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INTERACTIONS BETWEEN HYALURONAN AND SURFACE ACTIVE SUBSTANCES

INTERAKCE HYALURONANU A POVRCHOVĚ AKTIVNÍCH LÁTEK

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1 INTRODUCTION

With increasing number of patients with cancer increases the interest of the researchers who would like to discover methods for successful treatment. The researchers focus on the problem of treatment already attacked cells in human organisms. The development of nanotechnology, the branch of science focusing on nanoparticles and their application help them to find the right way for solving the problems in medicine, surgery, cosmetics etc. The investigators would like to take advance of substances which occur naturally in the organism to deliver drugs exactly to the required place in the tissue. The process is called controlled drug delivery or targeted drug delivery. However, the main disadvantage is the hydrophobic nature of most used or potential drugs (cytostatics) because the human body contains about 60% of water.

How can be the drug, which is toxic for healthy cells, transported directly to the cancer cells and effectively perform its role? The active substance is usually coated with a safe colloid- or nanomaterial which is the barrier between the drug molecule and the extracellular fluids. One of the solutions is to use a combination of a natural hydrophilic polysaccharide, hyaluronan, which occurs in a human body and has unique properties not only from the view of interaction with the cell receptors and surface active molecules. The mechanism of the process can be briefly described as follows. The hyaluronan molecules play a role of the protection and a delivery shell which gets in contact with the right cell and surfactant aggregates – micelles – provides the hydrophobic domain for the solubilization of the drug. Thanks to the negative charge on hyaluronan chain we have suggested to use cationic surfactants because of relatively strong electrostatic interactions between the charged parts of the components. However, interesting results can be obtained also with the negatively charged or amphoteric surfactants. The main task is to prove the existence the hydrophobic domain formed as the result of hyaluronan – surfactant interaction for solubilizing the drug.

The experimental work is based on the previous published experience and knowledge and facilities of the home faculty. The physicochemical methods for the characterization of the described interactions were mainly surface tension (Du Noüy ring and maximum bubble pressure method) and isothermal titration calorimetry with an addition of conductometric and potentiometric titration of hyaluronan and amino acids. The obtained results of the experiments with amino acids serve as a base for the subsequent study of the interactions of amino acid-based surfactants instead of cationic surfactants with hyaluronan. For this purpose, two amino acids were used as model substances because the charged amino groups should play a similar role from the point of view of their interactions with hyaluronan as cationic surfactants do.

2 STATE OF THE ART

2.1 Phase behavior of surfactant–hyaluronan system

Hyaluronan-surfactant interactions were studied in several previous papers. Because of the negatively charged hyaluronan chain most of the authors used cationic surfactants. Yin et al. [1] worked with hyaluronan and both nonionic and anionic surfactants. The association between hyaluronan and surfactants was studied using a pyrene fluorescence method. The effect of saccharide was also investigated. The data indicated that the interactions between hyaluronan and nonionic surfactants are extremely weak.

In the 1990`s Swedish researches studied hyaluronan and its phase behavior with alkyl trimethylammonium bromides (tetradecyl and hexadecyl derivative were the most used types). They used both aqueous and electrolyte solution (NaCl or NaBr).

In the first study [2] of that series directed to the investigation of the interactions between hyaluronan and different classes of amhiphilic substances they studied the described system in aqueous solutions. The series of alkyl trimethylammonium bromides with chain lengths of 8, 9, 10, 12, 14 and 16 carbons in the alkyl group was used. They performed conductivity, solubilization, optical activity and ^{1}H NMR self-diffusion measurements. They observed the phase separation and precipitation (except surfactants with 8 and 9 carbons in chain). The initial precipitates were opalescent or formed a milky opaque dispersion. Toward higher concentration of surfactant the two-phase dispersion again turns more and more transparent until finally a clear one-phase region was reached. They suggest that formation of mixed micelles between the surfactant molecules and hyaluronan is taking place with the carboxylate groups facilitating surfactant association by decreasing the electorstatic repulsion between the cationic head group of the surfactants. In conclusion, for the long chain cationic surfactants, electrostatic interactions have a strong influence on a self-association due to the very low bulk electrolyte concentration.

In the following papers [3]-[7] the authors focus on construction of the phase diagrams of hyaluronan and tetradecyltrimethylammonium bromide (TTAB) in water and sodium bromide solution. The phase diagrams contain a two-phase region, which is surrounded by a continuous isotropic one-phase region. In the absence of added salt separation occurs into one dilute phase and one concentrated in both polyelectrolyte and surfactant. Addition of low concentration of NaBr leads to a reduction of the two-phase region in the phase diagram and at higher salt concentration the phase separation no longer occurs. At high salt concentration, phase separation also occurs but it is of a totally different nature than observed at no or low concentration. It involves separation into surfactant-rich and polymer-rich solutions. In addition, the longer the surfactant alkyl chain the larger the two-phase separation region was observed.

The gel formation in aqueous system of a polyanion and an oppositely charged surfactant is described in ref. [6]. The intention of this work is to study gels and to try to establish their major structural aspects. The main object of the study is the system of hyaluronan and alkyl trimethylammonium bromides (from 10 to 14 carbon atoms in the alkyl chain). The experimental techniques were X-ray diffraction, solubilization, ^{1}H NMR relaxation and self-diffusion. The viscosity of the dilute phase was close to that of pure water, while the concentrated phase was highly viscous. The observed rheology in the gels is largely

dependent on the polyelectrolyte molecular weight. All gels in this paper behaved as isotropic. The authors conclude that cationic surfactants of mentioned types form micelle like clusters adsorbed to the hyaluronan chain in dilute solution and, moreover, this appears probable also in the gels. This idea was supported by the solubilization measurement with the water-insoluble dye Orange OT. The dye does not dissolve in a binary system hyaluronan-water, therefore it is clear that the gels contain the hydrophobic domains.

The only one paper about surface tension of a hyaluronan-surfactant system published by the Swedish group in ref. [8]. Herslöf et al. studied the properties of that system also by viscosity measurement and they monitored the phase equilibrium properties for increasing sodium chloride concentration at neutral pH where all the carboxylic groups on the polymer chain are completely ionized. In the absence of salt, the system hyaluronan-TTAB-water forms a one phase solution only for very low or very high TTAB content and the phase separation occurs in the intermediate range. By addition of suitable electrolyte (NaCl, NaBr) this solubility gap can be suppressed and the phase separation eventually eliminated. The surface tension results showed the decrease of CMC in the presence of NaCl and hyaluronan in the TTAB solution. The viscosity results showed that with increasing salt concentration increases viscosity.

2.2 Surfactant micellization studied by ITC

For purpose of this thesis, ITC was used to determine CMC of pure surfactant solutions and to study the interactions between hyaluronan and two cationic surfactants. The process of surfactants micellization was studied by ITC method in a lot of papers.

The demicellization of some cationic surfactants was studied by Beyer et al. [9]. They determined not only CMC and ΔH_{demic} but also changes of entropy ΔS_{demic} and the Gibbs free energy change ΔG_{demic} for demicellization using the pseudophase-separation model and the mass-action model. The influence of the counterion binding was investigated after the comparison measurement of the demicellization of CTAB in water and in salt. The results were summarized according the alkyl chain length of the surfactants.

The authors in [9] define the CMC as a midpoint of the transition range. To determine the midpoint of the demicellization process, the first derivative of the reaction heat with respect to the total detergent concentration was calculated. The asymmetric shape of the demicellization curve is discussed as a result of the enthalpy of the counterion binding of chloride and bromide to the micelles after reaching the CMC. As expected, the CMC decreases with increasing alkyl chain length. Furthermore, the counterion binding in general increases with increasing alkyl chain length for ionic surfactants.

The lowering of the CMC in the presence of additional salt is well known and discussed in many other papers, e.g.[10],[11],[12],[13]. Increasing the salt concentration reduces the electrostatic repulsion between the charged groups and therefore favors the aggregation process inducing a decrease of the CMC [11]. The increasing ionic strength induces a decrease of the CMC, which implicates a micelle formation at lower total surfactant concentration, when the ionic strength is increased. The reason for this behavior is that the higher ionic strength decreases the surface charge of the micelles by shielding the charges as a result of conterion adsorption, and as a consequence, it becomes energetically more favorable for the monomers of surfactant to self-associate into larger aggregates [9]. The increasing

ionic strength decreases the CMC, which is caused by electrostatic field and, thus, reduced polarity and solubility of the monomers.

Temperature and salt-induced micellization of dodecyltrimethylammonium chloride in water and sodium chloride solution was studied by Šarac et al. [14]. The heat changes at the micellization were measured using a VP-ITC microcalorimeter of MicroCal Inc.

