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The influence of quarternary salt on hyaluronan conformation and particle size in solution

Lenka Gřundělová[a,b], Aleš Mráček[a,b,*], Věra Kašpárková[b,c], Antonín Minařík[a,b], Petr Smolka[a,b]

[a] Tomas Bata University in Zlín, Faculty of Technology, Department of Physics and Material Engineering, nám. T.G. Masaryka 275, 760 01 Zlín, Czech Republic
[b] Centre of Polymer Systems, University Institute, Tomas Bata University in Zlin, Nad Ovcirnou 3685, 760 01 Zlín, Czech Republic
[c] Tomas Bata University in Zlín, Faculty of Technology, The Department of Fat, Surfactant and Cosmetics Technology, nám. T.G.Masaryka 275, 760 01 Zlín, Czech Republic

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ABSTRACT

Interaction of hyaluronan (NaHy) with the quaternary salt, benzalkonium chloride (BAC), was studied. Based on the DLS experiments, viscometry and surface tension measurements executed on hyaluronan samples with two molecular weights of M_w = 1.8 MDa and M_w = 0.35 MDa, the hypothesis was proposed suggesting that at certain BAC concentrations, hyaluronan can form aggregates, which lead to increase of the polymer coil size measured as *z*-average diameter. Moreover, it was confirmed that within the whole range of BAC concentrations, repeated variations in size and conformations of polymer coils occur, being connected with the critical micellar concentration of BAC and with hydrophobic interactions of non-polar segments of BAC with hyaluronan hydrophobic domains. Tensiometry, DLS and viscometry data support the assumption that variations of thermodynamical "favourability" of BAC–BAC or NaHy–BAC interactions take place in hyaluronan solutions, based on the BAC concentration.

1. Introduction

The physical and physico-chemical properties of hyaluronic acid and its sodium salt – hyaluronan (NaHy) in solutions are of great interest in various industrial branches, e.g. pharmaceutics, cosmetics, tissue engineering and many others. The behaviour of NaHy in solution strongly depends on the conformation of its polymer chain, which is influenced by molecular weight, polydispersity and the unique chemical composition of the NaHy molecule. Sodium hyaluronate is a natural polysaccharide with a regularly alternating disaccharide units of D-glucuronic acid and *N*-acetyl-D-glucosamin that can be found in various body fluids, tissues and extracellular matrix. The first description of this substance was performed in 1934 by Meyer and Palmer (1934). Conformation of NaHy in highly diluted aqueous (<1%) solution is driven by hydrogen bonds acting mostly between water and polysaccharide molecules. With a slight increase of the concentration of NaHy in solution, also inter- and intramolecular interactions must be considered. NaHy molecule forms ordered secondary structure, containing hydrophobic domains (patches). From the physical point of view, the hydrophobic domains are presented in the backbone, and hydrophilic groups are located in the side branches of the NaHy molecule (Scott, 1992). According to Scott and co-authors (Scott, Cummings, Brass, & Chen, 1991; Heathly & Scott, 1988), NaHy may aggregate and create tertiary structures due to bonding between the hydrophobic patches (see Fig. 1), forming thus two-fold helix based aggregates which are stable at physiological temperatures in water (Heathly & Scott, 1988). In solutions with a very low concentration of high molecular NaHy, presence of intramolecular interactions based on the principle described above can be expected (Heathly & Scott, 1988), which will affect size of the polymer coil.

The theory of chain interactions in dilute and semi-dilute solutions of polyelectrolytes, studied from the point of view of statistical physics and the scaling concept, can be found in Dobrynin, Colby and Rubinstein, (1995), Dobrynin and Rubinstein (2005), and information about behaviour of NaHy chains in semidilute solutions based directly on the interpretation of dielectric measurements data present Vuletić, Babić, Ivek, Grgičin, and Tomić (2010).

From the hydrophobic and hydrophilic nature of the NaHy molecule follows that in aqueous solution NaHy will be very sensitive to the presence and concentration of any surface

* Corresponding author at: Tomas Bata University in Zlín, Faculty of Technology, Department of Physics and Material Engineering, nám. T.G. Masaryka 275, 760 01 Zlín, Czech Republic. Tel.: +420 576035110.
E-mail address: mracek@ft.utb.cz (A. Mráček).

