VYSOKÉ UČENÍ TECHNICKÉ V BRNĚ
BRNO UNIVERSITY OF TECHNOLOGY

FAKULTA CHEMICKÁ
CENTRUM MATERIÁLOVÉHO VÝZKUMU

FACULTY OF CHEMISTRY
MATERIALS RESEARCH CENTRE

INTERACTIONS BETWEEN HYALURONAN AND SURFACE ACTIVE SUBSTANCES

DIZERTAČNÍ PRÁCE
DOCTORAL THESIS

AUTOR PRÁCE
AUTHOR

Ing. JITKA KROUSKÁ

BRNO 2012

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INTERAKCE HYALURONANU A POVRCHOVĚ AKTIVNÍCH LÁTEK

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DOCTORAL THESIS

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BRNO 2012

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Purkyňova 464/118, 61200 Brno 12

Doctoral thesis Assignment

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Interactions between Hyaluronan and Surface Active Substances

Doctoral thesis assignment:

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Deadline for doctoral thesis delivery: 31.8.2012

Doctoral thesis is necessary to deliver to a secreatry of institute in three copies and in an electronic way to a head of doctoral thesis. This assignment is enclosure of doctoral thesis.

Ing. Jitka Krouská Student	prof. Ing. Miloslav Pekař, CSc. Head of thesis	prof. Ing. Miloslav Pekař, CSc. Head of institute

In Brno, 1.9.2011

prof. Ing. Jaromír Havlica, DrSc.
Dean

ABSTRACT

Influence of hyaluronan on surfactant micellization is studied by different physico-chemical methods. For this purpose, two cationic surfactants were chosen: tetradecyltrimethylammonium bromide (TTAB) and cetyltrimethylammonium bromide (CTAB). Isothermal titration calorimetry was used to determine enthalpy of micelle (aggregate) formation and tensiometry was useful for the description of the surface properties of the prepared samples. The different molecular weight of hyaluronan and the different alkyl chain length of the surfactant are taken into account. The results are evaluated to obtain the values of critical micelle or aggregation concentration of surfactants and the question of possible application of these systems as drug delivery systems is also discussed.

ABSTRAKT

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.

KEYWORDS

hyaluronan, surfactant, surface tension, isothermal titration calorimetry, critical micelle concentration

KLÍČOVÁ SLOVA

hyaluronan, surfactant, povrchové napětí, izotermická titrační kalorimetrie, kritická micelární koncentrace

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DECLARATION

I declare that this thesis has been compiled by myself and on my own and I cited all my information sources completely and correctly. The thesis is in terms of its content a property of the BUT Faculty of Chemistry and its usage for commercial purposes is subject to a prior consent of the supervisor and the dean.

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Ing. Jitka Krouská

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CONTENT

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 THEORETICAL PART

2.1 Hyaluronan

2.1.1 Structure and properties of hyaluronan

Hyaluronan was first described by Meyer and Palmer and co-workers in 1934 as a sodium salt of hyaluronic acid [1]-[2]. They proposed the name "hyaluronic acid" from hyaloid (vitreous) and uronic acid, which was found in the structure. It is a natural, linear, unbranched and negatively charged polysaccharide with repeating dissaccharide units of D-glucuronate and N-acetyl-D-glucosamine linked by β(1-4) and β(1-3) bonds [3][4] Therefore, hyaluronan belongs to a family of glucosaminoglycans [5]. It is a unique biopolymer with a huge amount of viscoelastic and moisturising properties as itself. It has a high molecular weight, usually at about millions of Daltons [6]. The negative charge character is due a carboxylic group of glucuronic acid residue. This structure determines hyaluronan to behave as a polyelectrolyte (polyanionic) in solution at physiological pH [7]. Hyaluronan molecule assumes an expanded random coil structure in physiological solutions. The domain structure of hyaluronan has important and interesting consequences [8]. The main advantage of this domain is its high hydrophilic character due to an extensive hydration shell which is based on hydrogen bond principle. Small molecules such as water, electrolytes and nutrients can diffuse through the solvent within the domain. On the other hand, large molecules such as proteins, are partially excluded because of their larger hydrodynamic sizes in solution.

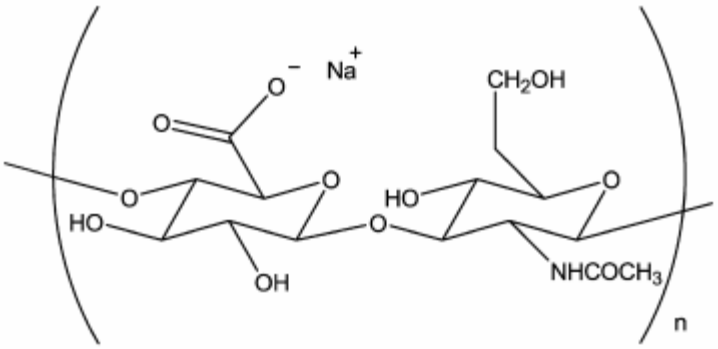


Fig.1 *Hyaluronan structure* [3].

Other important properties are its biocompatibility and biodegradability. It occurs, as a critical component, in biological fluids, both pericellular and extracellular matrices and tissues of vertebrates, e.g. cartilage, vitreous humour of the eye, etc. [6]. The main hyaluronan fraction is localized in the skin tissue [1]. It plays a vital role in many biological processes such as tissue hydration, proteoglycan organization in the extracellular matrix and cell differentiation [9]. In the last two decades, the interest in this molecule has grown because of its associative properties and also for its great potential in pharmaceuticals, cosmetics, coatings and recovery [5].

The purification of hyaluronan from biological sources has been an interest of many scientists for decades. The first isolation and characterization of hyaluronan was described by Meyer et al. already in 1934 [2]. A polysaccharide with high molecular weight was obtained by methods avoiding strong hydrolytic agents from the vitreous humor of cattle eyes. Their starting material was the acetone precipitate of fresh cattle vitreous humor. They obtained 0.73 g of hyaluronan from 100 eyes. This yield contained other material such as $CaSO_4$. As constituents they recognized uronic acid, amino acid and possibly pentose. Later, in 1942, E. Balazs used hyaluronan for commercial use as a substitute for egg white in bakery products [10]. Subsequently, he patented a process for purifying hyaluronic acid from rooster combs in 1970`s [11].

Hyaluronan is produced by certain strains of bacteria so it is the reason for its commercial extraction nowadays. The most used bacterial strains are *Streptococcus equi* and *Streptococcus zooepidemicus* [1], shown in Fig.2. The bacterial production of hyaluronan enables to achieve larger quantities of this macromolecule. Hyaluronan produced by *S. equi* has a lower molecular weight than *S. zooepidermicus* does. The yield is about 4 grams of hyaluronan per one liter of the cultivated solution. The final product, white powder or fibrous aggregate, is obtained with some impurities such as proteins, nucleic acids, bacterial endotoxines and water (between 5 and 10%). The molecular weight may vary and is required according to the next pharmaceutical or medical application.

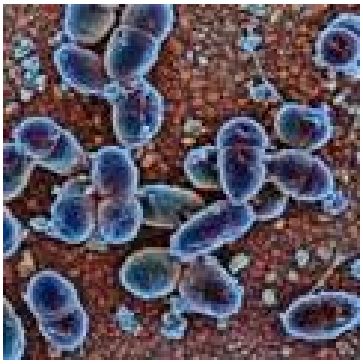


Fig.2 *Streptococcus zooepidemicus* [12].

At present, some alternative sources for hyaluronan production have been found. One is a genetically modified bacterial strain *Bacillus subtilis* [6]. This strain, shown in Fig.3, is able to produce hyaluronan in the 1 MDa range. One advantage is that *B. subtilis* is easily cultivable on a large scale and does not produce exo- or endotoxins. Moreover, it does not produce hyaluronidase, an enzyme that could degrade the synthesized hyaluronan.

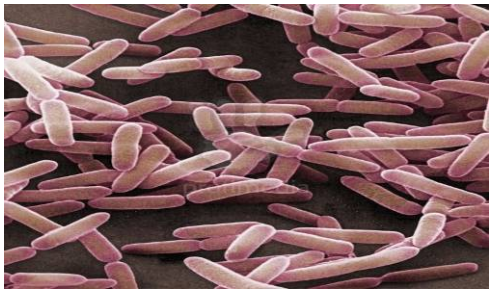
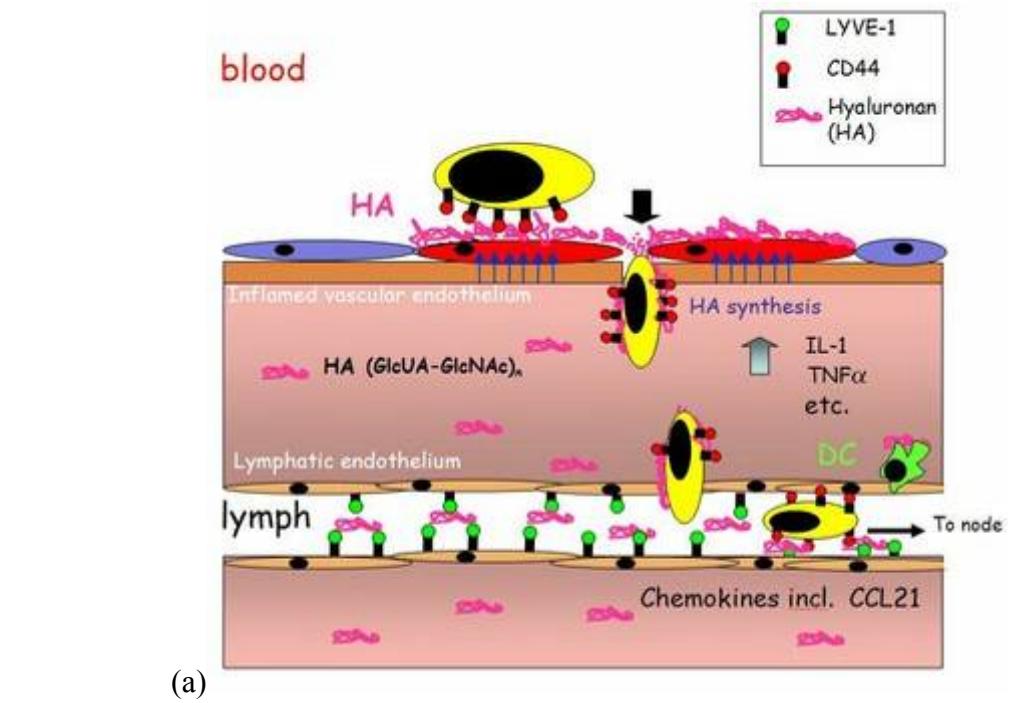


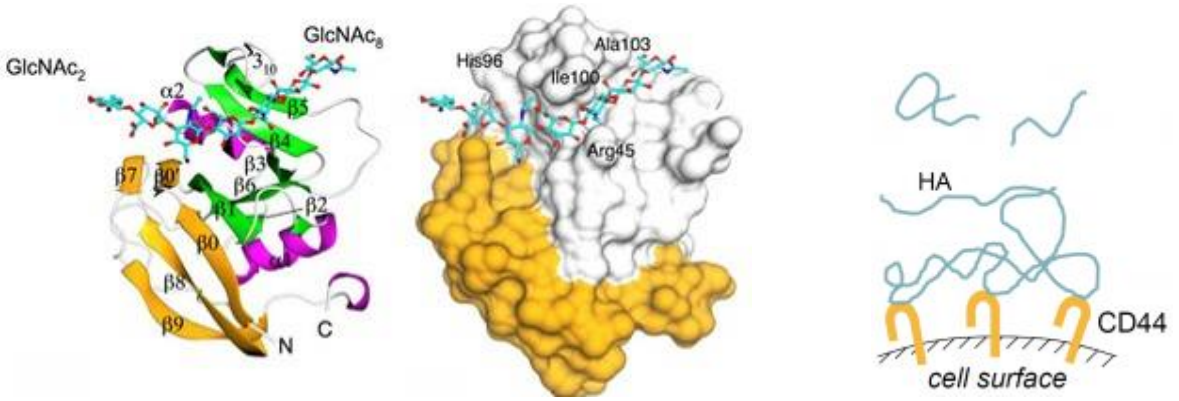
Fig.3 *Bacillus subtilis* [13].

2.1.2 Hyaluronan-binding receptors

There are some proteins in the cell membranes called receptors which are responsible to bind cells to hyaluronan. The assembly and retention of the pericellular matrix of many cells involves interactions between receptor CD44 (cluster determinant) and hyaluronan. It is thought to be the primary receptor for hyaluronan and to date it is the best characterized transmembrane hyaluronan receptor. The CD44 receptor is a glycoprotein responsible for cell-cell interactions, cell adhesion, migration and pathological tumor metastasis. RHAMM receptor (Receptor for HA-Mediated Mobility) has been found on the cell surfaces. It binds hyaluronan via short amino acids sequence containing multiple basic amino acids. Other activities and functions of hyaluronan can be explained by other hyaluronan-binding receptors: layilin, hyaluronic acid receptor for endocytosis (HARE) or lymphatic vessel endocytic receptor LYVE-1 [8][10][14][15].



(a)



(b) (c)

Fig.4 *Hyaluronan and leukocyte trafficking (a) and crystal structure of the CD44 hyaluronan binding domain complexed with HA8 (b)* [16]*; interaction of*

hyaluronan with CD44 on the cell surface (c) [17].

2.1.3 Hyaluronan in targeted drug delivery

A review published by Ossipov [18] summarizes the information about the nanostructured hyaluronic acid-based materials for active delivery to cancer. The vast majority of clinically used traditional drugs are low-molecular-mass compounds that are able to penetrate both tumor and normal cells by diffusion, which does not completely exclude the systemic toxicity. This means dangerous side effects such as nephrotoxicity or neurotoxicity. Thus, anticancer agents are typically given with the inclusion of treatment-free periods to allow the recovery of normal cells. On the other hand, the half-life of the most low-molecular-mass drugs (i.e. < 500 Da) is short in the blood stream, forcing them to be used at their maximum tolerated dose.

Consequently, several creative drug delivery systems have been created to improve the therapeutic index of traditional drugs. The significant potential show systems based on nanoparticles. They can be injected directly into the systemic circulation without the risk of blocking blood vessels. The diameters of possible particles differ from 10–70 nm which can flow through even very small capillaries. On the other hand, larger particles with diameters from 70 to 200 nm have prolonged circulation time which is good to avoid their clearance and removing from the body.

Polymeric drug delivery including not only homopolymers but also dendritic macromolecules and amphiphilic block copolymers can spontaneously assemble into spherical, cylindrical or vesicular shapes. Polymeric chains can be physically or chemically crosslinked, forming hydrogels. All these nanoobjects can act as carriers for cytotoxic agents or they can be made cytotoxic themselves in response to stimuli such as light, heat or pH. Such nanoparticles physically incorporate drugs in their interior or adsorb the cytotoxic agents on their surface. The other possibility is that low molecular mass drug can be covalently conjugated to a nanocarrier through a releasable linker. However, more recent studies have shown that polymeric drug carriers encounter difficulties in interacting with target cells after extravasation because macromolecules or other nanocarriers are internalized by endocytosis, which is a relatively slow process requiring high concentration of the nanocarriers in extracellular space. To improve the targeting efficiency of nanoparticles, the use of active

targeting based on the use of biomolecular recognition molecules has been introduced to provide a greater degree of specifity.

The author in ref. [18] later describes hyaluronan nanogels, their composition and behavior in the bloodstream. It is summarized that the production of nanostructured materials from hyaluronic acid can tailor many properties for specific applications, such as solubility, biodistribution, biocompatibility, biodegradability and drug release.

Hyaluronan cannot be used directly for targeted drug delivery to carry nonpolar substances because it is a highly hydrophilic biopolymer with massive hydration shell. Many efficient drugs are hydrophobic, therefore hyaluronan must be chemically modified (hydrophobized) or interact with some substances which can form any hydrophobic space for solubilizing these drugs. For this purpose, surfactants are used to ensure formation of micelles, simple self-assembly structures. The advantage here is to use oppositely charged surfactant and polymer because of the occurrence of electrostatic interactions between micelles and the polymer chain. On the other hand, the disadvantage of using surfactants is their toxicity as itself so the main aim in the field of targeted drug delivery research is to find a molecule with surfactant nature and simultaneously, with antitoxic effect on the humans. One possible solution is to use hylans. Hylans are a family of chemically modified hyaluronans [19]. They include hylan fluids, gels, microparticles and membranes. Hylan fluid is hydrophilic, polyanionic hyaluronan derivative with excellent biocompatibility. It can provide the basis of various release systems therefore it is one of the possible transport agents in the controlled drug delivery research. Hylans are produced by chemically crosslinking hyaluronan chains.

Hyaluronan and hylans can act as vehicle molecules for controlled and localized delivery of biologically active molecules (nonpolar therapeutic agents) to provide their in vivo release [20]. The structure of such a drug delivery system from hyaluronan can be described as follows. A colloid structure, e.g. a micelle of an oppositely charged surfactant, with a hydrophobic domain which is suitable for the drug solubilization, is electrostatically bound to the core of a hydrophilic hyaluronan chain.

As mentioned above, hyaluronan can interact with cell receptors and thus, the complex of hyaluronan and the hydrophobic domain is able to enter the cancer cell and release the drug inside. The complex can find just the tumor cells with no influence on the healthy cells.

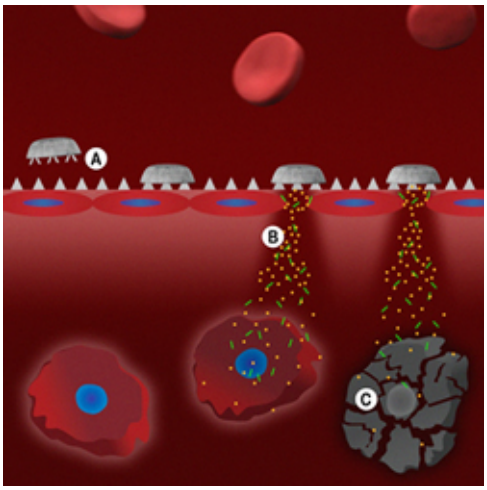


Fig.5 *A new multi-stage drug delivery system delivers therapeutic or diagnostic agents directly at the site of a tumor or other problem area. (A) shows the injected nanocarrier landing on the inner wall of a tumor-associated blood vessel, (B) the release of nanoparticles that penetrate both the blood vessel wall and the tumor cell membrane and, (C) the delivery to the tumor of doses of a cancer killing medication* [21].

Another way how to use hyaluronan in drug delivery is to prepare its chemically modified derivatives to obtain a water insoluble macromolecule. The advantage of such a derivative is that it can be used as a drug delivery system directly as itself. There are several previous studies about hydrophobically modified hyaluronan [22]-[24], each of them describes a different kind of derivative. The chemical modification can change the physiological functions or biodegradability of hyaluronan which brings advantages in further application.

2.2 Surfactants

2.2.1 Definition of surfactants

Surface active agents (surfactants) are molecules with tendency to adsorb at surfaces and interfaces. The term surface indicates that one of the phases is gas, usually air. The term interface denotes a boundary between any two immiscible phases. The driving force for a surfactant to adsorb at an interface is to lower the free energy of that phase boundary. It is a common property of any surfactant. The interfacial free energy per unit area represents the amount of work required to expand the interface. The term interfacial tension is often used instead of interfacial free energy per unit area. Thus, the surface tension of water is equivalent to the interfacial free energy per unit area of the boundary between water and the air above it. When that boundary is covered by surfactant molecules, the surface tension (or the amount of work required to expand the interface) is reduced. The denser the surfactant packing at the interface, then the larger the reduction in surface tension [25].

Surfactants are molecules with both hydrophilic and hydrophobic part. The hydrophilic one is called a head and can be without a charge or charged. The hydrophopic is called a tail and mostly consist of a carbon chain. The chain length of the tail and the charge of the headgroup are the main factors influencing the properties of any surfactant. Thus, the basic classification of surfactants is based on charge or molecular size. Surfactants can be cationic, anionic, amphoteric or nonionic – it depends on the charge of the headgroup. Surfactant can be divided also according to their molecular weight to molecular (up to 1000 g/mol) or polymeric with molecular weight about 10 000 g/mol.

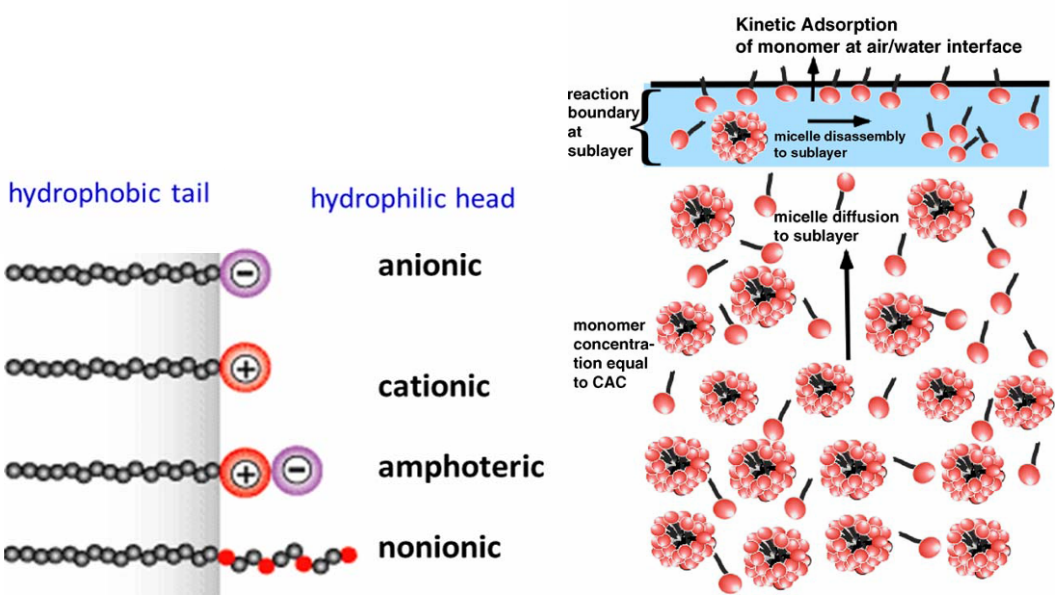


Fig.6 *Possible charges on surfactant headgroups and behavior of surfactant monomers and micelles in aqueous solution* [26],[27].

2.2.2 Critical micelle concentration

When a surfactant is added to aqueous solution surface tension decreases till defined surfactant concentration. During this process the molecules of the surfactant diffuse in the bulk and perform a layer at the surface. After an addition of certain amount of the surfactant there is no more area free for other molecules of the surfactant and the surface of the solution is saturated. At this point the solution reaches its critical micelle concentration (CMC).

It is the basic property of surfactants to form those self-assembly structures called micelles above the critical concentration of surfactant. Every surfactant has a different value of CMC. The idea of micelle formation and behavior in solution is shown in Fig.6 and Fig.7. The mixture obtains not only the surfactant molecule but also the micelles.

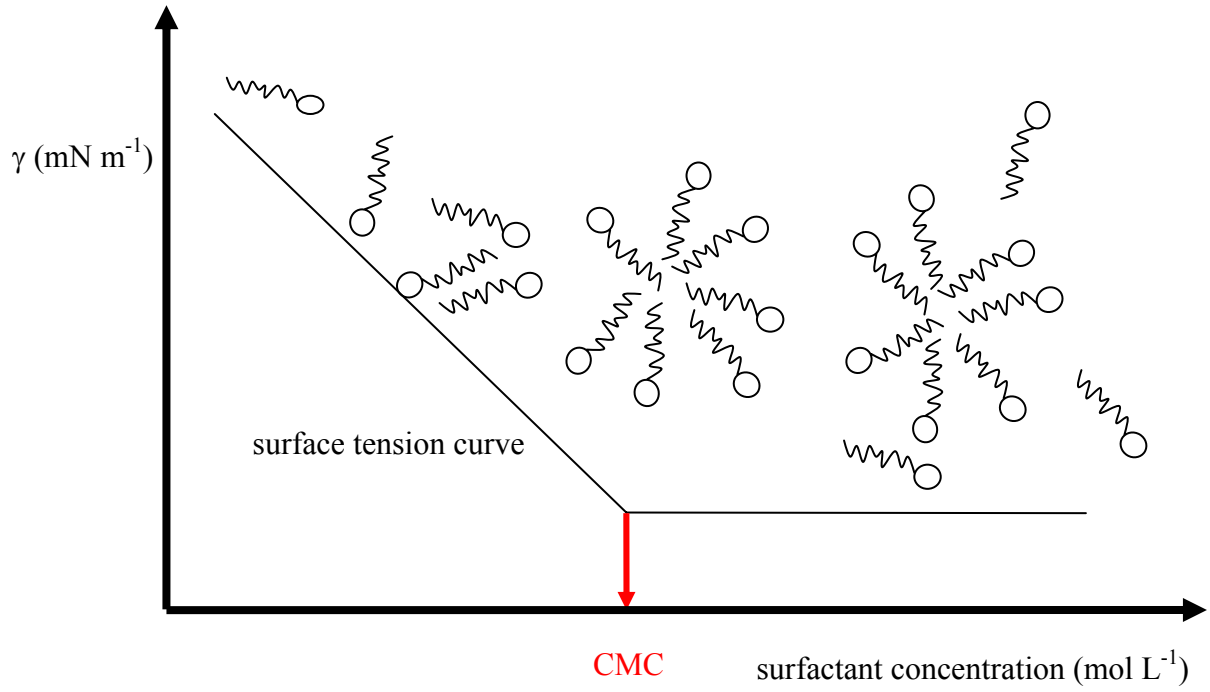


Fig.7 *Forming micelles in surfactant solution – relation between surfactant behavior and surface tension curve.*

The value of CMC is one of the characteristic information about surfactants. A schematic behavior of surfactant monomers according to increasing surfactant concentration in solution is described in Fig.7. At low surfactant concentration the monomers are adsorbed at the surface. With increasing surfactant concentration the first aggregation occurs and CMC is obtained (red arrow). After more addition of surfactant more micelles are formed but the value of surface tension does not decrease any more because the surface is saturated.

The knowledge of CMC allows us to declare that above this concentration the hydrophobic domains are formed. This is the reason why to use interactions between surfactant or micelles, respectively and hyaluronic acid to provide a suitable carrier for drugs.

2.3 Surfactant–polymer interactions

Combination of hyaluronan with surfactant may lead to formation of associates in which the surfactant hydrophobic domains solubilize hydrophobes and hyaluronan plays a role of biocompatible carrier and targeting agent.

Polymer-surfactant interactions are controlled by a balance between hydrophobic and electrostatic interactions and are modulated by temperature and ionic strength [28].

In ref. [25] authors describe interactions between surfactants and polymers. The combined occurrence of polymers and surfactants is found in many fields: cosmetics, detergents, paint, foods etc. The combination of surfactant with hyaluronan leads to a complex which has both hydrophilic and hydrophobic part. The hydrophobic part is based on micelles formed above

the critical micelle concentration (CMC). The interaction between surfactant and polymer, in this case negatively charged hyaluronan, may be described by two basic theories. The first one is about the interaction in terms of a strongly co-operative association of binding of the surfactant to the polymer, the second describes a micellization of the surfactant on or in the vicinity of the polymer chain.

As regards structure in these systems, the structure of a "pearl-necklace model" is widespread (Fig.8). The surfactant clusters are formed along the polymer chain. Electrostatic interactions are expected if both surfactant and polymer are charged. The intensity of this interaction is stronger in the case of oppositely charged substances. However, we must also take into account the repulsive interactions between charged surfactant molecules or charged polymer molecules and the charges of counterions.