The CMC and enthalpy of micellization strongly depend on the nature of counterion and alkyl chain length. Moreover, the magnitude of the heat capacities of micellization increases with increase in temperature, with longer alkyl chain length and with size of the counterion. In this paper, the CMC was determined according the Philip´s criterion [15]. According the pseudo-phase separation model [16]-[19], the standard Gibbs free energy of micellization in water was calculated from the relation

$$\Delta G_{mic}^{0} = (1+\beta)RT\ln X_{CMC}\,, \tag{4}$$

where β is the degree of micelle ionization and X_{CMC} is the mole fraction of the surfactant at the CMC. The degree of micelle ionization was determined by electrical conductivity measurement and has been published earlier by Perger et al. in [20]. The temperature changes and its influence on ΔH_{mic} are discussed. There is a variation of ΔH_{mic} with temperature (278.15–318.15 K) in all the systems. The formation of micelles is an endothermic process at low temperatures and exothermic at higher temperatures. There is a balance between the enthalpy and entropy in the micellization process: large changes in entropy and enthalpy with increasing temperature result in moderate decrease in the Gibbs energy. It can be explained in terms of the hydrophobic effect. Hydrophobic hydration of this type of surfactant monomers is characterized by the presence of ordered clathrate-like water structures surrounding the non-polar alkyl chains. Upon micelle formation these structures are destroyed since the alkyl chains are removed from water and structured hydration water is released in the bulk. The process is endothermic and is associated with positive entropy change. The hydration of head groups is also readjusted according to the surface charge density as a consequence of monomer association and counterions condensation. The negative contribution to the ΔH_{mic} is identified with the transfer of the hydrocarbon chains into the micelles. It restores the hydrogen bonding structure of water around micelles.

Mosquera et al. [21] worked with the self-association of n-hexyltrimethylammonium bromide in aqueous solution as a function of temperature and electrolyte concentration. They combined conductivity, microcalorimetry and ultrasound velocity measurement to obtain the CMC in water and at different electrolyte concentration. The influence of temperature on the CMC was investigated using conductometric methods. The thermodynamic parameters were calculated using basic relations. The authors showed the small aggregates of the surfactant (3–4 monomers form micelle). The additional measurements proposed a highly organized micellar structure with a large exposure of alkyl chains to the solvent.

Moulik et al. [22] studied the difference between the van`t Hoff and calorimetric enthalpies of micellization for the series of surfactants. The determination using the van`t Hoff equation is based on the quantitative evaluation of the equilibrium constant at different temperatures. The comparison on these two methods is required for the complete energetic information about the studied system. Nonionic amphiphiles have evidenced close agreement between the two procedures because of their weak nearly similar and neutral head group. Ionic amphiphiles produced greater enthalpy differences. For both types of amphiphiles, micellar aggregation

related free energy and hence enthalpy contributions were much smaller. For ionic surfactants, the counterion binding process shares the overall thermodynamics of the self-association process. The van`t Hoff method produces the heat associated with the micellization process whereas calorimetry uses the total heat of all the processes occurring in the system, micellization and others. Thus, results from these two procedures differ.

Two papers from the 1980`s by Woolley et al. [23] and Dearden et al. [24] are based on the studies of alkyltrimethylammonium bromides of different alkyl chain length at four different temperatures in water and sodium bromide solution. In [22] they measured the heat of surfactant dilution and real data were joined by a fit curve according mathematical relations. The small or bigger differences between obtained data and the fit are well discussed. The densities and heat capacities of the same surfactant are determined in [24]. These data were used to calculate apparent molar heat capacities.

Several methods, e.g. ITC, surface tension, conductance or fluorescence, were used by Moulik et al. [25] to study the miceller properties of cationic surfactants (alkyltrimethylammonium bromides) in pure and mixed states. The amount of adsorbed surfactants on the surface at various concentrations was calculated using the Gibbs adsorption equation and than, for the surfactant mixtures in water, the surface excess and surface pressure were determined. Their conclusion from the determination of standard free energy of adsorption is that the adsorption of mixed amphiphiles is more favorable that the pure amphiphiles.

Stodghill et al. [26] compare two surface-aggregate theories published earlier in [27] and [28]. There is a different view on the formation of micelles at increasing concentration of the surfactant. The paper focuses on the micellization properites of DTAB, TTAB and CTAB in water. It was proved that the CMC increases within a series of surfactants as the tail length decreases. It is also noted that with increasing chain length ΔG_{mic} becomes more negative. The ITC experiments with CTAB and TTAB are similarly discussed by Bach et al. [28].

Different types of enthalpograms are described by Bijma et al. [29]. They present the general characteristics for plots of measured heat by ITC against injection number for a range of the ionic surfactants. The enthalpogram of type A is formed by two straight lines of points which intersect at the CMC. Type B shows a slightly steeper rise of the first line, it means at low injection numbers. Type C is more complicated, there is an increasing trend at the beginning with a sharp peak and followed by a steep decrease at higher injection numbers. It is caused by the apparent molar enthaplies of both monomers and micelles on the composition of the solution.

2.3 Surfactant–polymer system studied by ITC

In the last decades, interactions between polyelectrolytes (especially biomacromolecules such as polysaccharides) and oppositely charged surfactants have attracted a great deal of interest due to their applications in the biological industry [30]. Bao et al. studied interactions between ionic surfactants and polysaccharides in aqueous solutions. They used six different combinations of neutral, positively and negatively charged polysaccharides (methylcellulose, chitosan and κ-carrageenan, respectively) with anionic and cationic surfactants (SDS and CTAB, respectively). The most interesting part from the view of the suitability for the thesis

is the interaction between negatively charged κ-carrageenan and CTAB. This kind of a mixture exhibits a strong association dominated by electrostatic attraction between the ionized groups on the polyelectrolyte and the charged headgroups of the surfactant. The data showed a titration curve with two plateaus. The lower plateau represents the highly endothermic demicellization process of CTAB and the Columbic interaction which is highly exothermic. The endothermic interactions means that the electrostatic interaction between κ-carrageenan and CTAB could not overcome the enthalpy changes of demicellization because it is quite large. The lower plateau is followed by a steep increase. After a sharp peak, the endothermic plateau occurs. It is attributed to the hydrophobic binding of CTAB to the polymer. The dissociation of CTAB could take place to produce CTAB monomers that bind hydrophobically to the polymer. When the polymer chain is saturated with the surfactant monomers, the enthalpy curve decreases dramatically and merges with the dilution curve at high concentration of CTAB. To sum it up, CTAB shows strong interaction with oppositely charged polymer due to the electrostatic interactions. In addition, κ-carrageenan induced the large reduction of the high endothermic enthalpy changes caused by the demicellization of CTAB.

A calorimetric study of interactions between hyaluronan and low molecular weight surfactants dodecyltrimethylammonium bromide (DTAB) and SDS was published by Chytil et al. in [31]. In the case of SDS, the only significant impact of added hyaluronan is the lowering of CMC in water. As expected, in the system of DTAB and hyaluronan the strong binding interactions were observed. The binding process did not occur immediately but after the optimal distance and steric conditions achieved between the species. Their results suggest that the hyaluronan-DTAB binding is carried out via the surfactant micelles which form the aggregates with the hyaluronan chain. The results showed the shift of the micellization process of DTAB in the presence of hyaluronan to the lower surfactant concentration and a large endothermic peak was observed. The experiments were additionally performed in NaCl solution and in the buffer of the pH 5. Both salt and buffer environment induced the decrease of the endothermic peak and the shift to the higher surfactant concentration.

The interactions between dodecyltrimethylammonium bromide and cationic polymers – neutralized poly(acrylic acid) and methacrylic acid/ethyl acrylate copolymers were investigated using ITC by Wang et al. [32]. In the initial stage of the titration the cationic headgroups of the surfactant individually bind to the anionic carboxylate groups on the polymer chains due to electrostatic attraction. The thermodynamic parameters derived from ITC measurements suggest that the electrostatic binding is an endothermic process driven by entropy. The addition of salt shows the electrostatic repulsion between surfactant headgroups and attraction between oppositely charged polymer chains and surfactant molecules, which favors the formation of free micelles, and weakens the binding of surfactant onto the polymers.

