Carbohydrate Polymers 76 (2009) 443–448

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Effect of new hydrophobic modification of hyaluronan on its solution properties: evaluation of self-aggregation

Martin Chytil [a,b,*], Miloslav Pekař [a]

[a] Institute of Physical and Applied Chemistry, Faculty of Chemistry, Brno University of Technology, Purkyňova 118, 612 00 Brno, Czech Republic
[b] CPN Ltd., Dolní Dobrouč 401, 56102, Czech Republic

ARTICLE INFO

Article history:
Received 23 October 2008
Accepted 5 November 2008
Available online 13 November 2008

Keywords:
Hyaluronan
Hydrophobic modification
Surface tension
Self-aggregation
Rheology
Viscosity

ABSTRACT

Solution properties of novel hyaluronan (HA) derivatives were investigated and their self-aggregation evaluated for drug delivery purposes. The HA had been grafted by C–8 and C–12 chains onto –OH groups of D-glucuronic acid via carbamate bond at low and high degrees of substitution (DS), so that the carboxyl groups of the polysaccharide were preserved. Surface tension, rheological, and viscosity properties were studied. All the derivatives displayed surface-activity by means of a decrease of surface tension (γ) of their solutions, and a breakpoint on $\gamma - \log C$ dependence suggests an aggregates formation. The high-molecular derivatives of high DS exhibited the most efficient surface activity and shear-thinning rheological behavior comparing to the shorter derivatives of low DS. The similar Mark–Houwink–Sakurada plot coefficient, $\bar{a} = 1.06$, for both the native and modified HA manifests that the modification did not meaningfully alter the stiffness of the HA chains.

1. Introduction

Hyaluronic acid (HA) is a naturally occurring linear high-molecular weight polysaccharide composed of repeating β-(1 → 4)-D-glucuronic acid β-(1 → 3)-*N*-acetyl-D-glucosamine disaccharide units (Cowman & Matsuoka, 2005; Lapčík, Lapčík, De Smedt, Demeester, & Chabreček, 1998) with a wide range of molecular masses from several hundred to 10 million g mol^{-1}. HA possesses one carboxyl group per disaccharide unit, giving it polyelectrolyte character, which has many consequences in its roles in living organisms and in applications. At physiological pH, hyaluronic acid occurs predominantly as sodium hyaluronate and both forms are referred as hyaluronan (Vercruysse & Prestwich, 1998). HA can be found primarily in the extracellular matrix of all higher organisms, especially in connective tissues, synovial fluid, eye vitreous, and is also produced by certain strains of bacteria.

The biological functions of HA include maintenance of viscoelasticity of liquid connective tissues, such as joint synovial fluid, because of HA supramolecular assembly with proteoglycanes in these tissues and its unique rheological properties. HA controls the hydration and water transport in tissues due to its immense ability to retain water (Forsberg, 1996 etc.). Low-molecular weight HA is in turn involved in numerous receptor-mediated roles in cell detachment, mitosis, migration, tumor development, metastasis, inflammation, etc. (Cowman & Matsuoka, 2005; Jaracz, Chen, Kuznetsova, & Ojima, 2005). The interactions with the cell-surface receptors are the basis for drug delivery of native HA (Prehm, 2002). Other applications of HA include ophthalmic drug delivery, viscosity enhancement of eye drops (Ludwig & Ooteghem van, 1989), visco-surgery, visco-supplementation of the synovial fluid and cartilage in treatment of joint diseases, wound healing, etc.

Despite the utilization of HA in drug delivery and many other medical applications, its repertoire of applications can be further broaden by a modification of its numerous functional groups to change and control its physicochemical properties (Prehm, 2002; Vercruysse & Prestwich, 1998). The aim is to enhance HA affinity to non-polar active agents and hereby increase their availability in the parts of organism, in which they need to be delivered. This could be achieved via forming micelle-like aggregates by the modified HA that comprise hydrophobic core formed by apolar segments, attached on the HA backbone, in aqueous media. Into these apolar domains, a non-polar bio-active agent could be incorporated. The reason for using HA for such modifications is its biocompatibility, natural occurrence in the human organism, and its

Abbreviations: DS, degree of substitution; HA, hyaluronic acid, hyaluronan; HMHA, hydrophobically modified HA; a_M, surface area occupied by each surfactant molecule at the saturation of the interface; c_T, critical concentration; c^*, coil-overlap concentration; γ, surface tension of solution; γ_0, surface tension of solvent; Γ_{max}, excess surfactant concentration at the interface; k_H, Huggins coefficient; η_{ap}, apparent viscosity; η_{sp}, specific viscosity; η_{red}, reduced viscosity; [η], intrinsic viscosity; pC_{20}, efficiency of reduction in surface tension; Π_{C_T}, effectiveness of reduction in surface tension.

* Corresponding author. Address: Institute of Physical and Applied Chemistry, Faculty of Chemistry, Brno University of Technology, Purkyňova 118, 612 00 Brno, Czech Republic. Tel.: +420 541 149483.
E-mail address: chytil@fch.vutbr.cz (M. Chytil).

0144-8617/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2008.11.003

specific interactions with cell-surface receptors, providing the delivery of bio-active agent highly selective. Indeed, we must remember that heavy substitution, and particularly of carboxyl groups, may significantly alter the HA properties and thus hamper the considered applications.

