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Phase Behavior of Agarose in Binary Solvents

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ABSTRACT: The thermoreversible gelation of agarose has been investigated in four different aqueous binary solvents: water/dimethyl sulfoxide, water/*N*,*N*-dimethylformamide, water/*N*-methylformamide, and water/formamide. The thermal behavior through the gelation/melting temperatures and the gelation/melting enthalpies has been studied, and phase diagrams have been subsequently established as a function of agarose concentration and solvent composition. The elastic modulus has been determined as a function of the same parameters. The results are discussed by considering different possible models. It is suggested that agarose/water/cosolvent ternary complexes can account for the experimental results.

Introduction

Thermoreversible gels prepared from agarose, a polysaccharide extracted from marine seaweeds, receive wide applications in biological sciences as a support for chromatography analysis but also as a medium for growing as perfect as possible protein single crystals.[1] Despite its every day use, both the gelation mechanism and the gel structure are still subjects of controversy. For instance, the helical conformation first proposed to be a double helix[2] is now seriously questioned. Foord and Atkins[3] claim that single helices can equally well account for the diffraction data, especially as those are relatively scarce. Similar conclusions have been recently reached from small-angle neutron scattering investigations carried out for determining the agarose chain conformation in the sol state.[4] The fact that the chains are very rigid with a conformation close to single helices as those proposed by Foord and Atkins has led one to call into question both the very existence of double helices and the gelation mechanism (helix alignment instead of helix intertwining[4]).

In addition, the same neutron scattering study[4] has also suggested that the formation of an agarose/solvent complex, in the present case with water and dimethyl sulfoxide (DMSO), is a possibility to be contemplated.

Investigations into thermoreversible gels produced from synthetic polymers have shown that the solvent type plays a major role in the gelation phenomenon.[5] It was accordingly felt that a similar study was timely if additional information were to be gained with agarose gels, especially for finding out whether the occurrence of agarose/solvent complexes was relevant or not. For this purpose, the gelation of agarose in a series of aqueous binary solvents has been carried out. Apart from DMSO, the three other solvents belong to a chemical series: *N*,*N*-dimethylformamide, *N*-methylformamide, and formamide. The knowledge of the existence of such complexes may have a direct bearing upon the determination of the appropriate crystallization conditions necessary for growing protein single crystals.

Experimental Section

(1) Materials. The two agarose samples used in this study are of differing molecular weights. They were kindly provided by Hispanagar (Burgos, Spain). Their molecular weights as determined by viscometry measurements ($[\eta] = 0.07M^{0.72}$ from ref 6) and their water content are as follows: AG1, $M_v = 1.12 \times 10^5$, water content = 12.7%; AG2, $M_v = 3.5 \times 10^4$, water content = 15.4%. The sulfate content provided by the manufacturer is 0.1%. The methyl content as measured by proton NMR is less than 0.7%. No L-galactose 6-sulfate was detected by ^{1}H NMR and ^{13}C NMR.

The solvents were purchased from Aldrich and were used without further purification. Bidistilled water was used. The following abbreviations will be employed throughout the paper: dimethyl sulfoxide, DMSO; *N*,*N*-dimethylformamide, DMF; *N*-methylformamide, MF; formamide, FOR.

(2) Thermal Analysis. The thermal analysis investigation was carried out on a DSC30 from Mettler using the TA9000 system for processing the raw data. Two types of systems were investigated so as to establish their binary (water/cosolvent) or ternary (agarose/water/cosolvent) phase diagrams:

(a) Water/Cosolvent Systems. Different water/cosolvent mixtures were prepared in test tubes beforehand. About 10 mg was then transferred into a "volatile sample" pan, which was hermetically sealed. The sample was then cooled to the required temperature at a rate of 2 °C/min in order to achieve crystallization. The different thermal events together with their corresponding enthalpy were obtained by heating the sample at a rate of 2 °C/min.

(b) Agarose/Water/Cosolvent Systems. Solutions of the desired concentrations were prepared in 20 cm^3 flasks by gently adding agarose powder to the binary solvent while stirring and then by heating to the appropriate temperature. Dissolution usually took about 20−30 min. Gels were obtained by a quench to room temperature (to −20 °C for the high contents of cosolvent). A piece of gel of approximately 20 mg was finally introduced into a "volatile sample" DSC pan. Once hermetically sealed, the pans were heated to melt the gel. After waiting for equilibration just above the gel melting point for 5 min, the solution was cooled at a rate of 2 °C/min and the DSC trace recorded to determine the gelation temperature T_{gel} together with the gel formation enthalpy ΔH_{gel}. After a minimum of 24 h of ageing, measurements were taken by heating the pan at a rate of 2 °C/min so as to obtain the gel melting temperature T_m and the melting enthalpy ΔH_m.

