# Anomalous fluorescence in supercooled organic liquids: Correlation with glass/phase transition

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**Anomalous fluorescence of some molecules in organic solvents at low temperature provides a new tool to probe the microscopic dynamics and glass transition in liquids below the freezing point. The fluorescence lifetime of aluminium and zinc complex of 8-hydroxyquinoline increases by an anomalous mechanism in a specific temperature interval: 100–160 K in ethanol, 140–240 K in dimethyl sulphoxide. In ethanol, the temperature dependence of fluorescence lifetime while cooling is different from that while heating. The observed fluorescence anomaly correlates well with the glass/phase transition temperatures of ethanol.**

GLASSY liquids do not ordinarily crystallize at the freezing point. They become supercooled liquids and glasses below the freezing point. Glass transition is a common phenomenon to organic liquids, biological, polymeric and amorphous materials[1]. The physical properties and function of the 'soft' materials are attributed to their glassy character. A microscopic or molecular level explanation of the glass behaviour has not emerged. Being one of 'the deepest and most interesting unsolved problems in solid state theory'[2], the structure and microscopic dynamics of molecules in supercooled liquids and glasses are the subject of many recent review articles[1,3–6].

At the heart of the glass behaviour is the non-Arrhenius type temperature dependence of experimental relaxation rates[1,3,6]. Temperature dependence of molecular relaxation rate or viscosity in supercooled liquids does not obey the Arrhenius equation. This important discrepancy arises due to dynamical and spatial heterogeneity spanning a wide range of time and length scale. Discovery of new molecules and molecular properties that show non-Arrhenius temperature dependence provides fresh experimental data on the puzzling glass behaviour[5]. Malachite green, a cationic fluorescent molecule has been used in a previous study to probe glass transitions in liquids[7]. Ionic molecules have a tendency to aggregate in nonpolar liquids, and hence a neutral molecular probe may be more suitable. We report here that the fluorescence lifetime of two neutral metal complexes, Tris(8-hydroxyquinolinato) aluminium (III) ($Alq_3$) and *bis*(8-hydroxyquinolinato) zinc(II) ($Znq_2$), varies anomalously with temperature in frozen organic liquids. The fluorescence anomaly in ethanol correlates well with the phase diagram of the solvent. Anomalous fluorescence provides a relatively simple fluorescence decay-based method to probe microscopic non-Arrhenius dynamics in supercooled liquids.

Figure 1 shows the fluorescence decays of $Alq_3$, $Znq_2$, and nile red in ethanol at 300 and 77 K respectively. It was found that the fluorescence lifetime at 77 K increased substantially for $Alq_3$ (3.6 times) and $Znq_2$ (10.6 times), but only marginally for nile red (1.2 times). The fluorescence decay fits well to a single exponential function at 300 K and multi-exponential function at 77 K. The values of lifetimes and amplitudes are given in the caption to Figure 1. Similar results are observed for the fluorescence decays of the three molecules in dimethyl sulphoxide (DMSO).

Figure 2 shows the plot of fluorescence lifetime with temperature for $Alq_3$ and $Znq_2$ in ethanol from 300 to 80 K. The lifetime increases gradually as the temperature decreases from 300 K to the freezing point (158.5 K), and more steeply below the freezing point, from 160 to 120 K. Data shown in Figure 2 are reproducible when cooled from 300 to 80 K. However, on heating the sample from 80 to 300 K, the fluorescence lifetime was not identical to that measured while cooling, especially in the temperature interval 160–120 K. The inset in Figure 2 shows the data obtained for $Znq_2$ while cooling and heating. While heating, the fluorescence lifetime decreases very sharply at 120–125 K and again at 160–170 K, which are close to the glass transition temperature[8] ($T_{g1}$ at 125 K) and freezing point (158.5 K) of ethanol. Hysteresis of the type seen here, while heating and cooling, is not surprising for glassy materials. But, a sharp phase-transition-like decrease in fluorescence lifetime for $Znq_2$ in ethanol in the heating cycle is rather unique.

