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ULTRASONIC AND DENSITOMETRIC CHARACTERIZATION OF HYALURONAN AND ITS INTERACTION WITH SURFACTANT

CHARAKTERIZACE HYALURONANU A JEHO INTERAKCÍ S TENZIDY ULTRAZVUKOVOU SPEKTROSKOPIÍ A DENSITOMETRIÍ

Ph.D. THESIS

AUTOREFERÁT DIZERTAČNÍ PRÁCE

AUTHOR **Ing. ANDREA KARGEROVÁ**
AUTOR PRÁCE
SUPERVISOR prof. Ing. MILOSLAV PEKAŘ, CSc.
VEDOUCÍ PRÁCE

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KLÍČOVÁ SLOVA

hyaluronan, hexadecyltrimethylamonium bromid, tetradecyltrimethylamonium bromid, ktitická micelární koncentrace, systém hyaluronan-tenzid, Ultrazvuková spektroskopie s vysokým rozlišením

MAIN DOCUMENT AVAILABLE AT Institute of Physical and Applied Chemistry, Faculty of Chemistry, Brno University of Technology, Purkyňova 118, 612 00 Brno, Czech Republic

CONTENT

1 INTRODUCTION

Hyaluronan is a natural biopolymer occurring in the human body and it is used as controlled drug delivery transport system, as a material for scaffold in tissue engineering, as a component of a bandage by wound healing processes, as an alternate fluid in human joints, as a space filling matter in plastic surgery and last but not least as a hydration matter in cosmetics.

Hyaluronan is considered as a polymeric carrier which is soluble in water or physiological solution and has specific receptors for the binding to the right molecules, for application in target delivery. Thanks to negative charge on hyaluronan chain we used cationic surfactants. There are existing strong electrostatic interactions between negatively charged hyaluronan carboxyl group and positively charged surfactant head group. Surfactant forms micelles, where a non-polar drug can be inserted. Drug carriers, which could contain hyaluronan, as a target delivery could be medicament for cancer. The thesis investigates the interactions between surfactants and polymer for the possible following application for target delivery.

Theoretical part summarizes the research of hyaluronan and method high resolution ultrasonic spectroscopy. The experimental part is divided in three parts. The first part (preliminary part) studies the behaviour of hyaluronan in water, measurement of pH, preliminary density measurement. The aim of this part of the work is to characterize the dissolution and storage stability of hyaluronan. The second part (experimental part I) is focused on the study of hyaluronan in wide range of molecular weights and as broad range of its concentration. The density and ultrasonic velocity is measured in solutions in temperature range from 25 °C to 50 °C. In the third part (experimental part II), high resolution ultrasonic spectroscopy is used for a detailed study of interactions between hyaluronan and two cationic surfactants tetradecyltrimethylammonuim bromide (TTAB) and hexadecyltrimethylammonuim bromide (CTAB) either in water or in sodium chloride solution at physiological concentration (0.15 M).

2 STATE OF ART

2.1 Hyaluronan and density meausurement

The solution properties of hyaluronan are well documented, particularly with respect to the chain structure and size, rheology, and electrolyte-related properties. Rinaudo [9] states that there is nothing much remarkable in the behaviour of hyaluronan in solution. It is a typical semi-flexible polyelectrolyte with the properties dependent on the concentration and on the 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 [10] 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 the predicted change from short extended chains to longer chains coiled into spheres as the molecular weight increases. 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 et al. [11] 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 analyzed and discussed. Some density data are reported in the paper [12] 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 °C and 50 °C. No analysis of density data was given.

García-Abuín et al. [12] studied the influence of the polymer concentration, temperature, electrolyte presence and use of co-solvents on the rheological behaviour of hyaluronan water solutions. Vibrating tube densitometer and a sound analyser Anton Paar DSA 5000 was used for the measurement of the density and sound speed of hyaluronan. They discuss the dependence of density and the speed of sound on concentration of hyaluronan and on the temperature. The value of the density and of the speed of sound increases with the concentration of hyaluronan. This behaviour might indicate slight interactions among the polymer-solvent and polymer-polymer molecules. They measured the influence of the temperature on the density value and the sound speed too, in the range of temperature 10-50 °C. The value of the density decreases and the value of the sound speed increases with the rise of temperature.

The effects of temperature and concentration on carboxymethylcellulose [16], chitosan [17] and polyethylene glycol [18]-[20] have been studied by density measurements. Some authors calculated apparent molar volumes or adiabatic compressibility from the measured densities and ultrasonic velocities for study of interactions of glycine with polyethylene glycol [21], study of system β-cyclodextrin-alkyltrimethylammonium bromides [22] or chitosan [17].

In our work the adiabatic compressibility of the aqueous solution of hyaluronan was calculated from the density and ultrasonic velocity data which are obtained by measuring at oscillating-U-tube densimeter and it have been compared with other works [14] [15].

Our interest in density data of hyaluronan solutions comes from the studies of hyaluronan and its interactions measured by means of high resolution ultrasonic spectroscopy. The ultrasonic measurements are usually accompanied by the measurements of density in order to enable the calculations of compressibility from ultrasonic velocity and density. There are several studies on ultrasonic propagation in hyaluronan solutions. The speed of sound of hyaluronan with molecular weight 1.43 MDa is reported in ref [12] for the density and is not further analysed. Suzuki and Uedaira [13] and Davies et al. [14] used compressibilities for the study of hyaluronan hydration.

2.2 Polymer-surfactant systems

Surfactants and water soluble polymers have very broad ranges of applications [1],[23]. The main types of the polymer-surfactant interactions can be relatively weak interactions between the polymer chains and the surfactant groups or strong electrostatic interactions between oppositely charged polyelectrolytes and surfactant head groups. The kinetics of surfactant binding onto polymers, as relaxation times, and the thermodynamic quantities controlling polymer-surfactant interactions, is presented and discussed in the reference [24]. There is used lot of biopolymers as cellulose [24],[25], carboxymethylcellulose [26], chitosan [25],[31], DNA [27]-[28], gelatin or lysozyme [29],[30].

Bao et al. [26] studied the interactions between surfactants and polysaccharides in the aqueous solutions. They used different six combinations of neutral, positively and negatively charged polysaccharides as methylcellulose, chitosan and κ-carrageenan with anionic (SDS) and cationic (CTAB) surfactants.

Hyaluronan is a very hydrophilic polymer surrounded by a massive hydration shell. Due to the presence of dissociable (carboxyl) group on its basic unit, hyaluronan has a character of a polyelectrolyte. At physiological pH, the carboxyl groups are predominantly ionized, the hyaluronan behaves like a polyanion and can interact with or associate cationic counterions to maintain charge neutrality. Hyaluronan interactions with positively charged surfactants were thus studied as a specific case of polyelectrolyte-surfactant interactions [2]. Hyaluronan-surfactant complexes containing hydrophobic domains formed by surfactant molecules and bound to hyaluronan chain can also be a potential carrier system for water-insoluble pharmaceutics agents. A series of papers by Swedish research groups provided a detailed study on phase behaviour of systems containing water, hyaluronan, alkyl trimethylammonium bromides (tetradecyl derivative was the most studied type) and salt (mostly NaBr) [3],[4],[32],[33]. Binding of surfactant to hyaluronan was detected for surfactants with alkyl chain consisting from at least ten carbon atoms. The binding was found to be considerably weaker than for most other carboxyl-containing polyelectrolytes, due to the low linear charge density of hyaluronan. Thalberg with Lindman [3],[4] studied interactions between hyaluronan and alkyltrimethylammonium bromide of various chain lengths (8, 9, 10, 12, 14 and 16 carbons in the alkyl group) by phase separation, conductivity and NMR self-diffusion. Their results indicate that there is needed certain minimum concentration of surfactant for marked formation of hyaluronan-surfactant complexes.

Below this concentration, the only general electrostatic interactions take place. The binding of surfactant to hyaluronan was detected for surfactants with least ten carbons in the alkyl chain. In the case of the surfactants with shorter chain then 10 carbons in alkyl chain, there is energetically preferred formation of free micelles to binding surfactant to hyaluronan. Their results of measuring showed that very low concentrations of surfactant suffice to binding surfactant to hyaluronan. The value of concentration of surfactant is bellow critical micelle concantration of certain surfactant. With the growing number of carbons in alkyl chain, the value of concentration of surfactant decrease.

Most of the authors used cationic surfactants because of the negatively charged hyaluronan chain. System tetradecyltrimethylammonium bromide-hyaluronan in water and salt (NaCl, NaBr) was published in papers [3],[6],[25].

The aim of the reference [34] was to obtain additional information on hyaluronan-surfactant interactions in solutions with physiological salt concentration (0.15 M NaCl) by tensiometry and fluorescence probe technique. Reference [34] found effect of low (90 kDa) and high (1400 kDa) molecular weight hyaluronan on the critical micelle concentration as determined by pyrene and surface tension methods. They used non-ionic (Tween 20, Octyl-β-$_{D}$-glucopyranoside) anionic (SDS), cationic (TTAB, CTAB, CTAT) and zwitterionic (Cetylbetaine, Betadet THC2) surfactants. The dependence of surface tension on surfactant concentration in the physiological solution showed no or small effect of hyaluronan (of any molecular weight) on the surface activity and particularly on the micellization. The value of the critical micelle concentration was practically not affected by the addition of hylauronan in tensiometry measurement. Fluorescence results showed, that althought the presence of sodium chloride may supress interactions between oppositely charged polyelectrolyte and surfactant, the interactions are still present in some hyaluronan-surfactant systems. The greatest influence of hyaluronan was observed on Tween 20 and hexadecyltrimethylammonium bromide. In the presence of hyaluronan, the critical micelle concentration was significantly decreased in Tween 20 and increased in the case of CTAB in comparing in the absence of hyaluronan. For both surfactants the micellization region was substantially broadened in comparing without hyaluronan.

The interactions of hyaluronan with dodecyldimethylamine oxide (DDAO) and another dimeric surfactant (dimeric quarternary ammonium surfactant 12-8-12, 12 carbon atoms in the chain, 8 carbon atoms in the spacer) was studied in [35],[36]. From a light scattering measurements of hyaluronan in aqueous sodium chloride solution have been calculated molecular weight, second virial coefficient, radius of gyration, and hydrodynamic radius.

Pisárčik [35] with his colegues calculated the number of positive charge of dimeric surfactant unit per one negatively charged hyaluronate disaccharidic unit in hyaluronan-surfactant complex. First they calculated the number n_{ps} of dimeric surfactant units in hyaluronan aggregate as

$$n_{ps} = \left(M_{ps} - M_{p}\right)/M_{s0} \tag{1}$$

where M_{ps} is the molecular weight of hyaluronan-surfactant complex, M_{p} is the molecular weight of hyaluronan without surfactant addition, and M_{s0} is the molecular weight of surfactant dimer.

The number of disaccharide unit n_p

$$n_p = M_p/M_{p0} \quad (2)$$

where M_{p0} is the molecular weight of one disaccharide unit of hyaluronan (390.3 g/mol). Then they could calculate the ratio n - number of positive charge of dimeric surfactant unit per one negatively charged hyaluronate disaccharidic unit in hyaluronan-surfactant complex as follows

$$n = 2n_{ps}/n_p. \quad (3)$$

There is a slight excess of positive surfactant charges per one negatively charged disaccharidic unit in the region around critical micelle concentration and the hyaluronan-surfactant complex is not far from electroneutrality. The excess adsorption of surfactant is observed at high surfactant concentrations.

