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SHORT COMMUNICATION

Aggregation behavior of novel hyaluronan derivatives—a fluorescence probe study

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Abstract Aggregation properties of hydrophobized hyaluronan with different molecular weights and degrees of substitution were studied using pyrene and perylene as fluorescence probes. Both probes in contrast to native biopolymer confirmed aggregation of modified hyaluronan. The critical aggregation concentration (cac) was determined by the pyrene I_1/I_3 and perylene fluorescence intensity method. The cac value varied both with the molecular weight and the degree of substitution and was between 0.610 and 0.003 $g \cdot L^{-1}$. Pyrene polarity scale confirmed formation of hydrophobic domains.

Keywords Hyaluronate derivatives · Fluorescence probes · Critical aggregation concentration · Core hydrophobicity

Introduction

Polysaccharides and their derivatives have become major components in the development of biocompatible and biodegradable materials with many areas of applications (e.g., tissue engineering, drug delivery). Chemical modification, which does not affect the biodegradability and does not suppress biological activity, can lead to further expansion of medicine and engineering applications [1, 2].

Hyaluronan (HA) is a major component of pericellular and extracellular matrices [3]. It is a linear polymer formed by repeating disaccharide units composed of the disaccharide unit formed by D-glucuronic acid-1-*β*-3-*N*-acetylglukosamine. It plays an important role in stabilizing the extracellular matrix in many tissues by binding to specific proteins called hyaladherines. The main hyaluronan fraction is localized in the skin tissue [4].

Synthesis of hyaluronan derivatives is generally based on the esterification on the D-glucuronic subunit [5]. Recently, preparation of new type derivatives that are subject of this study was reported [6]. Modification was made on the secondary hydroxyls of the glucuronic subunit. This way of modification leaves all carboxylic groups free in contrast to common derivatives prepared via esterification on the COOH group and enables to achieve high substitution degrees while maintaining polyelectrolytic character. Amphiphilic hyaluronan polyelectrolyte is thus obtained (hydrophobized hyaluronan, hHA), which is supposed to be still water-soluble and to aggregate in aqueous solution forming micelle-like structures with a non-polar core that will be able to entrap hydrophobic species, e.g., drugs.

In this work, first results on studying their aggregation capabilities are reported. Aggregation behavior of amphiphiles can be studied by non-polar fluorescence probes, which can be solubilized into this core. Fluorescence probe techniques have been used successfully in the study of a wide range of surfactants [7–9]. They are able to determine not only the critical micellar or aggregation concentration (cac) but also the polarity index of probe's microenvironment [10–12] and effective viscosity of the micellar core [13, 14].

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Pyrene I_1/I_3 ratio method is a widely used method to determine the cac value for many surfactant-based systems. Its unique response to the microenvironment polarity is well known and described [15]. In this method, the ratio of the fluorescence intensity at 373 nm (I_1) and at 383 nm (I_3) is plotted against the logarithm of the aggregating molecule concentration. Below the cac, the pyrene I_1/I_3 ratio does not change in a wide range of concentration. Near the cac value, this ratio sharply decreases with increasing concentration up to a final, nearly constant, value.

To confirm the results obtained with pyrene, we used also the perylene fluorescence method [16]. Using different probes in aggregation studies is not common. Perylene is non-fluorescent in aqueous environment. Fluorescence intensity of the perylene increases with the number of non-polar domains formed in the solution. No fluorescence is observed until these domains are present in solution. When the domains are formed, a sharp increase in the fluorescence is observed. Straight lines can fit these two trends and the concentration-coordinate of their point of intersection define the cac value directly.

Materials and methods

Sodium hyaluronate and its alkyl derivatives (Fig. 1) were obtained from CPN (Dolní Dobrouč, Czech Republic). Details on the synthesis of derivatives and their molecular characteristics have been published elsewhere [6]. Hyaluronans were of the following weight-average molecular weights: 97, 560, and 1,630 kg·mol^{-1}.

Derivatives were of the weight-average molecular weights of 44, 134, 183, 360, and 1,470 kg·mol^{-1}, and their substitution degrees were in the range of 10–70%. Substitution degree is defined as the ratio of the mole of substituents per mole of the disaccharide unit [6], e.g., SD 100% means one alkyl chain per each disaccharide unit in the hyaluronate chain. Molecular weights were determined by size-exclusion chromatography/multi-angle laser light scattering and the substitution degree is defined from the ^{1}H NMR spectra (for details, see [6]). All the molecular parameters were determined and provided by the producer. The hyaluronate samples were dissolved in doubly distilled water to the concentration 2 g·L^{-1}. This stock solution was stabilized by addition of sodium azide (p.a., Lachema) in final concentration 10^{-3} mol·L^{-1}. The alkyl-type abbreviation comes as the first followed by the original molecular weight (before the derivatization) and the substitution degree. For example, D 134/10 means C_{10}-derivate of the molecular weight 134 kg·mol^{-1} and with the substitution degree 10%.

