode (LED) as the modulated light source. The intensity decays were best fit by a sum of two exponentials, and we obtained a longer mean lifetime at 4 °C (<τ>=491.8 ns) as compared to that at 25 °C (<τ>= 435.1 ns). The anisotropy decay data showed a single rotational correlation time, which is typical for a spherical molecule, and the results showed a longer rotational correlation time at 4 °C (2,574.9 ns) than at 25 °C (2,070.1 ns). The use of RuBDc enabled us to measure the rotational correlation time up to several microseconds. These results indicate that RuBDc has significant potential for studying hydrodynamics of biological macromolecules.

Keywords Long-lifetime metal-ligand complex · Macromolecular dynamics · R17 bacteriophage · Rotational diffusion · Light-emitting diode

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Introduction

Long-lifetime metal-ligand complexes (MLCs), which display decay times ranging from 100 ns to more than 10 μs, show several characteristics that make them versatile biophysical probes. Because of the large Stokes' shift, the MLCs do not display significant radiative or nonradiative homotransfer [1–3]. Consequently, they do not self-quench even for a protein randomly labeled with several MLCs. In general, the MLCs display good water solubility and high thermal, chemical, and photochemical stability [1–3]. Most MLCs display polarized emission, making them useful for microsecond hydrodynamics [1–19]. Finally, if needed, the long lifetimes of the MLCs allow the use of gated detection, which can be employed to suppress interfering autofluorescence from biological samples and can thus provide increased sensitivity [20].

Dynamics of biological macromolecules have occasionally been measured using phosphorescence. But, MLCs have some advantages over the phosphorescent probes. In general, phosphorescence is completely quenched by dissolved oxygen, whereas the fluorescence from MLCs is usually measured in the presence of oxygen. Besides, phosphorescent probes are relatively limited, however, there are a diverse collection of MLCs. A variety of Os, Re, and Ru complexes have been synthesized and utilized to probe microsecond dynamics of proteins [4–11], lipid vesicles [5, 12–14], and DNA [15–19]. The Ru-MLCs appears to be one of the most promising MLCs because of a combination of favorable photochemical and photophysical properties. The Re-MLCs display high fundamental anisotropy and lifetimes as long as several μs, but their absorption and emission wavelengths are much shorter than the Ru-MLCs. The Os-MLCs are characterized by long-wavelength excitation and emission, with typical emission maxima near 750 nm, but display a much shorter lifetime and lower quantum yield

and fundamental anisotropy. Because $[Ru(bpy)_2(dcbpy)]^{2+}$ (bpy = 2,2′-bipyridine, dcbpy = 4,4′-dicarboxy-2,2′-bipyridine) (RuBDc) showed the largest fundamental anisotropy among the Ru-MLCs [7, 9], this luminophore was chosen for probing macromolecular hydrodynamics in the present study. RuBDc has been used to monitor the rotational motions of high-molecular-weight (MW) proteins such as concanavalin A (MW 102,000) [9], HSA (MW 65,000) [7, 9], ferritin (MW 500,000) [9], immunoglobulin (Ig) G (MW 150,000) [9, 11], and IgM (MW 900,000) [11].

To evaluate further the usefulness of RuBDc for macromolecular dynamics, we measured the intensity and anisotropy decays of RuBDc conjugated to R17 bacteriophage which has a MW of approximately 4×10^6 [21–25]. All measurements were done using an inexpensive blue light-emitting diode (LED) as the modulated light source. LEDs are easily modulated up to hundreds of MHz [26]. The combination of the use of a long-lifetime Ru(II) MLC with blue LED excitation allowed us to perform time-resolved intensity and anisotropy decay measurements with a simpler and lower-cost instrument.

Materials and methods

Materials The *E. coli* (# 25868) and phage R17 were purchased from ATCC (Manassas, VA, USA). The PF_6^- salt of succinimidyl ester of RuBDc was from Sigma-Aldrich (St. Louis, MO, USA), and the chemical structure is shown in Fig. 1. Trypticase soy broth (TSB) and agar (TSA) media were obtained from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). Sepharose CL-4B and Sephadex-G25M columns were from GE Healthcare (Piscataway, NJ, USA). Centriprep YM-50 and Centricon YM-100 concentrators were procured from Millipore (Billerica, MA, USA). Coomassie plus protein assay reagent was provided by Pierce (Rockford, IL, USA). All other chemicals were of the reagent grade, and water was deionized with a Milli-Q.