Bai et al. [33] studied the interaction behavior of a hydrophobically modified polyelectrolyte and oppositely charged surfactants in aqueous solution. They used a synthesized polymer based on dextran and anionic sodium alkyl sulfates and cationic alkyltrimethylammonium bromide/chloride. When the alkyl chain length of the surfactant changes, the behavior of the polymer-surfactant solution presents a great variation. The critical aggregation concentration (CAC) at which the surfactant begins to bind to the polymer chain is much lower than the CMC and increases with increasing polyelectrolyte concentration. This is in contrast to the

behavior for uncharged polymer/surfactant system, where the CAC decreases only slightly and is only weakly dependent on the polymer concentration. After reaching the CAC the system becomes a little turbid but without any precipitation. Later, a clear phase separation occurs and a new phase is a dispersion of a viscous fluid. After several days at rest, the samples formed a gel-like phase on the bottom. The authors also made a phase diagram of that mixture with a solution region, cross-linking mixed micelle solution, a region with precipitation and solution, gel phase and a region with surfactant-rich mixed micelle and/or free surfactant micelle. That 3-D diagram shows the phase boundaries and the effect of the surfactant alkyl chain length on the interaction. Consequently, the mechanism of interaction between the surfactant and polymer is described in details (self-assembly process, cross-linking, phase separation, redissolution, formation of micelles).

New challenges for pharmaceutical formulation and drug delivery systems characterization using ITC are summarized in [34]. Bouchemal describes here ITC as a technique applicable to determine the stability constants, stoichiometry, interaction enthalpies, entropies, Gibbs free energies and heat capacity changes. The author describes more possible interactions which can be studied by ITC (drug-surfactant, protein-surfactant, polyelectrolyte complexes, polyeletrolyte-protein complexes, etc.)

The calorimetric studies of synthesized hydrophobically modified cationic polysaccharides based on dextran mixed with CTAB or CTAC are published by Bai et al. [35]. The titration curves show rapid decrease in CAC of a mixture in contrary to the CMC of pure surfactants CTAB or CTAC. They take the CAC and the CMC as the break point on the titration curve, it means at the end of the first straight line. The hydrophobically modified cationic polyelectrolytes proved to be useful in the studies of the hydrobhobic effect on polymer/surfactant association. The authors evaluated the data according the standard procedure after obtaining the plot ΔH vs. surfactant concentration. The CMC and CAC, respectively, are determined as the breakpoint of the linear part of the upper plateau and the linear fit of the decreasing part of the plot. Enthalpy of micelle formation is set as the distance between the linear fits of the upper and the lower plateau in the plot. For better illustration of the evaluation see Fig.8 bellow. In addition, the shapes of enthalpograms are in agreement with the theory in [29].

3 AIM OF THE WORK

The thesis is focused on the interactions between hyaluronan and surfactants of different charge and nature with the main emphasis focused on cationic surfactants. One of the main aims is to determine the critical concentrations (micelle or aggregation) of the surfactants in the absence and presence of hyaluronan of different molecular weight. The influence of added hyaluronan on the surfactant aggregation properties is studied, particularly in relation to the surface activity, in other words, differentiating interactions of surfactant molecules present in the surface layer and in the solution bulk. Knowledge of behavior of the hyaluronan-surfactant system is crucial for the possible application of such a complex in the targeted drug delivery.

The thesis should bring a contribution to answering the questions about applications of surfactants in combination with hyaluronan for using in drug delivery. The results are discussed with respect to the molecular weight of hyaluronan and its concentration, ionic strength of the environment, surfactant alkyl chain length and finally, with respect to different measurement method.

4 EXPERIMENTAL PART

The main document of the thesis is divided into three main parts and two of them are summarized in the following text.

4.1 Hyaluronan and surfactants

Hyaluronan of different molecular weights was purchased from Contipro Biotech Ltd., Czech Republic. Surfactants CTAB and TTAB were purchased from Sigma Aldrich, Germany. The experiments were performed in water.

4.2 Surface tension

Surface tension of concentration series of surfactants were measured with a platinum/iridium Du Noüy ring (diameter 9.545 mm) on tensiometer KSV Sigma 701 at room temperature. The duration of each experiment was set to 10 minutes, the surface tension value for every sample was obtained as an average value from that time.

Surface tension was further determined by maximum bubble pressure method using tensiometer BPA-800P. The instrument was set to the standard measuring method, i.e. with the increasing gas-flow velocity from 10 ms up to 10 s.

Stock solutions of hyaluronan were prepared at concentration 1.5 g/l and 22.5 mg/l by dissolving solid hyaluronan in water under slow stirring for 24 hours at room temperature in closed vessel to ensure the complete dissolution. These stock solutions of hyaluronan were used to prepare samples for surface tension measurement with constant hyaluronan concentration 15 mg/l and 1000 mg/l. Stock solutions of hyaluronan were stabilized by NaN_3 (0.05% w/v). Stock solutions of surfactants were prepared by dissolving solid surfactant in water at concentration 20 mM.

Samples with varying surfactant concentration and constant hyaluronan concentration were prepared by mixing hyaluronan stock solution (10 ml) and surfactant stock solution and subsequently diluting with water to the final volume 15 ml. Samples with hyaluronan were stirred for 24 hours before measurement, samples with pure surfactant were stirred for 4 hours.

4.3 Microcalorimetry

The first part of the ITC experiments was measured at Faculty of Chemistry and Chemical Technology, University of Ljubljana in Slovenia, using a microcalorimeter TAM 2277 (Thermometric, Sweden). The heat of micelle formation of both surfactants CTAB and TTAB and heat of aggregation with hyaluronan was measured at 25°C. The measuring cell was filled with 2 ml of water in case of CMC determination and with 2 ml of hyaluronan (concentration 0.1% w/v) in case of CAC determination. The titration experiments consist of 20 injections of 10 μl aliquots of the surfactant solution into water. The reference cell was filled with water. Dilution of hyaluronan during the titration was neglected.

The second part of the experiments was performed using a modular microcalorimeter TAM III (TA Instruments, USA) at the home faculty. The titration cell has volume of 1 ml, it was filled with 800 μl of water or hyaluronan (concentration 0.1% w/v) solution and during the titration experiment 40 injections of 5 μl aliquots of surfactant stock solution was added. For this purpose, both surfactants CTAB and TTAB were used to study their micellization properties and the interaction with hyaluronan of different molecular weight (70, 110 and 1700 kDa) and its two concentrations 15 and 1000 mg/l. All the experiments were performed at 25°C in aqueous solution.

5 OVERVIEW OF MAIN RESULTS

5.1 Surface tension

As expected from the previous published experience and knowledge, there exist strong electrostatic interactions between negatively charged hyaluronan carboxyl group and positively charged surfactant head group. The existence of these interactions is clearly evident observing turbidity, opacity and further, at higher surfactant concentration, precipitation of hyaluronan-surfactant system up to the formation of hyaluronan gel.

5.1.1 Interactions between hyaluronan and CTAB

Concentrations of CTAB in the samples for surface tension measurement with and without addition of hyaluronan are summarized in Tab. 1. Large number of samples was prepared in the case of the mixture hyaluronan-surfactant for the better observation of the phase separation which occurred in the premicellar region.

Tab. 1 Concentrations of CTAB for surface tension measurement.

no HyA

sample number	c (mM)
1	0.001
2	0.01
3	0.05
4	0.075
5	0.1
6	0.5
7	1
8	2.5
9	5
10	10

with presence of HyA (1000 mg/l)

sample number	c (mM)
1	0.001
2	0.005
3	0.01
4	0.02
5	0.04
6	0.08
7	0.1
8	0.15
9	0.2
10	0.25
11	0.3
12	0.35
13	0.4
14	0.8
15	1
16	2
17	5

Surface tension was measured at room temperature, every sample was measured three times and an average value with a standard deviation was calculated.

The study of surface activity of pure hyaluronan was the object of my diploma thesis [36]. The results showed that hyaluronan itself does not decrease surface tension and in the mixture with cationic surfactant the decreasing is very slow when compared to the behavior of pure surfactant. Moreover, the surface tension values corresponding to the samples of CTAB with added hyaluronan are considerably lower (Fig.1) than those values for pure CTAB.

