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# Calorimetric and light scattering study of interactions and macromolecular properties of native and hydrophobically modified hyaluronan

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ABSTRACT

The paper presents a new investigation of a native and a hydrophobically modified hyaluronan (HMHA) $C_8$NH-HA100 interactions with surfactants SDS, DTAB, and a dye polarity probe Coomassie Brilliant Blue (CBB), and their macromolecular properties in aqueous environment using isothermal titration calorimetry (ITC) and SEC-MALLS. A novel alkylated HA derivative was prepared by the introduction of octyl chains onto –OH groups of the HA D-glucuronic acid unit via carbamate bond at a high degree of substitution preserving the HA carboxylic groups. Large and abrupt endothermic enthalpy changes ($\Delta H$) during the titration of HA by DTAB evidence a strong electrostatic interaction and a formation of complex aggregates between HA and DTAB micelles. Heat effects of HA–SDS titration are moderate and reveal a lowering of the surfactant CMC in the presence of HA. The $C_8$NH-HA100 displays binding with CBB in terms of exothermic enthalpy changes, which is ascribed to hydrophobic interactions, while such binding is not observed for HA. The assessment of the polysaccharides backbone stiffness and the coefficient of the Mark–Houwink–Sakurada plot (MHS), *a*, by SEC-MALLS shows a little alternation of the HA backbone stiffness after its modification.



## 1. Introduction

Hyaluronan, sodium salt of hyaluronic acid, (HA) is a linear natural polysaccharide of the glycosaminoglycans family. Its chemical structure comprises disaccharide units composed of D-glucuronic acid and *N*-acetyl-D-glucosamine, which are alternatively linked through β-1,3 and β-1,4 glycosidic bonds (Cowman & Matsuoka, 2005; Lapčík, Lapčík, De Smedt, Demeester, & Chabreček, 1998). High-molecular weight HA occurs mainly in connective tissues of vertebrates where it interacts with other bio-molecules, such as proteoglycanes and proteins creating a resilient network matrix of high viscoelasticity. On the other hand, low-molecular weight HA is involved in many fundamental cell processes due to its specific interactions with cell-surface receptors (Jaracz, Chen, Kuznetsova, & Ojima, 2005). In organism HA comes into a contact with a number of molecules, macromolecules as well as small ions, oligomers, water, etc. Therefore, it is of a great importance to deal with HA interactivity with various kinds of molecules regarding its medicinal and pharmacological applications, such as drug delivery, where HA has particularly found its biggest utilization (Brown, 2008; Vercruysse & Prestwich, 1998).

The attention of this paper is focused on interactions of native and modified HA with some other types of molecules in order to investigate the interactivity of the polysaccharides in general and for medicinal and/or pharmacological purposes, namely drug delivery. The compounds selected for such an investigation were surfactants, sodium dodecyl sulfate (SDS) and dodecyltrimethyl ammonium bromide (DTAB), and a polarity dye probe, Coomassie Briliant Blue (CBB).

HA is a strongly hydrophilic molecule, whereas most drugs, e.g. cytotoxic agents, are poorly soluble in water. Thus, surfactants are often employed in the formulation to improve the active ingredient's (drug) solubility and bioavailability (Fukada, Suzuki, & Seimiya, 1999). Moreover, the interactions of, generally, polyelectrolytes with oppositely charged surfactant micelles have found utilizations in some specific industrial applications, e.g. colloid flocculation in water clearing, and also they represent a model for the interactions between polyelectrolytes and oppositely charged colloids in general (Thalberg & Lindman, 1989). Despite its utilization in drug delivery, native HA has been also a subject for various chemical modifications, which have offered a number of advantages over simple HA-drug admixtures, such as controlled-release, cell targeted properties, or improved water solubility than relative to the parent drug (Vercruysse & Prestwich, 1998). Since the HMHA

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has been designed for the use as a novel drug delivery system, the present work is also focused on the study of the interactivity of highly substituted C-8 carbamate derivative of HA with a polarity probe CBB in order to detect any hydrophobic interactions between the species and assess the derivative potential to interact with mostly water-insoluble bioactive compounds, e.g. anticancer drugs.

There is an array of methods that have been used in the study of HA's interactivity, e.g. dye solubilization methods, conductivity (Thalberg & Lindman, 1989), viscometry or rheology (Herslöf, Sundelöf, & Edsman, 1992; Pisárčik, Soldán, Bakoš, Devínsky, & Lacko, 1999) turbidimetric titrations (Kayitmazer, Seyrek, Dubin, & Satggemeire, 2003), surface tension measurements (Santos, Nome, Zanette, & Reed, 1994), fluorescent probes (Kakehi, Kinoshita, & Yasueda, 2003; Yin, Yang, Ge, & Yuan, 2005), light scattering methods, etc. In this work, isothermal titration calorimetry (ITC), which particularly has found a great favor in the study of binding between biological molecules because it enables to quantify thermodynamic parameters associated with these interactions directly and precisely (Ladbury & Chowdhry, 1996), is applied. So far, only few papers studying interactions of hyaluronan with some other molecules using ITC have been published. Some of them characterize interactions of mostly HA oligosaccharides and inflammation-associated hyaluronan-binding protein TSG-6 (Blundell et al., 2007; Kahmann et al., 2000), or polymeric HA with some drugs (Santos, Manzanares, Murtomäki, & Kontturi, 2007). The ITC experiments are supported by the solubilization method using the Coomassie Brilliant Blue dye polarity probe. For comparison of the macromolecular behavior of HA and C-8 alkylated HA the method of size-exclusion chromatography with multiangle laser light scattering and viscosity detector (SEC-MALLS) is utilized to determine the dimension of their macromolecules as a function of their molar mass, Mark–Houwink–Sakurada plot parameters, and stiffness of their backbones in order to find whether the applied modification has significantly altered these properties.

Thus, the major aims of this paper are the verification of the ITC method for the study of the interactivity of polymeric hyaluronan with different types of surfactants, and investigation of the difference in reactivity between native HA and HMHA by means of calorimetric titrations with a dye polarity probe. The presence of hydrophobic domains on both polymers, and thus their potential ability to interact with water-insoluble molecules by means of ITC, as well as the difference between native and modified HA macromolecular behavior in terms of *a* coefficient of the Mark–Houwink–Sakurada plot and the persistence length have also been inquired.

## 2. Materials and methods

### 2.1. Materials

A bacterially produced HA of molecular weight $M_W = 1.69 \times 10^6$ g mol$^{-1}$ and HMHA designated as $C_8$NH-HA100 were provided by CPN, Ltd. (Dolní Dobrouč, the Czech Republic). The derivative of HA was synthesized by the activation of HA by cyanogen bromide followed by the reaction with octyl-amine on the $C_2$ or $C_3$ of D-glucuronic acid, forming carbamate bond between the alkyls and –OH groups of HA (Fig. 1a) as reported by Mlčochová et al. (2006). The determination of the derivative molar mass (MM), $M_W = 4.95 \times 10^5$ g mol$^{-1}$, as well as its degree of substitution, DS = 100%, are also reported there. The DS represents a number of HA structure units bearing the alkyl chain per overall number of HA structure units, i.e. DS of 100% means that 100 of 100 disaccharide units contain octyl chain.

