Carbohydrate Polymers 82 (2010) 498–503

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

An alternative DSC approach to study hydration of hyaluronan

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ARTICLE INFO

Article history:
Received 7 April 2010
Received in revised form 5 May 2010
Accepted 7 May 2010
Available online 21 May 2010

Keywords:
Hyaluronan
Hydration
DSC
Water evaporation

ABSTRACT

Differential scanning calorimetry (DSC) was used to determine the number of water molecules in the hydration shell of hyaluronan of different molecular weights and counterions. First, traditional experiments including freezing/thawing of free water in semi-diluted solutions were carried out leading to the determination of melting enthalpy of freezable water. Non-freezing water was determined using extrapolation to zero enthalpy. For sodium hyaluronan within the molecular weight range between 100 and 740 kDa the hydration shell was determined as 0.74 g g^{-1} HYA. A larger hydration shell containing 0.84 and 0.82 g g^{-1} HYA was determined for hyaluronan of 1390 kDa in its sodium and protonized form, respectively. Second, melting enthalpy of freezing water was further studied applying water evaporation experiments. Resulted plot of enthalpy vs concentration indicated an additional heat evolution process which occurs at specific concentration and decreases the measured evaporation enthalpy. The heat evolution was attributed to the mutual approaching of hyaluronan molecular chains, their mutual interactions and formation of the ordered hyaluronan structure which starts immediately when the hydration water is desorbed from the hyaluronan surface. The concentration at which the process occurred was related to "non-evaporable water" which was determined as 0.31–0.38 g g^{-1} for sodium hyaluronan and 0.84 g g^{-1} for its protonized form. The second approach provides additional information enabling a deeper insight into the problem of hyaluronan hydration.

1. Introduction

Hyaluronan (HYA) is a linear, unbranched, high molecular weight extracellular matrix polar polysaccharide belonging to the glycosaminoglycans class. HYA is composed of repeating polyanionic disaccharide units which consist of N-acetyl-D-glucosamine and D-glucuronic acid linked by a β 1–4 glycosidic bond. The disaccharides are linked by β 1–3 bonds to form HYA chains (Fig. 1). In vivo, it occurs exceptionally in the form of Na$^+$ salt. HYA polymers have extraordinarily wide range of use and often different biological functions depending on the molecular mass which can reach up to 10 MDa. Larger matrix polymers of HYA show space-filling, antiangiogenic, immunosuppressive effects and play an important role in tissue hydration (Kogan, Šoltéz, Stern, & Gemener, 2007). In contrast, the HYA segments of lower molecular weight are well known to have pronounced biological activities playing role for example in tumour diagnose.

Hydration and/or water holding capacity is probably one of the most important aspects of the HYA function. A perusal of literature shows a lot of works dealing with the determination of hydration shells and enumeration of water molecules surrounding HYA molecules in diluted and semi-diluted HYA/water solutions (Haxaire, Marechal, Milas, & Rinaudo, 2003a, 2003b; Joun, Rinaudo, Miles, & Desbrieres, 1995; Marechal, Milas, & Rinaudo, 2003; Yoshida, Hatakeyama, & Hatakeyama, 1992) and swelling of HYA in water (Mráček, Benešová, Minařík, Urban, & Lapčík, 2007) or in salt solutions (Mráček et al., 2008). There are several techniques and approaches, both experimental and theoretical, to shed light on water behaviour in the presence of HYA molecules. In this paper we focus on the differential scanning calorimetry (DSC), a method belonging to the family of thermo-analytical techniques.

The traditional and probably the only way of differentiation of water molecules in hydration shells using DSC is based on freezing/thawing experiments in which the difference in physical properties between freezable water in form of ice and non-freezable water that is tightly bound on the HYA surface is investigated. Accordingly, the water shells are categorized into three groups, i.e. non-freezing water (NFW), freezing-bound water (FBW) and free water (FW). NFW is strongly fixed to the HYA surface through the electrostatic interactions. The motion of NFW is limited and therefore such water cannot crystallize when cooled down (Wolfe, Bryant, & Koster, 2002) or crystallizes in time period which is far beyond the time framework of the experiment. It was stated that such water molecules are directly attached especially to the hydroxyl groups of HYA (Hatakeyama, Nakamura, & Hatakeyama, 2000). FBW is located in larger distance from a HYA molecule. It is

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doi:10.1016/j.carbpol.2010.05.022

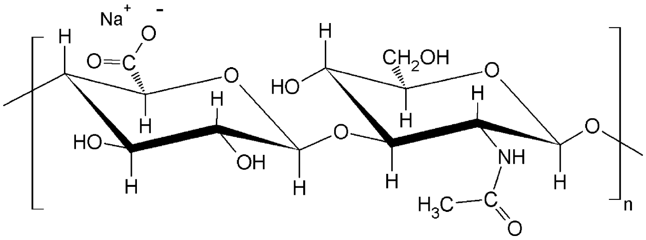

Fig. 1. Disaccharide unit of hyaluronan.