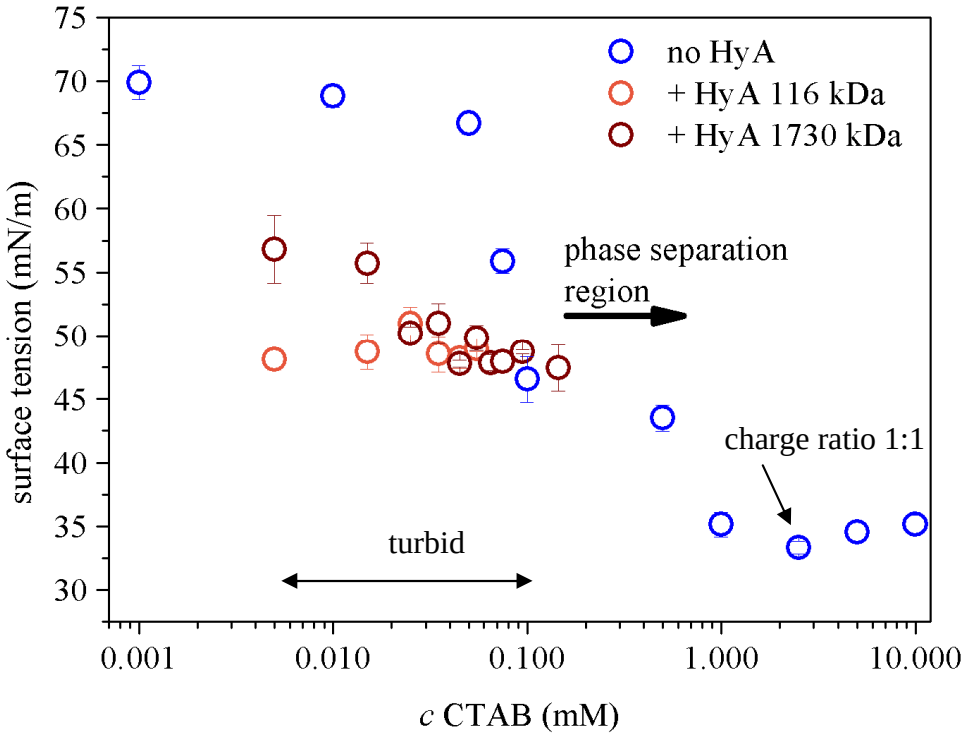

Fig.1 *Surface tension of CTAB in water and with addition of hyaluronan determined by the ring method (hyaluronan concentration 1000 mg/l) at 25°C.*

Fig.1 shows the detailed view on the interactions of hyaluronan of two molecular weights with CTAB at low surfactant concentration. When compared with results of pure CTAB in water, surface tension of the samples with the presence of hyaluronan is lower in general, which could be explained as the result of the expected interactions between hyaluronan and oppositely charged surfactant molecules in the surface layer. But the lowering of surface tension also proved the existence of remaining free surfactant molecules in the layer which are responsible for the decreasing trend.

As mentioned above in section 4.2, surface tension was simultaneously determined using two methods, briefly ring and bubble method so the results and the differences between them are discussed also from the point of view of the measurement method.

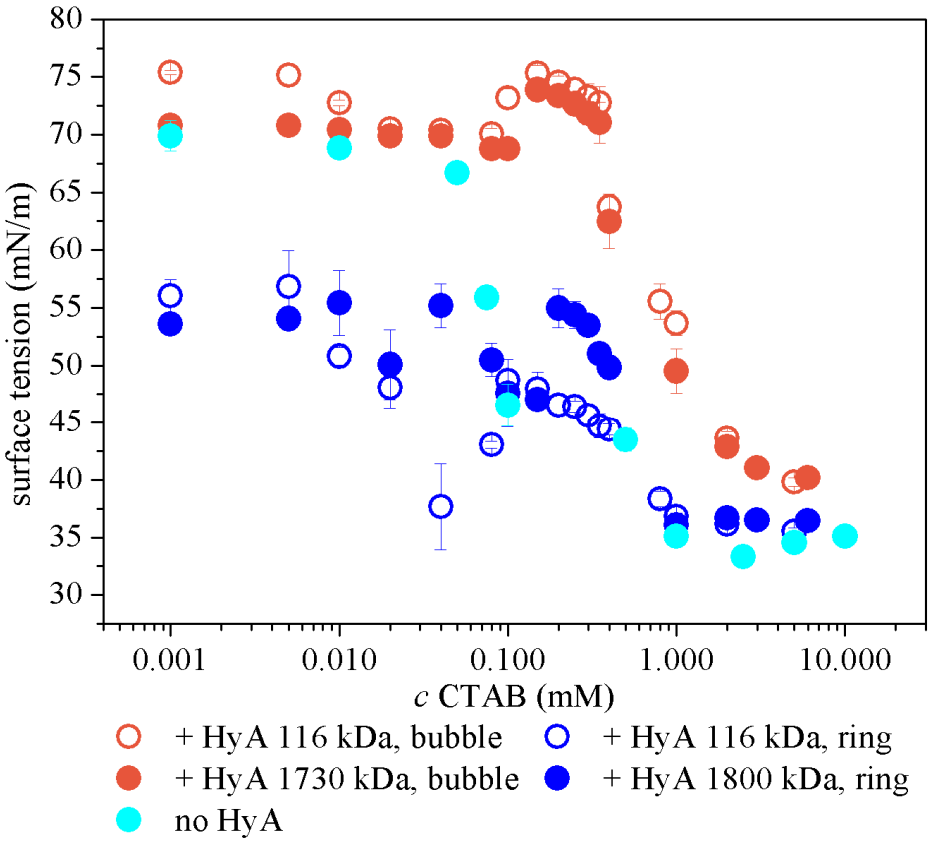

Fig.2 *Comparison of surface tension values obtained by the ring and maximum bubble pressure method (t_{life} = 0.3 s, CTAB with hyaluronan 116 and 1730 kDa, concentration 15 mg/l) at 25°C.*

When the curves in Fig.2 are compared from the point of measuring method, the maximum bubble pressure method determined the values at about 10 mN/m higher than the ring method. The reason is in the different physical principle of these methods. The ring method is based on the measurement on the surface of the sample at therefore this method can detect the free surfactant molecules which are not covered by the hyaluronan shell in the bulk.

So there is still some evidence of interaction between hyaluronan and CTAB proved by the ring method at low hyaluronan concentration. At low CTAB concentration the difference between pure surfactant and the mixture with hyaluronan is observed and than the curves, for both low and high hyaluronan molecular weight, comes together at the region of the CMC of pure CTAB. The theoretic shape of the surface tension curve is more or less maintained for all the data in Fig.2.

On the other hand, hyaluronan of a very low concentration 15 mg/l has an insignificant influence on the surface tension. The inspiration in choosing exactly this low hyaluronan concentration is described in ref. [37]. The author of this thesis studied the interaction of low concentrated hyaluronan (molecular weight 650 kDa) and CTAB by fluorescence probe technique with pyrene as a probe. The data from fluorescence and surface tension are compared in Fig.3.

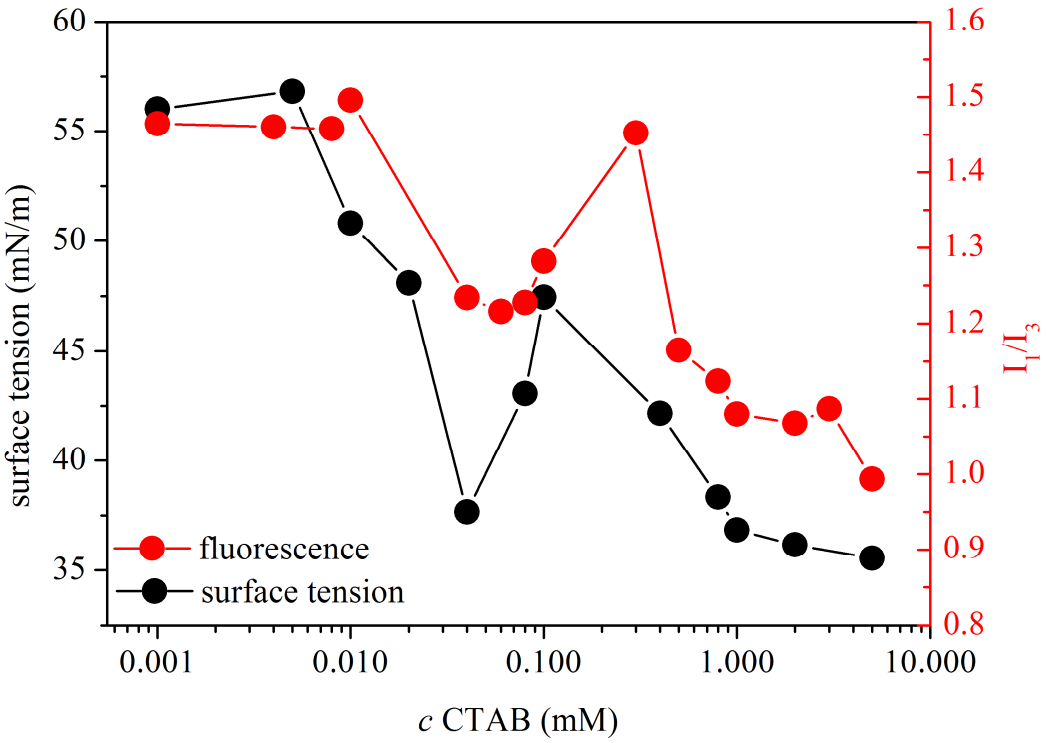

Fig.3 *Surface tension (ring method, black) and fluorescence (red) data of hyaluronan-surfactant system at 25°C. Hyaluronan 650 kDa, concentration 15 mg/l, I_1/I_3 is the polarity index.*

If the area between 0.1 and 0.4 mM is neglected, there is a typical sigmoidal curve with two break points corresponding to CAC and CMC, respectively. But there is an unexpected behavior observed in the surfactant concentration range 0.1–0.4 mM. The value of the polarity index I_1/I_3 increased up to the initial value close to 1.5. This behavior is caused by fluorescence of pyren in water. In that case the sample can not contain any hydrophobic domains suitable for pyrene solubilization, in other words, there are no aggregates or free micelles in the system. The author in ref. [37] discuss the situation using other parameters, one of them is intensity of fluorescence of the third vibrational band I_3 of pyrene. The intensity of fluorescence of samples above surfactant concentration 0.06 mM was at the same value as fluorescence corresponding to water. It confirmed once more the assumption that the sample contains pyrene at very low concentration or is completely without presence of pyrene.

