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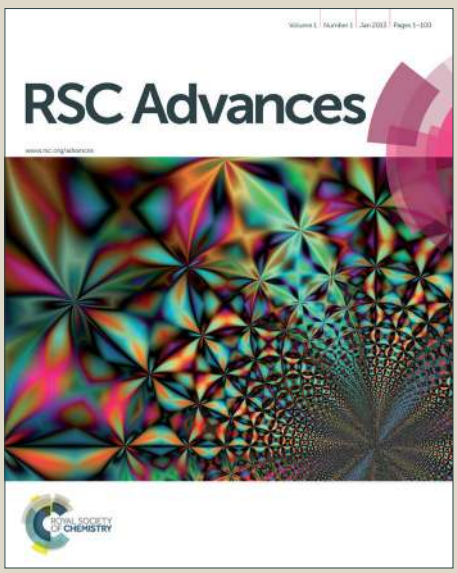

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Thermotropic Gelation Induced Changes in Micropolarity and Microviscosity of Hydrogel Derived from Glucose-Triazole-Hydrogenated Cardanol Conjugate: A Study Using Fluorescent Molecular Probe

JitendriyaSwain[a], *M. Kamalraj*[b], *H. Surya PrakashRao*[b], *Ashok K. Mishra*[a]*,

[a]*Department of Chemistry, Indian Institute of Technology Madras, Chennai –600036, INDIA*
[b]*Department of Chemistry, Pondicherry University, Pondicherry – 605014, INDIA*

Abstracts

Glucose triazole cardanol conjugate (GTHCC) is a supergelator in water: methanol (1:1) solvent mixture. Environmental scanning electron microscopy **(**ESEM) and fluorescence molecular probe based techniques were employed to understand the thermotropic gelation induced changes in micropolarity and microviscosity of GTHCC hydrogel in water: methanol (1:1) solvent mixture. ESEM analysis has shown the formation of spongy sheet like structure having micrometer size pores inside it. The fluorescence intensity and fluorescence anisotropy measurements of the polarity sensitive probe 8-anilino-1-naphthalene sulfonic acid (ANS) revealed a significant increase in the micropolarity and a significant decrease of microviscosity of GTHCC hydrogel between gel and sol phases. Time resolved fluorescence decay analysis of ANS has shown the presence of two different microenvironments in the system and thermotropic microenvironmental changes between gel and sol phases of GTHCC hydrogel.

Introduction

Hydrogels resulting from the molecular self-assembly through the non-covalent physical process is termed as a physical gel or molecular gel.[1]At the microscopic scale hydrogels are heterogeneous systems.[2] Differences in the micropolarity, microviscosity and complex structure is due to hydrophobic and hydrophilic parts of the hydrogels both of which contribute to the total gel microheterogeneity.[3,4] Estimation of thermotropic gelation induced changes in micropolarity and microviscosity of hydrogels is important in understanding the

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physicochemical behaviour of gels in the context of their wide applications in different fields.[3-8]In a recent work,[9] we studied the synthesis and gelation properties of a structurally simple, carbohydrate based glucose-triazole-hydrogenated cardanol conjugate (GTHCC) (Figure 1). GTHCC molecule has formed four different classes of gels, namely, (i) hydrogel from aqueous-protic solvent, (ii) organo gel from non-polar solvent (iii) gel from vegitable oil and (iv) gel from petroleum oil. In water/methanol (1:1) mixture, the conjugate has formed stable themoreversible supergelator, even at a very low gelator concentration of 0.03 % w/v. The intrinsic fluorescence of the GTHCC was found to be sensitive towards gel-sol transition process.[9]

To get insight into complex heterogeneous structure of the hydrogels, variety of different spectroscopic techniques are employed which include NMR, IR, UV, CD and fluorescence spectroscopy.[10-13]Among them, Fluorescent molecular probe-based techniques, due to their higher sensitivity and multiparametric nature, are particularly suitable for studying the microheterogenious nature and microenvironmental changes occurred between gel and sol phases of hydrogels.[14-17] In this study, polarity sensitive hydrophobic probe 8-anilino-1-naphthalene sulfonic acid (ANS) and environmental scanning electron microscopy (ESEM) were used to study the morphology, micropolarity, microviscosity and microenvironmental changes occurring between gel and sol phases of GTHCC supergelator (water: methanol (1:1)) (SI Figure 1).