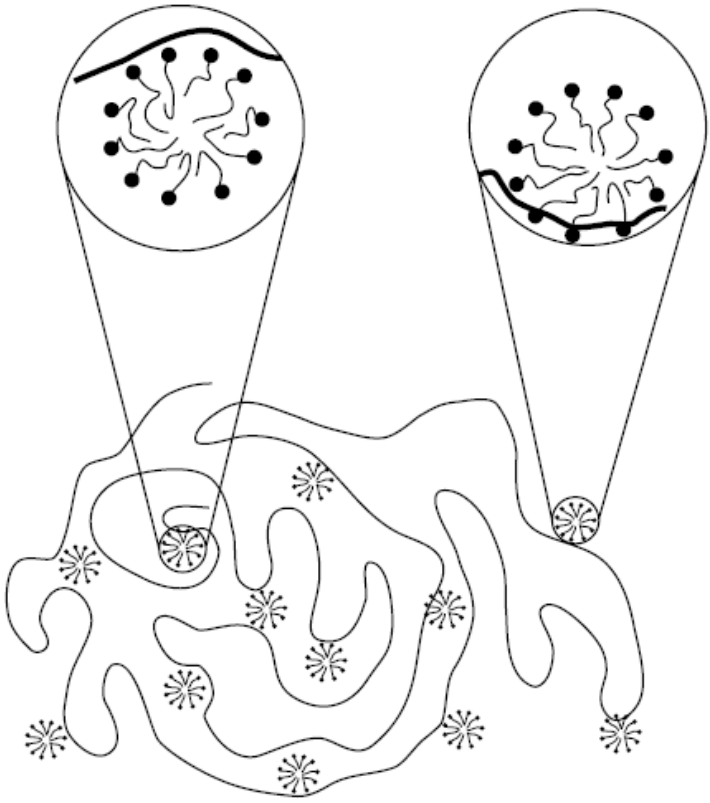


Fig.8 *Pearl-necklace model of surfactant-polymer association* [25].

The contribution of entropy associated with the increased concentration of counterions at the aggregate surface compared to the bulk is highly unfavourable for self-assembly and explains why ionic surfactants have higher CMCs than nonionics. A polymer may modify this entropy contribution in a number of different ways. If it is ionic and has a similar charge, then the electrolyte effect is relatively moderate. If the charge is opposite and it acts as a multivalent electrolyte, the interaction becomes very strong since the association between the polymer and micelle leads to release of counterions of both the micelles and the polymer molecules. A very similar effect occurs in mixtures of two oppositely charged polymers.

The added surfactant into the hydrophobically modified polymer solution will interact strongly with the hydrophobic groups of the polymer chain leading to the strengthened

association between polymer chains, and thus to an increased viscosity. The behavior can be simply described as shown in Fig.9.

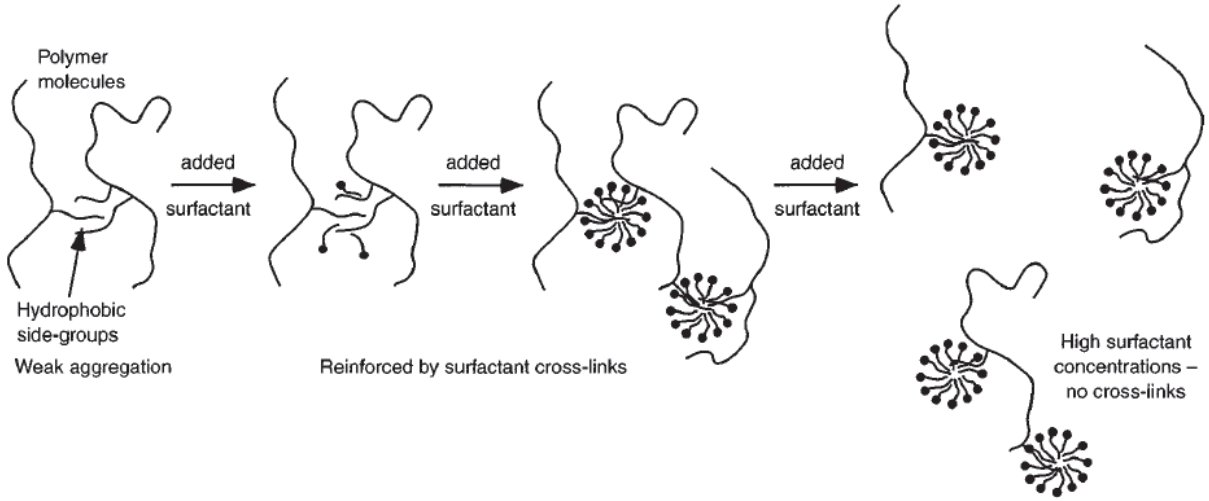


Fig.9 *The self-association of a hydrophobically modified water-soluble polymer after addition of a surfactant solution* [25].

The widespread method for characterization the system surfactant-polymer is surface tension measurement. It is based on the fact that surfactants lower surface tension of the solution and the break point on the surface tension curve against concentration is observed at critical aggregation concentration (CAC). The complex behavior of such a system is shown in Fig.10.

The values of surface tension are high at low surfactant concentration, the surfactant monomers are dissolved in the bulk and also cover the surface. Later, at higher surfactant concentration, first monomers start to form micelles and the minimum of surface tension is observed as a lower plateau. When no polymer is added to the solution (solid line) there is just one break point at the CMC (red point).

On the other hand, the addition of polymer causes interactions between surfactant and polymer chains (dashed line). The surface tension values are lower and the strength of the interaction depends mainly on the charges of surfactant and polymer. There are two break points (blue points) on the curve in the precipitation zone – the decreasing trend of surface tension is slower because the first aggregates are formed at critical aggregation concentration. Later, the second break is observed at the end of aggregation process. All the possible binding positions between the polymer chain and the surfactants micelles are occupied and the real micelles are formed in the solution. Finally, the two curves overlap each other at higher surfactant concentration.

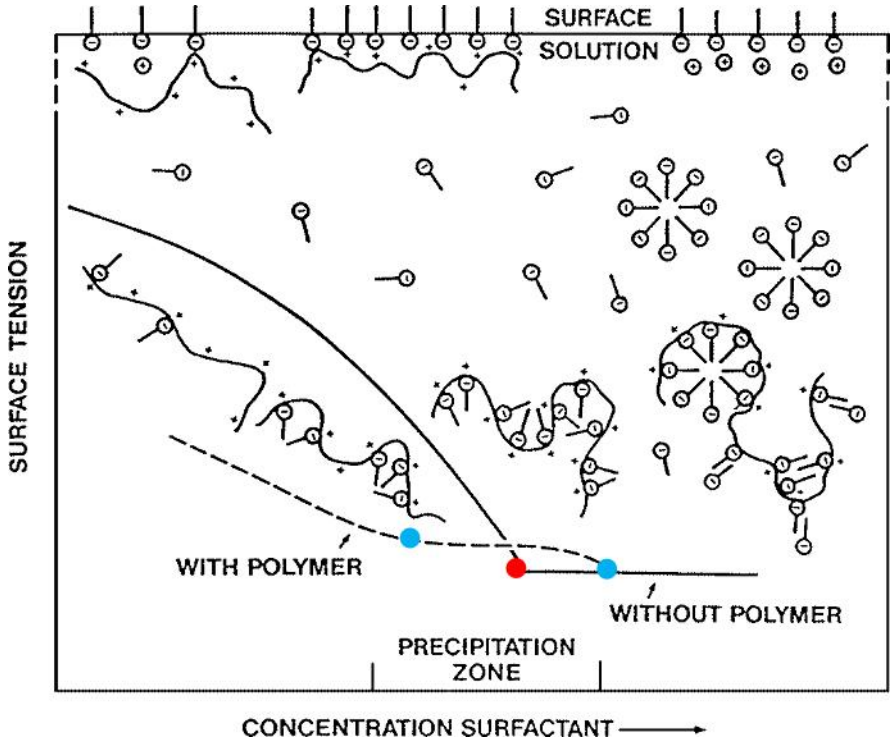


Fig.10 *Behavior of surfactant with addition of polymer in solution* [29].

For a mixture of oppositely charged polymer and surfactant, the formation of a concentrated phase with charge stoichiometric amounts of polymer and surfactant and dilute phase containing any excess of either polymer or surfactant becomes unfavourable if the polymer is hydrophobically modified. The reason is the tendency of the polymer to associate hydrophobically with the micelles in the concentrated phase. Then, this phase will lose charge stoichiometry and have a tendency to swell. Therefore, associative phase separation will occur only over a restricted concentration region for a mixture of a hydrophobically modified polyelectrolyte and an oppositely charged surfactant [25].

2.4 Methods for CMC determination

2.4.1 Surface tension

The detailed description can be found in [30]. Generally, surface tension γ is defined as the magnitude of the force F exerted parallel to the surface of a liquid divided by the length L of the line over which the force acts:

$$\gamma = \frac{F}{L} \quad . \tag{1}$$

The unit of surface tension is N m^{-1}, force per unit of length.

The most related equation connected to the definition of surface tension is the Gibbs adsorption equation

$$d\gamma = -\Gamma RTd\ln c\,, \tag{2}$$

where Γ is the surface concentration of the surfactant, R is the gas constant, T is the absolute temperature and c is the surfactant concentration.

Surface tension is a property of the surface of a liquid which causes it to behaves as an elastic sheet. It has the aim to reach the smooth surface with a minimum span. The surface requires the stage with a minimum of energy. So the surface of a liquid will be smooth in every situation due to the minimum of the surface area. The chemical and physical behavior of liquids cannot be understood without taking surface tension into account.

Tensiometry is a technique about measuring surface and interfacial properties of liquids. During surface tension measurement the cohesive energy, which is the result of attractive interactions among the molecules of the liquid, presents at a measured interface. The interactions of a molecule in the bulk of a liquid are balanced by an equal attractive force in all directions as is shown in Fig.11.

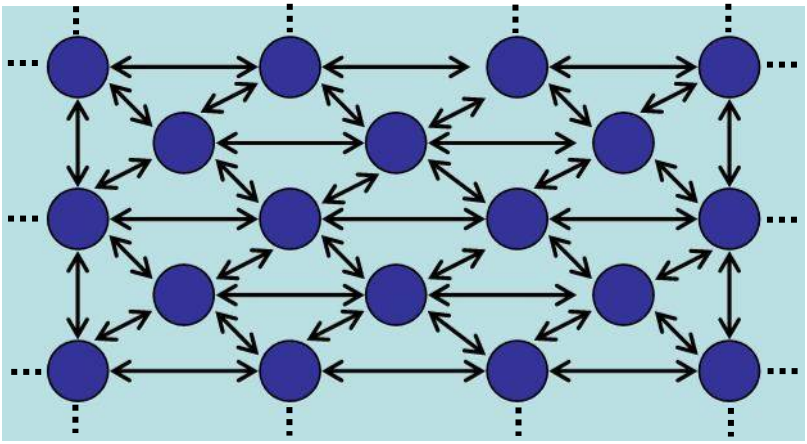


Fig.11 *Intermolecular forces between particles in liquids.*

One of the possible techniques of surface tension measurement is a Du Noüy ring measurement method. This method belongs to semistatic tensiometric methods. The ring is made of platinum and iridium (usually 90 % Pt and 10 % Ir). The ring is raised in the liquid until the contact with the surface is registered. The sample is then lowered again so that the liquid film produced beneath the ring is stretched. As the film is stretched a maximum force is experienced; this is recorded in the measurement.

Surface tension is directly calculated by the instrument using the following equation:

$$\gamma = \frac{F}{4 \cdot \pi \cdot r_p} \cdot \Phi\,, \tag{3}$$

$$\frac{\text{weight of hyaluronan in the stock solution (g)}}{\text{weight of one disaccharide unit (g/mol)}} = \text{number of disaccharide units (mol)}$$

where F is the force needed for pulling the ring from the interface, r_p is the radius of the ring and Φ is the dimensionless correction factor.

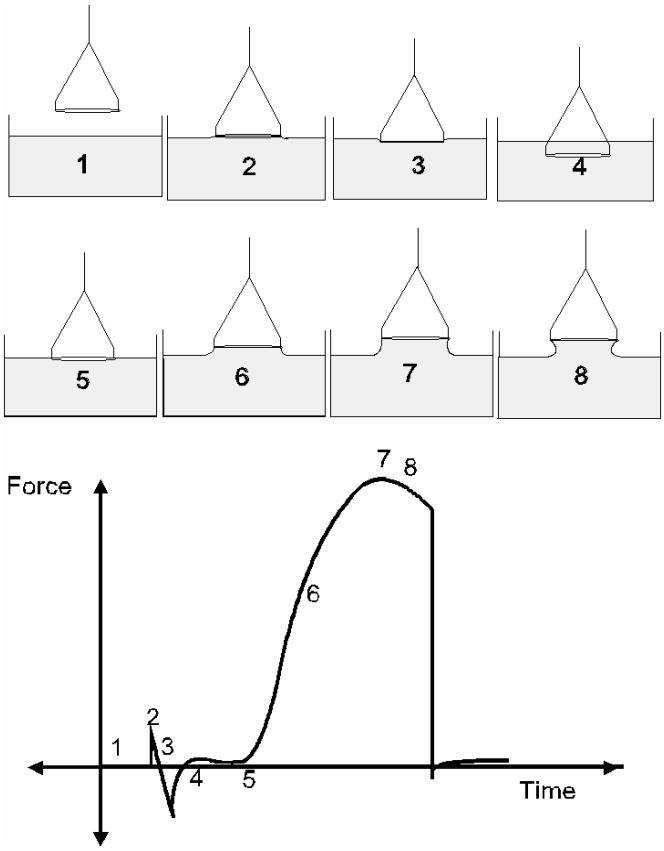


Fig.12 *Schematic diagram of the ring method measurement* [30].

The process is described as follows:

1. The ring is above the surface and the balance is zeroed.
2. The ring touches the surface and gets wetted resulting in some positive force.
3. Negative force is applied on the ring when it is pushed through the surface into the liquid.
4. Some positive force will remain because of the wetting of the vertical supports of the ring.
5. When pulled out of the liquid (by lowering the sample cup) the upper surface of the ring touches the liquid surface and the force starts to increase. This is because the surface tension of the liquid tries to prevent the ring from penetrating its surface.
6. The liquid is attached to the ring while pulling it up until the maximum force (described in point 7) is reached. At this point the volume of the liquid pulled up by the ring is also at its maximum causing the ring to detach from the liquid.
7. The maximum force needed to pull the ring from the liquid is proportional to the surface tension of the liquid. The greater is the force needed the greater is the surface tension.

The second method used for the characterization of the system hyaluronan-surfactant was a maximum bubble pressure method. It is mainly used for interfacial tension measurement. A

thin capillary is immersed in the liquid sample while air is bubbled out of the capillary. The liquid surface is automatically detected and the capillary is lowered to the appropriate level. The bubbles are continuously formed at the interface between the end of the capillary and the sample. As the pressure increases in the capillary, a bubble is gradually pushed into the liquid. The maximum bubble pressure is reached when the bubble is hemispherical and has the same diameter as the diameter of the capillary. The maximum pressure depends on the force exerted by the liquid, and hence its surface tension [31].

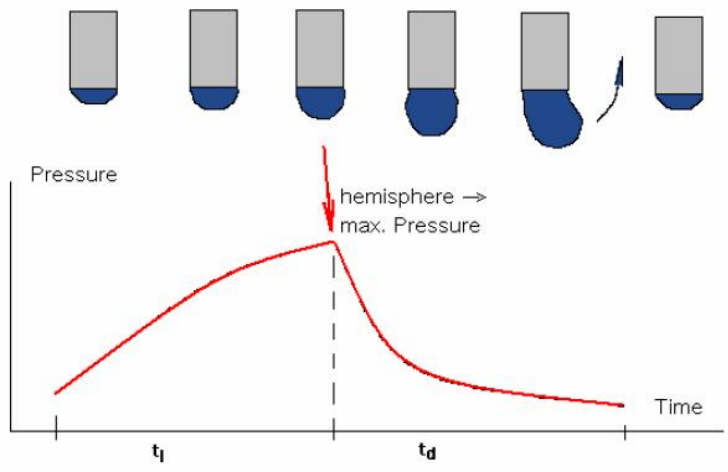


Fig.13 *Schematic diagram of bubble formation, t_l is time of life, t_d time of death of the bubble* [31].

2.4.2 Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) is a technique based on measurement of heat that is generated or absorbed in an interaction between two molecules. One of the most used applications can be found in the field of surfactant/polyelectrolyte interactions, where it is used as an additional measurement method to determine aggregation concentrations. Moreover, ITC is a very suitable technique for studying the association mechanisms in these systems and it allows evaluation of the thermodynamic parameters (e.g. Gibbs free energy, enthalpy, entropy, heat capacity) of the binding process [32]. Furthermore, ITC is used to measure binding isotherms for other systems such as ligand binding to proteins, polymers and colloidal particles.

Typically, in an ITC experiment aiming to study the micellization process, a micellar solution in the syringe is diluted in water contained in the sample cell. The concentration of the micellar solution in the syringe is chosen in such a way that, with increasing surfactant concentration in the sample cell, the CMC is reached during the experiment. Generally, the sigmoidal enthalpogram is obtained (Fig.15). It can be divided into three concentration ranges reflecting the following events published in ref. [33].

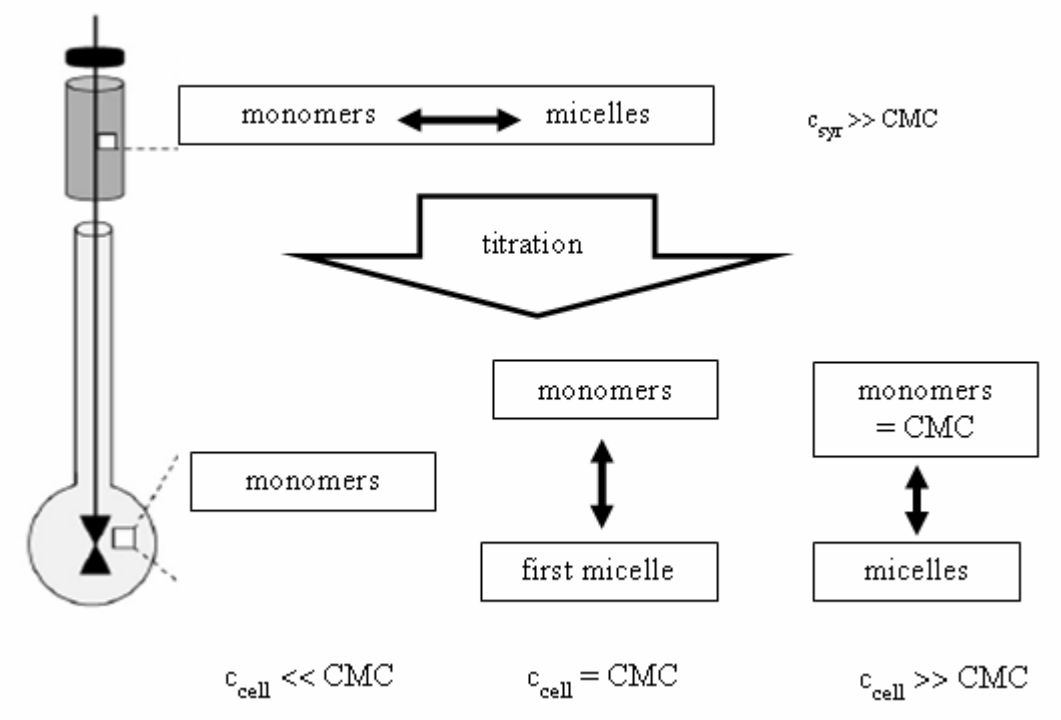


Fig.14 *Schematic process of the titration* [32],[33].

After the first injections, the final concentration of the surfactant molecules in the sample cell remains below their CMC and the weak enthalpic effects appear on account of the dissociation of the micelles to unimers and to the dilution of the resultant unimers. If more micellar solution of the surfactant is added to the sample cell, a clear increase or decrease in the heat is observed indicating that the added micelles are not dissociated; the CMC is progressively approached at each injection. The surfactant concentration in the sample cell is now above the CMC.

If more micellar solution is added beyond the CMC, the micelles no longer dissociate and the micellar solution is being diluted, leading to an observed heat output due to the micelles dilution. From the titration curve, and if the clear break of the curve is obtained, the ΔH_{mic} is calculated from the enthalpy difference of the two levels of the titration curve. The CMC is directly determined from the maximum value of the first derivative of the titration curve.

There are several papers about ITC and its applications in many areas such as polymer chemistry, nanotechnology or biochemistry. The analysis of thermodynamic parameters, combined with the study of physicochemical properties of the phenomena, allows an understanding of how the two molecules interact and why they do so [34]. ITC is the only technique capable of measuring CMC and the enthalpy of the micellization (ΔH_{mic}) of a surfactant in a single experiment without the necessity of any probe.

3 STATE OF THE ART

3.1 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. [35] worked with hyaluronan and both nonionic (Cremophor EL, Tween 80) and anionic (sodium dodecyl sulfate, SDS) surfactants. The association between hyaluronan and surfactants was studied using a pyrene fluorescence method. The effect of saccharide (glucose, lactose, mannitol) was also investigated. The data indicated that the interactions between hyaluronan and nonionic surfactants are extremely weak. The results showed an almost identical CMC value for Cremophor EL in the presence and absence of hyaluronan. The system hyaluronan-SDS showed lower values of CMC which indicated that hyaluronan acts similary as a low molecular weight electrolyte. This behavior was demonstrated by similar experiments with pure SDS (without hyaluronan) in sodium chloride solution. The authors concluded it as the attraction of the hydrophilic sulfonate groups of SDS toward the microdomains formed by the hydroxyl groups in the hyaluronan chain.

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 [36] 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 (Orange OT), 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 [37]-[41] 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. [40]. 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. [42]. 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. The viscosity changes upon addition of TTAB are due to binding of micelles to the polymer. As the TTAB concentration increases, there will be enough free micelles for the polymer chains to expand. This results in a viscosity increase.

3.2 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 (dodecyl-, tetradecyl- and cetyltrimethylammonium bromide) was studied by Beyer et al. [43]. They performed experiments in the temperature range between 20 and 60°C in 0.1 M NaCl at pH 6.4. 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 [43] 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. They proved typical behavior of surfactants at different temperatures. The minimum change for surfactants in salt was observed between 20 and 34°C. 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 enthalpy of demicellization increases with increasing alkyl chain lengths of the surfactants at constant temperature. The change of sign for the demicellization enthalpy from positive to negative values at room temperature was not observed for CTAB in salt. The Krafft point (for CTAB in salt solution) is a little above of 20–25°C which prevents measurements in this temperature range. This means that the determination of the CMC with calorimetry is difficult in this range.

The standards enthalpy and entropy of demicellization were calculated using the Gibbs-Helmholtz relation. The aggregation numbers of the surfactnats were calculated using the simulation method based on the mass-action model taking the counterion condensation degree into account. The comparison of the values obtained with the pseudophase-separation and the mass-action model, respectively, shows good agreement between the demicellization enthalpy values, as well as the Gibbs free energy and the entropy changes.

The lowering of the CMC in the presence of additional salt is well known and discussed in many other papers, e.g.[34],[44],[45],[46]. Increasing the salt concentration reduces the electrostatic repulsion between the charged groups and therefore favors the aggregation process inducing a decrease of the CMC [44]. 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 [43]. 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. [47]. 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 [48]. According the pseudo-phase separation model [49]-[52], the standard Gibbs free energy of micellization in water was calculated from the relation

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

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 [53]. 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. [54] 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. [55] 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. [56] and Dearden et al. [57] are based on the studies of alkyltrimethylammonium bromides of different alkyl chain length at four different temperatures in water and sodium bromide solution. In [55] 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 [57]. 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. [58] 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 then, 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. [59] compare two surface-aggregate theories published earlier in [60] and [61]. 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. [61].

Different types of enthalpograms are described by Bijma et al. [62]. 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. The constant value is not reached at the end in contrast to type A and B.

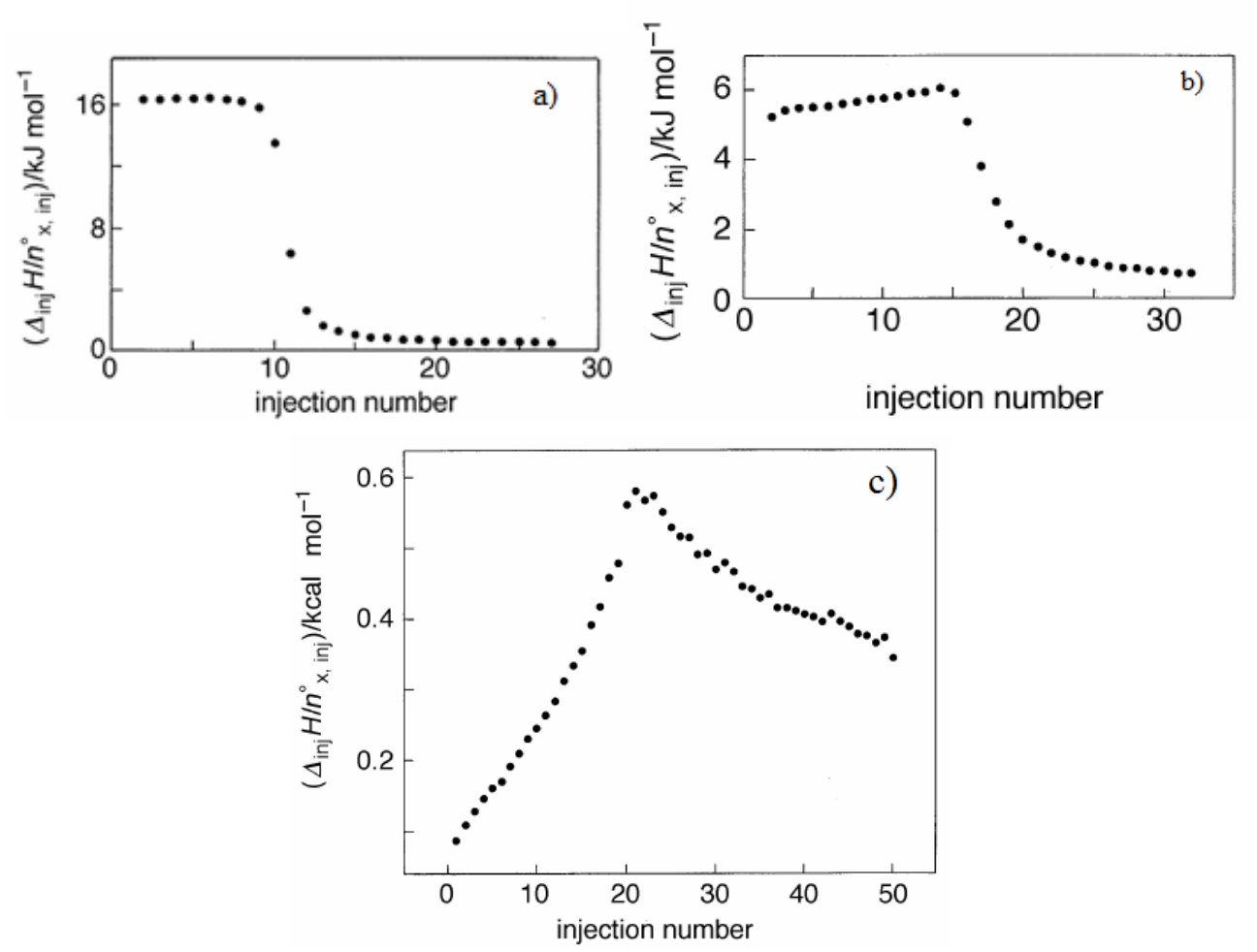


Fig.15 *Three possible types of ITC enthalpograms* [62].