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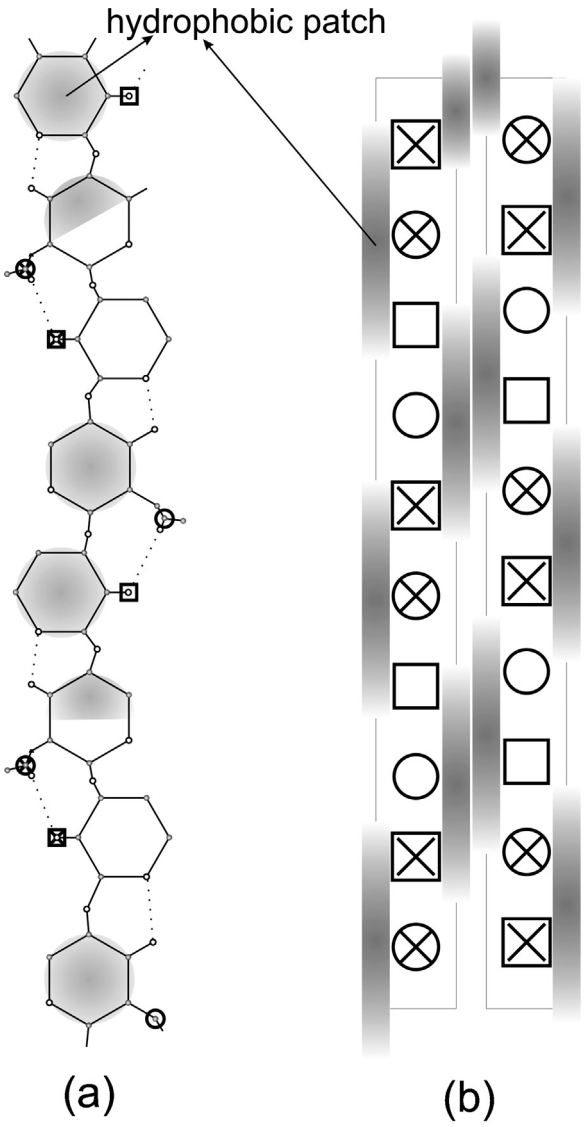

Fig. 1. (a) The hydrophobic patches stretching along three sugar units on alternate sides of polymer chain (circles – acetamido groups, squares – carboxylate groups), (b) Possible duplex between two molecules of hyaluronan (Scott, 1992; Scott, Cummings, Brass, & Chen, 1991).

active compound. Among the substances used for preservation of NaHy solutions, quarternary salts are commonly applied (Pisarcik, Soldan, Bakos, Devinsky, & Lacko, 1999; Pisarcik, Toyoko, Devinsky, Lacko, & Bakos, 2000; Pisarcik, Toyoko, Devinsky, & Lacko, 2001). These salts are classified as cation-active surfactants, capable of micelle formation at concentrations above their critical micelle concentrations. Hyaluronan dissolved in water, on the other side acts as a negatively charged polyelectrolyte and attracts cation-active surfactants, which may bond to specific sites in its structure. In solution, these bonded molecules can interact with each other and form micelles, thus effecting the conformation and coil size of NaHy. As a result, these changes will be reflected in the flow properties of the NaHy/BAC solution, which can be determined for example by viscosity measurements.

In order to calculate limiting viscosity number [η], the dilute solution viscometry data are usually processed using the Huggins equation. In addition to [η], another parameter derived from this equation is Huggins coefficient (k_H), which reflects binary interactions of polymer molecules, both hydrodynamic and thermodynamic. Huggins coefficient is thought to depend mainly on molecular weight and the strength of the polymer-solvent interaction and should be lower in good solvents and at higher molecular weights. Values of k_H for NaHy have been provided by several researches and were found to be prevailingly in the range of 0.35–0.45, as published in Bohdanecky and Kovar (1982). Another method capable of determining the conformation changes in NaHy chain due to the presence of BAC is dynamic light scattering, used for example by Pisarcik et al. (2001).

In this article, the effect of benzalkonium chloride (BAC) presence on the conformation of NaHy coil in phosphate buffered aqueous solution was studied by the means of dynamic light scattering (DLS) and viscometric methods. The critical micellar concentration (CMC) of BAC was determined by standard surface tension measurements and a new possibility of critical aggregation concentration (CAC) identification in more complex polysaccharides/surfactant systems using DLS was also presented. Moreover, the hypothesis explaining NaHy behaviour in the presence of different BAC concentrations was proposed.