2.2.1 Polymer-surfactant systems studied by HR-US

There are not many references about interactions of cationic surfactant with hyaluronan measured by means of high resolution ultrasonic spectroscopy. The main reference for this thesis is an article by Buckin et al. [28], where they worked with DNA polymer and cationic surfactant (C_{12}TAB). The complexes of cationic surfactant with DNA play important role in the construction of liposomal genetic delivery systems. They studied the complex formation by using combination of high-precision ultrasonic velocity and density measurements.

Buckin et al. [28] used the high resolution ultrasonic spectrometry to study the DNA interactions with cationic surfactants. Combining ultrasonic data with measurements of density they found a high value of the effect of surfactant binding to DNA on compressibility. This could not be explained by hydration changes only. It was considered as an indicator of the formation of micelle-like aggregates of surfactants on the DNA surface with a highly compressible core.

They obtained the compressibility effect in the micelle formation for three surfactants (C_{12}TAB, C_{14}TAB and C_{16}TAB). The compressibility in the formation of C_{12}TAB micelles in the solution was very close to the compressibility in the binding of C_{12}TAB to DNA. The C_{12}TAB molecules form structures on the DNA surface with the intrinsic compressibility closed to the compressibility of C_{12}TAB micelles in the solution and in the binding of C_{12}TAB to DNA, demonstrated the formation of micelles-like aggregates of C_{12}TAB molecules on the DNA surface. They expressed ultrasonic measurement as the concentration increment of ultrasonic velocity (A) which was determined by the relation:

$$A=(u-u_0)/(u_0 c\rho_0) \quad (4)$$

where u and u_0 were ultrasonic velocities in the solution and pure solvent, respectively, c was the concentration of the solute in mol/g, and ρ_0 was the density of water at 25 °C in g/cm^3, 0.997047 g/cm^3. In case of micelle formation, where depend of the concentration increment of ultrasonic velocity on the concentration of the surfactants in water was studied, the concentration increment of ultrasonic velocity was constant at low surfactant concentration which corresponds to the monomer form of surfactant. Above the critical micelle concentration, the value of the concentration increment of ultrasonic velocity

decreased as a result of the micelle formation. In the case of binding of surfactant with DNA, where was done the dependences of the concentration increment of ultrasonic velocity of DNA (A_{DNA}) on concentration of dodecyltrimethylammonium bromide in sodium bromide solution, the value of A_{DNA} at low surfactant concentration in the solution. The concentration increment of ultrasonic velocity decreased in the concentration range 0.5-2 mmol/l of the surfactant which indicated the binding of the surfactant to DNA. At concentration of surfactant above 2 mmol/l, there was a plateau in graph as a result of the saturation of all binding sites on the DNA surface. The polymer is completely saturated by the surfactant micelles and there is complex polymer-surfactant and free micelles in the system.

From the measurement of density has been calculated the apparent molar volume.

$$\Phi_v = M/\rho - (\rho - \rho_0)/(\rho_0 \rho\, c) \tag{5}$$

where ρ is the density of the solution and M is the molecular weight of the solute.

The values of the apparent molar adiabatic compressibilities of the surfactant and DNA ($(\Phi_{kS})_S$) were determined from the values of the concentration increment of ultrasonic velocity (A) and the values of the apparent molar volume (Φ_v). Then it can be calculated the compressibility effect in the binding of surfactant with DNA.

$$(\Phi_{kS})_S = 2\beta_{so}\,(\Phi_v - A - M/2\rho_0) \tag{6}$$

where β_{so} is the coefficient of adiabatic compressibility of the solvent.

In case of low molecular weight solutes, there are no cavities in their structure; the intrinsic compressibility, Km, is determined by the compressibility of covalent bonds and van derWaals radii. Km is small and negligible. For high molecular weight molecules, such as globular proteins and also for molecular aggregates, such as micelles or liposomes, the contribution of the value of the internal compressibility can be significant.

Kudryashov et al. [37] analysed the apparent adiabatic compressibility of surfactants in the free form in solution and in a micelle as a function of the surfactant length. They obtained the apparent molar adiabatic compressibility, Φ_{kS}, and the apparent molar volume, Φ_v, for a series of alkyltrimethylammonium bromides, C_8TAB, C_{10}TAB, C_{12}TAB, C_{14}TAB, and C_{16}TAB, from the ultrasonic measurement using high resolution ultrasonic spectroscopy. Because of high precision of ultrasonic technique it is possible to analyse the concentration behaviour of the apparent molar adiabatic compressibility of long surfactants near the critical micelle concentration, below and above critical micelle concentration.

They found out that values of the apparent molar adiabatic compressibility of alkyltrimethylammonium bromides in a micelle state, $(\Phi_{kS})_{mic}$, are large and positive for all of the surfactants.

$$\Phi_v = V_m + \Delta V_h \tag{7}$$

$$\Phi_{kS} = K_m + \Delta K_h \tag{8}$$

where Vm is the intrinsic volume of the solute molecule, Km is the intrinsic molar adiabatic compressibility of this solute volume; ΔV_h and ΔK_h represent, respectively, the difference between volume and compressibility of the hydration shell of a solute and volume and compressibility of the bulk water.

From the experimental data of Buckin et al. [38] as well as of Kudryashov et al. [37], the values of apparent molar adiabatic compressibilities of the surfactant in the micelle formation increase with the length of carbon chain, from C_{12}TAB to C_{16}TAB. The compressibility effect in the binding of C_{12}TAB molecules to DNA is nearly the same as the compressibility effect of the micelle formation, caused by that the hydration contribution to the effect of binding is small. At low concentrations of surfactant, in the dependence of the apparent molar volume, Φ_v, on the concentration of the surfactant, the value of Φ_v is constant which means that the surfactant is in the momomer form. At concentrations above critical micelle concentration, the value of the apparent molar volume increases as a result of the micelle formation [7],[37].

For the determination of the critical micelle concentration and of the micelle formation, Buckin et al. [28] introduced the concentrantion increment of ultrasonic velocity (A). Main experiment observable in the ultrasonic measurements is determined by the Equation (9).

The concentrantion increment of ultrasonic velocity is constant at low surfactant concentration, which means that the surfactant is in the monomer state. By adding of surfactant to the solution, the micelles begin to form from the value of critical micelle concentration.

Different surfactants have different concentration increment of ultrasonic velocity and different value of critical micelle concentration. The value of concentration increment of ultrasonic velocity, A, increases with the lengh of carbon chain and critical micelle concentration is lower for the surfactants with a chain legth of 12-16 carbon atoms [28],[38].

The reference [8] demonstrates the potential of high resolution ultrasonic spectroscopy in evaluating aggregation-degaggregation behaviour of self-assemling polymer Poloxamer. The results shows that polymer aggregation process can be successfully monitored using both ultrasonic parameters of sound speed and attenuation. The sound attenuation data were able to identify both transitions of the micellization and the gelation. Both parameters were in very good agreement with differential scanning calorimetry (DSC).

Andreatta et al. [40] used high resolution ultrasonic spectroscopy for determination of the critical nanoaggregate concentration of asphaltenes and of the critical micelle concentration of ionic surfactant (CTAB, SDS) and nonionic surfactant (Tween 80, Brij 35) in both water and organic solvents (toluen). They determinated critical micelle concentration of the mentioned surfactants by the measuring of ultrasonic velocity. The comparison of ultrasonic curves for ionic surfactants in water with nonionic surfactants in water showed very different behaviour. From the density measurements they calculated the apparent specific volumes of surfactants and of asphaltene nanoaggregate. The combination of high resolution ultrasonic spectroscopy and densitometer allows calculating the compressibility of the monomers and the micelles.

Hickey et al. [41] published an analysis of the phase diagram and microstructural transitions in a microemulsion system containing phospholipid in which titration arrangement with the detection of ultrasound parameters was used. They demonstrated that break points (points of abrupt changes in slope) on dependences of ultrasonic velocity on the concentration of titrant are located on phase boundaries and can be used to construct phase diagram. Knowledge of other physical parameters of the microemulsion system enabled to make also a quantitative characterization of various detected microphases or the state of water.

Similar study on compositionally different microemulsion system was published by the same group later [42]. Singh and Yadav [44] investigated interactions between poly(N-vinyl-2-pyrrolidone) and sodium dodecyl sulphate using a less sensitive ultrasound equipment operating at single frequency [44]. Ultrasonic velocity as a function of the surfactant concentration exhibited several breaks which were attributed to different molecular organization in the system – for instance, the formation of premicelle associates or their binding to the polymer chain. The authors also noted that ultrasound titration did not substantiated previous claims based on conductivity or viscosity data.

3 AIM OF THE WORK

This thesis is focused on the study of physico-chemical interactions of hyaluronan whose molecular weight is from 10 to 1750 kDa with cationic surfactants. These interactions are studied by means of using uncommon technique named high resolution ultrasonic spectroscopy (HR-US). The densitometry measurement is the indispensable complement to ultrasonic technique enabling to calculate compressibility from ultrasonic velocity and density. The interactions in these systems are important especially for the design of drug delivery systems to human body; the study of the system surfactant-hyaluronan could be possible using as new carrier systems for drug delivery.

In the theoretical part of the thesis, there is mapping the current state of knowledge in the carrier system based on the interaction polyelectrolyte-surfactant. This part is focused on hyaluronan, surfactants and surfactant-hyaluronan system and also in methods using for the discovery of these systems. The previous knowledge of density and ultrasonic measurement of polymers and surfactant-hyaluronan system is summarized in the state of the art. The working out of the theroretical part and the state of the art is basis for the experimental part. The first aim of the experimental part is basic study of hyaluronan in the dependence on its molecular weight and on elevated temperature (25-50 °C) by measuring of density and of ultrasonic velocity with densitometer DSA 5000M. The second aim is to determine the critical micelle concentration and critical aggregation concentration of the surfactants in the absence and in the presence of hyaluronan of different molecular weight and study interactions between surfactants and hyaluronan by means of method high resolution ultrasonic spectroscopy.

This thesis should conduce to recognition applications of surfactants in combination with hyaluronan for using in drug delivery. The results are discussed with regard to the molecular weight of hyaluronan and its concentration. Other aspects are surfactant alkyl chain length and two solution media (water, sodium chloride).

4 EXPERIMENTAL PART

4.1 Hyaluronan and surfactants

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. Following products were used in this study: 10-30, 110-130, 300-500 and 1750-2000 kDa. The surfactant CTAB was get from Sigma Aldrich, TTAB was obtained from Fluka and sodium chloride (NaCl) from Lachner. The surfactant solutions were prepared by dissolving of surfactant powder (TTAB, CTAB) in water or 0.15 M NaCl. The solutions were prepared by weighing their components. Ultrapure deionized water from PURELAB water purification system was used for the preparation of all samples. PURELAB Option R7/15 was from ELGA (Great Britain).

4.2 Densitometer

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). 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 densito-meter 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, CzechRepublic).

4.3 High resolution ultrasonic spectroscopy

Ultrasonic velocity and attenuation were measured at seven selected frequencies in the range from 2.5 to 17.5 MHz using HR-US 102T ultrasonic spectrometer (Ultrasonic Scientific, Ireland) in titration regime. This device is equipped with two cells enabling the single-cell or differential measurements.

