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Short communication

The effect of hyaluronan on the aggregation of hydrophobized amino acids—A fluorescence study

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ABSTRACT

The influence of the addition of native hyaluronan of different molecular weights (1.36 MDa and 106 kDa) on the aggregation behavior of hydrophobically modified amino acids in aqueous solution and in 0.15 M NaCl was investigated using pyrene as a solubilization probe. Hyaluronan decreased the critical aggregation concentration in aqueous solution in the case of amino acids modified by a single alkyl chain whereas no change was observed in physiological solution. The aggregation of amino acids modified by two alkyl chains was insensitive to the presence of hyaluronan.

1. Introduction

Hyaluronan is a naturally occurring polysaccharide with a very simple structure but unusual properties and a variety of functions (Prehm, 2005). It can be viewed as a homopolymer of a basic disaccharide unit consisting of D-glucuronic acid and D-*N*-acetylglucosamine. The biopolymer occurs mostly as a sodium salt and the name hyaluronan is used to describe both acidic and salt forms. The biological or physiological functions of hyaluronan are mostly determined either by its physical and physico-chemical properties or by interactions with hyaluronan-binding proteins. In synovial fluid, the vitreous humor, or the umbilical cord, it plays a significant mechanical and structural role, while its presence in the extracellular matrix can trigger important responses due to its interactions with receptors or proteins. Hyaluronan is a very hydrophilic polymer surrounded by a massive hydration shell. At physiological pH, the carboxyl groups are predominantly ionized, and hyaluronan behaves like a polyanion, which can interact or associate with cationic counterions to maintain charge neutrality. Because it is a ubiquitous polysaccharidic component of tissues and body fluids, it does not confer immunological reactions and is metabolized in the lysosomes of certain cells. Hyaluronan is an ideal candidate for use as a carrier of drugs. Many drugs are of a hydrophobic nature and are not compatible with hydrophilic hyaluronan. Various strategies are reported to overcome this problem and can be grouped either as chemical or physical approaches (Liao, Jones, Forbes, Martin, & Brown, 2005; Ossipov, 2010). The physical approach is based on physical interactions between hyaluronan and a suitable partner capable of forming hydrophobic domains. Typical examples are complexes formed by hyaluronan and cationic surfactants (Herslöf, Sundelöf, & Edsman, 1992; Thalberg & Lindman, 1989; Thalberg, Lindman, & Karlström, 1991). Traditional cationic surfactants may be problematic due to their harmful effects on cells and their toxicity in general. The use of amino acids may be a solution to this problem, providing they have a hydrophobic chain and behave like surfactants. Amino acid-based surfactants may be prepared from selected combinations of amino acids or by the hydrophobization (alkylation) of suitable amino acids (Pons, Morán, Infante, Pinazo, & Pérez, 2011; Zhao, 2009). In this work we report on hyaluronan interactions with several new types of alkylated amino acids (Fig. 1) investigated by the fluorescence probe method. All amino acids were prepared as alkyl-esters and converted to hydrochlorides to improve aqueous solubility. Modification was performed with both single alkyl chain (on monocarboxyl amino acid) and double alkyl chains (on dicarboxyl amino acid) to simulate surfactants with one or two nonpolar tails. To the best of our knowledge these systems have been almost completely neglected so far and there is no information on the surfactant-like behavior of investigated amino acid derivatives. Only Niikura, Nambara, Okajima, Matsuo, and Ijiro (2010) used a fluorescence labeled derivative of Glu-C_{10} in their study of cell membrane permeability, and Ser-C_{12} was prepared and tested as a transdermal penetration enhancer (Valivety, Gill, & Vulfson, 1998; Vavrova, Hrabalek, Dolezal, Holas, & Zbytovska, 2003).