2. Materials and methods

Sodium hyaluronate (NaHy) samples of two molecular weights, M_w = 1.8 MDa and M_w = 0.35 MDa were kindly provided by Contipro, Ltd. (Dolní Dobrouč, Czech Republic). Cationic surfactant benzalkonium chloride (BAC) (≥95.0%) and sodium chloride were purchased from Fluka. Phosphate buffer (pH = 7.36) was prepared by dissolving appropriate amount of Na_2HPO_4 and NaH_2PO_4 (both purchased from Sigma–Aldrich) in de-ionized water. Stock solution of NaHy in phosphate buffer was prepared in concentration of 0.1% (w/w) by slowly adding polymer to the buffer solution under continuous stirring, followed by 24-h dissolving at 50 °C. The stock solution of hyaluronan with added BAC was then used for surface tension, particle size and viscosity measurements.

2.1. Surface tension measurement

The critical micellar concentration of BAC was determined by surface tension measurement. For a series of BAC concentrations ranging from 0.032 mmol L^{-1} to 0.89 mmol L^{-1}, surfactant was dissolved in phosphate buffer and de-ionized water, respectively. The surface tension of the first sample set was measured immediately after preparation. For the second set of samples, the measurement was performed after one day equilibration of the prepared surfactant solutions. The surface tension measurement was performed at 25.0 ± 0.1 °C with a K100MK3 Tensiometer (Krüss), utilizing the Wilhelmy plate method.

2.2. Dynamic light scattering measurement (DLS)

Solution of NaHy and NaHy with BAC (0.32–4,2 mmol L^{-1}) was characterized by dynamic light scattering. Zetasizer Nano ZS (Malvern) equipped with 4 mW He–Ne laser operating at the wavelength of 633 nm was used for the measurement of hydrodynamic diameter (*z*-average) at 25 °C. The intensity of scattered light was observed at the angle of 173°. Prior to each measurement, the samples were filtered through 0.22 μm pore-size PTFE Syringe filters (Millipore). The mean hydrodynamic diameter and the polydispersity index (PDI) were obtained. Each sample was measured in triplicate.

2.3. Kinematic viscosity measurement

The kinematic viscosity measurement on NaHy solutions with the same BAC concentration as for DLS measurements, was carried out in a Ubbelohde capillary viscometer (0.53 ± 0.01 mm diameter).

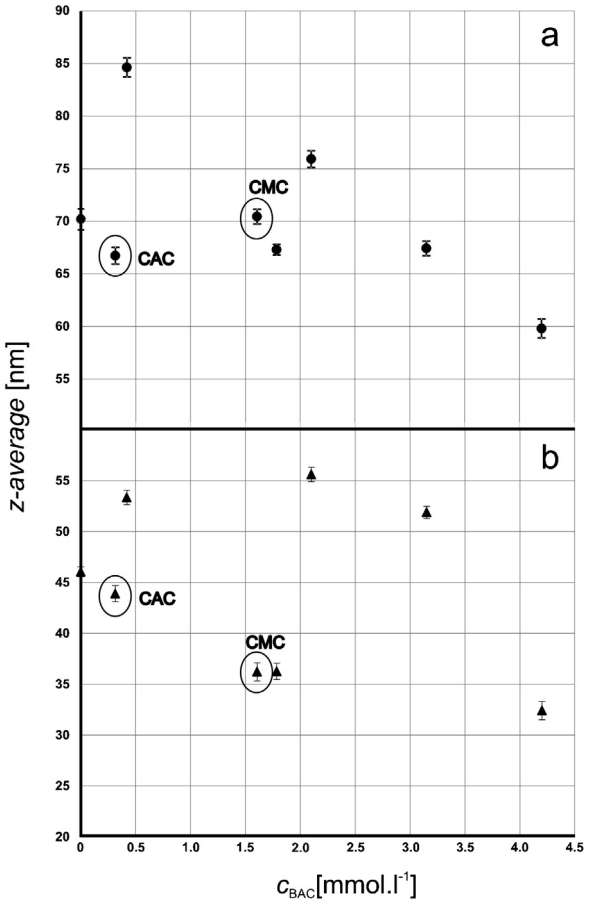

Fig. 2. Relation between NaHy (constant weight fraction NaHy/phosphate buffer) coils *z*-average and BAC concentration in phosphate buffer, (a) M_w = 1.8 MDa (b) M_w = 0.35 MDa.