The phase diagram of ethanol below the freezing point has recently been reported[8]. It indicates the existence of supercooled liquid and crystalline and/or glassy phases below 160 K. In particular, glass transition temperatures were identified at 159 K (freezing point, FP), 125 K ($T_{g1}$, 'rotator' phase or plastic crystal) and 95 K ($T_{g2}$, orientational glass). The temperature range of 160–120 K, where the fluorescence lifetime of $Alq_3$ or $Znq_2$ was observed to increase anomalously correlates well with the region of supercooled liquid, between the freezing point and $T_{g1}$ in the phase diagram of ethanol[8]. In this interval, ethanol is a translationally and rotationally active super-cooled liquid. The transition to the rotator phase (rotationally active molecules in bcc lattice) begins at 125 K. The temperature interval in which the fluorescence anomaly occurs (160–120 K) is approximately the same as the interval between melting point (159 K) and $T_{g1}$ (125 K) of ethanol. There is, however, no sharp change of lifetime, either in $Alq_3$ or $Znq_2$ to indicate the transition temperatures in the cooling cycle. On the other hand, there are sharp changes in the lifetime in the heating cycle (inset, Figure 2) which occurred approximately

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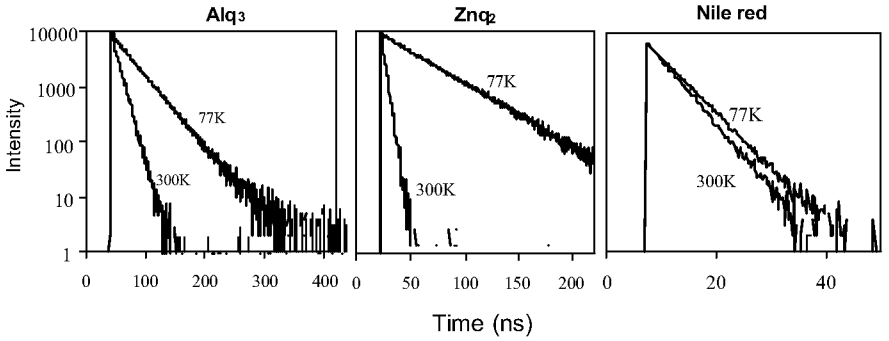


**Figure 1.** Fluorescence decays of $Alq_3$ (100 μM), $Znq_2$ (120 μM), and nile red (12 μM), in ethanol (> 99.9% v/v) at 300 K and 77 K. $Alq_3$ and $Znq_2$ were prepared by the procedure as described for $Alq_3$ (ref. 12). Nile red (Sigma Chemicals, USA) was used as received. The samples (~ 0.5 ml) were degassed by 3–4 freeze–pump–thaw cycles under vacuum (< $10^{-3}$ Torr) and subsequently sealed in glass vials. Fluorescence decays were obtained using a time-correlated single photon-counting (TCSPC) set-up[13] using a mode-locked Ti-sapphire laser, and a fast photodiode, MCP-PMT and fast electronics modules. Samples were excited at 310 nm and the emission wavelengths were as follows: $Alq_3$ (515 nm), $Znq_2$ (545 or 560 nm) and nile red (630 nm). Fluorescence decays thus obtained were fitted to single or multiple exponential functions by standard algorithms[13,14]. For multi-exponential decays, the average fluorescence lifetime is calculated using the lifetimes ($\tau$) and amplitudes ($\alpha$) as, $\tau_{av} = (\Sigma\alpha_i\tau_i)/(\Sigma\alpha_i)$, $\alpha_i > 0$. The fit of the fluorescence decays shown in the figure to single, double or triple exponentials gave the following results for lifetimes (and fractions): $Alq_3$ [10.6 ns (100%) at 300 K; 38.65 ns (97%) and 2.57 ns (3%) at 77 K]; $Znq_2$ [3.6 ns (100%) at 300 K; 38.43 ns (81%), 8.08 ns (5%) and 1.37 ns (14%) at 77 K]; Nile red [3.66 ns (100%) at 300 K; 4.51 ns (92%) and 3.03 ns (8%) at 77 K].