(3) Mechanical Testing. Determination of the elastic modulus *E* was achieved on an Instron 4301 through the analysis of the stress−strain curves obtained on compression. The following relation was used:

$$\sigma = E \times \epsilon \qquad (1)$$

in which ϵ is the strain ($\epsilon = (l - l_0)/l_0$, where l_0 is the sample's

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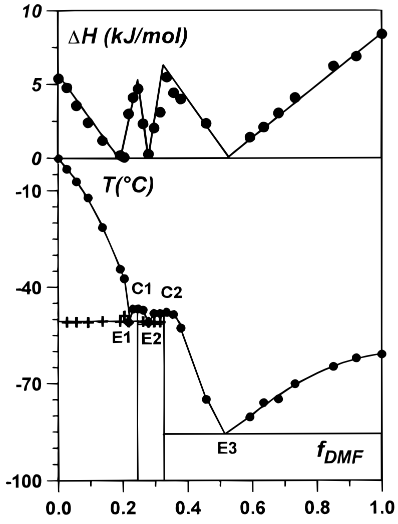

Figure 1. Temperature–concentration phase diagram for *N*,*N*-dimethylformamide/water (below) and Tamman's diagram (above). The same symbol is used for corresponding enthalpies and transition temperatures. Two complexes, C_1 and C_2, and three eutectics, E_1, E_2, and E_3, can be seen. The concentration is given in mole fraction.

initial height) and σ the stress. Experiments were carried out at a speed of 10 mm/min.

Gels were prepared by pouring hot, homogeneous solutions into a cylindrical test tube of 17-mm diameter and about 15-cm length. The gel cylinders were then guillotined out with a razor blade so as to produce samples of 17-mm length and 17-mm diameter suitable for mechanical measurements. A special setup described elsewhere[7] was used for cutting the gels in order to obtain highly parallel faces.

Results

I. Phase Diagrams. (1) Binary Phase Diagrams: Water/Cosolvent Systems. The water/DMSO phase diagram was already established by Rasmussen and MacKenzie.[8] These authors came to the conclusions that two complexes are formed with the following stoichiometries: $1DMSO/2H_2O$ and $1DMSO/3H_2O$. There exist converging indications that the interactions are strong enough to be kept in the liquid state.[9]

We have established the phase diagrams for the three other systems, i.e., DMF/water, MF/water, and FOR/water.

In the case of DMF/water, the phase diagram shows the existence of two *congruently-melting compounds* with the following stoichiometries: $1DMF/3H_2O$ and $1DMF/2H_2O$ (see Figure 1). The stoichiometries are confirmed by the Tamman's plots. Similar studies had been carried out by Kessler et al.[10] on the same mixture. These authors, however, missed the $1DMF/2H_2O$ compound. The water/DMF system is decidedly quite similar to the water/DMSO system in that they form congruently-melting compounds of identical stoichiometries.

As can be seen in Figure 2, the system water/MF exhibits the occurrence of a compound characterized by a singular point (intermediate case between a *congruently-melting* and an *incongruently-melting* compound). The stoichiometry of this compound is $1MF/2H_2O$ as ascertained by the Tamman's plot.

The system water/FOR, unlike the other three systems, does not form any compound. Figure 3 shows the occurrence of a simple eutectic with no solid solutions, which means that formamide molecules cannot incorporate into the crystalline lattice of water and vice versa. The eutectic composition is about 1/1. This result agrees with findings of Kessler et al.[11] These authors report also the occurrence of a peritectic but we did not see anything of the kind during our investigations.

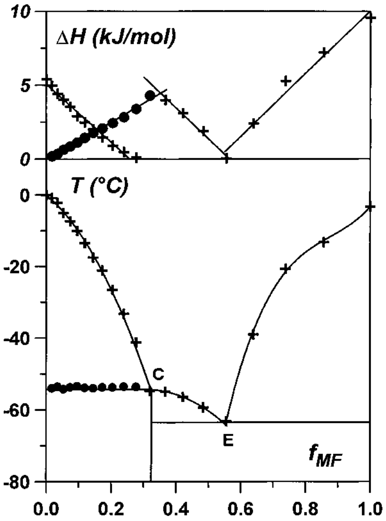

Figure 2. Temperature–concentration phase diagram for *N*-methylformamide/water (below) and Tamman's diagram (above). The same symbol is used for corresponding enthalpies and transition temperatures. One complex (singular point) and one eutectic are seen. The concentration is given in mole fraction.