Sodium dodecyl sulfate (SDS) (≥99.0%), dodecyltrimethylammonium bromide (DTAB) (Sigma Ultra ~99%) and Coomassie Brilliant Blue R (CBB, Fig. 1b) (75% of the dye content) were purchased from Sigma Aldrich. Salts, NaCl, $Na_2HPO_4 \cdot 2H_2O$ and citric acid (all of p.a. grade) were purchased from Merck. Milli-Q water was used for the preparation of all solutions.

### 2.2. Preparation of solutions

The 0.2 and 0.1% (w/w) hyaluronan and 0.5% (w/w) $C_8$NH-HA100 stock solutions were prepared by addition of appropriate amount of dry polysaccharides into smaller volume of Milli-Q water and let swollen at room temperature for several minutes. Afterwards, the rest of water was added up to a desired concentration at gently stirring and the solution was left stirred for at least 24 h in order to obtain well-homogenized solution. The stock solutions were afterwards diluted with Milli-Q water, or filtered NaCl solution or citric acid/$Na_2HPO_4$ buffer (pH 2.5, 5) down to desired concentrations

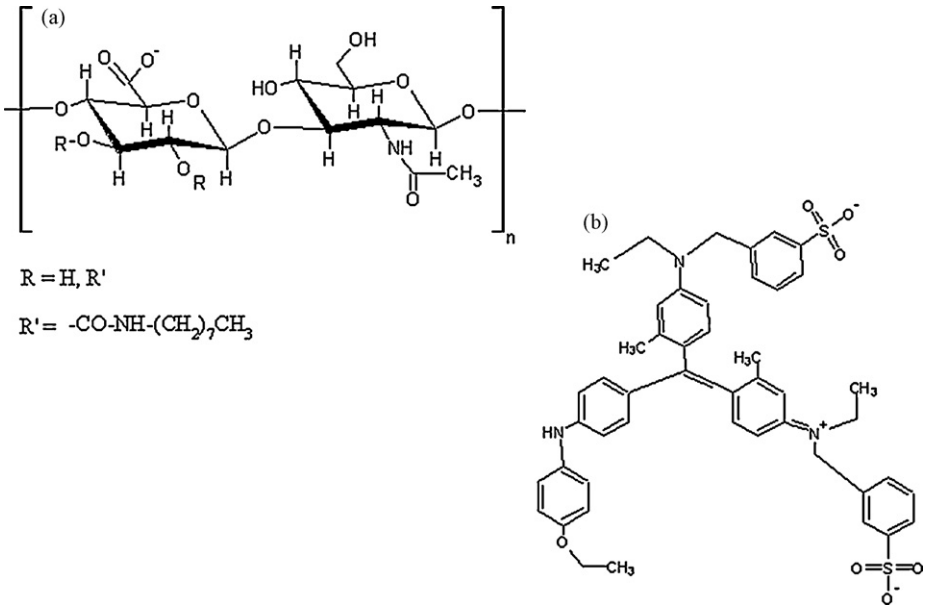


**Fig. 1.** (a) A structure formula of HA (when R=H) and alkylated derivative of HA, $C_8$NH-HA100, and (b) a dye polarity probe Coomassie Brilliant Blue (CBB).

and ionic strength and agitating for at least 2 h. All solutions were stored in the refrigerator at approximately 4 °C prior to the use. The surfactant and dye stock solutions were prepared in the same way as the polysaccharides but being stirred for 2 h only due to their low-molecular weight. If needed, these solutions were further diluted to desired concentrations with solvent.

### 2.3. Isothermal titration calorimetry

Each solution as well as solvent was degassed for at least 5 min prior to the measurements. An isothermal titration calorimeter (*VP-ITC, Microcal Inc., Northampton, MA, USA*) was used for measuring enthalpies of mixing at 30 °C. The reference cell of the calorimeter contained only MQ-water and the sample cell solvent or a solution of a macromolecule. Firstly, one 5 μL aliquot of ligand for removing the bubbles was sequentially injected into the 1.4 mL reference cell containing either solvent or a solution of the polysaccharide followed by twenty-four 10 μL aliquots of ligand. Each injection lasted 20 s, and there was an interval of 300 s between successive injections. The solution in the cell was stirred at the speed of 290 rev. $min^{-1}$ throughout the experiment. Measurements were carried out in duplicate and the results reported as the average. Data were processed using the software provided by the manufacturer (ORIGIN).

### 2.4. Solubilization of CBB in the polysaccharide solutions

The 50 μL of the CBB stock solution were injected into the $C_8$NH-HA100 solutions, so that the final concentration of the dye in such solutions was 0.026 g $L^{-1}$ ($3.06 \times 10^{-5}$ mol $L^{-1}$). These samples were then agitated on a rotational shaker for 24 h. The absorbance of CBB in the polysaccharide solutions was measured using Cary 50 Probe UV–vis spectrophotometer (Varian) in the range of wavelengths 500–800 nm against the appropriate solvent as a blank. Due to high turbidity of the HMHA solutions above the polymer CAC the absorbance of these solutions without the dye was also measured within the same range of the wavelengths and then deducted from the absorbance of CBB in the HMHA solutions. The measurement of each sample was repeated at least twice and the data were further treated as an average. The critical concentration corresponding to the onset of detection of some hydrophobic clusters formation was determined by plotting the CBB absorption at 618 nm ($A_{618}$) versus the HMHA concentration as described by Duval-Terrié, Huguet, and Muller (2003).

### 2.5. Size-exclusion chromatography (SEC) with online multiangle laser light scattering (MALLS) and viscometer (VISC) detector

The apparatus comprised an HPLC system consisted of a solvent reservoir, online degasser, HPLA isocratic pump, autoinjector, precolumn, and three columns (serially connected) of TSK G-6000PWXL, 5000 PWXL, and 4000 PWXL. The column outlet was connected to a Dawn DSP multiangle laser light scattering photometer (Wyatt, USA) ($\lambda_0$ = 633 nm) followed by Optilab DSP differential refractometer (P-10 cell) and finally a Viscotek TDA 301 viscosity detector. The flow rate was 0.4 mL $min^{-1}$. The injected volume was 100 μL, and the sample concentration was adjusted to obtain the best possible light scattering signal without influencing RI profile (overloading). Samples were filtered (pore size 0.80 μm) prior to injection. Data from the light scattering and differential refractometers were collected and processed using Astra (v. 4.70.07) software (Wyatt, USA), whereas data from the viscometer were collected and processed by the TriSec (v. 3.0) software. Data from both software packages (slice results) were exported to an Excel spreadsheet for further processing.