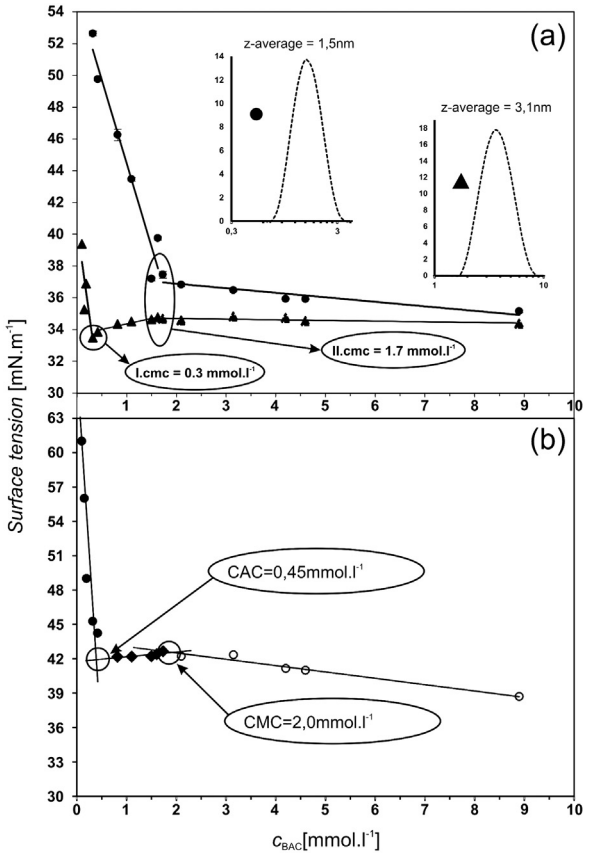

Fig. 3. (a) Surface tension of BAC in de-ionized water (● *z*-average = 1.5 nm) and phosphate buffer (▲ *z*-average = 3.1 nm), (b) surface tension of NaHy–BAC (NaHy M_w = 1.8 MDa) in phosphate buffer solution.

The viscometer was placed into a water-cooled bath with the temperature 25.0 ± 0.1 °C. The ratio between viscosity of NaHy solution with added BAC (η) and NaHy solution of the same concentration without BAC adition (η_o) was defined as $\eta_r = \eta/\eta_o$.

2.4. Limiting viscosity number

NaHy solution with concentration of 0.1% in phosphate buffer was added 0.32 mmol L^{-1} BAC. The polymer/BAC solution was gradually diluted with phosphate buffer in order to obtain five solutions differing in concentrations. Values of limiting viscosity number (LVN) [η] and Huggins parameter k_H were then determined from the flow times measured with these polymer/BAC solutions and the linear least square regression of the η_{sp}/c vs *c* dependence was used for the [η] and k_H calculation (*c* is polymer concentration [g L^{-1}], η_{sp} is specific viscosity). Correspondingly, the measurements were performed with NaHy added all studied BAC concentrations ranging from 0.42 mmol L^{-1} to 4.2 mmol L^{-1}, one by one, and values of [η] and k_H were determined for each of the BAC addition.

3. Results and discussion

Substantial variation of the *z*-average diameter can be observed in Fig. 2a and b depicting the dependence of the sodium hyaluronate particle sizes versus concentration of the used surfactant, benzalkonium chloride for both samples differing in molecular weights (M_w = 1.8 MDa and M_w = 0.35 MDa).

It can be stated that NaHy with both molecular weights used, dissolved in phosphate buffer without BAC forms a random coil with the mean diameter of 70 nm, resp. 46 nm. This confirms to generally accepted finding that in aqueous solutions with excess salt, NaHy chains have an expanded random coil conformation as would be expected for a flexible polyelectrolyte (Herslöf, Sundelöf, & Edsman, 1992). Nevertheless, the first addition of BAC, corresponding with the BAC critical micelle concentration (CMC) of 0.3 mmol L^{-1} in a buffered solution (Fig. 3) induced formation of BAC-polymer associates resulting in contraction (tighter packing) of the NaHy coils and lowering of hydrodynamic coil radius (see Fig. 2).

From the previous research it is well known that in the absence of polymer and below the CMC surfactant molecules (with the exception of the monomolecular film present at the liquid/gas boundary) can exist only as individual entities, while above the CMC they begin to aggregate into micelles (Wennerström & Lindman, 1979). In the presence of NaHy and at low surfactant concentration, no micellization appeared, as positively charged BAC molecules bound to negatively charged groups of NaHy. Generally, this process proceeded until all the accessible, negatively charged groups of NaHy were occupied by BAC and critical aggregation concentration (CAC) was reached. With further increase of BAC concentration micelle formation in the solution can be expected accompanied by a process involving surfactant molecules bound on the NaHy chain, leading to the shrinkage/contraction of the polymeric coil (Fig. 4a–c). This fact is supported by surface tension measurement of NaHy–BAC in phosphate buffer (Fig. 3b). The CAC of NaHy–BAC complex at 0.45 mmol L^{-1} BAC addition was observed (Fig. 3b), which corresponds with results of NaHy *z*-average increase detected at 0.42 mmol L^{-1} BAC concentration (Fig. 2).

