Carbohydrate Polymers 106 (2014) 453–459

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Densitometry and ultrasound velocimetry of hyaluronan solutions in water and in sodium chloride solution

A. Kargerová, M. Pekař*

Brno University of Technology, Faculty of Chemistry, Materials Research Centre, Purkyňova 118, Brno CZ-61200, Czech Republic

ARTICLE INFO

Article history:
Received 17 October 2013
Received in revised form 6 January 2014
Accepted 7 January 2014
Available online 16 January 2014

Keywords:
Compressibility
Density
Hyaluronan
Hydration
Ultrasound velocity

ABSTRACT

The densities of hyaluronan solutions in water and 0.15 M NaCl were measured in the temperature range from 25 to 50 °C for the hyaluronan molecular weights from 10 to 1750 kDa. The density increased linearly with concentration and decreased with temperature. The data were fitted by the equation describing the density as a linear function of concentration and a quadratic function of temperature. The effect of molecular weight was negligible and thus single equation was sufficient to describe all data. The apparent and partial specific volumes were calculated from the density data including their extrapolated values to infinite dilutions. The measurement of ultrasound speed in the same solutions under the same conditions enabled to calculate the compressibility and its dependence on concentration and temperature. The compressibility decreased with both the concentration and the temperature but the effect of the concentration was only slight mild. The compressibility was used to estimate the hydration numbers which slightly decreased with increasing temperature and concentration. The addition of NaCl changed only the numerical values of density and ultrasound velocity while not changing the character of their dependence on temperature and concentration. Measured and calculated data indicate that hyaluronan does not disturb the specific water structure in the studied concentration range and support the idea of the existence of water clusters or nanodroplets hydrating the hyaluronan chains in solution.

1. Introduction

Hyaluronan is a linear polysaccharide built from regularly alternating monosaccharides, glucuronic acid and N-acetylglucosamine, which form a basic disaccharide unit (Lapčík, Lapčík, Smedt, Demeester, & Chabreček, 1998). It is an abundant biopolymer with a wide range of naturally occurring molecular masses from several hundreds to 10^7 g/mol. The highest concentrations of hyaluronan occur in synovial fluid, vitreous body, skin and in certain specialized tissues such as umbilical cord and rooster comb. The largest amounts are found in the intercellular matrix of skin and in musculoskeletal tissues (Kogan, Šoltés, Stern, & Gemeiner, 2006). Due to its biocompatibility and non-immunogenicity hyaluronan is an attractive material for various cosmetic and medical applications (Kuo, 2006) and is produced industrially mostly by biotechnological processes. The solution properties of hyaluronan are well documented, particularly with respect to the chain structure and size, rheology, and electrolyte-related properties. Rinaudo (2009) states that there is nothing very remarkable in the behaviour of hyaluronan in solution. It is a typical semi-flexible polyelectrolyte with the properties dependent on concentration and molecular weight. Even at low concentrations the zero shear viscosity is high and the complex viscosity remains non-Newtonian which contribute to much higher apparent molecular weight of entangled hyaluronan chains. The review (Cowman & Matsuoka, 2005) summarizes the studies of hydrodynamic properties of hyaluronan in neutral aqueous solutions in the presence of physiological NaCl concentration as the expected behaviour of a high molecular weight linear semi-flexible polymer. The dependence of the intrinsic viscosity on hyaluronan molecular weight follows two straight lines – higher slope is found in the low molecular weight region. This is in accord with the predicted change from short chains in somewhat extended shape to longer chains which are coiled into hydrodynamic spheres as the molecular weight increases. The persistence length which is a measure of the intrinsic stiffness of the chains (which is the same for both short and long chains) is used to explain the change in the slope and is reported to be between 4 and 10 nm. The unusual high viscosity of hyaluronan solutions arises from the huge hydrodynamic volume and also from transient interchain interactions. The significant nonideality found for hyaluronan solutions could be predicted by simple models for hydrodynamic interactions between polymer chains.

The studies on the density of hyaluronan solutions are scarce. Gómez-Alejandre, Blanca, Usera, Rey-Stolle, and

* Corresponding author. Tel.: +420 541149330; fax: +420 541149398.
E-mail address: pekar@fch.vutbr.cz (M. Pekař).

0144-8617/$ – see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2014.01.020

Hernández-Fuentes (2000) measured the density of high molecular weight hyaluronan (1.5 MDa) either in water at different pH or in the presence of several inorganic salts. In water and in $CaCl_2$ solution they also determined the effect of temperature. Their main interest was the determination of the partial specific volume at infinite dilution and the density data were not analysed and discussed. Some density data are reported in the paper by García-Abuín, Gómez-Díaz, Navaza, Regueiro, and Vidal-Tato (2011) but its main aim was the study of viscosimetric behaviour of hyaluronan in aqueous and water-alcohol solutions. Only single hyaluronan sample of high molecular weight (1.43 MDa) was used in this work and the density was reported for five concentration points at 20 and 50 °C. No analysis of density data was given.