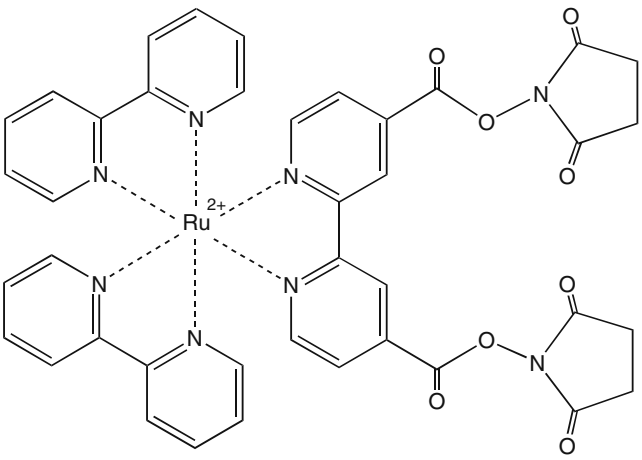

Fig. 1 Chemical structure of succinimidyl ester of $[Ru(bpy)_2(dcbpy)]^{2+}$

Growth and purification of R17 The growing of *E. coli* (ATCC # 25868) and infection with R17 followed the procedure of Kolakofsky [27], and purification of the virus was carried out by the procedure of Valegård et al. [28] although with several modifications. Briefly, *E. coli* (ATCC # 25868) was grown in TSB medium until a density of 6×10^8 CFU/ml was reached. $CaCl_2$ was added to a final concentration of 5 mM, and the cells were infected with phage R17 at a multiplicity of infection of 0.5 and incubated for another 5 h. Lysis was completed by addition of lysozyme (5 mg/l) and $CHCl_3$ (5% *v*/*v*) in the presence of EDTA (1 mM) and DNase I (1 μg/l). Cell debris was pelleted at 8,000 g for 10 min, at 4 °C, and discarded. The bacteriophages were separated by ultracentrifugation at 110,000 g for 5 h. The last step of the purification was a gel filtration chromatographic run on a Sepharose CL-4B column. The column was equilibrated and eluted with buffer A (100 mM NaCl, 0.1 mM $MgSO_4$, 0.01 mM EDTA, 0.02% sodium azide and 10 mM Tris-HCl, pH 7.4). The purity of the R17 was analyzed by SDS/polyacrylamide gel electrophoresis. The phage concentration was determined spectrophotometrically. The extinction coefficient of a 1 mg per ml solution of purified phage is 8.03 absorbance units at 260 nm [27].

Labeling of R17 with $[Ru(bpy)_2(dcbpy)]^{2+}$ R17 was labeled with the succinimidyl ester of RuBDc by adding a three-fold molar excess of Ru-NHS in N,N-dimethylformamide to 1 ml of slowly stirred R17 suspension in 0.05 M carbonate buffer (pH 9.2) followed by an incubation for an hour at room temperature. Centriprep YM-50 and Centricon YM-100 concentrators were used to change buffer A into 0.05 M carbonate buffer. The resulting labeled R17 was separated from the free dye by passing the solution through a Sephadex G-25 M column, using buffer A, pH 7.4. The dye/virus protein molar ratio of the Ru-R17 conjugates was determined by measuring the absorbance of the Ru-R17 conjugates at 467 nm ($\varepsilon_{467\ nm}$=12,000 $M^{-1}cm^{-1}$) and by separately determining the protein concentration with Coomassie plus protein assay reagent. Bovine serum albumin was utilized as a protein standard. The dye/virus protein molar ratio of Ru-R17 conjugate was 0.51. R17 is a member of single-stranded RNA bacteriophages that infect male strains of *E. coli*. Because the capsid of these phages is composed of 180 copies of the coat protein and one copy of the maturation or A protein [29], the MW of virus protein was assumed to be that of the coat protein (approximately 14,000). We performed our measurements immediately after labeling.