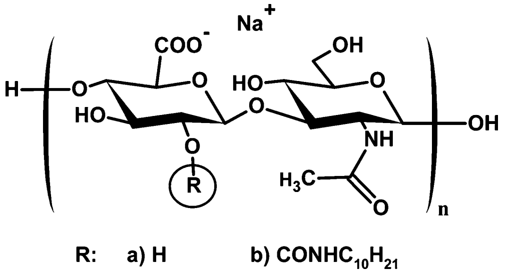

Fig. 1 Schematic structure of the sodium hyaluronate (**a**) and its C_{10} alkyl derivative

Sodium dodecylsulfate (p.a., Lachema) was dissolved in water to obtain concentration of 2×10^{-2} mol·L^{-1}.

Pyrene and perylene (fluorescence grade) were obtained from Fluka, acetone p.a. from Lachema.

The hyaluronate samples were listed in correspondence to their characteristics. Stock solutions of pyrene and perylene were prepared in acetone in final concentration 10^{-4} mol·L^{-1}. Probe stock solution was introduced into a vial and acetone was evaporated. The concentration of both probes in final samples was set to 5×10^{-6} mol·L^{-1}. The stock solution of HA or hHA was introduced into the vial with the probe, diluted to the desired concentration, and the resulting solution was sonicated for 4 h and stored during next 20 h. The fluorescence emission spectra were monitored with a luminescence spectrophotometer (AMINCO-Bowman, Series 2) at 293.15±0.1 K. The excitation and emission slit widths were set to 4 nm, and the excitation wavelength was 335 and 408 nm for pyrene and perylene, respectively.

The experimental data, i.e., the pyrene I_1/I_3 ratio (y) dependency on concentration (x), were evaluated using nonlinear fitting with Boltzman's curve containing four parameters—the maximum (a), the minimum (b), the inflex point (x_0), and the width of the step change (Δx; Eq. 1).

$$y = \frac{a-b}{1+e^{\frac{(x-x_0)}{\Delta x}}} + b. \tag{1}$$

The data were fitted using the nonlinear curve fitting with Origin 75. The cac was obtained from the inflex point of the nonlinear fitting. This is denoted as the cac_1 point. Alternatively, we determined also the cac_2 point defined as (cf. Fig. 2)

$$\mathrm{cac}_2 = x_0 + 2\Delta x. \tag{2}$$

Figure 2 shows the typical dependency of the I_1/I_3 on the logarithm of the concentration in a model surfactant system, sodium dodecyl sulfate, with two possible points, cac_1 and cac_2, which can determine the critical aggregation concentration. Its critical aggregation concentration, 8.2×10^{-3} mol·L^{-1} in aqueous solution at 293.15 K, via surface tension experiments was reported elsewhere [17]. Obtained

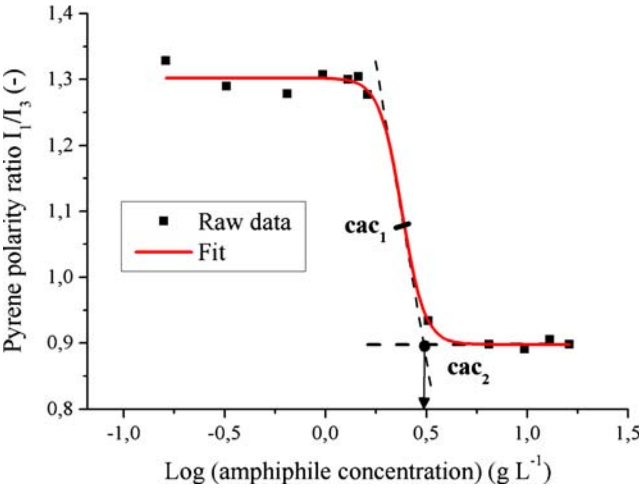

Fig. 2 Typical dependency of the pyrene polarity ratio on the concentration of an amphiphile (sodium dodecyl sulfate)

cac values from the fit are 8.3 and 10.2×10^{-3} mol·L^{-1} for cac$_1$ and cac$_2$, respectively. It is obvious that the cac$_1$ value is closer to surface tension value and it seems to be more relevant as cac point.