Our interest in density data of hyaluronan solutions comes from the studies of hyaluronan and its interactions measured by means of high resolution ultrasound spectroscopy (Sarvazyan, 1982, 1983; Buckin, Kankiya, Sarvazyan, & Uedaira, 1989). The ultrasound measurements are usually accompanied by the measurements of density in order to enable the calculations of compressibility from ultrasound velocity and density. The densitometer used in this study allowed also the measurement of the ultrasound velocity although of lower resolution and precision. Besides the density we thus report also the data on ultrasound velocity. There are several studies on ultrasound propagation in hyaluronan solutions. The speed of sound is reported in García-Abuín et al. (2011) for the same sample and conditions as described above for the density and is not further analysed. Suzuki and Uedaira (1970) and Davies, Gormally, Wyn-Jones, Wedlock, and Phillips (1982, 1983) used the compressibilities for the study of hyaluronan hydration.

In this study we used a wide range of hyaluronan molecular weights and as broad range of its concentration as possible to measure the density of solutions and the ultrasound velocity in solutions in details which have not been explored. From the measured data the additional parameters like the partial specific volume, the compressibility or the hydration number were calculated. The solutions were prepared either in water or in aqueous solution of NaCl at the physiological concentration (0.15 M).

2. Materials and methods

Hyaluronan of several molecular weights was obtained from Contipro Biotech (Czech Republic). It is produced biotechnologically and extracted from the cell walls of the bacteria *Streptococcus zooepidemicus*. This producer offers a broad range of molecular weights in predefined range of molecular weights. The following products were used in this study: 10–30 kDa, 110–130 kDa, 300–500 kDa and 1500–1750 kDa; particular molecular weights (determined by the producer using SEC-MALS) of particular samples from each range used in this study are given in Table ST0 in Supplementary information. Sodium chloride of p.a. purity was obtained from Lachner (Czech Republic).

The hyaluronan solutions were prepared at the concentration ranges reported in Tables ST1a-d and ST2a-d in Supplementary information by dissolving the original hyaluronan powder slowly in water or in 0.15 M NaCl in a closed vessel. The selected concentration range was dependent on the molecular weight and was selected in order to enable the sample injection into the densitometer without problems (not too high viscosity and no entrapment of bubbles). The solutions were prepared by weighing their components. The solutions were stirred for 24 h at the room temperature to ensure the complete dissolution; the preliminary density measurements made after up to 14 days of dissolution confirmed that 24 h are sufficient to obtain stable and reproducible results. Ultrapure deionized water from PURELAB water purification system (Option R7/15; ELGA, Great Britain) was used for the preparation of all samples.

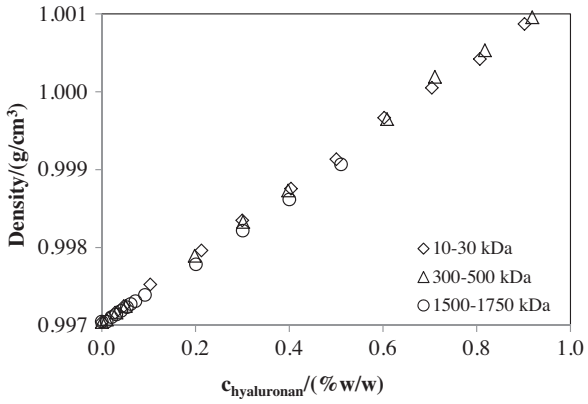

Fig. 1. The dependence of density on hyaluronan concentration at 25 °C. Solutions of hyaluronan of different molecular weights in water.

The density and ultrasound velocity were measured for all molecular weights in the temperature range from 25 to 50 °C using the densitometer DSA 5000 M (Anton Paar, Austria) with the accuracy of density measurement of 0.000005 g/cm^3. DSA 5000 M is equipped with a density cell and a sound velocity cell with the temperature controlled by a built-in Peltier thermostat. Both the density and the velocity were measured simultaneously. The temperature was controlled with integrated Pt 1000 temperature sensor with the accuracy of 0.001 °C. The calibration of densitometer was performed at 20 °C using air and water. The samples were degassed using the syringe and then they were injected into U-shaped borosilicate glass tube that was excited electronically to vibrate at its characteristics frequency. It had to be ensured that the U-tube was properly filled and that no gas bubbles were present.

The density and velocity measurements of each molecular weight range, at each concentration and for each temperature were made at least in triplicates. The data fitting and the statistical analyses were made with QC.Expert 3.3 software (TriloByte, Czech Republic).

3. Results and discussion

All the measured data on density and ultrasound velocity are collected in Tables ST1a-d, ST2a-d, ST6a-d and ST7a-d in Supplementary information. First, the result obtained for the solutions in water is discussed.

3.1. Density

The density increased with increasing concentration and decreased with increasing temperature. The data for all molecular weights are collected in Table ST1a-d in Supplementary information. The effect of molecular weight on the density was negligible. The example of the concentration dependence on the density at 25 °C in water is given in Fig. 1, where also the independence on the molecular weight is seen. The linear concentration dependence was typical for all temperatures. The example of the temperature dependence is shown in Fig. 2. The temperature dependencies were slightly curved the reason of what was the temperature effect on the density of pure solvents (see Figure SF1 in Supplementary information).