With increase in the CMC, the technique requires the use of more concentrated solutions. In some cases, it may be possible to overcome this concentration dependence by using a

relatively high concentration of added salt in combination with a strong exo- or endothermicity of micelle formation.

3.3 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 [63]. 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 [64]. 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. [65]. 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.

The differential enthalpy curves of the surfactant into polymer at different salt concentration show the decreasing hight of the endothermic peak with increasing salt concentration (0–1 M NaCl). It means that he binding is weakened by the electrostatic attraction between the surfactant and polymer. The second peak observed in the titration curve corresponds to the micellization of polymer-bound surfactant molecules when the surfactant concentration reaches a critical value. The thermodynamic parameters were extracted from the phase pseudo-phase separation model and they suggest that both the electrostatic binding and micellization are enthalpy opposed and driven by entropy. This work also confirms the theory about the attribution of positive entropy which causes the disruption of water structure in micelles surroundings.

Bai et al. [66] 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 [33]. 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. [67]. 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.36 bellow. In addition, the shapes of enthalpograms are in agreement with the theory in [63].

4 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.

In addition, the study of the interactions between hyaluronan and charged amino acids serves as a model system which could substitute the commonly used hyaluronan-surfactant domains in the targeted drug delivery. Obtained knowledge from this part of the thesis will be an objective of the following research.

5 EXPERIMENTAL PART I – SURFACE TENSION

As mentioned previously, the thesis focuses on the interactions between hyaluronan and several types of surfactants in water and sodium chloride solution. Part of results from the first part of this thesis was published in ref. [68]. The aggregation properties of surfactants of different types and their interactions with hyaluronan were studied in 0.15 M NaCl solution at room temperature by tensiometry and fluorescence probe technique. The conclusions are discussed in 5.3.1.

Surface tension measurement method was further used for a detailed study of interactions between hyaluronan and two cationic surfactants tetradecyltrimethylammonuim bromide (TTAB) and cetyltrimethylammonuim bromide (CTAB) in aqueous solution. This part of the work is summarized in 5.3.2.

5.1 Materials

Information about hyaluronans is listed in Tab. 1 and Tab. 2

Tab. 1 Specification of hyaluronan for surface tension measurement in 0.15 M NaCl solution.

Mw	Lot number	Supplier
90 kDa	050607	Contipro Biotech Ltd., Czech Republic
1.4 MDa	210306-D2	

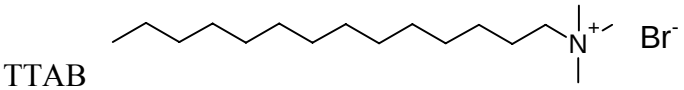
Tab. 2 Specification of hyaluronan for surface tension measurement in water.

Mw	Lot number	Supplier
110–130 kDa (116*)	210-493	Contipro Biotech Ltd., Czech Republic
1750–2000 kDa (1800*)	211-180	
1500–1750 kDa (1730*)	210-636	

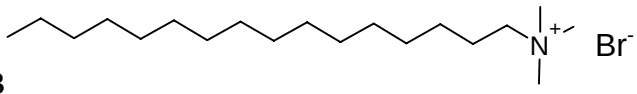
* *Numbers in brackets correspond to precise molecular weight obtained by HPLC/SEC-MALS analysis provided by the supplier.*

Surfactants were of the best available quality and were used as received without further purification. The detailed information is summarized in Tab. 3.

Tab. 3 Specification of surfactants for both surface tension measurement and microcalorimetry.



TTAB



CTAB

	Mw	Lot number	Supplier
TTAB	336.4 g/mol	1377175	Sigma Aldrich, Czech Republic
CTAB	365 g/mol	117K0732 059K0041 BCBC4707V	

5.2 Methods

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 1 g/l (~ 0.1% w/v ~ 1000 mg/l) and 15 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 being measured, samples with pure surfactant were stirred for 4 hours.

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 which is shown in Fig.16. 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.

a)  b) 

Fig.16 *Tensiometer KSV Sigma 701* [69] *(a) and a measuring vessel with the ring (b).*

The tensiometer for maximum bubble pressure determination BPA-800P is shown in Fig.17 a). The instrument was set to the standard measuring method, i.e. with the increasing gas-flow velocity from 10 ms up to 10 s. As shown in Fig.17 b), the only disadvantage can be the formation of foam at the surface due to the presence of concentrated surfactant in the sample.

a) 

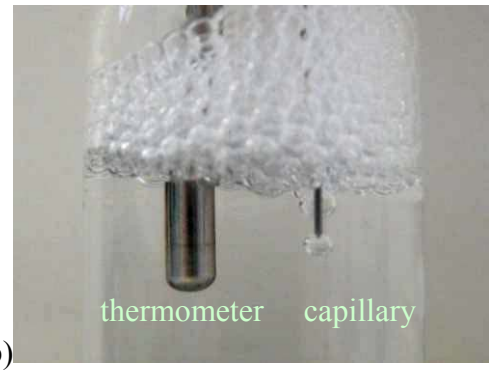
b) 

Fig.17 *Tensiometer BPA-800P (a) and the bubble formation during the measurement (b).*

5.3 Results and discussion

5.3.1 Surface tension of samples in physiological solution

The obtained results from surface tension measurement of samples in physiological solution are summarized in ref. [68], see the attached research article in Appendix I. The most important conclusions and comments are listed here in the following text.

The value of the CMC of CTAB in 0.15 M NaCl was practically not affected by addition of hyaluronan. Surface tension measurement method indicated that hyaluronan influenced only the interactions within the CTAB surface layer and did not involve into the micellization process. This conclusion could be the result of screening the electrostatic interactions between hyaluronan and CTAB by the presence of NaCl in physiological solution which seems to be more effective in the bulk solution than in its surface layer.

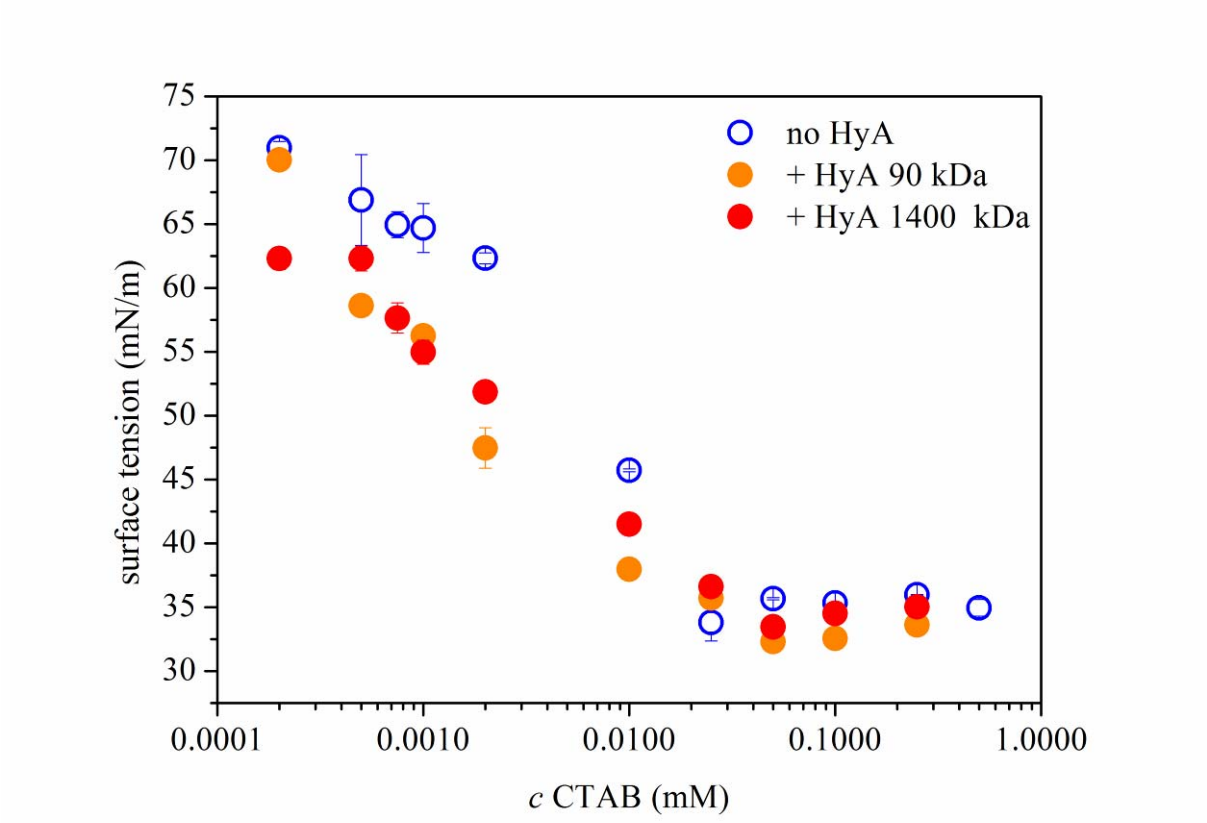


Fig.18 *Surface tension of CTAB with hyaluronan 90 and 1400 kDa in 0.15 M NaCl solution at 25°C, ring method.*

Interestingly, hyaluronan affected surface tension in the premicellar region, especially in the case of CTAB (see and cf. Fig.18 and Fig.19). The surface tension is lower in comparison to the pure surfactant solution. Besides the mentioned electrostatic repulsion effects it might be also caused by sterical or excluded volume effects, and perhaps also due to hydrophobic effect – the lowering is more remarkable for CTAB which has longer alkyl chain then TTAB and in the case of TTAB it is observed only in the presence of the long chain hyaluronan (Fig.19). Moreover, the lower surface tension may be also caused by stronger binding of sodium from the added salt to hyaluronan as counterions. Nevertheless, molecules of surfactants are bound to hyaluronan chain which increases surface activity of such a formation.

Surface properties of hyaluronan solution as itself were studied in ref. [30] with the result that it does not show any surface activity.

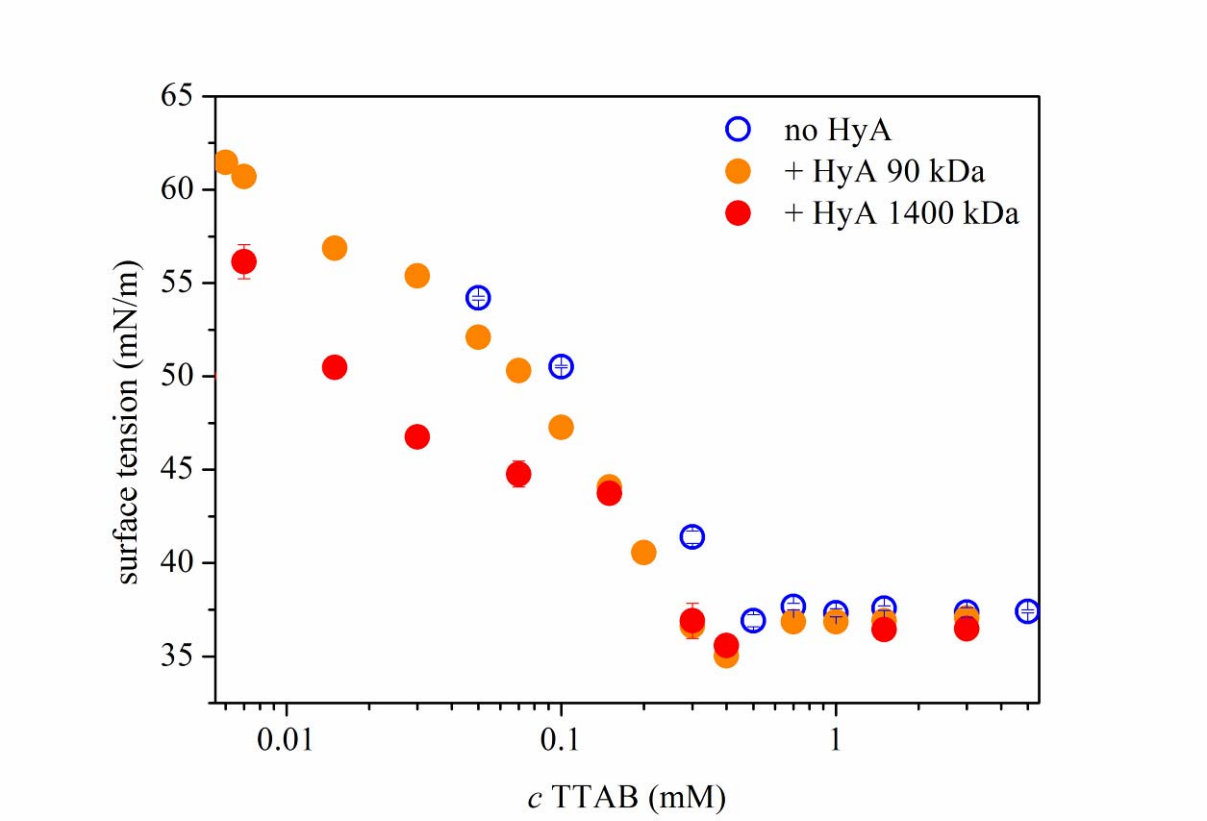


Fig.19 *Surface tension of TTAB with hyaluronan 90 and 1400 kDa in 0.15 M NaCl solution at 25°C, ring method.*

The published paper [68] describes interactions of ten different surfactants with hyaluronan and the results concerning its effect on critical micellar (aggregation) concentration are summarized in Tab. 4. The greatest difference between pure surfactant in physiological solution and surfactant with the addition of hyaluronan was found in fluorescence data for nonionic Tween 20 and cationic CTAB. The situation with Tween 20 was surprising because of its nonionic nature. The interactions with CTAB were expected, they manifested in increased CMC values or, in other words, in the shift of solubilization capability to higher surfactant concentrations as a result of the hydrophobic interactions of a long surfactant alkyl chain with low-polarity parts of hyaluronan backbone.

From the point of view of fluorescence, no solubilization was detected at cationic surfactant concentrations below the CMC in saline solution using pyrene as a probe. Moreover, the nile red emission maxima showed broadening of the micellar region which means that the probe molecules may be located in environments of different polarity. Hyaluronan of both molecular weights increased the CMC of CTAB detected by the inflex point on the pyrene polarity index curve.

Tab. 4 Effect of low (LMW, 90 kDa) and high (HMW, 1400 kDa) molecular weight hyaluronan on the critical micellar concentration (CMC) as determined by fluorescence (pyrene as a probe, upper values) and surface tension (bottom values) methods in ref. [68], in 0.15 M NaCl at 25°C.

surfactant	CMC (mmol/l)		
	no HyA	LMW HyA	HMW HyA
sucrose monolaurate	0.288 ± 0.006	0.26 ± 0.05	0.25 ± 0.03
	0.25 ± 0.02	0.26 ± 0.03	0.3 ± 0.1
n-dodecyl-β-D-maltoside	0.160 ± 0.001	0.150 ± 0.003	0.160 ± 0.002
	0.149 ± 0.007	0.136 ± 0.006	0.19 ± 0.08
octyl-β-D-glucopyranoside	20.2 ± 0.1	19.9 ± 0.3	19.9 ± 0.3
	20.6 ± 0.4	19 ± 1	21 ± 2
Tween 20	0.048 ± 0.001	0.014 ± 0.001	0.014 ± 0.002
	0.072 ± 0.003	0.053 ± 0.004	0.05 ± 0.02
SDS	1.04 ± 0.01	0.740 ± 0.001	0.80 ± 0.03
	0.87 ± 0.05	0.7 ± 0.1	0.7 ± 0.2
TTAB	0.52 ± 0.03	0.51 ± 0.03	0.61 ± 0.01
	0.49 ± 0.02	0.58 ± 0.01	0.5 ± 0.2
CTAB	0.062 ± 0.002	0.12 ± 0.02	0.11 ± 0.02
	0.030 ± 0.004	0.025 ± 0.006	0.040 ± 0.001
CTAT	0.056 ± 0.001	0.072 ± 0.001	0.15 ± 0.05
	0.036 ± 0.004	0.099 ± 0.007	0.058 ± 0.008
cetyl betaine	0.016 ± 0.001	0.009 ± 0.001	0.021 ± 0.005
	0.009 ± 0.001	0.010 ± 0.001	0.051 ± 0.003
Betadet THC2	0.14 ± 0.01	0.10 ± 0.01	0.100 ± 0.001
	0.13 ± 0.03	0.10 ± 0.03	0.27 ± 0.03

To sum it up, interactions between hyaluronan and CTAB showed in increasing CMC and broadening of micellization regions as detected by both pyrene and nile red probes. When compared the aggregation of CTAB and TTAB, the results showed that the aggregation of CTAB is much more affected by the presence of hyaluronan which could be attributed to the longer surfactant alkyl chain.

Surface tension measurements with hyaluronan confirmed no hyaluronan surface activity [70] up to the concentration 2 g/l. All measured data for the dependence of the surface tension on surfactant concentration in physiological solution showed no or small effect of hyaluronan on

surface activity and particularly on the value of the critical micelle concentration. Hyaluronan of both molecular weights slightly decrease the surface tension of CTAB in physiological solution in the premicellar region. However, the CMC is not affected. Tensiometry thus indicates that hyaluronan influences only the interactions within the CTAB surface layer and does not affect in the micellization process. It is the result of screening the electrostatic interactions between hyaluronan and CTAB by the presence of NaCl in physiological solution which seems to be more effective in the bulk solution than in its surface layer. Similar decrease of surface tension in the premicellar region was observed also for SDS and for TTAB in the presence of high molecular weight hyaluronan.

In addition, the greatest differences in behavior of pure surfactant in saline solution and surfactant with added hyaluronan were found for nonionic Tween 20 and cationic CTAB. Specifity of Tween 20 was attributed to its structure where a sugar-based polar head provides weak physical interactions. Fluorescence data also demonstrated differences between the results obtained with different fluorescence probes which are caused by their different location and solubility in the studied system.

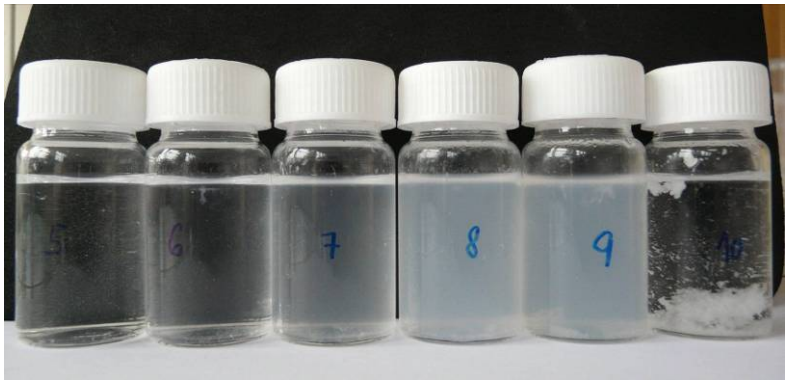
When the CMC values in Tab. 4 are compared from the point of view of measuring method, they differ more or less within every surfactant because the fluorescence curve are very broad and it made some uncertainty to identify or calculate the right position of CMC. The other reason for that is also the influence of the fluorescence probe in the samples.

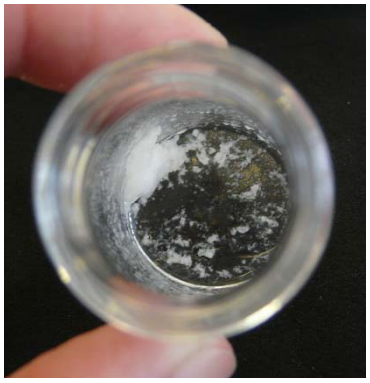
5.3.2 Surface tension of samples in water

5.3.2.1 Preliminary experiments

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. This behavior is illustrated in Fig.20 for TTAB. The observation of samples brought up the following questions: What is the surface tension of the liquid phase (after removal of phase separated particles, precipitate or gel)? Is there any free surfactant after the phase separation capable of decreasing the surface tension?

a)





b)

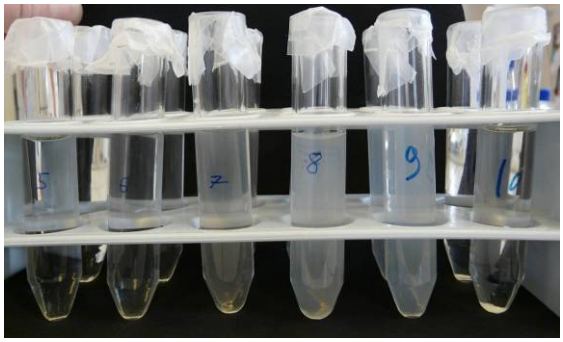


Fig.20 *Samples of TTAB with hyaluronan (M_w 1730 kDa, 1000 mg/l) before (a) and after (b) centrifugation. For surfactant concentrations see Tab. 5.*

The samples for this analysis were prepared in the glass vials and then centrifuged in the glass test tubes under 4000 rpm for 10 minutes. Concentrations of TTAB in the series of samples are summarized in Tab. 5, concentration of hyaluronan is 1000 mg/l.

Tab. 5 Concentration of TTAB in the samples for centrifugation, concentration of hyaluronan was 1000 mg/l; solution in water.

sample number	c (mM)	charge ratio surfactant : HyA
5	0.5	0.2
6	0.75	0.3
7	1	0.4
8	2	0.8
9	2.5	1
10	5	2

The charge ratio surfactant : HyA mentioned in Tab. 5 and Tab. 6 corresponds to the ratio of concentration of charges on the surfactant molecule and hyaluronan. Whereas both surfactants TTAB and CTAB have one positive charge in their structure and hyaluronan has one negative charge per one disaccharide unit, it is necessary to calculate the concentration of charges on hyaluronan chain according the following relation (the weight of one disaccharide unit is 400 g/mol):

$$\frac{\text{concentration of hyaluronan in the solution (g/l)}}{\text{weight of one disaccharide unit (g/mol)}} = \text{concentration of disaccharide units (mol/l)}$$

Consequently, the charge ratio surfactant : HyA is calculated as the ratio of molar surfactant concentration and concentration of disaccharide units.

The ratio of charges gives the information about the possible free or occupied hyaluronan charged groups, in other words, the ratio higher than 1 indicates the presence of potentially free molecules of surfactant in the system which are suggested to be responsible for lowering the surface tension at higher surfactant concentration in the samples with addition of hyaluronan. For example, the sample 10 in Fig.20 shows the phase separation at the charge ratio 2.

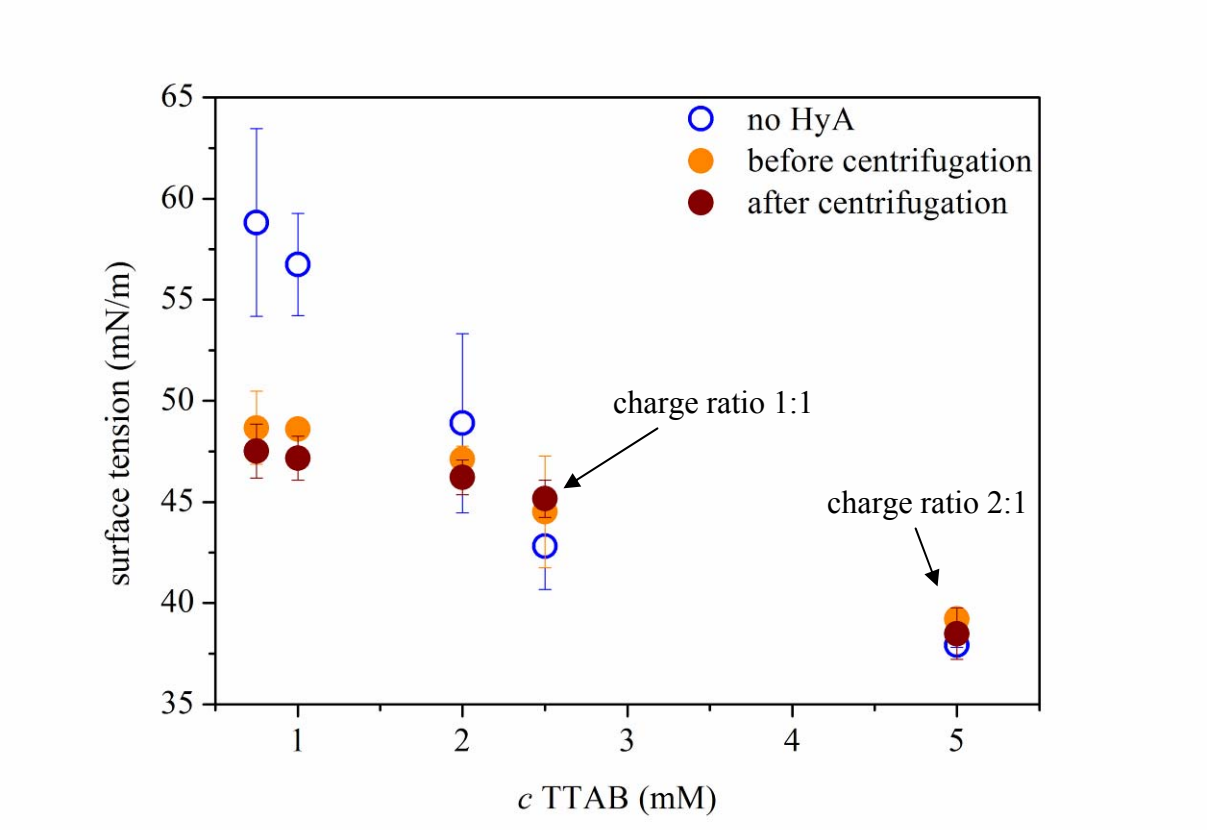


Fig.21 *Surface tension of TTAB with hyaluronan 1730 kDa (1000 mg/l) before and after the centrifugation at 25°C, ring method.*

The separation of the solid phase (precipitates, gel) was efficient only in the last sample with TTAB concentration of 5mM. The other samples did not show any optical changes – they remained with the opaque and cloudy character. Surface tension was measured before and

after the centrifugation and the results of this experiment are shown in Fig.21 together with the data for pure surfactant in water.

During the measurement of the samples with high surfactant concentration with the solid phase some parts of that precipitation stick to the ring and the repeated measurements (every sample was measured three times) was performed without these precipitations because the ring was cleaned and flamed after every experiment. We were afraid that the missing precipitation will cause the rapid change in the surface tension values. But the results showed that there was still a huge amount of surfactant monomers in the surface layer which were responsible for decreasing of surface tension.

To sum it up, surface tension of centrifuged samples decreased very slightly and furthermore, there is practically no influence on the CAC determination. Due to this fact, no samples were centrifuged before being measured even though they contained the precipitation.

Fig.21 indicates that even in the case of samples with precipitated hyaluronan-surfactant complex (TTAB concentration of 5 mM) there is still sufficient amount of surfactant in the liquid surface layer giving as low surface tension as in the case when no hyaluronan is present which is in agreement with the calculated charge ratio in Tab. 5.

5.3.2.2 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. 6. 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. 6 Concentrations of CTAB for surface tension measurement.

no HyA		with presence of HyA (1000 mg/l)		
sample number	c (mM)	sample number	c (mM)	charge ratio surfactant : HyA
1	0.001	1	0.001	0.0004
2	0.01	2	0.005	0.002
3	0.05	3	0.01	0.004
4	0.075	4	0.02	0.008
5	0.1	5	0.04	0.016
6	0.5	6	0.08	0.032
7	1	7	0.1	0.04
8	2.5	8	0.15	0.06
9	5	9	0.2	0.08
10	10	10	0.25	0.1
		11	0.3	0.12
		12	0.35	0.14
		13	0.4	0.16
		14	0.8	0.32
		15	1	0.4
		16	2	0.8
		17	5	2

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 [30]. The results showed that hyaluronan itself does not decrease surface tension 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.22) than those values for pure CTAB. It is the similar situation to that discussed previously for the system containing CTAB in 0.15 M NaCl (Fig.18).

