ELSEVIER

0144-8617(94)00086-7

Carbohydrate Polymers **26** (1995) 11–18

0144-8617/95/$09.50+.00

Effect of preparation method on the hydration characteristics of hylan and comparison with another highly cross-linked polysaccharide, gum arabic

Shoji Takigami[a], Michiko Takigami[a] & Glyn O. Phillips[b]*

[a]*Department of Chemistry, Gunma University, Japan*
[b]*Newtech Innovation Centre, The North East Wales Institute, Clwyd, Wales, UK*

(Received 30 August 1994; revised version received 17 October 1994; accepted 18 October 1994)

The water binding characteristics of hylan are compared with another cross-linked polysaccharide *Acacia senegal* gum exudate (*A. senegal*) using differential scanning calorimetry. Both polysaccharide systems bind water effectively, and the transitions characteristic of two types of freezing-bound water can be distinguished from the melting or freezing of free water. There is evidence for the existance of metastable states of freezing-bound water within the two systems. Gum arabic binds considerably less freezing-bound water than hylan systems. *A. senegal* does not have the same ability as hyaluronic acid to form structured entangled networks which can incorporate water within the matrix. The hylan samples are of two types: hylan fluid where the hyaluronan chains are cross-linked with formaldehyde, and hylan gel where the cross-linking agent is vinyl sulphone. The hylan gel retains the freezing-bound state of water as a stable thermodynamic state *ca* 20–50% more effectively than hylan prepared from the freeze-dried solid prepared from either concentrated or dilute hylan fluid. The traps formed from freeze-dried hylan gel are also more stable. Hylan gel prepared by precipitation with isopropanol and freeze-dried is the most effective hylan sample for stabilising the freezing bound state. For this material even in $\sim$6% solution the vast majority of the water is retained in the freezing-bound form.

INTRODUCTION

Previously it was shown that the formaldehyde cross-linked hyaluronan (hylan) has an exceptional ability to interact with water. With water contents even up to 10 g water to 1 g hylan, almost all the water remains in the freezing-bound state when studied by differential scanning calorimetry (DSC) (Takigami *et al.*, 1993). This freezing-bound state of water can be distinguished from non-freezing and free water. The non-freezing water is intimately associated with the hydrophilic groups of hylan, and corresponds to 13 mol water per disaccharide unit of the hyaluronan chain. As might be anticipated, this amount of non-freezing water does not increase with water content after complete hydration of the sugar skeleton, and since hyaluronan and hylan have the same fundamental skeleton, both interact with equivalent amounts of non-freezing water. Our conclusion, therefore, was that it is the cross-linked nature of hylan, which itself incorporates the basic hyaluronan structure, which allows the system to form structured networks with water. Conditions in the highly concentrated aqueous–hylan systems which were employed are most favourable for the formation of hydrogen-bonded chain–chain entanglement networks. Structured into such networks, the water is 'bound' and behaves quite differently from free water. Due to the polysaccharide network this water cannot sterically form hexagonal ice, and, therefore, melts easier owing to the presence of such defects.

The hylan prepared by cross-linking hyaluronan chains with formaldehyde is water-soluble (Balazs *et al.*, 1987, 1988). Another form of hylan is formed if cross-linking is carried out with vinyl sulphone, which has the form of a highly hydrophilic gel, dispersable but not readily soluble in water (Balazs *et al.*, 1988).

One objective of this study is to compare these two forms of hylan, and examine the effect of the methods used to produce the hylan solid before hydration is commenced in the DSC experiments. Secondly, we seek to compare the hydration characteristics of hylan with a

---

*Author to whom correspondence should be addressed.

polysaccharide structure which has a densely cross-linked structure in its native state. We have selected the gum exudate from the *Acacia senegal* tree found in the Sabelian belt of Africa, and widely used in the food industry because of its versatility in acting as an emulsifier, stabiliser, encapsulator and texture controller, all of which involve interaction with water. A knowledge of its interaction with water is important in view of its own unique characteristics. It can also provide a direct comparison to establish whether hylan systems are unusually interactive in 'binding' water, compared to *Acacia senegal* gum, which is highly regarded as gum arabic (Randall *et al.*, 1988, 1989) in the food industry for its ability to form useful viscous and non-viscous systems with water. In this paper the designation *A. senegal* will be used for this gum.

METHODS, MATERIALS AND NOMENCLATURE

The DSC method and hydration procedures are identical to those described previously (Takigami *et al.*, 1993). The following designations are used for the weights of various types of water: W_{nf} non-freezing, W_{fb} freezing-bound and W_f free. The water content W_c is defined as:

$$W_c\,(g/g) = \frac{\text{Weight of water (g) of polysaccharide}}{\text{Weight of dry polysaccharide (g)}}.$$

Where the DSC curves show distinct and separate transitions for two types of freezing-bound water W_{fb1} and W_{fb2} etc. are used. The free water which enables these quantities to be calculated from the areas represented by the endothermic transitions is connected to these quantities by the relationship:

$$W_c = W_{nf} + W_{fb} + W_f.$$

The term hylan gel refers to the vinyl sulphone cross-linked material and hylan fluid to the water-soluble formaldehyde cross-linked hyaluronan. Both were supplied (Biomatrix Inc.) in solid form. Solid was prepared by freeze-drying hylan gel (GFD), by precipitation with isopropanol and subsequently freeze-dried (hylan gel GP), or by freeze-drying a dilute (HD) and a concentrated (HC) aqueous solution of the hylan fluid previously studied (Takigami *et al.*, 1993). *A. senegal* was supplied by the Gum Arabic Company (Sudan), designated Kordofan gum, which was authenticated by their agronomic specialists and subsequently characterised as previously described (Randall *et al.*, 1988, 1989; Osman *et al.*, 1993; Jurasek & Phillips, 1993). Hylan has a molecular weight of M_w 6×10^6 and polydispersity ~4, measured as previously described (Takigami *et al.*, 1993). The hylan gel would have a higher molecular weight, in view of its enhanced viscoelastic properties. However, in such a continuous network structure, M_w is not an appropriate parameter. The overall properties and rheological behaviour of hylan gel have been described (Balazs *et al.*, 1989).