Several authors have presented papers describing synthesis and characterization of various kinds of HA derivatives (Creuzet, Kadi, Rinaudo, & Auzély-Velty, 2006; Hirano et al., 2005; Šimkovič, Hricovíni, Šoltés, Mendichi, & Cosentino, 2000, etc.); however, these papers report about modifying HA mostly via its carboxyl or *N*-acetyl groups. In this work we present the characterization of novel HA derivatives prepared by linking of C–8 and C–12 chains with –OH groups of D-glucuronic acid of the polysaccharide via carbamate bond. This procedure enables to preserve the HA carboxyl groups, which is crucial for HA applications. Beside low degree of substitution (DS), which has been normally exploited in other studies, we focused our attention also on higher DS, since we preserved the carboxyl groups of HA, and discussed its potential in drug delivery. Therefore, the study concerns the formation of micelle-like aggregates by the HA derivatives presented, and with what influences such a behavior and how. Thus, the discussion about the effect of molar mass, DS, length of the attached alkyls and solution conditions are involved. After a detailed investigation of these effects and deep understanding of their mechanisms, we could predict and tailor the preparation conditions so that to achieve desired properties of the HA derivatives regarding to their applications. There is a variety of methods for such investigations; in this work, the methods of surface tension, rheology, and viscometry were employed.

2. Materials and methods

2.1. Materials

Bacterially produced HA of molecular weight ranging from 5.6×10^4 g mol^{-1} to 1.63×10^6 g mol^{-1} and hydrophobically-modified HA (HMHA) was provided by CPN Ltd. (Dolní Dobrouč, the Czech Republic). The HA was modified by, firstly, activation with cyanogen bromide (Vercruysse & Prestwich, 1998) and then the aduct of the activation subsequently reacted with alkyl-amine of various lengths and forming carbamate with HA, as described by Mlčochová et al. (2006). The HA derivatives studied contained C–8 or C–12 chains and their degree of substitution, DS, varied within the range from 3% to 100%. The DS corresponds to the percentage amount of disaccharide units bearing the alkyl chain. The DS and molar mass of the derivatives were determined by NMR and SEC-MALS as also described in the reference (Mlčochová et al., 2006). The supposed structure of the derivatives is given in Fig. 1 and their characteristics summarized in Table 1. All samples were prepared and studied in Milli-Q water, phosphate buffer containing KCl (I = 0.146 M, pH 7) or in NaCl solutions with concentration ranging from 0.05 to 0.25 M. All chemicals used for the preparation of the solvents were of p.a. purity obtained from Fluka.

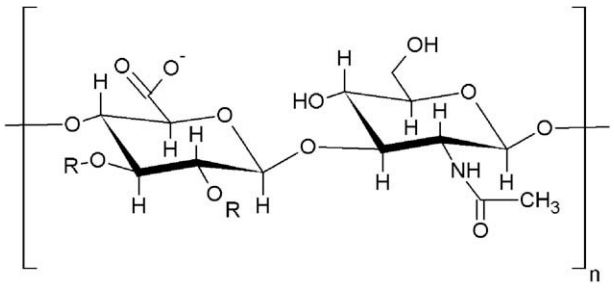

Fig. 1. Supposed structure formula of the HA carbamate derivative.

Table 1
Molecular weight and degree of substitution of the carbamate derivatives of the HA and molecular weight of the HA studied.

Sample	M_w (kg mol^{-1})	DS (%)
C_{12}–NH–HA32	32	3.3
C_{12}–NH–HA59	59	3.7
C_{12}–NH–HA74	74	4.7
C_{12}–NH–HA97	97	4.7
C_8–NH–HA5.8	370.5	5.8
C_8–NH–HA18.8	843.3	18.8
C_8–NH–HA100	494.7	100
HA59	59	–
HA74	74	–
HA97	97	–
HA560	560	–

2.2. Samples preparation

The appropriate amount of dry HA and HMHA were firstly added into solvent in order to obtain a stock solution and let gently stirred for at least 24 h at room temperature. For the preservation of the solutions, NaN_3 was added in the concentration of 0.05%–0.1%wt. After the proper homogenization, the stock solutions were diluted to desired concentrations with solvent and vigorously agitated for at least 4 h. The solutions were stored in the refrigerator at ca 4 °C before their use.

2.3. Surface tension measurements

The measurements of surface tension were performed at 24.2 ± 0.8 °C with tensiometer Krüss K-10 using the method of Du Noüy platinum ring. Before the measurement, the tensiometer was adjusted and calibrated onto water and then the appropriate solvent was measured. The measuring vessels were rinsed with a small amount of a measured solution prior to the measurement and each solution was then measured in 1-min intervals as long as at least five values of surface tension varied less than by 1%. These stabilized values of surface tension were further considered and utilized for other calculations.