thought it freezes and melts at lower temperatures than normal, bulk water, it is easy to be supercooled and further the melting enthalpy is also lower than for the bulk water due to its different crystal morphology. In fact, while frozen FBW is thought to consist of cubic ice, the FW ice is formed by hexagonal structures (Yoshida et al., 1992). FW behaves as normal pure water, because its structure is not influenced by the presence of HYA molecules, it means that, when frozen, the melting enthalpy is 334 J g^{-1} and the melting and freezing temperature is around 0 °C (Berthold, Desbrieres, Rinaudo, & Salomen, 1994; Joshi and Topp, 1992; Joun et al., 1995; Lui & Cowman, 2000; Takahashi, Hatakeyama, & Hatakeyama, 2000; Yoshida, Hatakeyama, & Hatakeyama, 1989, 1990; Yoshida et al., 1992; Yoshida, Hatakeyama, & Hatakeyama, 1993). Lui and Cowman (2000) reviewed previously published DSC approaches and made the first attempt to describe such behaviour mathematically. They derived equations allowing a precise determination of NFW and FBW while adopting the minimum value of fusion enthalpy change of 312 J g^{-1} previously reported by Yoshida (Yoshida et al., 1992). For the native HYA they determined about 44 g g^{-1} HYA as FBW and about 0.6 g g^{-1} HYA as NFW (Lui & Cowman, 2000). When related to number of water molecules per dissacharide HYA unit, the determined amount of NFW corresponds to 13.4 molecules. This value is rather different from 4 to 5 molecules that were theoretically derived using FTIR in dry HYA film (Haxaire et al., 2003a, 2003b; Marechal et al., 2003). The reason of such difference is that there are several undisputable limits to use DSC cooling/thawing experiments for precise enumeration of water in NFW shell (Wolfe et al., 2002). The main problem is the occurrence of an unknown amount of amorphous (Wolfe et al., 2002) and low density ice (probably associated with FBW) in frozen water which can bias the determined enthalpy of ice melting which in turn may consequently result in an overestimation of NFW. In addition, there are other experimental aspects which can influence the DSC results, such as the baseline distortion, or supercooling effect (Wolfe et al., 2002).

In order to overcome some of the disadvantages mentioned above, instead of melting enthalpy, the enthalpy of water evaporation from hyaluronan solution was measured in this work. Determined data were compared with results obtained by traditional HYA hydration experiments using DSC cooling/thawing experiments. The results obtained from the two different methods provided new information regarding the hydration of hyaluronan.

2. Materials and methods

2.1. Hyaluronan (HYA)

Bacterial HYA, specifically its Na^+ form (Na^+HYA) was kindly provided by CPN Company (Dolní Dobrouč, Czech Republic). HYAs with the following molecular weights were used: 100, 254, 740 and 1390 kDa.

The protonized form of HYA (H^+HYA) was produced as follows: 1390 kDa Na^+HYA was dissolved in water, transferred into a dialysis bag (cut off 3500 Da) and dialyzed against 0.1 mol L^{-1} HCl until Na^+ free. Then, the obtained product was dialyzed against milli-Q water until it became chloride-free. Quality of final product was controlled by thermogravimetry to determine the residual ash after burning in dynamic air atmosphere at 600 °C (i.e. 0%).

2.2. Preparation of HYA/water systems

Samples of approximately 10–20 mg (weighted with an accuracy of ±0.01 mg) were placed in aluminum sample pans (TA Instruments, Tzero® technology) and the excess of water (milli-Q) was added to HYA sample. Surplus water was allowed to evaporate slowly at room temperature until the desired water content was obtained. The pans were subsequently hermetically sealed and left to equilibrate at room temperature for 26 h as recommended by Takahashi et al. (2000). It was already published that the time interval is enough to reach a constant value of NF water in the HYA sample. Similar samples were used for freezing/thawing as well as for the evaporation experiments.

Water content (W_c) was defined as follows:

$$W_c = \frac{\text{grams of water}}{\text{grams of dry sample}}\,(\text{g}\,\text{g}^{-1}) \qquad (1)$$

2.3. Thermal analysis

Differential scanning calorimetry (DSC) was carried out using the TA Instruments DSC Q200 equipped with a cooling accessory RCS90 and assessed by the TA Universal Analysis 2000 software. The following thermal protocol was used for freezing/thawing experiments: start at 40.0 °C; cooling from 40.0 to −90.0 °C at 3.0 °C min^{-1}; isothermal at −90.0 °C for 2.0 min; heating from −90.0 to 30 °C at 3.0 °C min^{-1}. Flow rate of dynamic nitrogen atmosphere was 50 mL min^{-1}, as a sample holder was used hermetically sealed T_{zero} Al pan while sample was prepared as described above.