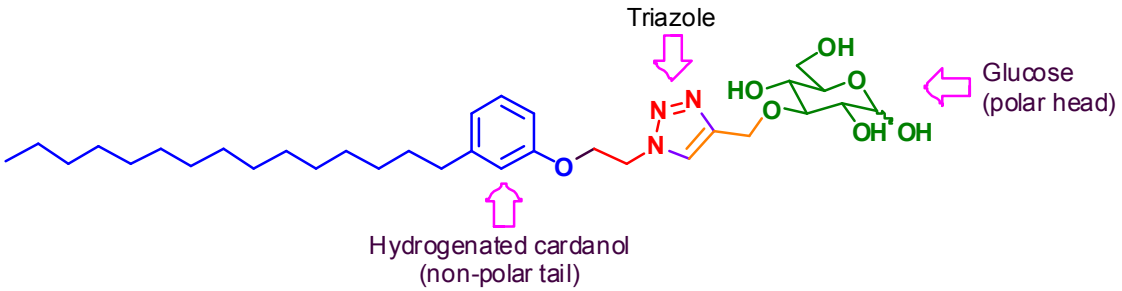

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Figure 1.Structure of the glucose-triazole-hydrogenated cardanol conjugates (GTHCC)

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Experimental Procedure

Material

8-Anilino-1-naphthalenesulfonic acid (ANS) was purchased from Sigma Chemical Co. (Bangalore, India). The Glucose triazole cardanol conjugate (GTHCC) was prepared according to the synthetic scheme disclosed in the previous report.[9] Spectroscopic grade methanol was used for the gelation studies. Triple-distilled water prepared using alkaline permanganate solution was used for the experiments.

Methods

Environmental scanning electron microscopy (ESEM) (Quanta 450) was employed to study the morphology of the hydrogel. The pressure was set at 4 Torr. Analyses were conducted at an accelerating voltage of 25 kV and a working distance range of 5 mm to 10 mm. Fluorescence emission and fluorescence anisotropy measurements were performed using Fluoromax-4 fluorescence spectrophotometer. The fluorescence lifetime measurements were carried out using Horiba Jobin-Yvon TCSPC lifetime instrument. 370 *nm* nano-LED was used as the light source for the experiments. The pulse repetition rate was set to 1 MHz, and the pulse width was ~1.1 *ns* for 370 *nm* LED. The instrument response function was collected using a scatterer (Ludox AS40 colloidal silica). The decay data were analyzed by a reconvolution method using DAS6 software of IBH. A value of χ^2, in-between 0.99 – 1.22 and symmetrical distribution of residual was considered as a good fit. The average fluorescence lifetime (τ_{avg}) values were calculated by the following equation[18]

$$\tau_{avg} = \left(\sum_{i=1}^{n} \alpha_i \tau_i\right) \Big/ \left(\sum_{i=1}^{n} \alpha_i\right)$$

Where τ_i is the individual lifetime with corresponding relative amplitude $\boldsymbol{\alpha}_i$.

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Estimation of micropolarity

A set of ANS solutions in UV-spectroscopic grade solvents tetrahydrofuran (THF), dioxane, dimethylsulfoxide (DMSO), acetonitrile, propanol, methanol were prepared. The fluorescence spectra were recorded and the emission frequency of ANS in different solvents was plotted as a function of E_T (30) [19] polarity scales for each solvent. The E_T(30) value for a solvent is defined as the transition energy for the longest wavelength absorption band of dissolved pyridinium-N-phenoxidebetaine dye, measured in kcal/mol. The emission maximum of ANS in GTHCC hydrogels (in cm^{-1} units) with variation of temperature was plotted with the respective solvent polarity values.