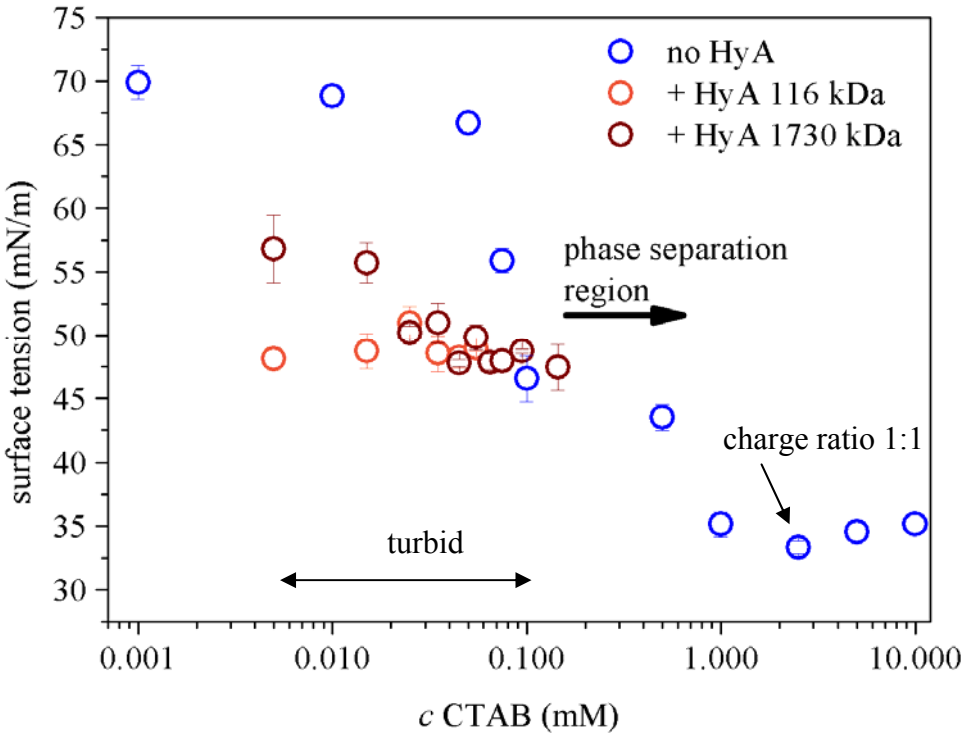


Fig.22 *Surface tension of CTAB in water and with addition of hyaluronan determined by the ring method (hyaluronan concentration 1000 mg/l) at 25°C.*

Fig.22 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. The arrow in Fig.22 indicates the beginning of the concentration region when the phase separation occurred.

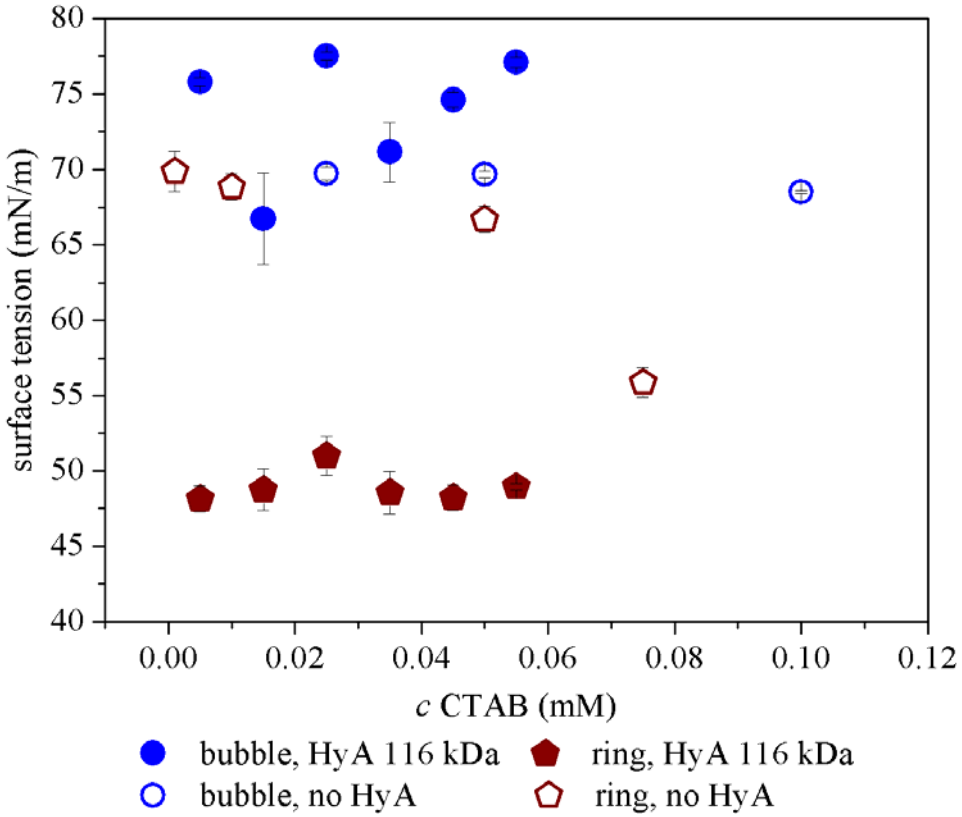


Fig.23 *Comparison of surface tension values obtained by the ring and maximum bubble pressure method, t_{life} = 0.7 s (CTAB with hyaluronan 116 kDa, concentration 1000 mg/l) at 25°C.*

As mentioned above in the section 5.2, 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.

The almost constant surface tension of CTAB – hyaluronan system is shown in Fig.23. The results obtained by maximum bubble pressure method showed higher values of the interfacial tension in the whole concentration range when compared with the ring method. It is probably caused by the effect of the bubble lifetime. In the bubble pressure method the lifetime is too short for rather long and large CTAB molecules to arrive at the fresh bubble surface and to decrease the surface tension. Addition of hyaluronan has no substantial effect on the results of bubble pressure method in contrast to the ring method. Again it is a consequence of slow surfactant movement, especially in the hyaluronan containing media with increased viscosity.

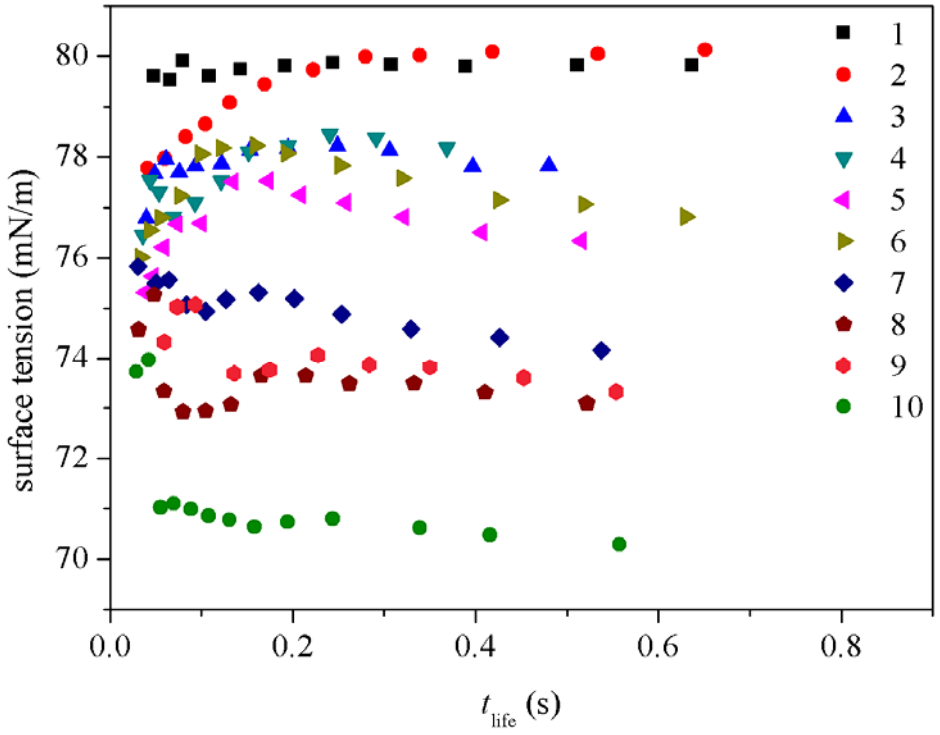


Fig.24 *Surface tension of CTAB with added hyaluronan (1800 kDa) at increasing time of life of the bubbles at 25°C. Surfactant concentrations increasing from 0.005 to 0.145 mM, for details see Tab. 7.*

Fig.24 shows surface tension of CTAB solutions in the concentration range far below its CMC and in the presence of high molecular weight hyaluronan as determined by the maximum bubble pressure method for various bubble lifetimes; the surfactant concentrations are listed in Tab. 7. Although the data are rather scattered they show decreasing surface tension with increasing surfactant concentration and mostly a weak or negligible dependence on the bubble lifetime. This is rather in contrast with behavior of CTAB aqueous solution, see Fig.25, where a surface tension decrease is observed even at very low surfactant concentrations at the lowest bubble lifetimes. Thus at very short lifetimes hyaluronan seems to hinder surfactant movement to the liquid surface and prevents reduction in surface tension.

Fig.25 also shows substantial decrease of surface tension at CTAB concentrations closer or around its CMC but the surface tension is still strongly dependent on the bubble lifetime and at very low lifetimes the reduction of the surface tension is not dramatic. More detailed discussion of these data is summarized in a bachelor thesis [71] where I participated as a consultant.

Tab. 7 Concentration series of samples with CTAB and hyaluronan (1800 kDa) for the measurement using the maximum bubble pressure method.

sample number	concentration of CTAB (mM)
1	0.005
2	0.015
3	0.025
4	0.035
5	0.045
6	0.055
7	0.065
8	0.075
9	0.095
10	0.145

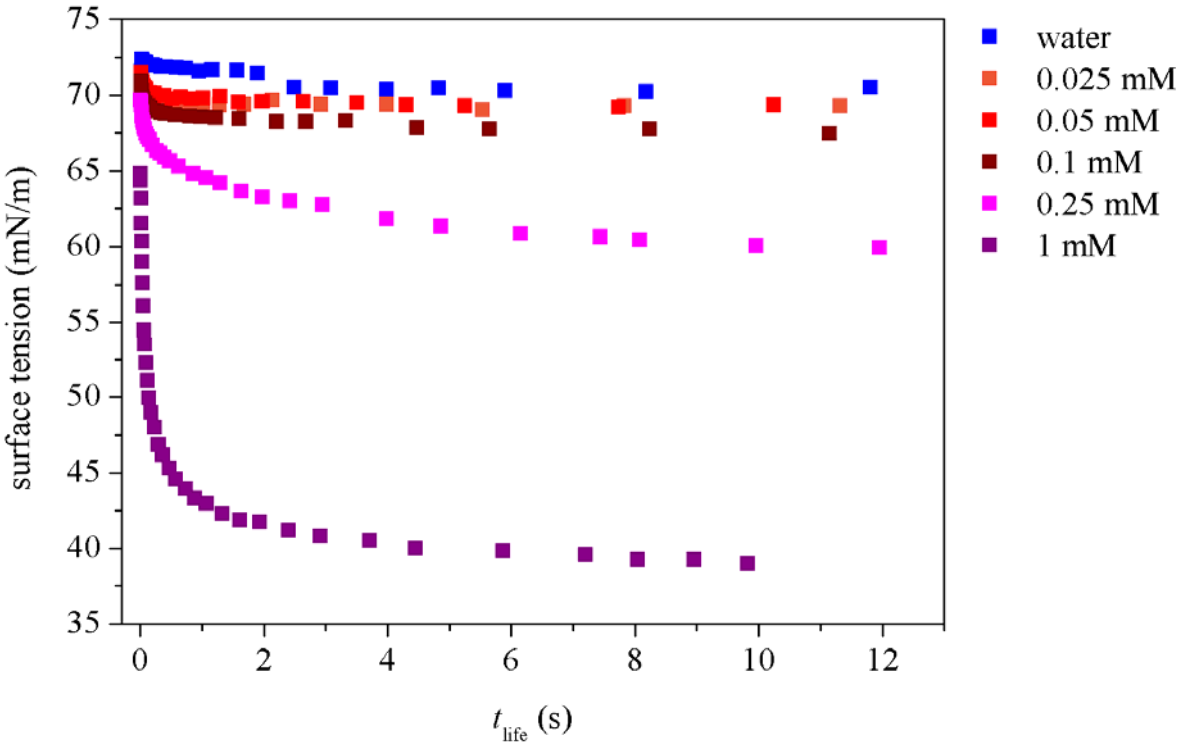


Fig.25 *Surface tension of water and CTAB without hyaluronan at increasing time of life of the bubbles at 25°C* [71].

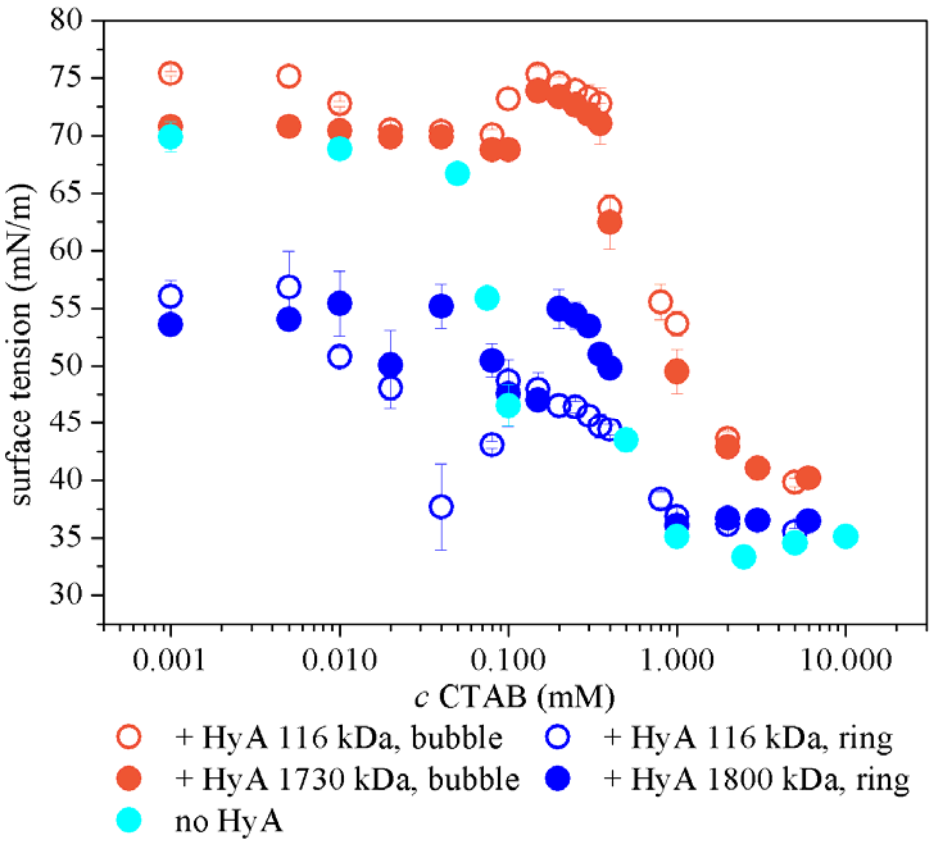


Fig.26 *Comparison of surface tension values obtained by the ring and maximum bubble pressure method (t_{life} = 0.3 s, CTAB with hyaluronan 116 and 1730 kDa, concentration 15 mg/l) at 25°C.*

The experiments concerning surface tension of surfactants and hyaluronan in this chapter were performed with two different hyaluronan concentrations 15 and 1000 mg/l. The reason for chosing two concentrations is explained bellow.

When the curves in Fig.26 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 then 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.26. The explanation corresponds to the previous observation of system (Fig.23) where the ring method indicated lower surface tension values in the premicellar region even in the case of lower molecular weight hyaluronan than the bubble method.

The inspiration in choosing low hyaluronan concentration 15 mg/l is described in ref. [72]. 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.27.

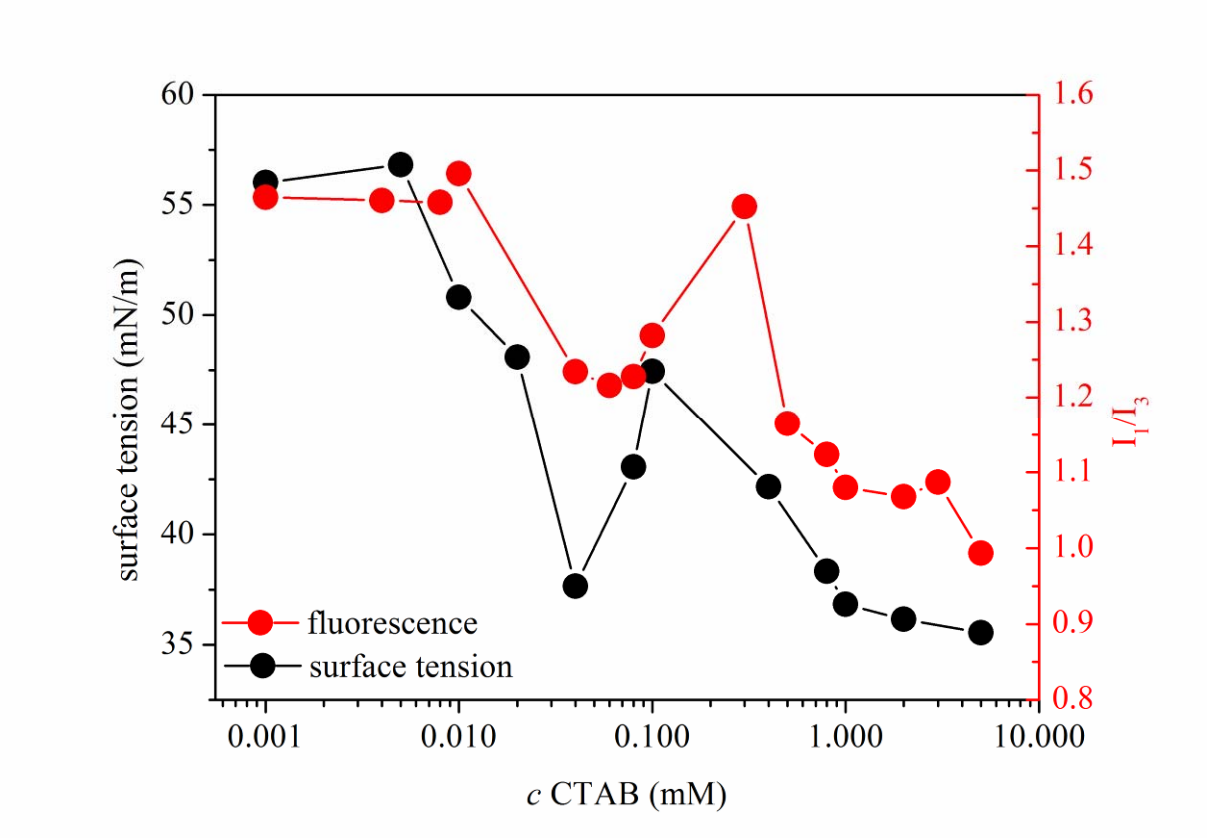


Fig.27 *Surface tension (ring method, black) and fluorescence (red) data of hyaluronan-surfactant system at 25°C. Hyaluronan 650 kDa, concentration 15 mg/l, I_1/I_3 is the polarity index.*

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. [72] 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 fluorescence plot in Fig.27 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 [72] 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. In fact, the similar shape of the surface tension curves are showed in Fig.26.

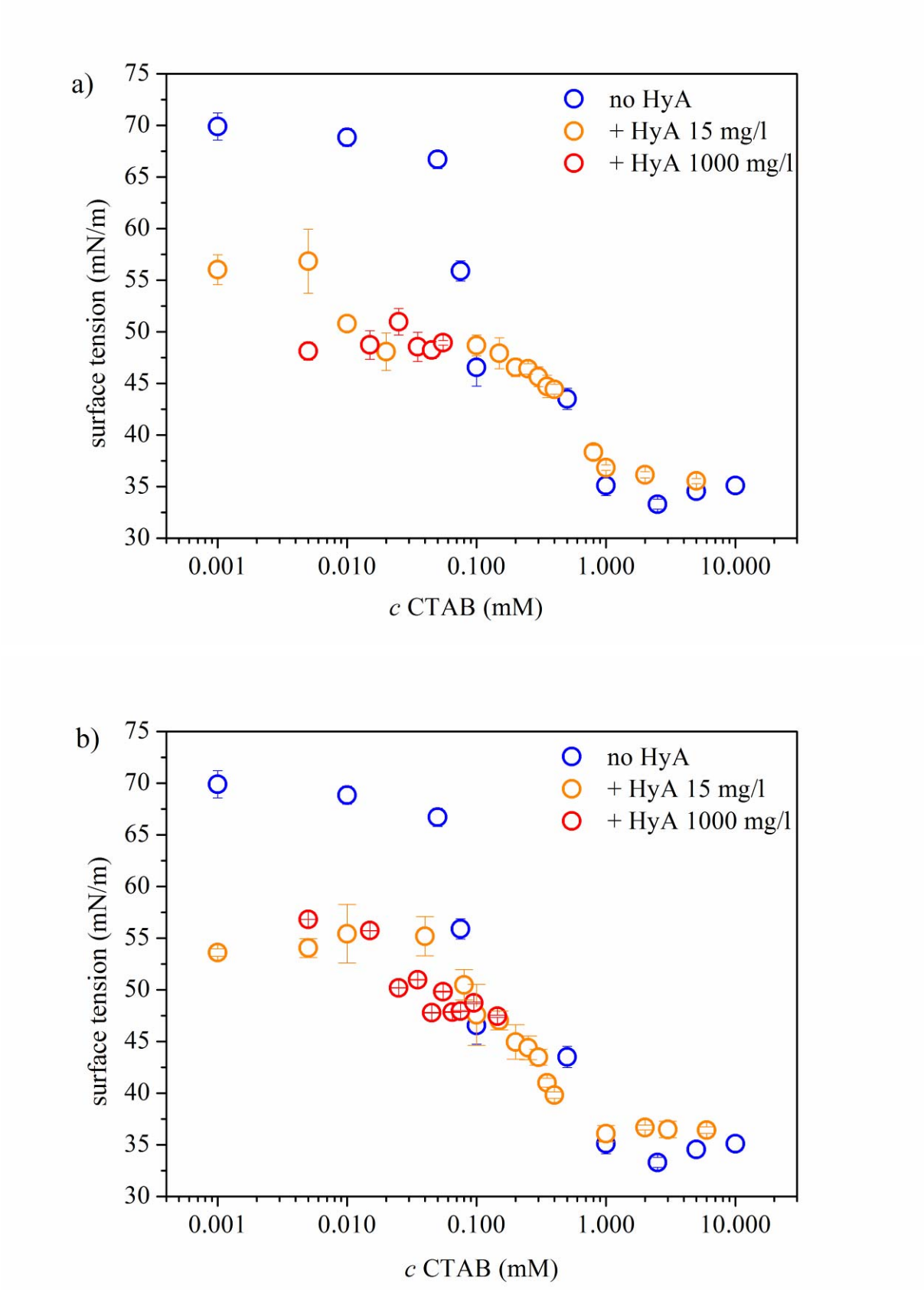


Fig.28 *Surface tension of CTAB and hyaluronan a) 116 kDa and b) 1730 kDa of both hyaluronan concentrations 15 and 1000 mg/l determined by the ring method at 25°C.*

Results obtained by the ring method for hyaluronan of two different molecular weights at two different concentrations are summarized in Fig.28. The first conclusion of the results shown in Fig.28 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.

5.3.2.3 Interactions between hyaluronan and TTAB

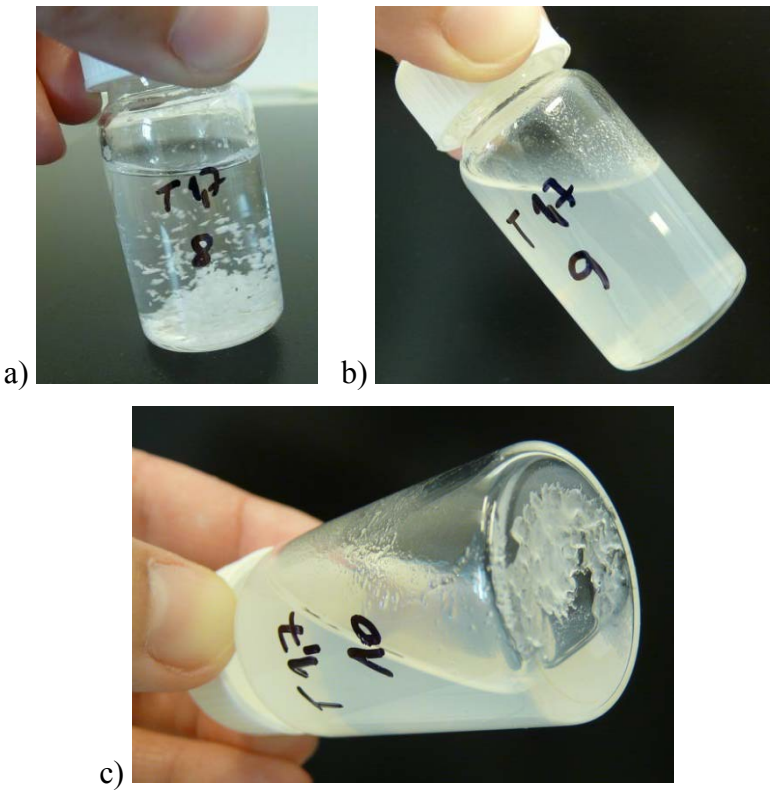


Fig.29 *Samples of TTAB with hyaluronan (1800 kDa, 1000 mg/l) in water with different surfactant concentration: a) 5, b) 7 and c) 10 mM.*

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. The last samples in the concentration series are shown in Fig.29. Sample 8 (conc. 5 mM of TTAB) is clear with small white flakes. Samples 9 and 10 (conc. 7 and 10 mM of TTAB) had milky opacity and the hyaluronan gel was sticked on the wall of the vial. However, the process of aggregation occurs in the concentration range and not just at one determined surfactant concentration.

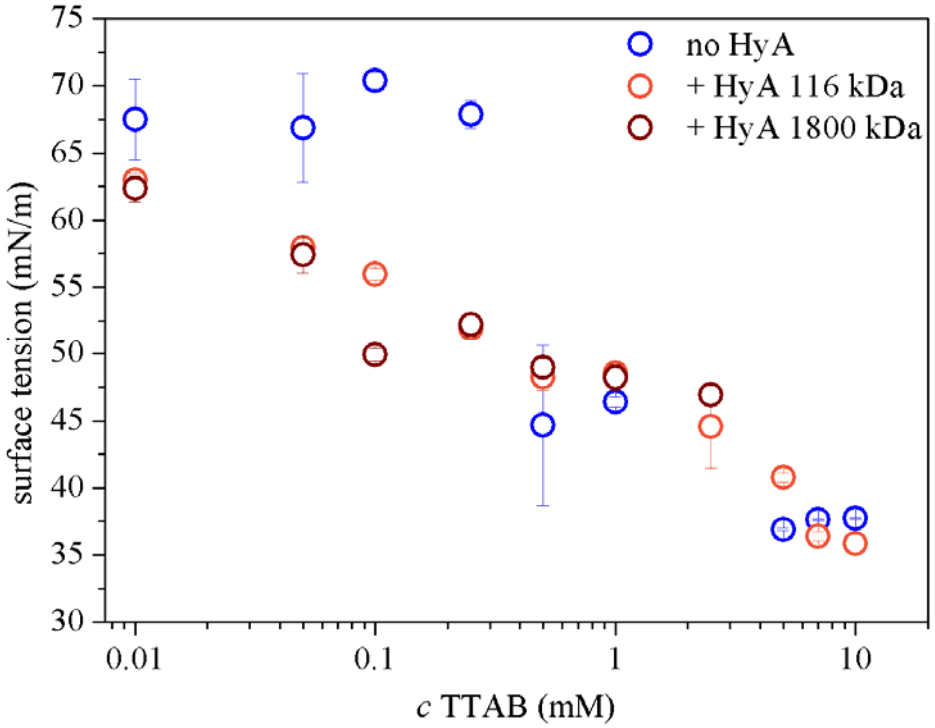


Fig.30 *Surface tension of TTAB and hyaluronan (116 and 1800 kDa, 1000 mg/l) determined by the ring method at 25°C.*

The results from surface tension shown in Fig.30 and Fig.31 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.

