J Fluoresc (2011) 21:1871–1877
DOI 10.1007/s10895-011-0883-6

ORIGINAL PAPER

Thermal Phase Transitions of Agarose in Various Compositions: A Fluorescence Study

Selim Kara · Ertan Arda · Fahrettin Dolastir · Önder Pekcan

Received: 21 October 2010 / Accepted: 23 March 2011 / Published online: 31 March 2011
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Abstract The effect of agarose content on thermal phase transitions of the agarose gels was investigated by using Steady State Fluorescence (SSF) method. Scattered light, I_{sc} and fluorescence intensity, I_{fl} were monitored against temperature during heating and cooling processes to investigate phase transitions. Two regions were observed during the heating and cooling processes. At the high temperature region, double helix to coil (h-c) transition took place. However, during the cooling process coil to double helix (c-h) transitions occurred at low temperature region. Transition energies were determined using the Arrhenius treatment, and found to be strongly correlated with the agarose content in the gel system. Transition temperatures were determined from the derivative of the sigmoidal transition paths and found to be increased by increasing agarose content in both cases.

Keywords Fluorescence · Agarose · Phase transition · Gels · Scattering

Introduction

Agarose is a polysaccharide isolated from agar which is produced by Rhodophyceae-type red algae. Food industry, pharmacy, tissue engineering and chromatography are some application areas of agarose gels [1, 2] which is a characteristic member of biogels. Gelation and thermoreversible sol–gel processes of biomacromolecules involve intra- and inter-molecular hydrogen bondings, electrostatic and hydrophobic interactions leading to different supramolecular structures, and therefore are of high intrinsic interest [3, 4].

Molecular structure of agarose is consisting primarily of β-1, linked D-galactose and α-1,4-linked 3,6-anhydro-α-L-galactose, and contains a few ionized sulfate groups which are not exist in idealized neutral form [5, 6]. It forms physically bonded thermoreversible gels when dissolved in water. Agarose is insoluble in organic solvents, and can not form a gel structure due to absence of hydrogen bonding of aqueous solutions. Generally, gelation occurs at temperatures below 40 °C, whereas the sol-state temperature seems to be around 90 °C [7]. According to general opinion, gelation takes place by replacement of galactose residues of agarose chains. As a result, ordered regions are formed, which are called as "junctions". Various experimental techniques reveal that physical properties of the gel or sol states change in a great manner. According to a gelation mechanism proposed by Tako and Namura, intramolecular hydrogen bonding occurs below the temperatures of 60 °C, whereas below 40 °C intermolecular hydrogen bonding takes place [8]. In this system water molecules are also bonded. As a result, stiffness of the chains increases. At higher temperatures, a structural destruction occurs due to breaking of molecular bonds. Some other studies which propose double-stranded helix formation and single helical model [3, 6, 9, 10] also support this model [7, 11]. Relation between the processes of gelation and of spinodal decomposition in the sol state leading to the formation of polymer-rich and poor zones has also been studied [12–14]. X-ray diffraction [6, 13], light scattering [12, 13], optical rotation [3, 7, 12], differential

S. Kara (✉) · E. Arda · F. Dolastir
Faculty of Science, Department of Physics, Trakya University, 22030 Edirne, Turkey
e-mail: skara@trakya.edu.tr

Ö. Pekcan
Faculty of Arts and Science, Kadir Has University, 34320 Cibali, Istanbul

scanning calorimeter (DSC) [15], small-angle neutron scattering (SANS) [16, 17] and dynamic viscoelastic measurements [15, 18] are the some major techniques used in the inspection of agarose gels.

Agarose and agarose-like biogel systems (e.g. carrageenans) have been widely studied over the last several decades to produce specific properties for specific applications. For example, the kinetics and equilibrium processes of the sol–gel transitions of agar or agarose gels as well as the effect of gelation conditions on the gel's microstructure and rheological properties like the effect of salts and ions [1, 19] and influence of thermal history [18], pore-size determination [2, 20] have been studied in past few years. It was observed that gelation of agar molecules results in a large sigmoidal increase in the magnitude of the sol's shear modulus [21, 22]. On reheating, the gel structure is destroyed and during the gel–sol transition, the shear modulus follows another sigmoidal path back to its initial value, forming a hysteresis loop [23]. Typically, agarose gels have much greater hysteresis of melting and setting with temperature [6]. The photon transmission technique was employed to study the hysteresis phenomena during the sol–gel and gel-sol transitions in carrageenan-water system [24]. The cation effect on the sol–gel and gel-sol phase transitions and some hybrids of that system was also investigated by the same technique [25–27]. Recently, fluorescence technique was used to study thermal phase transitions of κ-carrageenan in various salt solutions [28, 29].