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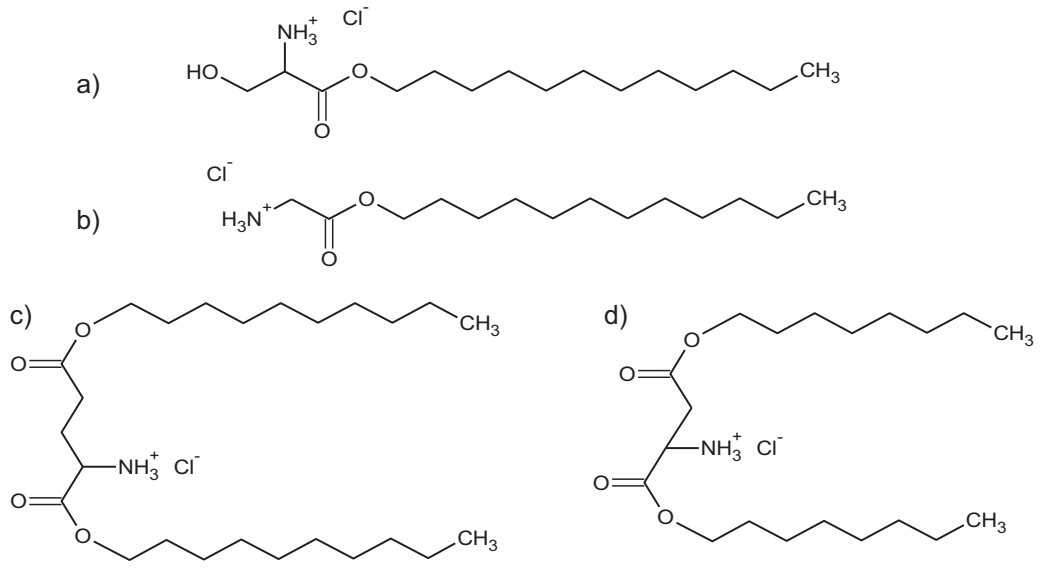

Fig. 1. Structures of amino acids used in this study: (a) serine dodecylester, Ser-C_{12}; (b) glycine dodecylester, Gly-C_{12}; (c) glutamate didecylester, Glu-C_{10}; and (d) aspartate dioctylester, Asp-C_8.

2. Material and methods

Native hyaluronan (HyA) of two different molecular weights (1.36 MDa and 106 kDa) and alkylated amino acids (see Fig. 1) were donated by CPN (Czech Republic). Modified amino acids were provided as developmental products of the same company to be tested for their aggregation behavior and their interactions with hyaluronan.

Samples were prepared in glass vials. First, the stock solution of the fluorescence probe (pyrene) in a volatile solvent (acetone) was added to glass vials and the solvent was evaporated. The final concentration of pyrene in the samples was 10^{-6} mol dm^{-3}. The stock solutions of relevant components prepared in water or in a model physiological solution (0.15 M NaCl) were then added to these pretreated vials. The samples were stirred for a minimum of 24 h on a magnetic stirrer.

Fluorescence spectroscopy with pyrene as a probe was used to study the resulting interactions and to determine the critical micellar or aggregation concentrations. This fluorescence probe reacts to the polarity of its microenvironment. The ratio of the first (373 nm) and third (384 nm) vibronic peaks in the emission spectra was used as a polarity parameter, denoted as EmPI (the emission polarity index). The ratio of fluorescence intensities at 333 nm and 338 nm in the excitation spectra of pyrene, denoted as ExPI (the excitation polarity index), was used as a second polarity parameter. Fluorescence emission and excitation spectra were recorded on an AMINCO Bowman Series 2 luminiscence spectrometer; see Fig. S1 in the supplementary material. The excitation wavelength was 336 nm and during excitation spectra measurement the emission wavelength was set at 392 nm. Measurements were performed at laboratory temperature (25 °C).