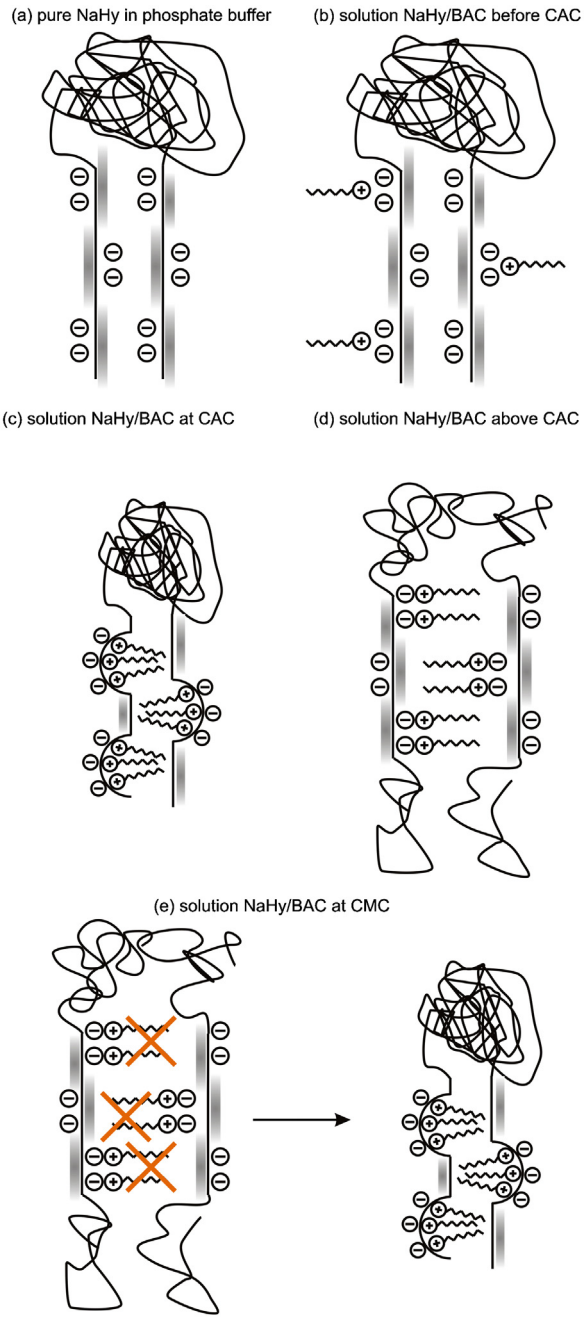

Fig. 4. Hypothesis of interaction between NaHy and BAC.

When moderately exceeding the CMC of added BAC (0.42 mmol L^{-1}), a significant expansion of NaHy–BAC aggregates/complexes occurs in samples with both molecular weights resulting in an increase of *z*-average diameter measured by DLS, which agrees with previously published results (Pisarcik et al., 2001). Here, a hypothesis could apply suggesting that thanks to interactions between NaHy hydrophobic patches (Scott, 1992; Scott, Cummings, Brass, & Chen, 1991) and BAC tails, rigid structures are formed, which tend to unpack the otherwise hydrophilic NaHy chain (see Fig. 4d). The third addition of BAC with concentration of 1.61 mmol L^{-1} causes steep drop of the *z*-average diameter in both studied samples. The above explanation can be also formulated according to Thalberg, van Stam, Lindblad, Almgren, & Lindman (1991), who attributed critical aggregation concentration of NaHy to presence of oppositely charged surfactants decyl- and dodecyl-trimethylammonium bromide below their CMC, as was indicated by the isotherms showing strongly cooperative binding. As explained later in the text, this BAC concentration is a point, where second type of micelles may form. Thus the thermodynamic conditions in the solution can lead to weakening of the hydrophobic interactions in favour of interactions between NaHy/BAC aggregate and solvent (see Fig. 4e). The data of the *z*-average diameter measurements obtained in the presence of even higher BAC concentrations suggest that the behaviour of NaHy in solution may follow similar pattern as proposed in Fig. 4b–e. In addition to CAC, the CMC of NaHy–BAC complex at 2.0 mmol L^{-1} BAC concentration was observed (Fig. 3b), which corresponds with results of NaHy *z*-average increase at 2.1 mmol L^{-1} BAC. The alternative interpretation of above results can be provided based on the conclusions presented by Bjoling, Herslof-Bjorling, and Stilbst (1995), who showed that NaHy molecules can be bridged by TTAB molecules, which are of similar character as BAC. This might also be explanation of the increasing *z*-average values in Fig. 2.

