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Letter to the Editor

Response to "A note on An alternative DSC approach to study hydration of hyaluronan"

The author of the note (Pekar, 2012) criticizes some issues reported in our recent paper (Prusova, Smejkalova, Chytil, Velebny, & Kucerik, 2010) and suggests alternative ways of data evaluation. Briefly, we used hyaluronan samples with certain water contents in order to determine non-freezing water content using the DSC technique. In these experiments samples were cooled down and then heated up. Samples containing more water than only non-freezing water showed an endothermal peak originating from a melting of the freezable water. The obtained enthalpies were treated according to the suggestions of Liu and Cowman (2000) and the non-freezing water contents, for the set of hyaluronan with different molecular weights and counterions, were determined. In the second part of our work, similar approach was used for water evaporation. The results unraveled a process apparently compensating the enthalpy needed for evaporation of water from hyauronan.

In this work, we would like to clarify some points which may have caused fundamental misunderstanding of our paper and some DSC principles leading to conclusions reported in Pekar (2012).

1. Freezing/cooling experiments

The influence of a biopolymers and other organic materials on ice melting has been published several times (e.g., Hatakeyama & Hatakeyama, 1998; Hatakeyama, Hatakeyama, & Nakamura, 1995; Hatakeyama, Quinn, & Hatakeyama, 1996; Hatakeyama, Yoshida, & Hatakeyama, 1987; Liu & Cowman, 2000; Schaumann, 2005; Takahashi, Hatakeyama, & Hatakeyama, 2000; Yoshida, Hatakeyama, & Hatakeyama, 1993). The assumption is that part of the water in the system water/hyaluronan does not freeze under experimental conditions due to the interaction between water and hyaluronan. Traditionally, this non-freezing water (NFW) content was determined as a water content at which any resolvable peak on the DSC record was observed. Naturally, due to the different sensitivities of DSC devices, the amount of NFW determined using different DSC devices can slightly differ. Based on these observations, Liu and Cowman (2000) derived an equation which simplifies this problem by extrapolation of determined enthalpies, normalized by hyaluronan mass, as a function of water content in hyaluronan, to a situation when no peak occurs on the DSC record. It is beyond the scope of this paper and beyond the scope of Prusova et al. (2010) to either criticize or extend equations suggested by Liu and Cowman (2000), but it is necessary to correct some statements reported by Pekar (2012) since those are based on erroneous understanding of the problem.

The author reports that "$\Delta H = f(m_w, m_p)$" and the determined melting enthalpy ΔH can be expressed as "$\Delta H = k_1 m_w + k_2 m_p$", where m_p and m_w are the mass of hyaluronan and water, respectively (Pekar, 2012). However, in Figure 1 in Pekar (2012) the author reports only "$\Delta H = f(m_w)$" while the influence of m_p, which is the second variable influencing ΔH, is for unknown reason overlooked (or assumed to be constant). However, m_w and m_p are interdependent. Then the trend in Figure 1 (Pekar, 2012) is treated as $\Delta H = f(m_w)$, the previously defined part "$k_2 m_p$" is replaced by coefficient "q" which does not seem to contain m_p since the equation after the normalization by m_p provides the "q" as"$(k_2 m_p)/m_p$". This inconsistency in description leads to author's confusion in further paragraphs of the commented paper which we would like to, at least partially, elucidate.

What one can actually read from the Figure 1 reported by Pekar (2012)? The only usable information is that the operator preparing the samples used water mass in the range from 5 to 20 mg and added appropriate amount of hyaluronan (or vice versa) to reach the desired water content W_c ($W_c = m_{water}/m_{hyaluronan}$ or $W_c = m_w/m_p$ using author abbreviations). Figure 1 in Pekar (2012) might lead to an erroneous conclusion that there is no detectable peak regardless the amount of m_p around 4 mg of water ($m_w = 0.004$ g in Fig. 1). In other words, according to Figure 1 (Pekar, 2012), keeping 4 mg of water in the pan and adding any amount of hyaluronan should always result in absence of a peak in DSC record. However, a sample containing 4 mg of water and 1 mg of hyaluronan, i.e. $W_c = 4$ gives already an intense peak (e.g. Figure 2 in Prusova et al. (2010)). That means that Pekar (2012) plotted the mass of the water chosen arbitrarily by the operator for sample preparation against determined enthalpy. It gives an apparently linear dependency which is however a result only of random choosing of m_w or m_p. This can be even easily demonstrated in case when the operator decides to use constant m_w during sample preparation, e.g. 5 mg ($m_w = 0.005$ g in Figure 1 (Pekar, 2012)) and changes only the m_p to get desired W_c. We try to illustrate this in Fig. 1 where we extracted the measured data around $m_w = 5$ mg of water in samples from data reported in Prusova et al. (2010). It can be seen that one m_w (more or less constant) gives different enthalpies. In fact, the same situation can be identified also in Pekar (2012), since in some cases m_w is the same and giving different ΔH, but this fact was ignored.