The density-concentration-temperature data were fitted for each molecular weight by two models – linear and quadratic in temperature – to compare the effect of including the slight curvature

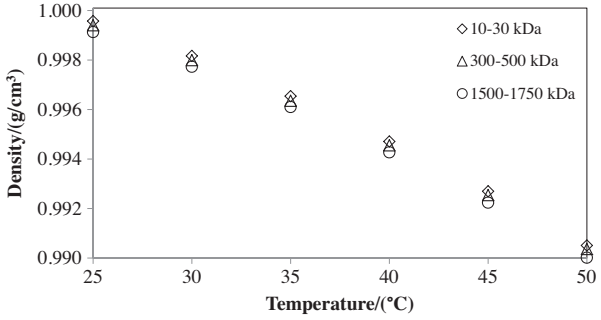

Fig. 2. The temperature dependence of density of hyaluronan solutions at the concentration of 0.5% (w/w). Solutions of hyaluronan of different molecular weights in water.

into the fitting equation. The latter model is called the quadratic model henceforth. The model equations are as follows:

$$\text{linear}: \quad \rho = a_0 + a_w c_w + a_t t$$

$$\text{quadratic}: \quad \rho = a_0 + a_w c_w + a_t t + a_{tt} t^2$$

where ρ is the density of hyaluronan solution in g/cm^3, c_w is the concentration of hyaluronan in grams per kilogram of solution, t is the temperature in °C and a_i denotes the (fitted) parameters. The results of regression fits were evaluated on the basis of following characteristics: multiple correlation coefficient (R), coefficient of determination (R^2), predicted coefficient of determination (R_p), mean quadratic error of prediction (MEP), and Akaike information criterion (AIC). These characteristics are collected in Table ST3 in Supplementary information and show that the quadratic model fits better the data. The parameters of quadratic models are given in Table 1 and the parameters of linear models in Table ST4 in Supplementary information for the sake of completeness.

Because the molecular weight did not show appreciable effect on the density the whole set of data over all molecular weights was fitted by one common equation. In this way a single equation was obtained which can serve for a reasonable estimate of density of hyaluronan solution at desired concentration and temperature which fall within their ranges used in this work and with hyaluronan molecular weights within the range from 10 up to 1750 kDa. The statistical characteristics of this overall fit are given in Table ST3 in Supplementary information and corresponding model parameters in the last row of Table 1.

Taking into account the smooth and (almost) linear dependence of the density on hyaluronan concentration and temperature it is concluded that no evident changes, e.g. in hyaluronan conformation, inter- or intrachain interactions, were detected by densitometry within used concentration and temperature ranges.

3.2. Volume characteristics

The measurements of density are usually used to calculate the volume characteristics of the solution and solvent. Perhaps the most frequently reported characteristic is the apparent specific volume defined as the difference between the solution and solvent volumes per unit solute mass; in our case:

$$V_{app} = \frac{V - V_0}{m_{hya}} \tag{1}$$

V is the solution, V_0 is the solvent volume, and m_{hya} is the hyaluronan mass in the solution. Expressing volumes in terms of densities the following relationships can be derived to calculate the apparent specific volume from measured densities:

$$V_{app} = \frac{m_0(\rho_0 - \rho)}{\rho\rho_0 m_{hya}} + \frac{1}{\rho} \tag{2}$$

$$V_{app} = \frac{m_0}{m_{hya}}\left(\frac{1}{\rho} - \frac{1}{\rho_0}\right) + \frac{1}{\rho} \tag{3}$$

where m_0 is the solvent mass in the solution, ρ_0 is the solvent and ρ the solution density. Both expressions gave identical values of V_{app} within the measurement precision of our data, as expected.

In thermodynamics, the effects of mixture components on mixture properties are rigorously described by partial quantities. They are defined as partial derivatives of a mixture property expressed as a function of relevant variables. In our case, the mixture property is the volume and the variable is the hyaluronan (solute) mass keeping the other variables (temperature, pressure) constant. The partial specific volume is then defined as follows:

$$\tilde{V}_{sp,hya} = \frac{\partial V}{\partial m_{hya}}; \quad T, p = \text{const.} \tag{4}$$

This definition can be expressed in terms of measured density using the solute mass fraction (w_{hya}):

$$\tilde{V}_{sp,hya} = \frac{1}{\rho} + (m_0 + m_{hya})\frac{\partial(1/\rho)}{\partial w_{hya}}\frac{\partial w_{hya}}{\partial m_{hya}} \tag{5}$$

Evaluating the second of the two partial derivatives, the final expression for calculating the partial specific volume from experimental data is obtained:

$$\tilde{V}^0_{sp,hya} = \frac{1}{\rho}\left[1 - \frac{1 - w_{hya}}{\rho}\frac{\partial\rho}{\partial w_{hya}}\right] \tag{6}$$

Note that this calculation requires the slope of the dependence of the solution density on hyaluronan mass fraction.