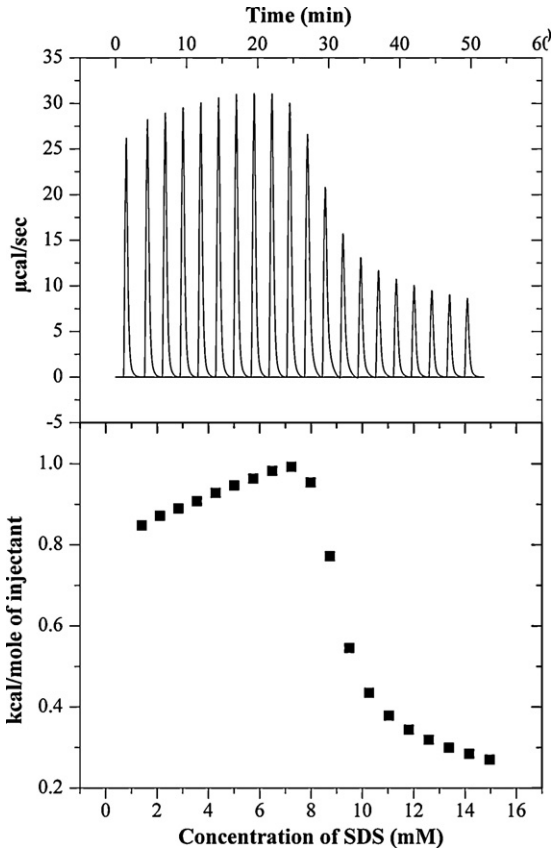


**Fig. 2.** Heat flow versus time profiles resulting from injection of 10 μL aliquots of 100 mM SDS into 1.4 mL calorimeter cell containing water at 30 °C (in the top) and dependence of enthalpy change per mole of SDS on the surfactant concentration in the calorimeter cell (in the bottom).

## 3. Results and discussions

### 3.1. Interactivity of native HA

#### 3.1.1. Influence of HA–SDS interactions on enthalpy changes

Fig. 2 shows the results of 100 mM SDS titration into water; the top part of the image corresponds to the heat flow recorded during the time of the surfactant titration, whereas the bottom part is the plot of enthalpy change ($\Delta H$) per mole of SDS, obtained by the integration of the heat flow over time and divided by SDS concentration, versus the surfactant concentration in the reaction cell. The titration of SDS induces relatively large and positive values of $\Delta H$, i.e. endothermic effect that is intensified as the surfactant concentration in the cell increases until a certain concentration, from which $\Delta H$ drops. Since the initial concentration of SDS in the syringe was appreciably above its critical micelle concentration (CMC), this endothermic effect is attributed to micelles dissociation and to hydrophobic effect (Thongngam & McClements, 2004) as the SDS concentration in the cell dropped below its CMC after the initial injection. This hydrophobic effect is caused by the exposure of the non-polar groups of SDS to water molecules and is temperature dependent (Thongngam & McClements, 2005). The demicellization is thermodynamically favorable below the CMC ($\Delta G < 0$) and because of the endothermic nature of enthalpy peaks, the demicellization must lead to the net increase in the overall entropy of the system; therefore, $T\Delta S > \Delta H$. This entropy increase has been attributed to the release of counterions associated with surfactant headgroups when micelles break down to monomers (McClements, 2000). After a certain number of injections $\Delta H$ drops, which is attributed to the formation of micelles, because the concentration

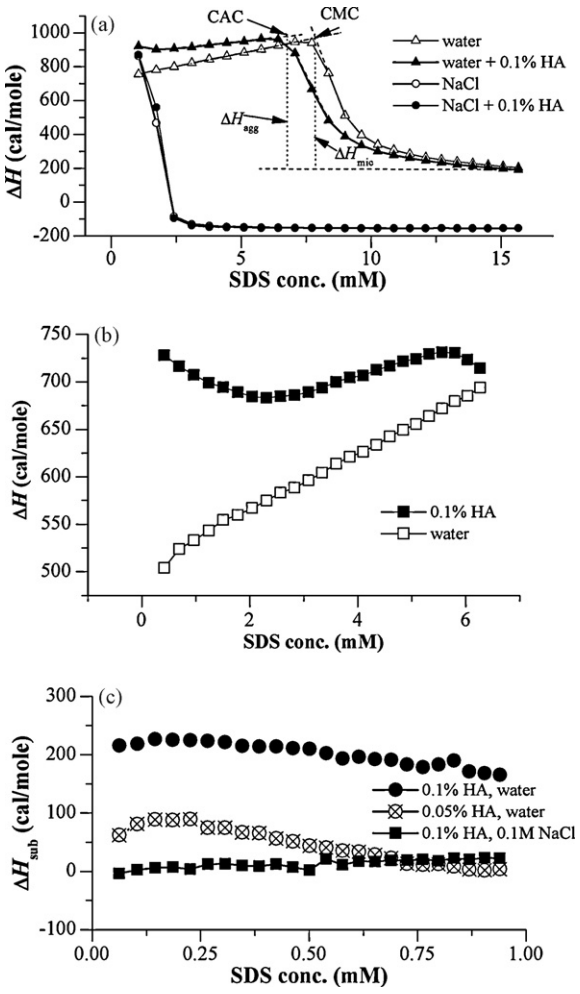


**Fig. 3.** Enthalpy changes per mole of SDS versus the surfactant concentration in the calorimeter cell for the titration of (a) 100 mM SDS into water or 0.1 M NaCl with or without the 0.1% (w/w) HA showing the determination of CMC, CAC, $\Delta H_{mic}$, and $\Delta H_{agg}$; (b) of 40 mM SDS into water and 0.1% (w/w) HA solution; (c) subtracted enthalpy changes for 6 mM SDS injected into aqueous and 0.1 M NaCl solution of 0.05% (w/w) and 0.1% (w/w) HA.

of SDS in the cell has exceeded its CMC. The CMC was determined as shown in Fig. 3a, as has also been reported by Bai, Goncalves, Gama, and Bastos (2008). The value 7.7 mM is in excellent agreement with that determined by other researchers (McClements, 2000; Wang, Han, & Yan, 1997).