Absorption and steady-state fluorescence measurements UV-visible absorption spectra were measured with a Hewlett-

Packard 8453 diode array spectrophotometer. Steady-state intensity measurements were carried out using a Cary Eclipse fluorescence spectrophotometer (Varian Inc., Palo Alto, CA, USA). The excitation was at 480 nm while emission was observed at 650 nm.

FD intensity and anisotropy decay measurements Measurements were performed with a Koala frequency-domain (FD) lifetime spectrometer (ISS Inc., Champaign, IL, USA) using a blue LED as the excitation source. A 480±20 nm interference filter and a 630 nm cut-off filter were used for isolating excitation and emission, respectively. Rhodamine B in water (τ=1.68 ns) was utilized as a lifetime standard. All measurements were performed at 25 °C.

The intensity decays were recovered from the FD data in terms of a multiexponential model using nonlinear least squares analysis [30, 31]:

$$I(t) = \sum_{i=1}^{n} \alpha_i e^{-t/\tau_i} \tag{1}$$

where the preexponential factor α_i is the amplitude of each component, $\sum \alpha_i = 1.0$, τ_i is the decay time, and n is the number of exponential components. Mean lifetimes were calculated by:

$$< \tau >= \frac{\sum_i \alpha_i \tau_i^2}{\sum_i \alpha_i \tau_i} = \sum_i f_i \tau_i \tag{2}$$

where f_i is the fractional steady-state contribution of each component to the total emission, and $\sum f_i$ is normalized to unity. f_i is given by:

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j} \tag{3}$$

The best fits were obtained by minimizing χ_R^2 values:

$$\chi_R^2 = \frac{1}{\nu} \sum_{\omega} \left[\left(\frac{\varphi_\omega - \varphi_{c\omega}}{\delta \varphi} \right)^2 + \left(\frac{m_\omega - m_{c\omega}}{\delta m} \right)^2 \right] \tag{4}$$

where ν is the number of degrees of freedom, and ϕ_ω and m_ω are the experimental phase and modulation, respectively. The subscript c is used to indicate calculated values for assumed values of α_i and τ_i, and $\delta\phi$ and δm are the experimental uncertainties.

The FD anisotropy decays were also analyzed in terms of the multiexponential model using nonlinear least squares analysis [32, 33]:

$$r(t) = \sum_i r_0 g_i e^{-t/\theta_i} \tag{5}$$

where g_i is the amplitude of the anisotropy component with a rotational correlation time θ_i, $\sum g_i = 1.0$, and r_0 is the anisotropy in the absence of rotational diffusion. The total anisotropy r_0 was a fitted parameter. The modulated anisotropy r_ω was calculated by:

$$r_\omega = \frac{\Lambda_\omega - 1}{\Lambda_\omega + 2} \tag{6}$$

where Λ_ω is the ratio of the amplitude of the parallel and the perpendicular components of the modulated emission.

Results and discussion

In this study we characterized the photophysical properties of RuBDc labeled to bacteriophage R17. Figure 2 shows the absorption and emission spectra of RuBDc conjugated to R17. As can be seen, RuBDc displays both long-wavelength absorption and emission maxima and the large Stokes' shift, with the emission shifting over 180 nm from the absorption maximum. The long-wavelength absorption allowed us to use a simple and inexpensive blue LED as the modulated light source. The long-wavelength emission is valuable because sample autofluorescence is minimized at longer wavelengths.

To determine whether RuBDc can yield useful information about macromolecular dynamics, we performed FD intensity and anisotropy decay measurements of RuBDc-labeled R17. The FD intensity decays of RuBDc conjugated to R17 are shown in Fig. 3. The intensity decays were best fit by a sum of two exponentials, and the result is summarized in Table 1. The mean lifetime values obtained at 4 and 25 °C were 491.8 and 435.1 ns, respectively. As expected, we observed increased decay times at 4 °C in comparison to 25 °C, which is probably due to the reduced collisional motions at the lower temperature.