Table 1
Parameters of quadratic model and their standard deviations for hyaluronan solutions of different molecular weights.

M_w (kDa)	Solvent	a_0 (g/cm^3)	a_w (10^{-4} kg/cm^3)	a_t (10^{-5} g/cm^3 °C)	a_{tt} (10^{-6} g/cm^3(°C)^2)
10–30	Water	$1.00152 \pm 8 \times 10^{-5}$	4.1 ± 0.03	−7.2 ± 1.1	−3.9 ± 0.15
110–130		$1.00124 \pm 39 \times 10^{-5}$	4.0 ± 0.03	−5.8 ± 2.1	−4.1 ± 0.28
300–500		$1.00149 \pm 26 \times 10^{-5}$	4.1 ± 0.03	−7.6 ± 1.4	−3.8 ± 0.19
1500–1750		$1.00110 \pm 9 \times 10^{-5}$	3.8 ± 0.02	−6.1 ± 0.5	−4.0 ± 0.06
10–30	NaCl[a]	$1.00795 \pm 20 \times 10^{-5}$	4.1 ± 0.01	−9.2 ± 1.1	−3.7 ± 0.15
110–130		$1.00806 \pm 22 \times 10^{-5}$	4.0 ± 0.02	−9.1 ± 1.2	−3.7 ± 0.16
300–500		$1.00800 \pm 16 \times 10^{-5}$	4.3 ± 0.02	−9.3 ± 0.9	−3.7 ± 0.12
1500–1750		$1.00786 \pm 16 \times 10^{-5}$	4.2 ± 0.04	−8.8 ± 0.9	−3.8 ± 0.11
10–1750	Water	$1.00131 \pm 17 \times 10^{-5}$	4.1 ± 0.013	−6.6 ± 0.9	−4.0 ± 0.12
10–1750	NaCl[a]	$1.00797 \pm 17 \times 10^{-5}$	4.1 ± 0.009	−9.1 ± 0.6	−3.7 ± 0.08

[a] 0.15 M in water.

Gómez-Alejandre et al. (2000) calculated hyaluronan partial specific volumes according to the following equation:

$$\bar{v}^0_{sp,hya} = \frac{1}{\rho_0}\left[1 - \frac{1}{\rho_0}\left(\frac{\partial\rho}{\partial w_{hya}}\right)^0\right] \quad (7)$$

where $\bar{v}^0_{sp,hya}$ is the hyaluronan partial specific volume at infinite dilution (we will call it the limiting partial specific volume here) and the derivative indexed by 0 is the limiting slope of the indicated dependence. This equation was called the rigorous relation but its justification is given neither in (Gómez-Alejandre et al., 2000) nor in Fabre, Tagle, Gargallo, Radic, and Hernandez-Fuentes (1989) to which the former refers. However, this equation can be arrived at as the zero-concentration limit of the partial specific volume, Eq. (6), when $\rho \to \rho_0$, $w_{hya} \to 0$, and the slope goes to its limiting value calculated at the point of zero mass fraction.

The values of the apparent and partial specific volumes are summarized in Tables ST1a-d and ST2a-d in Supplementary information. The apparent specific volume was calculated using the averages of the measured solvent and solution densities and of the corresponding hyaluronan masses. Its value and standard deviation were sensitive to measurements errors, particularly at low concentrations (and higher temperatures) where the solution density was very close to the solvent density. This is quite common situation (Kaulgud & Dhondge, 1988; Lo Surdo, Shin, & Millero, 1978) sometimes attributed also to the effect of dissolved gases in low concentrated samples (Kaulgud & Dhondge, 1988). Therefore only the values of V_{app} with the relative standard deviation not exceeding ca 20% are reported. The partial specific volume was calculated according to Eq. (6) using the averaged densities, averaged hyaluronan mass fractions and the slope of the straight line determined by fitting all experimental points. The values of the partial specific volume were determined much more reliably with the relatively standard deviation below 2% and at more consistent trends with concentration or temperature. Nevertheless, the numerical values of the averages of the two specific volumes were comparable and not too much different.

The partial specific volume slightly increases with increasing hyaluronan concentration which can be expected due to the increasing role and number of interactions with the solvent and inter-chain contacts. Analogical conclusions can be made also for the apparent specific volume. A moderate increase is observed also with the temperature growth; here the data for the partial specific volume are more conclusive. This could be expected and explained by the expansion of hyaluronan coils due to the increased mobility at elevated temperature. The effect of hyaluronan molecular weight on the specific volume was not detected. Thus the hyaluronan partial specific volume in water typically ranges, depending on concentration, between 0.587 and 0.594 cm^3/g at 25 °C and between 0.597 and 0.604 cm^3/g at 50 °C.