Despite its polyanionic character, the presence of 0.1% (w/w) HA in the reaction cell clearly influences the enthalpy–surfactant concentration profile (Fig. 3a). First of all, the values of $\Delta H$ are somewhat larger than those during the titration of SDS into pure water and slightly decreasing until the SDS concentration of ~3 mM (Fig. 3b). Above this concentration $\Delta H$ increases and continuous in the similar pattern as during the SDS titration into water. The larger enthalpy changes may arise from several contributions; nevertheless, we assume them to be mainly due to (i) electrostatic repulsions between the charged surfactant groups and the HA carboxylic groups, which is accompanied by the counterions release or their mutual interchange, and (ii) due to change in hydration of both the reacting species. The initial decrease in $\Delta H$ can be attributed to conformational changes of the polysaccharide molecules, since conformation changes are entropically unfavorable (O'Brien & Haq, 2004), and therefore go against the demicellization process that is in turn entropically favorable, causing an increase in $\Delta H$. The $\Delta H$ increases as long as another drop is occurred indicating a formation of the surfactant micelles. Nevertheless, the CMC of SDS was moderately lowered from 7.7 mM down to 6.7 mM in the presence of HA and the values of enthalpy change during the micellization ($\Delta H_{mic}$), or aggregation ($\Delta H_{agg}$) respectively, remained very alike for both cases, i.e. 635 cal mole$^{-1}$ without HA and 666 cal mole$^{-1}$, respectively, in the presence of HA (Fig. 3a). For the titrations studied in 0.1 M NaCl, the presence of HA was negligible in terms of the CMC (~1 mM regardless the presence or absence of HA) or $\Delta H_{mic}$ of SDS. There are several possible reasons for the reduction of the CMC. One is that salts are known to lower the CMC of ionic surfactants, and therefore any counterions associated with the HA molecules may have caused the reduction. Another possible mechanism is that by forming micelles, surfactant molecules are able to reduce the average distance between HA and surfactant molecules, which makes micellization thermodynamically favorable, i.e. it lowers the CMC, as it was also observed for, e.g. pectin–SDS interaction (McClements, 2000). Nevertheless, the $\Delta H$–concentration profile for HA–SDS interaction resembles rather the profile for the SDS titration into the solutions of pectin of the highest degree of methoxylation, i.e. with the lowest charge density, which gained rather weak interaction.

The large enthalpy peaks for the titration of 100 mM SDS can interfere with other contributions to the overall $\Delta H$. In order to isolate the effect of demicellization from the interaction of HA with SDS we reduced the concentration of the surfactant in the syringe below its CMC, i.e. down to 6 mM. The results of the titration of 6 mM SDS into 0.05 and 0.1% (w/w) HA aqueous and 0.1 M NaCl solutions are shown in Fig. 3c, showing the enthalpy changes of the surfactant titration into the HA solutions subtracted from those of the surfactant titration into appropriate solvent, i.e. $\Delta H_{sub}$, plotted versus SDS concentration. One can see that the presence of HA in water has still endothermic effect, which is opposite to the observation of similar SDS titration into pectin solution (McClements, 2000). Thus, our experiments do not indicate a binding as in the case of SDS and pectin. The only eligible effect of HA concentration results in approximately double values of $\Delta H_{sub}$ when the concentration of HA is raised from 0.05 to 0.1% (w/w). In NaCl solutions the endothermic effect of the surfactant titration in the presence of HA practically diminishes (Fig. 3a and c) and the $\Delta H_{sub}$ values are around zero cal/mole (Fig. 3c). Thus, these observations suggest electrostatic nature of the endothermic effect, evolved during the HA–SDS titrations.

### 3.1.2. *Influence of HA-DTAB interactions on enthalpy change*

The enthalpy–DTAB concentration profile for the titration of 150 and 95 mM DTAB into water (Fig. 4) is similar to that seen for 100 mM SDS since the concentration of DTAB in the syringe

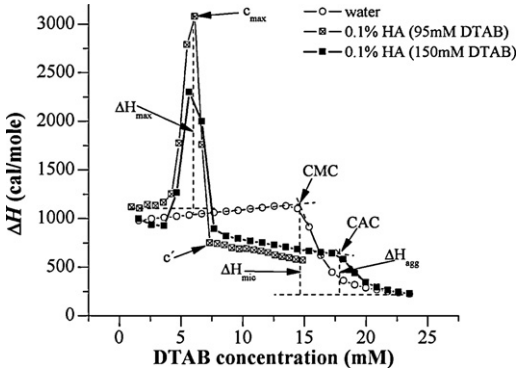


**Fig. 4.** Dependence of enthalpy change per mole of DTAB on the concentration of the surfactant in the reaction cell for the titration of 150 and 95 mM DTAB into water or 0.1% (w/w) HA at 30 °C with a construction of $c_{max}$, $c'$, CMC, CAC, $\Delta H_{max}$, $\Delta H_{mic}$, and $\Delta H_{agg}$ (see the explanation in the text).

was appreciably above its CMC. Under the presented conditions, the CMC of DTAB was determined as 14.3 mM, which is in very good agreement with the CMC range of 14.7–15.1 mM as reported in other studies (McClements, 2000).

The presence of 0.1% w/w HA in the reaction cell has a pronounced effect on the enthalpy–DTAB concentration profile (Fig. 4). Initially, $\Delta H$ values are constant or slightly decreasing, depending on the surfactant concentration, and slightly more positive (for 95 mM DTAB) than those for the injections of DTAB into water only. Beyond the DTAB concentration of ~4 mM, a dramatic growth in $\Delta H$ values into a large endothermic "peak" is observed, which is followed by an abrupt drop in the enthalpy changes even below the initial values and the values for the dilution of DTAB in water. As the injection of the surfactant continuous, the enthalpy changes are still moderately decreasing and still lower than those for the injection of DTAB into water as long as another drop in $\Delta H$ values is observed at the DTAB concentration of ~18 mM.

The immense increase in $\Delta H$ arose from inevitable electrostatic attractive forces between the positively charged headgroups of DTAB and the negatively charged carboxyl groups of the HA while forming an ionic pair. The formation of the ionic pair results in a release of the counterions associated with the polar groups of the reacting molecules, similarly as for the pectin–DTAB interaction (McClements, 2000). Since the binding reaction is endothermic, there must be an increase in the overall entropy of the system, so that the overall free energy is favorable ($\Delta G < 0$, so $T\Delta S > \Delta H$).

Interestingly, the $\Delta H$ values do not begin to increase immediately after the initial injections of DTAB but from the surfactant concentration of ~4 mM in water, i.e. after several injections of the surfactant. A number of different physicochemical processes may contribute to this observation, including (a) dissociation of micelles, (b) changes in surfactant–surfactant, (c) polymer–polymer interactions, and (d) changes in hydration or counterion distribution. Since the enthalpy changes are mostly constant up to ~4 mM DTAB and little more endothermic (also $\Delta H_{sub}$, Fig. 4b) than comparing to increasing $\Delta H$ values for the titration of DTAB into water, we assume that the hydrophobic effect, which is a result of the DTAB micelles dissociation, is partially suppressed by the presence of HA and/or there is another process which energetically compensates this hydrophobic effect, e.g. conformational changes. Most likely, the optimization of the distance between the surfactant and HA molecules, their mutual stoichiometry and steric conditions takes place before their mutual binding. Thus, we suppose the binding of particularly DTAB micelles onto the HA chains to take place while forming micelle-like clusters (Thalberg & Lindman, 1989) or aggregates, in which the DTAB micelles are adsorbed to the polymer chains. This hypothesis was supported by precipitation and formation of particles when the solution became turbid (visual observation). Therefore, the dramatic drop in $\Delta H$ values beyond the "peak" maximum ($c_{max}$) can be ascribed to the formation of the DTAB–HA clusters and most likely to the conformational changes of the HA polymer chains during the aggregation with DTAB, when the polymer chains wrap around the surfactant micelles in order to optimize the electrostatic interactions between the positively charged headgroups and the negatively charged carboxyl groups (McClements, 2000). The formation of complex aggregates of HA and cationic surfactants micelles before the surfactant CMC was supported by the observation of e.g. increased fluorescence intensity of a fluorescence probe (Santos et al., 1994), or by the solubilization of a non-polar dye (Thalberg & Lindman, 1989) in such a system.