The critical concentration of the HMHA, C_T, was determined as the intersection of two linear parts of the γ–log$C$ plot. Other surface-active properties such as efficiency, pC_{20}, and effectiveness, Π_{C_T}, of reduction in the surface tension, the maximum (excess) surfactant concentration at the air/water interface, $\Gamma_{max.}$, and surface area occupied by each surfactant molecule, a_M, were determined as reported by Zhang and Marchant (1996). The pC_{20} of the surfactant is defined by the value of the negative logarithm of the bulk concentration of the surfactant necessary to reduce the surface tension of the solvent by 20 mN/m, while the effectiveness, Π_{CMC}, is defined by the extent of the surface tension reduction attained at the CMC or C_T, respectively. The efficiency can be obtained from the surface tension plot and the simplified Gibbs adsorption equation:

$$d\gamma = -2.303 \cdot RT \cdot \Gamma \cdot d \log C \quad (1)$$

R represents the molar gas constant, T is the absolute temperature, and C represents molar concentration. Effectiveness, Π_{C_T}, was assessed by the difference of the surface tension of the pure solvent and surface tension of the HMHA solution at its appropriate C_T. The excess of the surfactant concentration, Γ_{max}, can be calculated from the slope of the γ–log$C$ plot, and the surface area, a_M, equals $1/\Gamma_{max} N_A$, where N_A is Avogadro constant.

2.4. Rheology experiments

Rotational rheological experiments were carried out using RheoStress Haake RS100 rheometer with the cone–plate (CP) geometry with the diameter of 60 mm and cone angle of 1°. The measurements were performed at 25 °C. Solutions of the polysaccharides of appropriate concentrations were gently injected onto the surface of the geometry, where they remained resting for at least 1 min to achieve equal temperature throughout the samples. The samples were then subjected to a steady-state shear experiment with the shear rates, $\dot{\gamma}$, ranging from 1 to 1000 s^{-1}.

2.5. Viscosity measurements

Viscosity measurements were performed using automatic micro-capillary viscometer AMVn Anton Paar with the capillaries diameters of 1.6 and 1.8 mm and the diameter of the steel ball of 1.5 mm. Series of HA and HMHA solutions were measured at 25 °C at various inclination angles of the capillaries from 40 to 70°. The rolling time of the ball through a certain part of the capillary filled with the measured solution was repeatedly measured (4 times) and recorded. From the average rolling time, the dynamic viscosity was calculated for all the repetitions. The viscosity parameters such as intrinsic viscosity, $[\eta]$, Huggins coefficient, k_H, and the coil overlap concentration, c^*, were calculated from this average viscosity over the whole range of the inclination angles used.

The c^* was determined as the intersection of the two linear parts of the log η_{sp}–log($c[\eta]$) plot as described by Arvidson, Rinehart, and Gadala-Maria (2006). The Huggins coefficient k_H was calculated from the slope of the linear fit of the η_{red}–w and η_{inh}–w plot according to Eqs. (2) and (3):

$$\eta_{red} = \frac{\eta_{sp}}{w} = [\eta] + k_H[\eta^2] \cdot w \quad (2)$$

$$\eta_{inh} = \frac{\ln \eta_{rel}}{w} = [\eta] + (k_H - 0.5) \cdot [\eta]^2 \cdot w \quad (3)$$

where w is the weight concentration (g L^{-1}) of a polymer and η_{rel} is the relative viscosity ($\eta_{rel} = \eta/\eta_0$) where η_0 is the viscosity of the solvent. The intrinsic viscosity, $[\eta]$, equals the limiting value of the reduced, η_{red}, or inherent viscosity, η_{inh}, to infinite dilution ($w \to 0$).

3. Results and discussions

3.1. Behavior of HMHA in aqueous and phosphate buffer solutions

3.1.1. Surface tension

The results of the surface tension measurements are presented in Fig. 2 by means of the concentration dependence of relative surface tension, γ/γ_0, that is the ratio of the solution surface tension and the solvent surface tension. Table 2 summarizes the surface-active properties determined. From the figure is obvious that our HA derivatives displayed surface active behavior by means of a decrease of the ratio γ/γ_0 with their concentration, unlike the native HA (Fig. 2a). The drop of γ/γ_0 for the 0.5%wt. HA solution is attributed to the increase of the solution viscosity and its gel-like (viscoelastic) character at this concentration as it is discussed in the literature (Ribeiro, Mata, & Saramago, 2007).

The breakpoint on the γ/γ_0–log$C$ dependence of the HA derivatives suggests a formation of aggregates, since the air/solution interface has been saturated by the polysaccharides molecules, and further addition of polymeric surfactant must therefore lead to aggregation, for the system to remain in thermodynamically favorable state. This breakpoint, or the concentration at which it occurs respectively, is related to the *critical micelle concentration*

