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

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## 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.

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## 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

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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
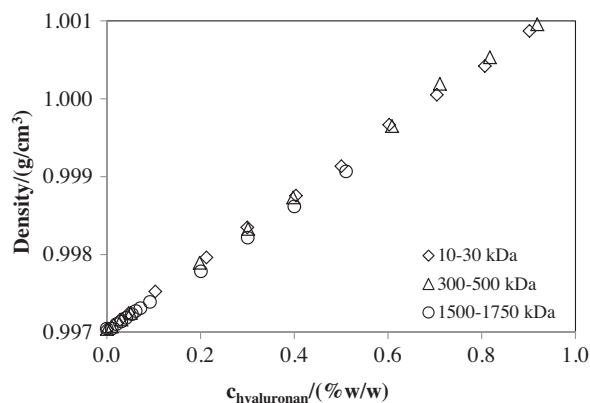


Fig. 1. The dependence of density on hyaluronan concentration at 25 °C. Solutions of hyaluronan of different molecular weights in water.

(Option R7/15; ELGA, Great Britain) was used for the preparation of all samples.

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