The drop in $\Delta H$ beyond the $c_{max}$ soon after alters its direction and becomes considerably much weaker exhibiting only a moderate decrease from the DTAB concentration of ~7.5 mM. Another abrupt change on the titration curve appears at the DTAB concentration of ~18 mM, resulting in a rapid decrease in the enthalpy changes, which indicates the formation of free surfactant micelles. Thus, behind the endothermic peak another addition of the surfactant leads to the dissociation of its micelles since the charge of HA has been previously neutralized by the formation of the HA–DTAB clusters. Nevertheless, $\Delta H$ values are still, even though moderately, decreasing. Therefore, the heat of some other process must again compensate the heat effect of the micelles dissociation, very likely conformational changes of HA–DTAB clusters and/or their dilution as long as a new CMC (or critical aggregation concentration, CAC) is reached. Furthermore, the enthalpy change related to the formation of free micelles, $\Delta H_{mic,agg}$, is determined as 918.8 cal mole$^{-1}$ in water comparing to 384.9 cal mole$^{-1}$ in the presence of the HA–DTAB aggregates. The CMC of DTAB in 0.1% w/w HA aqueous solution (17.9 mM, Table 1) is larger than in water (14.3 mM); nevertheless, the result from the deduction of the CMC from the concentration at the end of the endothermic peak, $c'$ (Fig. 3a) gives a smaller value (10.6 mM) than the CMC of the surfactant in water.

The HA–DTAB interactions are clearly influenced by the presence of low-molecular electrolyte, i.e. 0.05 M NaCl and 0.02 M citric acid/$Na_2HPO_4$ buffer of pH5 and 2.5. The most striking effect is that the large endothermic enthalpy changes ($\Delta H_{max}$) decrease and the concentration of the maximum enthalpy change, $c_{max}$, as well as the beginning of the binding are shifted to higher values than in water (Fig. 5a). At pH5, $\Delta H_{max}$ as well as $c_{max}$ are somewhat greater than that in 0.05 M NaCl, suggesting a bit weaker shielding of electrostatic attractions between the HA carboxyl groups and the DTAB headgroups in the buffered solution. Nevertheless, at pH 2.5 the large endothermic enthalpy peak practically diminishes due to low charge density of HA, although the CMC of the surfactant was lowered by the presence of 0.1% (w/w) HA.

For the same reason to reduce the heat effect from the dissociation of surfactant micelles as in the case of HA–SDS titrations, the initial concentration of DTAB in the syringe was reduced below its CMC, i.e. diluted down to 7.5 mM. The injection of 7.5 mM DTAB into 0.1% (w/w) HA is accompanied by the endothermic effect in contrast to the dilution of DTAB in pure water (Fig. 5b), and $\Delta H$ gradually decreases upon the continual addition of the surfactant. The same result was in fact obtained from the titration of 6 mM SDS into the

**Table 1**
Critical micelle concentration (CMC) or critical aggregate concentration (CAC), respectively, $\Delta H_{max}$ and the concentration of maximum enthalpy change, $c_{max}$, concentration at the end of maximum enthalpy change ($c'$) and difference CAC – $c'$ for DTAB titrated into various media with and without 0.1% (w/w) HA.

| | CMC/CAC (mM) | $c_{max}$ (mM) | $\Delta H_{max}$ (cal mole$^{-1}$) | $c'$ (mM) | CAC – $c'$ (mM) |
|---|---|---|---|---|---|
| Water | 14.3 | – | – | – | – |
| +0.1% HA | 17.9 | 6.1 | 2328.3 | 7.3 | 10.6 |
| 0.05 M NaCl | 9.9 | – | – | – | – |
| +0.1% HA | 13.7 | 8.5 | 1172.6 | 10.3 | 3.4 |
| Buffer (pH 5) | 12.2 | – | – | – | – |
| +0.1% HA | – | 9.1 | 1264.2 | 11.5 | – |
| Buffer (pH 2.5) | 12.9 | – | – | – | – |
| +0.1% HA | 10.5 | – | 12.3[a] | – | – |

[a] The maximum enthalpy change reached and subtracted from $\Delta H$ of DTAB dilution in solvent at the same concentration.

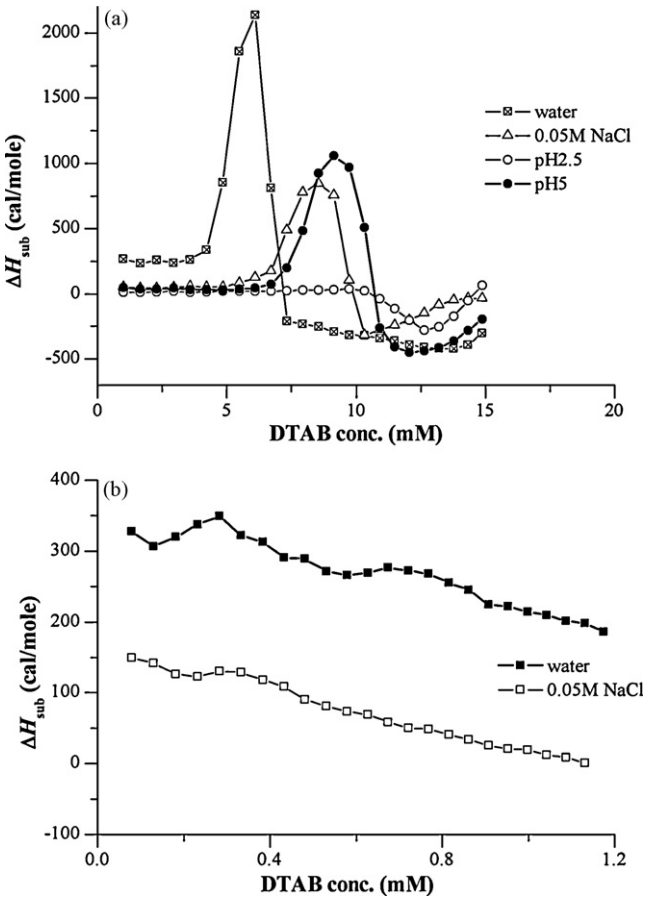


**Fig. 5.** (a) The subtracted enthalpy change, $\Delta H_{sub}$, per mole of DTAB against the surfactant concentration for the titration of 95 mM DTAB into water, 0.05 M NaCl, 0.02 M citric acid/$Na_2HPO_4$ buffer (pH 2.5, 5) containing 0.1% (w/w) HA. (b) $\Delta H_{sub}$ per mole of DTAB on its concentration for the titration of 7.5 mM DTAB into aqueous and 0.05 M NaCl 0.1% (w/w) HA solution.