The conclusions based on the DLS experiments are further supported with the discussed viscosity measurement results. As mentioned earlier, apart from the hydrophilic nature of the molecule as the whole, features NaHy also hydrophobic character along the chain backbone axis (Podzimek, Hermannova, Bilerova, Bezakova, & Velebny, 2010; Pisarcik et al., 1999, 2000, 2001). Possible reason of described behaviour can be the fact that in the phosphate buffer, the BAC molecules may form two types of micelles in contrast to the BAC molecules in the de-ionized water. This statement can be again supported by the surface tension measurements presented in Fig. 3a, where two breaks on the dependence C_{BAC} vs γ (surface tension) can be observed, being typical for different micelle types. The presence of two types of BAC micelles in buffer solution can be indicated by DLS measurements, showing distribution curve of higher polydispersity (*z*-average diameter of 3 nm) compared with that of recorded in water (1.5 nm). The higher polydispersity might be a reason for presence of two micelle types that cannot be differentiated by DLS method. Formation of different micelle types was reported for cationic surfactants, quaternary salts dodecyltrimethylammonium bromide (DTAB), tetradecyltrimethylammonium bromide (TTAB) and hexadecyltrimethylammonium bromide for example by Mata, Varade, & Bahadur (2005). Based on viscometry measurements, the authors concluded that in addition to standard spherical micelles, formation of rod-like micelles with increased surfactant concentration occurs (Mata, Varade, & Bahadur, 2005). In the buffered solution of the current study, the micelles formed already at the concentration of 0.3 mmol L^{-1} and a second break related to the second micelle type formation occurs at the concentration of 1.7 mmol L^{-1}, which is approaching the CMC of BAC in the de-ionized water (see Fig. 3a). In literature, comparable results for surface tension measurements in similar surfactant systems in a buffered solution are also stated (Roure, Rinaudo, Milas, & Frollini, 1998; Podzimek et al., 2010; Pisarcik et al., 1999, 2000, 2001). As can be seen in Fig. 2, with the third addition of the BAC (1.6 mmol L^{-1}), which corresponds to the second break for BAC in the buffered solution (see Fig. 3b) and is identical with CMC of BAC in the de-ionized water (see Fig. 3a), a significant drop of the *z*-average diameter of NaHy occurs. The minor difference between the results for polymers with distinct molecular weights can probably be attributed to a lower mobility and lower reorganization ability of the NaHy sample with higher molecular weight and are seen in Fig. 2. Accordingly, the polymer chain conformations incorporating the NaHy–BAC interactions are thermodynamically more convenient and preferred (see Fig. 4c–e). The fifth BAC addition (2.1 mmol L^{-1}) results in repeated expansion of NaHy polymer coils. It is probably the same effect as with the second BAC addition (0.42 mmol L^{-1}), i.e. exceeding the second CMC in the later

Table 1
Limiting viscosity number and Huggins coefficient of NaHy in buffer and BAC addition.

C_{BAC} [mmol L^{-1}]	[η][100 mL g^{-1}]	k_H
0	17.7	0.32
0.32	17.8	0.29
0.42	17.4	0.36
2.1	18.6	0.13
3.15	22.5	−0.23
4.2	25.1	−0.27

case. With further increase of BAC concentration in the NaHy solution the *z*-average values keep descending. This fact is discussed in connection with the Huggins constant and viscosity measurement later in the text (see Table 1 and Fig. 6). Fig. 5 presents a comparison of the particle size measurements for pure NaHy in a buffered solution, NaHy in a buffered solution with the highest BAC addition (4.2 mmol L^{-1}) and the same concentration of pure BAC in a buffered solution and in de-ionized water, respectively. As can be seen, the peak of BAC depicting *z*-average micelle diameter in a buffered solution corresponds with the peak of BAC micelles formed in a solution in the presence of NaHy. This means that at this BAC concentration, the NaHy polymer coils is entirely saturated with the BAC molecules and excess of not bound (free) BAC molecules form micelles coexisting with polymer-surfactant associate. The shift of the NaHy peak maximum with maximal BAC addition towards lower polymeric coil size values in comparison to pure NaHy (Fig. 5) can be also noticed which corresponds to the *z*-average diameter changes depicted in Fig. 2.