Similar situation appears when the ΔH is expressed for example as a function of $W_c = m_w/m_p$ ((Fig. 2 (in this work), n.b. samples with the same $W_c = 1.3$ (data come from Prusova et al. (2010)). Also in this case the picture shows that for the same W_c several different ΔH can be determined. Analogically to previous example, two samples with $W_c = 1$ which can be prepared either as 5 mg_w/5 mg_p or 20 mg_w/20 mg_p. Assuming the NFW content 0.7 $mg_{water}/mg_{hyaluronan}$ in sample 5 m_w/5 m_p will freeze 1.5 mg of water while in 20 mg_w/20 mg_p will freeze 6 mg of water giving naturally different not-normalized ΔH.

Pekar (2012) made the correct mathematical operation $\Delta H = k_1 m_w + k_2 m_p \rightarrow \Delta H/m_p = k_1 m_w/m_p + k_2$. This is formally the equation used in our paper and developed by Liu and Cowman (2000) and when $\Delta H/m_p = f(m_w/m_p)$ is plotted, the author got the same results (Fig. 2) as was previously obtained by Prusova et al. (2010, in Figure 3).

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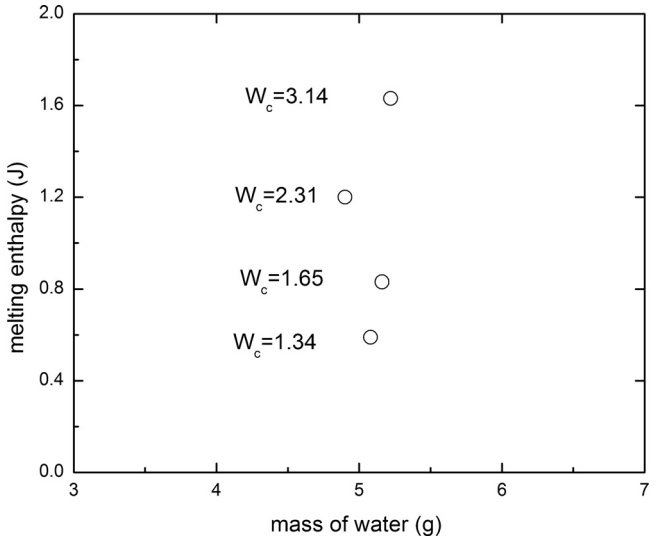

Fig. 1. Illustration of problem with the same ice melting enthalpy for samples with different W_c but the same water mass based on the approach reported by Pekar (2012).

Therefore, the statement of Pekar (2012) "This experimental observation was ignored in the paper though it seems to be of primary importance" cannot be taken into account since Figure 1 in Pekar (2012) reflects only the experimental conditions, ignores some variables, and the "linear" trend can be considered as an artifact depending on the choice of the person weighing the sample and the size of DSC pan. Therefore, to avoid similar serious data misinterpretation, the normalization of primary data is necessary.

Further Pekar (2012) states "It could be further demonstrated... that also the dependence of the measured heat per gram of the sample is a linear function of water mass fraction or that there is a clear dependence of the measured heat per gram of water on the water mass fraction (and linear up to about 70% of water). All this was ignored in the commented paper." As follows from previous paragraphs, the dependences should not be created arbitrarily expecting any physical meaning of dependencies or apparent dependencies, since it can lead to the erroneous conclusions. It is fair to say that NFW can be obtained also by plotting the $\Delta H/m_w$ against the W_c, while obtaining some non-linear trend. However, we used the approach of Liu and Cowman (2000) who demonstrated that the slope of obtained linear dependency has the meaning of the ice melting enthalpy. It is worth to note, that only in case the NFW content is known (and assumed to be constant also for higher water contents) the enthalpy can be normalized by freezable water content and information about the melting enthalpy of ice at specific W_c can be obtained (Kučerík et al., 2011). Further, if the sample is uniformly hydrated, the dependency $\Delta H/m_p = f(W_c)$ is linear which allows to obtain non-freezing water content from the extrapolation. This is advantageous mainly in case of DSC devices with lower sensitivity and it gives a hint about the amount of hydrating water. The nonlinearity, on the other hand, might imply the non-uniform hydration and allows the conclusions about other material's properties (Kučerík, Bursáková, Průšová, Grebíková, & Schaumann, 2012).