The purpose of this paper is to study the thermal phase transitions of agarose gel system at a molecular level by using fluorescence probe. Scattered light, I_{sc} and fluorescence intensity, I_{fl} were measured against temperature to monitor phase transitions and determine transition temperatures. Sol–gel and gel-sol phase transition energies were also determined. It is observed that both sol–gel and gel-sol transition temperatures and energies were found to be strongly correlated to the agarose content in the water–agarose system.

Experimental

Agarose Type 1-B (Sigma A0576) and pyranine (8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt, Fluka 56360) were used to prepare the gel samples by dissolving them in hot water without any further purification. Eight different agarose concentration (50, 75, 100, 150, 200, 250, 300 and 400 mg) gels were prepared during the experiments. Normally, agarose gels are in opaque white color at room temperature. However, when they are heated, their appearances change from opaque to transparent. On the other hand pyranine containing gel samples which are used in our experiments transform from opaque green to transparent green during heating. The pyranine concentration was kept at 2×10^{-4} M. An amount of 10 cc of distilled water was used for the preparation of the samples. During sample preparation, the heated agarose solutions were held at 90 °C and continuously stirred by a magnetic stirrer for a period of 20 mins. The measurements of fluorescence and scattering intensities were carried out using a Varian Cary Eclipse Fluorescence Spectrophotometer equipped with temperature controller. The pyranine in the samples was excited at 421 nm during in-situ experiments and variation in the fluorescence intensity was monitored at 511 nm as a function of temperature. The scattering intensities were collected at 421 nm. PMT voltage of the spectrometer was set to 600 Volts to obtain the optimum intensities. The excitation and emission spectra of pyranine with 100 mg agarose are shown in Fig. 1. Pyranine possesses a singlet ground state, which has not been affected by the agarose gel during the fluorescence measurements i.e. no spectral shift of pyranine's ground state is observed by the inclusion of the agarose.

Thermal phase transition observations were performed with a $1\times1\times4.5$ cm quartz cell equipped with a peltier type thermoelectric heat reservoir. The stirred sol state gels at 90 °C were rapidly transferred into the quartz cell. Before the measurements, the cell is first rapidly heated up to 95 °C and then cooled to 15 °C so that the sample in the cell was distributed uniformly. Then the sample was reheated up to 95 °C with the rate of 2 °C/min to observe the solid–liquid (gel–sol) transition. Cooling of the agarose sol from 95 °C to 15 °C was then performed at the same rate to detect the liquid–solid (sol–gel) transition. Both the scattered, I_{sc} and fluorescence intensities, I_{fl} were monitored against temperature. The measurements for every sample were repeated at least three times to prove the reproducibility. The maximum error on temperature measurements was found to be around 0.5 °C and a maximum of 2% error was detected during the repeated light intensity measurements due to the preparation and experimentation conditions of the samples.

Results and Dıscussıon

Temperature variation of I_{sc} and I_{fl} between15 to 95 °C for various agarose gels prepared with different concentrations is shown in Fig. 2a and b, respectively. In all cases, I_{sc} increased upon cooling of the agarose gels, indicating that the turbidity of the gel increased considerably (see Fig. 2a). During cooling, double helices are formed through the association of agarose molecules and then the double helices are aggregated to higher ordered assemblies to create a three dimensional network. During gelation, agarose-water system starts to form two phases with different network concentrations, which creates concentra-