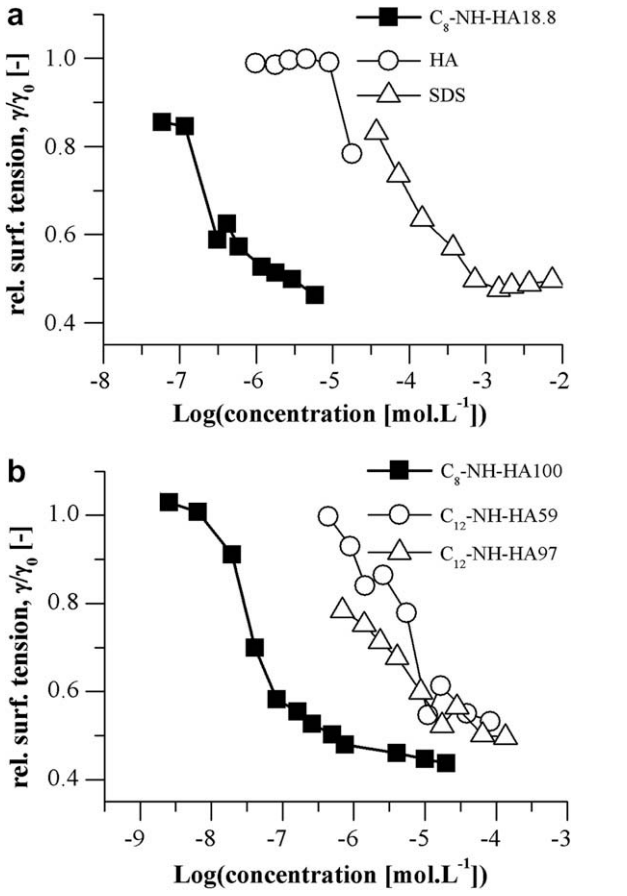

Fig. 2. Relative surface tension, γ/γ_0, of sodium dodecyl sulfate (SDS), native HA (5.6×10^5 gmol^{-1}) and HMHA marked as C_8–NH–HA18.8, as a function of the samples concentration in phosphate buffer (I = 0.146 M, pH 7) (a). Comparison of C–8 and C–12 HA derivatives surface-activity in phosphate buffer (b), influence of molar mass, the length of the alkyl chain, and DS; for the characteristics of the samples see Table 1. All lines were only to guide the eye.

(CMC) for simple low molecular surfactants (Hunter, 2001), such as sodium dodecyl sulfate (SDS), that start to self-associate into micelles at their CMC. Nevertheless, amphiphilic polymers that show such a behavior associate rather into micelle-like aggregates, as reported by several authors (Chen, Chen, Hu, & Wang, 2005; Geciova et al., 1995, etc.). The concentration at which this formation begins is then called the critical concentration instead. Nevertheless, some authors (Gang-Biao, Daping, Kairong, & Haihua, 2006) rather use the term critical aggregation concentration (CAC). For our samples, we tended towards the term critical concentration.

The fact of forming micelle-like aggregates is a crucial condition for the applications of the presented derivatives in drug delivery. Despite showing the breakpoint on the γ/γ_0–log$C$ plot, γ/γ_0 of the HMHA solutions continued in the, however rather moderate, decrease even beyond the critical concentration (C_T), unlikely for low-molecular surfactants, SDS. This finding supposes as if the air/solution interface of the derivatives solutions was not totally saturated by the HMHA molecules.

As it can be seen mainly from Table 2, the self-assembling behavior of our samples depended on the length of the alkyl chain, degree of substitution, DS, and molecular weight (M_W). One can well particularly distinguish different behavior of low-molecular weight C–12 derivatives of low DS from that of a high-molecular weight highly modified HA, C_8–NH–HA100, (Fig. 2b). The C–12 derivatives displayed generally significantly larger critical concentration C_T and were less efficient in reduction of the surface ten-

Table 2
Surface-active properties for C_{12} and C_8 HMHA in the phosphate buffer (I = 0.146 M) and for C_8–NH–HA100 in water and in NaCl solutions of various concentrations.

Sample	C_T (g L^{-1})	$C_T \times 10^6$ (mol L^{-1})	pC_{20} (−)	Π_{C_T} (mN m^{-1})	Π'_{C_T} (%)	$\Gamma_{max} \times 10^6$ (mol m^2)	a_M (A^2)
C_{12}–NH–HA32	0.49	15.3	5.08	27.9	42.3	4.79	34.7
C_{12}–NH–HA59	1.19	20.2	5.42	29.6	47.5	2.31	71.9
C_{12}–NH–HA74	0.66	8.9	5.33	23.8	36.6	2.31	71.9
C_{12}–NH–HA97	1.41	14.5	5.31	29.2	45.2	3.52	47.5
C_8–NH–HA5.8	0.23	0.62	6.36	22.5	31.5	2.95	56.3
C_8–NH–HA18.8	0.55	0.65	6.60	31.8	45.1	4.87	34.1
C_8–NH–HA100	0.17	0.34	7.08	35.1	50.3	4.26	39.0
0.00 M NaCl	0.10	0.20	6.91	40.2	23.1	2.79	59.4
0.05 M NaCl	0.12	0.24	7.25	49.6	32.3	3.02	55.0
0.10 M NaCl	0.15	0.30	7.27	49.4	31.4	2.68	61.9
0.10 M NaCl*	0.20	0.40	6.69	42.3	24.0	2.40	69.2
0.15 M NaCl	0.13	0.26	6.89	48.8	25.9	3.38	49.1
0.25 M NaCl	0.10	0.20	6.83	42.8	22.5	2.85	58.3

* Corresponds to the sample prepared in pure water into which concentrated NaCl solution was added up to the overall salt concentration of 0.1 M.

sion, resulting in lower pC_{20} and Π_{C_T} comparing to C_8–NH–HA100 and other C–8 derivatives. These results are in accordance with previous observations for other HM polysaccharides or polymers (Duval-Terrié, Huguet, & Miller, 2003; Gang-Biao et al., 2006; Jeong, Kang, Yang, Park, & Kim, 2005), stating that the higher the number of hydrophobic groups grafted to a macromolecule, the greater ability to self-associate in aqueous environment.