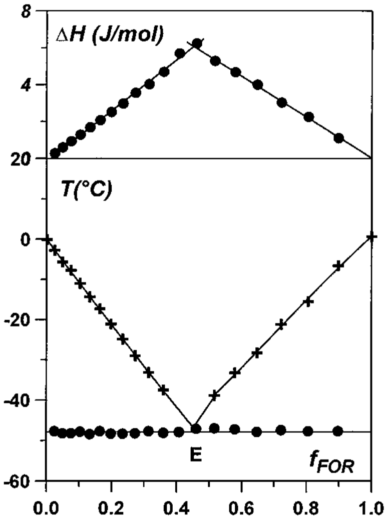

Figure 3. Temperature–concentration phase diagram for formamide/water (below) and Tamman's diagram (above). The same symbol is used for corresponding enthalpies and transition temperatures. Only a eutectic transformation is seen. The concentration is given in mole fraction.

Worth noticing is that gradual replacement of CH_3 groups by hydrogen atoms on the organic solvents under consideration strongly modifies their propensity to form compounds with water. When no CH_3 group remains, as is the case for formamide, compound formation no longer exists. It is known that CH_3 groups tend to repel electrons, leading to the appearance of fractional charges on the oxygen atoms of the C=O and the S=O groups. The more CH_3 groups there are, the higher the magnitude of these fractional charges. It may be so that complexation with water should be favored when increasing the value of these fractional charges. Cowie and Toporowski came up with the same conclusions for the mixture water/DMSO.[9]

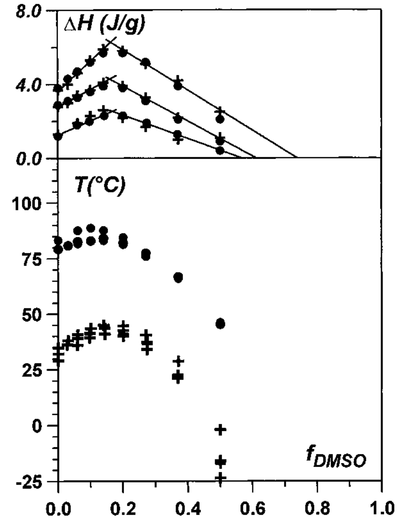

Figure 4. Temperature–solvent composition phase diagram for agarose/water/dimethyl sulfoxide gels (below). Gelation and melting temperatures are displayed. Tamman's diagram is drawn above for gelation (●) and melting (+) enthalpies from 5 to 15% agarose concentrations (sample AG2). Compositions are given in mole fraction.

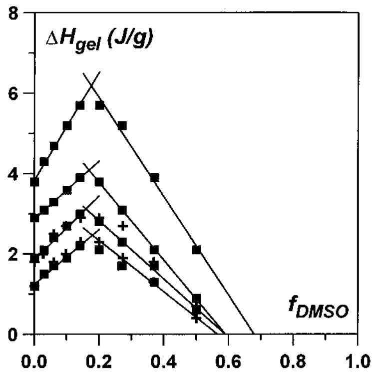

Figure 5. Gelation enthalpies obtained for different agarose concentrations (5, 7, 10, and 15%, respectively) and agarose samples: (+) AG1 ($M_w = 1.12 \times 10^5$); (■) AG2 ($M_w = 3.5 \times 10^4$).

Conversely, the complexation propensity with water does not seem to be linked to the dielectric constant[12] as this parameter decreases from formamide (110) to DMF (37) (the measured dielectric constant for MF is much larger due to associations and does not reflect the intrinsic value). Manifestly, complex formation is not linked to hydrogen bondings as it is seen to increase in spite of the elimination of the hydrogen bonds formed by the amino hydrogens.