Results from viscosity measurements in terms of limiting viscosity number and Huggins coefficient k_H are presented in Table 1. The interpretation of k_H values, in general, is not simple and can be considered even more complicated when association of NaHy with BAC arises. However, the above discussion on the changes of NaHy coil conformation due to the presence of different concentrations of BAC based on DLS measurements can be correlated with results

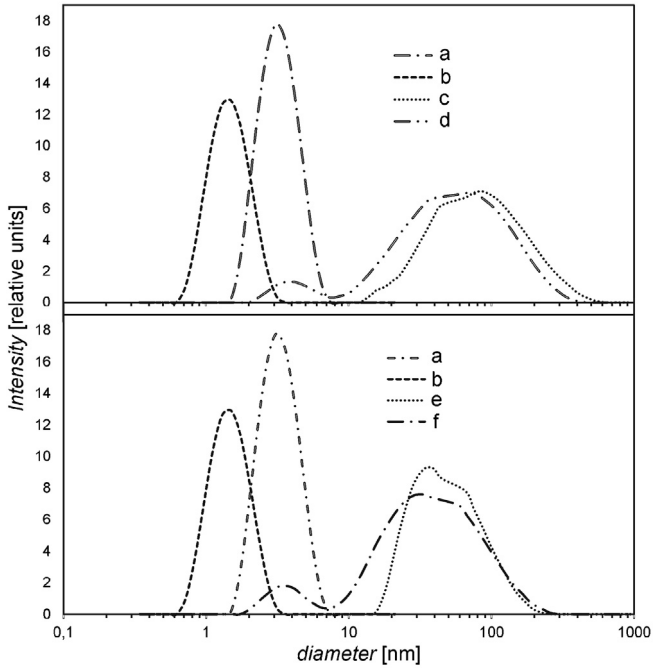

Fig. 5. Particle size measurement (a) BAC in buffered solution, (b) BAC in de-ionized water, (c) pure NaHy (M_w = 1.8 MDa) in buffered solution, (d) NaHy (M_w = 1.8 MDa) in buffered solution with BAC (c = 4.2 mmol L^{-1}), (e) pure NaHy (M_w = 0.35 MDa) in buffered solution, (f) NaHy (M_w = 0.35 MDa) in buffered solution with BAC (c = 4.2 mmol L^{-1}).

from k_H determination. Taking into account the fact that polymer chain of NaHy shows a microdomain structure with hydrophobic part present (Scott & Heatley, 1999; Scott, Cummings, Brass, & Chen, 1991), it can be assumed that in aqueous solvents the hydrophilic parts are flexible while the segments formed of NaHy–BAC associates are more rigid. The conformation parameters of such polymer (k_H) can be, in principle, influenced by additional factors such as chain stiffness, interaction between the flexible and stiff segments or intermolecular hydrodynamic interactions of each of the part with the solvent.

Based on the theory, the value of k_H is positive showing the Huggins' plot of η_{sp}/c vs. c with a positive slope (Herslöf et al., 1992). This situation was also observed for pure NaHy in saline buffered PBS, where the η_{sp}/c vs c dependence shows an expected course with k_H value of 0.32, which is in good correlation with the values published in literature (Bohdanecky & Kovar, 1982). Correspondingly, the NaHy solution with 0.32 mmol L^{-1} and 0.42 mmol L^{-1} BAC, showed positive slope of the aforementioned dependence, with k_H values of 0.29 and 0.36, respectively (Table 1). Here the similar effect as for *z*-average particle diameter, namely a shallow drop and subsequent increase of k_H, can be observed. However, after the BAC concentration started to increase, the change of the η_{sp}/c vs c took place. Addition of 2.1 mmol L^{-1} BAC caused k_H drop to 0.13 and when increasing surfactant concentration above 3.15 mmol L^{-1}, an unusual course of the η_{sp}/c vs c plot was observed, showing lower reduced viscosities with the increase of the polymer concentration. This leads to negative Huggins coefficients obtained through a linear fit. The observed effect is unusual, though not unknown in the literature. The corresponding behaviour was observed for polydimethylsiloxane–polyurea copolymers and was explained by the presence of the two blocks showing the different flexibility and by very strong interactions of polymer with the solvent (Yilgor, Ward, Yilgor, Atilla, 2006). Effect of decreasing of Huggins coefficient and diffusion coefficient of swelling was also observed on the hydrophobically modified derivatives of hyaluronic acid by Lapčík, Benešová, Lapčík, De Smedt, & Lapčíková (2010) and Mráček, Benešová, Minařík, Urban, & Lapčík (2007). The negative k_H observed in current study can be then attributed to interactions between the hydrophobic part of the NaHy chain and the BAC bound on the polymer. Moreover the changes in polymer-solvent interactions may also take place and influence the k_H value.