Perylene data evaluation was based on a fit of two linear trends. From equations of these straight lines, "*x*-coordinate" of the point of intersection was evaluated as the cac.

Results and discussion

Aggregation properties

First measurements were focused on possible aggregation behavior of native hyaluronan in aqueous solution. Three molecular weights (97, 560, and 1,630 kg·mol^{-1}) were selected to investigate possible aggregation of native hyaluronan using the pyrene fluorescence method. Figure 3 shows that the I$_1$/I$_3$ ratio, also called the polarity index, ranges from 1.37 to 1.45 through a wide concentration range. Value of the polarity index in this concentration range can be taken as constant and invariant. Therefore, from the hydrophobic polarity probe point of view, no aggregation behavior was observed in these solutions. Hydrophobic patches supposed to exist along HA chain [18] do not form domains able to solubilize non-polar substances like pyrene.

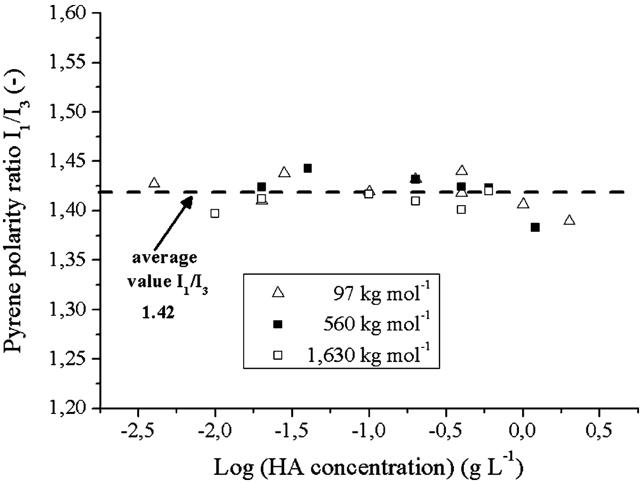

Fig. 3 Plot of the I$_1$/I$_3$ vs. log C$_{\text{HA}}$ for native hyaluronan in aqueous solution of different molecular weights

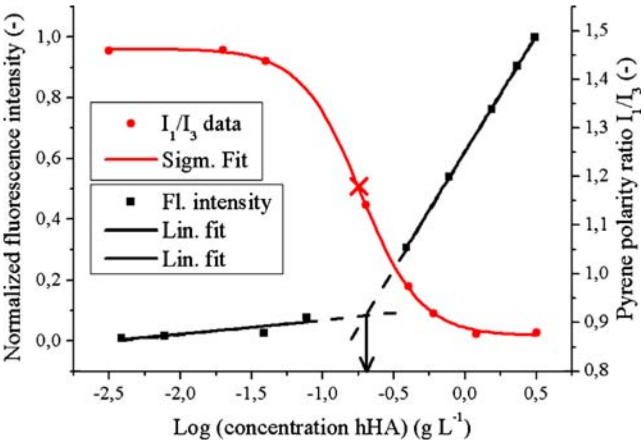

Fig. 4 Plot of the normalized integral fluorescence (I_F) and the I$_1$/I$_3$ vs. the log C$_{\text{HA}}$ for the D134/30 sample. The perylene data are fitted with two lines. The pyrene data are fitted by sigmoid curve with marked cac$_1$ (*X*). The point of intersection (*down arrow*) from perylene dependence (*x*-coordinate value=−0.747, which is equal to 0.179 g·L^{-1}) is identical with the pyrene cac$_1$ point (*x*-coordinate value=−0.750, which is equal to 0.178 g·L^{-1})

On the contrary, hyaluronate derivatives manifested clear aggregation behavior. Example of results obtained for D134/10 is presented in Fig. 4. As explained above, there are two possible cac points on the concentration dependency of the pyrene I$_1$/I$_3$ ratio. Aguiar et al. [15] suggested a condition to select the cac value from the pyrene I$_1$/I$_3$ ratio. If the $x_0/\Delta x$ (cf. Eq. 1) is less than 10, the cac point is determined by the *x*-coordinate of the inflex point x_0—cac$_1$ in our case. Table 1 examples determined values of the x_0/Δx ratio of hyaluronate derivatives. All of them passed the "less than 10 condition".