(2) Ternary Phase Diagrams: Agarose/Water/Cosolvent Gels. As expected from previous works,[4,13] the gel melting point in *agarose/water/DMSO gels* goes through a maximum for a composition of about 0.16 mole fraction of DMSO (see Figure 4). Similarly, the gel formation and the gel melting enthalpies, represented above the temperature–composition diagram of Figure 4, are maximum at about the same composition (0.16 mole fraction approximately). Gel melting enthalpies and gel formation enthalpies are virtually identical. They can be fitted on either side of the maximum by straight lines. Also, these results are not dependent upon the agarose molecular weight, as can be seen in Figure 5. This shows that up to an agarose concentration of 150 g/L (0.15 g/cm^3) the maximum of the enthalpy as a function of concentration is still visible at about the same binary solvent composition. As already reported by several authors,[13–15] the gel melting point and the gelation temperature are little dependent upon the agarose concentration. In all the composition range, the undercooling, that is the difference between the melting temperature and the gelation temperature, is about $\Delta T = 50$ °C.

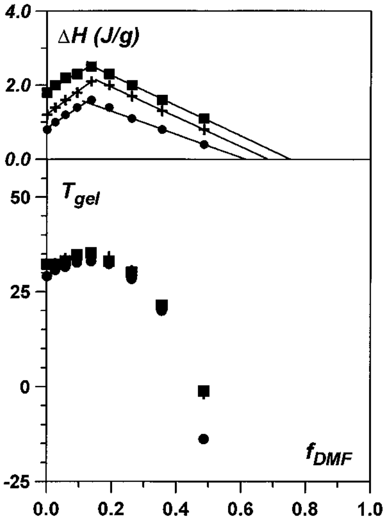

Figure 6. Temperature–solvent composition phase diagram for agarose/water/*N*,*N*-dimethylformamide gels (below). Only gelation temperatures are displayed. Tamman's diagram is drawn above for enthalpies from 3, 5, and 7% agarose concentrations (sample AG1). Compositions are given in mole fraction.

Similar results are observed for gels produced from *agarose/water/DMF* mixtures (Figure 6). Here only the gelation temperature and gelation enthalpy have been investigated. A maximum for these two parameters is seen for a composition of about 0.15 mole fraction of DMF. Again the enthalpies vary linearly on either side of the maximum.

The case of gels produced from *agarose/water/MF* mixtures possesses one unique feature in this series. While a maximum is observed for the gelation and melting enthalpies, peaking at about 0.17 mole fraction of MF with a linear variation on either side of the maximum, no such behavior is observed with the gelation and the melting point (Figure 7). These points decrease monotonously on increasing the MF content. Here again the undercooling is about the same as in agarose/water/DMSO gels and agarose/water/DMF gels.

Finally, neither the melting/gelation temperatures nor the melting/gelation enthalpies exhibit any maxima in the agarose/water/FOR system (Figure 8). While gelation and melting temperatures display a monotonous decay with increasing formamide content, the corresponding enthalpies are virtually constant.

II. Elastic Modulus. The determination of the elastic modulus was achieved in the four organic solvents presented above, for different water/cosolvent compositions and for agarose concentrations ranging from 2 to 20 g/L (2×10^{-3} to 2×10^{-2} g/cm^3).

Typically, the modulus does not virtually depend upon the organic solvent nor does it depend upon the composition of the binary mixture. Here two plots are given to illustrate this statement: the first one gives results obtained in a binary solvent exhibiting a maximum for both the gel melting point and the gel formation/melting enthalpy (water/DMF) together with results obtained in a binary solvent wherein such maxima are not seen (water/FOR); the second plot shows the effect of the binary solvent composition.

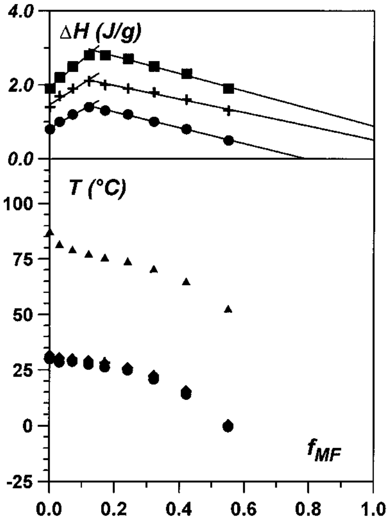

Figure 7. Temperature–solvent composition phase diagram for agarose/water/*N*-methylformamide gels (below). Gelation and melting temperatures are displayed. Tamman's diagram is drawn above for enthalpies from 3, 5, and 7% agarose concentrations (sample AG1). Compositions are given in mole fraction.