The measuring cell was filled with hyaluronan solution using a calibrated 1 ml Hamilton syringe. The titration accessory of HR-US 102T was equipped with 50µl Hamilton syringe which was used for the dosage of the titrant. Solution of TTAB and CTAB in water or in 0.15 M NaCl was used as a titrant. The titrant solution was injected to the sample automatically in 2-µl steps by the computer-controlled titration accessory of ultrasound spectrometer. Ultrasonic velocity and attenuation profiles measured in water or in 0.15M NaCl were subtracted from corresponding profiles in hyaluronan sample, in order to remove the effect of temperature fluctuations and fine differences in the resonance of the cells on measured values of ultrasonic parameters. The measured data were processed using Titration Analysis software (Ultrasonic Scientific, Ireland) which also includes a correction for dilution. Each measurement was done in triplicate at least and the values were averaged.

5 OVERVIEW OF MAIN RESULTS

5.1 Measurement of density and ultrasonic velocity of hyaluronan

5.1.1 Density of hyaluronan solutions

The density increases with the increasing concentration and decreases with the increasing temperature. The dependence of density on concentration of hyaluronan and the influence of the temperature on the density are seen in Figure 1. The effect of the temperature is much more perceptible than the effect caused by concentration of hyaluronan. The temperature dependences were slightly curved the reason of what was the temperature effect on the density of pure solvents.

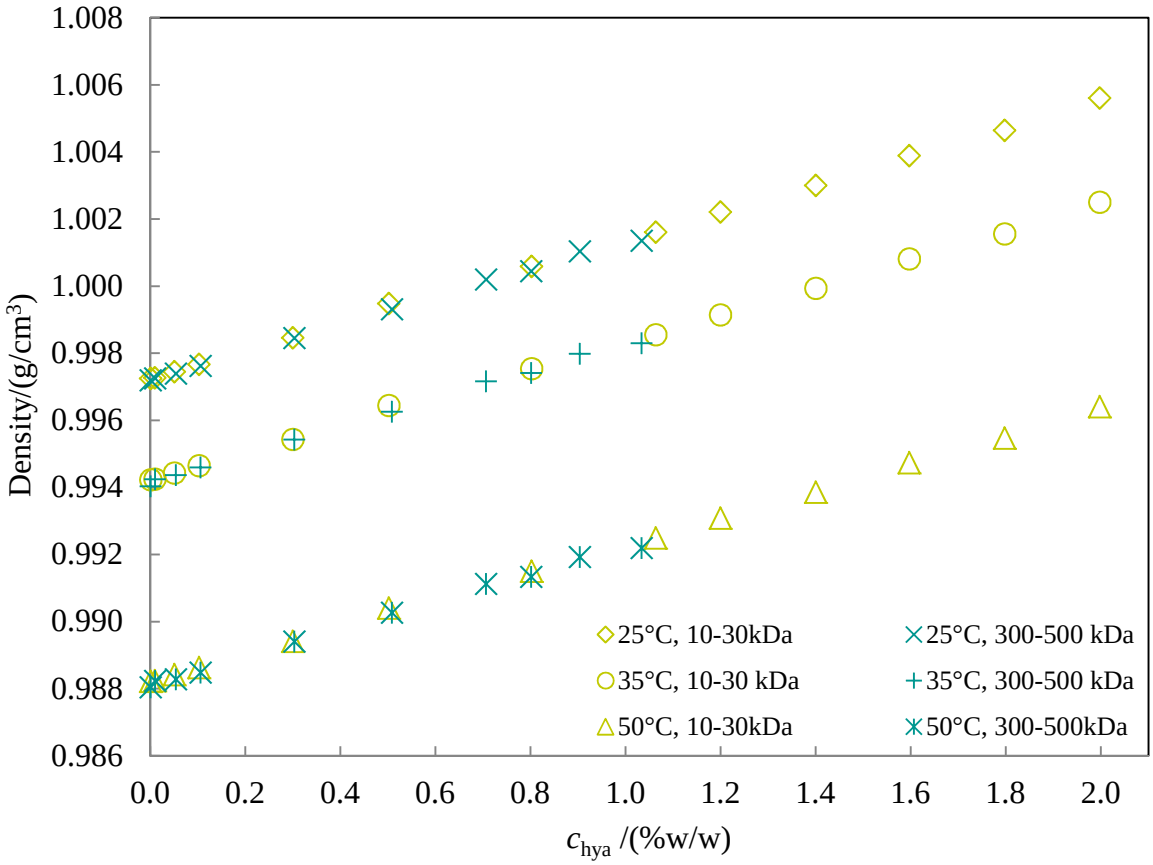

Figure 1. The temperature and concentration dependence of density of hyaluronan solutions (10-30 kDa and 300-500 kDa) in water.

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

linear: $\rho = a_0 + a_w c_w + a_t t$

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

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.

Because the molecular weight did not show appreciable effect on the density (Figure 2) 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 the desired concentration and temperature which fall within their ranges used in this work and with hyaluronan molecular weight within the range from 10 up to 1750 kDa.

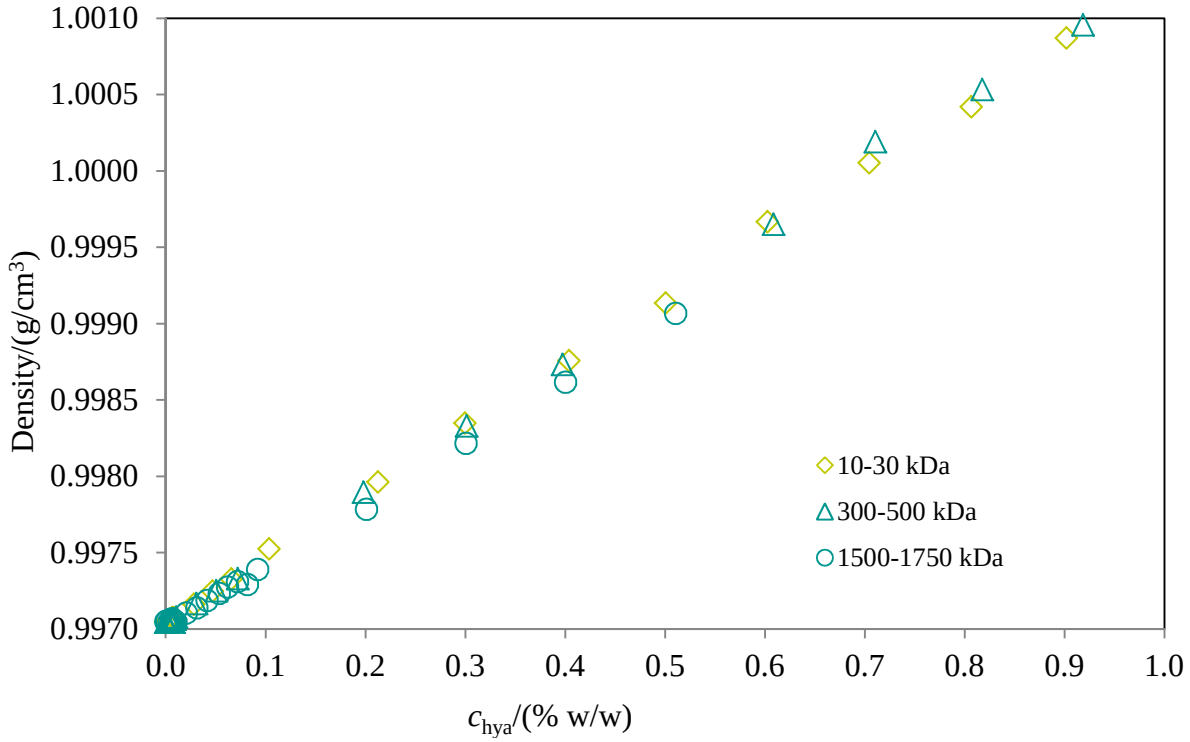

Figure 2. The dependence of density on concentration of hyaluronan at 25 °C. The hyaluronan water solution of three molecular weights (10-30 kDa, 300-500 kDa and 1500-1750kDa) in water.

These results can be additionaly used in the linear and quadratic equations to calculate directly density of hyaluronan solution at certain hyaluronan concentration and temperature (applicable for both water and 0.15 M NaCl environment) without a very time-consuming measurement:

$\rho_{H_2O} = 1.00662+0.00041c_w-0.00036t$

$\rho_{NaCl} = 1.01297+0.00041c_w-0.00037t$

$\rho_{H_2O} = 1.00131+0.00041c_w-0.00007t+0.000004t^2$

$\rho_{NaCl} = 1.00797+0.00041c_w-0.00009t+0.000004t^2$

5.1.2 Ultrasonic velocity of hyaluronan solutions

The ultrasonic velocity increases with hyaluronan concentration linearly as for many other solutions of low concentration [5]. The dependence on temperature at given concentration also increases but is slightly curved what corresponds to the temperature dependence of ultrasonic velocity in water. The molecular weight of hyaluronan practically has no effect on the concentration line of hyaluronan.

As already noted by [12] the effect of temperature is more significant than the effect of concentration (Figure 3). The negligible effect of hyaluronan molecular weight (Figure 3) is indicative of the principal role of hyaluronan basic disaccharide unit in determining the properties of hyaluronan solutions – its molar amount at a given hyaluronan mass concentration is independent on the molecular weight.

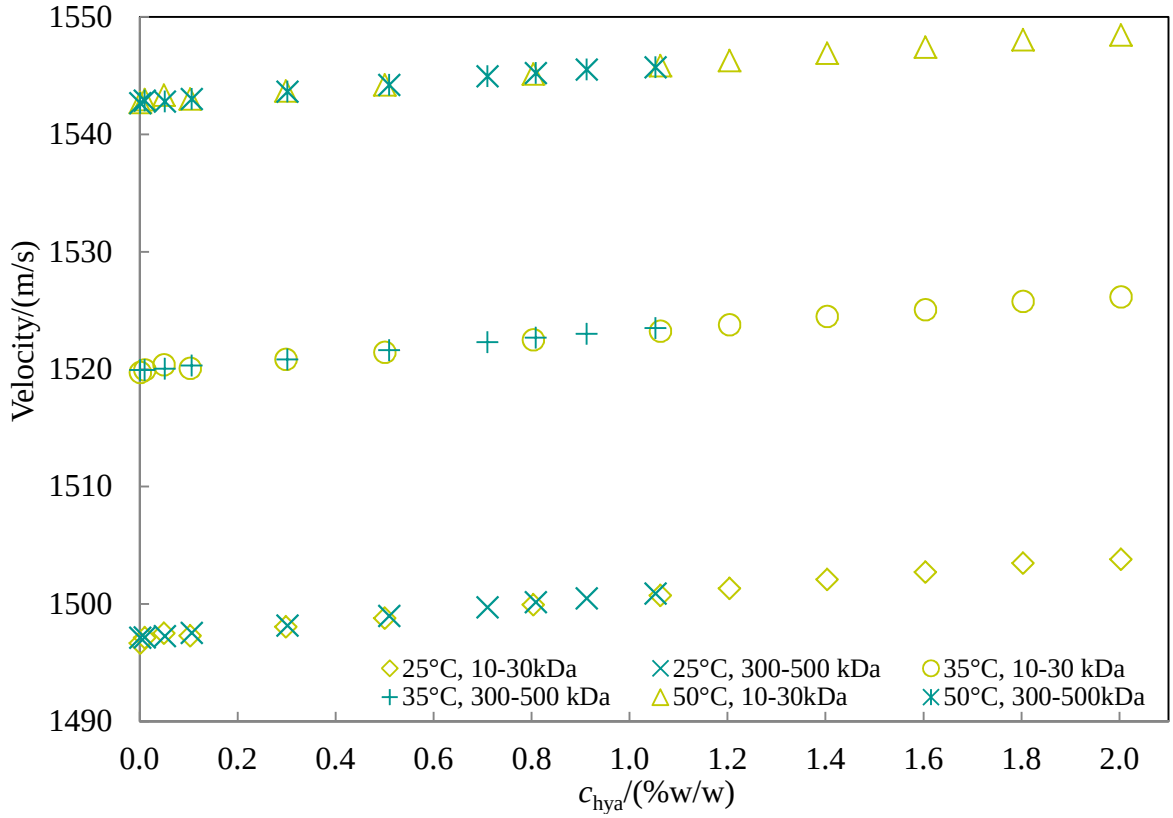

Figure 3. The temperature and concentration dependence of velocity of hyaluronan solutions (10-30 kDa and 300-500 kDa) in water.