The following thermal protocols were used for the measurement of evaporation enthalpy: equilibration at 27.0 °C; cooling from 27.0 to −40.0 °C at 10.0 °C min^{-1}; isothermal at −40.0 °C for 2.0 min; heating from −40.0 to 250.0 °C at 3.0 °C min^{-1} and switching the flow rate of nitrogen from 50 mL min^{-1} to 5 mL min^{-1}. Immediately before the measurement, the hermetic lid (necessary for the sample preparation) was perforated using a sharp tool and the measurement was carried out straightway.

Selected samples in different concentration ranges were measured in triplicate to determine the statistical significance. Standard deviation never exceeded 7%; typically it was below 5%.

To obtain precise water content, thermogravimetry (TA Instruments, Q5000IR) was used to determine the equilibrium moisture content as a weight loss in the temperature interval 25–220 °C under dynamic atmosphere of nitrogen 25 mL min^{-1}. That information was used during the HYA/water sample preparation.

3. Results and discussion

3.1. Freezing/thawing experiments

First of all, the DSC of HYA/water systems was carried out for different water concentrations; examples of DSC records for low concentrations are given in Fig. 2. Fig. 2 shows the heating run, i.e. ice melting records for HYA (740 kDa) with various concentrations of water; the dotted line represents the hypothetical straight baseline which should only serve for a better recognition of processes occurring during the melting of ice in frozen HYA/water mixture. The determination of enthalpies presented here was carried out using a slightly different approach taking into account the cold crystallization, baseline shift and non-linearity according to the literature recommendations (Riesen, 2007).

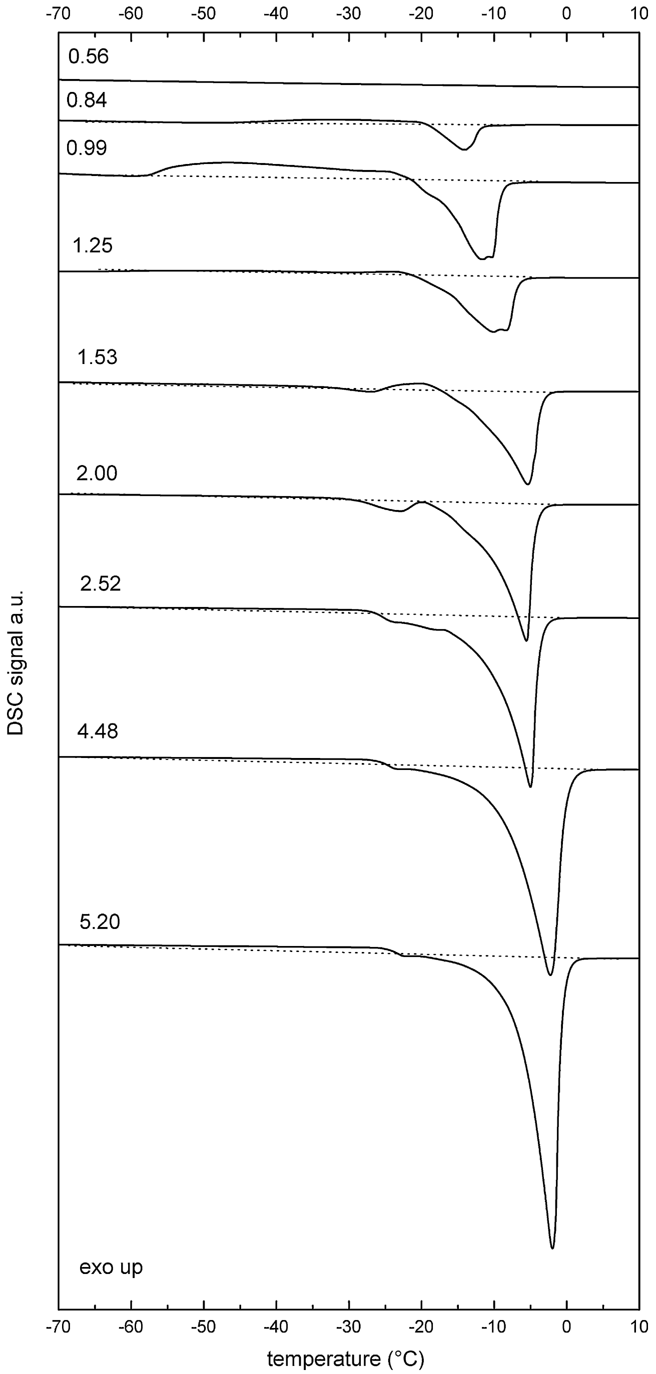

Fig. 2. DSC melting records for HYA (740 kDa).