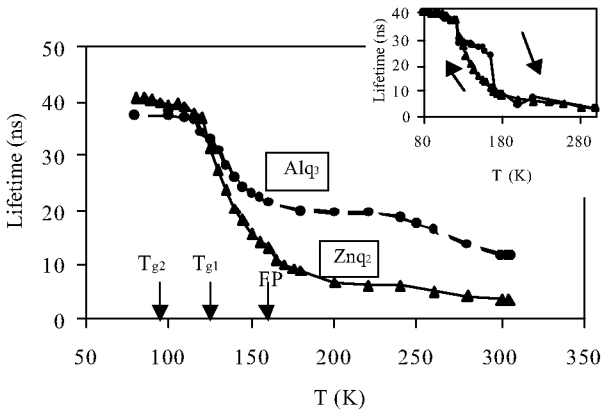


**Figure 2.** Variation of average lifetime of $Alq_3$ and $Znq_2$ with temperature in ethanol when the sample is cooled from 300 to 80 K. The sample in the optical cryostat was cooled (or heated) between 300 and 80 K in 5 or 10 K interval and equilibrated at the set temperature for at least 5 min before measurement. The freezing point (FP, 158.5 K), and glass transition temperatures at 125 K ($T_{g1}$) and 95 K ($T_{g2}$), are indicated. (Inset) Variation of lifetime of $Znq_2$ with temperature when the sample is cooled from 300 to 80 K (upward arrow) and heated from 80 to 300 K (downward arrow). The curve drawn through the points is a guide to the eye.

(± 5 K), coinciding with melting point and $T_{g1}$. These sharp changes indicate phase transitions in the heating cycle. Presumably, pure phases (crystallites) of ethanol have formed in the glass when the solvent is cooled to 80 K and heated upwards.

The fluorescence lifetime ($\tau_f$) is defined by the radiative ($k_r$) and nonradiative ($k_{nr}$) rate constants of the excited molecule: $\tau_f = (k_r + k_{nr})^{-1}$. The increase in fluorescence lifetime, as observed for $Alq_3$ and $Znq_2$ in frozen ethanol, is either due to decrease of $k_r$ or $k_{nr}$ or both. It was observed that the steady state fluorescence intensity (a measure of quantum yield, $\varphi$) of $Alq_3$ increased by 1.8-fold in the temperature region of 160–120 K. In the same interval the fluorescence lifetime also increased by a factor of 1.8. The ratio of the quantum yield to the fluorescence lifetime is unchanged. This confirms that $k_r$ is independent of temperature. Therefore, the increase of fluorescence lifetime of $Alq_3$ and $Znq_2$ between 160 and 120 K in ethanol is attributed to the decrease of nonradiative rate of $Alq_3$.

The temperature dependence of nonradiative rate of molecules in condensed media is generally consistent with Arrhenius mechanism involving an activation process[9]. That is, the nonradiative rate, consists of two parts: the temperature-independent part, $k'_{nr}$ and the temperature-dependent part, $k_{nr}$ that obeys eq. (1).

$$k_{nr} = k^0_{nr} e^{-\Delta E/RT}. \quad (1)$$

In eq. (1), $\Delta E$ is the activation energy. We verified whether the results for $Alq_3$ obey eq. (1). For this purpose, $k_{nr}$ was calculated as follows. The fluorescence lifetime measured at 77 K in ethanol ($\tau_0 = 39.2$ ns) is taken to be the limiting maximum value for $Alq_3$ in this solvent. The limiting value of lifetime has contributions only from the radiative rate and temperature-independent part of nonradiative rate. $k_{nr}$ at higher temperatures was calculated using $\tau_f$ and $\tau_0$ as follows: $k_{nr} = (\tau_f^{-1} - \tau_0^{-1})$, where $\tau_f$ is the measured lifetime. Figure 3 shows the plot of $\ln(k_{nr})$ vs $1/T$. Data in Figure 3 clearly show that the variation of $\ln(k_{nr})$ is nonlinear in the temperature region 220–110 K and linear for $T > 220$ K. $T > 220$ K is the

high-temperature fluid region where molecules are rotationally and translationally active. In this interval, the nonradiative rate obeys Arrhenius equation and the activation energy is calculated to be 0.056 eV. This is less than the activation energy (0.143 eV) associated with the temperature dependence of the viscosity of ethanol in the same temperature range. The activation energy of 0.056 eV must therefore be associated with an intramolecular nonradiative process of $Alq_3$.