These results can be additionaly used in the quadratic equations to calculate directly ultrasonic velocity of hyaluronan solution at certain hyaluronan concentration and temperature (applicable for both water and 0.15 M NaCl environment) without a very time-consuming measurement:

$v_{H_2O} = 1.415+0.0031c_w+4.06t-0.29t^2$

$v_{NaCl} = 1426+0.0032c_w+3.96t-0.29t^2$

5.1.3 Calculated parameters – volume characteristics, compressibility and hydration numbers

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 sodium chloride solution the values are slightly lower. The ultrasonic velocity was primarily used to calculate the compressibility. The compressibility decreased with both the hyaluronan concentration and the temperature, the influence of the temperature was stronger.

The hydration numbers determined from the compressibility data were typically about 20 and only slightly dependent on concentration.

5.1.4 Effect of NaCl on density, velocity and calculated parameters

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. There is negligible effect of molecular weight on all measured or calculated properties.

5.2 Hyaluronan-surfactant systems

The aim of this chapter is study of the system hyaluronan-surfactant in concentration of 15 mg/l and 1000 mg/l of hyaluronan in water and sodium chloride solution. These systems were investigated mainly with high resolution ultrasonic spectroscopy.

5.2.1 The critical micelle concentration in water and 0.15 M NaCl

The critical micelle concentration is an important parameter for the development of products and processes containing surfactants. The critical micelle concentration of alkyltrimethylammonium bromides with different long alkyl chain were studied using tensiometric, fluorimetric [34], and calorimetric [46],[47], viscometric and conductometric [48],[49],[50] methods. In our work as well as in other works [37],[28],[39], the critical micelle concentration was studied by measurement of density and ultrasonic velocity.

First, titrations with pure surfactants in water or 0.15 M NaCl were performed for the purpose of verification and comparison. Critical micelle concentrations and compressibility could be obtained and compared with literature. Further, the titration profiles in the absence of hyaluronan provided the base for investigation of its effect caused by the interactions with surfactants. The measured velocity profiles are shown in Figure 4 and Figure 5. The velocity increases with increasing surfactant concentration due to the increasing number of relatively rigid surfactant molecules and especially due to their hydration shells composed of less compressible water molecules [45]. The increase is linear and the point of abrupt change of the slope determines the critical micelle concentration. In water, the slope behind the critical micelle concentration is close to zero while in the NaCl solution it is still positive but smaller than below this concentration. The decreased slope is a combined result of the formation of micelles with compressible core, the release of hydration water from surfactant monomers, and the formation of new hydration shells around micelles. In the salt solution also the chloride ions are incorporated into micellization forming more rigid micelle structures and their increasing number causes the continuous velocity increase observed behind the critical

micelle concentration. In the salt solution also the chloride ions are incorporated into micellization forming more rigid micelle structures and their increasing number causes the continuous velocity increase observed behind the critical micelle concentration.

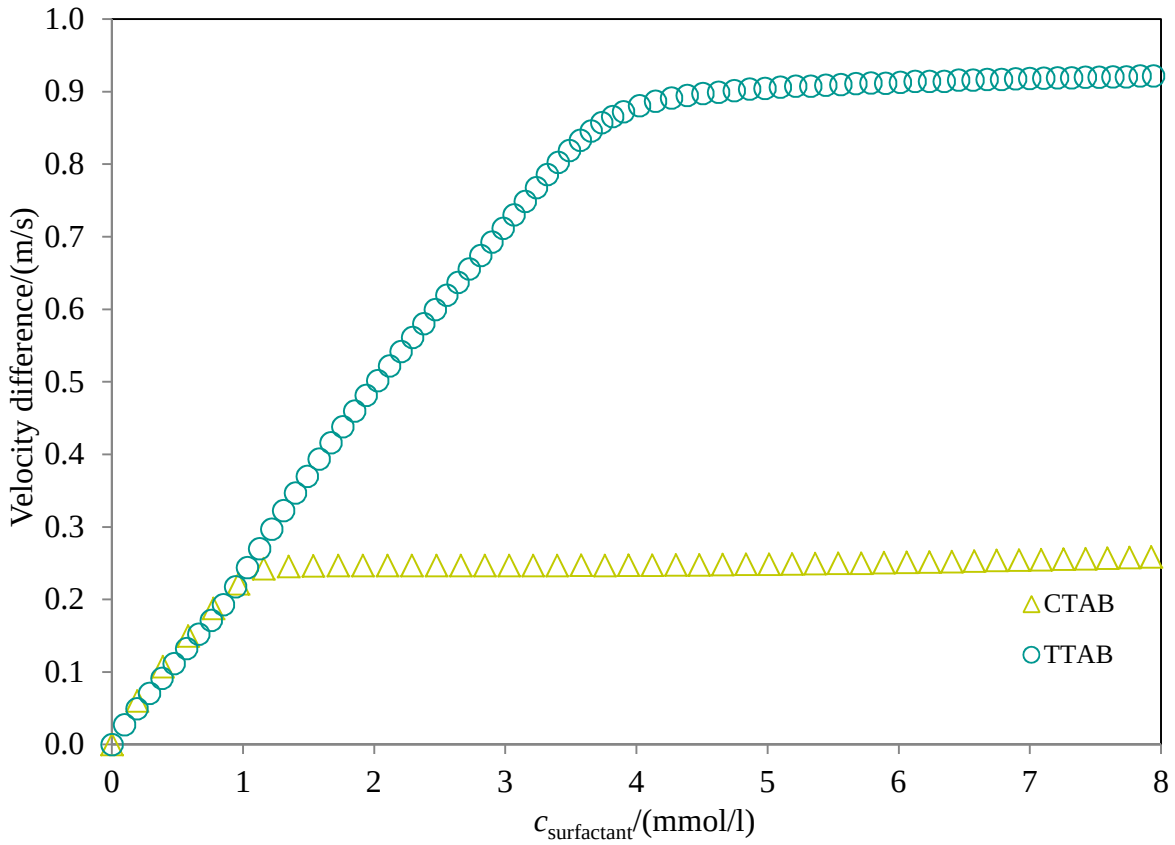

Figure 4. *The determination of the critical micelle concentration of surfactants TTAB and CTAB in water at 25 °C, at frequency 15MHz.*

Table 1. *The critical micelle concentration, CMC, of the surfactants TTAB and CTAB in water and sodium chloride solution at 25 °C measured by means of HR-US 102T, comparing with the CMC from literature.*

Surfactant/environment	CMC mmol/l (this work)	CMC mmol/l [references]
TTAB/water	3.7	3.7 [51], 3.7 [37] , 3.5 [48], 3.8 [22], 3.7 [28], 3.7 [39]
CTAB/water	1.0	0.9 [22],[28],[37] ,[38],[46],[48]; 1.0 [51]
TTAB/0.15M NaCl	0.58	0.52 [34]
CTAB/0.15M NaCl	0.07	0.06 [34]

In water and below the critical micelle concentration the velocity profiles of both surfactants are very close. In the micelle state the velocity in the TTAB system is several times higher than in CTAB. The TTAB micelles are thus less compressible than the CTAB micelles which correspond to previous determinations of the apparent adiabatic compressibility of alkyltrimethylammonium bromides [37] and was attributed to the structure of the internal core

of micelles which was found to be close to that in pure hydrocarbon liquids. Similar situation was observed comparing the two velocity profiles in NaCl solution. As expected, salt did not penetrated into the micelle core and affected only the micelle surface composed of polar groups and their hydration which resulted in increasing velocity within the post-micelle concentration region of both surfactants. Increasing the salt concentration reduces the electrostatic repulsion between the charged groups and therefore favours the aggregation process inducing a decrease of critical micelle concentration.

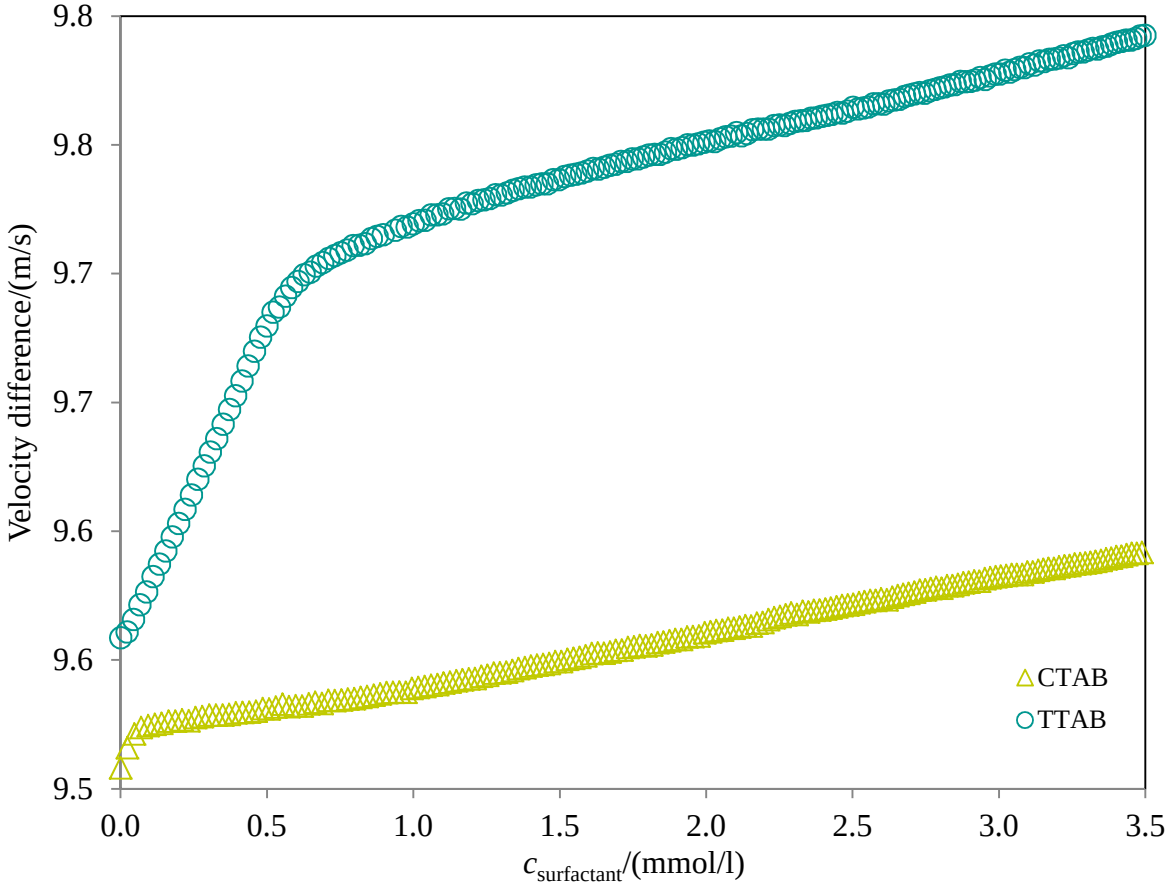

Figure 5. The determination of the critical micelle concentration of the surfactants TTAB and CTAB in sodium chloride solution at 25 °C, at frequency 15 MHz.