Estimation of microviscosity

The estimation of microviscosity of hydrogels by fluorescence methods involves the use of steady state fluorescence anisotropy and fluorescence lifetimes measurements. The steady state fluorescence anisotropy and the fluorescence lifetime are related to microviscosity by Perrin's equation.[18]

$$\frac{r_0}{r_{ss}} = 1 + C(r)\frac{T\tau}{\eta} \quad \text{.....(1)}$$

Where, r_{ss} is the observed steady state fluorescence anisotropy; r_0 is the limiting anisotropy of the probe in the absence of any depolarizing processes such as rotational diffusion or energy transfer; T is the temperature in Kelvin; τ is the fluorescence lifetime of the probe in seconds; and C(r) is a parameter which relates to the molecular shape and the location of the transition dipoles of the rotating fluorophores. Equation (1) can also be written as [21]

$$\eta = \frac{C(r)T\tau r_{ss}}{\delta r} \qquad \text{Where} \quad \delta r = r_0 - r_{ss}$$

The term η, often given in unit of mPa.s, gives the microviscosity of the immediate environment around the fluorophore. The C(r) value for ANS was calculated using Perrin's

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equation, taking the fluorescence anisotropy ($\mathbf{r_{ss}}$)of ANS in glycerol at 45 ^{o}C as 0.31,[20]the corresponding fluorescence lifetime ($\boldsymbol{\tau}$)as 7.60 ns[20] and the limiting anisotropy (r_0) as 0.4[21].The calculated C(r) value was 2.6 x 10^4 Pa. K^{-1}. $\boldsymbol{\eta}$ values of GTHCC gel in aqueous methanolic solvents at different temperatures were calculated using this value of C(r) and the fluorescence lifetime component (τ_2) corresponding to ANS in the hydrophobic environment of the microheterogeneous gel medium.

Gelation Test

The gelation ability of GTHCC in water: methanol mixture was determined by weighing 5 g/Lit of compound and added to water: methanol (1:1) solvent mixture. Then the mixture was heated till the solution become transparent and allowed to cool at 23 ^{o}C-20 ^{o}C (SI Figure 1). Gel formation was checked by the tube inversion method.[22]

Result and Discussion

Morphology Study:

Environmental scanning electron microscopy **(**ESEM) was generally used to study wet, insulating materials such as polymers, hydrogels and living cell etc. without dehydration or metal coating.[23,24] The ESEM images of GTHCC hydrogel (Figure. 2, SI Figure 2) are shows that the gel surface possesses uniformly distributed spongy structure with micrometer size pores inside it. The hydrophobic interaction of long chain cardanol hydrophobic group and hydrogen bonding of hydrophilic triazole-glucose group of GTHCC compound (Figure. 1) in polar solvent mixture (water: methanol (1:1)) play a crucial role in enhancing the strength of the gel and formation of 3D networks.[9] The formation of numerous micrometer size small pores may result in great solvent holding capacity.

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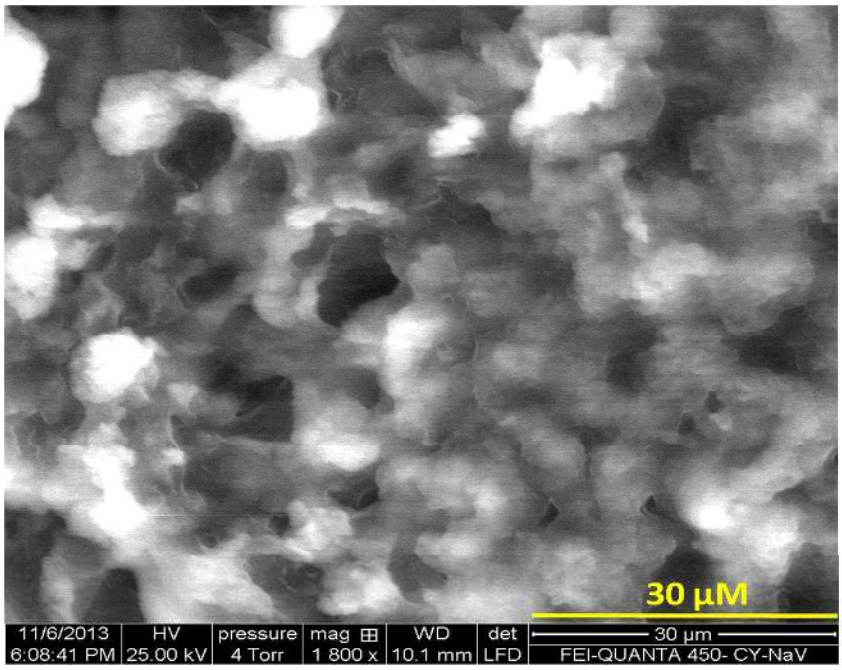

Figure 2. ESEM image of GTHCC gel in water: methanol (1:1)

Gelation processes in aqueous solvent are mostly driven by hydrophobic interactions.[1] Thus it would be informative to study the micropolarity and microviscosity properties of GTHCC hydrogel in water: methanol (1:1) mixture using hydrophobic fluorescent probe ANS with different fluorescence parameters.