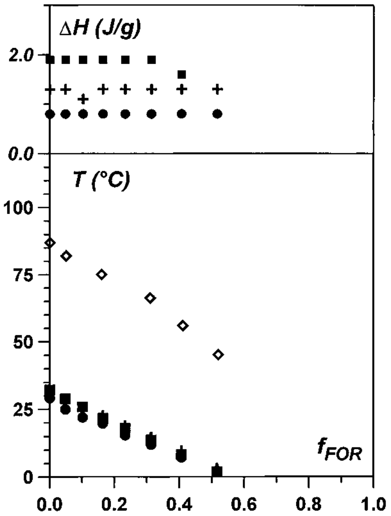

Figure 8. Temperature–solvent composition phase diagram for agarose/water/formamide gels (below). Gelation and melting temperatures are displayed. Tamman's diagram is drawn above for enthalpies from 3, 5, and 7% agarose concentrations (sample AG1). Compositions are given in mole fraction.

Figure 9 displays the modulus–concentration relation determined in water/DMF and water/FOR at a composition corresponding, in both solvents, to the mole fraction where a maximum of melting point and enthalpy is seen for water/DMF ($f_{DMF} = 0.13$). As can be seen, the discrepancy for moduli in either binary solvent is markedly low. Gels in water/DMF possess moduli slightly higher than those produced in water/FOR by about 20–30%.

In Figure 10, where a series of data obtained from agarose/water/MF gels are plotted, one perceives that the modulus can be regarded independent of the solvent composition.

In this range of agarose concentrations, a power variation is observed with an exponent of 2.1 ± 0.1, whose meaning has already been discussed elsewhere.[4]

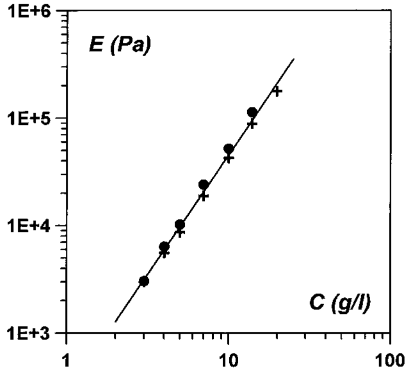

Figure 9. Modulus–concentration relation for agarose gels prepared in water/formamide (●) and water/*N*,*N*-dimethylformamide (+). In both cases, the solvent composition (mol fraction of cosolvent) is $f = 0.13$.

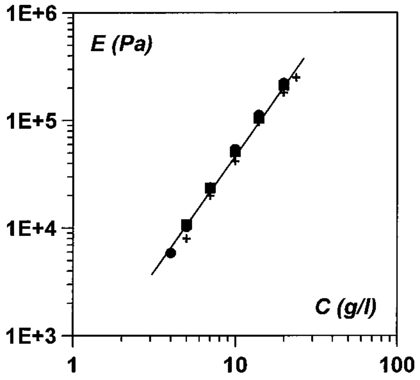

Figure 10. Modulus–concentration relation for agarose gels prepared in *N*-methylformamide. Solvent compositions (mole fraction of cosolvent) are $f = 0.12$ (●), $f = 0.24$ (■), and $f = 0.42$ (+).

Discussion

The maximum seen for the gel melting temperature as a function of solvent composition could be, in principle, accounted for by simply considering the three Flory interaction parameters. In the case of a polymer crystalline phase (subscript p) embedded in a binary solvent (subscripts 1 and 2), Flory has derived a relation between the melting point of the pure polymer, T_m°, and the actual melting temperature, $T_m^{1,2}$, at a given polymer volume fraction φ_p which is written[16,17]

$$\frac{1}{T_m^{1,2}} - \frac{1}{T_m^\circ} = -\frac{RV_p}{\Delta H_p V_1} F(\varphi_i,\chi_{ij}) \qquad (2)$$

in which

$$F(\varphi_i,\chi_{ij}) = -\varphi_1 - \frac{\varphi_2}{x_2} + \left(\varphi_1\chi_{1p} + \chi_{2p}\frac{\varphi_2}{x_2}\right)(1 - \varphi_p) - \chi_{12}\varphi_1\varphi_2 \qquad (3)$$

in which R is the gas constant, ΔH_p is the polymer melting enthalpy per mole, V_p and V_1 are the molar volume of the polymer and solvent S1, χ_{ij} are the different interaction parameters, x_2 is the ratio of the molar volumes of solvents S1 and S2, and φ_i are the different volume fractions related through

$$\varphi_p + \varphi_1 + \varphi_2 = 1 \qquad (4)$$

Relation 2 is only valid when no solvent is occluded within the polymer crystalline lattice, that is when no polymer–solvent complex is formed. The formation of such type of complex cannot be predicted by Flory's theory as the starting assumptions definitely preclude