5.2.2 Binding of the surfactant to hyaluronan in water and 0.15 M NaCl

Binding of surfactant to hyaluronan chain is observed mainly by technique high resolution ultrasonic spectroscopy, but it was also investigated by densitometer DSA 5000M. Data from densitometer were used for the calculation of compressibility.

Titration of hyaluronan solution hyaluronan by TTAB in water

The ultrasonic cell was filled with hyaluronan solution and titrant (surfactant) was injected stepwise in the solution and then the reference cell was filled with water/0.15 NaCl. Ultrasonic parameters (velocity, attenuation) of the solution were constantly monitored and the contribution of the titrant was subtracted using the reference cell data. Titration curve of water was shifted to to first point of curve of hyaluronan solution (Figure 6) for better comparing.

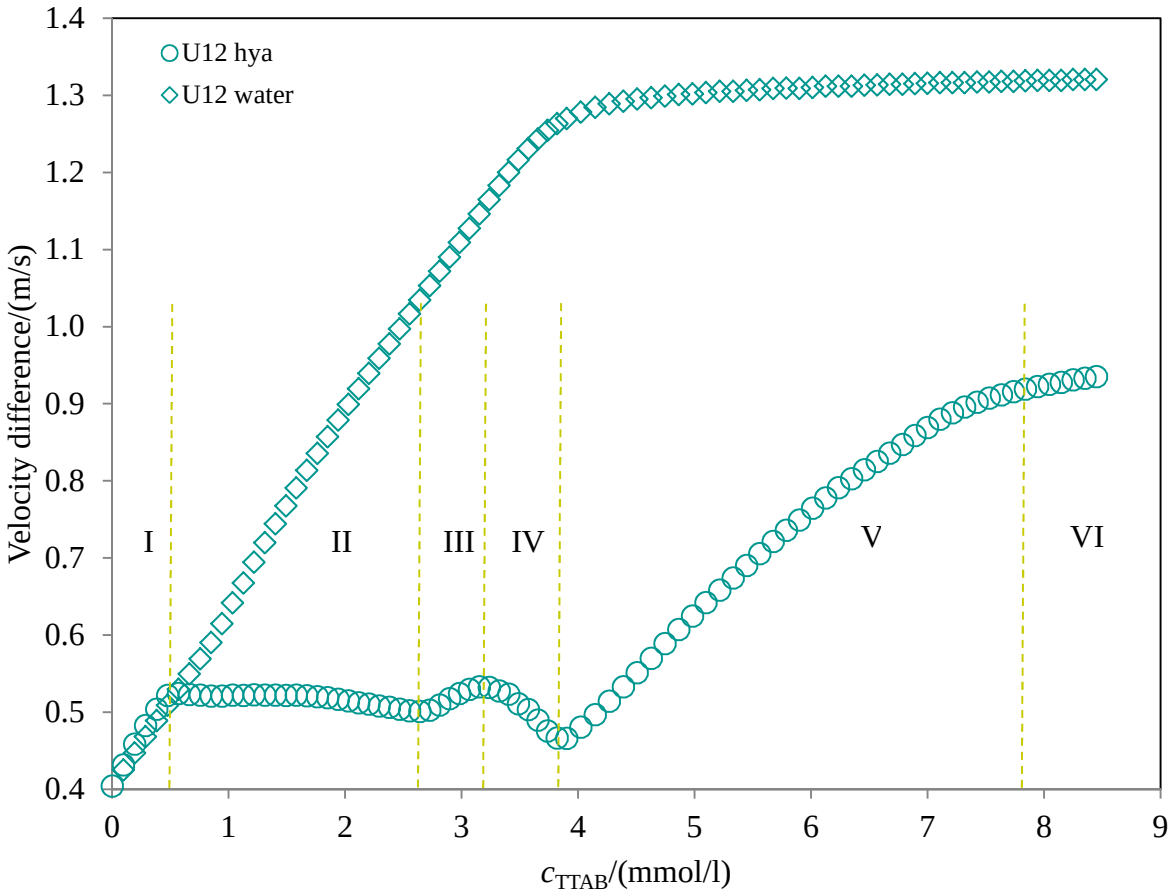

Figure 6. *The velocity titration profile of hyaluronan of concentration 1000 mg/l and molecular weight of 300-500kDa and titration velocity profile of water with TTAB, at frequency 15 MHz. The titration curve of water is shifted to fisrt point of hyaluronan curve.*

Figure 6 compares ultrasonic velocity titration curves obtained during the TTAB titration into water or hyaluronan solution (300-500 kDa, initial concentration of 1 g/l). The curve for the titration in water clearly shows the two parts corresponding to pre-micelle and post-micelle states. On the other hand, up to six parts can be found for the titration profile measured in hyaluronan solution. The first part is practically identical to the corresponding pre-micelle part of the titration profile obtained in water. Visually, this part corresponds to clear solution; the charge ratio is well below one. In this part no effect of added hyaluronan is thus observed which should be a result of the prevailing presence of free surfactant molecules; some single surfactant molecules could be loosely bound to hyaluronan without changing their compressibility.

In the second part the increase of ultrasonic velocity with the surfactant concentration stops at the presence of hyaluronan. On contrary, a very slight velocity decrease is observed. This part resembles the post-micelle part of the titration profile in water. Progressive opalescence up to milky clouding was observed visually. The charge ratio approaches one closely to the end of part II. Ultrasonic practically does not “see” the addition of surfactant molecules into the system. In this part binding of surfactant on hyaluronan in the form of micelles should prevail. Because the surfactant concentration is still below its normal

critical micelle concentration the micellization process is induced by the presence of hyaluronan, mainly by electrostatic interactions between oppositely charged groups on hyaluronan and surfactant. Two opposite effects contribute to the almost constant ultrasonic velocity in the second part. The formation of compressible micelles together with the release of the less compressible molecules of hydration water from the interaction sites decrease the velocity. Formation of new and rigid hydration shell on emerged micelles increases the velocity. The former effect thus more than compensates the latter.

The third part is characterized by re-increase of ultrasonic velocity upon the addition of surfactant. This part is somewhat similar to the pre-micelle part of the titration profile in water (with smaller slope). At the end of this part (around the local maximum of ultrasonic velocity) formation of macroscopic phase separated particles was observed visually. The charge ratio is slightly above one (around 1.2-1.3). In this part the binding of surfactant in the form of micelles is finished, free (unbound) surfactant molecules probably appear in the solution, electrostatic repulsion is removed and phase separation progresses to macroscopic level. Both the free surfactant molecules and rigid phase separated particles contribute to the velocity increase.

In part IV the velocity decreases this time more steeply than in the part II. The system progressively clarifies with increasing concentration of surfactant. In the same time the number of macroscopic separates decreases and at the end of part IV only a few particles can be observed. The decrease of velocity is thus attributable to the decreased size and number of phase separated particles which are more rigid than the liquid medium. Part IV ends when the surfactant concentration corresponds to its normal critical micelle concentration.

The velocity increases again during part V with a slope approximate to the slope of pre-micelle part of the titration profile in water. The increase goes on in part VI but with much lower slope similar to that of the post-micelle part of the titration profile in water. Visual inspection reveals no special changes or behaviour in both parts just slightly opalescent appearance. Due to the surfactant concentration formation of standard micelles should be expected in both parts. In part V the formed micelles probably interact with the previously formed hyaluronan-TTAB complexes, dissolve the macroscopically separated particles and form rather rigid structures apparently of microgel type which increase the ultrasonic velocity. When the interactions are completed part VI begins where mainly new free micelles are formed.

The measurement of ultrasonic attenuation is much less sensitive. From the transition between part I and II the attenuation is continuously increasing up to the transition between part III and IV. Then it steeply decreases in part IV and finally stays practically constant. Increasing attenuation indicates increasing heterogeneity of the system and formation of bigger particles scattering the ultrasonic wave. The step decrease corresponds to the dissolution of such particles and clarificiation of the system. Constant attenuation is found where no substantial heterogeneity evolves, i.e. when only really microscopic phase separation or structures are found.

Titration measurement in HR-US 102T was done in four molecular weights of hyaluronan and at eight frequencies in range 2.5-17.5 MHz. Effect of hyaluronan molecular weight on the velocity titration profiles was very small. The shapes were retained, small differences

were found in the values of the surfactant concentration corresponding to the boundaries between neighboring parts but they should be caused mainly by experimental uncertainities. The effect on the values of attenuation was stronger – the higher the molecular weight the higher the value of attenuation difference. This should be a result of more intensive ultrasonic scattering on more complex coiled structures formed from chains of higher molecular weight. The differences in the shapes of attenuation profiles were small.

In contrast to velocity, attenuation values were dependent on the frequency of ultrasonic which is typical for systems containing micro-heterogeneities capable of relaxation upon ultrasonic propagation [41],[42],[43]. Depending on the mechanism of relaxation ultrasonic waves of different frequency may be scattered to different amount. However, the curved shapes of attenuation profiles and their locations on the concentration axis were not affected by the frequency.

Titration of hyaluronan solution by CTAB in water

Titration profiles measured with CTAB are obviously different from those with TTAB. Some parts are missing in the velocity profile (Figure 7) and from this point of view the CTAB profile is simpler. On the basis of changes of the profile slope including its sign it is reasonable to assume that parts I and III are missing, and part V extends through much shorter concentration interval. The titration curve in the hyaluronan solution is from the first point different from that obtained in pure water. In comparison to TTAB, CTAB interacts with hyaluronan at much lower surfactant concentration, consequently, part I was not observed. Part II is characterized also by the evolution of turbidity and extends to concetrations well above the normal critical micelle concentration but it terminates also around the equivalent charge ratio. Visible macroscopic phase separation occurs here only in part IV and the steep velocity decrease in this part is mainly a result of particle setting out of the detection volume. The missing part III is probably a result of stronger cooperativity of CTAB binding on hyaluronan; perhaps no free surfactant molecules can be present in this case.

The precipitated particles were progressively dissolved during part V changing from precipitates through clot structures or swelled flocs to almost clear system in part VI where also the formation of free standard micelles can be expected.