Partial or apparent (specific, molar, molal) quantities are typically extrapolated to zero concentration to obtain a value which is supposed to be free of the effects of solute-solute interactions. The extrapolated value should thus reflect only the solute-solvent interactions (Junquera et al., 2002). The extrapolation of the apparent specific volume is usually problematic due to the high uncertainty and nonlinearity of data at very low concentrations and only the linear part of data at reasonably higher concentrations is used to be extrapolated, see e.g. Kaulgud and Dhondge (1988) or Lo Surdo et al. (1978); the same procedure was used in this work. The dependence of the partial specific volume on the hyaluronan concentration was linear and the extrapolation thus was without problems. Eq. (7) is, in fact, also a kind of the extrapolation to the infinite dilution. Due to the linearity of partial specific volume versus hyaluronan concentration the limiting partial specific volume $\bar{v}^0_{sp,hya}$ calculated from this equation is numerically equal to the extrapolated value of the partial specific volume $\bar{V}^0_{sp,hya}$; the latter has much lower standard deviation (owing to the fitting procedure). All the zero concentration volumes are shown in Tables 2 and ST5 in Supplementary information together with their dependence on the temperature. The values slightly increase with temperature and the extrapolated apparent specific volume is somewhat lower than its partial counterparts. Durchschlag and Zipper (1994) as well as Davies et al. (1982) reported the value 0.556 cm^3/g for the extrapolated partial specific volume of sodium hyaluronate at 25 °C with no other experimental details. Gómez-Alejandre et al. (2000) determined the limiting partial specific volume of sodium hyaluronate of a molecular weight of 1500 kDa and it changed from 0.512 cm^3/g at 25 °C to 0.561 cm^3/g at 40 °C. Our values are somewhat higher; due to the lack of original data in the referenced works it is difficult to discuss these differences, we can only point to a narrower concentration range used in ref. by Gómez-Alejandre et al. (2000) and to Haxaire, Maréchal, Milas, and Rinaudo (2003a, 2003b) who reported the density of (solid) hyaluronan to be 1.61 g/cm^3 which gives the specific volume of 0.621 cm^3/g. Further, Perkins, Miller, Hardingham, and Muir (1981) determined the value of the hyaluronan partial specific volume, 0.58 cm^3/g, which is in excellent agreement with our results.

Table 2
Extrapolated partial specific volume of hyaluronan of different molecular weights dissolved in water.

t (°C)	$\bar{V}^0_{sp,hya}$ [a] (10^{-3} cm^3/g)			
	10–30 kDa	90–130 kDa	300–500 kDa	1500–1750 kDa
25	587	587	587	587
30	589	589	589	589
35	592	592	592	592
40	594	594	594	594
45	596	595	595	595
50	597	597	597	597

[a] Standard deviation was less than 5×10^{-5} cm^3/g.

3.3. Ultrasound velocity

The velocity increases (see Table ST6a-d in Supplementary information) with hyaluronan concentration linearly as for many other solutions of low concentration (Povey, 1997); see the example in Fig. 3. The dependence on temperature at given concentration is also increasing but is slightly curved what corresponds to the temperature dependence of ultrasound velocity in water. There is practically no effect of hyaluronan molecular weight (cf. Fig. 3).

The increased velocity with increased concentration is caused by dissolved rigid polymeric chains and by the hydration shell formed

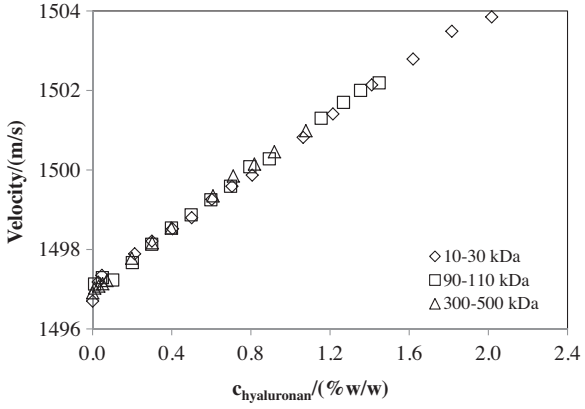

Fig. 3. The dependence of ultrasound velocity on concentration of hyaluronan at 25 °C. Solutions of hyaluronan of different molecular weights in water.

around them. The higher the rigidity of medium, the higher the speed of ultrasound propagation in it (Povey, 1997). The hydration water is known to have lower compressibility than bulk water (Buckin et al., 1989) therefore the ultrasound velocity in systems containing hydrated molecules increases. As already noted by García-Abuín et al. (2011) the effect of temperature is more significant than the effect of concentration. This is probably caused by water as a solution medium namely by the response of its specific compressibility to temperature described below. Dissolving hyaluronan does not interfere with the specific properties of water regardless its supposed massive hydration shell. The negligible effect of hyaluronan molecular weight is indicative of the principal role of hyaluronan basic disaccharidic unit in determining the properties of hyaluronan solutions (the properties measured in this work, at least) – its molar amount at a given hyaluronan mass concentration is independent on the molecular weight.

3.4. Compressibility and hydration numbers

The compressibility (β) as calculated from measured density (ρ) and ultrasound velocity (u) using the well-known equation, usually called Laplace equation: $u = 1/(\sqrt{\beta\rho})$ and is given in Table ST6a-d in Supplementary information. Because both the density and the velocity increase with hyaluronan concentration the compressibility of hyaluronan solutions is a decreasing function of concentration at all temperatures; however the decrease is only mild. More concentrated solutions are thus slightly tougher, more rigid what can be explained by increased amount of hydrated structures, increasing excluded volume effects and perhaps also by interchain interactions.