Results presented in Table 1 also show that for the pure NaHy and NaHy with two lowest concentrations of BAC, the values of [η] remained almost unchanged and were in good agreement with those typically observed for high molecular weight polymers.

As stated in Krause, Bellomo, & Colby (2001), NaHy solutions in phosphate-buffered saline are viscoelastic liquids with a concentration-dependent viscosity typical for polyelectrolytes with excess salt. However, increasing the BAC concentration above 2.1 mmol L^{-1} resulted in gradual increase of limiting viscosity number caused by the above described behaviour of the NaHy–BAC complex. Theoretically, the increase of [η] is inherently connected with increase of polymer molecular weight, formation of agglomerates or coil expansion caused by the preferential polymer-solvent interactions. Here, the reason [η] increase can be found in the change of NaHy chain character caused by the BAC presence.

In addition to limiting viscosity number, viscosity ratio η/η_0 was calculated, relating kinematic viscosity of NaHy solution with added BAC (η) and pure NaHy solution of the same concentration (η_0). The data, hence, represent the relative contribution of the BAC to the overall viscosity behaviour of the NaHy coils (Fig. 6). The results show that the addition of 0.32 mmol L^{-1} and 0.42 mmol L^{-1} BAC caused only an insignificant change of relative viscosity. However further increase of BAC concentration induced gradual lowering of η/η_0 ratio indicating thus an overall reduction of the hydrodynamic radius of the coil generated by the surfactant

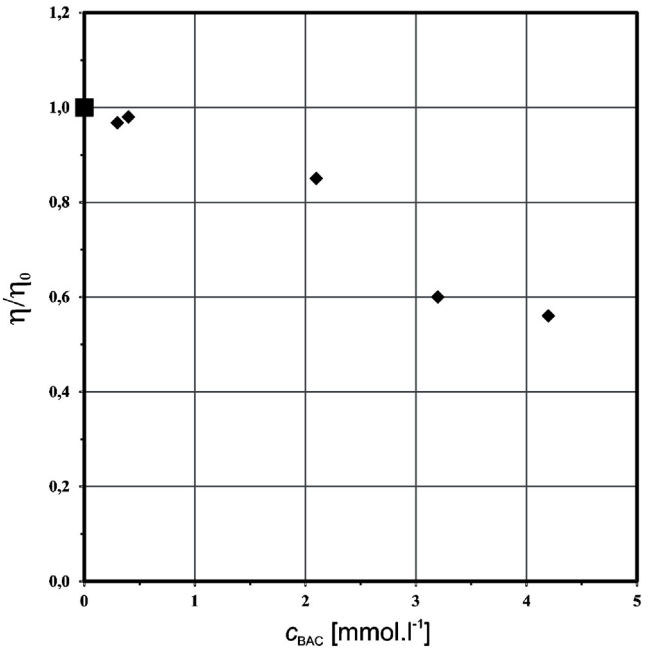

Fig. 6. Relation of relative viscosity to BAC concentration.

presence. This effect is a consequence of the chain shrinkage caused by the adsorption of BAC molecules, or BAC micelles, on the polymer chain. The viscosity results hence support data recorded by DLS.

4. Conclusion

Based on the obtained, the hypothesis of hydrophobic interactions of NaHy with BAC hydrophobic "tails" leading to statistically significant variations in size and conformation of NaHy polymer coils with increasing BAC concentration was proposed. This study can extend the theory of hydrophobic "patches" according to Scott and co-authors (Scott, 1992; Scott, Cummings, Brass, & Chen, 1991). In further experiments, thermodynamic aspects of interactions, namely Gibbs energy of van der Waals bonds between NaHy and BAC will be developed in more details, with the help of previously published method of Mráček (Mráček, 2010; Mráček et al., 2008), which should confirm the herein described hypothesis of NaHy interactions and quaternary salts in general.

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