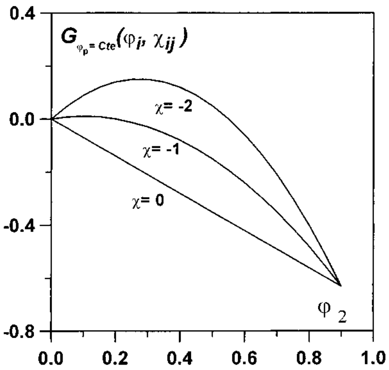

Figure 11. Variation of the function $G_{\varphi_p=\mathrm{Cte}}(\varphi_i,\chi_{ij})$ as a function of the volume fraction of cosolvent for an agarose volume fraction $\varphi_p = 0.1$. χ_{12} parameters as indicated.

this case. Further, the crystal size should be infinite although the relation applies if this size is not dependent upon solvent composition.

Upon increasing the binary solvent volume fraction, i.e. $\varphi_1 + \varphi_2$, the polymer melting point $T_m^{1,2}$ should decrease. Upon altering the composition of the binary solvent while keeping the polymer volume fraction constant, two cases are worth considering:

(i) The solvent 1−solvent 2 interaction is weak. As a result, the melting temperature varies monotonously with solvent composition. If one increases the content of the best solvent, then the melting temperature will decrease and vice versa.

(ii) The solvents exhibit strong interaction. If the interaction parameter χ_{12} is negative and large enough, then the melting point can be seen to go through a maximum upon altering the composition of the binary solvent. This can be evaluated by examining *at constant agarose volume fraction* the melting point depression in one solvent related to the melting point depression in the binary mixture. In one solvent, the melting point depression is written

$$\frac{1}{T_m^1} - \frac{1}{T_m^\circ} = -\frac{RV_p}{\Delta H_p V_1}[-(1-\varphi_p) + \chi_{1p}(1-\varphi_p)^2] \quad (5)$$

Subtracting (5) from (2) gives the melting point depression with respect to the pure solvent at constant agarose volume fraction:

$$\frac{1}{T_m^{1,2}} - \frac{1}{T_m^1} = -\frac{RV_p}{\Delta H_p V_1} G_{\varphi_p=\mathrm{Cte}}(\varphi_i,\chi_{ij}) \quad (6)$$

in which the function $G_{\varphi_p=\mathrm{Cte}}(\varphi_i,\chi_{ij})$ is written

$$G_{\varphi_p=\mathrm{Cte}}(\varphi_i,\chi_{ij}) = -\varphi_1 - \frac{\varphi_2}{x_2} - \chi_{12}\varphi_1\varphi_2 + \left(\varphi_1\chi_{1p} + \chi_{2p}\frac{\varphi_2}{x_2} + 1\right)(1-\varphi_p) - \chi_{1p}(1-\varphi_p)^2 \quad (7)$$

In order to obtain $T_m^{1,2} > T_m^1$, it suffices that this function be positive. An example for the variation of $G_{\varphi_p=\mathrm{Cte}}(\varphi_i,\chi_{ij})$ with volume fraction of one of the solvents is given in Figure 11. By choosing a hypothetical binary solvent consisting of a poor solvent ($\chi_{1p} = 1$) and of a good solvent powerful enough to dissolve crystals at room temperature ($\chi_{2p} = -1$), it can be seen that part of the function $G_{\varphi_p=\mathrm{Cte}}(\varphi_i,\chi_{ij})$ becomes positive for $\chi_{12} > -1$. In other words, the binary solvent behaves as a poorer solvent than the poorest solvent taken alone.

The latter case has been contemplated by Watase and Nishinari[13] to account for the increase of agarose melting point in water/DMSO mixtures. On the basis of the reported existence of strong DMSO−water associations already present in the liquid state,[9] these authors assume that the interaction between water and DMSO is strong enough to produce the melting point behavior seen in DMSO−water mixtures.