The compressibility decreases also with growing temperature at given hyaluronan concentration and, once again, the effect of temperature is more significant than the effect of concentration. In a typical liquid the compressibility increases with temperature as the structure becomes more open. The decrease of the compressibility with elevated temperature is among the peculiar properties of liquid water (see the overview by Chaplin, in Web references). In fact, the adiabatic compressibility of water decreases up to 64 °C where it has a minimum. This behaviour is explained by the equilibrium between the clusters formed by water molecules through their hydrogen bonding. Basically, two cluster structures are in equilibrium – the more open or expanded structure and the more ordered or collapsed structure. Both structures are formed from twenty 14-molecule tetrahedral units. The expanded structure creates icosahedral water clusters and can collapse into the puckered central dodecahedron forming the collapsed structure. In liquid water, the cluster equilibrium shifts towards more closed structure (the collapsed cluster). As the temperature increases, the water structure is less open at these higher temperatures and the capacity for it to be compressed decreases (Kell, 1975).

Hyaluronan does not disturb this peculiar property of liquid water. This is rather surprising, taking into account the accepted view on hyaluronan high hydration and thick hydration shell. The huge hydrodynamic volume is considered to be among the causes of the high viscosity of hyaluronan solutions (Cowman & Matsuoka, 2005). It could be supposed that at ambient temperatures a rigid, less compressible hydrated structure is formed which is weakened at elevated temperature as the decrease of viscosity indicates. A simple and straightforward explanation of the compressibility-temperature behaviour could be that the cluster structure of water is not essentially disturbed by the interactions with hyaluronan and the formation of its hydration shell. Another explanation can be the establishing of equilibrium between water in hydration shell and water clusters (perhaps less sized) remaining in the bulk and still responsible for the compressibility decrease. This equilibrium should be shifted to the (closed) clusters at elevated temperature. This explanation could be modified by imaging direct interactions, mediated by hydrogen bonding, between hydration layer and surrounding water clusters which are weakened by increased temperature. Still another explanation can be the relatively week bonding of water molecules in the hydration shell – even moderate increase in the temperature releases sufficient amount of water molecules from the hydration shell to re-establish the expanded-closed structure equilibrium in the aqueous environment. The hydration of hyaluronan was studied in details in refs. Haxaire et al. (2003a, 2003b) and Maréchal, Milas, and Rinaudo (2003) using advanced infrared spectroscopy. Although they did not study the hyaluronan solutions but the hydration of the solid hyaluronan films placed in the atmosphere of controlled moisture our results seem to confirm their conclusions on the hydration mechanism. They found out that at high moisture contents the hydration takes place by the arrival of nanodroplets of water molecules (containing at least 50 molecules) extending along the hyaluronan chain.

The effect of hyaluronan molecular weight is negligible, again, which further confirms the principal role of the basic disaccharidic unit in controlling the measured and calculated properties.

The compressibility can be used to estimate the hydration numbers, i.e. the number of water molecules in hydration shell. An overview of acoustic methods for the determination of hydration numbers was published recently (Burakowski & Glinski, 2011). Here the simple and general Pasynski method was applied which gives the following equation to calculate the hydration numbers, n_h: $\eta_h = (\eta_{H_2O}/\eta_{dimer})(1 - (\beta_{sample}/\beta_{H_2O}))$, where η_{H_2O} and η_{dimer} are the molar amounts of water and the dimer of hyaluronan, respectively, β_{H_2O} and β_{sample} is the compressibility of water and sample, respectively. The value of 401.299 g/mol was used for the molecular weight of disaccharidic unit of hyaluronan (sodium form) in calculations.

The Pasynski method assumes zero compressibility of water molecules in hydration shells and simple additivity of compressibility with respect to number of (compressible) molecules. The resulting hydration numbers should better be considered as estimates than as exact true values; all acoustic methods involve similar assumptions (Burakowski and Glinski, 2011) and should be used preferably for the comparison purposes within a series of similar samples. The hydration numbers in water are given in Table ST6a-d in Supplementary information and are very weakly dependent on temperature and hyaluronan concentration. Mostly they decrease with increasing temperature and concentration which is typical for the hydration numbers calculated by this method. No effect of the hyaluronan molecular weight could be observed. The hydration numbers in water are around 20. Haxaire et al. (2003a, 2003b) determined the hydration numbers up to about 30 depending on the moisture content in the atmosphere hydrating hyaluronan films. The hydration numbers of about 20 were observed at the very high moisture content, in relative units at almost 95% moisture. At the same time this corresponds to the onset of the hydration mechanism D (for details see Haxaire et al., 2003a, 2003b) which should reflect the arrival of water nanodroplets mentioned above. Our hydration numbers which should include the water molecules in close hydration shell with zero or very low compressibility (i.e. probably the shell formed at the onset of the mechanism D) thus seem to be in reasonable agreement with infrared study regardless the inherent assumptions of the Pasynski method. The weak dependence of estimated hydration numbers on the temperature does not support the explanation of compressibility-temperature curves which would be based on thinning the hydration shell with increasing temperature.