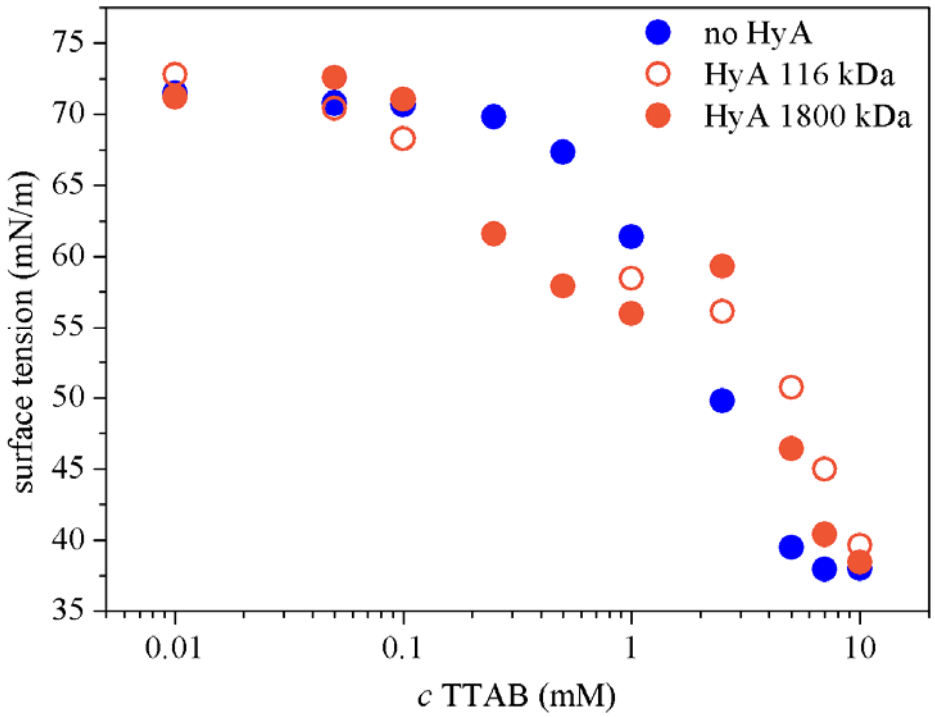


Fig.31 *Surface tension of TTAB and hyaluronan (116 and 1800 kDa, 1000 mg/l) determined by the maximum bubble pressure method at 25°C, t_{life} = 0.5 s (the error bars are smaller than the symbol signs).*

The lowering of surface tension determined with the maximum bubble pressure method was observed in the whole concentration range as shown in Fig.31. 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. In addition, the surface tension values do not show any increasing trend in the system with added hyaluronan at low surfactant concentration as was observed in the case of CTAB (Fig.26). It should confirm the idea that the increasing of surface tension could be caused by sufficiently long nonpolar surfactant chain (see above section 5.3.2).

6 EXPERIMENTAL PART II – MICROCALORIMETRY

This part of the thesis is divided into two parts because the work was performed using two different models of the same microcalorimeter (older type TAM 2277 and new type TAM III) at different universities. The aim was to obtain the basic thermodynamic characteristics of the interactions between hyaluronan and surfactant. The results are summarized as a manuscript of a research article in Appendix II.

6.1 Isothermal titration calorimetry performed using TAM 2277

6.1.1 Materials

Hyaluronans used for microcalorimetric measurements are listed in Tab. 8. The specification of the surfactants was mentioned previously in Tab. 3.

Tab. 8 Specification of hyaluronans for microcalorimetry.

Mw	Lot number	Supplier
70–120 kDa (87*)	209-073	Contipro Biotech Ltd., Czech Republic
110–130 kDa (116*)	210-493	
1700–2000 kDa (1800*)	211-180	

* *Numbers in brackets correspond to precise molecular weight obtained by HPLC/SEC-MALS analysis provided by the supplier.*

6.1.2 Method

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) which is shown in Fig.32. 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.

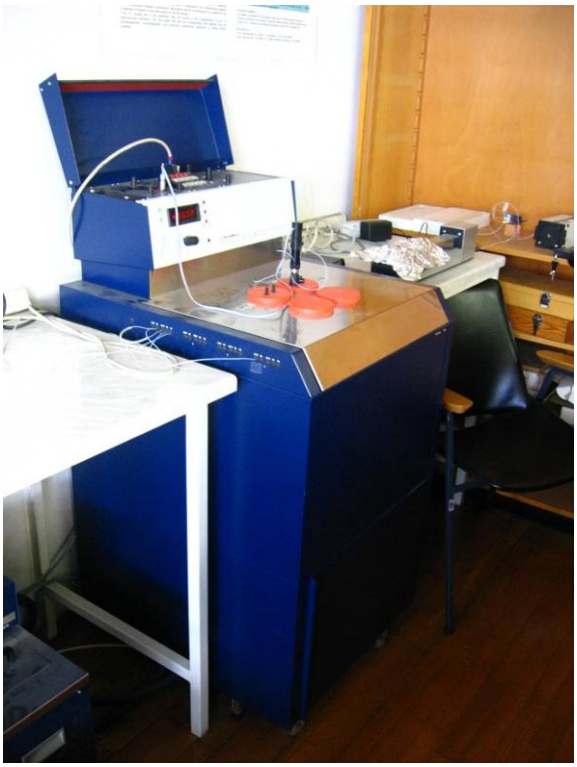


Fig.32 *Microcalorimeter TAM 2277.*

6.1.3 Results and discussion

The results from the titration experiments reflecting the interactions between CTAB and TTAB with two different molecular weight of hyaluronan performed using the microcalorimeter TAM 2277 are shown in Fig.33. The results obtained using both microcalorimeters TAM 2277 and TAM III were in a good agreement so the discussion of all the results is summarized below in chapter 6.2.3.

The shapes of the enthalpograms were compared with the published types presented in ref. [62] and the best comparison was found with the type A and B shown in Fig.15. For Type A plot the recorded heat of injection changes sharply between two parts of the titration curve over which the recorded heats are effectively independent of the composition of the solution in the sample cell. Type B plot shows the change which is less sharp and both parts of the plot indicate the dependences on heat on solution composition.

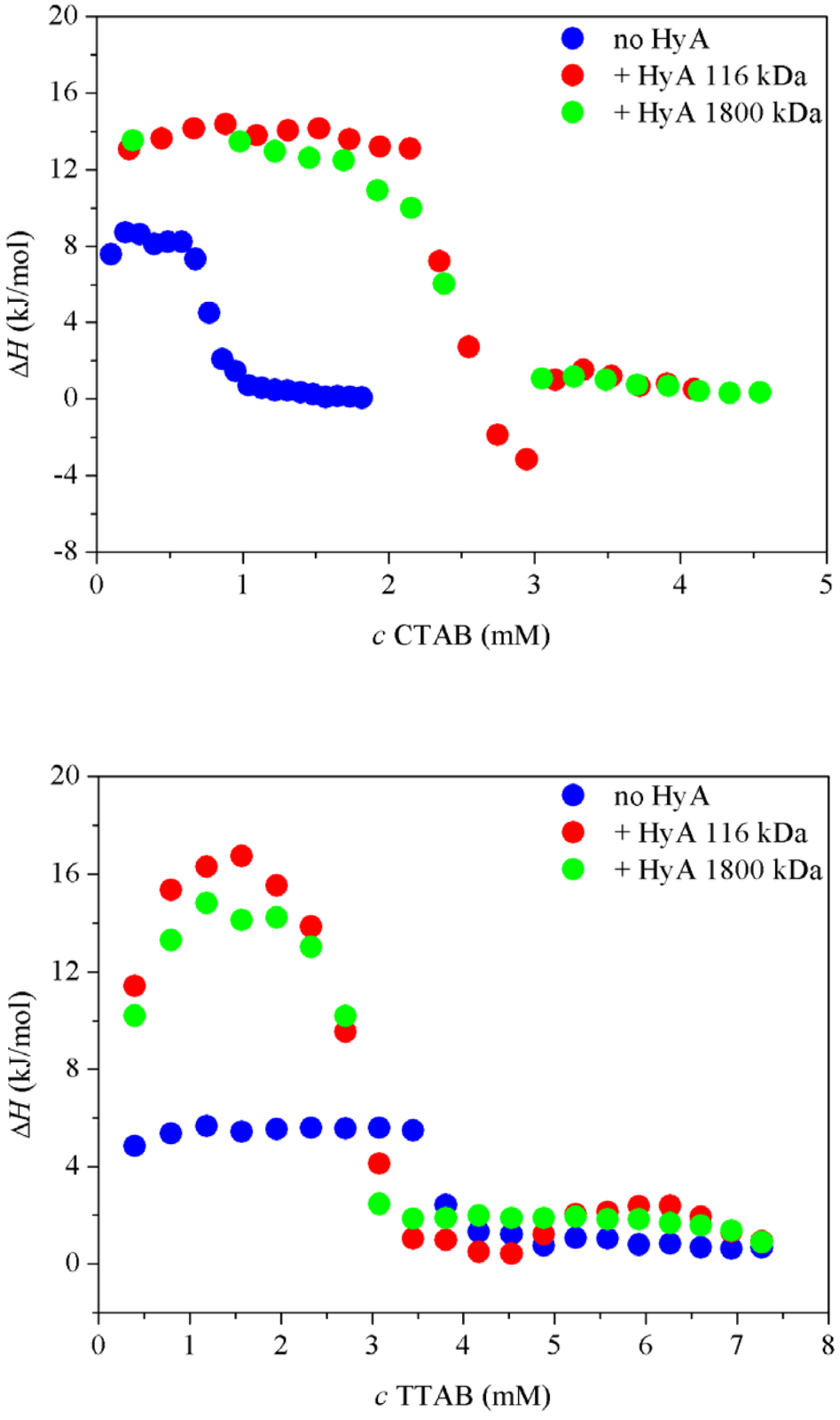


Fig.33 *Titration of CTAB (upper) and TTAB (lower) with hyaluronan at conc. 1000 mg/l in water at 25°C.*

6.2 Isothermal titration calorimetry performed using TAM III

The second part of the experiments was performed using a modular microcalorimeter TAM III (TA Instruments, USA, Fig.34) at the home faculty.

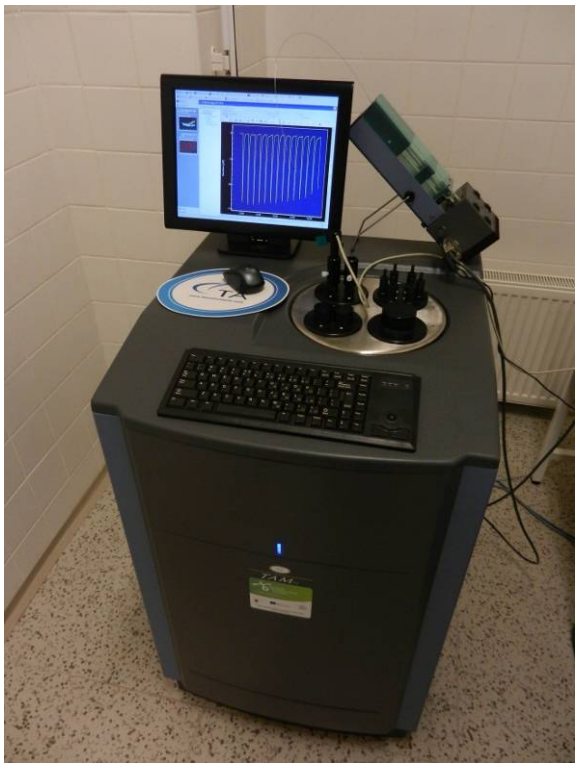


Fig.34 *Microcalorimeter TAM III.*

6.2.1 Materials

The specification of surfactants and hyaluronan for microcalorimetry is described above in Tab. 3 and Tab. 8.

6.2.2 Method

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 (87, 116 and 1800 kDa) and its two concentrations 15 and 1000 mg/l. All the experiments were performed at 25°C in aqueous solution.

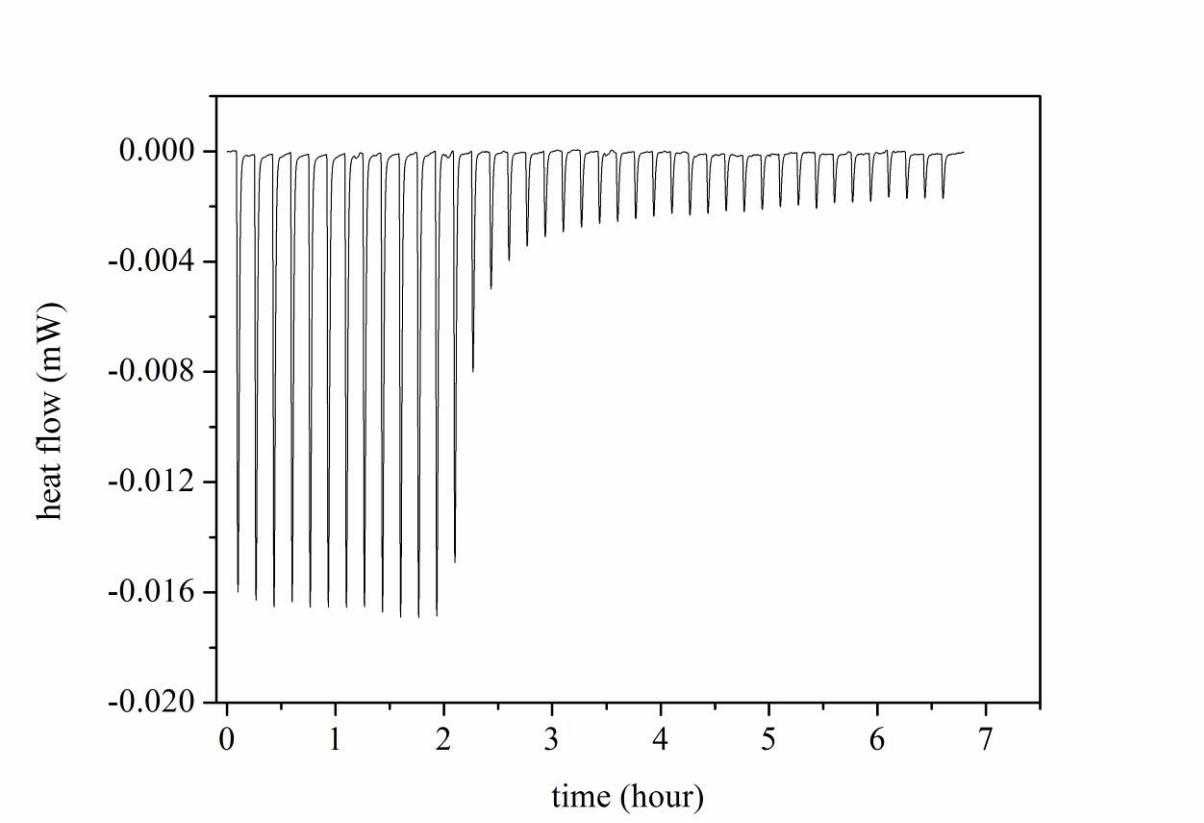


Fig.35 *Typical titration record from the microcalorimeter TAM III (titration of TTAB 50 mM into water at 25°C).*

An example of the raw data (endothermic character) is shown in Fig.35. The stock solutions of surfactant were prepared at concentration at least ten times higher that 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.

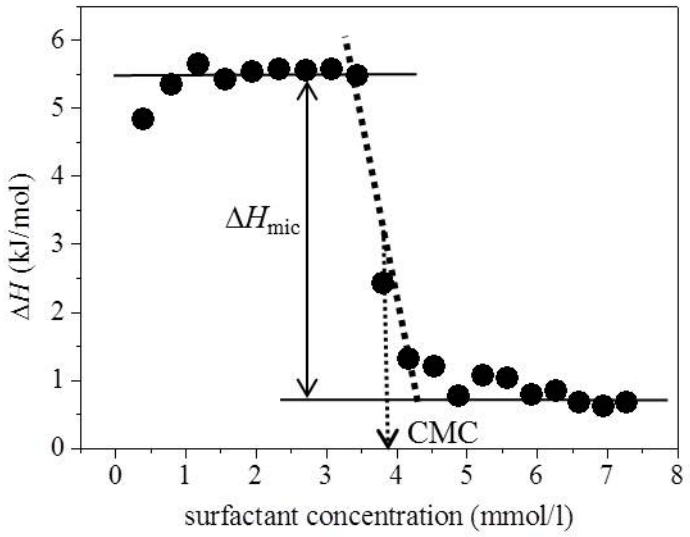


Fig.36 *Scheme of the evaluation of CMC and ΔH_{mic} from the enthalpogram.*

The peak areas from Fig.35 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.36 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 in the inflection point of the enthalpogram (dotted arrow in Fig.36).

6.2.3 Results and discussion

6.2.3.1 Interactions of hyaluronan and CTAB

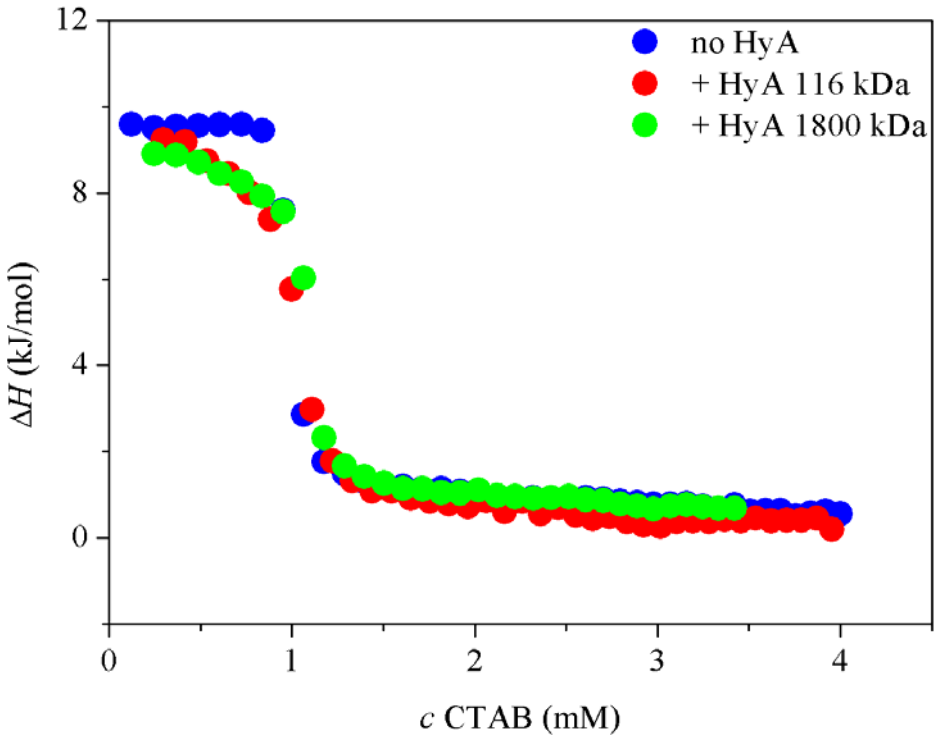


Fig.37 *Interaction of CTAB with hyaluronan (conc. 15 mg/l) of different molecular weight in water at 25°C.*

As expected, the low concentration of hyaluronan did not have any significant influence on the shape of the titration curve (Fig.37). 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 [73].

In contrary, very interesting interaction behavior of this system was observed by fluorescence measurement [72]. With respect to the smooth shape of the resulting curves in Fig.37 this behavior should be coupled with a very slight heat effect which was not detected using the ITC technique.

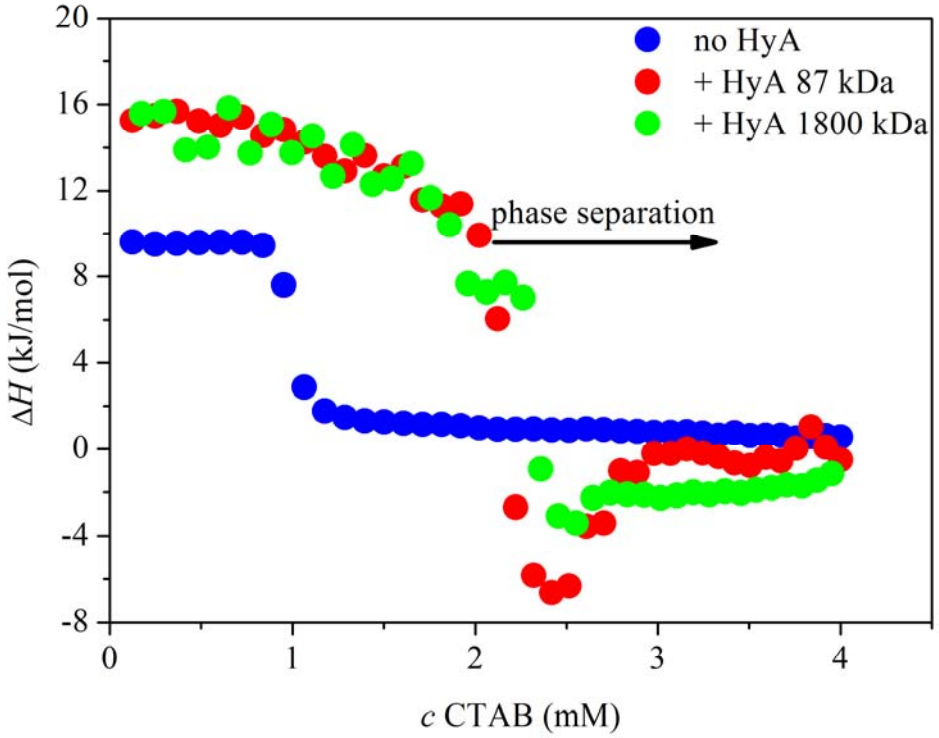


Fig.38 *Interaction of CTAB with hyaluronan (conc. 1000 mg/l) of different molecular weight in water at 25°C.*

The effect of concentrated hyaluronan on the aggregation with CTAB is shown in Fig.38. There are three main differences when results in Fig.37 and Fig.38 are compared. The upper plateau in Fig.38 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 up to 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. Then, 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.38 is not put to the same concentration as previously in Fig.22.

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. 9. 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. More detailed discussion can be found in the manuscript attached in Appendix II.

Tab. 9 Enthalpy of micellization and critical aggregation concentrations 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%

6.2.3.2 Interactions of hyaluronan and TTAB

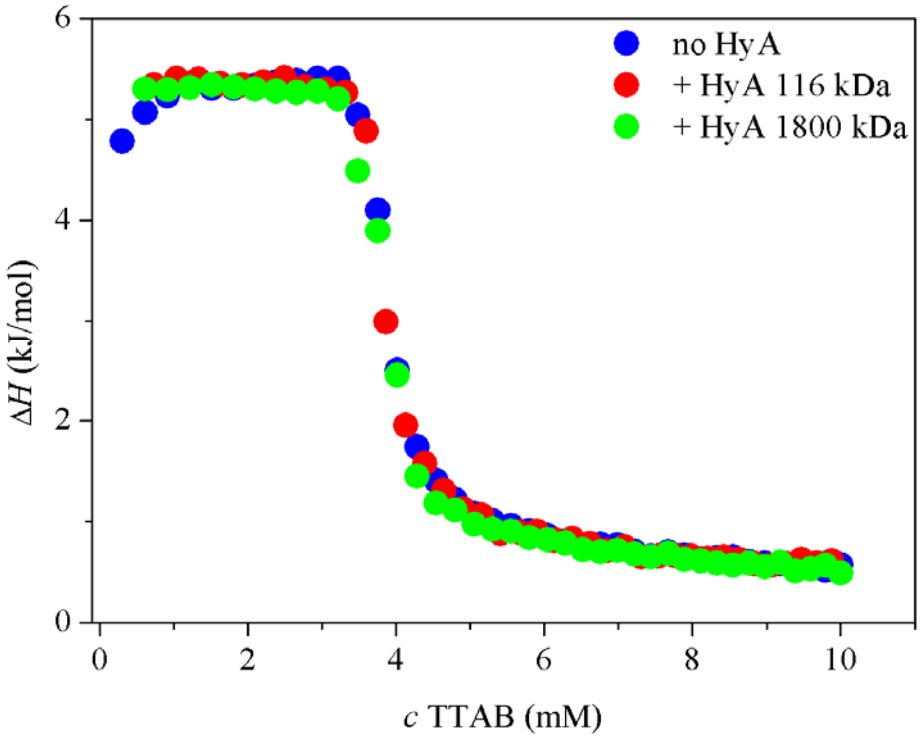


Fig.39 *Interaction of TTAB with hyaluronan (conc. 15 mg/l) of different molecular weight in water at 25°C.*

The plot in Fig.39 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 measurable effect on the aggregation properties of TTAB (as described above in the case of CTAB, Fig.37). The value of the CMC is in a good agreement with the literature value which is 4–5 mM in water [73]. 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.

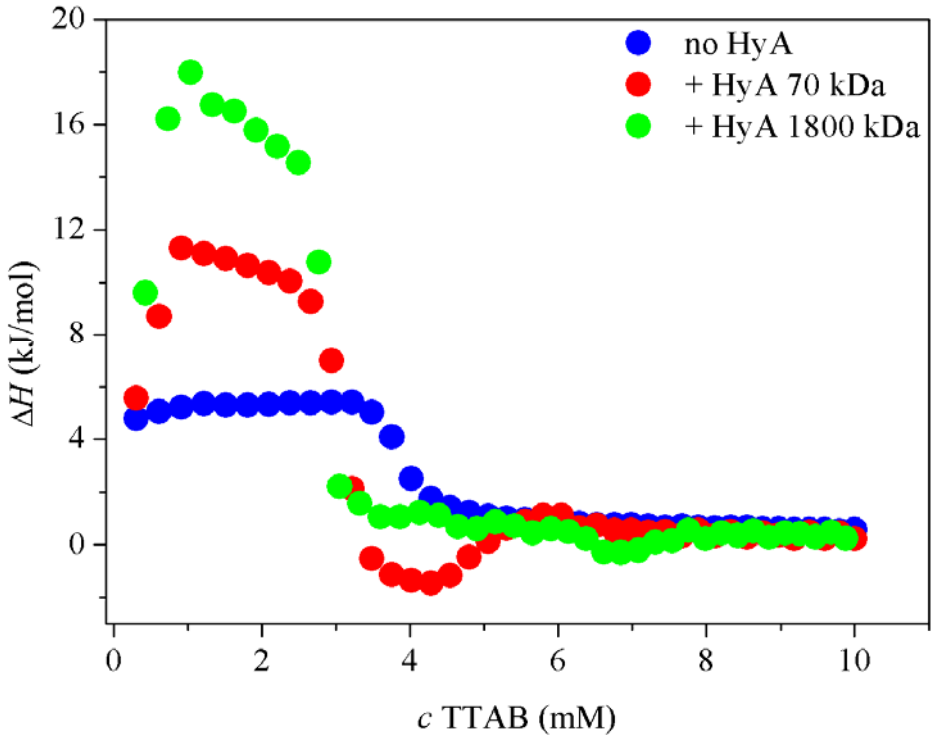


Fig.40 *Interaction of TTAB with hyaluronan (conc. 1000 mg/l) of different molecular weight in water at 25°C.*

The situation with high hyaluronan concentration changes dramatically. When the shapes of the titration curves of the system hyaluronan-surfactant in Fig.39 and Fig.40 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.39 and Fig.40 are discussed below.