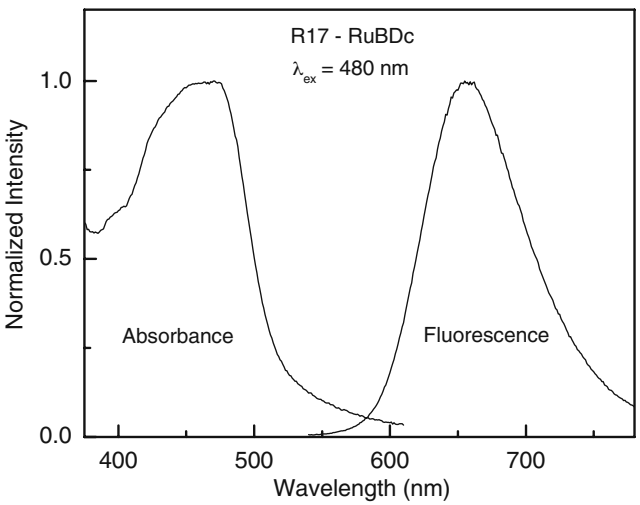

Fig. 2 Absorption and emission spectra of $[Ru(bpy)_2(dcbpy)]^{2+}$ conjugated to R17 bacteriophage

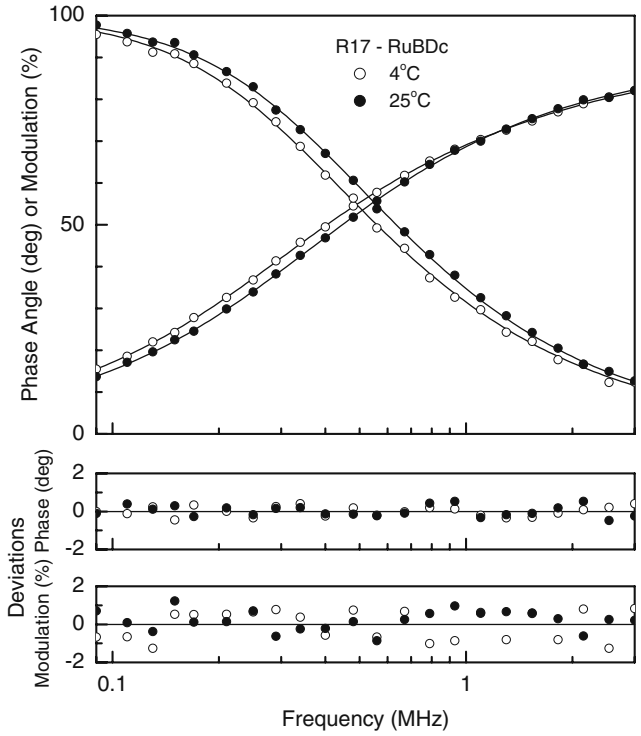

Fig. 3 Frequency-domain intensity decays of $[Ru(bpy)_2(dcbpy)]^{2+}$ conjugated to R17 bacteriophage. The symbols in the first panel represent the measured phase and modulation values. The *solid lines* show the best multiexponential fits to the data. The middle and lower panels show plots of the residuals between the experimental data and the fitted curve