RESULTS AND DISCUSSION

A representative set of DSC heating curves for soluble hylan (HD) are shown in Fig. 1. The W_c values are listed, and a comparable behaviour is observed to that previously reported for hylan (Takigami *et al.*, 1993). At W_c 0·62, the two endothermic peaks representing the melting of free and freezing-bound water are observed near 260 K (T_{fb1}) and the free water at 273 K (T_f). A second freezing-bound water transition can be observed near 250 K, particularly at higher W_c values (T_{fb2}), which does not significantly shift to higher melting temperatures at increasing W_c values. As W_c is increased T_{fb1} shifts towards T_f and eventually overlap, making it impossible to distinguish individual transitions. We have used this W_c value where the bound water merges with free water as an indication of the relative capacity of differing polysaccharides to bind water (Table 1 in Takigami *et al.*, 1993).

The behaviour on cooling of the same hylan system was studied, where the observed transitions are due to

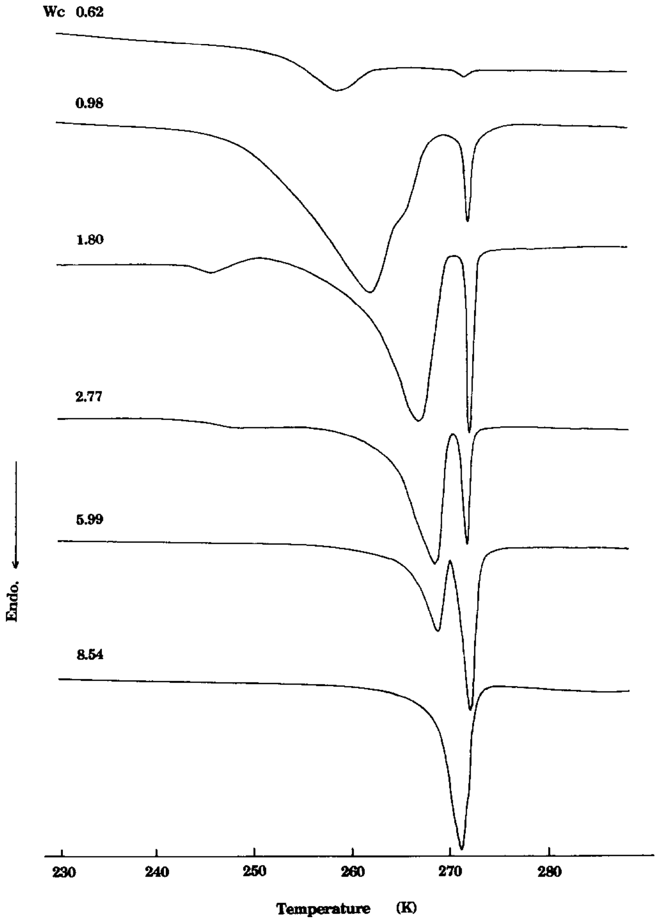

Fig. 1. DSC heating curves for water–hylan (HD) systems.

the formation first of amorphous ice, which on heating crystallises exothermically from the glassy state, and subsequently melts endothermically. Figure 2 shows repeated heating and cooling of the sample with W_c 0·98. On the first cooling cycle an exothermic transition relating to the crystallisation of the ice occurs. First, only the transition relating to formation of amorphous ice is observed at 254 K (T_f). When such amorphous ice is heated it melts endothermically and the transitions due to the freezing-bound and free water are evident. When the cooling–heating cycle is repeated there is sufficient ice for us to observe both exothermic crystallisation and endothermic melting. The overall behaviour and indication of more than one transition within the broad envelope, which is non-symmetrical with pronounced shoulders, point to metastable states of water existing in the various hylan–water systems. At the higher water contents, as the cooling and heating cycles are repeated immediately after one another, the amount of free water increases and the amount of bound water decreases (Fig. 3). A similar behaviour is observed for repeated cooling cycles (Fig. 4). There are also shifts in the position of the freezing temperatures of the free water (T_f) due probably to super-cooling effects but not such a significant difference in the repeated melting temperatures (T_{fb1} and T_{fb2}). The repeated heating and cooling cycles change the structure of the junction zones indicating the transient nature of the trapping sites.

The overall behaviour of the changes in transition temperature of the various transitions is shown in Fig. 5.