At first, there is a sharp increase of ΔH at concentration when solution becomes visually turbid. This was observed for both molecular weights of hyaluronan and more intensive for 1800 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 showed 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 precipitate.

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. 10 Enthalpy of micellization and critical aggregation concentrations 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. [67] 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.

7 EXPERIMENTAL PART III – AMINO ACIDS

This part of the experimental work seemingly stands a little bit farther from the previously described topics. The common idea is the interaction between positively charged nitrogen atom from the amino acid and negatively charged hyaluronan because some modern surfactants can be based on the structure of amino acids. The experimental work was focused on the simple titrations of 6- aminocaproic acid and L-lysine by strong acid with and without presence of hyaluronan. During the titrations protonation of acids occurs. The aim was to compare the titration curves in the presence and absence of hyaluronan and on this basis to elucidate amino acids-hyaluronan interactions.

7.1 Materials

Both amino acids L-lysine and 6-aminocaproic acid (6-aminohexanoic acid) were used as received without further purification. The detailed specification is listed table below (Tab. 11) HCl was purchased from Lach-Ner, s.r.o., Czech Republic. Hyaluronan of two molecular weights (116 and 1800 kDa) was purchased from Contipro Biotech, Ltd., the specification is listed in Tab. 2.

Tab. 11 Specification of amino acids.

amino acid	Mw	Lot number	Supplier
L -lysine	146.2 g/mol	1393705	Sigma Aldrich, Czech Republic
6-aminocaproic a.	131.2 g/mol	38K0716	

Purity: L-lysine 98%, 6-aminocaproic a. 99%

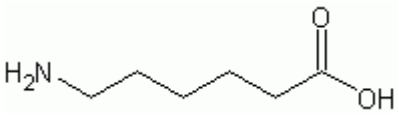


Fig.41 *Structure of 6-aminocaproic acid.*

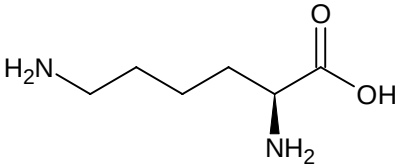


Fig.42 *Structure of L-lysine.*

7.2 Method

The stock solutions of both amino acids were prepared at concentration 40 mM in water. The reason for choosing this concentration is described below. The precise amount of the powder was dissolved in water under continuous stirring for 4 hours. The stock solutions of hyaluronan were prepared at concentration 0.1% w/v by dissolving solid hyaluronan in water under slow stirring for 24 hours at room temperature in closed vessel to ensure the complete dissolution. The titration of the amino acids and hyaluronan was performed using a potentiometric and a conductivity titration which were controlled by an automatic titrator Shott TitroLine alpha plus together with a pH meter Mettler Toledo. Conductivity and pH of the stirred samples was recorded simultaneously during the experiment as shown in Fig.43 and Fig.44. The results were evaluated in a combined plot of containing pH vs. volume of HCl and conductivity vs. volume of HCl, respectively.

The titration of hyaluronan was performed using 20 ml of hyaluronan solution and the experiments with the mixture of hyaluronan and amino acid were performed using 20 ml of hyaluronan and 3 ml of amino acid. The mixture of hyaluronan and amino acid was stirred max. for 5 min before the measurement.

Fig.43 *Automatic titrator connected to a pH meter.*

Fig.44 *Schematic view of the electrodes and the drip tip (a blue pH electrode on the left, a black conductivity electrode on the right).*

7.3 Results and discussion

First experiments focused on the visual study of the system hyaluronan-amino acid from the point of view of possible phase separation. Stock solution on amino acids was successively added to 0.1% solution of hyaluronan until the molar concentration of amino acid is higher than concentration of carboxyl groups of hyaluronan. No phase separation of the final sample solution was observed when low and high molecular weight hyaluronan was used. pH values decreased very slightly.

Further, pH of aqueous solutions of amino acid and the system hyaluronan-amino acid was lowered to approximately 1.5 using a titration by 0.1 M HCl to obtain the complete protonation of the system. The detailed description of the experiments is given below.

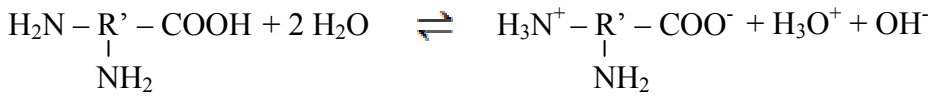
Pure 6-aminocaproic acid and L-lysine solutions have pH equal to 7 and 10, respectively. This can be explained taking into account their dissociation behavior in water:

a) amino acid with one amino group (6-aminocaproic acid)

$$H_2N - R - COOH + 2\ H_2O \rightleftharpoons H_3N^+ - R - COO^- + 2\ H_2O$$

b) amino acid with two amino groups (L-lysine)

b1)



b2)

The dissociation of L-lysine in water proceeds according the scheme b1) up to completely ionized the form in scheme b2) where basic pH is expected due to the presence of hydroxy groups. If the ionization proceeds via the scheme described in b1), pH of the solution must be neutral which is not in agreement with the real measured values.

The results of the titrations of 6-aminocaproic acid and L-lysine are summarized in Fig.45 and Fig.46, respectively. The titration curves of 6-aminocaproic acid and L-lysine can be explained as follows. The protonation of 6-aminocaproic acid starts on the amino group where a proton appears forming a charged $-NH_3^+$ group. Later, at lower pH, the carboxyl group which was dissociated as $-COO^-$ forms –COOH group. The lowering of pH in the mixture of 6-aminocaproic acid and hyaluronan of both molecular weights has a sharp slope, the equilibrium occurred at lower pH but the consumption of HCl to obtain the constant pH is approximately the same. When compared to L-lysine the reaction is only prolonged with the protonation of the second amino group which forms the second step during the titration.

As expected, conductivity curves corresponding to pure hyaluronan and its mixture with both amino acids indicated the presence of more charged groups in the sample which was expressed by the incomparably higher values of conductivity than amino acid as itself. On the other hand, after the comparison of the conductivity curves of hyaluronan and the mixture with amino acids could be concluded that there were proved interactions between the functional groups of hyaluronan and amino acids which were expressed in lower values of conductivity of the samples containing both hyaluronan and amino acids.

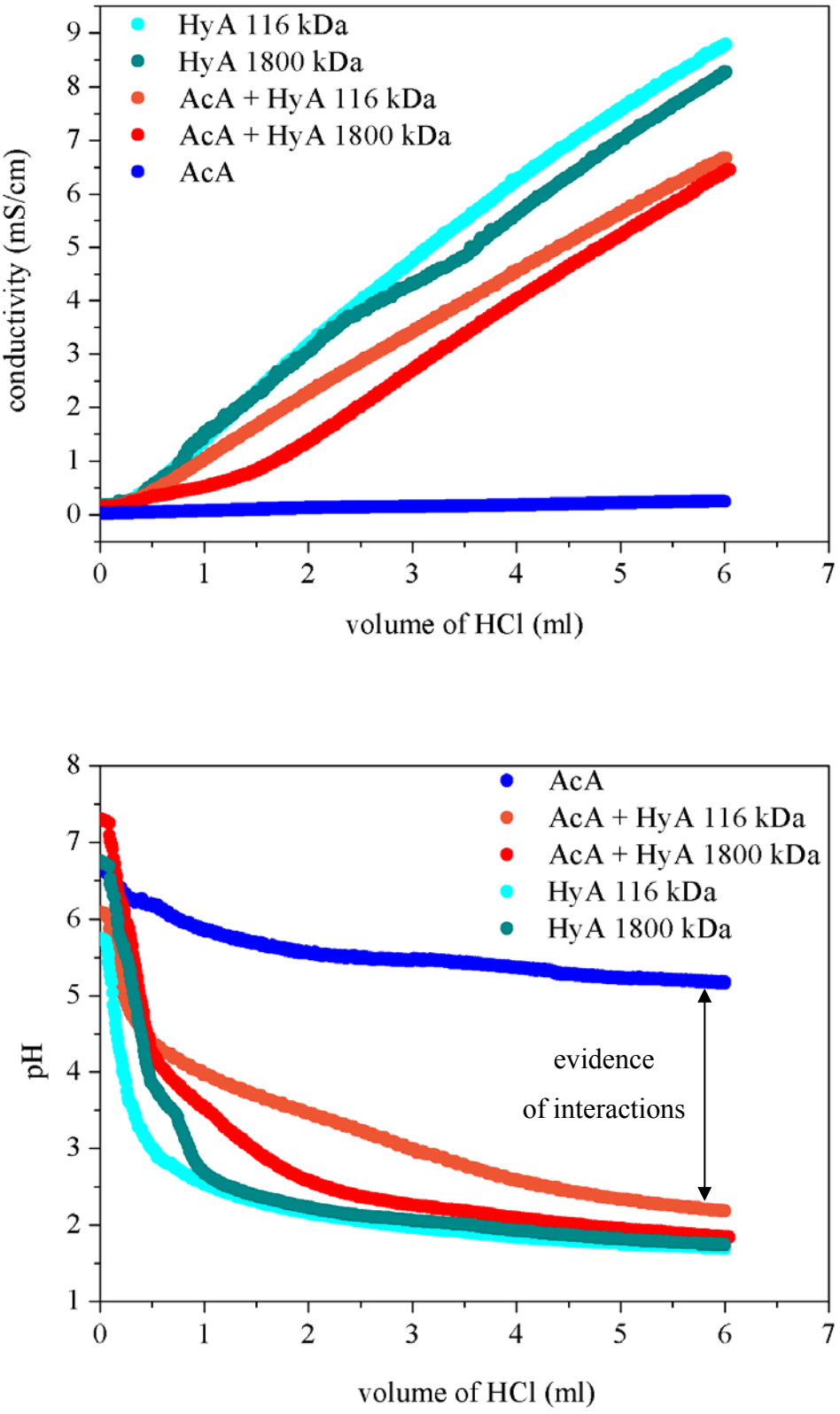


Fig.45 *Titration of 6-aminocaproic acid, hyaluronan and its mixture at 25°C using 0.1 M HCl.*

The titration was performed with 20 ml of amino acid solution at the beginning of the experiment. Amount of moles of charged groups $-COO^-$ and $-NH_3^+$ in 20 ml of amino acid (concentration 40 mM) is 0.8 mmoles. If the consumption of 0.1 M HCl for the titration is 6 ml, there are 0.4 mmoles of H^+ in this volume. So the excess of amino acid ensures that the titration curves really correspond to behavior of just amino acid in the sample.

The titration curves of 6-aminocaproic acid, hyaluronan and its mixture at 25°C is shown in Fig.45. 6-aminocaproic acid has pK_a values 4.4 (-COOH) and 10.7 ($-NH_2$) [74] so the required distribution of protonated amino group $-NH_3^+$ and carboxylic group -COOH without the charge occur at pH value lower than 4.4. This is the reason for the lower limit of pH (lower than pH = 1) reached at the end of the experiments. The difference in the distance between the titration curves in both pH-plots in Fig.45 and Fig.46 is the evidence of the interactions between amino acid and hyaluronan. In addition, pH of pure amino acids decreases slowly when compared to the rapid decreasing in the case of the mixture with hyaluronan. Basic $-NH_3^+$ groups from amino acids participate in the interactions with hyaluronan therefore the lowering of pH corresponds to the addition of HCl.

Further, the change of pH of pure hyaluronan of both molecular weights (pK_a = 3) appeared with steeper slope at the beginning of the titration than the mixture with amino acids as well, because the charge is concentrated just around the hyaluronan acetyl group forming $-NH_2^+$-CO-CH_3. This is illustrated in both conductivity and pH plots in Fig.45 and Fig.46.

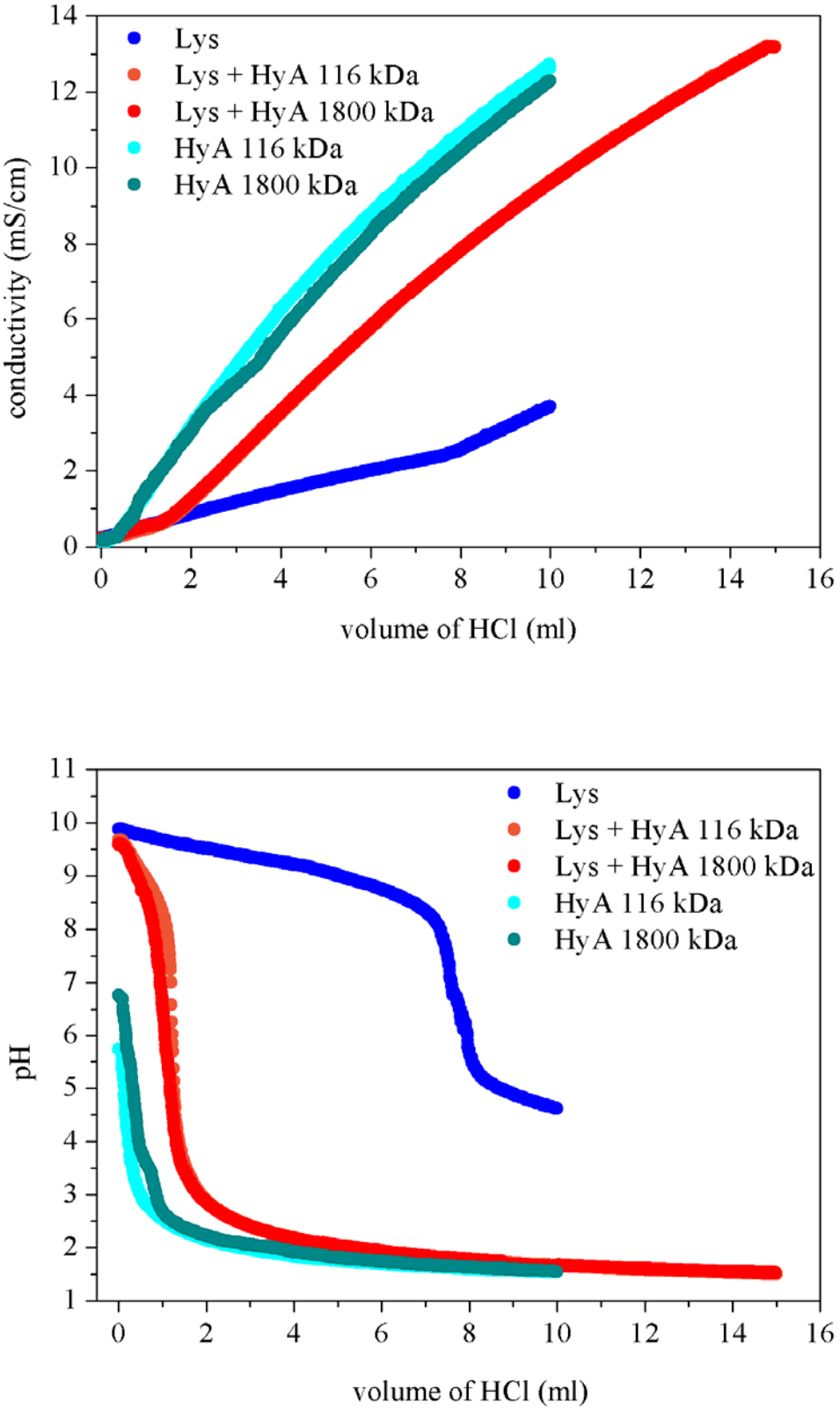


Fig.46 *Titration of L-lysine, hyaluronan and its mixture at 25°C using 0.1 M HCl.*

The results from the titration of L-lysine (pK_a 2.18, 8.95, and 10.53 [75]) are summarized in Fig.46. The titration curves corresponding to the titration of hyaluronan are the same as showed in Fig.45. Although the amino acids molecules differ in one amino group, the conductivity curves of L-lysine and 6-aminocaproic acid look the same. The different shapes of the curves are observed just in the pH plots where L-lysine forms the S-curve in both cases – with and without addition of hyaluronan.

To sum it up, the interactions of two amino acids with hyaluronan did not show any disadvantages from the point of view of possible precipitation or phase separation of the system but the question is if the protonated system can be used in contact with living organisms at such a low pH.

8 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. The first part of the thesis focused on the surface tension measurement of different types of surfactants in 0.15 M NaCl solution with added hyaluronan at 25°C. The aggregation concentrations of CTAB and TTAB were practically not affected by addition of hyaluronan and no phase separation was observed.

Surface tension measurement of the same systems 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 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.

The last part of the experimental work focused on the ionization of amino acids and their interaction with hyaluronan. The effective interactions were proved by the titration with HCl. The results from this part of the thesis form the essential knowledge about the interactions between amino acids and hyaluronan and it is the challenge for the following studies of this topic.

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10 LIST OF ABBREVIATIONS

HyA	Hyaluronic acid
TTAB	Tetradecyltrimethylammonium bromide
CTAB	Cetyltrimethylammonium bromide (Hexadecyltrimethylammonium bromide)
SDS	Sodium dodecyl sulphate
Mw	Molecular weight, g/mol
Da	Dalton, unit of MW
CD44	Type of HA receptor
RHAMM	Type of HA receptor
HARE	Type of HA receptor
LYVE-1	Type of HA receptor
CMC	Critical micelle concentration
CAC	Critical aggregation concentration
γ (mN/m)	Surface tension
F	Force exerted parallel to the surface
L	Length of the surface
Γ	Maximum surface concentration
R	Gas constant
T	Absolute temperature
c (M, mM)	Molar concentration, mol l^{-1}, mmol l^{-1}
w	Mass concentration, g l^{-1}, mg l^{-1}
w/v	Concentration ratio - mass vs. volume
r_p	Radius of the Du Noüy ring
Φ	Correction factor for surface tension calculation
t_l, t_{life}	Time of life of a bubble
t_d	Time of death of a bubble
ITC	Isothermal titration calorimetry
c_{syr}	Concentration in the titration syringe
c_{cell}	Concentration in the measuring cell
ΔH	Change of enthalpy, kJ mol^{-1}
ΔH_{mic}	Enthalpy of micellization
ΔH_{demic}	Enthalpy of demicellization

ΔG_{demic}	Gibbs energy of demicellization
ΔS_{demic}	Entropy of demicellization
$\Delta H_{inj}/n°_{x,inj}$	Enthalpy of injection per mole of monomer on injection number
β	Degree of micelle ionization
X_{CMC}	Mole fraction of a surfactant at CMC
I_1/I_3	Pyrene polarity index (ratio of fluorescence intensities at 373 and 383 nm)
AcA	6-aminocaproic acid
Lys	L-lysine

11 LIST OF PUBLICATIONS AND ACTIVITIES

Impacted publications:

1. HALASOVÁ, T., J. KROUSKÁ, F. MRAVEC and M. PEKAŘ. Hyaluronan-surfactant interactions in physiological solution studied by tensiometry and fluorescence probe techniques. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*. 2011, vol. 391, no. 1-3, p. 25–31. ISSN 09277757.

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Given speeches:

4. KROUSKÁ, J.; PEKAŘ, M. *Polyelectrolyte-surfactant interactions by isothermal titration calorimetry*. 24th Conference of the European Colloid and Interface Society, Praha. 2010.

5. KROUSKÁ, J.; HALASOVÁ, T.; MRAVEC, F.; PEKAŘ, M., BEŠTER-ROGAČ, M. *Surfactants in hyaluronan solution.* 18th International Symposium on Surfactants in Solution, Sebel Albert Park, Melbourne, Australia. 2010.

Conference contributions:

6. KROUSKÁ, J; PEKAŘ, M. Tensiometric study of surfactant micellization induced by hyaluronan. In *Book of abstracts.* 26th Conference of the European Colloid and Interface Society, Malmö University, Sweden. 2012

7. KROUSKÁ, J.; PEKAŘ, M. *Surface tension of hyaluronan-surfactant systems.* In *Book of abstracts*. 25th Conference of the European Colloid and Interface Society, Technical University Berlin, Berlin, Germany. 2011.

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10. KROUSKÁ, J.; HALASOVÁ, T.; MRAVEC, F.; PEKAŘ, M. *Determination of critical micelle concentration in hyaluronan - surfactant system.* ChemZi. 2009. vol. 9, no. 5, p. 133–134. ISSN 1336-7242.

APPENDIX I

HALASOVÁ, T., J. KROUSKÁ, F. MRAVEC and M. PEKAŘ. Hyaluronan-surfactant interactions in physiological solution studied by tensiometry and fluorescence probe techniques. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*. 2011, vol. 391, no. 1–3, p. 25–31. ISSN 09277757.

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Hyaluronan-surfactant interactions in physiological solution studied by tensiometry and fluorescence probe techniques

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Surfactants

ABSTRACT

Interactions of hyaluronan of two molecular weights with surfactants of different types were studied in NaCl solution. Results showed that although the presence of NaCl may suppress interactions between oppositely charged polyelectrolyte and surfactant, the interactions are still present in some hyaluronan-surfactant systems regardless the surfactant ionic nature. These interactions were demonstrated mainly by fluorescence probe techniques whereas tensiometry detected only minor effects. Fluorescence data showed that formation of aggregates (micelles) occurs rather in a certain interval of surfactant concentrations than in a single point, especially in the presence of hyaluronan. The greatest influence of hyaluronan was observed on nonionic Tween 20 and cationic cetyl trimethylammonium bromide.

1. Introduction

Hyaluronan is a naturally occurring polysaccharide, commonly found in connective tissues in the body such as vitreous, umbilical cord and joint fluid [1]. As a component of the extracellular matrix, hyaluronan plays an important role in lubrication, water sorption, water retention, and a number of cellular functions such as attachment, migration, and proliferation [2–6]. Hyaluronan is therefore an attractive building block for new biocompatible and biodegradable polymers that have applications in drug delivery, tissue engineering, and viscosupplementation [7–9]. It is a negatively charged monotonic co-polymer with repeating disaccharide unit composed of D-glucuronate and N-acetyl-D-glucosamine residues linked by β(1–4) and β(1–3) bonds, which are connected to unbranched chains [10].

Hyaluronan is a unique molecule not only from the view of physical properties but also from the view of its biological properties. It is of great interest that such a simple molecule that hyaluronan can exercise so many biological functions. In tissues, hyaluronan is mostly found as a high-molecular compound. In addition to these macromolecules there are also products of hyaluronan degradation in tissues, fragments with much lower molecular weight. Just in different molecular weights of hyaluronan lies the diversity of its biological functions [4,5].

Cells posses several specific receptors recognizing hyaluronan, among them CD44 is probably the most important, which further enhances its potential for application in targeted drug delivery systems. Hyaluronan is highly hydrophilic biopolymer with massive hydration shell and cannot be directly used to carry nonpolar substances. Because many efficient drugs, e.g. for fighting cancer, are hydrophobic, hyaluronan has been chemically modified, hydrophobized, to induce micelle-like properties or directly conjugated with hydrophobic biologically active molecules (drugs). Besides chemical modifications which could change also biological activity or compatibility of hyaluronan, physical interactions with suitable molecular partner may be another way to solubilize hydrophobes in the presence of hyaluronan. For example, combination of hyaluronan with surfactant may lead to formation of associates in which the surfactant hydrophobic domains solubilize hydrophobes and hyaluronan plays a role of biocompatible carrier and targeting agent.

Hyaluronan-surfactant interactions were subject of several previous studies. Because of negative hyaluronan charge almost all of them used cationic surfactants. Only Yin et al. [11] reported some results obtained with anionic and nonionic surfactants. Hyaluronan interactions with cationic surfactants were studied as a specific case of general polyelectrolyte-surfactant interactions to elucidate their phase behavior and physical causes of their interactions including the effect of electrolytes. Little is still known about solubilization in hyaluronan-surfactant associates, especially at physiological ionic conditions.

Detailed study on phase behavior of systems containing water, hyaluronan, alkyl trimethylammonium bromides (tetradecyl derivative was the most studied type) and salt (mostly NaBr)

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was published in a series of papers by Swedish researchers [12–18]. They found that there is a certain cationic surfactant concentration below which only general electrostatic interactions take place and only above it marked formation of hyaluronan-surfactant complexes can be observed. Their results indicated strong cooperativity of surfactant binding on hyaluronan macromolecule resulting in binding in the form of micelle-like clusters in which both hyaluronan carboxylates and background electrolyte anions participate as counterions. Increasing electrolyte (salt) concentration disfavored formation of hyaluronan-bound micelles in comparison to free micelles. The authors constructed phase diagrams for studied systems which essentially contain an area of homogeneous single phase surrounding two-phase region. Phase separation was observed as formation of precipitate or gel-like phase upon increasing surfactant concentration. Increasing concentration of added salt reduced the two-phase area unless the salt concentration was very high when phase separation occurred again. Binding of surfactant to hyaluronan was detected for surfactants with alkyl chain consisting from at least ten carbon atoms. The longer the surfactant alkyl chain the larger two-phase region was observed.

Yin et al. [11] used pyrene fluorescence to investigate interactions of (high molecular weight) hyaluronan with anionic sodium dodecyl sulfate (SDS) and nonionic Cremophor EL and Tween 80 in water. Practically no interactions between hyaluronan and nonionics were detected whereas certain decrease of SDS critical micellar concentration was observed. Hyaluronan in SDS solutions thus acts similarly as low molecular weight electrolyte as demonstrated by similar experiments with surfactant solutions free of hyaluronan but containing sodium chloride. This was explained by interactions of SDS polar heads with domains formed by hyaluronan hydroxyls.

The aim of this work was to obtain additional information on hyaluronan-surfactant interactions in solutions with physiological salt concentration by tensiometry and fluorescence probe techniques. Tensiometry gives information primarily on the solution surface layers and interactions therein and indirectly information on the bulk solution, especially on micellization (aggregation). Fluorescence probe informs on its local environment in solution, on domains where it can be dissolved or solubilized in solution. From this information deductions on aggregation in solution can be also made and on solubilizing properties of domains formed by aggregates. All this information is a base for design of hyaluronan-surfactant systems for targeted delivery of nonpolar biologically active substances (e.g., drugs). Fluorescence technique is not limited here to pyrene, perhaps the most popular probe for aggregation studies and determining critical micellar concentrations. Nile red was used as an additional, comparative probe that can also provide additional information on solubilizing hydrophobic domains [19–21]. Though pyrene is considered to be nonpolar its limited solubility in water is still high enough to produce remarkable fluorescence signal. Nile red fluorescence is quenched by water [22,23] therefore only those molecules give sufficient fluorescence intensity that is in the water-free domains. Pyrene as well as nile red can interact with charged parts of ionic surfactants. This means that both probes are located in the micellar palisade layer which still contains some water molecules. Whereas pyrene fluorescence occurs also from aqueous environment the nile red fluorescence from the water-rich palisade layer should be quenched. In the case of non-ionic surfactant also hydrogen bond interactions can be expected between nile red and surfactant. Direct interaction of this probe with surfactant can decrease its fluorescence intensity.

2. Materials and methods

Hyaluronan of two molecular weights (90 kDa and 1.4 MDa, i.e. the low and high molecular weight, respectively) was purchased from CPN, Ltd., Czech Republic. Surfactants tetradecyl trimethylammonium bromide (TTAB, cationic), cetyl trimethylammonium bromide (CTAB, cationic), cetyl trimethylammonium p-toluene sulfonate (CTAT, cationic), sucrose monolaurate (nonionic), octyl-β-D-glucopyranoside, and Tween 20 (nonionic) were from Sigma–Aldrich (Czech Republic); sodium dodecyl sulfate (SDS, anionic) and n-dodecyl-β-D-maltoside (nonionic) were purchased from Fluka (Czech Republic). Zwitterionic betaine surfactants Betadet THC 2 and cetyl betaine were purchased from Chemos (Czech Republic). All surfactants were of the best available quality and used as received without further purification.