Suzuki and Uedaira (1970) used in their ultrasound measurement different and rather complex methods arriving at the hydration numbers of about 9 or 18 (for potassium hyaluronate)

depending on the level of complexity of the method and its presumptions. The value 9 is reported as an averaged value also by Davies et al. (1983) who used yet another calculation method which should determine the number of water molecules directly interacting with hydrated substrate.

3.5. Effect of NaCl

The addition of NaCl resulted in different numerical values of measured densities or ultrasound velocities (cf. Tables ST2a-d and ST7a-d in Supplementary information) but did not change their dependency on concentration or temperature. The numerical difference is rooted in the difference of pure solvents – in the density of water and NaCl solution and in the speed of ultrasound in these two media, see Figures SF1 and SF2 in Supplementary information. If this difference is accounted for by proper shifting of concentration or temperature dependencies, common curves are obtained for both solvents as illustrated in Figure SF3 in Supplementary information. The partial volumes determined in the NaCl solution for hyaluronan of the two lower molecular weights are comparable with corresponding values in water. Increasing hyaluronan molecular weight causes some differences in partial volumes – those determined in the NaCl solution are lower than corresponding values in water. This can be explained by shielding the repulsive electrostatic interactions between dissociated functional groups on hyaluronan by added low molecular electrolyte. The hyaluronan chains then adopt more compact shapes in electrolyte solutions (Cowman & Matsuoka, 2005) and if the chains are sufficiently long this is reflected in lower partial volumes. The formation of compact structures is confirmed by lower compressibilities in NaCl solution although the effect of electrolyte ions cannot be neglected. The hydration numbers are comparable in both solvents. The reference (Gómez-Alejandre et al., 2000) reported increased limiting partial volume of hyaluronan in NaCl solution and rationalized this finding by the formation of more open structures in the presence of added small ions. Again, due to the lack of original data in that reference it is difficult to discuss this difference but our compressibility measurements do not support the idea of more open structures.

4. Conclusions

The density of hyaluronan solutions in water or in 0.15 M NaCl was linearly dependent on the concentration at any used temperature and increased with the concentration. Increasing temperature decreased the density of a solution of given concentration; the temperature dependence was slightly curved and slightly deviated from linearity. The molecular weight of hyaluronan had negligible effect. Therefore all the data through all molecular weights, concentrations and temperatures used could be satisfactorily fitted with a single equation (one for each solvent); linear for concentration and quadratic for temperature. The equation can be used for reliable estimate of the density of hyaluronan solutions in the concentration range 0–2% (w/w), the molecular weight range 10–1750 kDa and the temperature range 25–50 °C. The densities were used to calculate several specific volume characteristics. The hyaluronan partial specific volume in water typically ranges, depending on concentration, between 0.587 and 0.594 cm^3/g at 25 °C and between 0.597 and 0.604 cm^3/g at 50 °C; in NaCl solution the values are slightly lower. The ultrasound velocity was primarily used to calculate the compressibility. The compressibility decreased with both the hyaluronan concentration and the temperature, the influence of the latter was stronger. The compressibility data indicated that hyaluronan – regardless of its supposed huge hydration – does not disturb the specific structure of water which is responsible for peculiar properties of water. Taking into account also the smooth and linear dependences of density and ultrasound velocity on the hyaluronan concentration it seems that the hyaluronan hydration, at least in diluted solutions, includes some type of bonding of relatively large water clusters (nanodroplets) to hyaluronan chains. The hydration in solution should thus follow especially the last step of hydration mechanism proposed for solid hyaluronan films (Haxaire et al., 2003a, 2003b; Maréchal et al., 2003). The hydration numbers determined from the compressibility data were typically about 20 and only slightly dependent on concentration. The addition of NaCl caused the changes in numerical values of measured or calculated quantities but did not change the character of their concentration or temperature behaviour. The salt thus did not cause the detectable changes in hyaluronan conformation or structure, except for the formation of rather more compacted coils at higher molecular weights. A negligible effect of molecular weight on all measured or calculated properties pointed to the principal role of the basic disaccharide unit of hyaluronan in determining these solution properties.

To conclude with a single phrase we can re-confirm the statement by Rinaudo: there is nothing too much remarkable in the behaviour of hyaluronan in solution (Rinaudo, 2009). Perhaps except that despite of its proclaimed massive hydration shell hyaluronan seems not to disturb the specific structure of water. Maybe that large water clusters bound to or entrapped within the hyaluronan chains form the structure responsible for the high viscosity of even diluted hyaluronan solutions.

Acknowledgements

This work was supported by the COST action CM1101, project No. LD12068 (Ministry of Education, Czech Republic). Materials Research Centre at Faculty of Chemistry, BUT is supported by project No. CZ.1.05/2.1.00/01.0012 from ERDF.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.01.020.