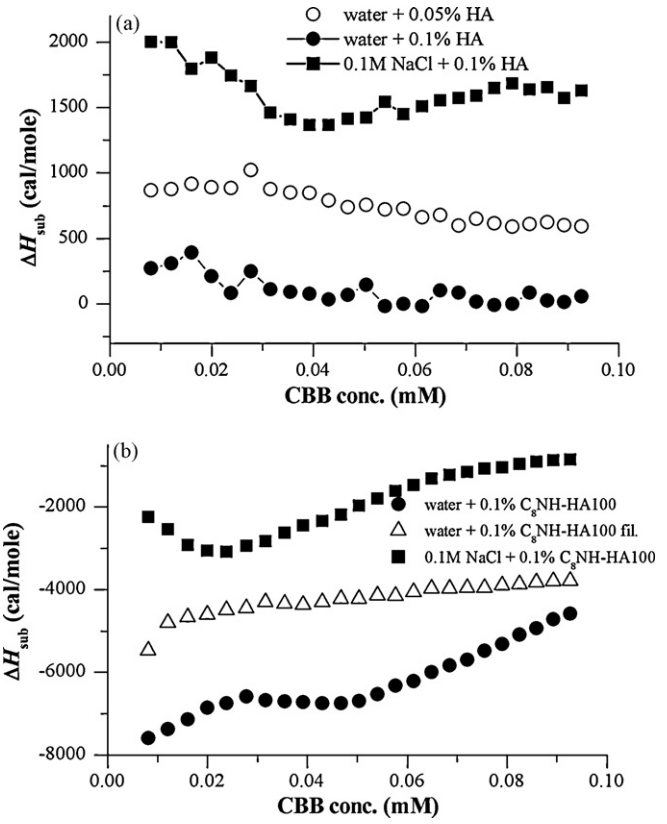


**Fig. 6.** (a) Plot of $\Delta H_{sub}$ per mole of CBB versus the dye concentration in the reaction cell for the titration of 0.36 g $L^{-1}$ (0.436 mM) CBB into aqueous 0.05 and 0.1% (w/w) HA solution, and 0.1 M NaCl 0.1% (w/w) HA solution, and (b) for the titration of 0.36 g $L^{-1}$ (0.436 mM) CBB into unfiltered and filtered aqueous solutions of 0.1% (w/w) $C_8$NH-HA100, and 0.1% (w/w) $C_8$NH-HA100 solution in 0.1 M NaCl.

HA solution but with a bit lower values (Fig. 3c). The presence of 0.05 M NaCl in the reaction cell causes the decrease in $\Delta H$ to negative values indicating net favorable redistribution of the hydrogen bond network (O'Brien & Haq, 2004). However, when 0.1% (w/w) HA is present the titration is still endothermic comparing to that in the absence of the polymer, thus $\Delta H_{sub} > 0$ and has also a decreasing trend (Fig. 5b). Thus, the curves most likely do not show interaction in terms of binding but rather exhibit the dilution of the individual molecules, change in their hydration and very likely the optimization of the distance between HA and DTAB. This assumption is in accordance with the observation of the first titrations of 95 mM or 150 mM DTAB, respectively, which show some kind of "lag" before the binding to HA.

### 3.1.3. Interactions of HA with a polarity probe CBB

Since CBB has been used as a probe for detection of hydrophobic clusters formation in the solutions of amphiphilic polysaccharides (Duval-Terrié et al., 2003) and HA has a potential for delivery of mostly water-insoluble drug, the purpose of this study was to investigate whether there are any hydrophobic interactions between HA and this type of probe.

The injection of 0.36 g $L^{-1}$ CBB into water induces large positive values of $\Delta H$ that are gradually decreasing as the titration continuous (not shown) and soon the decrease becomes less steep. Fig. 6 displays the values of the enthalpy changes produced during the titration of the dye into the HA solutions subtracted from those of the titration of CBB into the solvent ($\Delta H_{sub}$). When HA is present in the reaction cell (Fig. 6a), the titration curves exhibit a very similar shape as in the absence of the polymer with a clear endothermic effect and no abrupt changes, indicating no binding between CBB and HA. Interestingly, the titration into 0.05% (w/w) HA aqueous solution was more endothermic than that into 0.1% (w/w) HA solution. The endothermic nature of $\Delta H$ is very likely due to water rearrangement, release of counterions from the carboxylic groups of HA, and/or hydrophobic forces considering the presence of aromatic rings in the dye structure.

On the other hand, the injection of CBB into 0.1 M NaCl solution, either in the absence or presence of HA, gains negative $\Delta H$ values. Nevertheless, the subtracted values $\Delta H_{sub}$ (Fig. 6a) show that the effect of the presence of HA is again endothermic, and the titration curve of different shape than without the polymer, being decreasing up to the CBB concentration of ~0.04 mM. From this concentration on, the curve becomes slightly increasing till the end of the injection. These effects may again reflect the solvent rearrangement, most likely conformational changes of HA, and rather dilution of the species than real interactions between them.

## 3.2. Interactivity of modified HA, $C_8$NH-HA100

### 3.2.1. Interactions of $C_8$NH-HA100 with CBB

The titrations of CBB in the presence of $C_8$NH-HA100 evolve apparently different effects in comparison with those in the presence of native HA (Fig. 6b). The most striking difference is that the enthalpy changes are considerably negative, both in water and 0.1 M NaCl, and some abrupt alternations of the course of the titration curves can be observed. The exothermic origin of $\Delta H$ suggests a binding of the dye onto $C_8$NH-HA100, due to the presence

of hydrophobic moieties on either molecules, as postulated for the interaction of polysaccharides with surfactants (McClements, 2000) or drugs (Santos et al., 2007). However, our titration curves are not easy to interpret since they do not exhibit a simple trend. The $\Delta H$ values for the titration in water firstly increase, then reach more or less a constant plateau in the middle of the titration, and eventually rise again until the end of the titration. This shape is not typical for a binding curve of one species to another, which is often sigmoidal (Ladbury & Chowdhry, 1996; O'Brien & Haq, 2004). Since $C_8$NH-HA100 is a hydrophobically modified HA (HMHA) and its concentration 0.1% (w/w) in the solution is above its CAC, it contains aggregates and is turbid. Thus, the initial increase in $\Delta H$ might be ascribed to a rearrangement of the HMHA aggregates before the interaction between the HMHA and the dye takes place.