Fluorescence Intensity Study:

The hydrophobic fluorescent molecular probe ANS has been widely used to sense the micropolarity of the various organized media like micelles, lipid bilayer membranes, hydrogels etc.[25-28] ANS is almost non-fluorescent in polar medium and highly fluorescent in non-polar medium.[27,28] Highly polar environment promotes the intramolecular charge transfer in ANS leads to smaller quantum yield and a red shift in emission but in a non polar environment absence of intramolecular charge transfer leads to high quantum yield and a blue shift in emission.[27,28]

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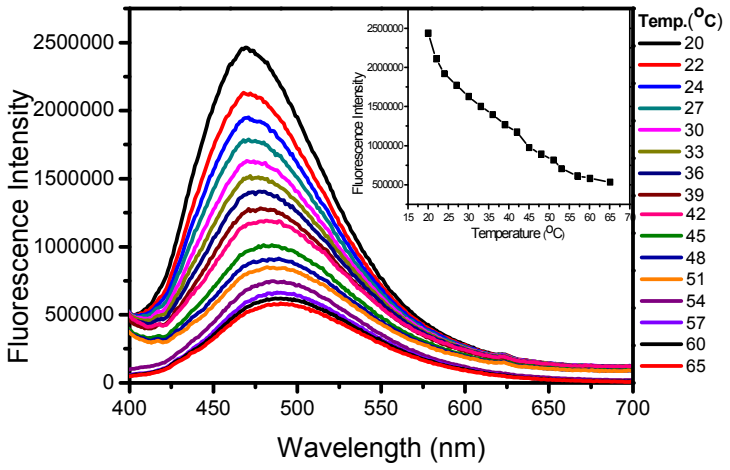

Figure 3. Plot of fluorescence Spectra of ANS in GTHCC hydrogel (water: methanol) with increase in temperature, ([ANS] = 4 μM, (λ_{ex}= 380 nm), Water: Methanol (1:1), [GTHCC] =2mg/mL.

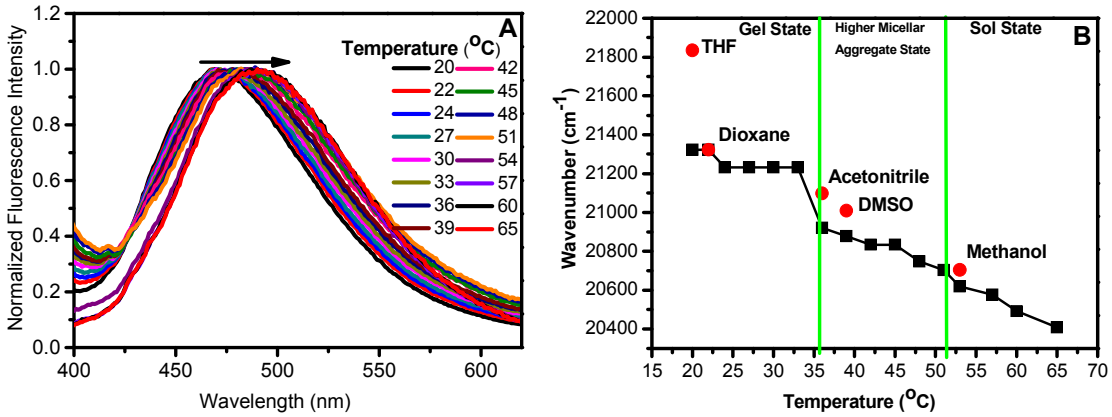

Figure 4.(A) Plot of normalized fluorescence Spectra of ANS in GTHCC hydrogel (water: methanol) with increase in temperature (B) Plot of fluorescence emission maximum (λ_{max}) of ANS against temperature in GTHCC hydrogel (water: methanol). [ANS] = 4 μM, (λ_{ex}= 380 nm), Water: Methanol (1:1), [GTHCC] =2mg/mL.