The C–12 derivatives had a very similar DS, ranging from 3.3% to 4.7%, and thus the largest difference among them can only arise from their molar masses. From Fig. 2b one can see that these samples differed slightly with each other, except the C_{12}–NH–HA59 which exhibited lower values of γ/γ_0 than the others. Their efficiency and effectiveness in the reduction of γ were very similar or changed irregularly as it is seen in Table 2. The only parameters, in which the C–12 HMHA differed significantly, was the maximum surfactant concentration at the air/solution interface, Γ_{max}, and therefore also the area occupied by each of their molecules at this interface at the saturation state, a_M, since these variable are related to each other (see Materials and methods section). The lowest Γ_{max}, was observed for the C_{12}–NH–HA59 and C_{12}–NH–HA74 and then it was larger for C_{12}–NH–HA97. For a_M, this dependence is inversed, considering its definition, i.e. it decreased for the case of C_{12}–NH–HA97. The decrease in a_M for larger derivatives suggests a contraction of the HMHA molecules, due to higher number of hydrophobic segments, and thus enhanced hydrophobic interactions.

Comparison of C–8 HA derivatives of much larger M_W (Table 2) revealed that despite having higher DS and molar mass, the derivative C_8–NH–HA18.8 exhibited the critical concentration similar to that of C_8–NH–HA5.8. Nevertheless, it reduced the surface tension more efficiently. On the other hand the sample marked as C_8–NH–HA100 exhibited the lowest C_T and most efficient surface activity of all the HA derivatives studied. Generally, as DS and molar mass increased, the area, a_M, in turn tended to decrease, as it was observed for C–12 derivatives, and thus, supporting the statement that larger molecules of more substituted HA occupy more compact conformation at the air/solution interface. However, $a_{M,C8-NH-HA18.8} < a_{M,C8-NH-HA100}$, despite larger molar mass. This observation then suggests that too high M_W tends rather to a somewhat decrease in C_T, as long as DS is not appropriately increased simultaneously, since with the growth in molar mass the number of polar groups also increases, which opposes the aggregation. Furthermore, high enough DS ensures efficient reduction in surface tension. However, high substitution most likely strengthens the polysaccharide chain stiffness, so that it becomes less flexible than the less substituted polysaccharide, and therefore occupies a bit less compact structure at the air/solution interface (compare C_8–NH–HA18.8 and C_8–NH–HA100).

3.1.2. Rheological properties

The results of the rheological measurements of the HMHA soluitons are given in Fig. 3. Fig. 3a shows the comparison of the rheology between native HA and HMHA marked as C_8–NH–HA100 of similar molecular weights, 5.6×10^5 or 4.95×10^5 g mol^{-1}, respectively. Both samples exhibited shear-thinning behavior, i.e. the decrease in viscosity as the shear rate, γ, was raised. Nevertheless, the derivative displayed lower values of apparent viscosity and slightly weaker shear-thinning (less steep decrease of η_{ap}) than the native HA. This can be attributed to somewhat lower molar mass of the

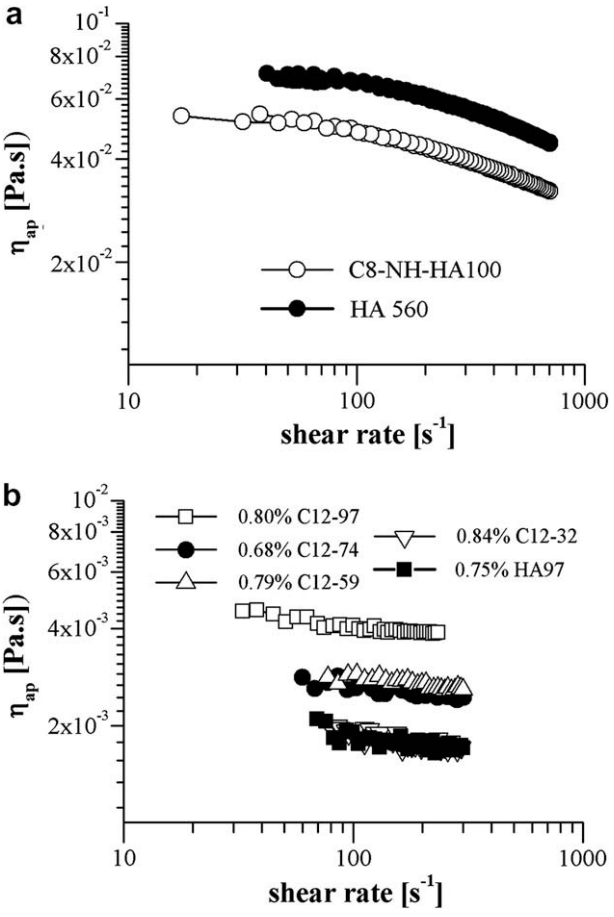

Fig. 3. Viscosity curves of 1%wt. HA 560 and C_8–NH–HA100 aqueous solutions at 25 °C (a). viscosity curves of the C–12 derivatives and HA97 solutions in phosphate buffer at 25 °C (b).

HMHA but also to the presence of hydrophobic segments in aqueous environment which reduces the number of hydrogen bonds between HA –OH groups and water molecules that are responsible for high viscosities of HA solutions (Cowman & Matsuoka, 2005).