It can be seen that around W_c = 0.5 no peak occurred on the heating curves. It seems that all the water molecules occurred in NFW shell. Since at this concentration the number of water molecules per HYA disaccharide unit (400 g mol^{-1}) is approximately 11, it is likely that the water molecules are strongly bound to the HYA skeleton and low temperature does not affect their mutual interactions. It means that low temperature did not cause the water desorption from HYA molecule, water molecules were not separated to form ice crystals which melt when heated up. Increase in concentration of water in HYA sample brought about the appearance of events associated with the presence of freezable water. There is a weak exothermal event that occurs at W_c = 0.84 and can be attributed to cold crystallization of supercooled water starting around −48 °C followed by melting around −22 °C. Increase in water content to W_c = 0.99 showed the enlargement of crystallization peak before the ice melting. Then two separate endotherms appeared at W_c = 1.53; first one starting at −32 °C and the other one starting at −20 °C. Those are not any longer separated at W_c = 2.52 and above that concentration, where again the crystallization appeared followed by a single melting peak with the onset around −30 °C. From Fig. 2 it can be further observed that there is a general tendency for the onset temperature of melting peak to slightly increase with increasing water content in the sample. Such finding is quite typical and is in accordance with the observations reported in the earlier papers (Hatakeyama & Hatakeyama, 1998); Yoshida et al., 1992). The HYA samples of molecular weight 100, 253 and 1396 kDa gave similar records and are not reported here.

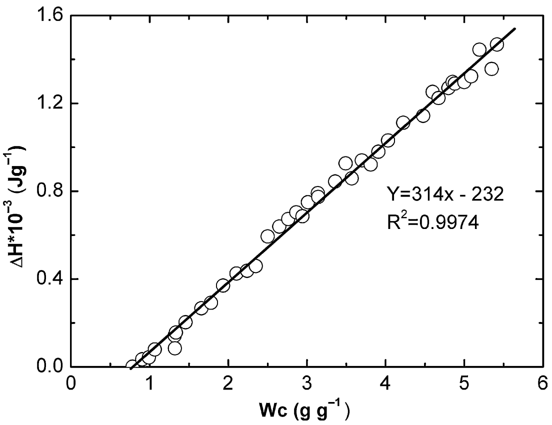

Fig. 3. Dependency of the enthalpy change associated with the melting endotherm in the HYA (740 kDa) solutions, normalized to the HYA weight, as a function of water content in HYA.

As previously suggested by Liu and Cowman, the observed enthalpy of melting was first normalized dividing by the weight of the dry HYA mass and then plotted against the respective W_c (Fig. 3). In this way, the NFW content was determined from the x-intercept (Lui & Cowman, 2000). Obtained values of NFW and parameters of linearization are listed for all samples in Table 1. It can be seen that the NFW content was constant for HYA of molecular weight from 100 to 740 kDa and was always determined as 0.74 g of water per gram of HYA. A larger hydration shell consisting of 0.84 g g^{-1} NFW was found for 1390 kDA HYA.

The same experiments were carried out using H$^+$ form of HYA (H$^+$HYA). The protonized form showed different behaviour in comparison with Na$^+$ form. In fact, it can be easily identified in Fig. 4 that ice around H$^+$ HYA form melts at significantly higher temperature than that in Na$^+$HYA form. Although the onset of the melting is not exactly at 0 °C as for pure water, the temperature is significantly shifted to higher temperatures. Determination of NFW was carried out in the same way as suggested in Fig. 3 and brought result of about 0.82 g g^{-1}.

Table 1
Content of hydration water for HYA of different molecular weight and counterion from cooling/thawing experiments. n_{NFW} is the number of water molecules per disaccharide unit, NFW stands for non-freezing water (in g of water per 1 g of HYA) determined using the approach reported in Lui and Cowman (2000).

Sample	^{a}NFW	$^a n_{\mathrm{NFW}}$	Parameters *a*; *b*	Confidence coefficient R^2
100	0.74	16.5	312; −230	0.9984
254	0.74	16.5	315; −234	0.9988
740	0.74	16.5	314; −232	0.9974
1390	0.84	18.7	302; −255	0.9976
H$^+$	0.82	17.2	329; −270	0.9979

[a] Recalculated to the molecular weight of Na$^+$ and H$^+$ form.