In order to examine the binding of the dye to the monomers of the HMHA, the polymeric solution was filtered (pore size 0.8 μm) and the titrations repeated. The resulting enthalpy changes are still negative, even though less than those for unfiltered solution; nevertheless, the titration curve displays no abrupt changes. Thus, this observation suggests no binding of the dye to the $C_8$NH-HA100 monomers but rather dilution of the dye only.

The injection of the dye into 0.1 M NaCl solution with HMHA gains negative $\Delta H$ values firstly decreasing, and soon after the titration curve resembles sigmoidal or pseudosigmoidal shape, however with considerably a less steep increase in $\Delta H$ (Fig. 6b). The curve tends to saturate at the end of the titration; thus from these observations, we assume again a binding of the dye to the polymer or into its aggregates, respectively. Because the curve begins to increase earlier than during the titration in water, the binding in salt solution starts at a lower concentration of CBB (~0.02 M) than in water (~0.05 M).

As CBB has been used for the detection of hydrophobic clusters in the solutions of hydrophobically modified polysaccharides (Duval-Terrié et al., 2003), we have also performed such an investigation in $C_8$NH-HA100. The absorbance of the dye at 618 nm ($A_{618}$) remarkably increases above the critical aggregation concentration (CAC) of the polymer (Fig. 7a), which is accompanied by the shift in the absorbance maximum wavelength towards longer values (~620 nm; Fig. 7b), while below the CAC the absorbance maximum remains at ~585 nm. These features indicate a formation of hydrophobic domains in the very vicinity of the dye as it is building into them once the CAC has been reached alike during the titration of CBB into unfiltered 0.1% (w/w) solution of $C_8$NH-HA100.

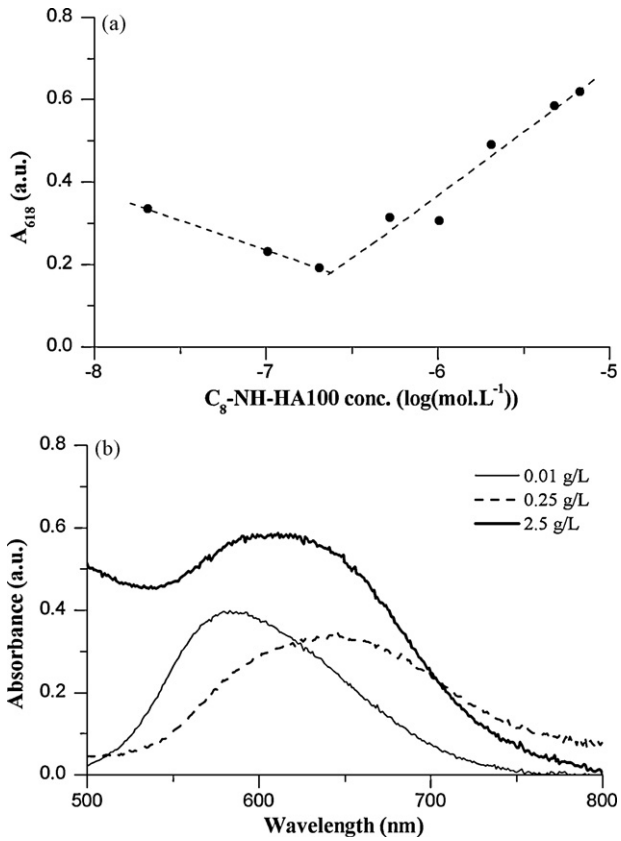


**Fig. 7.** Solubilization of CBB in the 0.15 M NaCl solutions of $C_8$NH-HA100 (a) as the function of the polysaccharide concentration, and (b) the absorbance spectra of CBB in the 0.15 M NaCl $C_8$NH-HA100 solutions for a low polysaccharide concentration (0.01 g L$^{-1}$) and above the polysaccharide CAC. The spectra show the characteristic shift of the dye's maximum absorbance wavelength when in a hydrophobic microenvironment.

### 3.3. Comparison of HA and HMHA macromolecular behavior by SEC-MALLS

Fig. 8 shows the results of one of the three SEC-MALLS measurements performed on the native HA and $C_8$NH-HA100. The solution behavior of the polysaccharides was investigated in the electrolyte containing 0.05 M NaCl, 0.025 M $Na_2SO_4$ supplied with 0.005 M EDTA of the total ionic strength $I = 0.14$ M. The edition of EDTA is routinely used in the laboratory (NOBIPOL) to prevent aggregation in Ca-binding polymers.

The results are presented as the double logarithmic plots of radius of gyration (Fig. 8a), $R_g$, and intrinsic viscosity (Fig. 8b), $[\eta]$, versus the molar mass of the sample, i.e. $R_g$–$M_W$, $[\eta]$–$M_W$. The $R_g$–$M_W$ plots for the HA and the HMHA differed practically in the constants only and can be well described by power law functions: $R_g = 8.45 \times 10^{-2}(M_W{}^{0.525})$ for HA, or $R_g = 6.41 \times 10^{-2}(M_W{}^{0.538})$ for $C_8$NH-HA100, respectively. The power law exponents are bit lower than those reported by Mendichi, Šoltés, and Schieroni (2003), i.e. ~0.6, and the constants are larger. The differences may arise from different experimental conditions used in the reference, consisted particularly of 0.15 M NaCl and 37 °C. The $R_g$ ranged from ~90 to ~270 nm for HA or from ~40 to ~120 nm for the HMHA, respectively.

The double logarithmic plots of $[\eta]$–$M$ overlapped with each other for certain molar masses and linked together with a slight tendency to bend at low molar masses. This non-linearity has already been observed by several authors (Mendichi et al., 2003); nevertheless, the data were fitted by a power law function, since we had only two samples of not too different molar masses. The procedure revealed the following equations: $[\eta] = 4.24 \times 10^{-1}(M_W{}^{0.600})$ for HA and $[\eta] = 1.35 \times 10^{-1}(M_W{}^{0.682})$ for $C_8$NH-HA100, thus showing again a close similarity of the samples. Higher coefficient of Mark–Houwink–Sakurada plot (MHS) for the HMHA, 0.682, manifests the curvature of the plot. The value 0.6 calculated for the HA is in excellent agreement with that calculated by Mendichi et al. for the HA of $M_W > 10^6$ g mol$^{-1}$, whereas the values of the coefficient for the HMHA is somewhat lower than that fitting the region of HA molar masses, $10^5 < M_W < 10^6$ g mol$^{-1}$, i.e. 0.778, which can also be attributed to different experimental conditions but also to the fact that the HMHA exhibited a broader molar mass distribution than native HA and also the modification must be taken into consideration. The average molar masses of the samples were determined as $(1.691 \pm 0.077) \times 10^6$ g mol$^{-1}$ for HA and $(4.514 \pm 0.059) \times 10^5$ g mol$^{-1}$ for the HMHA, respectively, and the intrinsic viscosity $[\eta] = (2146 \pm 104)$ mL g$^{-1}$ or $[\eta] = (874 \pm 59)$ mL g$^{-1}$, respectively.