On the contrary, the C_{12}-bearing derivatives exhibited practically Newtonian behavior or very weak shear-thinning (Fig. 3b) due to their much lower molar mass than that of C_8–NH–HA100. The values of apparent viscosity increased with the derivatives' molar mass; however, C_{12}–NH–HA59 and 74 displayed the same rheological behavior, which is most likely the result of somewhat lower concentration of the latter sample. The 0.75%wt solution of native HA ($M_W = 9.7\times10^4$ g mol^{-1}) showed similar behavior to the C–12 derivatives, but also, and surprisingly, the same values of η_{ap} as the 0.84%wt solution of C_{12}–NH–HA32 ($M_W = 3.2 \times 10^4$ g mol^{-1}). Considering these observations and the fact that the concentrations of the derivatives were above their critical concentrations, we suppose the hydrophobic effect, which leads to aggregation, to contribute to the enhancement of the viscosity of the low molecular weight derivatives solutions. Nevertheless, this effect seems to be overwhelmed by the effect of increasing molar mass, as the HA chains overlap with each other, and, in addition, increasing DS that leads to the reduction in the viscoelasticity of HA solutions.

3.1.3. Viscosity behavior

Fig. 4 demonstrates the comparison of viscosity behavior between the native and modified HA by means of the plot of the reduced viscosity of the polysaccharides solutions, η_{red}, versus their concentration. The curves show little difference between the native HA and the derivatives; nevertheless, from the molar mass of 7.4×10^4 g mol^{-1} and concentration of ~4 g L^{-1} a moderately steeper increase in η_{red} and bending of the plot can be seen for the native HA. The same result was observed for the intrinsic viscosity (Table 3), i.e. it was a bit larger for native HA than for its modified analogue of the same molar mass. This result would suggest a slight contraction of the HMHA molecules due to hydrophobic effect on the alkyl chains (Simon, Dugast, Le Cerf, Picton, & Muller, 2003). However, this decrease can be also due to the change of molar mass per unit contour length of the polysaccharide, resulting from the grafting of the HA backbone by hydrophobic segments, which causes that the HMHA chain becomes shorter than that of the native HA of the same molar mass. The Huggins coefficient, k_H, which indicates the origin of the interactions in the system, is very similar for the C–12 derivatives and the HA of lower molar mass, ranging from ≈0.40 to 0.48, which are the typical values for polymers in good solvents (Arvidson et al., 2006).

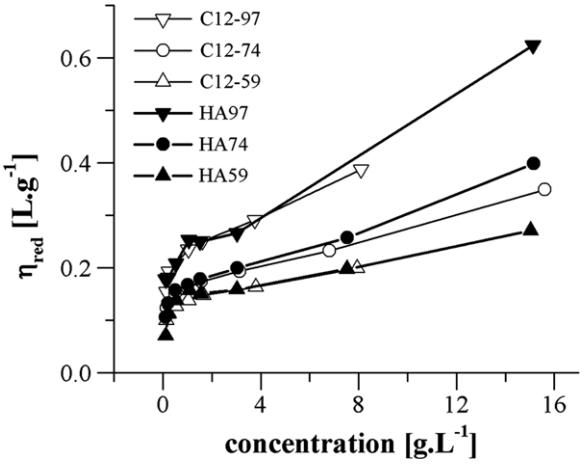

Fig. 4. Reduced viscosity of C–12 derivatives and the HA of the same molar masses (see Table 1) as a function of the polysaccharides concentration in the phosphate buffer (pH 7, I = 0.146 M) at 25 °C.

Table 3
The viscosity parameters for HA and C_{12} and C_8 HMHA in the phosphate buffer (I = 0.146 M) and for C_8–NH–HAl00 in NaCl.

Sample	$[\eta]$ (mL g^{-1})	k_H	$1/[\eta]$ (g L^{-1})	c^* (g L^{-1})
HA59	136.0	0.417	7.36	–
HA74	159.2	0.476	6.28	–
HA97	234.6	0.222	4.27	–
HA560	602.2	0.486	1.66	2.02
C_{12}–NH–HA32	69.6	0.427	14.38	–
C_{12}–NH–HA59	133.3	0.434	7.51	–
C_{12}–NH–HA74	155.8	0.432	6.42	–
C_{12}–NH–HA97	217.2	0.403	4.60	–
C_8–NH–HA100	477.5	0.573	2.09	1.63
0.05 M NaCl	514.6	0.646	1.94	–
0.15 M NaCl	513.2	0.314	1.95	–

The Huggins coefficient was determined using two fits according to Eqs. (2) and (3), and in some cases the values of k_H from these two fits differed with each other, which is most likely the reason of the coefficient drop for 9.7×10^5 g mol^{-1} HA. The coefficient a of the Mark–Houwink–Sakurada plot (MHS), i.e. the double logarithmic plot of $[\eta]$–M_W, was determined for both the HA and C–12 derivative of the molar masses, $M_W \leqslant 1 \times 10^5$ g mol^{-1}. The value of the coefficient, a, was very similar for both the polysaccharides ($a_{HA} = 1.11$, $a_{HMHA} = 1.01$) meaning that the conformation and the stiffness of the HA chains was not altered significantly after the modification by C–12 chains for the given DS and molar mass. The value of the coefficient determined here is in a good accordance with that reported by Cowman & Matsuoka (2005); Mendichi, Šoltés, & Schieroni (2003), etc., although our samples had rather a broad molar mass distribution.