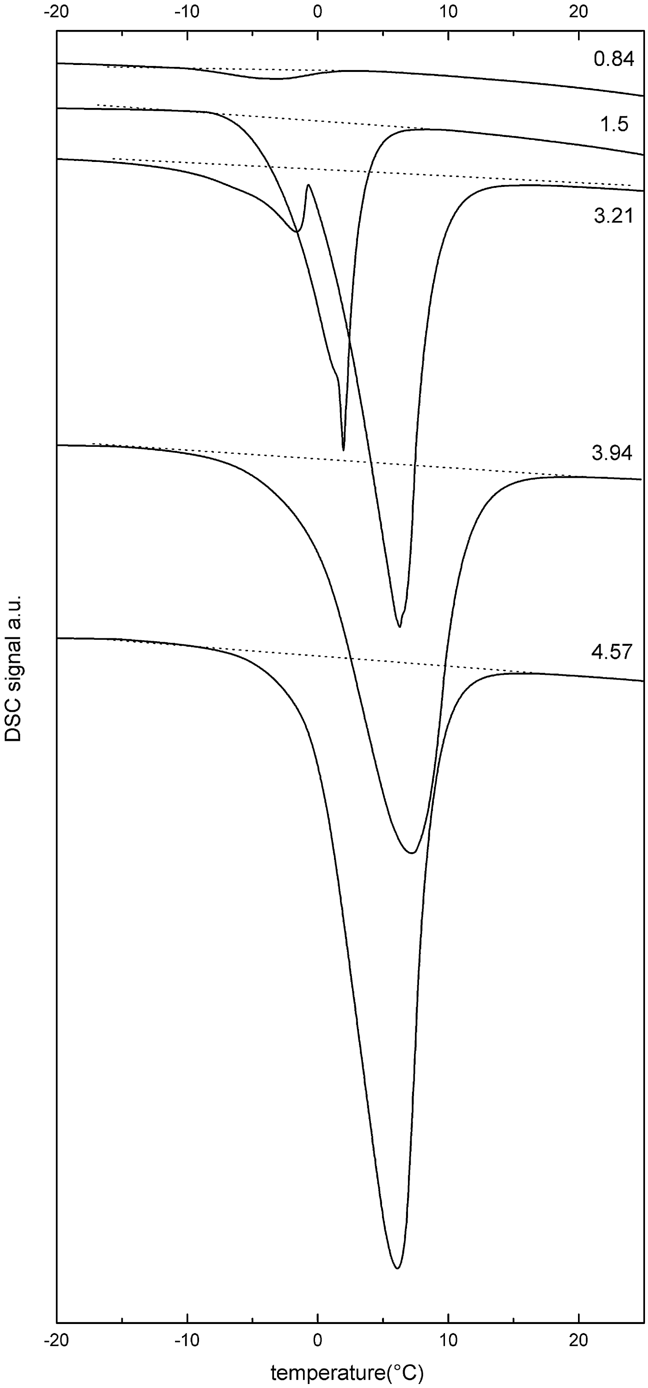

Fig. 4. DSC melting records for H^+HYA.

3.2. Evaluation experiments

Samples with the same water content as used in the freezing/thawing experiments were measured to determine the enthalpy of evaporation of water from the mixture with HYA. Simply, before the experiment was carried out, the lid was carefully perforated by a sharp pin; the sample was then cooled down and heated up to 220 °C. The reason to apply the freezing segment before the evaporation was due to easier identification of the onset of evaporation (Fig. 5).

The heating rate was chosen reasonably slow to evaporate as much as possible of the water present in the sample before its boiling. Again, the enthalpy of processes was assessed and elaborated as described above. In Fig. 5 there is given a representative DSC evaporation record for HYA 740 kDa. The cooling curve, depicted in the upper part of the figure, shows an event corresponding to the freezable water crystallization. Conversely, heating curve shown at the bottom part of Fig. 5 reveals two endothermic peaks, where the first one corresponds to the melting of water in the sample while the other broad endothermic peak can be attributed to the water evaporation. Fig. 6 shows the comparison of water/HYA samples with various W_c. As expected, the peak temperature and peak area is shifted with increasing water content in the samples.

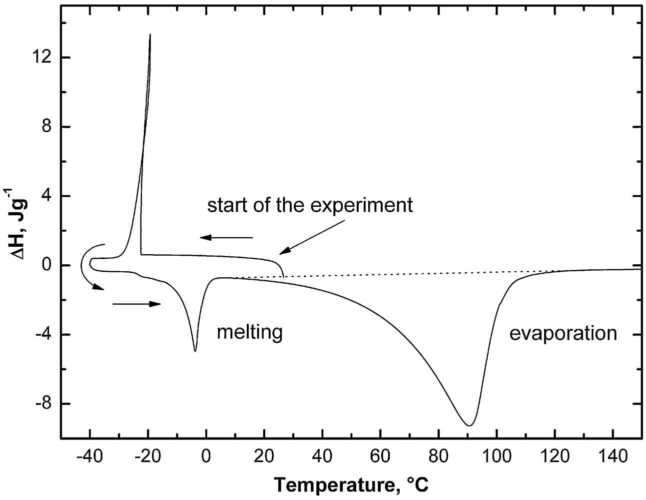

Fig. 5. DSC record for the determination of evaporation enthalpy for HYA (740 kDa), $W_c = 1.94$.

Fig. 7 shows typical dependency of evaporation enthalpy normalized to the mass of dry Na^+ forms of HYA or H^+HYA with molecular weights 101, 740 and 1390 kDa. There can be seen a linear decrease of the enthalpy with decreasing W_c; a break occurs around the value $W_c = 0.35$. Since at this concentration the water molecules are supposed to be bound more tightly to the HYA molecule, it is natural to assume that the energy necessary for its evaporation should be higher than that for bulk water. As a result, the enthalpy should be higher and the slope of the dependency should be in reverse direction to that shown in Fig. 7, i.e. more steeply decreasing. Therefore, it seems that at this concentration the consumption of energy necessary for evaporation is compensated by a process or processes in which the energy is evolved.