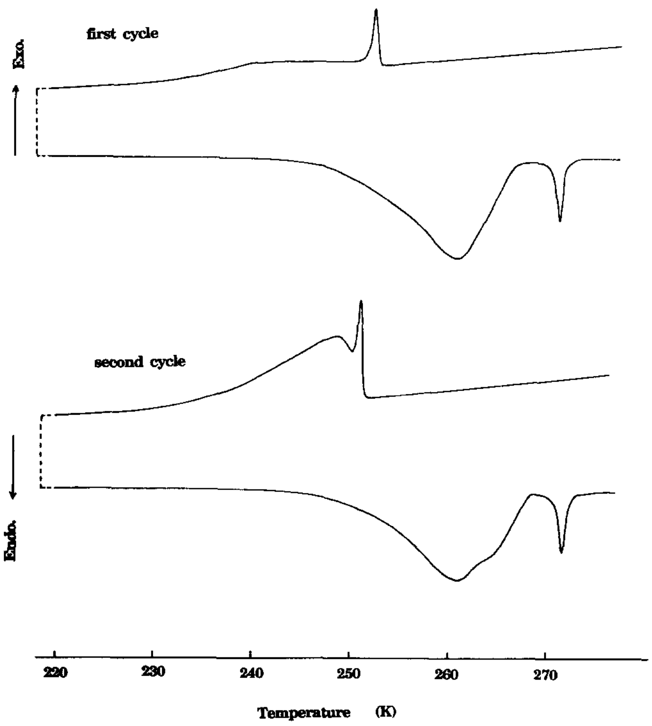

Fig. 2. Repeated heating and cooling DSC curves for water–hylan (HD); (W_c = 0·98).

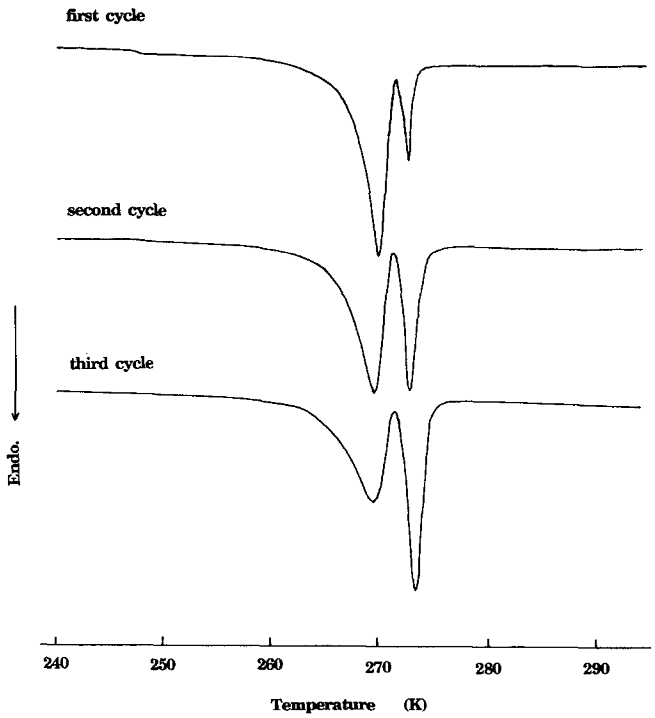

Fig. 3. Effect of repeated heating and cooling cycles on the melting of water–hylan (HD): W_c = 3·89.

If the first cycles only are considered, the pattern which we observed previously (Takigami *et al.*, 1993) is repeated. T_{fb2} is largely immobile, despite being held within the matrix. This water appears to have a structure intermediate between non-freezing water and the bulk of freezing-bound water, thus reducing its mobility (Nakamura *et al.*, 1983). As W_c increases, sorption of the water continues and the freezing-bound water builds up as a structured entity, and retains its distinctive character different from free water. The enthalpy of melting of this structured water remains throughout less than free water due to defects in the freezing-bound ice. The transition temperature gradually increases until it finally approaches that of free water at 273 K. As previously described (Takigami *et al.*, 1993), the amounts of the various types of water with increasing W_c were calculated (Fig. 6). The major portion of the water within the range of W_c examined remains in the bound state. The slope of the curve is less than one, indicating that the enthalpy of melting of the water in the freezing-bound state remains consistently less than free water. The non-freezing water, which does not change with water content after complete hydration of the sugar skeleton, corresponds to ~13 mol water bound in the non-frozen state to each disaccharide unit of hylan. This is water bound tightly by the OH groups of the saccharide units and the sodium ions associated with the anionic sites of hylan. Our previous compressibility and high precision densimetric methods indicated a similar hydration pattern (Davies *et al.*, 1983)

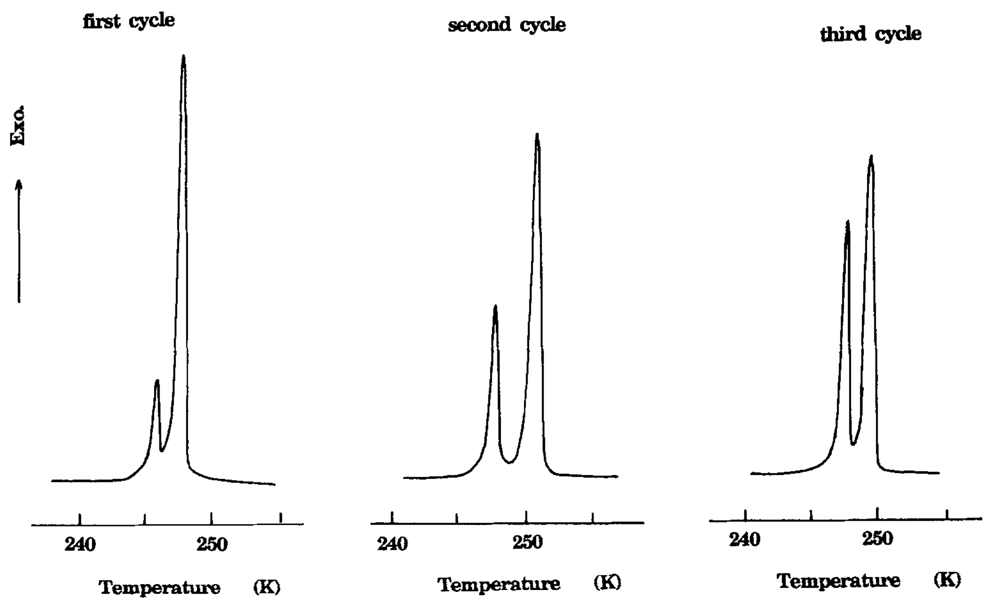

Fig. 4. Effect of repeated heating and cooling cycles of water–hylan (HD): W_c = 3·89.

for hyaluranan. This would be anticipated since both systems contain the same saccharide units. Thus, the overall pattern reported previously (Takigami *et al.*, 1993) is confirmed. Once these groups' OH groups and hydrated counterions are hydrated, no further change occurs with increasing water content.