Not only does the melting point behavior show a maximum, but so does the gel melting enthalpy. The increase of magnitude of ΔH can reach factors of 1.5−2 depending upon agarose concentration. Watase and Nishinari argue that this indicates an increase of "crystallinity" of the agarose phase (i.e. the agarose-rich phase, which is the framework of the gel) as ΔH is supposed to be directly related to this parameter of the gel sample. To increase the degree of "crystallinity" to this extent would require in the agarose/water gels either the existence of a high concentration in the agarose-dilute phase or high disorder in the agarose-rich phase so that gradual addition of DMSO would produce either an increase of the network concentration or an ordering of the original amorphous parts. Both processes should markedly affect the gel mechanical properties such as the magnitude of the elastic modulus, which is directly linked to the concentration of the agarose-rich phase and to its rigidity. Increasing gel "crystallinity" should, therefore, result in a substantial increase in elastic modulus. In fact, altering the composition of the binary solvent is seen to have no significant effect on the mechanical properties of water/cosolvent gels. The results reported here also show virtually no difference between gels prepared from three different water/MF compositions in a large range of agarose concentrations. Similarly, the nature of the organic solvent has practically no influence on the value of the elastic modulus; only a slight downward shift of about 20−30% is observed in agarose/water/FOR gels.

Another experimental observation deserves discussion. In the system *agarose/water/methylformamide*, the gel formation and gel melting enthalpies go through a maximum while the melting and the gelation points do not. Evidently, melting point behavior points toward an overall increase of solvent quality as opposed to enthalpy variation. In our opinion, this set of remarks should lead one to question seriously the relevancy of Watase and Nishinari's view to account for the behavior of agarose gels prepared from binary solvents. Clearly, while the melting point behavior could be understood in the framework of relation 2, a hypothetical increase of "crystallinity" to the extent required here appears to be unrealistic.

We shall now present an alternative view which considers the formation of *ternary agarose/water/organic solvent complexes* for explaining the existence of maxima, particularly with the melting/gelation enthalpies. We therefore suggest that *ternary complexes* are formed with DMSO, DMF, and MF while no complex occurs with FOR (only agarose/water complex will be considered here).

The basis for considering complexes relies in the first place upon recent neutron scattering data obtained while studying the agarose chain conformation in water/DMSO mixtures.[4] These results showed that the experimental mass per unit length has actually an apparent value much lower than the lowest value achievable for agarose under a hypothetical all-extended conformation. Such a result can be accounted for by contemplating the existence of agarose/solvent complexes.

Other hints point toward the existence of complexes, particularly with water, such as the residual amount of water with "dried" agarose. As is given in the Experimental Section, our Samples contain about 14 ± 1.5% of residual water. This translated into moles of water per mole of agarose gives a figure of about 3. Taking into account the paucity of the diffraction patterns from agarose gels, the existence of an *agarose/water complex* cannot be dismissed and remains therefore a possibility to be explored.

Another set of arguments relying upon the binary systems (water/cosolvent) can be put forward for supporting the existence of such complexes. Since DMSO, DMF, and MF are capable of forming compounds with water, why should they not do so with agarose, the molecular structure of which contains four OH groups per glucosidic residue? If, as will be explained below, complex formation is highlighted through the presence of a maximum in the gelation/melting enthalpies, then there is a remarkable coincidence with the corresponding binary systems: complex formation occurs in water/DMSO, water/DMF, and water/MF but not in water/FOR.

The thermal behavior can be explained by means of a hypothetical ternary phase diagram as represented in Figure 12 for a given temperature. In this diagram, a minimum of three hypothetical complexes is considered: one *agarose/water complex* defined at C_w and two *agarose/water/cosolvent complexes* defined at C_{ws1} and C_{ws2}. On the line $L_{ws}C_{ws1}$, there exist two phases, *agarose/water/cosolvent complex 1 + liquid*, while on the line C_wC_{ws}, two complexes, *agarose/water complex + agarose/water/cosolvent complex 1*, can be formed. Finally, on the dashed line $C_{ws1}C_{ws2}$, two complexes, *agarose/water/cosolvent complex 1 + agarose/water/cosolvent complex 2*, should be present. Under this set of conditions, a system at a concentration defined at point O will produce three phases: an *agarose/water complex* phase, an *agarose/water/cosolvent complex 1* phase, and a *liquid* phase. By applying the lever rule, the following ratios give the proportion of each phase in the sample:

$$\phi_{\mathrm{w}} = \frac{\mathrm{Oa}}{\mathrm{Oa} + \mathrm{Ob} + \mathrm{Oc}}$$

$$\phi_{\mathrm{ws1}} = \frac{\mathrm{Oc}}{\mathrm{Oa} + \mathrm{Ob} + \mathrm{Oc}} \quad \phi_{\mathrm{liq}} = \frac{\mathrm{Ob}}{\mathrm{Oa} + \mathrm{Ob} + \mathrm{Oc}} \tag{8}$$