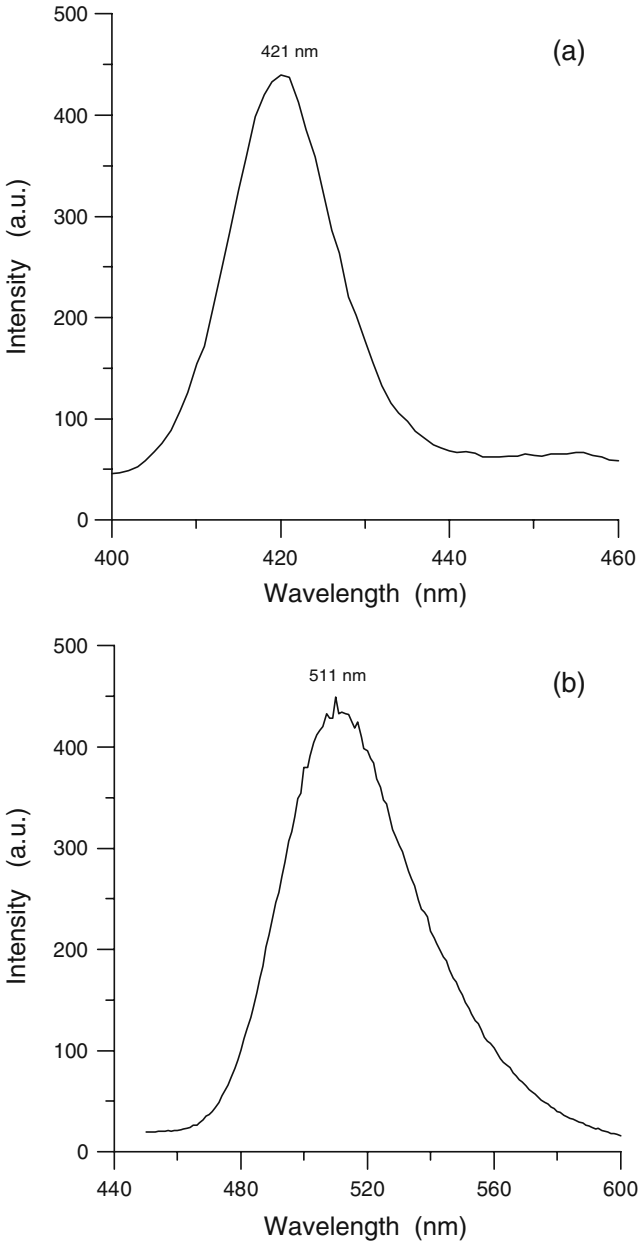

Fig. 1 **a** Excitation and **b** Emission spectra of pyranine in the 100 mg agarose gel sample

tion fluctuations. In other words, double helix aggregates are formed as a separate phase by excluding water from their domains. As a result, the contrast between agarose and water phases plays a role for scattering the light. On reheating, initially the double helix aggregates are destroyed and then the double helices are decomposed to agarose molecules which results in the destruction of the gel structure. As the agarose-water system becomes homogeneous, the scattered light intensity decreases (see Fig. 2a) and the system becomes fully transparent.

On the other hand, the fluorescence intensity, I_{fl} presented exactly the reverse behavior compared to I_{sc}. The temperature dependence of the fluorescence intensities, I_{fl} between 15 and 95 °C are plotted in Fig. 2b for all agarose samples. It is seen that fluorescence intensities, I_{fl} present a dramatic increase during heating for all samples under consideration. When the agarose samples were cooled, the fluorescence intensity, I_{fl} decreased dramatically by showing a nice hysteresis combined with sigmoidal transition curves i.e. low temperature back transition occurred.

Here, one expects to see the decrease in I_{fl} at high temperature, due to quenching of excited pyranine in the liquid-like, viscous medium i.e. to elaborate the above