Fluorescence data usually show a characteristic sigmoidal behavior, which can be fitted by a Boltzmann curve. Fig. S2 in the supplementary material shows the Boltzmann curve and its parameters. The equation of the Boltzmann curve is

$$y = \frac{A_1 - A_2}{1 + e^{x - x_0/\Delta x}} + A_2 \quad (1)$$

where y is the value of EmPI or ExPI, x is the concentration of hydrophobically modified amino acid, A_1 and A_2 are the asymptotic limits of the sigmoidal curve, x_0 is its inflection point, and Δx is the interval of the parameter x where the decrease in y is found (Aguiar, Carpena, Molina-Bolívar, & Ruiz, 2003).

3. Results and discussion

Gly-C_{12} and Ser-C_{12} showed very similar behavior both in water and in 0.15 M NaCl. The pyrene probe confirmed the formation of hydrophobic domains above a certain alkyl amino acid concentration in both media. These domains are believed to be a result of surfactant-like aggregation, i.e. of the formation of micelles or micelle-like aggregates, and the corresponding concentration is called the critical micellar concentration. A representative example of the measured polarity indices for Gly-C_{12} is presented in Fig. 2; similar results for Ser-C_{12} are presented in Fig. S3 in the supplementary material. The critical micellar concentration (cmc; see also Table 1) is decreased in the presence of a low molecular weight electrolyte, which corresponds to the standard behavior of ionic surfactants and is the result of the screening of electrostatic repulsions between ionic polar heads of the surfactant by

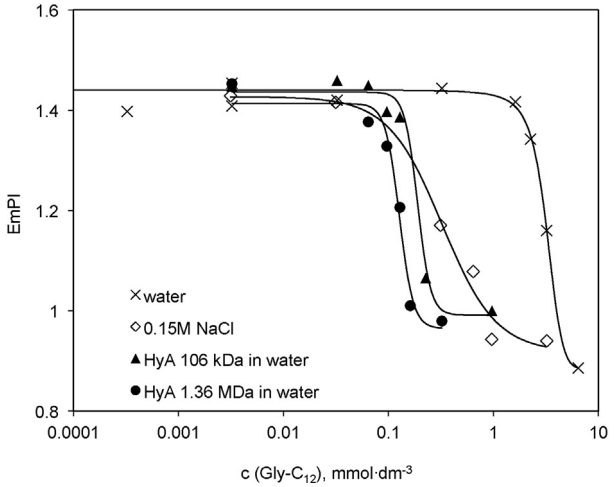

Fig. 2. Dependency of EmPI on the total concentration of Gly-C_{12} in water and in 0.15 M NaCl. Influence of the addition of hyaluronan of different molecular weights to solutions in water. The concentration of hyaluronan was 0.1% (w/v), the temperature was 25 °C.

Table 1
Values of the cmc or cac for systems of hydrophobically modified amino acids without and with added hyaluronan in water and 0.15 M NaCl. The concentration of hyaluronan was 0.1% (w/v), the temperature was 25 °C. No aggregation of Glu-C_{10} was detected up to its solubility limit.

Hydrophobically modified amino acid	Method	cmc or cac (mmol dm^{-3})					
		In water			In 0.15 M NaCl		
		No HyA	HyA 106 kDa	HyA 1.36 MDa	No HyA	HyA 106 kDa	HyA 1.36 MDa
Gly-C_{12}	EmPI	3.20	0.19	0.13	0.32	0.19	0.19
	ExPI	3.17	0.16	0.13	0.32	0.19	0.19
Ser-C_{12}	EmPI	2.22	0.14	0.07	0.25	0.29	0.18
	ExPI	2.25	0.11	0.07	0.29	0.29	0.25
Asp-C_8	EmPI	0.08	0.08	0.13	0.15	0.13	0.20
	ExPI	0.10	0.08	0.13	0.18	0.08	0.20