It is also interesting that the velocity difference measured in the TTAB-hyaluronan system approaches the values measured for TTAB micelle system and the values are very close for both systems starting at TTAB concentration of about 8 mmol/l. On contrary the velocity difference in the CTAB-hyaluronan system is always higher than in corresponding hyaluronan-free solutions. CTAB thus forms complexes with hyaluronan which are more rigid, less compressible than CTAB micelles whereas the opposite is found for TTAB.

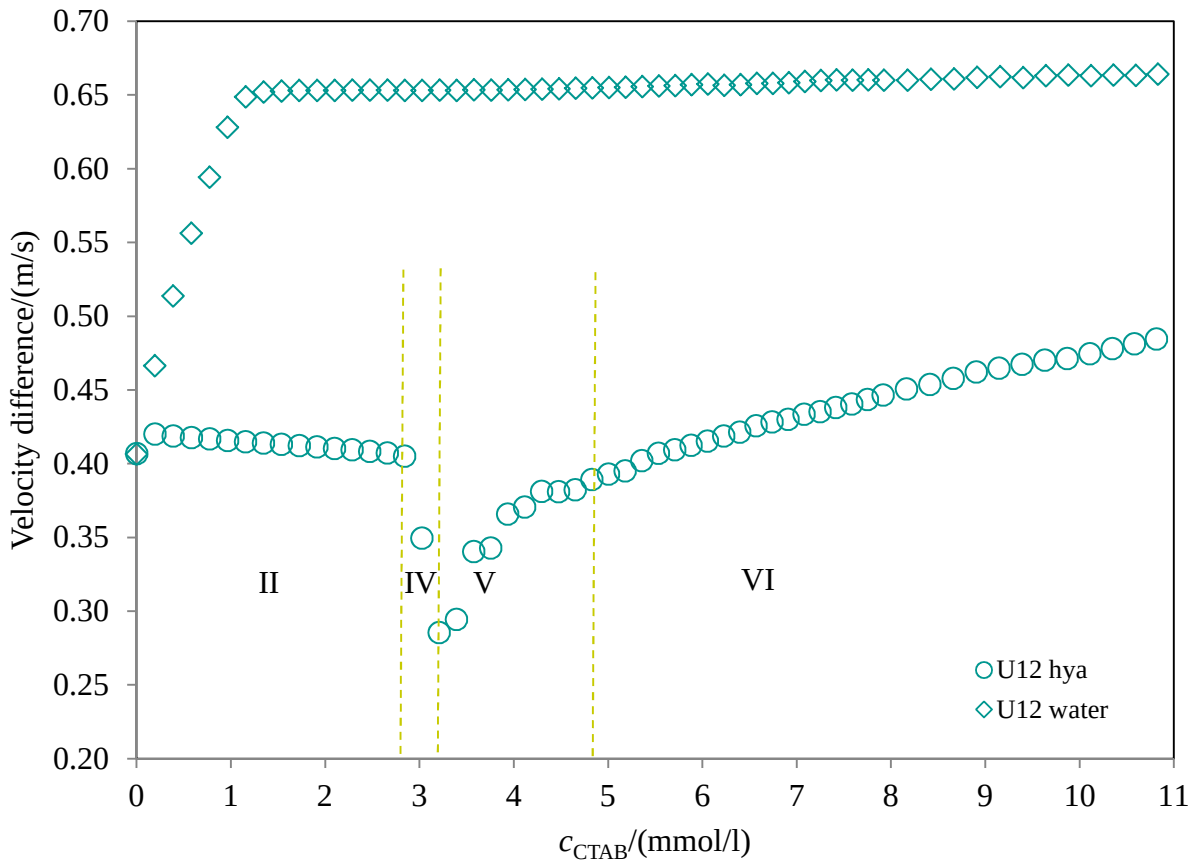

Figure 7. *The velocity titration profile of hyaluronan of concentration 1000 mg/l and molecular weight of 300-500kDa and the velocity titration profile of water with CTAB, at frequency 15 MHz. The titration curve of water is shifted to first point of hyaluronan curve.*

The titration profile of ultrasonic attenuation measured for CTAB is also different from that for TTAB. It continuously and slightly increases up to the boundary between parts II and IV (remember the missing part III). Then it sharply increases (in contrast to the system with TTAB) up to the boundary between parts V and VI. In the part VI it continues to increase at first, but with lower slope, and then is essentially constant. The sharp increase reflects the increased heterogeneity due to the phase separation. The relatively high final value of attenuation, comparing to TTAB system, shows more heterogenous structure capable of more intensive scattering of ultrasonic wave formed probably by rather rigid and translucent micro-gel or nano-gel particles.

The effect of hyaluronan molecular weight on the shape of all measured velocity titration profiles was very small. The shapes were retained, some differences were found in the values of the surfactant concentration corresponding to the boundaries between neighboring parts and in the velocity values within the same part of the profile. The differences are seen particularly in parts III-VI and should be ascribed to the effects of differences in conformations of hyaluronan chains of different molecular weight [10]. On average, the two preparations of lower molecular weight differed from the two preparations of higher molecular weight which gave very similar profiles. The effect on the values of attenuation was stronger – the higher the molecular weight the higher the value of attenuation difference. This should be a result of more intensive ultrasound scattering on more complex coiled structures formed from chains of higher molecular weight. The differences in the shapes of attenuation profiles were small. Visually, the smaller the hyaluronan molecular weight the less perceptible opacity was observed.

Titration measurement in HR-US 102T was done in four molecular weights of hyaluronan and at eight frequencies in range 2.5-17.5 MHz. Effect of hyaluronan molecular weight on the velocity titration profiles was very small. The shapes were retained, small differences were found in the values of the surfactant concentration corresponding to the boundaries between neighboring parts but they should be caused mainly by experimental uncertainities. The effect on the values of attenuation (Figure 8) was stronger – the higher the molecular weight the higher the value of attenuation difference. This should be a result of more intensive ultrasound scattering on more complex coiled structures formed from chains of higher molecular weight. The differences in the shapes of attenuation profiles were small.

In contrast to velocity, attenuation values were dependent on the frequency of ultrasonic which is typical for systems containing micro-heterogeneities capable of relaxation upon ultrasonic propagation [41]-[43]. Depending on the mechanism of relaxation ultrasonic waves of different frequency may be scattered to different amount. However, the curved shapes of attenuation profiles and their locations on the concentration axis were not affected by the frequency as is illustrated on typical example in Figure 8. The effect of ultrasonic frequency on both velocity and attenuation titration profiles is very similar for TTAB and CTAB .

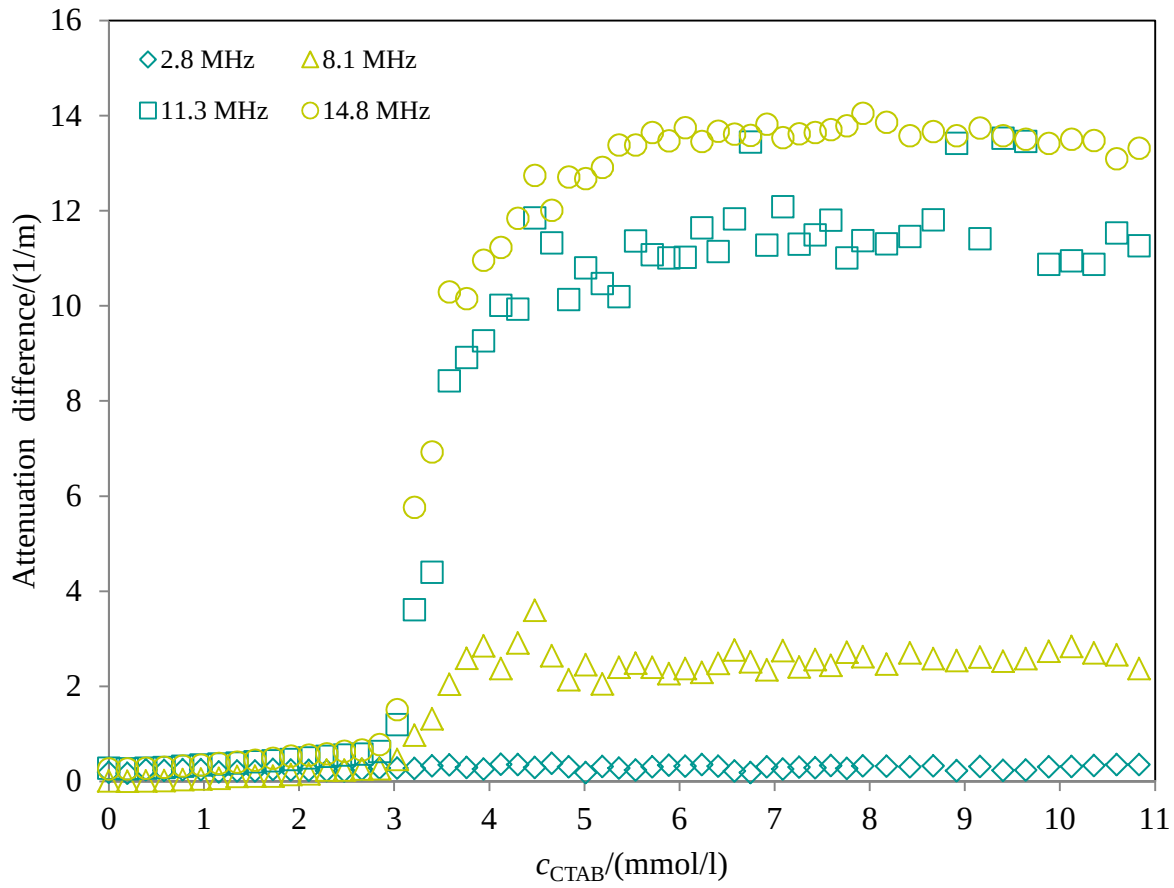

Figure 8. *The effect of frequency on ultrasonic attenuation profile of hyaluronan-CTAB system in water, at 25 °C, at frequencies 2.8-14.8 MHz. Hyaluronan 1000mg/l 300-500 kDa.*

The titration of hyaluronan solution by TTAB in sodium chloride solution

The measurement of the solution of hyaluronan in 0.15 M NaCl was done the same way as the measurement in water but results are different. The breaks in the curves of the plot are not so noticeable; in case of hyaluronan solution with the molecular weight 10-30 kDa the curve is linear without any breakpoint. The interactions of the system hyaluronan-surfactant in 0.15 M NaCl are so slight. In case of hyaluronan 90-110 kDa, the critical micelle concentration and range of the binding of the surfactant to the polymer is noticeable. In the experiment with hyaluronan 300-500 kDa and 1500-1750 kDa is noticeable break in the curve of ultrasonic velocity and expressive change in the trend of the curve of the attenuation.