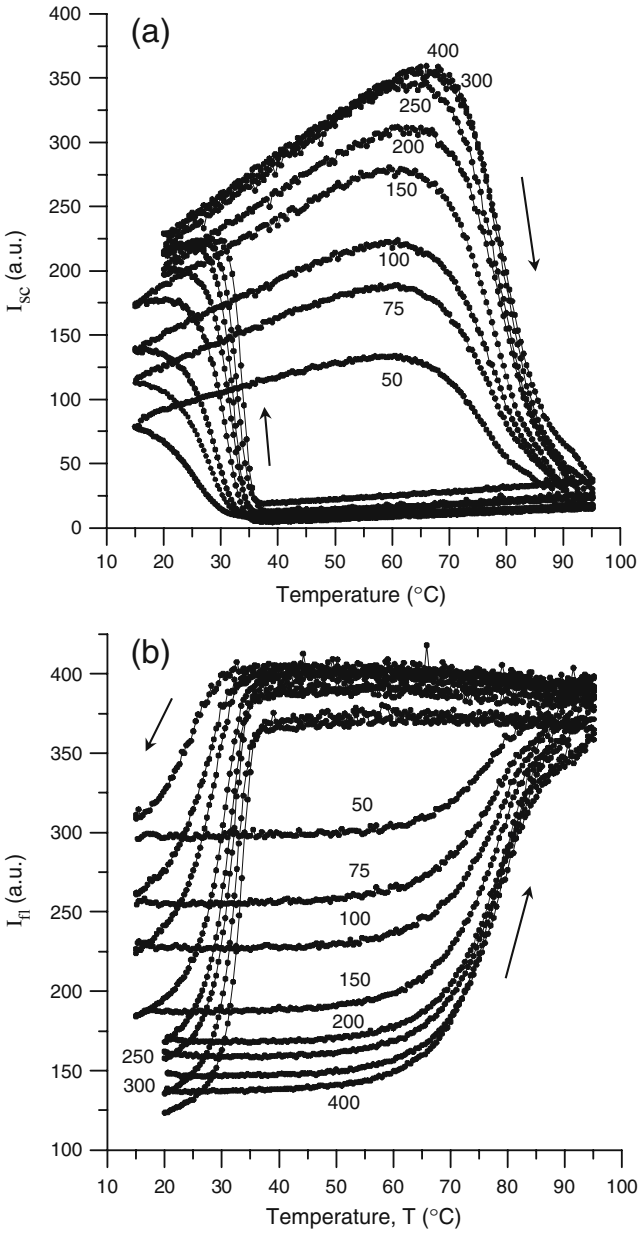

Fig. 2 **a** Scattering and **b** Fluorescence light intensities of the agarose gel samples during the heating and cooling processes. Numbers on each curve represents the agarose content in mg

results; the observed fluorescence intensity, I_{fl} has to be corrected by taking into account the behavior of scattered light intensity, to produce the real change in the fluorescence intensity due to environmental variations during thermal phase transitions. The corrected fluorescence intensity, I_{cor} can be obtained from the I_{fl}/I_{pn} ratio, where I_{pn} is the penetrated light which acts like a light source and it is assumed to behave like $1/I_{sc}$. The reason behind this correction is the variation of the turbidity and/or viscosity of the gel during phase transitions i.e. one has to produce the corrected fluorescence intensity, I_{cor} to eliminate the effect of physical appearance of the gel and to obtain the meaningful results for the fluorescence quenching mechanisms. Here, the observed fluorescence intensity, I_{fl} is in fact the convolution of the penetrated light intensity, I_{pn} and the desired fluorescence intensity (corrected intensity, I_{cor}) from the excited pyranine, where it is assumed that I_{pn} is inversely proportional to the scattered light intensity, I_{sc}. Fig. 3 presents the light intensities in the fluorescence cell and Fig. 4a and b show the behavior of the corrected fluorescence intensities during heating and cooling of the agarose-water system for the samples of 100 and 300 mg agarose content, respectively.

Usually, critical temperatures can be produced from the inflection points of the sigmoidal curves of thermal phase transitions [24, 25]. The sol–gel and gel-sol transition temperatures (T_{sg} and T_{gs}) were determined from the peak positions of the first derivative of the I_{cor} curves. The plots of dI_{cor}/dT versus T for all agarose samples are shown in Fig. 5a and b. It is known that the gelation process involves the transformation of agarose molecules from coil to helical conformation and the subsequent helix aggregation. Both the formation of the helices and helix aggregation occur in a narrow temperature range, resulting in a sharp dI_{cor}/dT

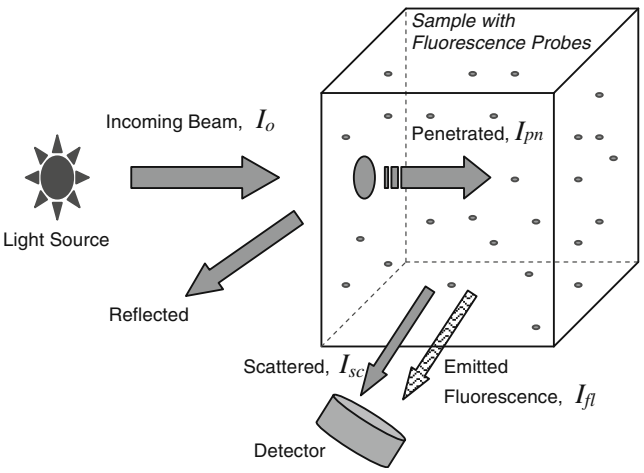

Fig. 3 Model principle of the light-sample interaction in the fluorescence cell, Penetrated (I_{pn}), Scattered (I_{sc}), Fluorescence (I_{fl}) and Corrected (I_{cor}) intensities