Stock solutions of hyaluronan in physiological solution (0.15 mol/l NaCl) were prepared in concentration of 0.5% (w/v) by slowly adding solid hyaluronan to the salt solution under stirring, followed by 24 h stirring in closed vessel to ensure complete dissolution. Hyaluronan stock solution was then used to prepare samples for tensiometry and fluorescence measurements with final constant hyaluronan concentration of 0.1% (w/v).

Samples with varying surfactant concentration and no hyaluronan were prepared by simple diluting surfactant stock solution prepared in 0.15 mol/l NaCl at concentration about ten times higher than its critical micellar concentration in this salt solution. Samples with varying surfactant concentration and containing hyaluronan (0.1%, w/v) were prepared by mixing hyaluronan (10 ml) and surfactant stock solutions and diluting with the physiological solution to the final volume of 15 ml (no phase separation was observed). The samples with hyaluronan were stabilized with NaN_3 (0.05%, w/v) and stirred overnight.

Surface tension measurement was performed with a platinum/iridium Du Noüy ring (diameter 9.545 mm) on tensiometer KSV Sigma 701 at room temperature.

Aggregation and solubilization properties were studied by means of two fluorescence probes – pyrene and nile red. Stock solutions of both fluorescence probes were prepared in volatile solvent – acetone (10^{-4} mol/l). An amount of stock solution to obtain final concentration of pyrene or nile red in samples equal to 10^{-6} mol/l was added to glass vial and acetone was evaporated under reduced pressure. Then 3 ml of hyaluronan or hyaluronan-surfactant solution was added to vial with fluorescent probe and incubated for 24 h at laboratory temperature.

Fluorescence emission spectra were recorded on AMINCO Bowman Series 2 fluorescence spectrometer. In the case of pyrene the excitation wavelength was 336 nm and emission scan was acquired in the range from 360 to 540 nm. Nile red was excited at 550 nm and emission spectra were recorded from 610 to 700 nm.

All measurements were done in duplicates, at least. Confidence intervals in table and error bars (shown when not smaller than the symbols) in figures represent the standard deviation.

Pyrene experiments were evaluated by plotting the intensity ratio I_1/I_3 (the pyrene polarity index or the ratio of fluorescence intensities at 373 and 383 nm) against surfactant concentration [24]. Typical sigmoidal curve was obtained indicating formation of nonpolar domains (micelles with hydrophobic cores) solubilizing pyrene molecules. Ideally, a sharp step change would be observed at the critical micellar concentration. However, the change was usually more or less broadened indicating micellization (aggregation) region (concentration range within which the polarity index decreases) rather than single point. Curves were fitted by Boltzmann equation and inflex point in the micellization region was considered as an estimate of critical micellar concentration. From the Boltzmann equation also points (concentrations) of beginning and end of the micellization region, i.e. the width of this region, can be obtained.

Nile red fluorescence spectra were evaluated by two methods. The first one was plotting normalized values of the total integral of fluorescence intensity (in the wavelength range 610–700 nm)

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Table 1
Effect of low (LMW) and high (HMW) molecular weight hyaluronan (HyA) on the critical micellar concentration (CMC) as determined by pyrene (upper) and surface tension (bottom) methods.

Surfactant	CMC (mmol/l)		
	no HyA	LMW HyA	HMW HyA
Sucrose monolaurate	0.288 ± 0.006	0.26 ± 0.05	0.25 ± 0.03
	0.25 ± 0.02	0.26 ± 0.03	0.3 ± 0.1
n-Dodecyl-β-D-maltoside	0.160 ± 0.001	0.150 ± 0.003	0.160 ± 0.002
	0.149 ± 0.007	0.136 ± 0.006	0.19 ± 0.08
Octyl-β-D-glucopyranoside	20.2 ± 0.1	19.9 ± 0.3	19.9 ± 0.3
	20.6 ± 0.4	19 ± 1	21 ± 2
Tween 20	0.048 ± 0.001	0.014 ± 0.001	0.014 ± 0.002
	0.072 ± 0.003	0.053 ± 0.004	0.05 ± 0.02
SDS	1.04 ± 0.01	0.740 ± 0.001	0.80 ± 0.03
	0.87 ± 0.05	0.7 ± 0.1	0.7 ± 0.2
TTAB	0.52 ± 0.03	0.51 ± 0.03	0.61 ± 0.01
	0.49 ± 0.02	0.58 ± 0.01	0.5 ± 0.2
CTAB	0.062 ± 0.002	0.12 ± 0.02	0.11 ± 0.02
	0.030 ± 0.004	0.025 ± 0.006	0.040 ± 0.001
CTAT	0.056 ± 0.001	0.072 ± 0.001	0.15 ± 0.05
	0.036 ± 0.004	0.099 ± 0.007	0.058 ± 0.008
Cetyl betaine	0.016 ± 0.001	0.009 ± 0.001	0.021 ± 0.005
	0.009 ± 0.001	0.010 ± 0.001	0.051 ± 0.003
Betadet THC2	0.14 ± 0.01	0.10 ± 0.01	0.100 ± 0.001
	0.13 ± 0.03	0.10 ± 0.03	0.27 ± 0.03

against surfactant concentration. The integrals (I_r) were normalized with respect to maximum value which was usually measured for the highest surfactant concentration, i.e. the normalized values ranged from 0 to 1 within each measured system. At low surfactant concentrations the integral intensity is zero or very low and not changing with concentration. At a certain concentration the intensity starts to increase, usually linearly. This is the point of the critical micellar concentration (the intersection of two linear branches). In the second method dependence of the maximum emission wavelength on surfactant concentration was plotted giving similar sigmoidal curve as the pyrene polarity index – mathematical treatment was then similar as for this index.

3. Results and discussion

3.1. Surface tension

Measurement of surface tension detects, in fact, surfactant accumulation on the liquid surface (adsorption). Indirectly it provides information on surfactant behavior in solution, specifically on its micellization which is supposed to start when the sharp drop of surface tension with increasing surfactant concentration stops. Measurements with hyaluronan solutions confirmed no hyaluronan surface activity [25] up to the concentration of about 2 g/l which is consistent with findings of Riberio et al. [26]. All measured data for the dependence of the surface tension on surfactant concentration in physiological solution showed no or small effect of hyaluronan (of any molecular weight) on surface activity and particularly on micellization, i.e. the value of the critical micellar concentration as determined by tensiometry was practically not affected by the addition of hyaluronan, see Table 1. Examples of tensiometry results are given in Fig. 1 for sucrose monolaurate and CTAB. In the case of sucrose monolaurate all three data curves are practically identical and are typical examples of most measured surface tension data. Thus from the point of view of tensiometry there are no specific interactions between hyaluronan and sucrose monolaurate (and most of the other surfactants) and the surface and micellization behavior of this surfactant is not influenced by the presence of hyaluronan chains. CTAB is oppositely charged than hyaluronan therefore some electrostatic interactions could be expected. Fig. 1b shows that hyaluronan of both molecular weights seems to slightly decrease the surface tension of CTAB physiological solutions in the premicellar region. However, the value of the critical micellar concentration (CMC), as determined by tensiometry, is not affected. Tensiometry thus indicates that hyaluronan influences only the interactions within the CTAB surface layer and does not intervene in the micellization process. No effect on micellization is a result of screening the electrostatic interactions between hyaluronan and CTAB by the presence of NaCl in physiological solution which seems to be more effective in the bulk solution than in

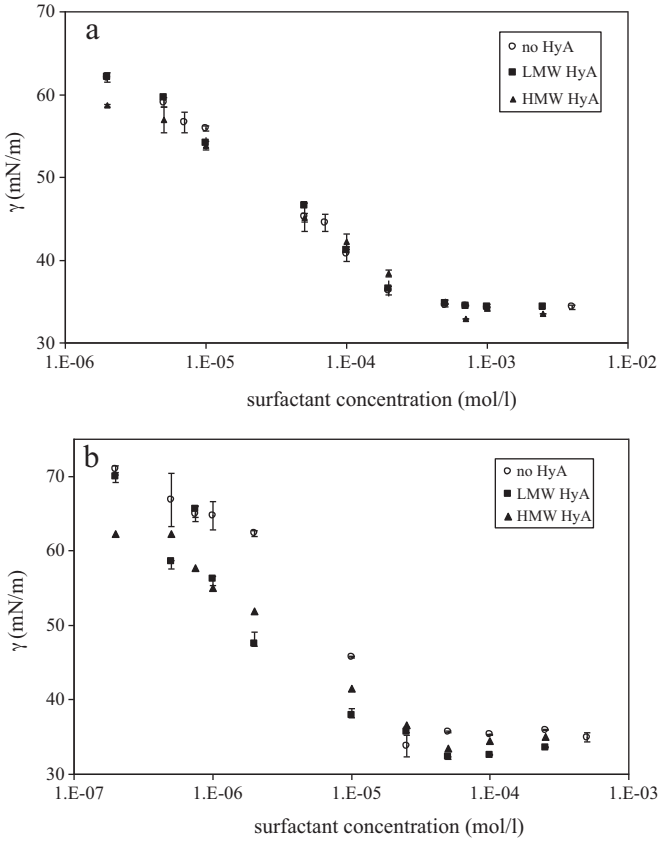

Fig. 1. Effect of low (LMW) and high (HMW) molecular weight hyaluronan (HyA) on surface tension of sucrose monolaurate (a) and CTAB (b) solutions.

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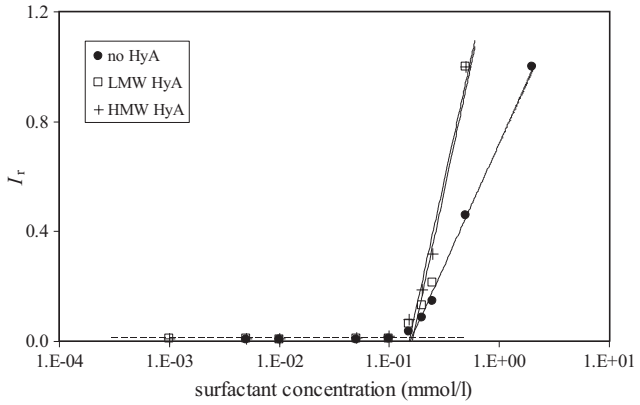

Fig. 2. Effect of low (LMW) and high (HMW) molecular weight hyaluronan (HyA) on nile red integral intensity dependence on dodecyl maltoside concentration.

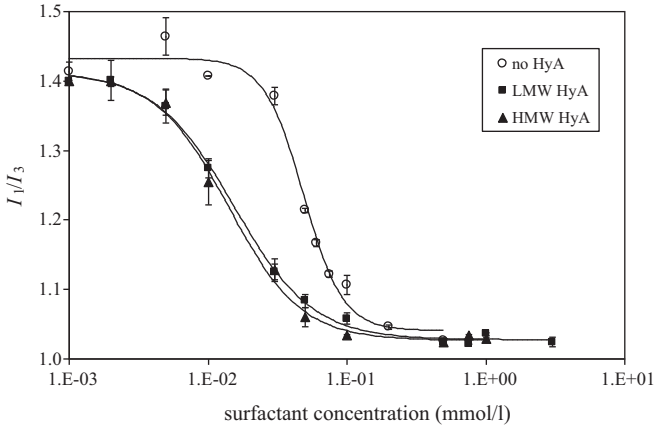

Fig. 3. Effect of low (LMW) and high (HMW) molecular weight hyaluronan (HyA) on pyrene polarity index dependence on Tween 20 concentration. Solid lines show fits by Boltzmann curve.

its surface layers. Because the CMC value, as determined by tensiometry, was not influenced by hyaluronan, it probably does not change the amount of CTAB on the solution surface in the premicellar region but changes only interactions in this layer which are the cause of (lowered) surface tension. The lower surface tension may be also caused by larger binding of sodium from the added salt to hyaluronan as counterions. Similar decrease of surface tension in premicellar region was observed also for SDS and for TTAB in the presence of high molecular weight hyaluronan. Specific results obtained for Tween 20 are discussed below.

3.2. Fluorescence probes – nonionic surfactants

Fluorescence probes, in contrast to tensiometry, provide information directly from the bulk solution or even directly from the formed micelles or from their close vicinity. Pyrene is considered to be insoluble in water. However, it has some though limited solubility in water and its location in water is detected by the high value of the pyrene polarity index. Fluorescence of nile red is quenched in water and its emission maximum wavelength decreases with decreasing polarity.

Critical micellar concentrations determined by the pyrene probe method are summarized in Table 1 and data obtained by all three different fluorescence approaches can be seen in Table S1 (Supplementary material) but the whole measured curves (concentration dependences) are more convenient for discussion of results.

First, the results for nonionic surfactants will be discussed. Data for dodecyl maltoside measured with pyrene and for the nile red emission maximum showed no effect of hyaluronans. Nile red integral intensity detected no change in critical micellar concentration but revealed increased slope of intensity dependence on concentration after formation of micelles (see Fig. 2). Increased fluorescence intensity is supposed to be caused by increased number of solubilized nile red molecules. Hyaluronan thus influences the solubilization capacity of dodecyl maltoside probably through the influence on shape, size or number of its micelles.

Pyrene and nile red curves measured for sucrose monolaurate showed no differences when hyaluronans were added.

Pyrene data for octyl glucopyranoside showed slight broadening of micellization region and slight increase of CMC upon addition of hyaluronan with no specific effect of its molecular weight. The CMC increase was observed also on the nile red emission maximum curve but here the effect of the high molecular weight hyaluronan was more distinct. Nile red intensity data showed increasing CMC value in the order of solutions with: no hyaluronan < low < high molecular weight hyaluronan. Hyaluronan also slightly increased slope of intensity dependence on concentration in post micellar region. Octyl glucopyranoside is relatively small molecule (monosaccharide with relatively short alkyl) what probably facilitates interactions of single surfactant molecules with hyaluronan through their hydroxyl groups disturbing thus the micellization process.

Pyrene data for Tween 20 revealed significant effects of hyaluronan of both molecular weights on the behavior of this surfactant in physiological solution (see Fig. 3). In the presence of hylauronans, the CMC was substantially decreased and the concentration interval where micellization occurs was significantly broadened. Very similar results were obtained also for the nile red emission maximum measurements (the curves are not shown – their shapes are very similar to those for pyrene in Fig. 3). On contrary, the nile red integral intensity showed increased CMC upon addition of hyaluronans; this increase was a little bit higher for the low molecular weight hyaluronan. The latter also gave somewhat smaller slope of intensity dependence on concentration in post micellar region, i.e. decreased solubilization. The apparently contradictory results can be explained by different photophysical basis of detection by the two different fluorescence probes quoted in introduction. Pyrene polarity index in the region of its decrease from the value corresponding to aqueous medium to the value corresponding to low polarity environment is composed from signals coming from pyrene molecules located in the formed micelles, from free pyrene molecules in water, and probably also from ("premicellar") aggregates formed by hyaluronan-surfactant interactions which consist of domains of higher polarity (higher water contents) than regular Tween micelles. Similarly, the nile red emission maximum measures the polarity of environment within which the nile red molecules are located or solubilized; the shift of this maximum is detectable even when the fluorescence intensity is very low, i.e. when most nile red molecules are surrounded by water that quenches their fluorescence. On contrary, the nile red fluorescence intensity is principally determined by the number of nile red molecules located in non-aqueous environment, i.e. solubilized within the nonpolar aggregates or micelles. Sufficient number of solubilized molecules, i.e. of micelles with the true non-aqueous interior, is formed in the presence of hyaluronan only at sufficiently high surfactant concentration. This probably occurs when interactions between Tween 20 and hyaluronan are "saturated", formation of hyaluronan-surfactant premicellar aggregates (of higher polarity than standard Tween micelles) is essentially completed and free Tween micelles start to be formed.

In contrast to the other used sugar-based surfactants, Tween 20 has relatively bulky polar head with several end hydroxyl groups on oligoethylene glycol chains, projecting from the head like

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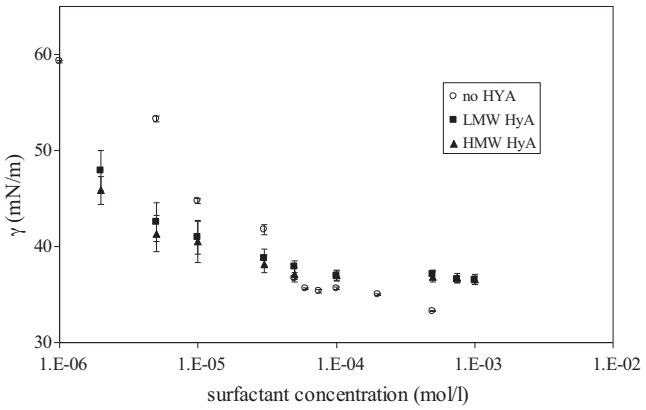

Fig. 4. Effect of low (LMW) and high (HMW) molecular weight hyaluronan (HyA) on surface tension of Tween 20 solutions.

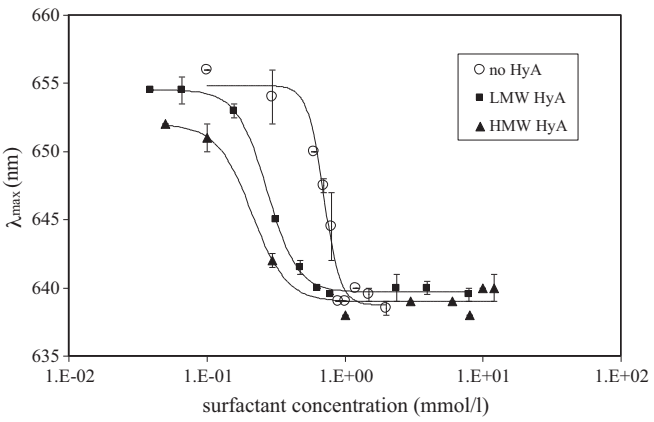

Fig. 5. Effect of low (LMW) and high (HMW) molecular weight hyaluronan (HyA) on dependence of nile red emission maximum wavelength on SDS concentration. Solid lines show fits by Boltzmann curve.

"antennas". One cause for formation of aggregates in the presence of hyaluronan may thus be steric exclusion by voluminous hyaluronan coils. Further, Tween "antenna" hydroxyl groups may participate in hydrogen bonding with hyaluronan hydroxyls or even carboxyls. Molecular modeling of hyaluronan hydration demonstrated numerous hydrogen bonds between hyaluronan disaccharide units and water molecules – some of them even form water bridges between adjacent hydroxyls of monosaccharide subunits [27,28]. Water thus can participate in the premicellar aggregate formation or can be entrapped within their structure. Formation of premicellar aggregates is not reflected in the value of critical micellar concentration as determined by surface tension but some effect of hyaluronan on the surface tension of Tween 20 physiological solutions can be seen (Fig. 4). Below the regular CMC value the solution surface tension is lower in the presence of hyaluronans which could confirm the hypothesis on hydrogen bonding between hyaluronan and surfactant which occurs also in the surface layers and decreases the surface energy. Constant and higher surface tension behind the regular CMC in the presence of hyaluronans could point to steric exclusion by hyaluronan of additional surfactant molecules to enter the surface layer and forcing them to form free micelles only.

Yin et al. [11] reported no interactions of hyaluronan with similar nonionic surfactant, Tween 80. However, this refers to experiments carried out in water. Sensitivity of hyaluronan conformation to ionic strength is well documented, e.g., by rheological properties of its solutions [29,30]. Also the hydration of hyaluronan chain and hydrogen bonds formed between hyaluronan functional groups and water molecules are influenced by the presence of added electrolyte ions [28]. Even Yin et al. [11] present influence of NaCl on the pyrene I_1/I_3 ratio in the presence of hyaluronan but give it only for five selected surfactant concentrations and do not show the whole curve. Nevertheless, the trends observed by Yin et al. upon addition of NaCl correspond to those seen in Fig. 3. Presence of NaCl thus influences interactions between hyaluronan and Tween surfactants despite of their nonionic nature.

3.3. Fluorescence probes – anionic surfactants

Representative data of pyrene polarity index measured for ionic surfactants can be seen in Figs. S1–S3 (Supplementary material). SDS was the only one anionic surfactant tested. Pyrene curves measured for SDS showed slight broadening of micellization region and slight decrease of CMC in the presence of hyaluronan. Yet more expressive broadening and CMC decrease was indicated by nile red emission maximum (Fig. 5). Nile red integral intensity showed almost no change of CMC but a change of the shape of its dependency on surfactant concentration in post micellar region. Solutions without hyaluronan had usual linear shape while in the presence of hyaluronans S-shape appeared indicating some saturation of nile red solubilization. Decreased CMC is consistent with findings of Yin et al. [11] where aqueous solutions (no added salt) were studied; because there was the added salt in our case the results seem to confirm hypothesis on hydrogen bonding nature of the CMC decrease [11]. The saturation of nile red solubilization probably points to formation of smaller micelles in the presence of hyaluronan with limited space to dissolve relatively large nile red molecule.

3.4. Fluorescence probes – cationic surfactants

Three cationic surfactants were included in this study. Pyrene polarity index curves measured for TTAB in the presence of hyaluronan were close to that obtained when no hyaluronan was present. Similar results were obtained from the nile red emission maximum curves, except somewhat broadened micellization region found especially when the high molecular weight hyaluronan was present. Nile red integral intensity data were also close for all three systems except slightly increased slope in the postmicellar region in the presence of the low molecular weight hyaluronan. Hyaluronan is known to induce aggregation in aqueous TTAB solutions at lower surfactant concentrations than is the CMC which is explained by surfactant cooperative binding on the biopolymer backbone and forming micelle-like aggregates. Salts are known to depress these interactions. Pyrene data thus show that the added salt probably completely screened interactions and the formed aggregates are free micelles of (non-bound) TTAB in saline solution. In other words, no solubilization was detected at surfactant concentrations below the CMC in physiological solution. Similar conclusions can be drawn from the nile red intensity data. Broadening of dependencies measured for the nile red emission maxima show that the probe molecules may be located in environments of different polarity. Whereas pyrene is a "pure" hydrocarbon of limited solubility in water, nile red is a heterocyclic compound containing also nitrogen atoms and in the solution can interact with heteroatoms of the other components also when it is not solubilized in nonpolar domains.

In the case of CTAB, hyaluronan of both molecular weights increased the critical micellar concentration detected by the inflex point on the pyrene polarity index curve. Also the micellization region was broadened especially in the case of the high molecular weight preparation (Fig. 6). Similar results were obtained from the nile red emission maximum. Increased CMC was also demonstrated by the nile red integral intensity data but here no differences were detected between the two hyaluronans. Interactions between

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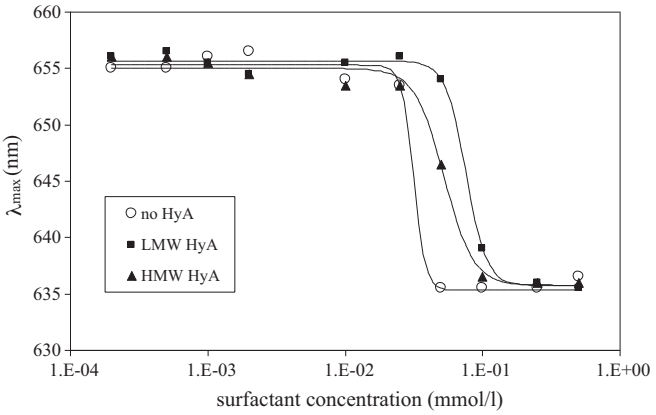

Fig. 6. Effect of low (LMW) and high (HMW) molecular weight hyaluronan (HyA) on dependence of nile red emission maximum wavelength on CTAB concentration. Solid lines show fits by Boltzmann curve.

hyaluronan and CTAB are demonstrated by increased CMC and broadening of micellization regions as detected by the pyrene and nile red sigmoidal curves. Aggregation of CTAB, in comparison with TTAB, is much more affected by the presence of hyaluronan which could be attributed to the longer surfactant alkyl chain. Increased CMC of CTAB in the presence of hyaluronan can be caused by stronger hydrophobic interactions between longer cetyl chain and hydrophobic patches on hyaluronan backbone [31,32]. Hydrophobic interactions are supposed not to be screened by the added salt as much as is the case of electrostatic interactions. Hydrophobically bound surfactant molecules are then not able to participate in micelles formation.

Pyrene polarity index curves measured for CTAT only and for CTAT with the low molecular weight hyaluronan are similar except slightly higher value of this index for the former in the premicellar region. Presence of the high molecular weight hyaluronan again shifted the inflex point to higher surfactant concentrations and broadened the micellization region. Nile red emission maximum showed increased CMC values for both hyaluronans, higher for the low molecular weight preparation; the high molecular weight type gave very narrow micellization region. The nile red integral intensity showed the same and increased CMC in the presence of both hyaluronans. CTAT results are thus somewhere between the results for TTAB and CTAB. Introducing different types of polar head (aromatic, with sulfate counterion) into cationic surfactant did not substantially change its interactions with hyaluronan and solubilization properties of formed aggregates.

3.5. *Fluorescence probes – zwitterionic surfactants*

Finally, two amphoteric surfactants were tested. Nile red data obtained for cetyl betaine were similar regardless the presence of hyaluronan. Only pyrene polarity index showed a minor decrease of CMC in the presence of the low molecular weight hyaluronan. Thus no significant hyaluronan-cetyl betaine interactions were observed in physiological solution. In the case of Betadet THC 2 nile red intensity showed certain difference – increased CMC in the presence of both hyaluronan samples. Betadet is a commercial cosmetic product based on disodium cocoamphodiacetate with unspecified additional ingredients. Hyaluronans added to this composition thus did not intervene its interactions too much except changing the number or size of aggregates capable to solubilize nile red.

4. Conclusions

Although the presence of NaCl may suppress interactions between oppositely charged polyelectrolyte and surfactant results of this work showed that they are still present in some hyaluronan-surfactant systems regardless the surfactant ionic nature. These interactions were demonstrated mainly by fluorescence probe techniques whereas tensiometry detected only minor effects. Fluorescence data demonstrated that formation of aggregates (micelles) occurs rather in a certain interval of surfactant concentrations than in a single point. It is thus more appropriate to speak about micellization (aggregation) region than single critical micellar (aggregation) concentration, especially in the presence of hyaluronan because its main effect on fluorescence data was broadening of concentration interval in which the fluorescence probe starts to be and is solubilized in nonpolar domains. Broadening of this interval usually changes the value of its mid-point which can be considered as a point estimate of the critical micellar (aggregation) concentration. Tensiometry did not detected changes in the critical micellar concentration, only in several systems slight decrease of surface tension in the premicellar region was observed in the presence of hyaluronan.

The greatest differences between surfactant physiological solution and surfactant + hyaluronan physiological solution were found for nonionic Tween 20 and cationic CTAB. This was rather surprising for the former, first because of its nonionic nature and second because of no such differences were observed for the other nonionic surfactants. Specificity of Tween 20 was attributed to its structural features – relatively bulky and sugar-based polar head capable of weak physical interactions, preferably hydrogen bonding, with hyaluronan chain. Interactions of hyaluronan with CTAB, an oppositely charged molecule, were expected, however, in contrast to aqueous solution, in physiological solution used here they manifested in increased CMC or, more precisely, they shifted solubilization capability to higher surfactant concentrations. This is probably a result of hydrophobic interactions of sufficiently long surfactant alkyl chain with low-polarity parts of hyaluronan backbone which are promoted in physiological solution where the electrostatic interactions are screened.