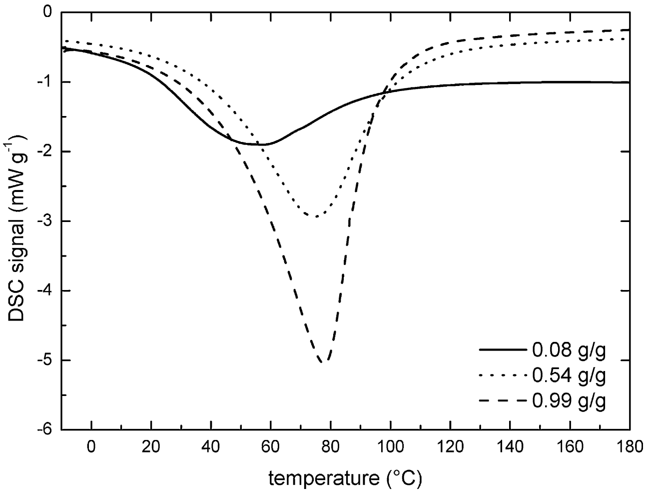

Fig. 6. Comparison of evaporation profiles of water/HYA (740 kDa) samples of different concentrations.

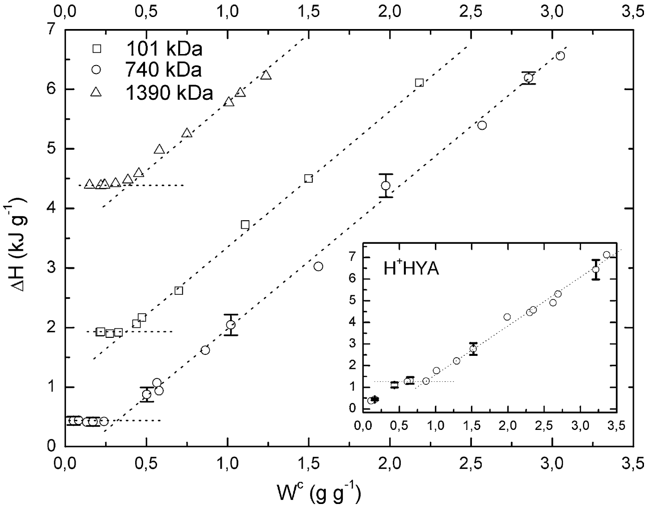

Fig. 7. Dependences of normalized enthalpy of evaporation on concentration. For better resolution, determined enthalpies of 101 and 1390 kDa HYA were shifted by 2 and 4 kJ g^{-1}, respectively.

A similar dependence was obtained also for sample of molecular weight of 254 kDa. Unlike the low molecular HYA samples, the sample of 1390 kDa did not show a perfectly clear break and instead, the dependency showed only a slow decrease of slope (Fig. 7). Nevertheless, even such a kind of dependency allowed us to proceed a rough estimation of the intersection of the two lines as suggested in Fig. 7 for low molecular weight HYA fractions. Table 2 summarizes the parameters of linear regression of points before the break occurred and the intercepts with linear region. Using those intercepts, the hydration numbers which have the meaning of "non-evaporable" water were determined (Table 2).

3.3. Comparison of methods applied

Data presented in this work confirmed earlier results that the nature and distribution of ice present in the HYA system depends on W_c (Yoshida et al., 1992). It has been also previously stated that the water-binding capacity is directly related to the molecular weight of the molecule (Sutherland, 1998). However, that statement was not confirmed in this work, where HYA having molecular weight from 101 to 740 kDa showed similar water binding capacity and different value was observed only for 1390 kDa (Table 1). A number of theories have already been reported as possible explanation for this difference such as for example influence of the molecular chain dynamics hindering the self-diffusion of water from the free movement during the nucleation (Wiggins, 1995) or occurrence and composition of glassy ice and low density ice with unknown melting enthalpy and unpredictable behaviour (Wolfe et al., 2002) In fact, in order to overcome problems associated with ice formation, the evaporation experiments were carried out. Comparison of results reported in Tables 1 and 2 shows that except for the H$^+$HYA the determined NFW content was substantially lower than value of non-evaporable water obtained by freezing/thawing experiment. However, some aspects should be clarified also in this case. First of all, while in general only a linear decrease of enthalpy of evaporation would be expected due to the progressively decreasing water content, in case of HYA at certain concentration the break occurs (Fig. 7). Such a break could be accounted for the appearance of a process which is associated with energy release competing with energy consumption necessary for water evaporation, or in the case of the last hydration layer, water desorption. That means that the total enthalpy measured by DSC is not linearly decreasing at lower concentration range, and instead it shows more or less constant values (except H$^+$HYA). Presumably, the reverse enthalpy balance should be expected since the hydration water is strongly tight on the HYA skeleton and thus would need more energy to be desorbed from the charged surface. A possible explanation could be found in the conformation and molecular movement of HYA segments. HYA was described as crowded random coil molecules in liquid state but after the evaporation of water, in solid state, it is predominantly a single helical conformation containing 3 disaccharides per helical turn. However, the number of disaccharides per helical turn and formation of single or double helix depends on the character of counter ions. Structural conformation can be understood as stretched coiled telephone cords stabilized by H-bonds linking adjacent sugar residues across both glycosidic linkages (Cowman & Matsuoka, 2005). Since the dissolution of HYA in water is accelerated by elevated temperature, it seems that the formation of some intermolecular and intramolecular H-bonds is thermodynamically slightly more favoured in comparison with water/HYA interaction. Considering this, the resulting supramolecular arrangement (entropy) in solid state is then better organized than the conformation of HYA in solution. The higher organization causes the energy release, which in turn results in the break dependence depicted in Fig. 7. Such explanation is further supported by Fig. 6 where the low water content sample $W_c = 0.08$ showed the evaporation peak at around 60 °C which is still in the temperature range, where the bulk water evaporates (Fig. 6). If the water was adsorbed on the HYA molecule, the desorption temperature would be higher than that of evaporation of bulk water. This again supports the idea of the occurrence of an additional exothermic process compensating for the enthalpy of water desorption at low water concentrations.