Further, it is stated: "It is a matter of further analysis to find whether this is a result of improper experimental design or really of some cause". In fact, the value of non-freezing water reported in Prusova et al. (2010) is very similar to results from different techniques dealing with hydration such as time domain NMR (Prusova, Vergeldt, & Kucerik, 2013) and polarization-resolved femtosecond-infrared and Terahertz time-domain spectroscopies (Hunger, Bernecker, Bakker, Bonn, & Richter, 2012).

Pekar (2012) also reported that "The linear model presented here tells that nothing is measured also when the water and biopolymer contributions are compensated: $\Delta H = 0 \Leftrightarrow k_1 m_w = -k_2 m_p$." The original equation $\Delta H = k_1 m_w + k_2 m_p$ suggests that the k_2 parameter has to have the magnitude of energy in J/g, negative to k_1 parameter, i.e. it is the exothermal energy. We hypothesize that this exothermal energy is related to the hydration heat evolved during the wetting of hyaluronan surface. In fact this enthalpy cannot be determined directly using DSC since it is lost during the equilibration phase 24 h prior to the measurement but it is reflected in the formation of NFW.

The empirical model for determination of NFW developed by Liu and Cowman (2000) is formally the same as $\Delta H/m_p = k_1 m_w/m_p + k_2$ and describes the situation for semi-diluted systems. As follows from the equations the main determinant of change of ice character is its melting enthalpy; the slope was not changing in the low water content interval and in accordance with previous observations (e.g., Hatakeyama & Hatakeyama, 1998) it was used for the determination of non-freezing water content.

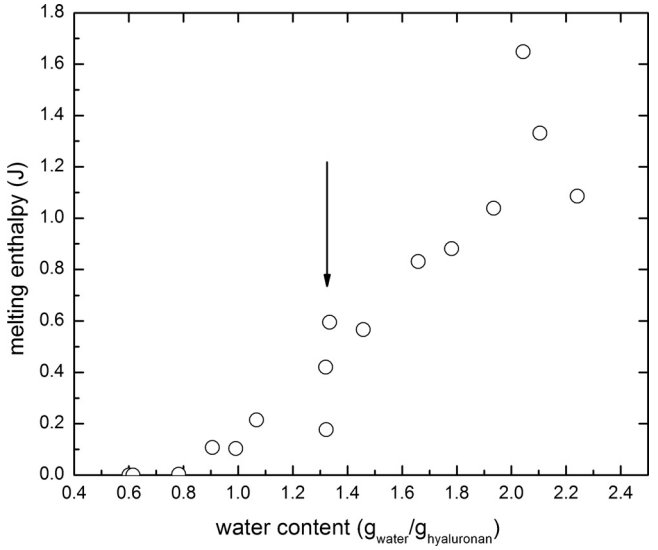

Fig. 2. Non-normalized enthalpy in dependency on W_c. Normalized data were published in Prusova et al. (2010).

2. Evaporation experiments

In the second part of the note, the author criticizes the approach used for the evaluation of the vaporization enthalpies determined during water evaporation from the hyaluronan sample. The author also speculates about the origin of the break on the dependency of the evaporation enthalpy, normalized by the dry sample mass, in dependency on W_c reported in the second part of Prusova et al. (2010). It is stated that some works about application of thermal analysis of hyaluronan have been overlooked and some examples of the articles dealing with the water vaporization from clay minerals are cited. As a model system, a system of rigid balls is considered (Pekar, 2012).

First, it was not perfectly correct to state that "Use of thermoanalytical investigation of the water evaporation is neither original nor new approach in study of biopolymers hydration". We agree that thermal analysis has been used frequently for such purpose but the way of data evaluation has not been published before. In fact the normalization of enthalpy by m_p was the reason of discovering of plasticization point since the other type of normalization

does not provide such possibility as demonstrated in Mlcoch and Kucerik (2013).