Compared with the hylan freeze-dried from dilute solution (HD) the soluble hylan prepared by freeze-drying a concentrated solution (HC) behaves similarly. Our objective in making the comparison was to establish whether the method of preparation could influence the extent of water binding. This does not appear to be the case, although we have observed that the sample structure produced by different methods of sample preparation can influence the stability of the freezing-bound water. In view of the similarity in behaviour, we are not reporting DSC heating curves for hylan (HC). However, in Fig. 7 which compares the behaviour of all the systems studied, the results for hylan (HC) are included.

The DSC heating curves for hylan gel (GFD) after freeze-drying to give the solid are shown in Fig. 7. Although the transitions are similar, giving the two types of bound water in the presence of non-freezing and free water, it is evident that the structures are more coherent. The transitions are sharper and more symmetrical. Even up to W_c 12·69 it is still possible to distinguish the bound water transition from the shoulder at 273 K which is associated with the free water melting. Moreover, the more highly cross-linked structure

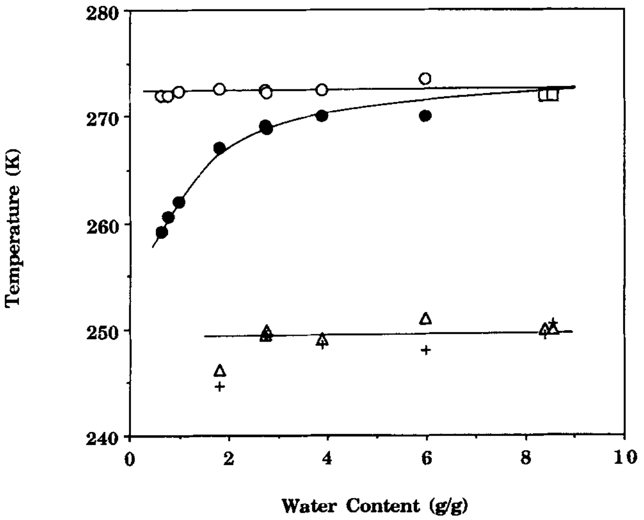

Fig. 5. Variation of melting and freezing temperatures with water content for hylan (HD) systems. (○) free water (T_w); (●) bound water (T_{fb1}); (△) bound water (T_{fb2}); (□) total freezing water ($T_{fb1} + T_w$); (+) freezing temperature (T_f).

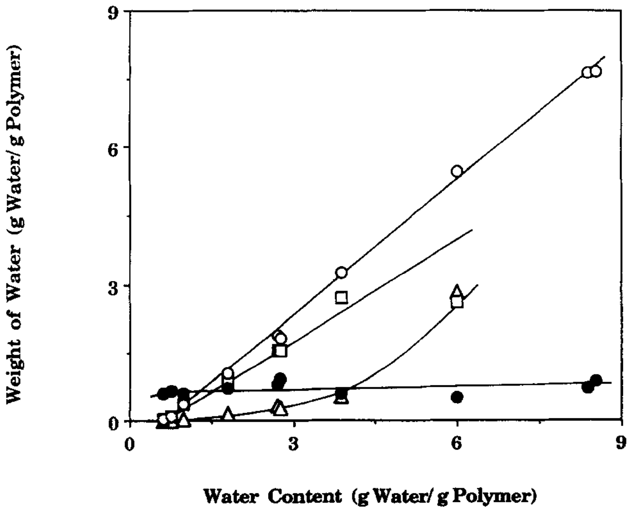

Fig. 6. Variation in content of various types of sorbed water with water content for hylan (HD). (○) total freezing water; (□) bound water; (△) free water; (●) non-freezing water.