The nonradiative rate does not fit the Arrhenius equation below 220 K, which may be explained by the fact that ethanol is a supercooled liquid below the melting point. Vogel–Tammann–Fulcher (VTF) equation is frequently used to fit non-Arrhenius type temperature dependence of relaxation rates in supercooled liquids and glasses[1,6]:

$$k = k^0 e^{-B/(T-T_x)}. \quad (2)$$

In eq. (2), $k^0$, $B$ and $T_x$ are constants. $T_x$ is approximately the glass transition temperature for the liquid. We examined the validity of the VTF equation for the temperature dependence of $k_{nr}$ for $Alq_3$ in the supercooled liquid regime. Data in the temperature range of 180–110 K fitted well to the VTF equation. Inset in Figure 3 shows the plot of $\ln(k_{nr})$ vs $(T-T_x)^{-1}$ for $Alq_3$, where $T_x = 95$ K. It may be noted that the fit includes data for 180 K, which is above the melting point. We used the second glass transition temperature ($T_{g2}$) for $T_x$, which gave a better fit for a larger number of data. From the slope of the linear plot we get the value of $B$ as 90 K. Assuming[1] that VTF is a modified form of Arrhenius equation, then $B = \Delta E/R$ and the activation energy is calculated to be 7.7 meV. This activation energy is small by several orders compared to viscosity-based apparent activation energy[1] (> 5 eV) for fragile liquids near the glass transition. The physical meaning of this very low activation energy will be clear only after the development of a suitable theory that connects nonradiative relaxation and the molecular dynamics in supercooled liquids. It may be concluded that the anomalous temperature dependence of the nonradiative rate of $Alq_3$ in the supercooled region obeys the VTF equation. This is in agreement with the previous finding, using malachite green as the probe, that the VTF equation holds good in the supercooled liquid region[7].

To our knowledge, a molecular level connection between the nonradiative rate of the fluorescent molecule and viscosity, diffusion coefficient(s) or collisions in supercooled or glassy state of liquids does not exist. It is well known[1] that viscosity ($\eta$) of the glass-forming liquids increases by 10–12 orders as the liquid is cooled from the fluid state at room temperature to glassy state at low temperature. Translational and rotational diffusion coefficient values of organic probe molecules (e.g. *o*-terphenyl)[10] were found to vary inversely as $\eta^{-x}$, where $0.5 < x < 1$. The dependence of the nonradiative rate of fluorescent molecules on the diffusion coefficient or viscosity in supercooled liquids is yet to be established. The models of molecular collisions that lead to Arrhenius-type equation with a single activation energy are not adequate. It is necessary to consider other models of molecular dynamics (e.g. separate contributions of rotational and translational dynamics to the nonradiative process with different activation energies) for a quantitative understanding of non-Arrhenius type relationship in supercooled liquids.

We have also observed the fluorescence anomaly in another organic solvent. Figure 4 shows the plot of fluorescence lifetime with temperature from 300 to 80 K in dimethyl sulphoxide (DMSO) for $Alq_3$, $Znq_2$ and nile red. The lifetimes of $Alq_3$ and $Znq_2$ show anomalously strong temperature dependence between 240 and 140 K. It may be noted that the temperature interval in which the fluo-

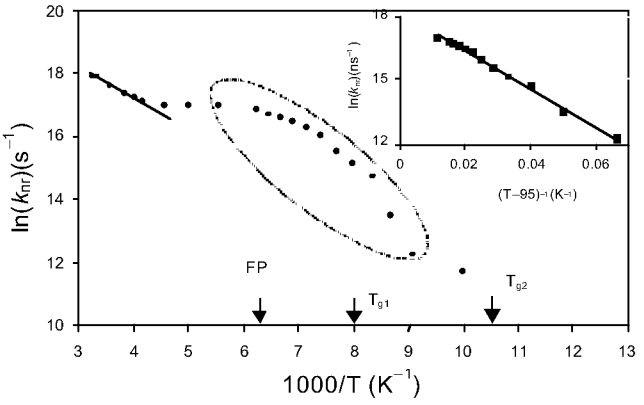


**Figure 3.** Plot of $\ln(k_{nr})$ vs $1/T$ for $Alq_3$ in ethanol obtained using data shown in Figure 2 (see text for definition of $k_{nr}$). Freezing point and glass transition temperatures of ethanol are indicated. The straight line shows that the data are linear above 240 K. (Inset) Plot of data from 180 to 110 K (within the ellipse) that agree well with the VTF equation (eq. (2), see text).