Assuming the monomolecular layer of water on the surface of HYA, the approaching of segments and subsequent formation of H-bonds can occur only when there is no molecule of water between two HYA segments. Accordingly, assumed reconformation process starts when the hydration water is being desorbed which means that the break necessarily indicates the desorption onset of the last hydration layer.

As it can be seen in Table 2, H$^+$HYA showed two times higher hydration number than HYA. The possible explanation has already been indicated in previous paragraphs (concerning the structure of HYA in solid state) and it is related to the secondary structure of HYA and H$^+$HYA in solid state. In fact, the presence of Na$^+$ ion brings about the occurrence of low-temperature melting ice when cooled down. It confirms that the presence of ions with different dimension and surface charge is crucial for the character of supramolecular structure. It seems that the Na$^+$ ion makes the structure more "rigid", and therefore the reported unfolding of the HYA chains during evaporation is easier when the H$^+$ ion is present.

Table 2
Content of hydration water for HYA of different molecular weight and counterion from evaporation experiments. n_b is the number of water molecules per disaccharide unit, NEW stands for the content of non-evaporable water in g per 1 g of HYA.

Sample	NEW	[a]n_{NEW}	[b]Parameters a; b	Confidence coefficient R^2
100	0.36	8.0	2224; −318	0.9998
254	0.36	8.0	2192; −279	0.9984
740	0.31	6.9	2251; −256	0.9981
1390	0.38	8.4	2040; −268	0.9894
H$^+$	0.84	17.6	2213; −609	0.9947

[a] Recalculated to the molecular weight of Na$^+$ and H$^+$ form.
[b] For the linear part of the increase.

4. Conclusion

Knowledge of HYA hydration is crucial for designing modification reactions such as crosslinking, hydrophobization etc.

In accordance with previous findings about hydration of ions (Zavitsas, 2001), the number of hydration water determined for HYA depends on the method and approach used. Nevertheless, as shown in this study, there exists an alternative approach which provides additional information enabling a deeper insight into the problem of HYA hydration when DSC technique is used. It is necessary to point out that the content of both NFW and non-evaporable water depends on the temperature since the origin of both is in the strong temperature-dependent water adsorption and therefore the content of both is different.

The evaporation approach seems to be a suitable option for the determination of hydration water in HYA or possibly also in other (bio)polymers. In our opinion, it provides more reliable results which are less biased by the unknown factors. Moreover, results published here revealed very important phenomena with interesting and exploitable consequences. In fact, during the evaporation, the concentration of water around 0.3–0.4 $\mathrm{g\,g^{-1}}$ seems to be very important for the character of HYA in dry state. In other words, this is the moment in which the HYA supramolecular structure can be simply influenced by the external factors (such as temperature, mechanical stress etc.) in order to obtain dry, non-modified or "native" HYA with specific properties. This is in accordance with the recent comment of Hargittai and Hargittai (2008) on the work of Laurent (1957) about importance of conditions under which HYA is prepared. In addition they also stressed out the observation of Scott (1998) who put in question the randomness of HYA coiling. The influencing of HYA structure in solid state by an external factor is a similar approach as frequently used in "crystal engineering". Such issue, however, is beyond the scope of this work and it will be solved in a special study.

Acknowledgement

This work was financially supported by the Ministry of Education, Youth and Sport of the Czech Republic project No. 0021630501.

References

Berthold, J., Desbrieres, J., Rinaudo, M., & Salomen, L. (1994). Types of adsorbed water in relation to the ionic groups and their counter-ions for some cellulose derivatives. *Polymer*, *35*, 5729–5736.

