Ternary Complexes in Gels from Agarose and from Chemically-modified Agarose

Jean-Michel Guenet*[1], Mohammed Dahmani[1,a], Mohamed Ramzi[1,b], Cyrille Rochas[2]

1= Institut Charles Sadron, CNRS UPR 022, 6 rue Boussingault F-67083 Strasbourg Cedex, France

2= Laboratoire de Spectrométrie Physique, Université J. Fourier-CNRS UMR 5588, F-38402 Saint-Martin d'Hères Cedex, France

SUMMARY: Thermodynamic data and mechanical measurements are shown for gels prepared in aqueous binary solvents (water/DMSO, water/DMF, water/methyl formamide and water/formamide). When electrostatic interactions, as opposed to hydrogen bonding, can be established with the cosolvent (DMSO, DMF, methyl formamide) we come to the conclusion that ternary complexes are formed (*agarose/water/cosolvent*). In the case of chemically-modified agarose (OH groups replaced by OCH_3 groups) we suggest that these cosolvents are directly involved in the formation of the gel.

Introduction

Agarose is a natural polymer extracted from seaweeds whose repeat unit (residue) consists *1,4-linked 3,6-anhydro-α-L-galactose* and *1,3-linked β-D-galactose* (see structural formulae 1 top). It forms gel in water and in some aqueous binary solvents where the cosolvent is dimethyl sulphoxide (DMSO) or a series of derivatives of formamide. The chain conformation of agarose in these gels is still a matter of debate. To be sure the diffraction pattern of dried and oriented gels displays but a few reflections which have been however assigned to a double helix[1)]. Ab-initio calculations confirmed the helical structure and suggested that single helices were not feasible. In fact, much of these conclusions were supported at that time from the fact that a double helix had already been proposed for κ-carrageenan in the gel state on the basis of a diffraction pattern containing a large number of reflections[2)]. It was then thought that polysaccharides would adopt the same type of helical conformation. While the existence of double helices in carraghenan gels is little disputed this is not the case for agarose. Recent studies by Atkins and Foord[3)], but also by Guenet et al.[4)] have reached the conclusion that single helices most problably characterize agarose instead of double helices. In addition, there is a high probability that agarose/water complexes are involved, as is seen in amylose[5)]

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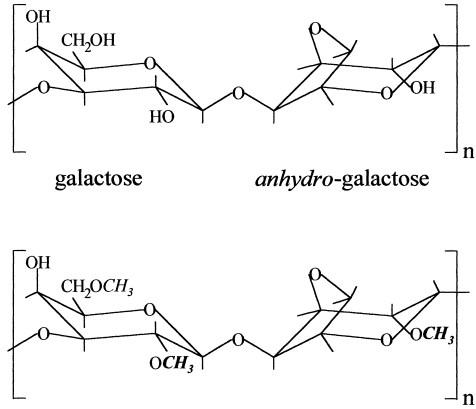
although no clear evidence from diffraction techniques is available so far. If this is the case, conformational calculations should take this into account for determining stable helical



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conformation. In this short paper we will present some thermodynamic evidence suggesting that ternary complexes are formed in aqueous binary solvent. Agarose but also chemically-modified agarose will be used. In the latter case chemically-modified agarose are obtained by replacing randomly OH groups by OCH_3 groups.

Sample characterization

The agarose sample (M0) and the chemically-agarose samples (M1 to M3) were kindly supplied by Hispanagar (*Burgos, Spain*). The three modified agarose samples used in this study are of differing degrees of modification (1 to 3): some hydrogens of the hydroxyl groups are replaced at random by OCH_3 groups (see structural formulae 1 bottom). Molecular weights were determined by viscometry measurements ($[\eta]= 0.07\ M_v^{\ 0.72}$ from *ref. 6*), and the average methyl content was measured by agarose hydrolysis.

M0 $M_v= 1.12\text{x}10^5$ $C_{12}H_{18}O_9$

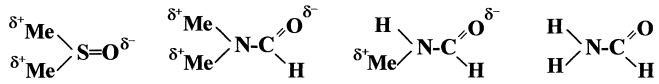
M1 $M_v= 9.87\text{x}10^4$ $C_{12}H_{17.46}(CH_3)_{0.54}O_9$

M2 $M_v= 1.03\text{x}10^5$ $C_{12}H_{17.32}(CH_3)_{0.68}O_9$

M3 $M_v= 1.02\text{x}10^5$ $C_{12}H_{17.07}(CH_3)_{0.93}O_9$

Temperature-composition phase diagrams for agarose

In addition to DMSO, a series of formamide derivatives has been used (see structural formula 2, from left to right DMSO, Dimethyl formamide (DMF), methyl formamide (MF) and formamide). The series of formamide shows increasing polarization when increasing the number of CH_3 groups.