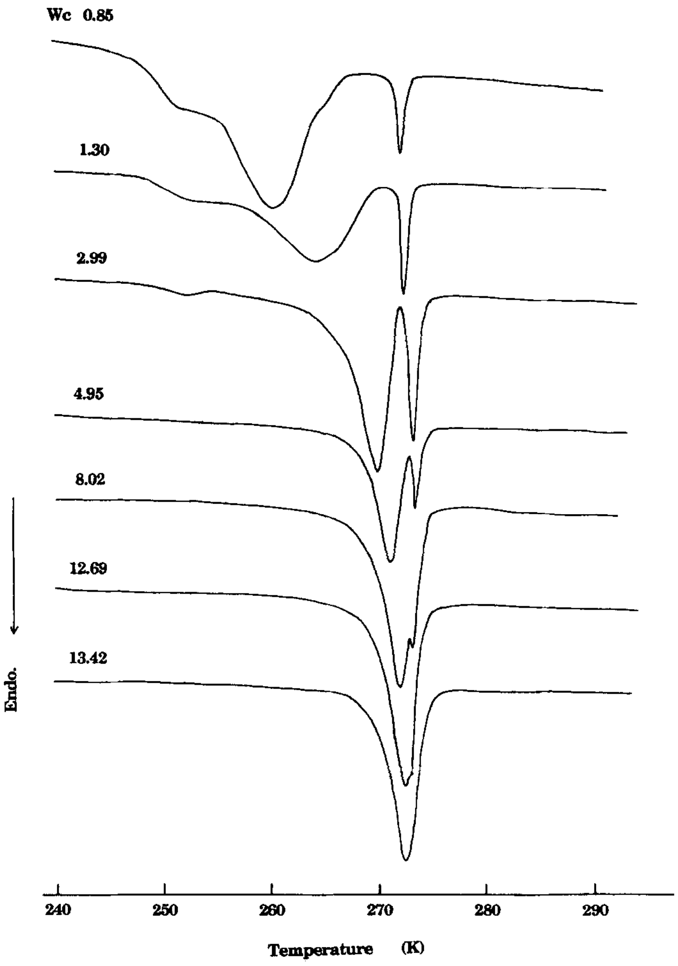

Fig. 7. DSC heating curves for water–hylan (GFD) systems.

appears also to confer greater stability to the freezing-bound state. The transitions as noted, are more discrete, more reproducible and are not profoundly influenced by repetition of the heating–cooling cycles (Fig. 8). The pattern shown in Fig. 8 is quite different from that of hylan HD at W_c 0·85 and 0·88. At least three types of water can be identified in the freezing-bound state, which are stable after repeated heating and cooling cycles. The gel structure clearly provides discrete and different types of traps than hylan HD. The changes in the temperature of the transitions of T_w, T_{fb1}, T_{fb2} and T_f with W_c are shown in Fig. 9 for hylan gel GFD, the freeze-dried vinyl sulphone cross-linked gel. The free water transition temperature is not achieved until $W_c > 12$, indicating the extremely high degree of water binding. The behaviour is better illustrated in the proportions of free, freezing-bound and non-freezing water (Fig. 10). This figure shows that initially the water binds to the hydrophilic OH groups and hydrated counterions (non-freezing), and that at water content of 12 g water to 1 g hylan the majority of the water remains in the freezing-bound state. Overall, the results testify to an extremely stable water-binding system. Hylan gel GFD, therefore, can retain the freezing-bound matrix as a stable thermodynamic state *ca* 20% more effectively than either hylan HC or HD.

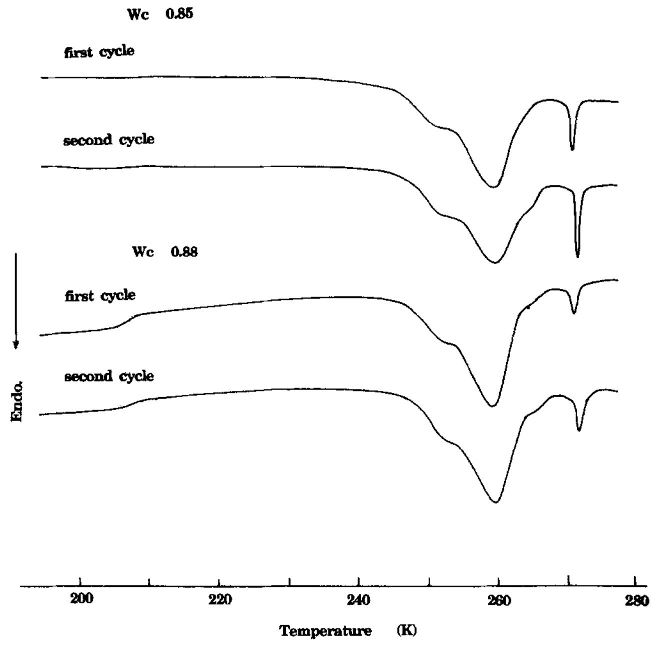

Fig. 8. Repeated heating and cooling cycles for water–hylan (GFD) systems.

Hylan gel (GP), prepared by precipitation with isopropanol and subsequently freeze-dried retains the freezing-bound state even more effectively than hylan gel GFD, as illustrated in Fig. 11. At W_c 0·15 no transition is observable, indicating that all the water is in the non-freezing state. There is a regular small increase in heat capacity. From W_c 0·79 to 1·38, the pattern has similar features to hylan gel GFD. However, the cold crystallisation is very marked for this system and the

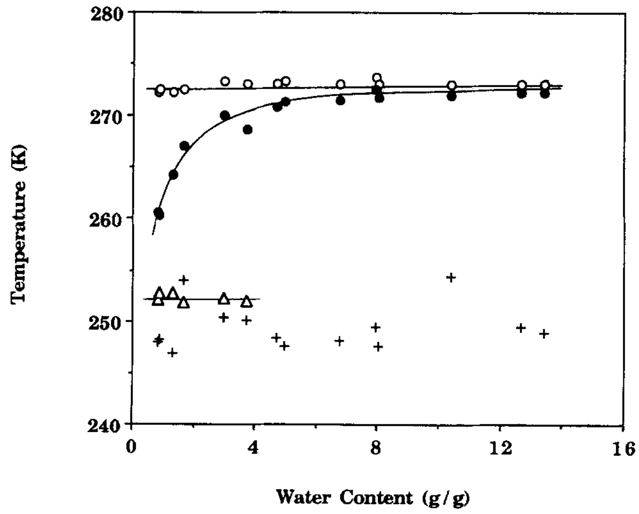

Fig. 9. Variation of melting and freezing temperatures with water content for water–hylan gel (GFD). (○) free water (T_w); (●) bound water (T_{fb1}); (▲) bound water (T_{fb2}); (+) freezing temperature (T_f).