References

Buckin, V., Kankiya, B., Sarvazyan, A., & Uedaira, H. (1989). Acoustical investigation of poly(dA).poly(dT), poly[d(A–T)].poly[d(A–T)], poly(A).poly(U) and DNA hydration in dilute aqueous solutions. *Nucleic Acids Research*, *17*, 4189–4201.

Burakowski, A., & Glinski, J. (2011). Hydration numbers of nonelectrolytes from acoustic methods. *Chemical Reviews*, *112*, 2059–2081.

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

Davies, A., Gormally, J., Wyn-Jones, E., Wedlock, D., & Phillips, G. (1982). A study of hydration of sodium hyaluronate from compressibility and high precision densitometric measurements. *International Journal of Biological Macromolecules*, *4*, 436–438.

Davies, A., Gormally, J., Wyn-Jones, E., Wedlock, D., & Phillips, G. (1983). A study of factors influencing hydration of sodium hyaluronate from compressibility and high-precision densimetric measurements. *Biochemical Journal*, *213*, 363–369.

Durchschlag, H., & Zipper, P. (1994). Calculation of the partial volume of organic compounds and polymers. *Progress in Colloid & Polymer Science*, *94*, 20–39.

Fabre, M., Tagle, L., Gargallo, L., Radic, D., & Hernandez-Fuentes, I. (1989). Partial specific volume and specific refractive index increment of some poly(carbonate)s and poly(thiocarbonate)s. *European Polymer Journal*, *25*, 1315–1317.

García-Abuín, A., Gómez-Díaz, D., Navaza, J., Regueiro, L., & Vidal-Tato, I. (2011). Viscosimetric behaviour of hyaluronic acid in different aqueous solutions. *Carbohydrate Polymers*, *85*, 500–505.

Gómez-Alejandre, S., Blanca, E., Usera, C., Rey-Stolle, M., & Hernández-Fuentes, I. (2000). Partial specific volume of hyaluronic acid in different media and conditions. *International Journal of Biological Macromolecules*, *27*, 287–290.

Haxaire, K., Maréchal, 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., Maréchal, 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.
Junquera, E., Olmos, D., & Aicart, E. (2002). Carbohydrate–water interactions of p-nitrophenylglycosides in aqueous solution. Ultrasonic and densitometric studies. *Physical Chemistry Chemical Physics*, *4*, 352–357.
Kaulgud, M. V., & Dhondge, S. S. (1988). Apparent molal volumes and apparent molal compressibilities of some carbohydrates in dilute aqueous solutions at different temperatures. *Indian Journal of Chemistry*, *27A*, 9–11.
Kell, G. S. (1975). Density, thermal expansivity, and compressibility of liquid water from 0° to 150 °C: Correlations and tables for atmospheric pressure and saturation reviewed and expressed on 1968 temperature scale. *Journal of Chemical and Engineering Data*, *20*, 97–105.
Kogan, G., Šoltés, L., Stern, R., & Gemeiner, P. (2006). Hyaluronic acid: A natural biopolymer with a broad range of biomedical and industrial applications. *Biotechnology Letters*, *29*, 17–25.
Kuo, J. W. (2006). *Practical aspects of hyaluronan based medical products*. Boca Raton: CRC Press.
Lapčík, L., Lapčík, L., Smedt, S., Demeester, J., & Chabreček, P. (1998). Hyaluronan: Preparation, structure, properties, and applications. *Chemical Reviews*, *98*, 2663–2684.
Lo Surdo, A., Shin, C., & Millero, F. J. (1978). The apparent molal volume and adiabatic compressibility of some organic solutes in water at 25 °C. *Journal of Chemical and Engineering Data*, *23*, 197–201.
Maréchal, Y., Milas, M., & Rinaudo, M. (2003). Hydration of hyaluronan polysaccharide observed by IR spectrometry. III. Structure and mechanism of hydration. *Biopolymers*, *72*, 162–173.
Perkins, S., Miller, A., Hardingham, T., & Muir, H. (1981). Physical properties of the hyaluronate binding region of proteoglycan from pig laryngeal cartilage. *Journal of Molecular Biology*, *150*, 69–95.
Povey, M. J. W. (1997). *Ultrasonic techniques for fluids characterization*. San Diego: Academic Press.
Rinaudo, M. (2009). Polyelectrolyte properties of a plant and animal polysaccharide. *Structural Chemistry*, *20*, 277–289.
Sarvazyan, A. P. (1982). Development of methods of precise ultrasonic measurements in small volumes of liquids. *Ultrasonics*, *20*, 151–154.
Sarvazyan, A. P. (1983). Ultrasonic velocimetry of biological compounds. *Molecular Biology*, *17*, 739–749.
Suzuki, Y., & Uedaira, H. (1970). Hydration of potassium hyaluronate. *Bulletin of the Chemical Society of Japan*, *43*, 1892–1894.

Further reading

Chaplin, M. *Water structure and science*. http://www.lsbu.ac.uk/water/index2.html Accessed 07.09.13.