Fluorescence data also demonstrated that there can be differences in results obtained with different fluorescence probes which are caused by their different location and solubility in studied system and by differences in the nature of their photophysical behavior. Pyrene seems to be a useful probe to detect the onset of micellization process and the width of concentration interval where this process occurs. Wavelength of the nile red fluorescence emission maximum can provide similar information whereas its intensity determines when a sufficient number of really hydrophobic domains is formed in studied system.

From the point of view of targeted delivery it is important to note that fluorescence probes demonstrated that solubilization properties of all tested surfactants were retained in the presence of hyaluronan, i.e. eventual hyaluronan-surfactant interactions do not destroy aggregates containing non-polar domains (micelles).

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.colsurfa.2011.05.035.

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APPENDIX II

Krouská, J., Pekař, M., Šarac, B., Bešter-Rogač, M.: Study of interactions between hyaluronan and cationic surfactants by calorimetry, turbidimetry, potentiometry and conductometry.

Type of publication: manuscript of research article

Study of interactions between hyaluronan and cationic surfactants by calorimetry, turbidimetry, potentiometry, and conductometry

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Abstract

Thermodynamic of micelle formation of cationic surfactants tetradecyltrimethylammonium bromide (TTAB) and cetyltrimethylammonium bromide (CTAB) with and without addition of hyaluronan of two molecular weights was studied in aqueous solution. The critical micelle concentration (cmc) and the enthalpy of micellization ($\Delta_{mic}H$) of the described system were determined using isothermal titration calorimetry (ITC), conductivity, turbidimetry and ionic selective electrode measurement. Results proved expected interaction between surfactant and oppositely charged polyelectrolyte.

Introduction

Interactions between polyelectrolytes and surfactants are subject of continuous interest in the last decades. Complexes and aggregates formed especially by oppositely charged polymers or biopolymers and surfactants are of considerable interest not only from the theoretical point of view but also due to their industrial or biological significance. Experimental data, theoretical explanations and practical applications on polymer-surfactant interactions were summarized in a book by Holmberg et al. [1]. This paper is focused on interactions between negatively charged sodium hyaluronate (hyaluronic acid, hyaluronan) and cationic surfactants.

Hyaluronan is a natural, linear, unbranched polysaccharide with repeating dissaccharide units of D-glucuronate and N-acetyl-D-glucosamine linked by β(1-4) and β(1-3) bonds [2],[3]. It occurs in connective tissues in the body such as skin, vitreous or join fluid. It is a unique biopolymer with a huge amount of viscoelastic and moisturising properties as itself [4]. The negative charge character is due to carboxylic group of glucuronic acid residue. This structure determines hyaluronan to behave as a polyelectrolyte (polyanion) in solution at physiological pH [5]. Other important properties are its biocompatibility and biodegradability. It plays a vital role in many biological processes such as tissue hydration, proteoglycan organization in the extracellular matrix and cell differentiation [6]. In the last two decades, the interest in this molecule has grown because of its associative properties and also for its great potential in pharmaceuticals, cosmetics, coatings and recovery [7]. Moreover, cells are able to recognize hyaluronan using their specific receptors in the cell membrane. The receptor CD44 is probably the most important. It makes hyaluronan very attractive molecule for application in targeted drug delivery systems [8].

Hyaluronan cannot be used directly for targeted drug delivery to carry nonpolar substances because it is a highly hydrophilic biopolymer with massive hydration shell. Many efficient drugs are hydrophobic and to deliver them, hyaluronan must be chemically modified (hydrophobized) to make hydrophobic domains for solubilizing these drugs [8],[10]. Because chemical modification can affect hyaluronan biological functions and biocompatibility an alternative approach uses physical interactions between hyaluronan and some hydrophobizing partner. For this purpose, surfactants can be used and surfactant micelles serve as solubilizing domains in the resulting complex, whereas hyaluronan provides water solubility, biocompatibility and targeting properties.

Hyaluronan-surfactant interactions were subject of several previous studies including our group (Thalberg et al. [11]-[14]; Herslöf et al. [15]; Björling et al. [16]; Yin et al. [17] and Halasová et al.

[18]). Hyaluronan interactions with cationic surfactants were studied as a specific case of general polyelectrolyte-surfactant interactions to elucidate their phase behavior and physical causes of their interactions including the effect of electrolytes and to determine critical aggregation concentrations under various solution chemistries. It was found that there is a certain cationic surfactant concentration below which only general electrostatic interactions take place and only above it marked formation of hyaluronan-surfactant complexes can be observed. Strong cooperativity of surfactant binding on hyaluronan macromolecule was observed resulting in binding in the form of micelle-like clusters in which both hyaluronan carboxylates and background electrolyte anions participate as counterions. Critical aggregation concentrations in water are well below (about an order of magnitude) the surfactant critical micelle concentrations and are increased by addition of low molecular weight electrolyte. There are no data on thermodynamics of interaction of hyaluronan with cationic surfactants. Therefore the main aim of this study was to study hyaluronan-surfactant interactions by means of isothermal titration calorimetry (ITC) and to compare results with outputs of other techniques, viz. conductivity, turbidimetry and ion selective electrode measurements.

Not only Bouchemal et al. [19] and Lapitsky et al. [20] describe ITC as a unique method for characterizing of polyelectrolyte-surfactant interactions from the thermodynamic point of view. It is focused on measurement of heat that is absorbed or generated in an interaction between two molecules. ITC is used to determine not only the aggregation parameters of surfactants but also other thermodynamic parameters such as stability constants, interaction enthalpies, entropies, Gibbs free energies and heat capacities. Moreover, it is the only method where critical micelle concentration (cmc) and enthalpy of micellization is determined without any other probe at the same time.

There are many authors who studied interactions between polyelectrolytes and surfactants from a calorimetric point of view. Wang et al. [21][22] focused on a system with oppositely charged polyelectrolyte and alkyl trimethylammonium bromide where electrostatic attractions where suggested. Interactions between different polysaccharides and ionic surfactants were reported by Bao et al. [23]. They used κ–carrageenan as a representative of anionic polysaccharides and CTAB as a cationic surfactant which is very close to our system. The results from ITC proved strong hydrophobic binding and electrostatic interactions between the polymeric chain and the surfactant molecules.

Materials

Hyaluronan of two molecular weights 116 kDa and 1.8 MDa (i.e. low and high molecular weight, respectively) was purchased from CPN, Ltd., Czech Republic. Cationic surfactants tetradecyltrimethylammonium bromide (TTAB) and cetyltrimethylammonium bromide (CTAB) were from Sigma–Aldrich (Czech Republic). Surfactants were of the best available quality and used as received without further purification.

Stock solutions of hyaluronan were prepared in concentration of 0.1% (w/v) by slowly adding the hyaluronan powder into water under slow stirring. The solution was stirred overnight to ensure complete dissolution. Surfactant stock solutions were prepared by simple dissolution of solid surfactant in triple distilled water under stirring for 4 hours. The concentrations of the surfactant solutions were approximately ten times higher than cmc.

Methods

Isothermal titration calorimetry

The cmc of pure surfactant was determined from the heat effects during the titration of surfactant stock solution into water. The reference and measuring cell were filled with 2 ml of water. Successive aliquots of 10 μl of surfactant stock solution were injected into the measuring cell by a motor-driven syringe and stirred. The total injected volume was 250 μl.

The critical aggregation concentration (cac) of the surfactant with added hyaluronan was determined using the similar procedure as described above. The reference cell was filled with 2 ml of water. The measuring cell was filled with 2 ml of hyaluronan solution and the amount of injected surfactant was the same as in cmc measurement. The stirring during the whole experiment is more than important because the precipitation of a surfactant-hyaluronan complex will occur. The dilution of hyaluronan during the experiment was neglected, its concentration was assumed to be constant at 0.1% (w/v).

The heat changes of the micellization process were measured using 2277 Thermal Activity Monitor microcalorimeter (Thermometric, Sweden) at constant temperature 298 K. The corresponding enthalpogram (enthalpy vs. concentration plot) was obtained by integration of the raw signal – peaks.

In the first part of the plot, the surfactant undergoes demicellization process of the micellar stock solution, surfactant is diluted. The resulting heat changes come from the demicellization of surfactant micelles, monomer dilution and the interactions of counterions. Starting from the point of micellization and continuing at still higher surfactant concentrations the solution undergoes micellar dilution with considerable enthalpy change. Finally, the enthalpy remains more or less constant with increasing surfactant concentration. As expected, the enthalpograms can be divided into three regions: initial and final horizontal part with constant enthalpy connected with the increasing one. The enthalpy of micellization corresponds to the difference between the final and the initial linear part of the enthalpogram as described in Fig. 1.

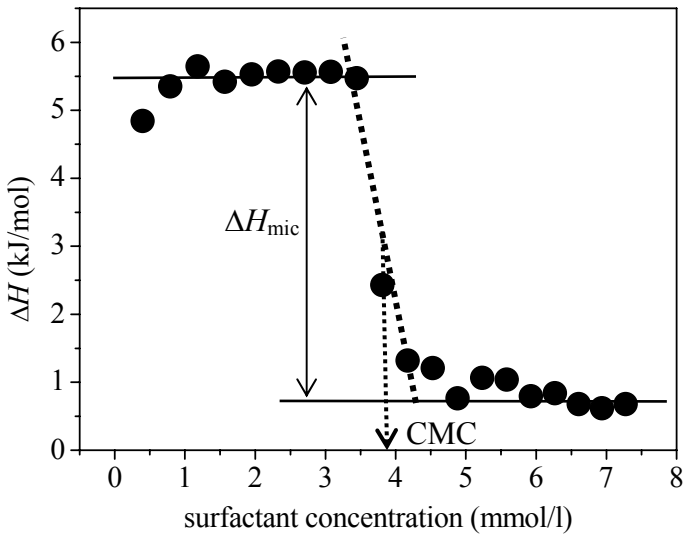

Fig. 1. Determination of enthalpy of micelle formation ($\Delta_{mic}H$) and the cmc from the experimental data. The distance between two plateaus corresponds to $\Delta_{mic}H$ and cmc is the inflection point on the curve.

Conductivity measurement

Conductivity of pure surfactants and surfactant-hyaluronan mixture was recorded with a PC-interfaced LCR Meter Agilent 4284 A connected to a three-electrode measuring cell. Temperature of the water bath was set to 298 K with reproducibility within 0.005 K. After measuring the resistance R of water or hyaluronan solution at a set temperature, successive aliquots of a stock solution of surfactant were added to water or hyaluronan by a programmable syringe pump (Model 1250, J-KEM Scientific, MO, USA), and the resistance of the solution was measured at 298.15 K.
A home-developed software package was used for temperature control and acquisition of conductance data. The measuring procedure, including corrections and extrapolation of the sample resistance to infinite frequency, has been described previously by Bešter-Rogač et al. [24]. The corresponding conductivities $\kappa_{overall}$ were obtained as $\kappa_{overall} = B/R$, the temperature dependence of the cell being taken into account as published by Barthel et al. [25]. Taking into account the sources of error (calibration, titration, measurements, impurities) the conductivities are accurate to within 0.5 %.
Pure water and hyaluronan solution before titration were treated as solvent and therefore the corresponding electrical conductivity, κ_{el} was subtracted from the overall measured electrical conductivities $\kappa_{overall}$. The contribution of the surfactant to the conductivity was calculated as $\kappa = \kappa_{overall} - \kappa_{el}$.

Turbidimetric titrations

Turbidimetric measurements were performed with a UV-VIS spectrophotometer Varian Cary 50 equipped with fiber optic probe with 1 cm optical path length at 420 nm. Blank corrections were made with surfactant or hyaluronan free systems.
Hyaluronan stock solution (1.8 MDa, 0.1 % w/v) was titrated with CTAB (10 mM) at room temperature. The mixture was stirred for 1 minute after each injection before the measurement. The total added volume of surfactant to 50 ml of hyaluronan was 5.92 ml.

Ionic selective electrode measurement (ISE)

Electrochemical potential E was measured using ionic surfactant electrode (6.0507.120, Metrohm). Hyaluronan stock solution (1.8 MDa, 0.1 % w/v) was titrated with CTAB (50 mM) at room temperature. The mixture was stirred for 1 minute after each injection before the measurement. The total added volume of surfactant to 50 ml of hyaluronan was 0.745 ml.

Results and discussion

Isothermal titration calorimetry

Thermodynamics of micelle formation of two cationic surfactants with different alkyl chain length was studied. Further, the influence of added polyelectrolyte, hyaluronan, was investigated. Cmc of both surfactants in water correspond to previously published data by Moulik et al. [26] and Beyer et al. [27]. Interesting results were obtained for mixtures of the two surfactants with hyaluronan. In the case of TTAB (Fig. 2), there are strong interactions at lower surfactant concentration below its regular cmc. Hyaluronan of both molecular weights increase enthalpy values in the endothermic region of the enthalpogram in comparison with pure surfactant solution. Enthalpy maximum seems to appear around 1.2 mmol/l of TTAB. This part of enthalpogram reflects interactions of individual surfactant molecules with hyaluronan chains and probably also formation of micelles on

hyaluronan. Then, the steep decrease follows similarly as for the pure surfactant solution but at lower surfactant concentrations than regular cmc. This is the region used to determine the cac. Finally, all three curves practically meet in a common plateau at higher surfactant concentration. Evidently, the added hyaluronan induces the aggregation of TTAB at lower concentrations that its cmc. Nevertheless, it was proved that both surfactants make precipitation with hyaluronan just at low surfactant concentration which supports an idea that surfactant monomers are binding on the hyaluronan backbone and the micelle-like aggregates are formed.

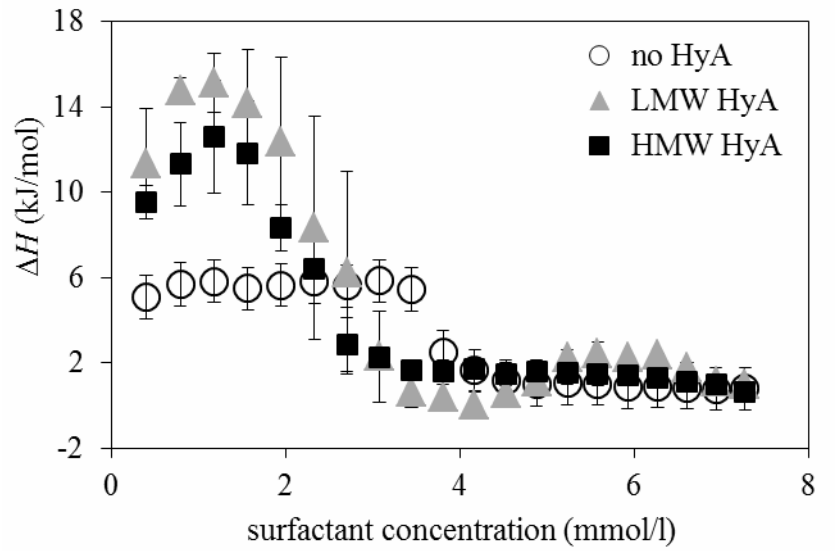

Fig. 2. Effect of low (LMW) and high (HMW) molecular weight hyaluronan (HyA) on cmc and normalized reaction enthalpy of TTAB in water.

The effect of hyaluronan addition to CTAB is completely different – enthalpograms are shifted to higher surfactant concentrations and the effect of hyaluronan molecular weight is more noticeable (Fig. 3). The enthalpy is higher for HMW hyaluronan at lower surfactant concentrations and the final plateau of all titrations is approximately at the same enthaply values.

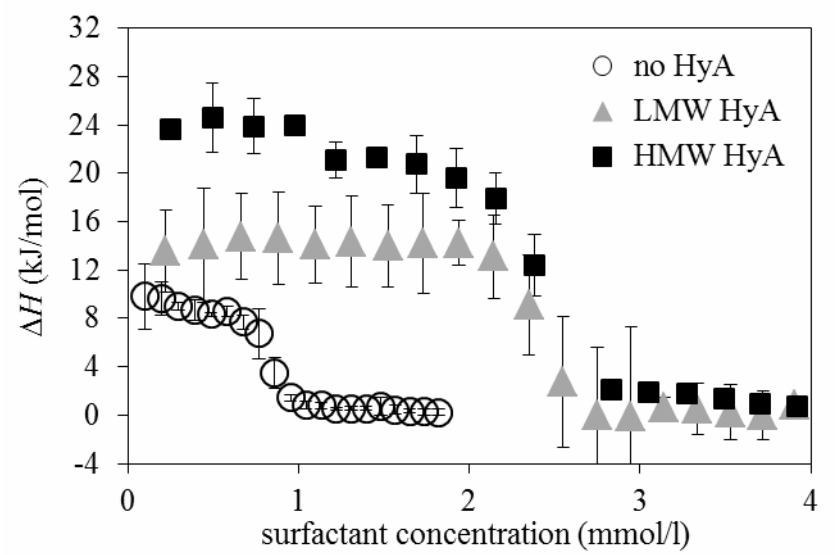

Fig. 3. Effect of low (LMW) and high (HMW) molecular weight hyaluronan (HyA) on cmc and normalized reaction enthalpy of CTAB in water.

The observed differences in the behavior of the two surfactants should be attributed to their alkyl chains because this is the only difference between them. The main contribution to hyaluronan-surfactant interactions is believed to be made by the electrostatic forces between the carboxylic groups on hyaluronan and the oppositely charged ammonium group on surfactants. ITC results indicate important contribution of interactions due to alkyl chains which can be hydrophobic or excluded volume (packing) effects. 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 such which is coupled with cloudy and opaque state of the samples.

Table 1
Critical micelle (cmc) or aggregation (cac) concentration and enthalpy of micellization of TTAB and CTAB, in the presence of low (LMW) and (HMW) molecular weight hyaluronan as determined using ITC method. The errors are accurate to within 5%.

	cmc, cac (mmol/l)			
	no HyA	LMW HyA	HMW HyA	references
TTAB	3.69	2.82	2.81	this work
	3.08			[26]
	4.11			[27]
	3.54			[28]
CTAB	0.76	2.43	2.34	this work
	0.80			[26]
	0.89			[27]
	0.89			[28]

	$\Delta_{mic}H$ (kJ/mol)			
	no HyA	LMW HyA	HMW HyA	references
TTAB	-5	-13	-13	this work
	-5			[27]
	-5			[28]
	-6			[29]
CTAB	-8	-14	-11	this work
	-9			[27]
	-11			[28]
	-11			[29]

Turbidimetry and ISE

By visual observation it was found that during titration of hyaluronan solution with surfactant solution the system becomes progressively clouded and finally phase separation occurs (pieces of precipitate are formed). Therefore turbidimetry was applied to investigate development of turbidity. Results are reported for CTAB only, conclusions for TTAB are similar. Fig. 4 shows that turbidity starts to increase already at CTAB concentration of about 0.07 mM, i.e. turbidity develops at such low concentrations where no (step) response on ITC records was measured. To further check this behavior titrations with surfactant ion selective electrode were made; results are also given in Fig. 4. The point of start of increasing turbidity corresponds to the point of the end of electrode potential increase. Both techniques thus indicate that binding of probably all surfactant molecules added

during titration starts at surfactant concentration of about 0.07 mM. Because the ISE response at lower surfactant concentrations is well below the response measured for surfactant without hyaluronan some surfactant molecules should bind to hyaluronan even at concentrations lower than 0.07 mM. This concentration could be thus viewed as some threshold for cooperative binding or binding in the form of micelles. This concentration is so low that no specific response was detected on ITC record. The step change on the ITC record resembling the change observed during micellization of pure surfactant (see Fig. 3) belongs to macroscopic phase separation and formation of precipitates. Data given in Tab. 1 for systems containing hyaluronan thus refer to phase separation aggregation. It is not easy to determine a single value of critical aggregation concentration precisely. It is better to speak about an interval of concentrations where the aggregation process occurs. From the point of view of turbidimetry and ISE aggregation starts from at least 0.07 mM and forms aggregates dispersing visible light and preventing surfactant molecules to travel to ISE and change its potential. However, even below this concentration a certain part of surfactant molecules should be bound to hyaluronan.

The ratio of charges on surfactant and hyaluronan can provide the information about the possible free or occupied hyaluronan charged groups in the system which can further indicate the presence of potentially free molecules of surfactant. The charge ratio surfactant : HyA can be calculated as follows. It corresponds to the ratio of concentration of charges on the surfactant molecule and hyaluronan. Whereas both surfactants TTAB and CTAB have one positive charge in their structure and hyaluronan has one negative charge per one disaccharide unit, it is necessary to calculate the concentration of charges on hyaluronan chain according the following relation (the weight of one disaccharide unit is 400 g/mol):

$$\frac{\text{concentration of hyaluronan in the solution (g/l)}}{\text{weight of one disaccharide unit (g/mol)}} = \text{concentration of disaccharide units (mol/l)}$$

Consequently, the charge ratio surfactant : HyA is calculated as the ratio of molar surfactant concentration and concentration of disaccharide units. As mentioned above, the aggregation determined by potentiometry and conductivity starts at 0.07 mM which corresponds to the charge ratio 0.028. This low value indicates that hyaluronan is in high excess and simultaneously, that the experimental results expressed by the titration curves showed that even the low surfactant concentration is high enough to signify the interactions with the polyelectrolyte chain.

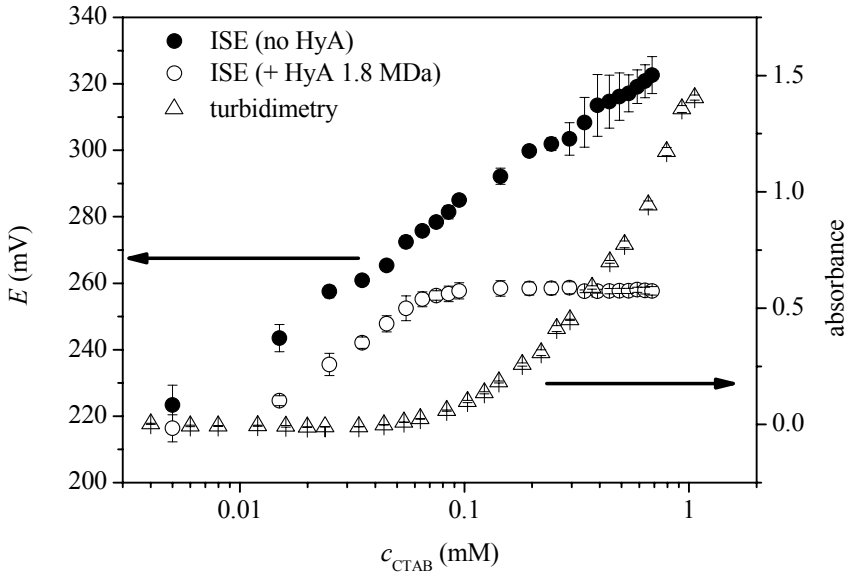

Fig. 4. Comparison of turbidimetric and ISE analysis of the system CTAB-hyaluronan (molecular weight 1.8 MDa) in water at 25°C.

Conductivity measurements

Conductivity measurements in the presence of hyaluronan were adversely affected by phase separation (precipitation) at higher surfactant concentrations. Fig. 6 shows representative example of the best results obtained. Conductivity data obtained for pure surfactant solutions correspond to expectations, the break point gives critical micellar concentrations agreeing with literature value, viz. 0.9 mM for CTAB and 3.7 mM for TTAB; reported values are 0.97 mM for CTAB [30] and 3.76 mM for TTAB [31]. Conductivity curves measured in the presence of hyaluronan are well below the curve measured for pure surfactants. They are continuously increasing and composed from two approximatelly linear main parts; the break point corresponds to cmc of pure surfactant. Thus the only indicator of the presence of hylauronan on conductivity data is the decreased conductivity. This might be caused by formation of micelles on hyaluronan surrounded by bromide counterions. Mobility of bound cationic surfactant molecules as well as of the counterions is thus decreased which results in decreased conductivity. Behind the break point, where precipitation occurs, conductivity should be attributed primarily to free micelles and ions in the supernatant.

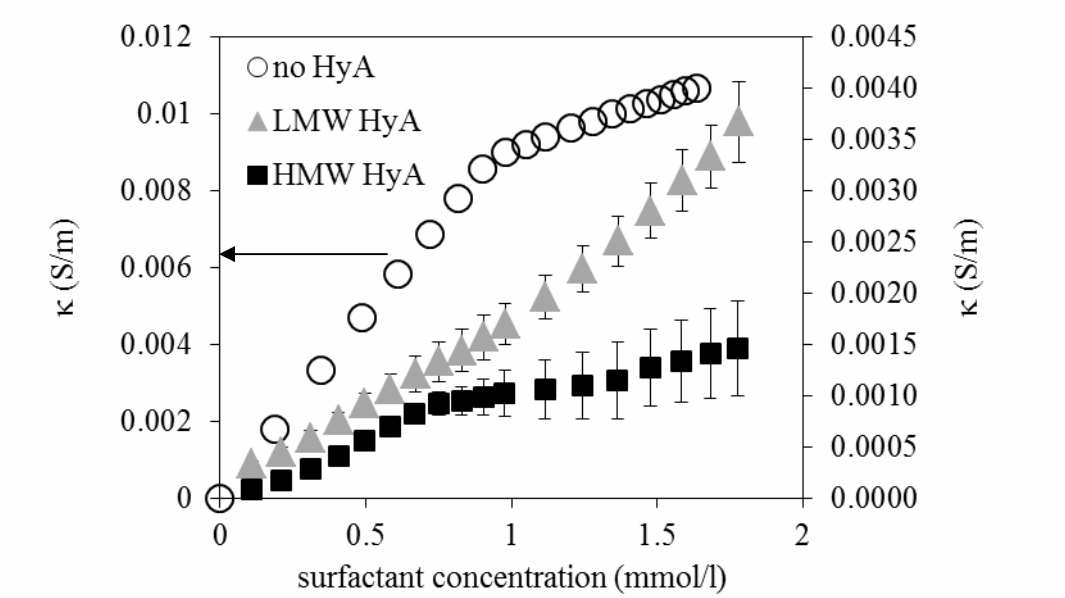

Fig. 5. Effect of low (LMW) and high (HMW) molecular weight hyaluronan (HyA) on conductivity of CTAB.

Conductivity in pure TTAB solution around the break point is about four times higher than analogical conductivity shown in Fig. 6 for pure CTAB solution. This is natural consequence of smaller and therefore more mobile molecules of TTAB. Similarly, the conductivity of CTAB-hyaluronan complexes is almost an order of magnitude smaller than conductivity ot complexes with TTAB (data not shown here). Also the difference between conductivity in the absence and in the presence of hyaluronan in the case of TTAB is smaller than shown in Fig. 6 for CTAB. This could indicate more intensive binding of CTAB to hyaluronan chain resulting in neutralization of more charges and in hindering mobility of more counterions.

Conclusion

The influence of added hyaluronan on micellization process of CTAB and TTAB was studied using calorimetry, turbidimetry, potentiometry, and conductometry. The interactions based on electrostatic attraction, hydrophobic effects and phase separation were proved also by visual observation of the samples with added hyaluronan. The samples changed with increasing surfactant concentration from clear to opaque, cloudy and finally, to phase separated at the end of the experiments. The transitions between the stages were detected using all the techniques but at different surfactant concentrations. ITC and conductometry detected just the last step concerning the phase separation while turbidimetry and potentiometry gave detailted information about the interactions at low surfactant concentration. The cmc values (surfactants without hyaluronan) are in good agreement with literature. Unfortunately, there are no published results from the same measurement methods for hyaluronan-surfactant system. In addition, ITC results indicated significant contribution of interactions due to alkyl chains caused by hydrophobic or excluded volume effects. Moreover, there was a shift of the aggregation region in the case of TTAB to lower surfactant concentration and the phase separation region with CTAB was shifted to higher concentrations.

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