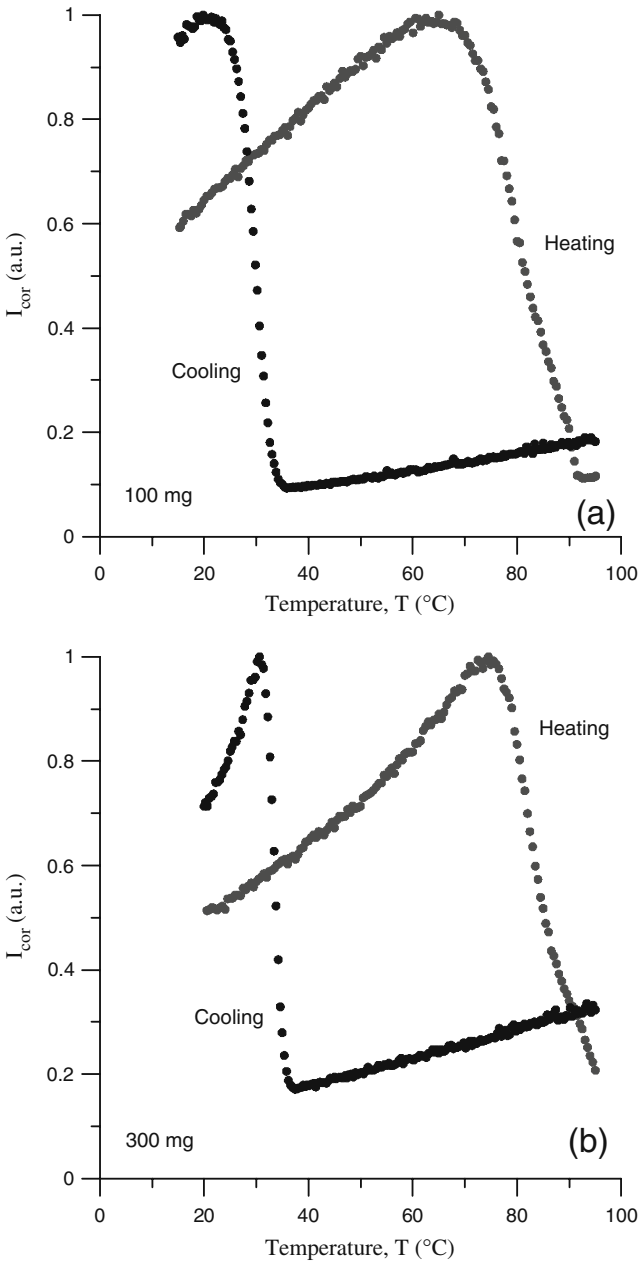

Fig. 4 Corrected Fluorescence Intensity, I_{cor} versus Temperature for heating and cooling processes of Agarose gel samples prepared with **a** 100 mg and **b** 300 mg

peak. On the other hand, melting of helical structure occurs in a broad temperature range. Figure 6a and b present the behavior of T_{gs} and T_{sg} temperatures versus agarose content, respectively, where the increase in agarose content resulted in an increase in T_{gs} and T_{sg} values. In other words, for high agarose content samples, the both sol–gel and gel-sol transitions require higher temperatures. It was observed that the value of T_{gs} is higher than that of T_{sg}, in agreement with previous optical rotation [30] and differential scanning calorimetry (DSC) [31] results on kappa carrageenan.