The occurrence of the interesting points in the plot in Fig.3 is explained as follows. When the sample was filled in to the measuring cuvette from the vial, the walls of the vials were covered by a gel layer which coated the pyrene molecules and consequently, pyrene was not placed in to the cuvette together with the liquid sample. The fluorescence results can be explained in a similar way as the results obtained by surface tension measurement. Surface tension temporarily increased because surfactant molecules are incorporated in the gel phase and thus, its surface activity is weaker. Further, with increasing surfactant concentration, surface tension decreases with increasing surface activity of free surfactant molecules.

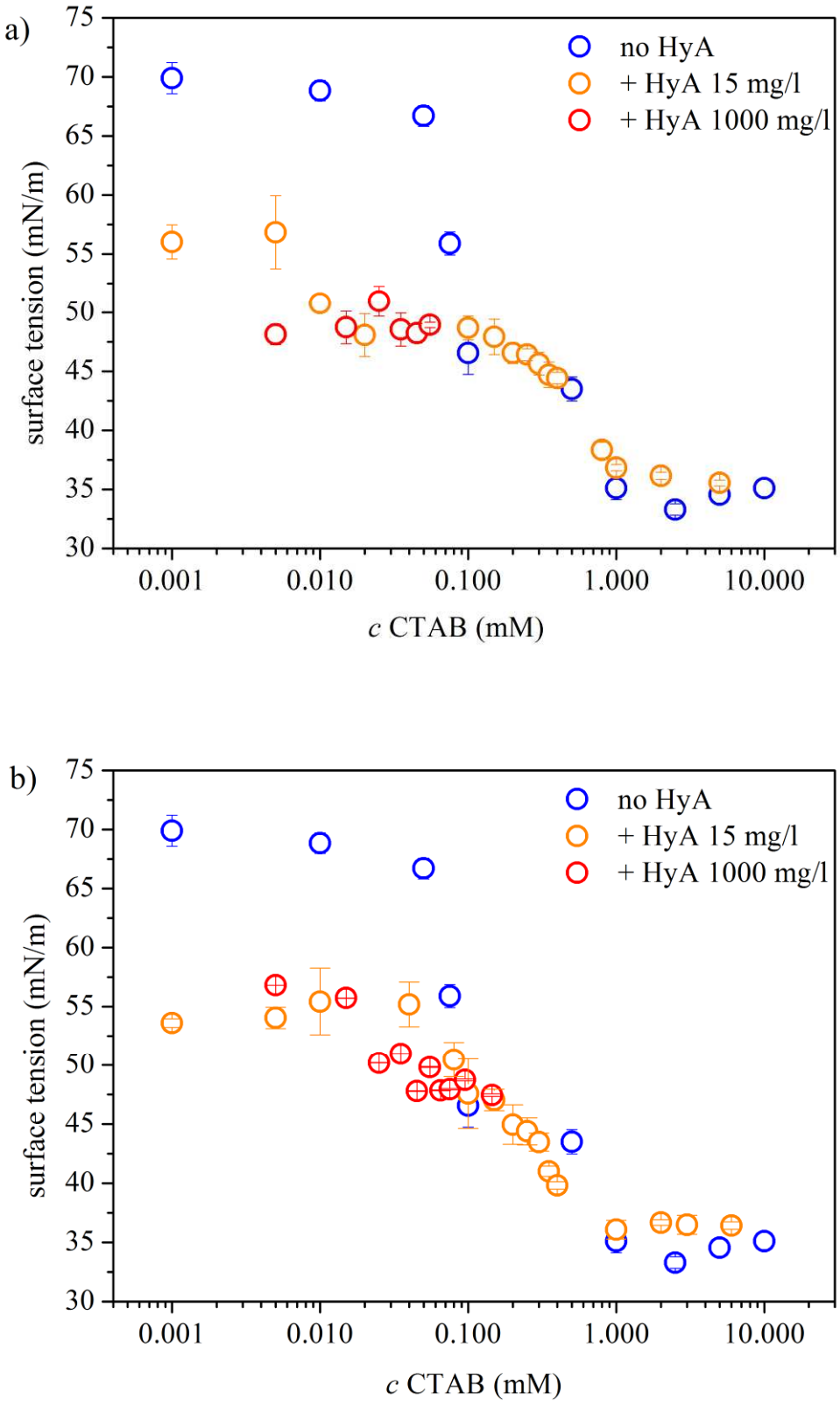

Fig.4 *Surface tension of CTAB and hyaluronan a) 116 kDa and b) 1700 kDa of both hyaluronan concentrations 15 and 1000 mg/l determined by the ring method at 25°C.*

The first conclusion of the results shown in Fig.4 is that there was no difference from the point of view of the molecular weight of hyaluronan observed. In both cases the present hyaluronan decreased the surface tension values at low surfactant concentration and further, the values corresponding to pure CTAB and diluted hyaluronan (15 mg/l) are practically the same. Moreover, there is no shift in the aggregation region of hyaluronan-surfactant system observed. In other words, the critical aggregation concentration of CTAB in the presence of diluted hyaluronan corresponds to the CMC of CTAB in aqueous solution.

5.1.2 Interactions between hyaluronan and TTAB

Hyaluronan-TTAB mixture showed similar behavior as was observed in the samples with CTAB. Low concentrated samples were clear and further, with increasing surfactant concentration, the precipitation was more and more considerable.

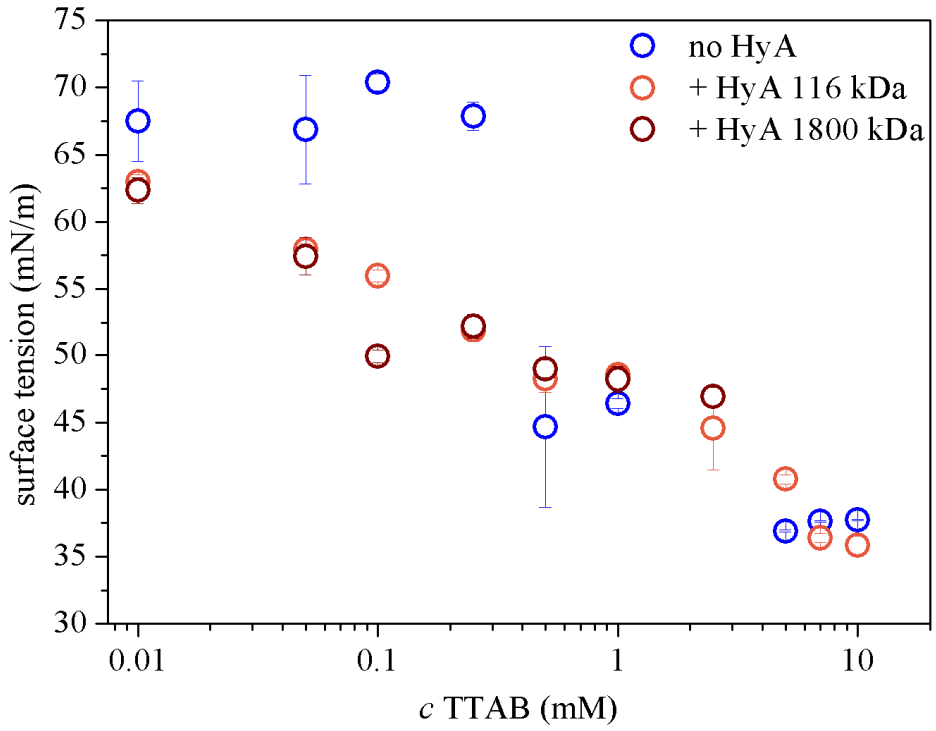

Fig.5 *Surface tension of TTAB and hyaluronan (116 and 1800 kDa, 1000 mg/l) determined by the ring method at 25°C.*

The results from surface tension shown in Fig.5 and Fig.6 describe the influence of hyaluronan on the aggregation properties of TTAB studied by both tensiometric methods. The addition of both hyaluronans into TTAB caused the lowering of the surface tension values at low surfactant concentration, there might be a linear decreasing in the case of hyaluronan 116 kDa.