Table 1 The representative samples of hydrophobized hyaluronan with the x_0, Δx, and criterion value obtained from curve fitting

Samples	x_0 (g·L^{-1})	Δx (g·L^{-1})	$x_0/\Delta x$	R^2
D44/10	0.54	1.42	0.38	0.99
D134/10	0.61	1.51	0.40	0.99
D183/30	0.15	1.55	0.10	0.98
D360/50	0.08	1.48	0.05	0.98
D1470/70	0.20	1.03	0.19	0.97

Table 2 Overview of the cac values for hHA samples from pyrene and perylene, in brackets, experiments

	M_W (kg·mol^{-1})	SD (%)			
		10	30	50	70
cac (g·L^{-1})	44	0.540 (0.520)	0.045 (0.050)	0.003 (0.003)	ND (ND)
	134	0.610 (0.600)	0.179 (0.180)	0.130 (0.140)	0.030 (0.025)
	183	0.190 (0.210)	0.150 (0.170)	0.110 (ND)	ND (ND)
	360	0.190 (0.200)	0.080 (ND)	0.110 (0.140)	ND (ND)
	1,470	ND (ND)	0.260 (0.280)	0.110 (0.130)	0.200 (0.160)

ND not determined

Perylene results confirmed the selection of cac$_1$ as the proper critical concentration. In Fig. 4, the integral perylene fluorescence intensity is plotted against the logarithm of hyaluronate concentration. The perylene data, resolved by two straight lines, lead to the cac value 0.179 g·L^{-1}, which corresponds to the cac$_1$ value determined by the pyrene method (0.178 g·L^{-1}). Accordingly, we used cac$_1$ for the evaluation of the pyrene data.

The cac values of various derivatives decrease when the substitution degree increases, with a small exception for samples D 1470 (Table 2). At constant substitution degree, there is no clear trend of cac change with molecular weight (Table 2). The greatest decrease of cac values with increasing substitution degree was observed for samples D 44. These samples have the shortest biopolymer chain. Aggregation is driven by hydrophobic interactions introduced by the alkylation of native hyaluronan backbone. Formation of aggregates is accompanied by reorientation of hyaluronan chains and also by changing its conformation. Short chains can be reoriented and deformed more easily than long ones; therefore, their cac values are more sensitive to the degree of substitution. But not only cac values are decisive for the final selection of the derivative, which can be used in future for an application in drug delivery system.

In comparison of cac values with another alkyl-derivative of HA [19], pyrene as the cac probe was used and the cac value was realized as the first decreasing of the pyrene polarity index dependency on the derivative concentration, our derivatives with high SD or MW have the same or lower cac values. Similarly, in the same orders of magnitude, range cac values of the alkyl-chitosan [20, 21], cholesteryl-chitosan derivatives [22], or heparin-deoxycholic acid derivative [23], where decreasing tendency of the cac on increasing SD, were observed.

Domain hydrophobicity

As the base for determination of the hydrophobicity of the non-polar core, the pyrene polarity scale (PPS) was selected [24]. The limiting values of the I$_1$/I$_3$ ratio from the concentration dependencies are shown in Table 3. Data indicate a general trend of increasing hydrophobicity (decreasing pyrene polarity parameter) with increase in the substitution degree, which is easily understandable and expectable. Closer inspection of data obtained for samples of the same molecular weight of the native hyaluronan does not always reveal simple trend (Table 3). This can indicate different distribution of alkyl substituents along the polysaccharide backbone of varying substitution degree, non-uniform alkyl distribution on highly substituted hyaluronan, and also different changes in conformational behavior of chains of various molecular weights modified by alkyls to different degrees.

Conclusion

The novel hyaluronan derivatives show surfactant-like aggregation behavior in aqueous solutions. Their critical aggregation concentrations can be modified by the molecular weight and substitution degree and ranges between 0.610 and 0.003 g·L^{-1}. Hydrophobic domains are formed with the relative hydrophobic index (PPS) nearby 0.85. This value is comparable to that of simple surfactants. Novel hyaluronate hydrophobized derivatives with preserved free carboxyl groups are thus potential candidates for preparing systems for targeted delivery of hydrophobic active substances.

Table 3 Overview of the hydrophobicity indexes for hHA samples

	M_W (kg·mol^{-1})	SD (%)			
		10	30	50	70
Pyrene polarity parameter	44	0.96	0.83	0.89	–
	134	0.88	0.86	0.79	0.82
	183	0.89	0.80	0.82	–
	360	0.96	0.83	0.8	–
	1,470	–	0.84	0.81	0.81

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References

1. Luo Y, Prestwich GD (2001) Hyaluronic acid-*N*-hydroxysuccinimide: a useful intermediate for bioconjugation. Bioconjugate Chem 12:1085–1088
2. Ghosh K, Shu XZ, Mou R, Lombardi J et al (2005) Rheological characterization of in situ cross-linkable hyaluronan hydrogels. Biomacromolecules 6:2857–2865
3. Lapčík L Jr, Lapčík L, De Smedt S, Demeester K (1998) Hyaluronan: preparation, structure, properties, and applications. Chem Rev 98:2663–2684
4. Tammi R, Tammi M (1998) Hyaluronan in the epidermis. Available via DIALOG. http://glycoforum.gr.jp/science/hyaluronan/HA04/HA04E.html. Accessed 10 Apr 2008
5. Bulpitt P, Aeschlimann DJ (1999) New strategy for chemical modification of hyaluronic acid: preparation of functionalized derivatives and their use in the formation of novel biocompatible hydrogels. Biomed Mater Res 47:152–169
6. Mlčochová P, Bystrický S, Steiner B et al (2006) Synthesis and characterization of new biodegradable hyaluronan alkyl derivatives. Biopolymers 82:74–79
7. Kalyanasundaram K, Thomas JK (1977) Environmental effects on vibronic band intensities in pyrene monomer fluorescence and their application in studies of micellar systems. J Am Chem Soc 99:2039–2044
8. Glushko V, Thaler MSR, Karp DC (1981) Pyrene fluorescence fine structure as a polarity probe of hydrophobic regions: behavior in model solvents. Arch Biochem Biophys 210:33–42
9. Lianos P, Zana RJ (1981) Fluorescence probe studies of the effect of concentration on the state of aggregation of surfactants in aqueous solution. Colloid Interface Sci 84:100–107
10. Offen HW, Turley WD (1982) Pyrene fluorescence spectra in micelles at high pressures. J Phys Chem 86:3501–3503
11. Ruiz CC, Sánchez FG (1994) Effect of urea on aggregation behavior of triton X-100 micellar solutions: a photophysical study. J Colloid Interface Sci 165:110–115
12. Molina-Bolívar JA, Aguiar J, Peula-García JM, Ruiz CC (2004) Surface activity, micelle formation, and growth of *n*-octyl-β-D-thioglucopyranoside in aqueous solutions at different temperatures. J Phys Chem B 108:12813–12820
13. Ruiz CC (2000) Fluorescence anisotropy of probes solubilized in micelles of tetradecyltrymethylammonium bromide: effect of ethylene glycol added. J Colloid Interface Sci 221:262–267
14. Crosas E, Egea MA, Reig FJ (2006) Spectroscopic techniques applied to the study of laminin fragments inserted into model membranes. Colloid Interface Sci 295:264–269
15. Aguiar J, Carpena P, Molina-Bolívar JA, Ruiz CC (2003) On the determination of the critical micelle concentration by the pyrene 1:3 ratio method. J Colloid Interface Sci 258:116–122
16. Mast RC, Haynes LV (1975) The use of the fluorescent probes perylene and magnesium 8-anilinonaphthalene-1-sulfonate to determine the critical micelle concentration of surfactants in aqueous solution. J Colloid Interface Sci 53:35–41
17. Elworthy PH, Mysels KJ (1966) The surface tension of sodium dodecylsulfate solutions and the phase separation model of micelle formation. J Colloid Sci 21:331–347
18. Scott JE, Heatley F (2002) Biological properties of hyaluronan in aqueous solution are controlled and sequestered by reversible tertiary structures, defined by NMR spectroscopy. Biomacromolecules 3:547–553
19. 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
20. Liu W, Sun SJ, Zhang X, De Yao K (2003) Self-aggregation bahavior of alkylated chitosans and its effect on the release of a hydrophobic drug. J Biomater Sci Polym Edn 14:851–859
21. Philippova OE, Volkov EV, Sitnikova NL, Khokhlov AR, Desbrieres J, Rinaudo M (2001) Two types of hydrophobic aggregates in aqueous solution of chitosan and its hydrophobic derivative. Biomacromolecules 2:483–490
22. Yingsong W, Lingrong L, Jian W, Zhang Q (2007) Preparation and characterization of self-aggregated nanoparticles of cholesterol-modified *O*-carboxylmethyl chitosan conjugates. Carbohydr Polym 69:597–606
23. Park K, Kim K, Kwon IC, Kim SK, Lee S, Lee DY, Byun Y (2004) Preparation and characterization of self-assembled nanoparticles of heparin-deoxycholic acid conjugates. Langmuir 20:11726–11731
24. Dong DC, Winnik F (1985) The Py scale of solvent polarities. Can J Chem 62:2560–2566