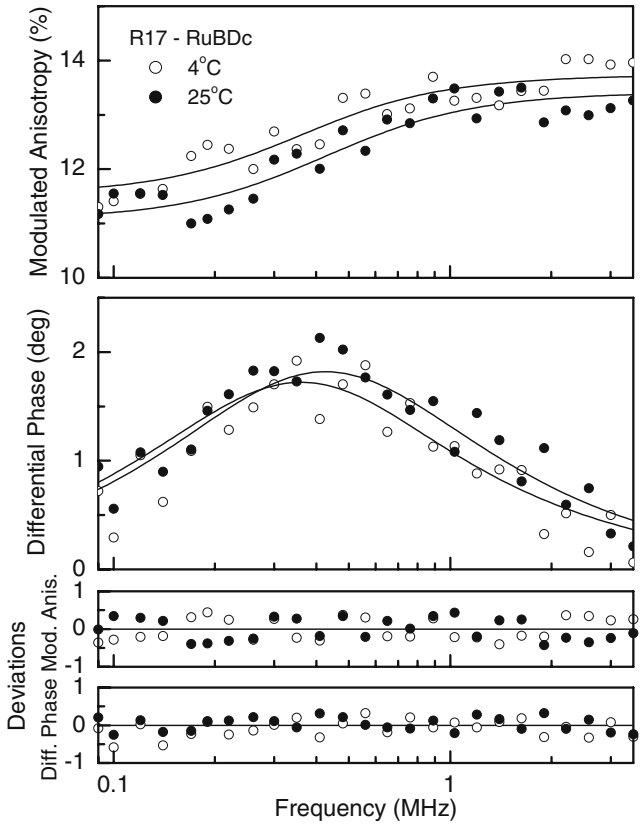

Fig. 4 Anisotropy decays of $[Ru(bpy)_2(dcbpy)]^{2+}$ conjugated to R17 bacteriophage. The symbols in the first and second panels represent the calculated modulated anisotropy and the measured phase shift values, respectively. The *solid lines* show the best single exponential fits to the data. The lower two panels show plots of the residuals between the experimental data and the fitted curve

In addition to the intensity decay measurements, we also measured the anisotropy decays of RuBDc conjugated to R17 at 4 and 25 °C (Fig. 4). Bacteriophage R17 has overall icosahedral symmetry. The virus has a spherical shape with a diameter of 230–280 Å [24, 34–36]. As expected for a spherical molecule [37], the anisotropy decay data were best fit using the single exponential model, and the result is summarized in Table 2. The rotational correlation times (θ) were 2,574.9 and 2,070.1 ns at 4 and 25 °C, respectively, and appear to be consistent with those expected for overall rotational diffusion of R17. In top panel of Fig. 4, we also see the modulated anisotropy values for RuBDc conjugated to R17. Of course, lower modulated anisotropy values were observed at 4 °C than those at 25 °C. At low frequency, the modulated anisotropy r_ω is equal to the steady-state anisotropy. The value of r_ω at low frequency was much larger than that of IgM which has a MW of 9×10^5 [11].

The measured θ value at 25 °C was compared with the values predicted for anhydrous virus (Table 3). The molecular volume (V) of the anhydrous virus can be calculated from its MW and partial specific volume ($\acute{v}$) using V=(MWx $\acute{v}$)/N where N is the Avogadro's constant.

Table 1 Multiexponential intensity decay analysis of $[Ru(bpy)_2(dcbpy)]^{2+}$ conjugated to R17 bacteriophage

Temperature (°C)	τ_i (ns)	α_i	f_i[a]	$\langle\tau\rangle$[a] (ns)	χ_R^2[b]
4	135.0	0.16	0.05	491.8	2.1
	509.8	0.84	0.95		
25	172.2	0.09	0.04	435.1	1.8
	445.6	0.91	0.96		

[a] Fractional intensities f_i and mean lifetimes $\langle\tau\rangle$ were calculated using Eqs. 3 and 2, respectively

[b] The χ_R^2 values were calculated by Eq. 4, and the standard errors of phase angle and modulation were set at 0.2° and 0.005, respectively

Table 2 Multiexponential anisotropy decay analysis of $[Ru(bpy)_2(dcbpy)]^{2+}$ conjugated to R17 bacteriophage

Temperature (°C)	θ_i (ns)	$r_o * g_i$	$\sum(r_o * g_i)$	χ_R^2[a]
4	2,574.9	0.137	0.137	3.4
25	2,070.1	0.134	0.134	3.0

[a] The standard errors of phase angle and modulation were set at 0.2° and 0.005, respectively

Table 3 Molecular volumes (V) and calculated rotational correlation times (θ) of anhydrous R17 bacteriophage

MW×10^{-6}	V [a] (nm^3)		r [b] (Å)		θ [c] (ns)	
	$\acute{\upsilon}$ = 0.670	$\acute{\upsilon}$ = 0.689	$\acute{\upsilon}$ = 0.670	$\acute{\upsilon}$ = 0.689	$\acute{\upsilon}$ = 0.670	$\acute{\upsilon}$ = 0.689
3.60 [22]	4,005	4,119	99	99	880	905
3.64 [23]	4,050	4,165	99	100	890	915
3.80 [23]	4,228	4,348	100	101	929	955
3.85 [23]	4,283	4,405	101	102	941	968
3.95 [23]	4,395	4,519	102	103	966	993
3.98 [23]	4,428	4,554	102	103	973	1,001
4.02 [24]	4,473	4,599	102	103	983	1,010
4.19 [25]	4,662	4,794	104	105	1,024	1,053