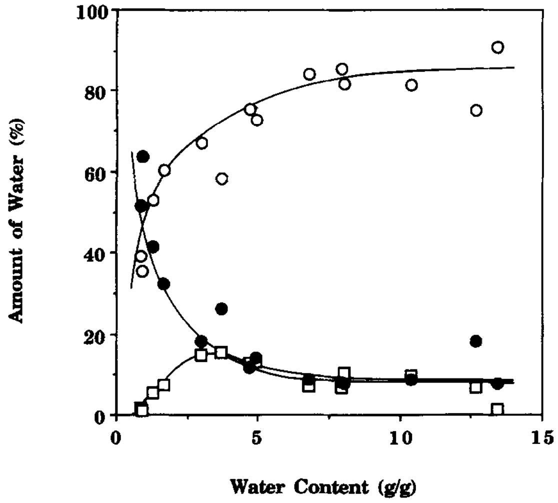

Fig. 10. The proportions of free, freezing-bound and non-freezing water in hylan (GFD) systems. (□) free water; (○) freezing-bound water; (●) non-freezing water.

exothermic crystallisation at 220–240 K is a dominant feature. Thereafter the freezing-bound water continues to dominate as W_c is increased. Even to W_c 15·75 (a 6% aqueous solution) the freezing-bound transition can be distinguished from free water. It is thus the most effective system for binding water in a stable thermodynamic state than any of the systems hitherto described. A comparison of the relative ability of the hylan samples to bind water can be obtained from the point where the free water transition is indistinguishable from that of bound water. The samples we have been studying are compared with other polymers in Table 1 (see Takigami *et al.*, 1993).

For hylan gel GP, the variations in melting and freezing temperatures with W_c are shown in Fig. 12. Heats of transition for the heating and cooling are shown in Fig. 13. The weights of bound-free, bound and non-freezing water with varying W_c are shown in Fig. 14. These follow the pattern previously described for hylan GFD. It is evident that the weight of bound water predominates throughout the range to the limit of the experiments at W_c 15·75. A detailed analysis of the behaviour of *A. senegal* compared with its component parts will be given elsewhere. Here we wish only to provide a quantitative comparison of water binding capacity with hylan.

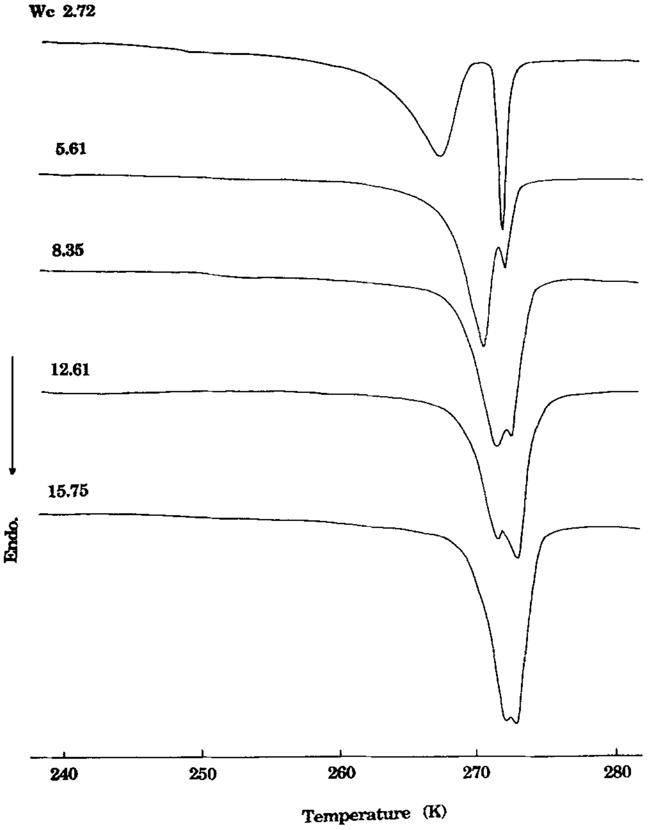

Fig. 11. DSC heating curves for water–hylan (GP) systems.

Table 1

Sample	W_c
Poly(2-hydroxystyrene)	0·080
Poly(2-acetoxystyrene)	0·026
Cellulose I	0·05
Cellulose II	0·06
Sodium carboxymethyl cellulose	0·08
Sodium polystyrene sulphonate	1·3
Sodium cellulose sulphonate	1·4
Sodium xanthan	2·0
A. senegal	3–6
Hyaluronan	3·0
Hylan fluid HC and HD	10·0
Hylan gel GFD	12·0
Hylan gel GP	15·75

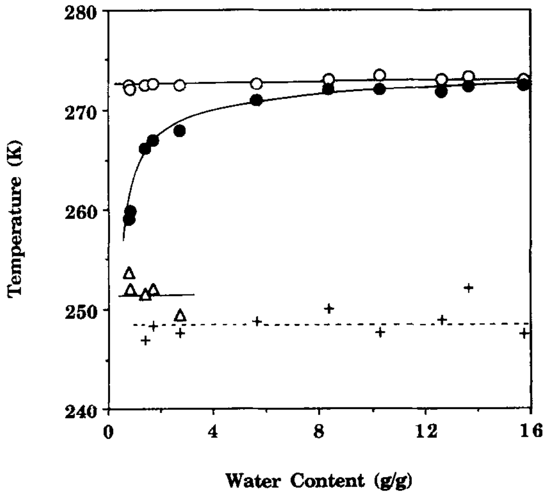

Fig. 12. Variation of melting and freezing temperatures with water content for hylan (GP). (○) free water (T_w); (●) bound water (T_{fb1}); (△) bound water (T_{fb2}); (+) freezing temperature (T_f).