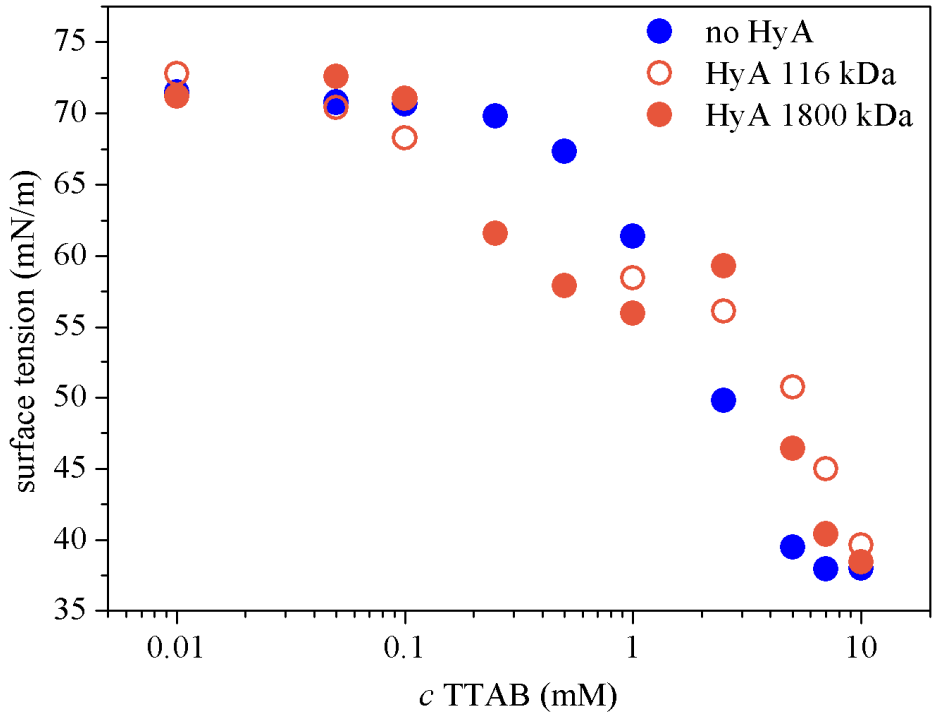

Fig.6 *Surface tension of TTAB and hyaluronan (116 and 1800 kDa, 1000 mg/l) determined by the maximum bubble pressure method at 25°C, t_{life} = 0.5 s (the error bars are smaller than the symbol signs).*

The lowering of surface tension determined with the maximum bubble pressure method was observed in the whole concentration range as shown in Fig.6. The interesting region is between 0.2 and 1 mM where samples with addition of hyaluronan show lower surface tension values than the samples of pure TTAB. On the other hand, the expected higher values in the phase separation region and after that (from concentration 2 mM) confirmed the interactions between TTAB and the polysaccharide chain. In other words, the surfactant monomers bound to hyaluronan are not able to adsorb on the forming bubble surface.

5.2 Microcalorimetry

An example of the raw data (endothermic character) is shown in Fig.7. The stock solutions of surfactant were prepared at concentration at least ten times higher than their CMC so initially, the surfactant undergoes demicellization followed by monomer dilution. When the surfactant concentration becomes close to the CMC and above, the solution undergoes micellar dilution with no further demicellization. This region is followed by the constant heat flow even though the concentration of the surfactant is still increasing.

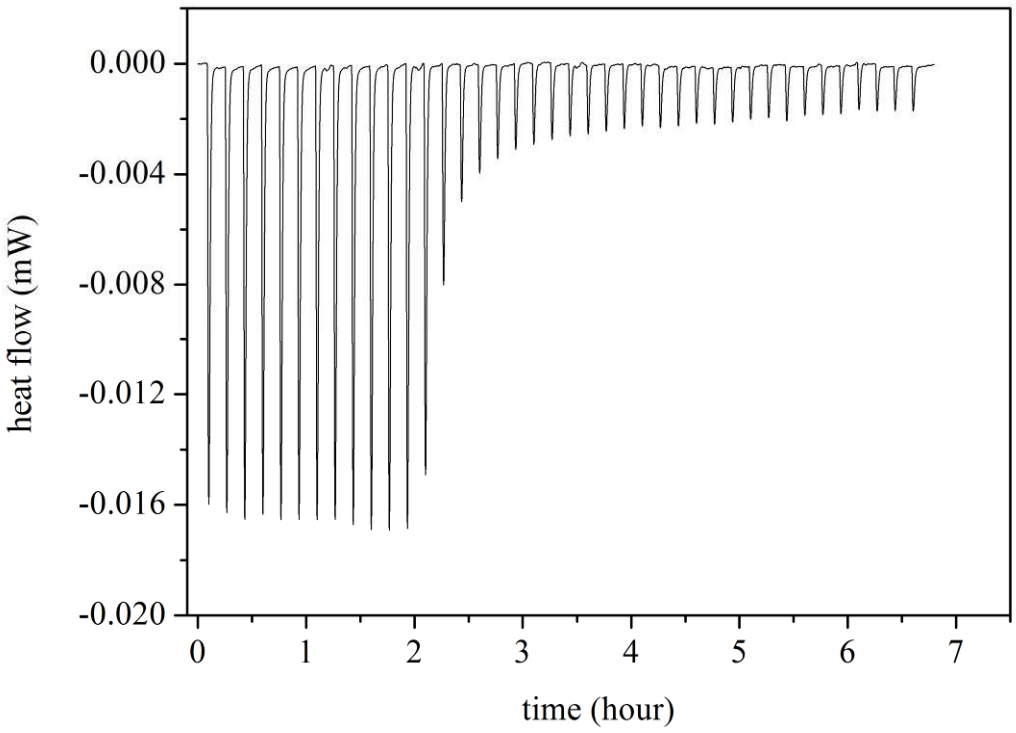

Fig.7 *Typical titration record from the microcalorimeter TAM III (titration of TTAB 50 mM into water at 25°C).*

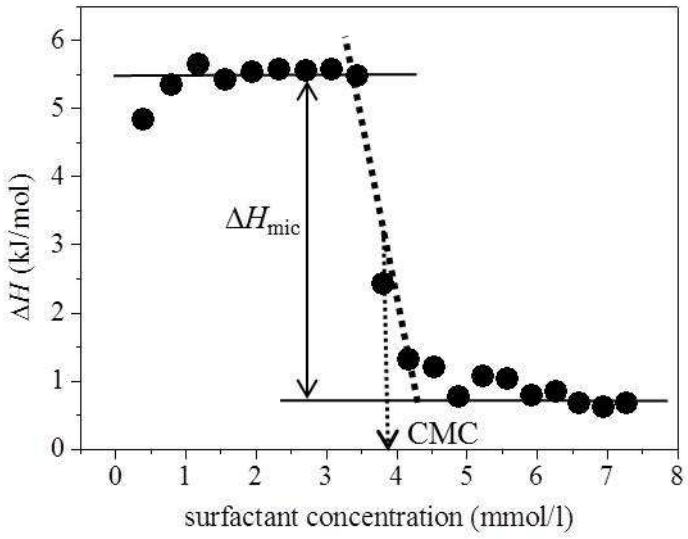

Fig.8 *Scheme of the evaluation of CMC and* ΔH_{mic} *from the enthalpogram.*

The peak areas from Fig.7 are integrated and the obtained heat of the reaction in units kJ/mol was used to make an enthalpogram (change of heat vs. concentration plot). Fig.8 shows the evaluation of the CMC and the heat of micelle formation ΔH_{mic} from that type of plot.The distance between the lower and upper plateau is considered as ΔH_{mic}. The character of all studied interactions was endothermic in general. The critical concentration CMC and CAC are determined as the crossing point of the upper plateau and the linear fit of the middle part of the enthalpogram (dotted line in Fig.8).