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Teemperature-composition phase diagrams are shown in figure 1 (*ref. 7*). Water/DMSO and water/DMF binary solvents give the same phase diagram: both the melting temperature and the melting enthalpies display a maximum for f_{DMSO} or $f_{DMF} \approx 0.2$. The maximum in the melting temperatures could be in principle accounted for by using Flory's theory[8]:

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

$$G_{\varphi_p}(\varphi_i,\chi_{ij}) = -\ \varphi_1 - \frac{\varphi_2}{x_2} - \chi_{12}\varphi_1\varphi_2 + (\varphi_1\chi_{1p} + \frac{\chi_{2p}\varphi_2}{x_2} + 1)(1 - \varphi_P) - \chi_{1p}(1 - \varphi_p)^2$$

where χ_{ij} is the interaction parameter between species *i* and *j*, and φ_i is the volume fraction of species *i*. If a strong interaction exists between the two solvents ($\chi_{12} < 0$) and if one is a good solvent whereas the second is a bad solvent then equation 1 may display a maximum. To be sure, water is a poor solvent while DMSO or DMF are good solvents. This explanation was put forward by Nishinari et al.[9].

Ramzi et al.[7] suggest, however, that the temperature-composition phase diagrams indicate the formation of a ternary complex: *agarose/water/DMSO* or *agarose/water/DMF* where the stoichiometric composition *water/cosolvent* is around *4/1*. Ramzi et al. argue that while the temperature maximum could be in principle explained using relation 1, the maximum observed for the melting enthalpies as a function of the composition would require the fraction of agarose gel involved in the elastic newtwork to vary with composition. As the gel elastic modulus is virtually invariant with the binary solvent composition this is not the case. Only the occurrence of a complex can explain this maximum. Further, in the case of methyl formamide there is no melting temperature maxipmum while one can observe a melting enthalpy maximum. Again, the elastic modulus is nearly invariant with solvent composition. Finally, no maximum is observed in formamide which therefore suggest the absence of a ternary complex