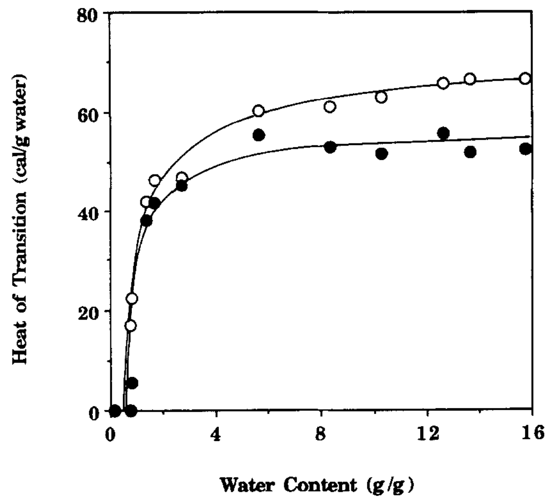

Fig. 13. Relationships between heat of transition and water content of water–hylan (GP) systems. (○) heating cycle; (●) cooling cycle.

It should, however, be noted that the behaviour resembles that of hylan gels (GFD) and (GP) rather than hylan (HC) or (HD). This can be observed at low W_c values particularly (W_c 0·55 for *A. senegal* in Fig. 15, compared with W_c 0·85 and 0·88 for hylan GFD in Fig. 8). In both instances three types of freezing-bound water can be distinguished. We point also to the cold crystallisation evident for *A. senegal*, a feature which will be discussed in detail elsewhere. At certain water contents ($W_w = 1{\cdot}28$) the relatively immobile freezing-bound water at $T_m \sim 225$ K is more pronounced than for hylan. Gum arabic is a highly complex polysaccharide, with a branched β-(1-3)-linked galactose backbone with branches linked through the 1,6-positions, with arabinose, rhamnose and uronic acids in ramified side chains (Osman *et al.*, 1993). It is a complex rather than a discrete molecule in that it is possible to fractionate (Randall *et al.*, 1988, 1989) by gel permeation chromatography the naturally occurring gum into several components: arabinogalactan protein (AGP) (about 10% of the whole) an arabinogalactan (AG) which comprises the bulk (~80%) of $M_w \sim 350{,}000$ and about 2% of GP, a lower molecular weight glycoprotein ($M_w \sim 50{,}000$). Using ion exchange and hydrophobic affinity chromatography combined with antibody screening, an even more complex pattern of sub-structures emerges (Osman *et al.*, 1993). The arabino galactan side-chains are attached to a common protein core, and now the 'wattle blossom' model (Connolly *et al.*, 1987, 1988) for the AGP structure is gaining experimental support. This globular hydrophilic highly cross-linked polysaccharide structure linked to a hydrophobic polypeptide backbone would appear to provide more discrete traps than the random coil hyaluronan for stabilising the freezing-bound water. The fact that hylan GFD resembles *A. senegal* in providing more trapping sites points further to its density cross-linked structure.

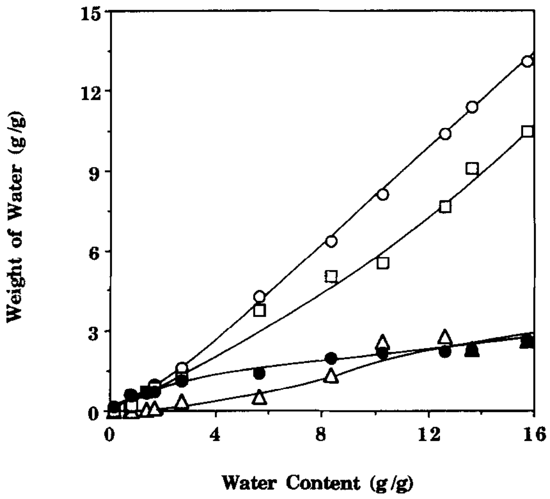

Fig. 14. Variation in content of various types of sorbed water content for hylan (GP). (○) total freezing water; (□) bound water, (△) free water; (●) non-freezing water.

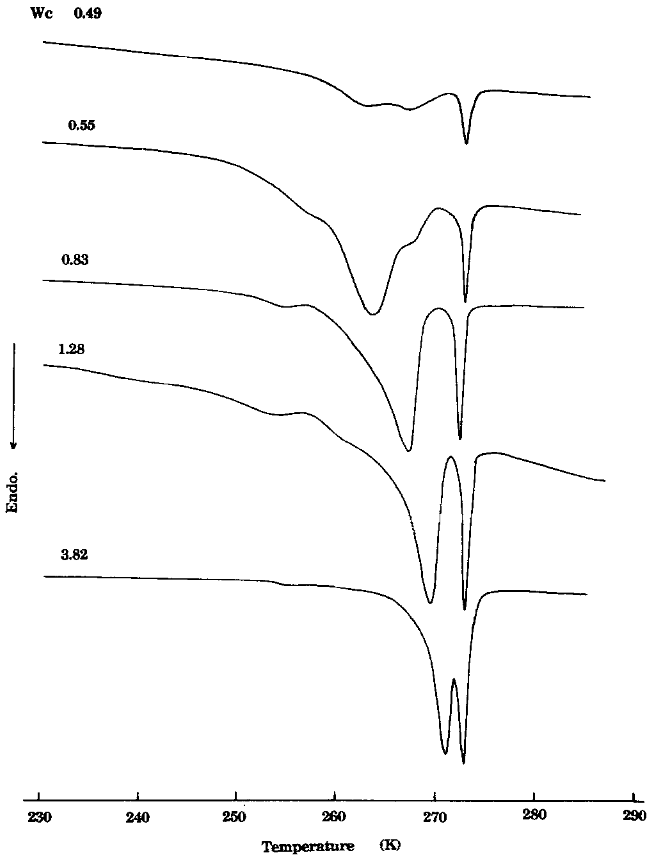

Fig. 15. DSC heating curves for water *A. senegal* systems.