The curvature of the MHS plot is a typical behavior of the so-called worm-like chain explained by the conformational change of HA from short and rigid chains at lower molar masses to more flexible chain at larger molar masses. The stiffness of the HA

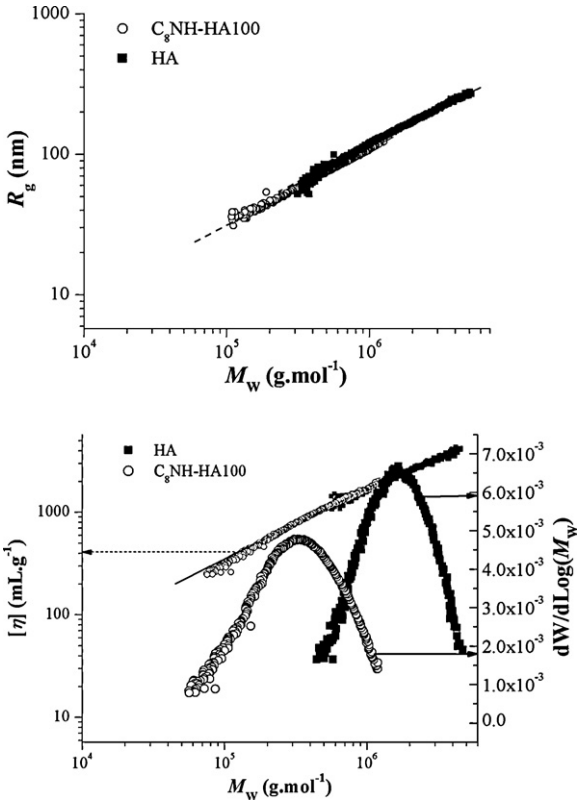


**Fig. 8.** Molar mass dependence of (a) radius of gyration, $R_g$, for HA and $C_8$NH-HA100, and (b) molar mass distribution and Mark–Houwink–Sakurada plot (MHS) for the same samples. The lines represent power law models fitted to the data.

chain can be estimated via the persistence length, $q$, using the $R_g$–$M_W$ dependence. According to the Odijk's model, the total persistence length, $q_T$, of worm-like polyelectrolyte chains is the sum of two contributions: $q_T = q_0 + q_e$, where $q_0$ is the intrinsic persistence length corresponding to an equivalent neutral chain in which all the electrostatic interactions are screened out. The second, $q_e$, is the electrostatic contribution to the total persistence length due to short-range interactions, which depend strongly on the ionic strength. Using the Odijk's model, the persistence length at infinite high or any ionic strength can be calculated, which for $I \to \infty$, $q_T \approx q_0$. Assuming the molar mass per unit contour length $M_{L,HA} = 410\,nm^{-1}$ and $M_{L,HMHA} = 523\,nm^{-1}$, then using the Odijk's model, the best superimposition between the experimental and the theoretical $R_g$–$M$ data was obtained for $q_{0,HA} = 6.6$ nm and $q_{0,HMHA} = 9.2$ nm, which somewhat differs from the value 7.5 nm found by Mendichi et al. Nevertheless, they are still relatively similar, comparing to several discrepancies from other authors.

Alternatively, the persistence length $q$ was determined using the molar mass dependence of the intrinsic viscosity according to Bohdanecký's procedure (Vold-Nygård, Kristiansen, & Christensen, 2006), in which the data were expressed as

$$\left(\frac{M_W^2}{[\eta]}\right)^{1/3} = A_\eta + B_\eta M_W^{1/2} \quad (1)$$

The $A_\eta$ and $B_\eta$ parameters are related to $M_L$ and $q$ according to the equation in the reference and can be easily estimated by a linear regression. Using this procedure, we have obtained the following results for our conditions: $q_{HA} = 9.5$ nm, $q_{HMHA} = 10.7$ nm, $M_{L,HA} = 440\,nm^{-1}$, $M_{L,HMHA} = 481\,nm^{-1}$. The persistence length is somewhat larger than that calculated by Mendichi et al. (6.8 nm) on the other hand $M_L$, particularly for the HMHA, is relatively in agreement with their data.

## 4. Conclusions

The first isothermal titration calorimetry study of interactions of polymeric native and hydrophobically modified HA with surfactants and a dye polarity probe is presented. The titrations of SDS into the HA solutions do not reveal any binding reaction on the contrary to the observations of SDS interaction with a similar polysaccharide pectin. The only significant impact of HA is a lowering of the surfactant's CMC from 7.7 to 6.7 mM in water. This effect is apparently governed by electrostatic repulsive forces since in 0.1 M NaCl solution the CMC of SDS remains practically unchanged in the presence of HA, being ~1 mM.

On the contrary, DTAB and HA exhibit a strong mutual binding interaction as a result of their cationic or anionic, respectively, character. Their binding is accompanied by a huge endothermic effect which arises from the release of counterions from their oppositely charged groups. Interestingly, HA–DTAB binding does not occur immediately but after the optimal distance and steric conditions have been achieved between the species, which is in contrast to the DTAB–pectin interactions showing binding of the surfactant at its very low concentrations. Our ITC results support the results from other methods, suggesting that the HA–DTAB binding is carried out via the surfactant micelles, forming complex aggregates with the HA chains. This interaction is partially suppressed by NaCl and the buffered solution of the pH 5 and it is practically screened out at the pH 2.5, and as a consequence, the endothermic effect diminishes.

The ITC results assume a binding of a polarity dye probe CBB to $C_8$NH-HA100 aggregates by means of a strong exothermic effect and pseudosigmoidal shape of its titration curve. In contrast, such a binding is not observed between CBB and native HA.

The results obtained from the ITC experiments prove the usability of ITC for studying interactions of the presented type; however, complementary results from other methods such as NMR, viscosity measurements, fluorescence, etc., are necessary.

The $R_g$–$M_W$ and $[\eta]$–$M_W$ plots are nearly identical for both the samples, suggesting that the modification of HA does not significantly alter its macromolecular behavior. The coefficients of these relations for native HA, namely of MHS plot, perfectly match those described in literature ($a = 0.6$), whereas the coefficient for $C_8$-NHHA100 was found somewhat smaller ($a = 0.68$), which is likely the result of less number of interactions between the HMHA chains and the solvent molecules due to the presence of alkyl chains on the HMHA backbone. However, the persistence length was found larger for the HMHA, being 9.2 nm using Odijk's model or 10.7 nm using Bohdanecký's procedure, respectively, as well as the persistence length for HA (9.5 nm) determined by Bohdanecký's procedure is larger than those found in the literature. The greater persistence length of $C_8$-NHHA100 suggests somewhat greater stiffness of the HMHA chain than that of the native HA. Nevertheless, the differences in the persistence lengths are still smaller than the discrepancies of their determination reported by other authors.

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