Figure 3 shows the fluorescence emission spectra of ANS in GTHCC hydrogel with increase in temperature. ANS in water: methanol (1:1) mixture shows a weak fluorescence emission at 502 nm (SI Figure 3). In the gel state, ANS shows a highly intense fluorescence emission. There is a decrease in emission intensity observed with increase in temperature (Figure 3). Figures 4 (A, B) shows the red shift in fluorescence emission maximum of ANS with increase in temperature. The fluorescence emission maximum of ANS is observed at

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468 nm in the gel state of GTHCC hydrogel (water: methanol (1:1)) at low temperature (20 ^{o}C) and with increase in temperature from 20 ^{o}C to 65 ^{o}C the fluorescence emission maximum shifted towards longer wavelength region from 21321 cm^{-1} (468 nm) – 20408 cm^{-1} (490 nm). The fluorescence of ANS as a good solvent polarity indicator is well known.[27,28]The quantification of micropolarity of GTHCC hydrogel microenvironments with increase in temperature was done by using ANS emission maximum (frequency cm^{-1}) in various solvents of different polarity (Figure 4B) and polarity E_T (30) parameter (SI Figure 4). [19] The polarity of the microenvironment form by GTHCC hydrogel is obtained from Figure 4B (SI Figure 4) indicates a non-polar hydrophobic microenvironment at lower temperature and slowly hydrogel meltdown to a more polar environment with increase in temperature.[19]The figure shows three distinct regions marked by changes in the slope: alcohol-like polarity above 52 ^{o}C, DMSO-like polarity between 38 and 52 ^{o}C and dioxane-like polarity below 38 ^{o}C. A study of fluorescence anisotropy and fluorescence lifetime of ANS in hydrogel microenvironments is expected to provide a clearer understanding of the issue.

Microviscosity Studies:

Microfluidity and microviscosity analysis give information regarding to the mobility of a molecule in a systems. Microfluidity describes the mobility of a single molecule undergoing diffusion inside the systems and microviscosity shows the internal resistance experienced by a molecule undergoing diffusion in a systems.[18,21] Figure 5 shows the variation of fluorescence anisotropy and microviscosity values of ANS in aqueous methanolic GTHCC gel medium with temperature.

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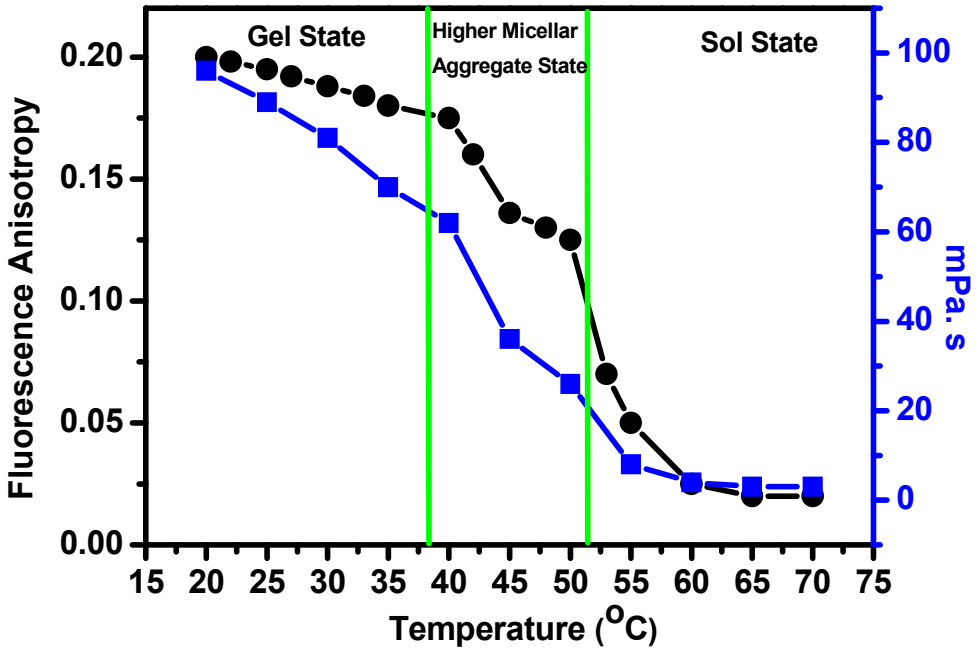