The intrinsic viscosity of C_8–NH–HA100 was lower than that of the 5.6×10^5 g mol^{-1} HA due to lower molar mass of the derivative (4.947×10^5 g mol^{-1}) and high DS (Table 3). Thus, one would expect the critical coil-overlap concentration, c^*, larger for the derivative than for the HA ($c^*_{HA} = 2.02$ g L^{-1}) due to smaller space-filling volume of the HMHA molecules. Nevertheless, we found it to be smaller, i.e. 1.63 g L^{-1}. According to some studies (Simon et al., 2003), $[\eta]$ decreases with DS and the c^* firstly decreases too, up to a certain DS, and then consequently increases above this value. This increase of c^* is attributed to predomination of intra-molecular associations in the system above certain DS; nevertheless, our sample exhibited lower c^*, which would mean that inter-molecular interactions predominate. However, we could neither directly compare this result with the HA of exactly the same M_W, nor we had more HMHA samples of the same molar mass and different DS to decide whether intra- or inter-molecular interactions prevailed in this case. In addition, C_8–NH–HA100 displayed the highest k_H (0.57) of all the samples and also turbidity of its solutions, which indicated enhanced polymer–polymer interactions. Nevertheless, neither this foundation tells us whether inter- and/or intra-molecular association predominates in this system, even though the turbidity suggests the existence of larger particles in the system.

3.2. Influence of NaCl and buffer

In order to investigate the ionic strength influence on the HA derivatives aggregation, the HMHA solutions were either prepared in NaCl or the salt was added. Fig. 5 shows the influence of 0.1 M NaCl on the surface tension of the solutions of C_8–NH–HA100, demonstrating lower values of γ/γ_0 after the addition of NaCl than the derivative was prepared directly in 0.1 M NaCl. The influence of the other salt contents is summarized in Table 2 for the surface tension, and in Table 3 for the viscosity measurements, respectively. Generally, the surface-active properties altered irregularly as the concentration of NaCl in the solutions increased, i.e. they

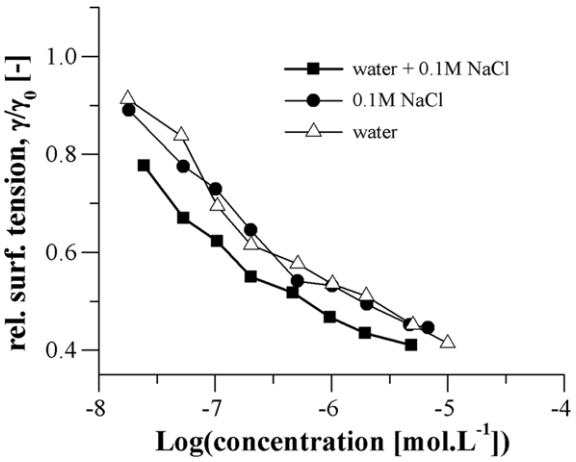

Fig. 5. The influence of 0.1 M NaCl solution and the concentrated NaCl solution added into the C_8–NH–HA100 solutions up to the overall ionic strength of 0.1 M on the surface tension of the polysaccharide as a function of its concentration.

mostly passed through a maximum at the ionic strength of 0.1 M and then decreased with the ionic strength towards the values of the parameters nearly the same as in salt free solutions.

The influence of NaCl on the sample's viscosity behavior was not so remarkable, resulting in the same intrinsic viscosities for the ionic strength studied. Nevertheless, the k_H value decreased with the ionic strength.

From the tables is clear a different influence of NaCl on the HA derivatives aggregation behavior than that of the buffer used. Firstly, the C_T and Γ_{max} of C_8–NH–HA100 derivative were larger and the conformation at the air/solution interface more compact in the buffer (I = 0.146 M, pH 7) than in 0.15 M NaCl; however the efficiency and effectiveness of reduction in the surface tension was like in 0.15 M NaCl. The intrinsic viscosity of the derivative was smaller in the buffer, suggesting shrinkage of the polysaccharide macromolecular domain comparing to that in NaCl of the same ionic strength. The variations may be attributed to a different hydration of the ions and their effect on the polysaccharide conformation.

4. Conclusions

The investigation of physical–chemical properties of the novel alkylated derivatives of HA in solution has been presented by means of surface tension, rheology and viscometry. The study has shown surface-activity for all studied HMHA and their self-association into micelle-like aggregates, which was accompanied by turbidity of the solutions of some samples. The higher the molar mass and DS, the lower the critical polymer concentration at which the self-association was observed and the more efficient reduction in the surface tension. This suggests the larger and more substituted polysaccharides more convenient for drug delivery purposes. Nevertheless, the molar mass and DS must be finely balanced, so that the derivative displayed efficient surface-activity and low critical concentration simultaneously. The large derivatives of high DS displayed also more compact conformation at the air/water interface than the smaller and less substituted ones due to stronger hydrophobic effect on the higher number of alkyls on the HA backbone. Viscous properties of C–12 derivatives were very similar to those of HA of the same molar mass ($M_W \leqslant 1 \times 10^5$ g mol^{-1}) showing also very similar value of the MHS plot coefficient, a = 1.01/1.11. This reflects that the HA chain stiffness and conformation was not substantially altered after its modification by C–12 chains. Higher value of the Huggins coefficient for the large and the most substituted C_8–NH–HA100 suggests enhanced polymer–polymer interactions in the aqueous environment and turbidity of its solutions indicates larger inter-molecular particles. Practically Newtonian rheological behavior of low molecular-weight derivatives of low DS turned into shear-thinning as the molar mass increased, despite the high DS. The influence of NaCl had rather irregular influence on the C_8–NH–HA100 aggregation properties, showing the largest alternation at 0.1 M NaCl.