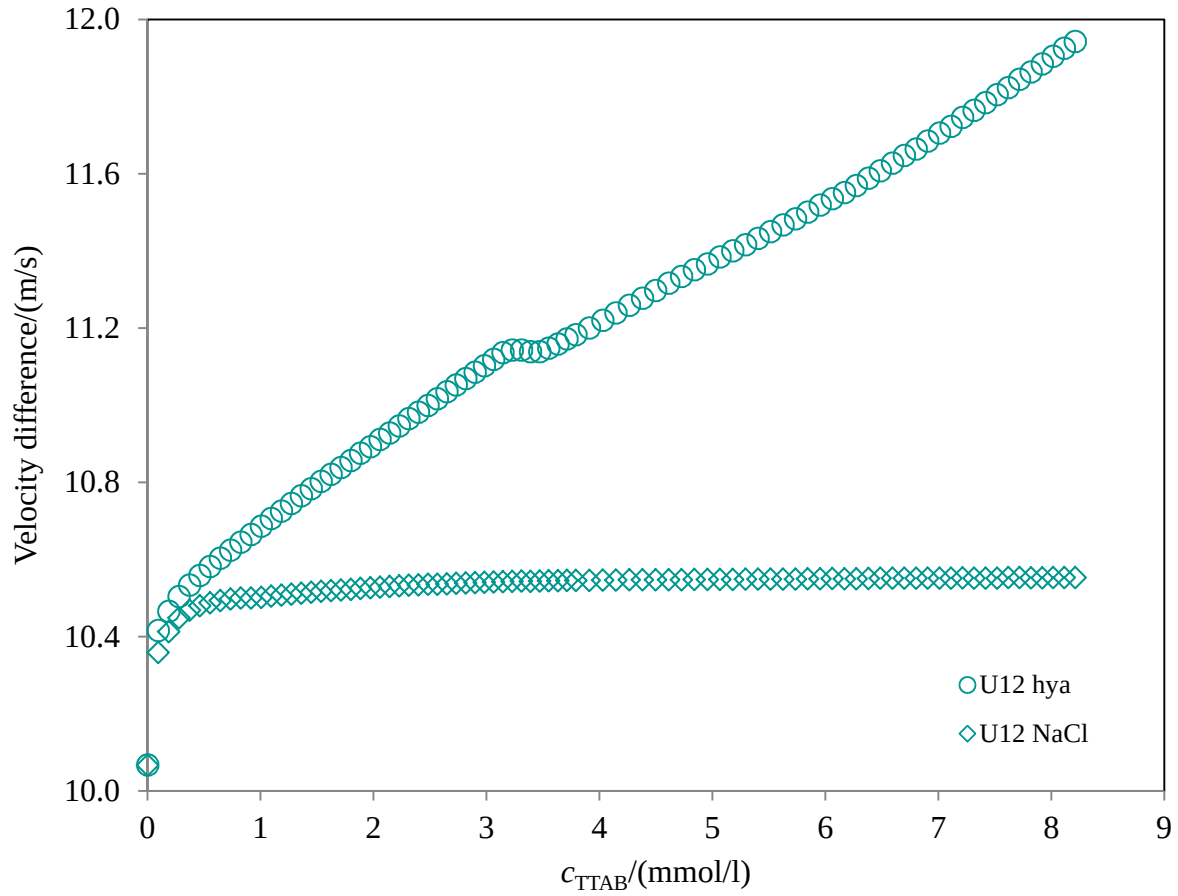

Figure 9. *The velocity titration profile of hyaluronan of concentration 1000 mg/l and molecular weight of 300-500kDa and titration curve of water with TTAB in 0.15M NaCl, at frequency 15 MHz. The titration curve of 0.15 M NaCl is shifted to first point of hyaluronan curve.*

The titration profiles were significantly changed by the presence of NaCl at the concentration of 0.15 M (the physiological concentration). Ultrasonic titrations of both surfactants into NaCl solution (without hyaluronan) confirmed the well-known decrease of critical micelle concentration in about one order of magnitude (Figure 5). In case of TTAB the titration profiles at the absence and presence of hyaluronan practically coincide at low surfactant concentrations (and show a linear dependence of the ultrasonic velocity on the surfactant concentration) which is the same observation as for the titrations in water. However, in the NaCl solution this coincidence survives up to the critical micelle concentration and even then the velocity increases at the presence of hyaluronan though with apparently somewhat smaller slope. This continuous increase is interrupted by a short interval of almost constant velocity which corresponds to the steep increase in the ultrasonic attenuation and to the visual observation of several macroscopically phase-separated particles dispersed in a milky system. The charge ratio at the start point of this increase is about 1.3. It is interesting that this short interval occurs at surfactant concentrations close to critical micelle concentration in water. The system is clear before and cloudy or milky after this interval.

The ultrasonic attenuation of TTAB-hyaluronan-NaCl system does not almost change up to the start of the short region of constant velocity where it sharply increases. The sharp increase continues behind the region of constant velocity and then the attenuation progressively levels

off and finally increases only moderately but continuously. In fact, the start of only moderate increase of attenuation corresponds to a weak bend on the velocity titration profile. The increased attenuation reflects the formation of heterogeneities – microaggregates of hyaluronan-surfactant complexes. The effect of ultrasound frequency is similar as described above – the velocity values are unaffected in contrast to the values of attenuation.

The shapes of titration profiles for CTAB in hyaluronan-NaCl system are much closer to those for TTAB than it was observed in water. The velocity increases before the critical micelle concentration but is higher than in the absence of hyaluronan, the slope of this increase is smaller behind this concentration and a narrow interval of almost constant velocity appears at much higher surfactant concentration than is the critical micelle. CTAB concentration corresponding to this constant interval is very similar as for TTAB as well as the charge ratio which is here around 1.3. Thus electrostatic interactions control this behaviour. The re-increase of the velocity behind this interval is clearly (in contrast to TTAB) composed from at least two more or less linear branches intersecting around the CTAB concentration of 6 mmol/l. The initial increasing part corresponds visually to a clear system, during the constant interval some flakes or flocs and evolution of turbidity can be observed. After this interval the system is cloudy.

The attenuation reflects the changes in velocity. Before the short region of constant velocity the attenuation increases moderately then sharply within this region and even behind it. Then it remains essentially constant up to the bend mentioned in the preceding paragraph which appears around the CTAB concentration of 6 mmol/l and above this concentration it increases again. The effect of ultrasonic frequency is still the same as given above.

Due to the higher velocity and increasing attenuation at low CTAB concentrations in presence of hyaluronan it cannot be excluded that the surfactant interacts with CTAB in NaCl solution even in its normal pre-micelle region forming some more rigid structures. It can not be concluded that micelle structures bound to hyaluronan are formed at this stage but taking into account the smaller slope of velocity increase during the normal post-micelle region (at CTAB concentration between ca 1 and 3 mmol/l) single surfactant or “minimicelle” binding should be prefered. The steep increase of attenuation reflects increasing heterogeneity in the system which results in separation of macroscopic particles (the flakes). The interval of almost constant attenuation corresponds to re-dissolving of big particles by the excess of surfactant and is followed by progressive formation of rigid (increasing velocity) microheterogeneous (increasing attenuation in cloudy system) structures probably of microgel type as can supposed due to the increased slope of the velocity profile (from the CTAB concentration of 6-7 mmol/l).

The effect of hyaluronan molecular weight is much more obvious in the presence of NaCl than measurements in water. The velocity profile for the lowest molecular weight (10-30 kDa) is practically linear, there is small change in attenuation and the sample clear throughout the whole range of applied surfactant concentration. Because the velocity is systematically increasing in presence of hyaluronan this should not directly indicate that no interactions between hyaluronan and surfactant are detected. Further, only below the critical micelle concentration (0.071 mmol/l CTAB) the profiles measured at the presence and at the absence of hyaluronan coincide. The velocity profile measured for the molecular weight of 110-130 kDa shows instead of the short interval of almost constant velocity a broader interval of “ïnflexing” behaviour during which also the attenuation markedly increases and opacity is

developed. For hyaluronan 300-500 kDa and for the highest molecular weight (1500-1750 kDa) the constant velocity interval and corresponding sharp increase of attenuation are shifted to higher surfactant concentrations, in case of 1500-1750 kDa to concentration 5-7 mmol/l. Again, frequency affects the values of attenuation (not the shape of profiles).

Our results suggest that in case of very low molecular weight hyaluronan in presence of salt, the surfactant cation may replace the polysaccharide counterion forming very small and phase non-separating structures of separate surfactant molecules bound to the biopolymer chain. An increased molecular weight of the hyaluronan leads to the formation of larger and coiled ball-like biopolymer structures with surfactant molecules bound preferably on their surface. The higher the structure, the larger its surface and the higher surfactant amount is needed to induce (micro)phase separation.

6 CONCLUSION

The experimental part was divided to three parts. The first part (preliminary part) followed up behaviour of hyaluronan in water, measurement of pH and preliminary density measurement. The aim of this part was to characterize the dissolution and storage stability of hyaluronan. The value of pH all measured solutions did not change with adding of surfactant. Sodium azide could not be used for preparation of hyaluronan solution because the stability is the same in the presence and absence of azide. So the samples without sodium azide were used in shorter time (three days after dissolving). As emerged from the density measurements of different ages of hyaluronan solution, there were no changes in the values of density in different days of measurement so measurements were performed in first day.

The second part (experimental part I) was focused on the study of hyaluronan in wide range of molecular weights and as broad range of its concentration. Density and ultrasonic velocity were measured in solutions in temperature range 25-50 °C by means of densitometer DSA 5000M.

The density and ultrasonic velocity of hyaluronan solutions in water or in 0.15 M NaCl was linearly dependent on the concentration at any used temperature and both quantities increased with the concentration. Increasing temperature decreased density and increased ultrasonic velocity of a solution of given concentration; the temperature dependence was slightly curved and slightly deviated from linearity. Therefore all the data through all molecular weights, concentrations and temperatures used could be satisfactorily fitted with a single equation (one or each solvent and each quantity); linear in concentration and quadratic in temperature. The equation can be used for reliable estimate of the density and ultrasonic velocity 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 sodium chloride solution the values are slightly lower. The ultrasonic velocity was primarily used to calculate the compressibility. The compressibility decreased with both the hyaluronan concentration and the temperature, the influence of the temperature was stronger. 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. There is negligible effect of molecular weight on all measured or calculated properties.

In the third part (experimental part II), high resolution ultrasonic spectroscopy was used for a detailed study of interactions between hyaluronan and two cationic surfactants tetradecyltrimethylammonuim bromide (TTAB) and hexadecyltrimethylammonium bromide (CTAB) either in water or in sodium chloride at physiological concentration (0.15 M).

The same values of ultrasonic velocity were measured both with densitometer DSA 5000M, and with ultrasonic spectrometer HR-US 102 and HR-US 102T.

Our results suggest that in the case of very low molecular weight hyaluronan in the presence of salt, the surfactant cation may replace the polysaccharide counterion forming very small and phase non-separating structures of separate surfactant molecules bound to the biopolymer chain. An increased molecular weight of the hyaluronan leads to the formation of larger and coiled ball-like biopolymer structures with surfactant molecules bound preferably on their surface. The higher the structure, the larger its surface and the higher surfactant amount is needed to induce (micro)phase separation.

The results clearly show differences in interactions of TTAB and CTAB with hyaluronan in water. Taking into account the molecular structure of these surfactants the differences should be caused by little bit longer alkyl chain of CTAB. In other words, besides the electrostatic contribution also the hydrophobic interactions play an important role especially in the case of CTAB which has longer hydrophobic tail. For example, hydrophobic component supports interactions at very low surfactant concentration. Ultrasonic titration profiles revealed that the behaviour of hyaluronan-surfactant system is more complex than simple one-phase or two-phase behaviour with a sharp boundary as could be inferred from phase diagrams. In the phase separation region several transitions were observed on the ultrasonic velocity profiles which reflect formation of colloidal structures of different rigidity. The number of detected transitions was richer for TTAB therefore increasing importance of the hydrophobic interaction contribution simplifies the details of phase separation behaviour.