At this point, we shall make three assumptions:

(i) the number of moles of water and of moles of water + cosolvent is the same for both complexes. This means that complex C_{ws1} is produced by replacing *n* moles of water by *n* moles of cosolvent. As a result, the line C_wC_{ws1} is parallel to the H_2O/solvent axis.

(ii) The liquid phase produced after gelation possesses the same composition as the binary solvent.

(iii) The melting enthalpy ΔH_{ws1} of complex C_{ws1} is taken larger than that of complex C_w. Due to the first assumption, we may expect virtually no entropic modification, which ultimately entails that the melting point is also higher ($T_{ws} = \Delta H_{ws}/\Delta S > T_w = \Delta H_w/\Delta S$). Obviously, this remark does not hold for agarose/water/MF systems.

In principle, we should be able to resolve experimentally the melting endotherm into two endotherms corresponding to the melting of either complex. The difference in melting point of about 10 °C, however, does not allow a clear differentiation of both meltings. As a result, the enthalpy determined experimentally is a combination of the melting enthalpy of both complexes. The same remark holds for the gelation enthalpies. Consequently, the experimental enthalpy reads

$$\Delta H_{\mathrm{exp}} = \phi_{\mathrm{w}}\Delta H_{\mathrm{w}} + \phi_{\mathrm{ws1}}\Delta H_{\mathrm{ws1}} \tag{9}$$

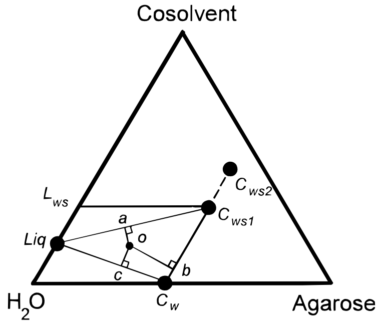

Figure 12. Ternary phase diagram at constant temperature. C_w, C_{ws1}, and C_{ws2} represent the different complexes. L_{ws} stands for the water/cosolvent composition at which the melting enthalpies are maximum. Point O represents a given agarose/water/cosolvent system which is shown here to phase separate into three phases: C_w, C_{ws1}, and a liquid phase. Projection of O onto the lines drawn between each phase determines line segments directly related to the proportion of each phase (see text for details).

Use of the above assumptions implies a linear increase of ΔH_{exp} when altering the water/cosolvent composition from 1/0 to $L_{ws}/1 - L_{ws}$, in agreement with the results presented herein. The *stoichiometry of the binary solvent* leading to the formation of complex C_{ws1} is then given by the maximum of the enthalpies, i.e. about six molecules of water and one molecule of cosolvent (DMSO, DMF, MF).

We hasten to add that, due to the three-dimensional character of the phase diagram, we cannot guarantee the uniqueness of this approach; other assumptions not thought of at the moment may possibly apply. Further investigations will be needed to settle this issue. The advantage of the model presented here lies in its simplicity for illustrating how complex formation is a relevant way to explain the body of the experimental results.

For concentrations higher than L_{ws}, two cases are liable to account for the observed variations of both the enthalpies and melting temperatures: formation of a complex with a higher content of cosolvent, in which case we would have $C_{ws1} + C_{ws2} +$ *liquid*, or only two phases $C_{ws1} +$ *liquid*, wherein the liquid phase would consist of solvent and free agarose chains. As has been discussed above, the fact that the elastic modulus does not significantly vary with solvent composition would rather favor the first hypothesis. Here again, further investigations will be needed to clarify these points.

Usually, one way for giving additional support to the existence of complexes would be through the observation of a maximum in the variation of the enthalpies as a function of agarose concentration at constant solvent composition. Such a study seems, however, difficult, even out of reach, as very high agarose concentrations should be obtained: for instance, 4 DMSO molecules per agarose residue would correspond to agarose concentrations of about 50%, and 4 water molecules still higher concentrations (about 80%). Clearly, other techniques, possibly spectroscopic methods, will be needed to give further support to the existence of ternary complexes.

References and Notes

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