Acknowledgments

The authors thank CPN, Ltd. (Dolní Dobrouč, the Czech Republic) for the material and financial support. This work was also supported by the Czech government funding – project MSM0021630501.

References

Arvidson, S. A., Rinehart, T. B., & Gadala-Maria, F. (2006). Concentration regimes of solutions of levan polysaccharides from Bacillus sp. *Carbohydrate Polymers, 65*, 144–149.

Chen, H., Chen, T., Hu, J., & Wang, Ch. (2005). Nano aggregates of biodegradable amphiphilic poly(hydroxylethyl aspartamide-*co*-propyl asparamide) grafted poly(D, L-lactide). *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 268*, 24.

Cowman, M. K., & Matsuoka, S. (2005). Experimental approaches to hyaluronan structure. *Carbohydrate Research, 340*, 791–809.

Creuzet, C., Kadi, S., Rinaudo, M., & Auzély-Velty, R. (2006). New associative systems based on alkylated hyaluronic acid. Synthesis and aqueous solution properties. *Polymer, 47*, 2706–2713.

Duval-Terrié, C., Huguet, J., & Miller, G. (2003). Self assembly and hydrophoibic clusters of amphiphilic polysaccharides. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 220*, 105–115.

Forsberg, N. (1996). Studies of cell and matrix components interacting with hyaluronan. Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine.

Gang-Biao, Jiang, Daping, Quan, Kairong, Liao, & Haihua, Wang (2006). Preparation of polymeric micelles based on chitosan bearing a small amount of highly hydrophobic groups. *Carbohydrate Polymers, 66*, 514–520.

Geciova, R., Flarbani, A., Delben, F., Liut, G., Urnani, R., & Cesaro, A. (1995). Physicochemical properties of hyaluronan and its hydrophobic derivatives – A calorimetric and viscometric study. *Macromolecular Chemistry and Physics, 196*, 2891.

Hirano, K., Sakai, S., Ishikawa, T., Avci, F. Y., Linhardt, R. J., & Toida, T. (2005). Preparation of the methyl ester of hyaluronan and its enzymatic degradation. *Carbohydrate Research, 340*, 2297–2304.

Hunter, J. R. (2001). *Foundations of colloid science* (2nd ed.). Oxford University Press.

Jaracz, S., Chen, J., Kuznetsova, L. V., & Ojima, I. (2005). Recent advances in tumor-targeting anticancer drug conjugates. *Bioorganic and Medicinal Chemistry, 13*, 5043–5054.

Jeong, J. H., Kang, H. S., Yang, S. R., Park, K., & Kim, J. D. (2005). Biodegradable poly(asparagine) grafted with poly(caprolactone) and the effect of substitution on self-aggregation. *Colloids and sufaces A: Physicochemical and Engineering Aspects, 264*, 187–194.

Lapčík, L., Lapčík, L., Jr., De Smedt, S., Demeester, J., & Chabreček, P. (1998). Hyaluronan: Preparation, structure, properties, applications. *Chemical Reviews, 98*(8), 2663–2684.

Ludwig, A., & Ooteghem van, M. (1989). Evaluation of sodium hyaluronate as viscous vehicle for eye drops. *Journal de Pharmacie de Belgique, 44*(6), 391–397.

Mendichi, R., Šoltés, L., & Schieroni, A. G. (2003). Evaluation of radius of gyration and intrinsic viscosity molar mass dependence and stiffness of hyaluronan. *Biomacromolecules, 4*, 1805–1810.

Mlčochová, P., Bystricky, S., Steiner, B., Machova, E., Koos, M., Velebny, V., & Krcmar, M. (2006). Synthesis and characterization of new biodegradable hyaluronan alkyl derivatives. *Biopolymers, 82*(1), 74–79.

Prehm, P. (2002). Hyaluronan. In E. J. Vandomme, S. De Baets, & A. Steinbückel (Eds.). *Biopolymers Polysaccharides I: Polysaccharides from prokaryotes* (Vol. 5, pp. 379–406). Weinheim: Wiley-VCH.

Ribeiro, W., Mata, J. L., & Saramago, B. (2007). Effect of concentration and temperature on surface tension of sodium hyaluronate saline solutions. *Langmuir, 23*, 7014–7017.

Simon, S., Dugast, J. Y., Le Cerf, D., Picton, L., & Muller, G. (2003). Amphiphilic polysaccharides. Evidence for a competition between intra and intermolecular associations in dilute system. *Polymer, 44*, 7917–7924.

Šimkovič, I., Hricovíni, M., Šoltés, L., Mendichi, R., & Cosentino, C. (2000). Preparation of water-soluble/insoluble derivatives of hyaluronic acid by cross-linking with epichlorhydrine in aqueous NaOH/NH_4 OH solution. *Carbohydrate Polymers, 41*, 9–14.

Vercruysse, K. P., & Prestwich, G. D. (1998). Hyaluronate derivatives in drug delivery. *Critical Reviews in Therapeutic Drug Carrier Systems, 15*(5), 513–555.

Zhang, T., & Marchant, R. E. (1996). Novel polysaccharide surfactants: The effect of hydrophobic and hydrophilic chain length on surface active properties. *Journal of Colloid and Interface Science, 177*, 419–426.