MW = molecular weight; $\acute{\upsilon}$ = partial specific volume; and r = radius. The numbers in brackets refer to those of references

[a] Calculated from MW and $\acute{\upsilon}$ using $V = (\mathrm{MW} \times \acute{\upsilon})/N$ where N is the Avogadro's constant. The $\acute{\upsilon}$ values were either 0.670 [25] or 0.689 [24]

[b] Calculated from V using $r = \sqrt[3]{3V/4\pi}$

[c] The rotational correlation times (θ) were calculated according to the Perrin equation $\theta = \eta V/RT$, where η is the viscosity, V is the molecular volume, R is the gas constant, and T is the absolute temperature. η was assumed to be 0.904 cP and was calculated by multiplying the viscosity of water at 25 °C (0.890 cP) by 1.016 [24]

The calculated V values of the anhydrous virus ranged from 4,005 to 4,794 nm^3 because of the discrepancy of the MW of R17 (Table 3). The radius (r) of the anhydrous sphere was calculated to be about 100 Å, which coincides with the value reported previously [38] (Table 3). Using these values and the Perrin equation, the θ values of the anhydrous virus were calculated, and the values were in the range of 880–1,053 ns (Table 3). Our observed θ value at 25 °C (2,070.1 ns) was much larger than the θ values calculated for the anhydrous virus. We also calculated the molecular volumes (V) and rotational correlation times (θ) of a hydrous sphere from the diameter data of R17 in the literatures [24, 34–36] (Table 4). The observed θ value (2,070.1 ns) was within the range of the θ values of hydrous virus (1,400–2,525 ns).

An important point of the present study is our use of a semiconductor light source for the FD intensity and anisotropy decay measurements. At present, very high-intensity LEDs are available across visible, UV, and IR wavelengths. LED excitation eliminates the need for expensive lasers, sophisticated optics and electro-optical light modulators, making FD intensity and anisotropy decay measurements much easier.

Conclusion

In the present study, we demonstrated the usefulness of RuBDc, a long-lifetime MLC, for probing macromolecular hydrodynamics. It is obvious that the rotational correlation time (θ) values reflect both the molecular size and shape of the R17 bacteriophage, as well as the viscosity. In addition, the use of RuBDc enabled us to measure the rotational correlation times up to several microseconds. However, it should be pointed out that it was not possible to accurately measure the overall rotational correlation times of R17 bacteriophage which has a MW of approximately 4×10^6. Information on the rotational motion is usually available over a time scale not exceeding three times the lifetime of the fluorophore, after which there is too little signal for accurate anisotropy measurement. Thus, it is not considered that the rotational correlation time values obtained in this study are very precise because they were far longer than three times of the intensity decay times. In addition, the lifetime of RuBDc is too short to measure the overall rotational correlation times of larger viruses than R17. The use of long-lifetime MLCs to measure dynamics of biological macromolecules is still in its infancy, and

Table 4 Molecular volumes (V) and calculated rotational correlation times (θ) of hydrous R17 bacteriophage

r (Å)	V [a] (nm^3)	θ [b] (ns)
115 [34]	6,371	1,400
125 [34]	8,181	1,798
132 [35]	9,634	2,117
133 [36]	9,855	2,165
140 [24]	11,494	2,525

r is radius, and the numbers in brackets refer to those of references

[a] Calculated from r using $V = 4\pi r^3/3$

[b] The rotational correlation times (θ) were calculated according to the Perrin equation $\theta = \eta V/RT$, where η is the viscosity, V is the molecular volume, R is the gas constant, and T is the absolute temperature. η was assumed to be 0.904 cP and was calculated by multiplying the viscosity of water at 25 °C (0.890 cP) by 1.016 [24]

additional MLCs with longer lifetimes, higher quantum yields, and larger fundamental anisotropies are yet to be developed.

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