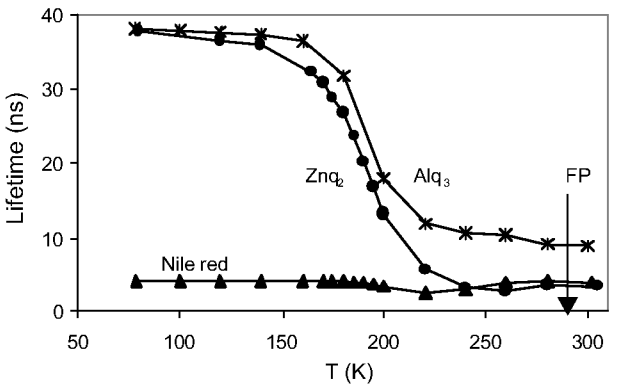


**Figure 4.** Variation of average lifetime of $Alq_3$, $Znq_2$ and nile red with temperature in dimethyl sulphoxide (HPLC grade). The sample is cooled from 300 to 80 K. The curve drawn through the points is a guide to the eye.

rescence anomaly is observed in DMSO is different from that in ethanol. The fluorescence lifetimes of $Alq_3$ and $Znq_2$ measured in DMSO while heating the sample from 80 to 300 K are identical to those obtained while cooling. This is markedly different from that observed in ethanol, where hysteresis and phase transition-like variations were observed (inset, Figure 2).

Nile red is a widely used neutral molecule whose fluorescence is used to probe the polarity, hydrogen bonding and solvent type[11]. However, as seen in Figure 4, nile red is not useful for investigating the glass transitions in 'frozen' liquids. $Alq_3$ and $Znq_2$ are more suitable for probing the microscopic dynamics in supercooled liquids. The long lifetime of these fluorescent molecules gives kinetic data for hundreds of nanoseconds (Figure 1). The potential usefulness of this kinetic information adds another dimension to the utility of the anomalous fluorescence for testing competing theories of glass transition. However, a molecular level connection between the non-radiative mechanism of the probe molecule in the supercooled liquid and glass transition region will have to be established.


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# Functional recombinant antibodies against human chorionic gonadotropin expressed in plants

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**Single-chain variable fragments, diabodies and chimeric antibodies (mouse variable domains and human immunoglobulin constant domains) were engineered by DNA recombinant technique and expressed transiently in tobacco leaves. The plants expressed the three types of antigen-binding moieties, accurately and faithfully. The yield obtained was 32 mg, 40 mg and 20 mg respectively, per kg of wet weight of leaves. The chimeric antibody had high affinity for human chorionic gonadotropin ($Ka = 1.9 \times 10^{10} M^{-1}$). All three forms of the recombinant antibodies expressed by plants inhibited the binding of hCG to receptor on Leydig cells.**

SEROTHERAPY has been employed for life-threatening infections since the 1890s for tetanus, diphtheria and rabies. Initially antibodies raised in horses were employed, which limited the repeat use of such antibodies for therapeutic interventions owing to sensitization caused in recipients, to the heterospecies proteins. In recent years antibodies used as a last resort at the terminal stage of infections are derived from human sera prepared from hyper-immunized donors. These are consequently expensive. However for snakebites, horse continues to be the source of serum for therapy. The contention of this article is to propose and demonstrate that humanized therapeutic antibodies can be made by recombinant DNA route. Furthermore, plants can be used for expression of the recombinant antibodies. Plants offer several advantages. Besides being eco-friendly, plant-derived immunoglobulins would be expected to be devoid of harmful endotoxins and animal viruses present in the commonly used prokaryotic or animal cell culture expression systems. We describe the successful production of recombinant antibodies against the human chorionic gonadotropin (hCG), employing plants as an expression system. Also illustrated are the different constructs of antigen-binding fragments that can be engineered and their relative merits

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