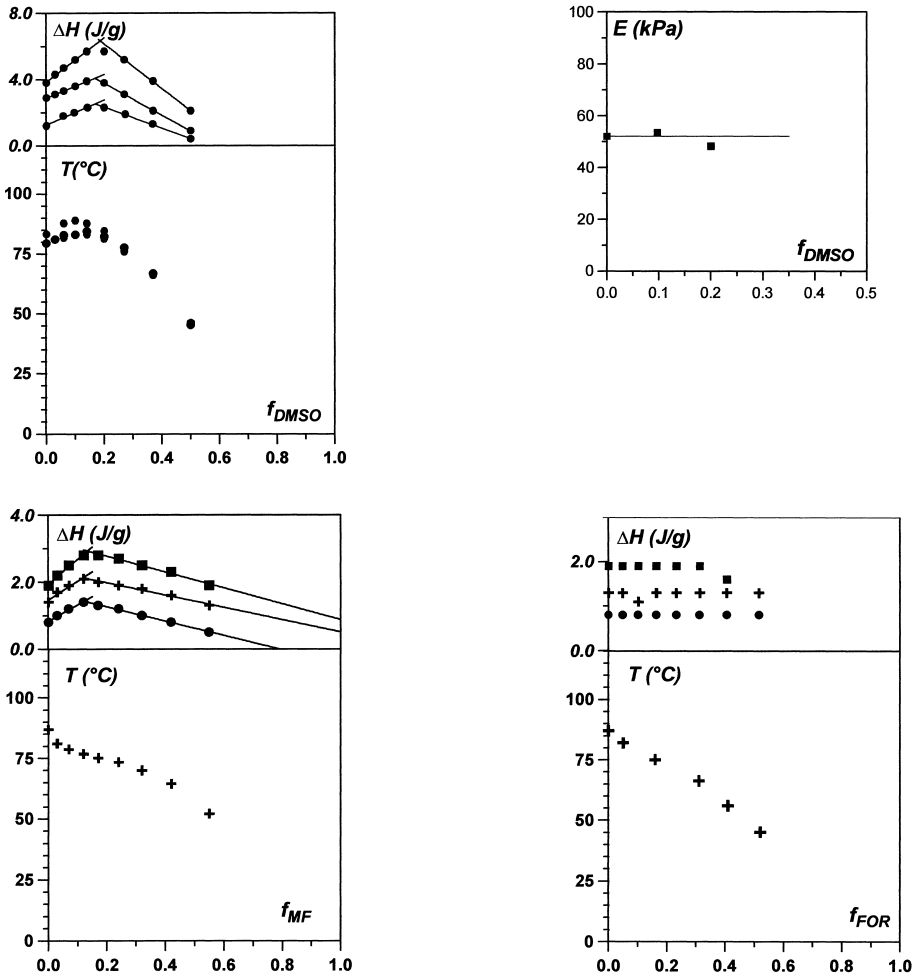

Fig. 1: Temperature-composition phase diagrams in water/DMSO, water/methyl formamide and water/formamide. f with the appropriate subscript stands for the mole fraction. Tamman's diagrams are also given (gel melting enthalpy ΔH vs f). Several agarose concentrations are been investigated (for further details see ref. 7). Upper figure *right*: evolution of the gel elastic modulus E as a function of water/DMSO composition.

in this solvent.

The contrast variation method in small-angle neutron scattering

As aforementioned, crystallographic investigation turns out to be powerless in the case of agarose gels for deciding whether complex formation occurs. The contrast variation method in

small-angle neutron scattering experiments is of interest in the preent question[10]. The scattered intensity reads:

$$I(q) = A^2 S(q)$$

where $S(q)$ is the structure factor and A^2 the contrast factor which depends upon the relative composition between hydrogenous and deuterated species ($q = 4\pi/\lambda \sin\theta/2$, θ= scattering angle). In other words by varying the proportion of hydrogenous and deuterated solvents we can find out at which isotopic composition the contrast becomes $A^2 = O$. The value that would be expected in the absence of complex can be easily calculated (pure agarose fibrils pervading through the solvent mixture) and compared to the actual value. If there is a strong discrepancy then the existence of a complex can be seriously envisaged.

Here we present data obtained for a DMSO mole fraction off-stoichiometry (this enhances the effect as the composition inside the fibrils and in the surrounding solvent differ markedly). To dtermine the extinction point the square root of intensity is plotted at various isotopic compositions for different scattering vectors[11].

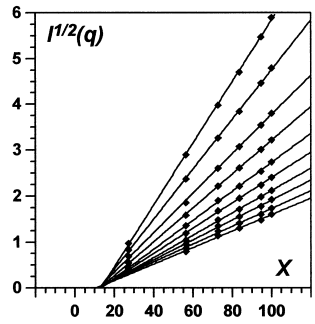

Fig 2: square root of the scattered intensity by agarose gels prepared in *water/DMSO 9/1* but where the isotopic composition of water (X= volume fraction of D_2O) is varied. The extinction point occurs for $X = 0.16$.

In the case shown in figure 2 if complex were absent the theoretical extinction should occur for a virtual volume fraction X= -1. This simply means that no extinction at all should occur unlike what is experimentally seen.

Allegedly, this method cannot give any clues as to the placement of solvent molecules with respect to the agarose residue nor does it give quantitative values such as the stoichiometric composition (for further reading see ref. 12). Yet, it gives support to the conclusions drawn from temperature-composition phase diagrams.

Interstingly enough, the propensity of forming ternary complex is not linked to the solvent potentiality of forming hydrogen bons (formamide) but to the capability of establishing electrostactic interactions (DMSO, DMF, MF). Chemically-modified agarose , where potential hydrogen bonds are replaced by electrostatic groups should allow to cast additional light on this issue.