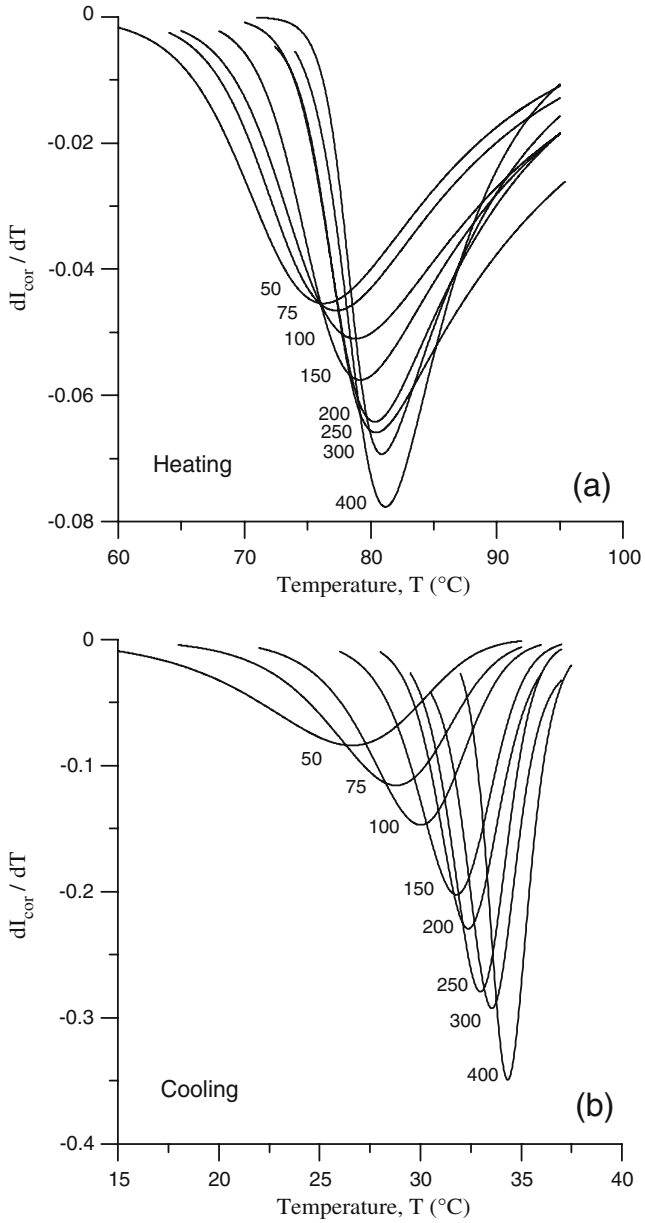

Fig. 5 Derivatives of the corrected fluorescence light, I_{cor} intensities with respect to temperature. The numbers on the curves present the agarose content in the sample in mg. The peak positions correspond to critical temperatures of **a** gel-sol (heating) and **b** sol–gel (cooling) transitions

Lower T_{sg} values compared to T_{gs} temperature are the origin of the hysteresis behavior during coil-helix and helix-coil transition loops. In other words, formation of helices from the coils is more possible and occurs at lower temperatures; however the disassociation of helices to coils requires higher temperatures. All these observations are in accord with the work of Arnott and his coworkers [7].

These thermal phase transitions can be explained by the energetic needs of coil-helix (c-h) and helix-coil (h-c) transitions. In order to produce these energy needs, Arrhenius treatment can be performed to the curves in Fig. 4 by using the following equation

$$I_{cor}(T) = I \exp(-\Delta E/kT) \quad (1)$$

where ΔE can be named as sol–gel and/or gel-sol transition energies, k is the Boltzmann constant and T is the temperature. The fits are presented in Fig. 7a and b for 100 and 300 mg agarose samples, respectively. Here assumption is made that molecular organization in agarose gel during thermal phase transition are monitored by fluorescence intensity. If system goes to the gel structure,