Figure 5. Plot for fluorescence anisotropy and microviscosity (mPa.s) of ANS in GTHCC hydrogel with variation of temperature, solvent taken water/methanol (1:1), (λex= 380 nm), ([GTHCC] =2mg/mL). (Error = 3%)

From a reasonably high value of r_{ss} (0.2) and the corresponding η (96 mPa.s) at 20 °C, the microviscosity values keep decreasing with increase in temperature and level off at very low values of r_{ss} (0.02) and η (3 mPa.s) above 65 °C. There is a reasonably good correlation between fluorescence anisotropy and corresponding microviscosity. Similar to the trend in figure 4, figure 5 shows three distinct regions marked by changes in the slope of variation of r_{ss} and η: above 52 °C, between 38 and 52 °C and below 38 °C.

It is interesting to note that both micropolarity (figure 4) and microviscosity studies (figure 5) indicate a close correspondence in their variation pattern with temperature, separable into the same three temperature ranges. These three regions closely correspond to three different states of aggregation of GTHCC in water/methanol (1:1) medium, as reported in a recent previous study[9].At low temperatures (~ 25 °C) GTHCC, known to be a super gelator in aqueous methanolic medium, forms non-flowing gel. ANS as a fluorescent molecular probe

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is known to report from the hydrophobic surface of a microheterogeneous system.[14,29] The presence of an extensive network of the hydrophobic surfaces required to support the hydrogel gel is clearly reflected through various fluorescence parameters of ANS: blue shift of emission maximum indicating non-polarity of the hydrophobic surface (dioxane-like in the E_T30 scale) and the appreciable high values of fluorescence lifetime (discussed subsequently) as well as fluorescence anisotropy indicating significant resistance to the motional dynamics of ANS at the interface.[14,29] The onset of gel melting at ~ 38^oC results in the formation of higher micellar aggregates[9]. In the temperature range 38 -52^oC, the breaking down of gel network increases the micropolarity and reduces the microviscosity around ANS. At temperatures above~52^oC the higher order aggregates breakdown to form simple micellar aggregation creating a transparent sol state[9]. Above 55 ^oC the extent of decrease of fluorescence anisotropy as well as microviscosity is fairly small and almost constant above 65 ^oC.

Fluorescence Lifetime Study:

Time resolved fluorescence decay dynamic is one of the most informative methods for studying the different microenvironments form in hydrogels during gelation process.[15] The ESEM images show the microheterogeneous nature of GTHCC hydrogel. Fluorescence lifetime of ANS is solvents and polarity sensitive.[31] Fluorescence lifetime of ANS decreases with the increase in polarity of the medium.[31] Thus fluorescence lifetime measurements of ANS would be a useful parameter to studies the microheterogeneity of the GTHCC hydrogel between gel and sol phase.

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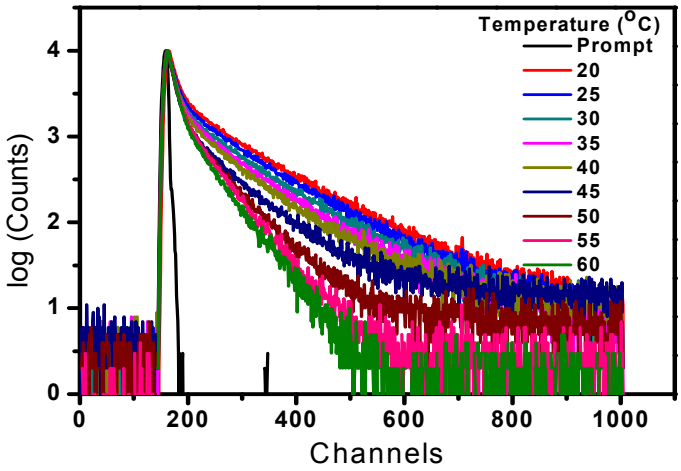

Figure 6. Fluorescence lifetime decay of ANS in GTHCC hydrogel {water: methanol (1:1)} with the variation in temperature; (λ_{ex} = 370 nm), [GTHCC] = 2 mg/mL, [ANS] = 4 μM, (Channels=0.11ns/chan.).