Regardless the chemical and physicochemical differences between clays and polysaccharides, differences in evaporation processes in these kinds of materials have been experimentally proven for example by Prado and Vyazovkin (2011). In Pekar (2012), author tried to simplify this problem (with no experimental support) assuming that the observed process might only be the interstitial water and overlooked some other important facts. Those are mainly: (i) the temperature range for evaporation in Prusova et al. (2010) was high enough to evaporate all the evaporable water and thus 0.34 g/g can hardly be considered as the interstitial water (as author confirms himself by "As far as I know, the producer's standard limits the moisture content in hyaluronan up to 5–10%."), (ii) the criticized break in Figure 7 was confirmed using different approach such as KAS method showing that the total evaporation enthalpy is still higher than the pure water evaporation (Kučerík et al., 2011), (iii) in case of evaporation some other normalization is possible e.g. using water content (Park, Venditti, Jameel, & Pawlak, 2007) but it does unravel a possible sudden change in sample's heat capacity as discussed in Mlcoch and Kucerik (2013) and does not reflect the role of hyaluronan in the intended way as reported in Figure 7 in Prusova et al. (2010), (iv) only DSC unlike the thermogravimetry measures the heat capacity-related processes and thus it can register also heat capacity changes during the hyaluronan drying which are in principle invisible for other thermoanalytical techniques, e.g. thermogravimetry. Using time domain NMR this particular water content has been identified as a plasticization point in hyaluronan (Prusova et al., 2013) which stresses out the importance of such kinds of measurement using DSC, providing that the changes in structure of hyaluronan are of interest, (v) if the process occurred because of the interstitial water then such a break would be observable also in other biopolymers. However, several polysaccharides have been studied by this method and only hyaluronan (under certain experimental conditions) showed such a break (Mlcoch & Kucerik, 2013).

The author stated "The results are presented and evaluated in the form of measured enthalpy divided by the weight of the dry polymer. There is no theory explaining why just this normalization is necessary or why is preferred. Taking into account also the analysis of the measured heat or of the heat per unit water mass is necessary to arrive at more realistic conclusions". Since we did not assume that this issue can be unclear and it was not the aim of the paper we did not put much effort to go in detail in Prusova et al. (2010). But similarly as in case of cooling/thawing experiments and derived equations (Liu & Cowman, 2000), it can be seen that the slope of this dependency is equal to the evaporation enthalpy, its dramatic decrease below 0.34 g/g clearly shows that less energy is needed for evaporation of water from hyaluronan. In accordance with the scope of the paper, the main aim was to demonstrate the existence of the break in the evaporation profile which was assumed and later also proved (Prusova et al., 2013), to be an important parameter of hyaluronan drying.

The only point we agree with the criticism of Pekar (2012) is that the term "non-evaporable water" which we used in Prusova et al. (2010) to describe the break in Figure 7 is not an appropriate nomenclature but this issue was clarified recently (Prusova et al., 2013).

3. Additional comments regarding principles of DSC

Here we would like to correct also some statements about DSC principle which is incorrectly described in Pekar (2012) and might lead to above-mentioned misunderstandings. Author claims: "The real "input" to DSC measurement is the sample weight and composition and the true output, in fact, is the measured heat ("Joules")" and ". . .when both weights are zero, no heat is measured". The heat flux differential scanning calorimetry (DSC) system used in Prusova et al. (2010) is based on the measurement of difference in temperature between sample and reference pans when both pans are exposed to the same temperature program; the enthalpy which corresponds to the peak area is therefore not "measured" but determined based on the previous calibration using standards. Also in case of power compensated DSC system the signal is the *difference* between power input to the sample and reference cells to keep the temperature difference between pans zero. Thus the general principle differs from that described in Pekar (2012). Under non-isothermal conditions, e.g. using constant heating rate, the zero line is the signal obtained when no pan is placed in the DSC furnace(s). If a sample and a reference are placed into measurement cell(s), the baseline position depends on the sample mass, its heat capacity and heating rate while the pan mass and heat capacity contributions are compensated. In the first part of Prusova et al. (2010) the experiments were carried out in hermetically sealed crucibles, the heating rate was constant and thus any change in the baseline is linked with a change in the heat capacity. Therefore the absence of either a peak (e.g. melting) or a step change (e.g. glass transition) in the DSC record indicates the absence of transition or reaction but the signal (baseline) corresponding to heat capacity is still measured. This situation is incorrectly mentioned as $\Delta H = 0$ in Pekar (2012) leading to an impression that when no process takes part "nothing" is measured. It is important to stress out that the appearance of the DSC peak, and its related area, is not necessarily caused by a process taking part in the whole sample. For further reading we recommend specialized literature (e.g. Hatakeyama & Quinn, 1999; Wunderlich, 2005).

To summarize briefly:

(1) the determined melting ΔH has to be normalized using dry mass and plotted against W_c as demonstrated by Liu and Cowman (2000) and earlier works since it reflects the influence of both water and hyaluronan content;
(2) the determined evaporation ΔH has to be normalized as well and plotted against the W_c to stress out the role of mass and the change in its physicochemical properties, normalization of ΔH by water content is also possible;
(3) any other plotting of data has to be chosen carefully to avoid confusing conclusions.

Therefore, most of the suggestions reported by Pekar (2012) cannot be accepted. However, we appreciate the opinion which allowed us to clarify in detail the principles of DSC measurements and its application in hydration studies which is not necessarily clear to everyone and for which there is usually lack of space in classical scientific papers.

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