Two questions of practical import need to be addressed from the overall studies on hylan and *A. senegal*:

- Are there significantly different mechanisms and amounts of water being bound by the various methods of preparing the hylans HD, HC, GFD and GP.

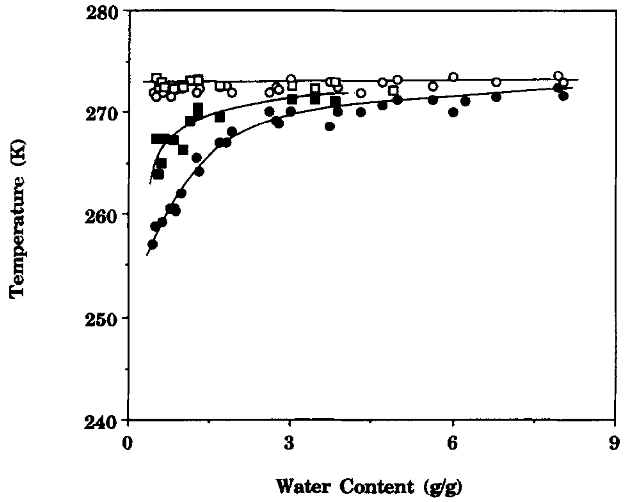

Fig. 16. Variation of melting temperature of sorbed water with water content for water–hylan and water–gum arabic systems. (○) T_w for hylan; (●) T_{fbI} for hylan; (■) T_w for gum arabic; (□) T_{fbI} for gum arabic.

- How does hylan compare with *A. senegal* in its overall capacity to bind water?

With regard to the first point, the uptake mechanism of the water is similar for the various hylan samples. However, as noted previously, the hylan gel is able to stabilise the water more coherently than the water-soluble fluid hylan produced by cross-linking hyaluronan with formaldehyde. Moreover, the cross-linked gel provides more individual stable traps for the freezing-bound water in a thermodynamically stable state up to W_c 12 (8% solution) and hylan gel (GP) up to W_c 15·75 (~6% solution). Evidently the dislocated solid state structure can be influenced by the methods of preparation.

Secondly, Fig. 16 demonstrates that when all the samples of hylan and *A. senegal* are compared, it is evident that hylan is able to bind considerably more water overall than *A. senegal*. As noted, $W_c > 10$ before the freezing-bound water approaches W_c for free water for the hylan HD and HC and $W_c > 12$ and 15·75 for hylan GFD. The corresponding value for *A. senegal* is between 3 and 6, confirming again the pre-eminent position of hylan in its ability to form hydrogen-bonded chain–chain entanglement networks in water. Such dyamic networks allow water to be incorporated into the structure, resulting in the high viscoelasticity that is the basis for the application of such materials in visco-supplementation therapy (Balazs *et al.*, 1988).

ACKNOWLEDGEMENTS

We are grateful to Dr Endre A. Balazs for providing the hylan samples for this investigation and for valuable assistance from him and his colleagues at Biomatrix Inc. We also acknowledge the invaluable discussions with Drs H. & T. Hatakeyama, who have maintained a supportive interest in this study.

REFERENCES

Balazs, E.A. & Leshchiner, A. (1989). In *Cellulosics Utilisation*, eds. H. Inagaki & G.O. Phillips. Elsevier Applied Science, pp. 233–241.

Balazs, E.A., Leshchiner, A., Leshchiner, A. & Band, P. (1987). United States Patent No. 4, 713448.

Balazs, E.A., Leshchiner, A., Wedlock, D.J., Cowman, M., Band, P., Laisen, N., Leshchiner, A. & Hoefeling, J. (1988). *Biomatrix Report 101*, Biomatrix Inc, Ridgefield, NJ, USA.

Connolly, S., Fenyo, J.-C. & Vandevelde, M.C. (1987). *Food Hydrocolloids*, **1**, 477–481.

Connolly, S., Fenyo, J.-C. & Vandevelde, M.C. (1988). *Carbohydr. Polym.*, **8**, 23–27.

Davies, A., Gormaly, J., Wyn-Jones, E., Wedlock, D.J. & Phillips, G.O. (1983). *Biochem. J.*, **213**, 363–9.

Jurasek, P. & Phillips, G.O. (1993). *Food Hydrocolloids*, **7**, 337–352 and previous papers in the series.

Nakamura, K., Hatakeyama, T. & Hatakeyama, H. (1983). *Polymer*, **24**, 871–874.

Osman, M.E., Menzies, A.R., Williams, P.A., Phillips, G.O. & Baldwin, T.C. (1993). *Carbohydr. Res.*, **246**, 303–309.

Osman, M.E., Williams, P.A., Menzies, A.R. & Phillips, G.O. (1993). *J. Agric. Food Chem.*, **41**, 71–76.

Randall, R.C., Phillips, G.O. & Williams, P.A. (1988). *Food Hydrocolloids*, **2**, 131–136.

Randall, R.C., Phillips, G.O. & Williams, P.A. (1989). *Food Hydrocolloids*, **3**, 65–69.

Takigami, S., Takigami, M. & Phillips, G.O. (1993). *Carbohydr. Polym.*, **22**, 153–160.