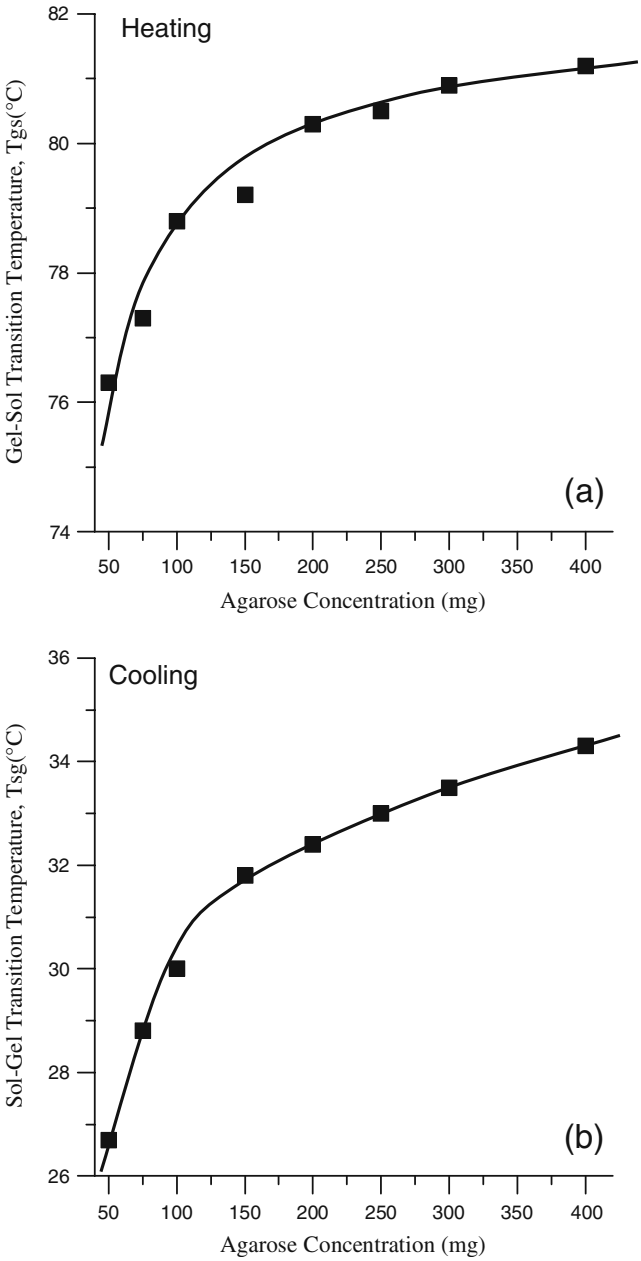

Fig. 6 Plot of **a** Gel-Sol and **b** Sol–gel Transition Temperatures versus agarose content obtained from I_{cor} data

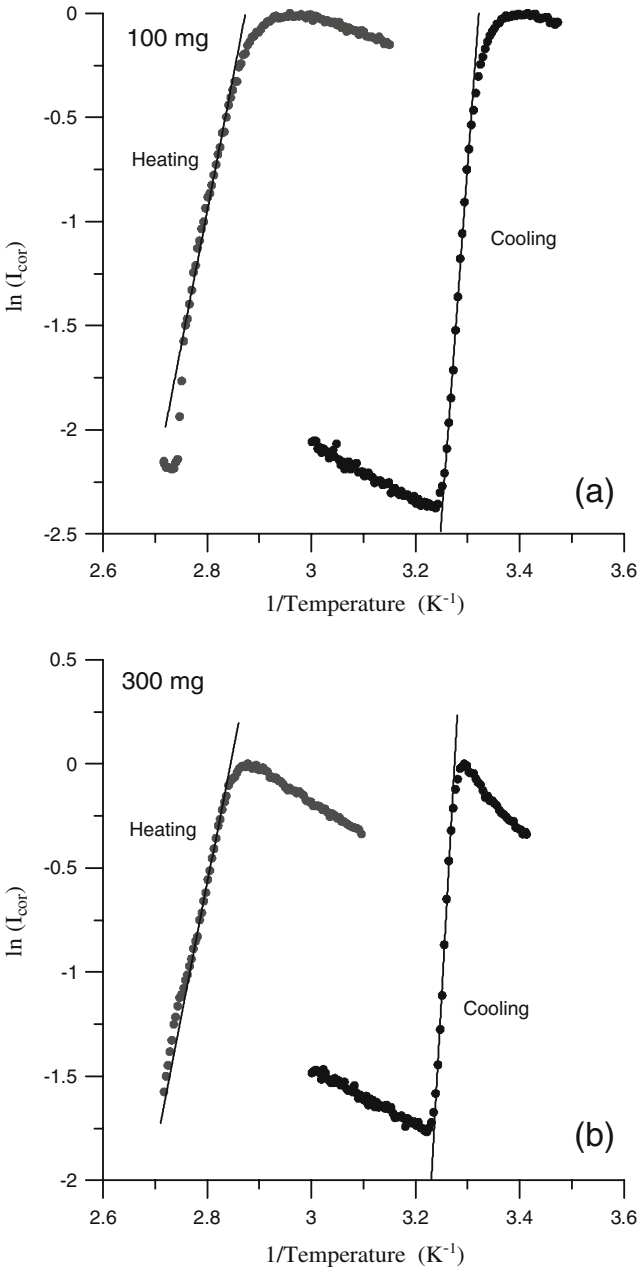

Fig. 7 Logarithms of Corrected Fluorescence Intensity, I_{cor} versus inverse Temperature for heating and cooling processes of Agarose gel samples prepared with **a** 100 mg and **b** 300 mg

I_{cor} increases; However, if system makes a back transition to the sol state then I_{cor} decreases. Under this assumption Eq. 1 now can be used to produce sol–gel and gel-sol transition energies. The produced energies are plotted versus agarose content in Fig. 8a and b for the heating (gel-sol) and cooling (sol–gel) processes, respectively. It is seen in Fig. 8a and b that gel-sol transition which is occurred at high temperature needs small energy to do this action, however sol–gel transition requires larger energy to perform its action at low temperature region.

Conclusion

All these results can be interpreted via model proposed by some authors [7, 11] and [3, 6, 9]. According to this model, there can be two levels of ordering of agarose in water. These orderings are in the form of double helices and coils. This model can be explained via the following scheme,

$$2H_2 \Leftrightarrow 4C \tag{2}$$

where C is the random coil and H_2 is the double helix. This model can predict the thermal phase transitions in Figs. 4 and 5. The coil to double helix (c-h) transition takes place during cooling, where I_{cor} increase dramatically at low

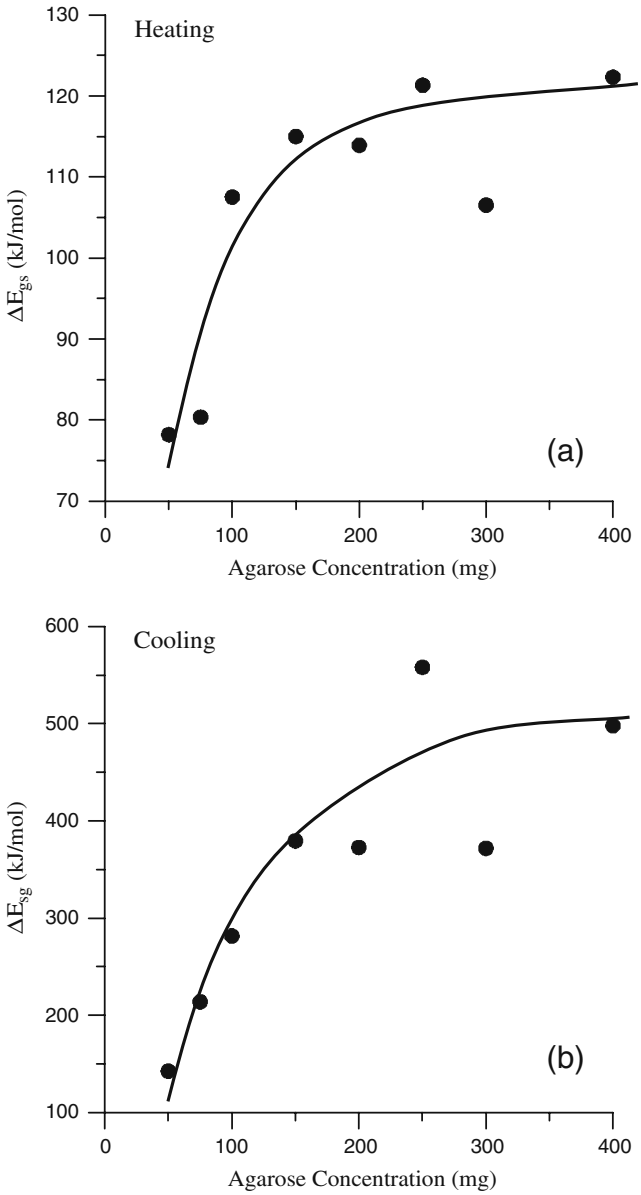

Fig. 8 Gel-sol and sol–gel transition energies obtained from I_{cor} data during **a** heating and **b** cooling processes

temperature region. In other words, during the (c-h) transition, the double helix aggregates form a separate phase by excluding water from their domains as a result agarose-water system forms two phases with different network concentrations. Quenching of excited pyranine molecules in this two phase systems has to cause increase in the fluorescence intensity due to their rigid environment i.e. increase in the corrected fluorescence intensity, I_{cor} predict that more rigid environment has been reached at low temperature, which results less quenching of excited pyranine molecules in this medium. When the system is heated back, the double helices are disappeared to the coils and system goes into the double helix-to-coil (h-c) transition. During (h-c) transition, decrease in I_{cor} can be explained by intensive quenching of pyranine molecules due to coiled-water environment.

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