Cowman, M. K., & Matsuoka, S. (2005). Experimental approaches to hyaluronan structure. *Carbohydrate Research*, *340*, 791–809.

Hargittai, I., & Hargittai, M. (2008). Molecular strcuture of hylauronan: an introduction. *Structural Chemistry*, *19*, 697–717.

Hatakeyama, H., & Hatakeyama, T. (1998). Interaction between water and hydrophilic polymers. *Termochimica Acta*, *308*, 3–22.

Hatakeyama, T., Nakamura, K., & Hatakeyama, H. (2000). Vaporation of bound water associated with cellulose fibres. *Termochimica Acta*, *352–353*, 233–239.

Haxaire, K., Marechal, Y., Milas, M., & Rinaudo, M. (2003a). Hydration of polysaccharide hyaluronan observed by IR spectrometry. I. Preliminary experiments and band assignments. *Biopolymers*, *72*, 10–20.

Haxaire, K., Marechal, Y., Milas, M., & Rinaudo, M. (2003b). Hydration of hyaluronan polysaccharide observed by IR spectrometry. II. Definition and quantitative analysis of elementary hydration spectra and water uptake. *Biopolymers*, *72*, 149–161.

Joun, N., Rinaudo, M., Miles, M., & Desbrieres, J. (1995). Hydration of hyaluronic acid as a function of the counterion type and relative humidity. *Carbohydrate Polymers*, *26*, 69–73.

Joshi, H. N., & Topp, E. M. (1992). Hydration in hyaluronic acid and its esters using differential scanning calorimetry. *International Journal of Pharmaceutics*, *80*, 213–225.

Kogan, G., Šoltéz, L., Stern, R., & Gemener, P. (2007). Hyaluronic acid: a natural biopolymer with a broad range of biomedical and industrial applications. *Biotechnology Letters*, *29*, 17–25.

Laurent, T. C. (1957). The amorphous X-ray diffractogram of hylauronic acid. *Arkiv for Kemi*, *11*, 513–518.

Lui, J., & Cowman, M. K. (2000). Thermal analysis of semi-diluted hyaluronan solutions. *Journal of Thermal Analysis and Calorimetry*, *59*, 547–557.

Marechal, Y., Milas, M., & Rinaudo, M. (2003). Hydration of haluronan polysaccharide observed by IR spectrometry. III. Structure and mechanism of hydration. *Biopolymers*, *72*, 162–173.

Mráček, A., Benešová, K., Minařík, A., Urban, P., & Lapčík, L. (2007). The diffusion process of sodium hyaluronate (Na-HA) and Na-HA-n-alkyl derivatives films swelling. *Journal of Biomedical Materials Research Part A*, *83A*, 184–190.

Mráček, A., Varhaníková, J., Lehocký, M., Grundelová, L., Pokopcová, A., & Velebný, V. (2008). The influence of Hofmeister series ions on hyaluronan swelling and viscosity. *Molecules*, *13*, 1025–1034.

Riesen, R. (2007). Choosing the right baseline. *User Com*, *25*, 1–6.

Scott, J. E. (1998). Chemical morphology of hyaluronan. In T. C. Laurent (Ed.), *The chemistry and medical applications of hyaluronan and its derivatives, Wenner–Gren international series* (pp. 7–15). London: Portland Press.

Sutherland, W. (1998). Novel and established applications of microbial polysaccharides. In *Institute of cell and molecular biology*. Edinburgh University., p. 16.

Takahashi, M., Hatakeyama, T., & Hatakeyama, H. (2000). Phenomenological theory describing the behaviour of non-freezing water in structure formation process of polysaccharide aqueous solutions. *Carbohydrate Polymers*, *41*, 91–95.

Wiggins, P. M. (1995). High and low density water in gels. *Progress in Polymer Science*, *20*, 1121–1163.

Wolfe, J., Bryant, G., & Koster, K. L. (2002). What is "unfreezeable water", how unfreezable is it and how much is there. *CryoLetters*, *23*, 157–166.

Yoshida, H., Hatakeyama, T., & Hatakeyama, H. (1989). Glass transition of hyaluronic acid hydrogel. *Kobunshi Ronbunshu*, *46*, 597–602.

Yoshida, H., Hatakeyama, T., & Hatakeyama, H. (1990). In J. F. Kennedy, G. O. Phillips, & P. A. Williams (Eds.), *Cellulose* (pp. 305–310). Chichester, UK: Horwood.

Yoshida, H., Hatakeyama, T., & Hatakeyama, H. (1992). *Polymer preprints, Japan* , p. 3026.

Yoshida, H., Hatakeyama, T., & Hatakeyama, H. (1993). Characterization of water in polysaccharide hydrogels by DSC. *Journal of Thermal Analysis*, *40*, 483–489.

Zavitsas, A. A. (2001). Properties of water solutions of electrolytes and nonelectrolytes. *Journal of Physical Chemistry B*, *105*, 7805–7815.