Table 1. Temperature dependence fluorescence lifetime data of ANS in GTC hydrogel {water: methanol (1:1)}; (λ_{ex} = 370 nm), [GTHCC] =2 mg/mL, [ANS] = 4 μM, (Channels=0.11ns/chan.). The corresponding residue distribution plots for fluorescence lifetime of ANS in GTHCC gel (water: methanol (1:1)) with the variation of temperature given in SI Figure 6.

Temperature (^{o} C)	τ_s(ns) (α_1)	τ_l (ns) (α_2)	τ_{avg}(ns)	χ^2
20	1.41(0.32)	12.63(0.68)	9.03	1.48
25	1.39(0.36)	12.20(0.64)	7.93	1.43
30	1.36(0.40)	11.61(0.60)	7.51	1.45
35	1.32(0.42)	10.63(0.58)	6.71	1.46
40	1.28(0.47)	9.80(0.53)	5.79	1.34
45	1.27(0.53)	8.64(0.47)	4.73	1.29
50	1.25(0.54)	6.86(0.46)	3.83	1.15
55	1.26(0.54)	6.07 (0.46)	3.47	1.22
60	1.21(0.57)	5.61(0.43)	3.10	1.02

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The fluorescence lifetime decay of ANS in GTHCC hydrogel (water: methanol (1:1)) shows a biexponential decay (Figure 6, Table 1). The two different lifetime components of ANS in GTHCC hydrogel clearly indicate the presence of two different microenvironments in the hydrogel. The lifetime decay of ANS in water: methanol (1:1) mixture shows a monoexponential decay with a lifetime of 1.18 ns (SI Figure 5), which is almost same as the lifetime of shorter lifetime components of ANS in GTHCC hydrogel (Table 1). Thus the shorter lifetime component of ANS in GTHCC hydrogel must be originated from the water: methanol (1:1) solvent medium. The longer lifetime component is expected to originate from the ANS bound to hydrophobic surface of GTHCC hydrogel, as surface bound ANS is known to have relatively lesser k_{nr} resulting in longer fluorescence lifetime and higher fluorescence intensity.[14, 29, 30] Figure 7A shows that the longer lifetime component of ANS decreases with increase in temperature where as the shorter lifetime component remains constant. This observation clearly indicates the changes in micropolarity of the microenvironments of GTHCC hydrogel between gel and sol phase.[31] The average fluorescence lifetime and fluorescence intensity of the ANS decreases with increase in temperature (Figure 7B), implying a possible increase in non-radiative energy loss of ANS in GTHCC hydrogel upon melting of gel.[31]

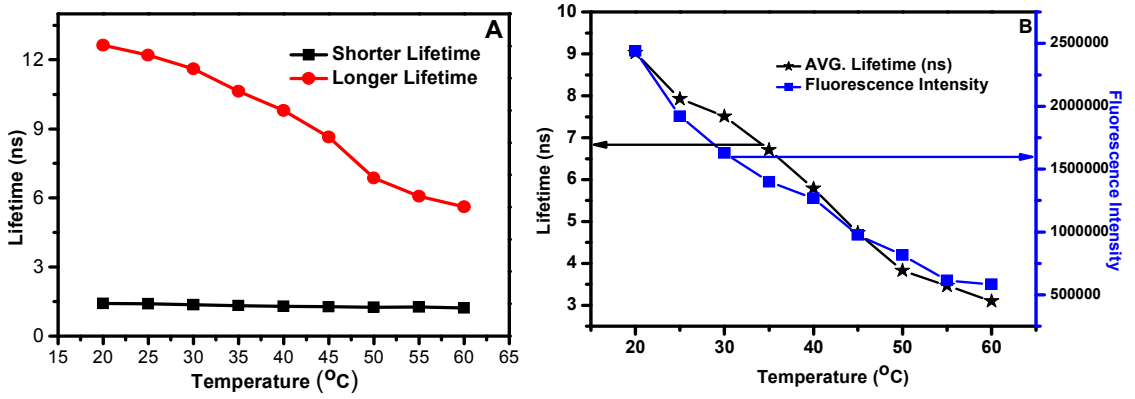

Figure 7.(A)Variation in the lifetime of longer and shorter fluorescence lifetime components of ANS decay with temperature in GTHCC hydrogel {water: methanol (1:1)} (B) Variation in the average

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lifetime and fluorescence intensity of ANS with temperature in GTHCC hydrogel {water: methanol (1:1)}; (λ_{ex} = 370 nm), [GTHCC] =2 mg/mL, [ANS] = 4 μM, (Channels=0.11ns/chan).

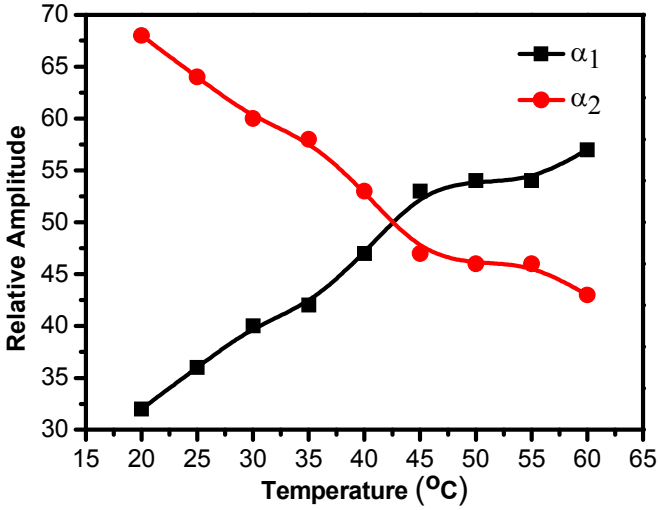

Figure 8. Variation in the relative amplitude of longer and shorter fluorescence lifetime components of ANS with temperature in GTHCC hydrogel {water: methanol (1:1)}; (λ_{ex} = 370 nm), [GTHCC] =2 mg/mL, [ANS] = 4 μM, (Channels=0.11ns/chan.).

Relative amplitudes of the fluorescence lifetime measurements indicate the fraction of emitted photon contributing towards emission from their respective environments. Figure 8 shows the changes in relative amplitudes of shorter and longer lifetime components of ANS in GTHCC hydrogel with increase in temperature (Table 1). Two different relative amplitudes of ANS indicate the distribution of emitting photon between the hydrophobic region and the solvent accessible region of the GTHCC hydrogel. In the gel state, the relative amplitude of shorter lifetime component (α_1) is less as compared to the relative amplitude of longer lifetime component (α_2) as the gel networks offers somewhat more rigid hydrophobic surface (supported by the microviscosity felt by the probes). α_1 increases and α_2 decreases with increase in temperature (Figure 8), indicating the melting of GTHCC hydrogel breaks the gel networks and free the bound solvent mixture in sol state at higher temperature leads to increase in the micropolarity of the medium.

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Conclusion

In this present work, we have used environmental scanning electron microscopy **(**ESEM) and different fluorescence parameters of 8-anilino-1-naphthalene sulfonic acid (ANS) to study the thermotropic gelation induced changes in micropolarity and microviscosity of GTHCC supergelator (water: methanol (1:1)). From a transparent micellar sol state at higher temperatures above 52 ^{o}C, progressive decrease in temperature results in progressive aggregation of GTHCC. The consequent progressive increase in hydrophobic domains is reflected in the concomitant decrease in micropolarity and increase in microviscosity as reported by two independent fluorescence parameters of ANS, hypsochromic shift of emission and increase of steady state fluorescence anisotropy. Time resolved fluorescence decay analysis showed the gelation process of GTHCC hydrogel involved in the formation of two different microenvironments: self-assembled molecular hydrophobic region and solvent accessible polar region, which was also supported by environmental scanning electron microscopy **(**ESEM).

Supporting Information

Demonstration of gel to sol transition process, ESEM image of GTHCC hydrogel in water: methanol (1:1), Fluorescence emission Spectra of ANS in water: methanol (1:1), Polarity E_T (30) plot, Fluorescence lifetime decay of ANS in water: methanol (1:1).

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