In water there is no significant effect of hyaluronan molecular weight similarly as in the previous studies on phase diagrams [4]. Thus the basic disaccharide unit and its interaction abilities are relevant for hyaluronan interactions with oppositelly charged surfactants whereas specific conformations or coil shapes of the biopolymer chain of various lengths are not so important. In the presence of sodium chloride at the concentration of 0.15 M the effect of molecular weight is appreciable. Salts should suppress the electrostatic interactions and this was reflected in much simpler titration profiles and less rich behaviour in two-phase region. However, the titration profiles measured in the NaCl solution were much closer for both surfactants than in water. Probably, the more collapsed conformations supposed to exist in the presence of electrolyte as compared to conformations in water prevented the access of surfactant molecules to the polyelectrolyte interaction sites and consequently restricted (cooperative) interactions between surfactant alkyl chains. In other words, the effect of salt on hydrophobic interactions was mediated by sterical reasons.

The measured values of the ultrasonic velocity had the same values from the HRUS 102, HRUS 102T and DSA 5000M.

The binding of surfactant to hyaluronan is portrayed in Figure 10. At low surfactant concentration its free monomers bind to the hyaluronan chain; later, with increasing surfactant concentration the micelles are formed and electrostatically bind to the chain. At the very high surfactant concentration, the free surfactant micelles are formed in the bulk solution because the hyaluronan chain is already fully occupied by previously formed micelles.

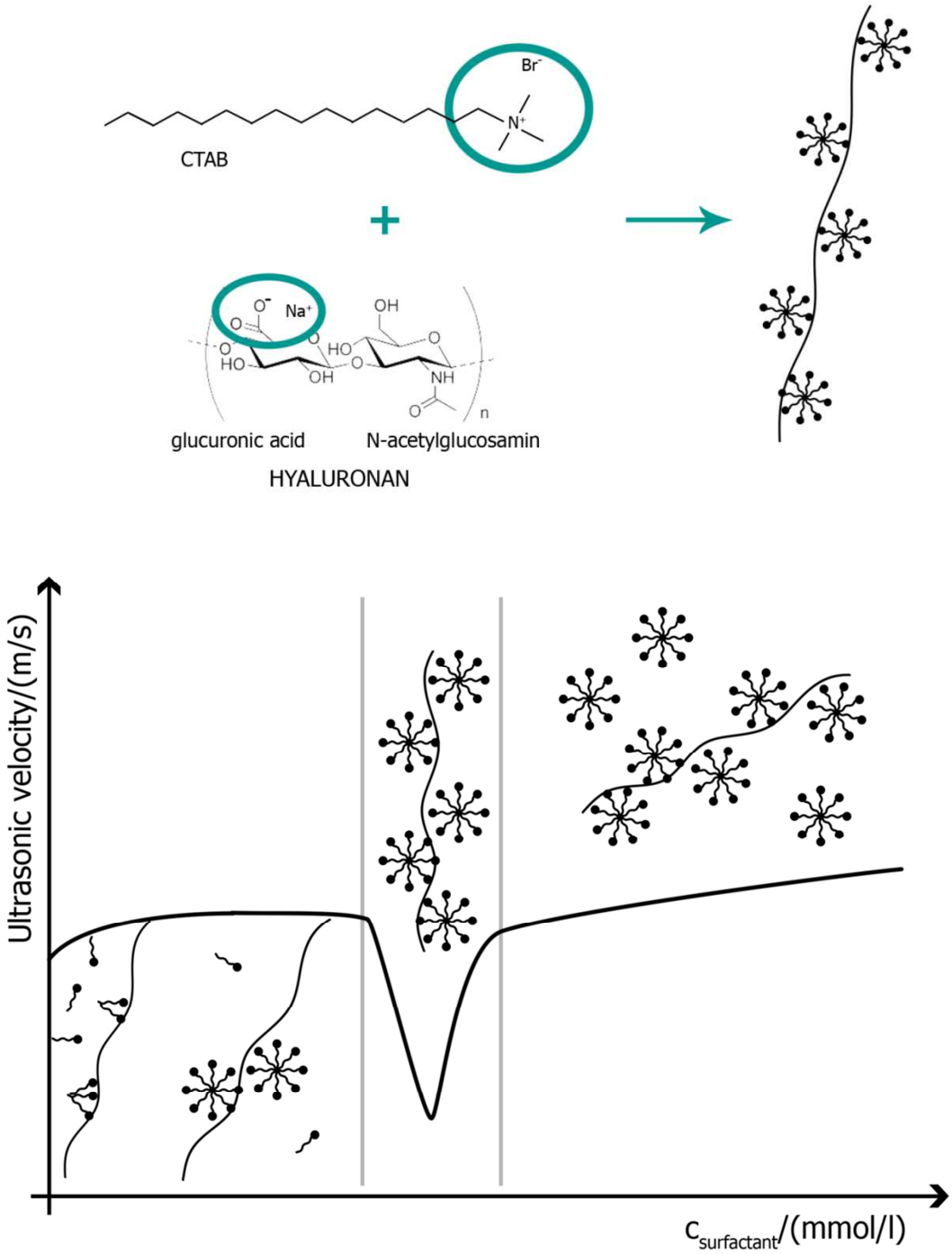

Figure 10. Illustration of the interactions between hyaluronan and surfactant CTAB in water.

High-resolution ultrasonic spectroscopy in titration regime enabled to reveal details of interactions between hyaluronan and oppositely charged surfactants. Due to its high sensitivity much more in-depth picture on interactions could be obtained when compared to other methods. Up to six different regions could be identified in a narrow interval of surfactant concentration corresponding to different states of hyaluronan-surfactant complexes formed by the interactions. These regions differed primarily in the shape of the dependence of ultrasonic velocity on surfactant concentration values of which are determined by the compressibility of structures formed in the system. The richness of titration profiles was depressed in salt solution where essentialy only two principal regions were observed. On the other side, the effect of hyaluronan molecular weight on the position of boundary between regions was more significant in the presence of salt. Very important effect on titration profiles in water had the length of the surfactant hydrophobic tail. Besides electrostatic also hydrophobic interactions are relevant for determining the behaviour of hyaluronan-surfactant systems and properties of formed complexes (aggregates).

The series of papers by Swedish research groups [3],[4] provided a detailed study on phase behaviour of systems containing water, hyaluronan, alkyl trimethylammonium bromides (static experiment). This thesis investigated directly study of interactions hyaluronan-surfactant (dynamic experiment). HR-US is very the excellent method for measurement of the interactions polymer-surfactant with very excellent results and resolutions which is seen from the comparison of measurement in DSA 5000M and in HR-US.

The other contribution to this thesis is particularly in detailed study of attributes of different samples of hyaluronan. Thus is thought the different molecular weights and concentration of hyaluronan, its behaviour in water and 0.15 M NaCl and at different temperature.

System hyaluronan-TTAB and hyaluronan-CTAB as dynamic experiment were performed in this thesis for the first time.

Our results of density and ultrasonic velocity and subsequently calculated parameters are published in this broad range for the first time.

Other experiments could be done in temperature around 37 °C.

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8 CURRICULUM VITAE

Name: Andrea Kargerová

Born in Usti nad Orlici, 18.7. 1983

E-mail: xckargerova@fch.vutbr.cz

Education:
Gymnazium Ceska Trebova 1996 – 2003
Charles University in Prague, Faculty of Pharmacy in Hradec Kralove, study programme Healthcare bioanalytics 2003 – 2006, Bachelor degree
Brno University of Technology, in Faculty of Chemistry, in Institute of Food Science and Biotechnology 2006 – 2009, Master degree
Faculty of Chemistry, Brno University of Technology, Doctoral degree 2009–2014

Career:
since 2010 Researcher in Laboratory of Biocolloids,Materials Research Centre (FCH BUT)

Training courses:
Measuring of IR vibration spectra (University College of Dublin, Ireland)

Impacted publications:

KARGEROVÁ, A., M. PEKAŘ. Densitometry and ultrasound velocimetry of hyaluronan solutions in water and in sodium chloride solution. *Carbohydrate Polymers*. 2014.

KARGEROVÁ, A.; PEKAŘ, M. High resolution ultrasonic spectroscopy for measuring of interactions of hyaluronan with surfactants contribution. hyaluronan with surfactants contribution. *Chemistry is life*. 2012. pp. 316-321. ISBN: 978-80-214-4545- 8.

KLUČÁKOVÁ, M.; BACHRATÁ, R.; KARGEROVÁ, A. Comparison of complexation properties of humic acids and simple organic ligands. In *X. Workshop of Physical Chemists and Electrochemists 2010.* pp.124-125. ISBN: 978-80-7375-396- 2.

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Given speeches:

KARGEROVÁ, A.; PEKAŘ, M. *Interaction of hyaluronan and CTAB studied by high resolution ultrasonic spectroscopy*. 9th International Conference on Hyaluronan. Oklahoma City, Oklahoma, 2013.

KARGEROVÁ, A.; PEKAŘ, M. *High resolution ultrasonic spectroscopy for measuring of interactions of hyaluronan with surfactants contribution. hyaluronan with surfactants contribution*. In Student conference Chemistry is life, 2012.

KARGEROVÁ, A.; PEKAŘ, M. *Ultrasonic spectroscopy of systems hyaluronan surfactant*. SIS Conference 2012. Edmonton.

9 ABSTRACT

This disertation thesis is focused on the study of physico-chemical interactions of hyaluronan (with molecular weights from 10 to 1750 kDa) with cationic surfactants measured using uncommon technique named high resolution ultrasonic spectroscopy. Densitometer was also used for the study of these interactions, in measuring of density and ultrasonic velocity of hyaluronan with different molecular weight in dependence on elevated temperature (25-50 °C). The aim is the determination of critical micelle concentration (CMC) and critical aggregation concentration (CAC) of the suractants in the absence and in the presence of hyaluronan with various molecular weights. Interactions in this system are important for the design of the systems for the targeted delivery, especially for the drugs. The experiments were made in water and sodium chloride solution. The significant breakpoint in the ultrasonic velocity showed changes in the system hyaluronan-surfactant.

ABSTRAKT

Tato disertační práce se zaměřuje na fyzikálně-chemické interakce hyaluronanu (molekulové hmotností od 10 do 1750 kDa) s kationickými tenzidy. Pro zkoumání a měření vzájemného působení byla použita technika s názvem ultrazvuková spektroskopie s vysokým rozlišením (HR-US). Při zkoumání interakcí byl též použit denzitometr, a to při měření hustoty a uktrazvukové rychlosti hyaluronanu o různé molekulové hmotnosti v závislosti na vybrané teplotě (25-50 °C). Práce se zaměřuje se na studium kritické micelární (CMC) a agregační (CAC) koncentrace tenzidů v přítomnosti a nepřítomnosti hyaluronanu o různé molekulové hmotnosti. Interakce hyaluronanu s kationickými tenzidy jsou důležité pro systémy s cíleným transportem, zejména léčiv. Měření interakcí bylo prováděno ve vodě a v roztoku chloridu sodného. V získaných datech lze pozorovat významné zlomy v ultrazvukové rychlosti, které nám ukazují změny v systému hyaluronan-tenzid.