the action of electrolyte ions. The effect of the addition of hyaluronan is dependent on the solvent composition. In water, hyaluronan decreases the critical micellar concentration in a similar way to low molecular weight electrolyte (see Fig. 2), whereas in 0.15 M NaCl essentially no effect is observed (Fig. S4 in supplementary material). The decrease in surfactant concentration at which surfactant aggregation is observed can be attributed to the standard behavior of surfactants in the presence of an oppositely charged polyelectrolyte (Holmberg, Jönsson, Kronberg, & Lindman, 2007) – the polyelectrolyte induces surfactant aggregation by binding the oppositely charged surfactant molecules on its backbone, and the aggregation is thus promoted at a lower concentration than the surfactant critical micellar concentration; this lower concentration is therefore called the critical aggregation concentration (cac; see Table 1). The molecular weight of hyaluronan has only a moderate effect – the critical aggregation concentration is somewhat lower in the presence of a high molecular weight preparation. The screening of electrostatic interactions in the presence of a low molecular weight electrolyte suppresses interactions between surfactant molecules and polyelectrolyte chains, and no substantial changes can be observed in fluorescence probe solubilization.

Values of cmc for classical C_{12} surfactants containing an ammonium group are in the range 14–23 mmol dm^{-3} at 25 °C (Mukerjee & Mysels, 1971), i.e. somewhat higher than those determined for Gly-C_{12} and Ser-C_{12}. For N-dodecyl-β-alanine hydrochloride, a cmc value of 10 mmol dm^{-3} (at 30 °C) is reported (Mukerjee & Mysels, 1971). The addition of 50 mmol dm^{-3} NaCl decreased the cmc of dodecylammonium chloride more than twice (Mukerjee & Mysels, 1971). Data obtained in this study thus accord reasonably with literature.

The molar ratio of modified amino acid to hyaluronan dimer unit (which is the corresponding charge ratio at the same time) at the cac can give an idea of the distribution of amino acid molecules along the hyaluronan chain. For low molecular weight hyaluronan in water, this ratio is around 0.079 for Gly-C_{12} and about 0.048 for Ser-C_{12}. This means that most hyaluronan dimers that form its basic repeating unit are not in contact with amino acids at the cac, and indicates that amino acids are bound in the form of micelle-like aggregates only to some of the dimers (their carboxyl groups). The ratios calculated for high molecular weight hyaluronan are somewhat lower, viz. 0.057 for Gly-C_{12} and 0.027 for Ser-C_{12}, which could be attributed to conformational effects – the more coiled longer hyaluronan chains impede access to interacting sites inside the coil. In 0.15 M NaCl the ratios are slightly higher, but because it is not possible to separate the effect of the low molecular weight electrolyte they are not discussed here.

The dependency of the emission polarity index on the total concentration of Asp-C_8 in water and in 0.15 M NaCl is shown in Fig. 3. In this case, the cmc is not decreased by the presence of the electrolyte; on the contrary, a moderate increase in cmc is observed. Estimated values of cmc are given in Table 1. Hydrophobically modified aspartic acid is structurally different from the two abovementioned amino acid derivatives, mainly in that it has two hydrophobic chains; their length is smaller than in the two previous cases. The presence of two non-polar chains is manifested in much lower cmc compared to Gly-C_{12} and Ser-C_{12}. These chains probably separate the charged polar heads from mutual contact, and shield their repulsive interactions and, consequently, also the effect of the added salt. Micellization is primarily driven by hydrophobic interactions of alkyl chains which are not affected by the electrolyte. A lower cmc for two-alkyl-chain surfactants in comparison with single alkyl analogs is well known for classical surfactants (Mukerjee & Mysels, 1971; Holmberg et al., 2007).

Octyltrimethylammonium bromide cmc is about 140 mmol dm^{-3} (at 25 °C) and is not affected by the addition of NaCl at a concentration of 100 mmol dm^{-3}; the cmc of nonionic octyl-β-D-glucoside is 25 mmol dm^{-3} (Mukerjee & Mysels, 1971). The effect of adding another alkyl chain to the surfactant structure can be estimated from comparison of the cmc values for dodecyl- and didodecyl dimethylammonium chloride – the latter has a cmc lower by more than two orders of magnitude (Mukerjee & Mysels, 1971). The behavior of Asp-C_8 thus also accords with classical surfactants.

The addition of hyaluronan to the Asp-C_8 solution had no significant effect either in water or 0.15 M NaCl (see Fig. 3 for solutions in water). This corresponds to the minor effect of NaCl on the aggregation of pure Asp-C_8 shown also in Fig. 3 and is a result of the

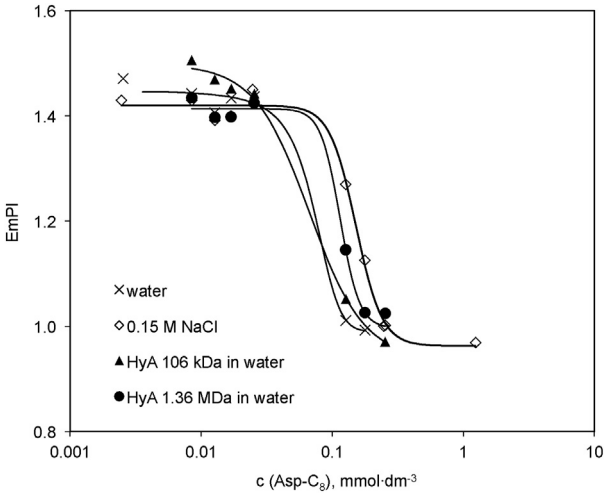

Fig. 3. Dependency of EmPI on the total concentration of Asp-C_8 in water and in 0.15 M NaCl. Influence of the addition of hyaluronan of different molecular weights to solutions in water. The concentration of hyaluronan was 0.1% (w/v), the temperature was 25 °C.

effective screening of electrostatic interactions between hyaluronan and Asp-C_8. Cac values are thus close to the corresponding cmc values (Table 1).

In the case of Glu-C_{10} we were not able to detect its aggregation by the pyrene method. Polarity indices had a constant value over the whole accessible concentration interval (the solubility of Glu-C_{10} was determined to be 0.086 mmol dm^{-3} at laboratory temperature, 25 °C), indicating the location of pyrene in the polar environment. In other words, no decrease in polarity indices analogical to that shown in all figures here was observed for Glu-C_{10}, and the same values were measured in water and 0.15 M NaCl in the presence and absence of hyaluronan. This is rather unexpected behavior particularly in comparison with the structurally similar Asp-C_8. The arrangement of alkyl chains in Glu-C_{10} is probably different, hinders packing into micellar aggregates, and decreases the solubility. The structure of glutamic acid (Metzler, 2001) suggests that the two alkyl chains in Glu-C_{10} are perpendicular, in contrast to Asp-C_8, where a parallel arrangement can be expected.

4. Conclusions

The aggregation behavior of four hydrophobically modified amino acids with different structures was studied in water and 0.15 M NaCl. In addition, the influence of the addition of low and high molecular weight hyaluronan was tested. Two of the used amino acids, monoalkylated glycine and serine, showed typical aggregation behavior known for most traditional surfactants. Above a certain concentration (the critical micellar concentration), aggregates with a hydrophobic core are formed. These aggregates are able to solubilize a nonpolar fluorescent probe and thus they should also be able to solubilize other nonpolar molecules. The presence of a low molecular weight electrolyte caused a decrease in the critical micellar concentration by about one order of magnitude. This is also typical behavior for ionic surfactants. The addition of hyaluronan caused a similar effect in water, not significantly dependent on the hyaluronan molecular weight. The effect of an electrolyte and hyaluronan can be thus attributed to electrostatic interactions. In 0.15 M NaCl, no change in aggregation behavior was observed after hyaluronan addition, which is because of the presence of the low molecular weight electrolyte that shields electrostatic interactions.

Aspartic acid modified by two alkyl chains also showed typical surfactant aggregation and solubilization behavior, but in this case such behavior was not affected by the presence of the low molecular weight electrolyte or hyaluronan. A similar derivative of glutamic acid was the least soluble preparation and no aggregation was detected by the fluorescence probe method up to the solubility limit.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.04.051.

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