5.2.1 Interactions of hyaluronan and CTAB

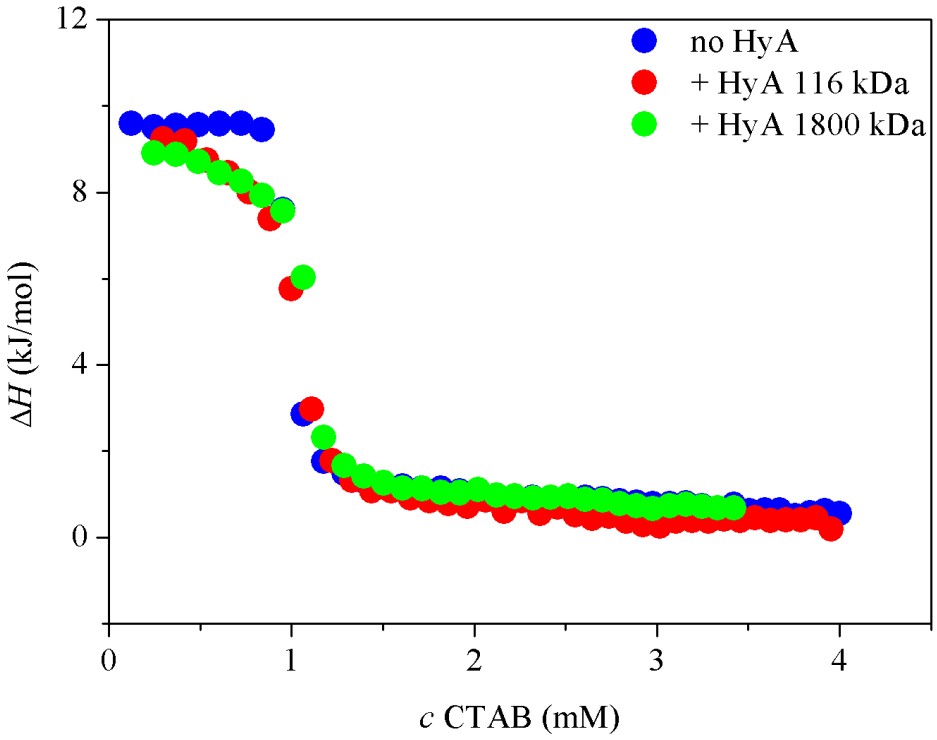

Fig.9 *Interaction of CTAB with hyaluronan (conc. 15 mg/l) of different molecular weight in water at 25°C.*

As expected, the low concentration of hyaluronan did not have any significant influence on the shape of the titration curve (Fig.9). Furthermore, the micellization and aggregation with both kinds of hyaluronan occur at the same surfactant concentration. The only difference is that the first part of the S-curve corresponding to the mixtures with hyaluronan has more intensive decreasing trend than the curve for pure surfactant which is almost constant. No precipitation during the titration was observed and it is also possible to speak about the agreement between the CMC and the CAC of CTAB. The value of the CMC is in a good agreement with the literature value which is 1 mM in water [38].

In contrary, very interesting interaction behavior of this system was observed by fluorescence measurement [37]. With respect to the smooth shape of the resulting curves in Fig.9 this behavior should be coupled with a very slight heat effect which was not detected using the ITC technique.

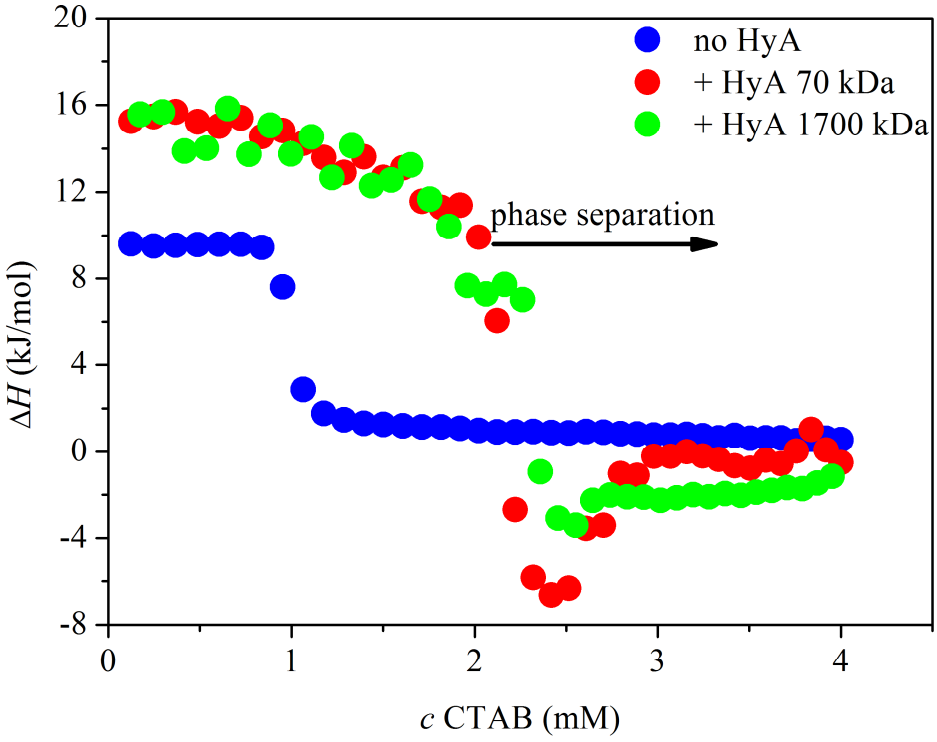

Fig.10 *Interaction of CTAB with hyaluronan (conc. 1000 mg/l) of different molecular weight in water at 25°C.*

The effect of concentrated hyaluronan on the aggregation with CTAB is shown in Fig.10. There are three main differences when results in Fig.10 and Fig.10 are compared. The upper plateau in Fig.10 begins at 16 kJ/mol, but the values of pure surfactant are approximately at 10 kJ/mol. The second point is the shape of the curves. The addition of hyaluronans of both molecular weights (at 1000 mg/l) decreased the ΔH values almost over the whole concentration range till 2 mM and then the region of precipitation follows. The curve changes from endothermic to exothermic character between 2 and 3 mM. In addition, the interaction between low molecular weight hyaluronan seems to be more intensive. Than, a region of dilution follows and no unexpected behavior is observed. But the gel which is formed in the titration cell is not diluted at all, the rest of the gel is still present after the end of the experiment.

From the point of view of visual observation it was determined that the samples become cloudy and opaque from CTAB concentration 0.1 mM but this change was not detected by ITC. It may be concluded that the formation of precipitates is not coupled with measurable heat effect or the effect is compensated by the heat of dilution. Therefore the arrow in Fig.10 is not put to the same concentration as previously in Fig.1

The last difference is the shift of the inflection point on the curves with addition of hyaluronan. In other words, the heat change observed as the inflection point of the curve is a result of the precipitation and not the micellization process in the titration cell. Moreover, the curves corresponding to both hyaluronan-surfactant systems overlap in their first parts. There

is also an evidence of the complicated thermodynamics of the interactions between the polyelectrolyte and surfactant resulting in the worse linearity of first plateau. The results from this chapter are listed in Tab. 2. Additionally, it is must be highlighted that the ITC technique is sensitive to detect the phase separation of our system and not the beginning of the interaction between the substances.

Tab. 2 Enthalpy of micellization and critical aggregation concentration of CTAB obtained by the microcalorimetric method.

	ΔH_{mic}* (kJ/mol)	CMC, CAC (mM)
no Hya	-8	0.95 ± 0.03
HyA 15 mg/l		
HyA 116 kDa	-6	0.84 ± 0.02
HyA 1800 kDa	-6	0.94 ± 0.02
HyA 1000 mg/l		
HyA 87 kDa	-12	2.02 ± 0.05
HyA 1800 kDa	-14	2.32 ± 0.06

* the errors are smaller than 5%

5.2.2 Interactions of hyaluronan and TTAB

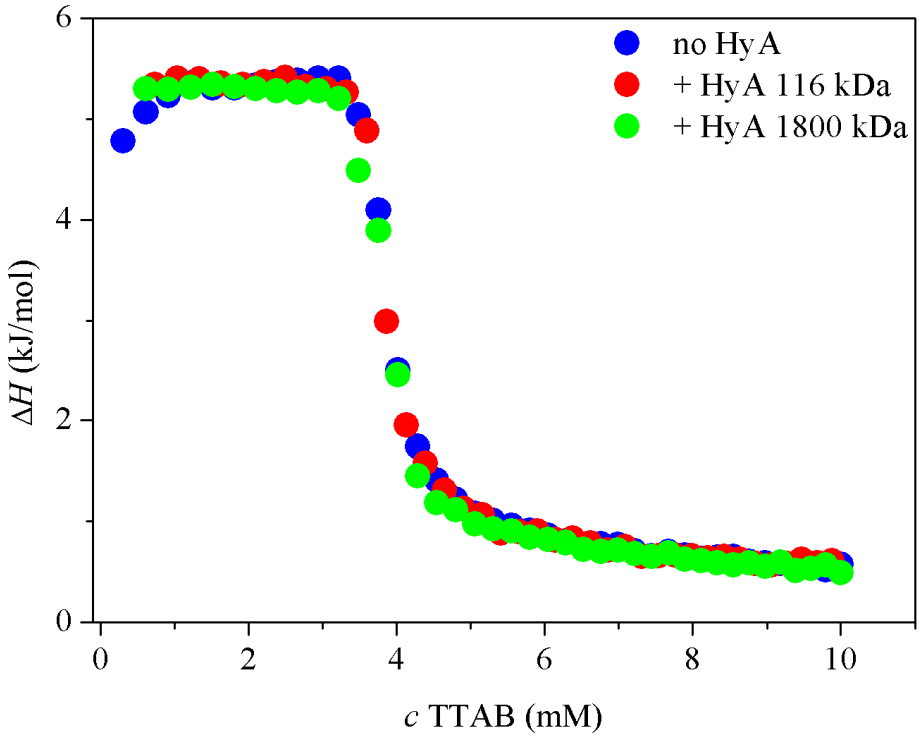

Fig.11 *Interaction of TTAB with hyaluronan (conc. 15 mg/l) in water at 25°C.*

The plot in Fig.11 summarizes the results from the titration of TTAB into water and hyaluronan of different molecular weights at its concentration 15 mg/l. The data corresponding to the titration of TTAB into hyaluronan overlap the data of the pure surfactant in the whole concentration range. It is evident, that the present hyaluronan at such a low concentration did not have any influence on the aggregation properties of TTAB (as described above in the case of CTAB, Fig.9). The value of the CMC is in a good agreement with the literature value which is 4–5 mM in water [38]. In addition, there was no phase separation of hyaluronan observed in the titration cell after the experiment. Therefore, the inflection point on the titration curve corresponds just to the micellization of the surfactant.

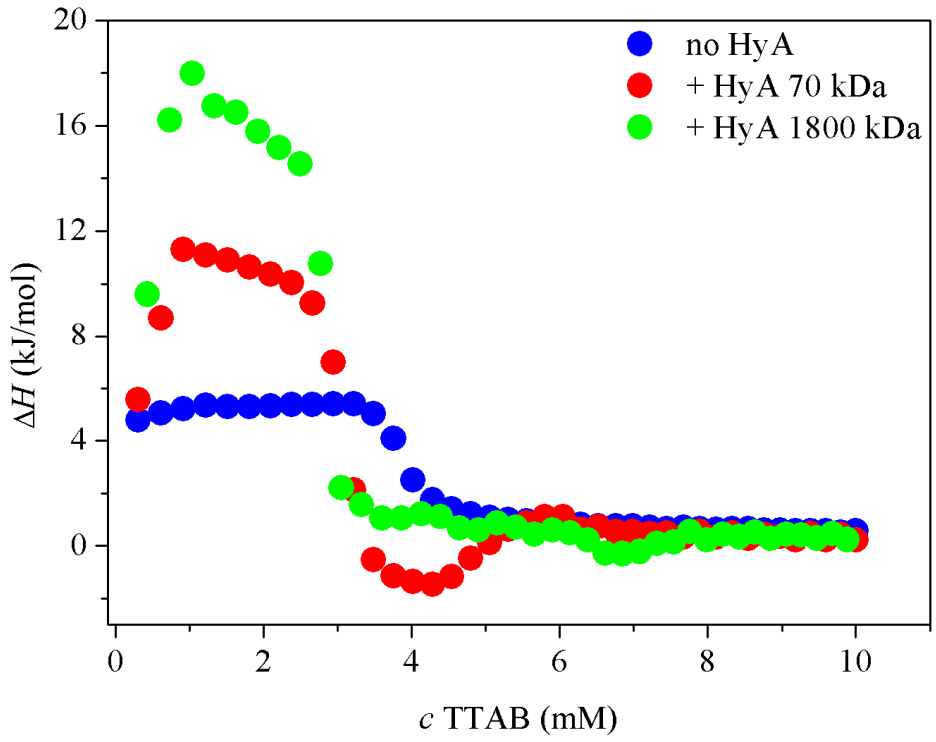

Fig.12 *Interaction of TTAB with hyaluronan (conc. 1000 mg/l) of different molecular weight in water at 25°C.*

The situation with high hyaluronan concentration changes dramatically. When the shapes of the titration curves of the system hyaluronan-surfactant in Fig.11 and Fig.12 are compared, it can be concluded that there is a significant effect of concentrated hyaluronan on the aggregation properties of TTAB. The main differences between the results shown in Fig.11 and Fig.12 are discussed below.

At first, there is a sharp increase in ΔH in Fig.12 observed for both molecular weights of hyaluronan and more intensive for 1700 kDa. After reaching a maximum at 1 mM, both plateaus decreased with the same slope to the same TTAB concentration which is approximately 3 mM. The low molecular weight hyaluronan made a local minimum at concentration 4 mM and further, another local maximum is observed at concentration 6 mM.

On the other hand, hyaluronan of higher molecular weight made a constant lower plateau up to 7 mM and then a local minimum is observed. This behavior should be again explained as the subsequent dilution of the existing precipitation.

The shift of the inflection point of both hyaluronan-surfactant curves is also evident, but the aggregation concentration is observed at lower surfactant concentration than the micellization of TTAB in water, in contrast to experiments with CTAB.

Tab. 3 Enthalpy of micellization and critical aggregation concentration of TTAB obtained by the microcalorimetric method at 25°C.

	ΔH_{mic}* (kJ/mol)	CMC, CAC (mM)
no Hya	-4	3.86 ± 0.02
HyA 15 mg/l		
HyA 116 kDa	-4	3.80 ± 0.03
HyA 1800 kDa	-4	3.91 ± 0.03
HyA 1000 mg/l		
HyA 70 kDa	-9	3.09 ± 0.04
HyA 1800 kDa	-14	2.85 ± 0.05

* the errors are smaller than 5%

To sum it up, the influence of the surfactant alkyl chain length is expressed in the shift of the aggregation region with simultaneous phase separation of both systems – hyaluronan of concentration 1000 mg/l with CTAB and TTAB. As mentioned previously, it is caused by the excluded volume and hydrophobic effects in the solutions. The lowering of the CAC in the case of TTAB is in a good agreement with the published theory in ref. [34] where the authors discuss the influence of the alkyl chain length on the shift of the CAC. On the other hand, the interactions are independent on the presence of hyaluronan in the case of its low concentration 15 mg/l. This technique probably indicates more considerable changes such as formation of a macroscopic precipitate, therefore the aggregation based on just turbidity or opacity of the solution may not be detected.

6 CONCLUSION

Interactions of negatively charged hyaluronan of different molecular weight and concentration with cationic surfactants and amino acids in aqueous solutions were studied by different physico-chemical methods with a common goal – to define the influence of hyaluronan on the aggregation properties of the surfactants. If the interaction between hyaluronan and surfactant is proved and the critical aggregation concentration of the surfactant is found, this system can be used as a solubilizing domain for an active substance in the drug delivery systems.

Two methods of surface tension measurement gave the information about the self-assembly both in bulk and on the surface, respectively. Surface tension measurement of the samples in water showed that hyaluronan decreases just the values of surface tension but the aggregation region is not affected. Lower values of surface tension are caused by the sorption of free surfactant on the surface because hyaluronan fills the bulk solution due to its hydrophilic character and therefore surfactant monomers are pushed up to the surface. However, interactions between hyaluronan chains and surfactant definitely occur in the premicellar concentration region. The effect of low and high (15 m/l and 1000 mg/l, respectively) hyaluronan concentration is approximately the same in the mean of decreasing the surface tension.

On the other hand, microcalorimetry was used to study the described interactions from the thermodynamic point of view. The data were evaluated in the form of the heat of micellization and critical aggregation concentrations. The electrostatic interactions and the phase separation was proved not just from the point of the shape of the titration curve but also from the point of the visual observation of the mixture hyaluronan-surfactant (hyaluronan concentration 1000 mg/l) in the measuring cell after the experiment reached. While the phase separation of hyaluronan with CTAB is shifted to the higher surfactant concentration, the separation of hyaluronan with TTAB is shifted to the lower surfactant concentration.

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8 CURRICULUM VITAE

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Physical Chemistry (thermodynamics): exercise classes for students from abroad

9 ABSTRACT

Vliv hyaluronanu na micelizaci tenzidů byl studován různými fyzikálně-chemickými metodami. Byly zvoleny dva kationaktivní tenzidy, a to tetradecyltrimethylammonium bromid (TTAB) a cetyltrimethylammonium bromid (CTAB). Metoda izotermické titrační kalorimetrie byla využita pro stanovení entalpie micelizace, tenziometrie popisuje povrchové vlastnosti daných vzorků. Je sledován také vliv různé molekulové hmotnosti použitého hyaluronanu a délka alkylového řetězce tenzidu. Výsledkem jsou hodnoty kritické micelární nebo agregační koncentrace tenzidu. V neposlední řadě se diskutuje využití agregátů hyaluronan-tenzid jako možné nosiče pro cílenou distribuci léčiv.