Temperature-composition phase diagrams for chemically-modified agarose

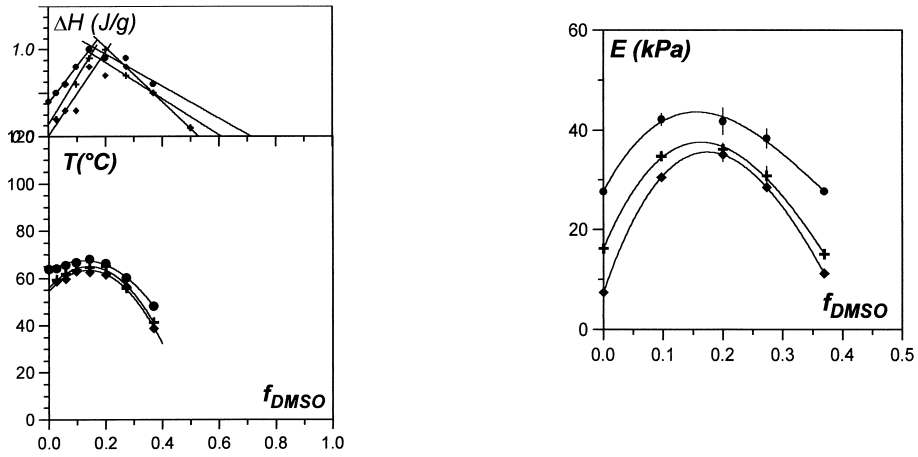

Fig.3: *Left*: Temperature-composition phase diagram for modified agarose in water/DMSO; ($C_{agarose}$= 30g/l) *right*: variation of the elastic modulus as a function of the DMSO mole fraction. For both figures = M1; [= M2; = M3.

As can be seen in fig.3, the shape of the phase diagram is similar to that of agarose, although some changes have appeared: 1) melting points have globally droped by about 25-30°C, and the melting enthalpy has drastically decreased in pure water, being almost close to 0 for M3. The ratio of the melting enthalpy at the maximum over that in pure water is much larger than 1.5 as was previously the case with agarose.

To account for these facts it is worth keeping in mind that the agarose chemical modification occurs at random. As a result, there is most probably a large distribution in the degree of modification. Manifestly, modification of this type prevents gelation as is shown by the drop of melting enthalpy. Here, all happens as if water was a better solvent than for agarose. Therefore, the melting point maximum as a function of DMSO composition cannot be explained at all in the framework of Flory's theory since both solvents act as if they displayed

an interaction parameter $\chi_{sp} < 0.5$. Our assumption is that highly modified agarose chains do not aggregate in pure water but do so when increasing the fraction of cosolvent (DMSO, DMF and MF). Clearly, the cosolvent promotes gelation as this is ascertained by the variation of the elastic modulus as a function of DMSO composition (see fig. 3). The modulus displays a maximum at about $f_{DMSO} \approx 0.2$ unlike what has been observed with agarose. We also note that the increase of the magnitude of the elastic modulus is larger with the most modified sample (by about 5 times with M3 against about 1.5 with M1). Here again we suggest that DMSO bridges chains together by electrostatic interactions as is show in fig. 4:

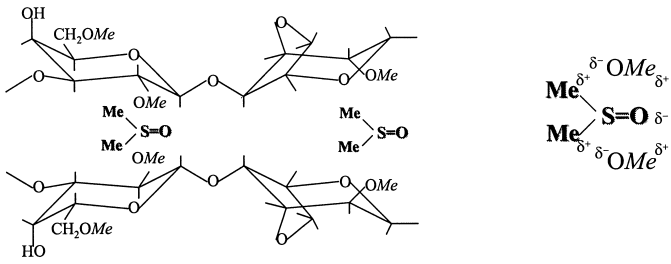

Fig. 4: *left*: A schematic model for the interaction between DMSO and the sections of the agarose residue bearing CH_3 groups. *right*: a possible way of how electrostatic interactions may occur. Here all the OH groups have been replaced just for the sake of clarity.

The removal of OH groups disrupt the formation of hydrogen bonds necessary for agarose chain aggregation. These hydrogen bonds are then replaced by electrostatic bonds through the addition of cosolvent.

Again there is a subtle balance between the fraction of water and DMSO. Here also a ternary complex is formed yet we have no clue as to where are located the water molecules. The results gathered here on chemically-modified agarose further highlight the role of electrostatic interactions.

Concluding remarks

The data reported here clearly suggest the occurence of ternary complexes both for agarose and for chemically-modified agarose. While it seems clear that the role of the cosolvent is to replace missing hydrogen bonds in the case of chemically-modified agarose, its effect in modified agarose is different. The cosolvent does not favour the aggregation of agrose chains as shown by the invariance of the elastic modulus. However, agarose gel stability is made higher by addition of DMSO since there is an increase of its melting point and of its melting

enthalpy. It remains now to find out how the cosolvent molecules interact with agarose in the ternary complex..

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a) present address: Université d'Oujda, Faculté des Sciences, 60000 OUJDA MAROC

b) Debye Institute, Van't Hoff Laboratory for Physical and Colloidal Chemistry,

Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands.