

Aggregation behavior of novel hyaluronate derivatives – a fluorescence probe study

Filip MRAVEC^{1,2}, Miloslav PEKAŘ¹, Vladimír VELEBNÝ²

(1) *Institute of Physical and Applied Chemistry, Faculty of Chemistry, Brno University of Technology, Purkyňova 118, 612 00 Brno, CZ*

(2) *CPN spol. s.r.o., Dolní Dobrouč 401, 561 02 Dolní Dobrouč, CZ*

tel.: +420 541 149 483

fax.: +420 541 149 389

filip.mravec@centrum.cz

www.fch.vutbr.cz/ipac ; www.hyaluronan.cz

Abstract: Aggregation properties of hydrophobized hyaluronan (hHA) in different molecular weights and degree of substitution were studied by pyrene and perylene fluorescence method. The critical aggregation concentration (cac) was determined by the pyrene I_1/I_3 and perylene fluorescence intensity method. The value of the cac for the hHA in aqueous solution was measured at different molecular weights and at 293.2 K. The cac value varied both with the molecular weight and the degree of substitution and was between 0.610-0.003 g·L⁻¹. Pyrene Polarity Scale confirmed formation of hydrophobic domains of the relative hydrophobic index about 0.85.

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 matrices³. It is a linear polymer formed by repeating disaccharide units composed of the disaccharide unit formed by *D*-glucuronic acid-1- β -3-*N*-acetylglucosamine. 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 tissue⁴.

Synthesis of hyaluronan derivatives is generally based on the esterification on the *D*-glucuronic subunit⁵. The derivatives that are subject of this study have been prepared by modification on the second carbon of the glucuronic subunit⁶. Because carboxylic groups remain still free, amphiphilic

(hydrophobized) polyelectrolyte is thus obtained (hydrophobized hyaluronan, hHA) in contrast to common derivatives prepared via COOH group. Consequently, it is supposed that the modified hyaluronan will be still soluble aggregate in aqueous solution to form micelle-like structures with a non-polar core that will be able to carry a useful hydrophobic species

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 surfactants⁷⁻⁹. They are able to determine not only the critical micellar or aggregation concentration (cac) but also the polarity index of probe's microenvironment¹⁰⁻¹² and effective viscosity of the micellar core^{13, 14}.

Pyrene I₁/I₃ 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 described¹⁵. In this method, the ratio of the fluorescence intensity at 373 nm (I₁) and at 383 nm (I₃) is plotted against the logarithm of the aggregating molecule concentration. Below the cac the pyrene I₁/I₃ 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.

For confirmation of results obtained with pyrene we used also the perylene fluorescence method¹⁶. Using different probes in aggregation studies are not common. The perylene can be also used in anisotropy measurements as viscosity probe because of its high quantum yield and relatively short lifetime. This probe is non-fluorescent in aqueous environment. The perylene measurements are quite simple to evaluate. The fluorescence intensity of the perylene increases with the number of non-polar domains 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 derivatives (Figure 1) were obtained from CPN Ltd. (Dolní Dobrouč, Czech Republic). Details on the synthesis of derivatives have been published elsewhere⁶. Hyaluronates were of the following molecular weights: 97, 560, and 1 630 kg·mol⁻¹.

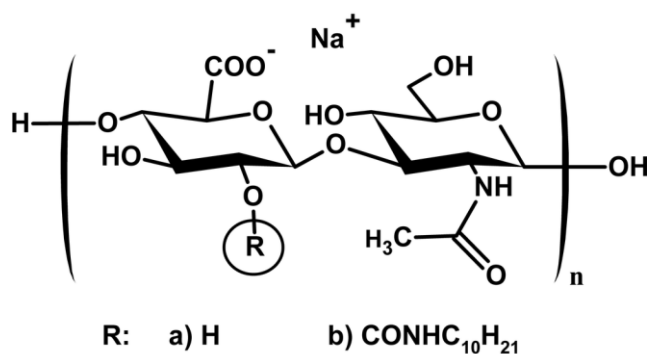


Figure 1 Schematic structure of the sodium hyaluronate (a) and its C₁₀ alkyl-derivative

Derivatives were of the molecular weights 44, 134, 183, 360, and 1 470 kg·mol⁻¹ and their substitution degrees were in the range from 10 to 70 %. Substitution degree is defined as the ratio of the mol of substituents per mol of the disaccharide unit, e.g. SD 100 % means one alkyl chain per each disaccharide unit in the hyaluronate chain. All the molecular parameters were determined and provided by the producer. The molecular weights were determined by SEC-MALLS and the substitution degree is defined from the ¹H NMR spectra⁶. 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.

Sodium dodecylsulfate (p.a., Lachema) was dissolved in water to obtain the concentration 2·10⁻² mol·L⁻¹.

Pyrene and perylene (fluorescence grade) were obtained from Fluka, acetone p.a. from Lachema. The hyaluronate samples were named in correspondence to their characteristics. 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 C₁₀-derivate of the molecular weight 134 kg·mol⁻¹ and with the substitution degree 10 %.

The stock solutions of pyrene and perylene were prepared in acetone. 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 hours and stored during next 20 hours. The fluorescence emission spectra were monitored with a luminiscence 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 nm and 408 nm for pyrene and perylene, respectively.

The experimental data, i.e. the pyrene I₁/I₃ ratio (*y*) dependency on concentration (*x*), were evaluated using non-linear 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) (Equation 1).

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

The data were fitted using the nonlinear curve fitting with Origin 75. The cac was obtained from the inflex point of the non-linear fitting. This is denoted as the cac_1 -point. Alternatively, we determined also the cac_2 -point defined as (cf. Figure 3)

$$cac_2 = x_0 + 2\Delta x \quad (2)$$

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 $\text{kg}\cdot\text{mol}^{-1}$) were selected to investigate their concentration dependencies in the presence of pyrene. Figure 2 shows that the I_1/I_3 ratio, called also the polarity index, ranges from 1.39 to 1.45 through a wide concentration range. Value of the polarity index in this concentration range can be taken as constant and invariant. So, from the hydrophobic polarity probe point of view no aggregation behavior was observed in these solutions.

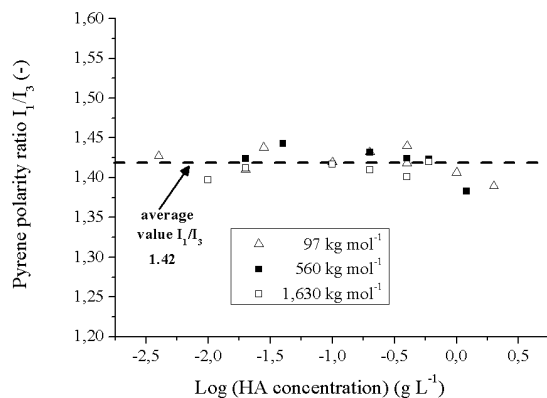


Figure 2. Plot of the I_1/I_3 vs. $\text{Log } C_{\text{HA}}$ for native hyaluronan in aqueous solution of different molecular weights.

Figure 3 the typical dependency of the I_1/I_3 on the logarithm of the concentration in a model surfactant system, SDS, with two possible points, cac_1 and cac_2 , which can determine the critical aggregation concentration.

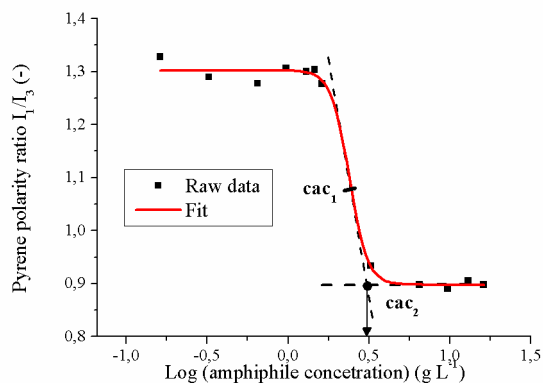


Figure 3 Typical dependency of the Pyrene Polarity Ratio on the concentration of an amphiphile (Sodium dodecyl sulphate).

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
-	-	-	-	-
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

On contrary, hyaluronate derivatives manifested clear aggregation behavior. Example of results obtained for D134/10 is presented in Figure 4. As explained above there are two possible cac points on the concentration dependency of the pyrene I_1/I_3 ratio. Aguiar and co-workers¹⁵ 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 summarizes determined values of the $x_0/\Delta x$ ratio of hyaluronate derivatives. All of them passed the “less than 10-condition”.

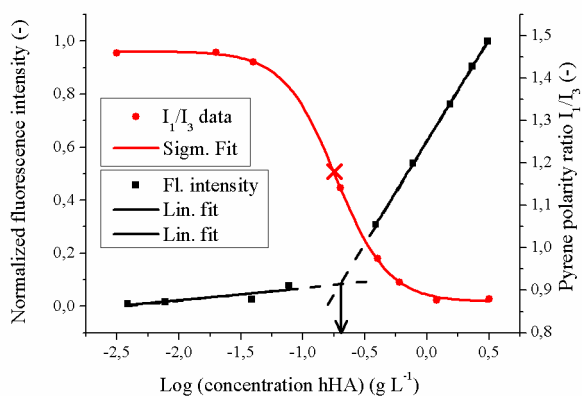


Figure 4. Plot of the normalized integral fluorescence (I_F) and the I_1/I_3 vs. the $\text{Log } C_{\text{HA}}$ for the D134/30 sample. The perylene data are fitted with two lines. The pyrene data are fitted by sigmoid

curve with marked cac_1 (X). The point of intersection (↓) from perylene dependence (x-coordinate value = -0.747, which is equal to $0.179 \text{ g}\cdot\text{L}^{-1}$) is identical with the pyrene cac_1 point (x-coordinate value = -0.750, which is equal to $0.178 \text{ g}\cdot\text{L}^{-1}$).

Perylene results confirmed the selection of cac_1 as the proper critical concentration. In Figure 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 $0.179 \text{ g}\cdot\text{L}^{-1}$, which corresponds to the cac_1 value determined by the pyrene method $0.178 \text{ g}\cdot\text{L}^{-1}$. Accordingly, we used cac_1 for the evaluation of the pyrene data.

The cac values of various derivatives show one type of trend. Dependency of the cac values on the substitution degree show that the cac value decreases when the substitution degree increases, with a small exception for samples D 1470. Through the individual substitution degree there is no observable trend depends only on the M_w . These results are summarizing in Table 2. The greatest decrease of cac values with increasing substitution degree show samples D 44. It is possible to relate this behavior to the fact that D 44 samples have the shortest chains and new types of interactions – hydrophobic – can much easily change chains orientation and conformation leading to their aggregation. But not only cac values have decisive influence on the final selection of the derivative, which can be use in future for an application in drug delivery system.

Table 2 Sum of the cac values for hHA samples.

	M_w ($\text{kg}\cdot\text{mol}^{-1}$)	SD (%)			
		10	30	50	70
cac ($\text{g}\cdot\text{L}^{-1}$)	44	0.540	0.045	0.003	-
	134	0.610	0.179	0.13	0.030
	183	0.190	0.150	0.110	-
	360	0.190	0.080	0.110	-
	1470	-	0.260	0.110	0.200

Domain Hydrophobicity

As the base for determination of the hydrophobicity of the non-polar core, the Pyrene Polarity Scale (PPS) was selected¹⁷. The limiting values of the I_1/I_3 ratio from the concentration dependencies are shown in Table 3. Data indicates a general trend of increasing hydrophobicity with increase in the substitution degree, which is easily understandable and expectable. Closer inspection of data obtained for samples coming of the same molecular weight of the native hyaluronan does not always reveal simple trend. Especially the samples of high substitution degree can deviate from simply decreasing trend. 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.

Table 3 Sum of the cac values 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	-
	1470	-	0.84	0.81	0.81

Conclusion

The novel hyaluronan derivatives show surfactant-like aggregation behavior in aqueous solutions. Their critical aggregation concentration can be modified by the molecular weight and substitution degree and ranges between 0.610-0.003 g·L⁻¹. Hydrophobic domains are formed with relative hydrophobic index (Pyrene Polarity Scale) nearby 0.85. This value is comparable with 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.

Acknowledgement.

We thank CPN Ltd Dolní Dobrouč (Czech Republic) for material and financial support. The research was supported also by the Ministry of Education of the Czech Republic, project. No. MSM 0021630501.

References

1. Luo, Y.; Prestwich, G. D. *Bioconjugate Chem.* 2001, *12*, 1085-1088
2. Ghosh, K.; Shu, X. Z.; Mou, R.; Lombardi, J.; Prestwich, G. D.; Rafailovich, M. H.; Clark, A. F. *Biomacromolecules* 2005, *6*, 2857-2865
3. Lapčík, L. jr.; Lapčík, L.; De Smedt, S.; Demeester, K. *Chem. Rev.* 1998, *98*, 2663-2684
4. Tammi, R.; Tammi, M. <http://glycoforum.gr.jp/science/hyaluronan/HA04/HA04E.html> 1998
5. Bulpitt, P.; Aeschlimann, D. *J. Biomed. Mater. Res.* 1999, *47*, 152-69
6. Mlčochová, P.; Bystrický, S.; Steiner, B.; Machová, E.; Velebný, V.; Krčmář, M. *Biopolymers* 2006, *82*, 74-79
7. Kalyanasundaram, K.; Thomas, J. K. *J. Am. Chem. Soc.* 1977, *99*, 2039-2044
8. Glushko, V.; Thaler, M. S. R.; Karp, D. C. *Arch. Biochem. Biophys.* 1981, *210*, 33-42
9. Lianos, P.; Zana, R. *J. Colloid. Interface Sci.* 1981, *84*, 100-107
10. Offen, H. W.; Turley, W. D. *J. Phys. Chem.* 1982, *86*, 3501-3503

11. Ruiz, C. C.; Sánchez, F. G. *J. Colloid Interface Sci.* 1994, *165*, 110-115
12. Molina-Bolívar, J. A.; Aguiar, J.; Peula-García, J. M.; Ruiz, C. C. *J. Phys. Chem. B* 2004, *108*; 12813-12820
13. Ruiz C. C. *J. Colloid Interface Sci.* 2000, *221*, 262-267
14. Crosas, E.; Egea, M. A.; Reig, F. *J. Colloid Interface Sci.* 2006, *295*, 264-269
15. Aguiar, J.; Carpena, P.; Molina-Bolívar, J. A.; Ruiz, C.C. *J. Colloid Interface Sci* 2003, *258*, 116-122
16. Mast, R. C.; Haynes, L. V. *J. Colloid Interface Sci.* 1975, *53*, 35-41
17. Dong, D. C.; Winnik, F. *Can. J. Chem.* 1984, *62*, 2560-2566