



Interactions of hyaluronan with oppositely charged surfactants in very diluted solutions in water

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ARTICLE INFO

Article history:

Received 14 November 2017

Received in revised form 11 January 2018

Accepted 26 January 2018

Available online 31 January 2018

Keywords:

Hyaluronan

Interactions

Surfactants

ABSTRACT

The phase behavior of aqueous systems containing hyaluronan, at concentrations between 2 and 100 mg/L, and oppositely charged surfactants was investigated. A fluorescence probe technique revealed the formation of micellar structures on the hyaluronan in homogeneous systems well below the surfactant standard, critical, micellar concentration. Moreover, regions of gel-phase separation were revealed. A detailed phase diagram was, thus, constructed in the very diluted region and the hyaluronan concentration was found to be the main parameter controlling the phase behavior, in contrast to the charge ratio. The stability of hyaluronan-surfactant aggregates in the homogeneous systems while in storage at 4 °C (up to three months), against dilution, salt addition and on heating-cooling (between 10 and 50 °C) was also investigated. The aggregates were stable while in storage or upon increasing and decreasing the temperature. The dilution of hyaluronan-surfactant complexes or the addition of 0.15 M NaCl led to their disintegration. Finally, systems prepared in a 0.15 M NaCl solution showed that interactions are suppressed and no aggregation below the standard critical micellar concentration was observed.

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1. Introduction

Hyaluronan is a naturally occurring polysaccharide formed by repeating disaccharide units composed of D-glucuronate and N-acetyl-D-glucosamine residues linked by $\beta(1 \rightarrow 4)$ and $\beta(1 \rightarrow 3)$ bonds, which are connected to unbranched chains. The name hyaluronan commonly denotes a sodium salt of hyaluronic acid which is the form that occurs in various living organisms, including human bodies. Hyaluronan is a principal component of the extracellular matrix and plays important roles in lubrication, water sorption, water retention, and a number of cellular functions, such as attachment, migration, and proliferation. In the human body, hyaluronan is found mainly in connective tissues, such as the vitreous, the umbilical cord, joint fluid, etc. [1]. Hyaluronan is, therefore, an attractive building block for preparing new biocompatible and biodegradable materials that could find applications in drug delivery, tissue engineering, viscosupplementation or cosmetics [2–5].

In an aqueous solution, the carboxyl group on hyaluronan is in a dissociated form giving, theoretically, each repeating unit a negative charge, consequently, hyaluronan can be generally described as a negatively charged polyelectrolyte. Interactions between polyelectrolytes and an oppositely charged surfactant are an area of vital scientific research. This is due to the theoretical significance of the understanding electrostatic and other (e.g., hydrophobic or excluded volume)

interactions between charged polymers and low molecular counterparts and also due to practical applications of polyelectrolyte-surfactant colloidal systems [6–9]. For instance, when prepared from biocompatible polymers, they can find applications in pharmaceutical and medical industries. They can stabilize encapsulated proteins or may be prepared as materials responsive to external stimuli [10]. Other application fields include detergency, the modification of rheological properties, or paints [11]. Also, their surface (interfacial) effects are important [12].

Hyaluronan-surfactant interactions were the subject of several previous studies. Perhaps the most detailed study was published in a series of papers by Swedish researchers [13–16] who investigated the phase behavior of systems containing water, hyaluronan, alkyl trimethylammonium bromides (a tetradecyl derivative was the most studied type) and salt (mostly NaBr). The binding of the surfactant to the hyaluronan was detected for surfactants with an alkyl chain consisting of at least ten carbon atoms. A ternary phase diagram of tetradecyltrimethylammonium bromide (TTAB)-hyaluronan (300 or 240 kDa)-water was constructed in the ref. [14]. It consisted of a droplet-shaped two-phase region emerging from the water apex of the triangular diagram and entirely enclosed by a one-phase area. The diagram shape demonstrated marked dissymmetry – for example, concentrated surfactant solutions almost immediately phase-separated upon the addition of hyaluronan in contrast to systems with high hyaluronan concentrations in which a rather large amount of surfactant could be added without phase separation. The effect of the surfactant alkyl chain length and the hyaluronan molecular weight was

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investigated in the ref. [17]. Hyaluronans of molecular weights of 250, 90, 60, and 23 kDa (weight average) were used. A decrease in the molecular weight resulted in a slight shift in position of the two-phase region while its size remained almost unaffected. This size was much more sensitive to the alkyl chain length of the surfactant – the longer the chain, the larger the size. Due to the prevailing electrostatic nature of the hyaluronan-cationic surfactant interactions, the phase behavior is affected by the presence of salt also. The effect of sodium bromide on the TTAB-hyaluronan system was described in the paper by Thalberg et al. [18]. The addition of low concentrations of NaBr reduced the size of the two-phase region of the phase diagram and this region disappeared at 250 mM NaBr. Another type of phase separation was observed with high NaBr concentrations (at and above 500 mM). Generally, oppositely charged polyelectrolyte-surfactant systems are characterized by association without added electrolyte, miscibility at intermediate electrolyte concentration and segregative phase separation at high ionic strength [7].

Fukada et al. [19] investigated the interaction between hyaluronan and decyltrimethylammonium bromide by rheological methods. Also, they presented a part of the phase diagram in a hyaluronan concentration region below 10 g/L and a surfactant concentration below 1 mol/L. The phase separation region was indicated when the surfactant concentration was between 0.03 and 0.35 mol/L and the hyaluronan concentration was above ca 0.05 g/L. Eventually, the authors focused their study on the isotropic region with a large surfactant excess.

In the referred experiments, concentrated solutions of hyaluronan and surfactants were usually investigated. Our preliminary fluorescence probe study of similar systems prepared in water did not reveal dependencies of fluorescence indices on the surfactant concentration, which are typical for the formation of micellar aggregates on polyelectrolyte chains, prior to the phase separation. In other words, the fluorescence data did not indicate the formation of hyaluronan-micelle aggregates in isotropic, clear solutions, which would occur well below the critical micellar concentration of a pure surfactant. From the previous studies, only ref. [20] reports on the solubilization of the fluorescence probe (pyrene), but in a single system of a high surfactant excess. Actually, our work in this area was initiated by an attempt of a hyaluronan producer to get hyaluronan-decorated micelles and test their applicability as delivery systems for hydrophobic substances. Therefore, we focused on interactions in a dilute (with respect to hyaluronan), and clear solutions that have not been directly investigated thus far. Some diluted solutions were mentioned by Fukada et al. [19] but they eventually studied rheological properties of concentrated systems only. Our main purpose was not to prepare delivery systems, but to investigate the diluted region for hyaluronan-surfactant interactions and for stability properties of the hyaluronan-micelle aggregates. In this study, we revealed that the phase behavior in diluted systems is not as simple as could be expected from previous studies.

2. Materials and method

2.1. Materials

Hyaluronan (a sodium salt of hyaluronic acid; HyA) of several chain lengths were purchased from Contipro, Ltd., Czech Republic. Here they are denoted as low (LMW, 75–100 kDa), medium (MMW, 650–750 kDa) and high (HMW, 1400–2000 kDa) molecular weight hyaluronan (the values in parentheses give the range of the weight of the averaged molecular weights of the used batches, determined by the producer using the SEC-MALLS technique). Cationic surfactant cetyltrimethylammonium bromide (CTAB) was purchased from Sigma-Aldrich, Czech Republic and carbethopendecinium bromide (Septonex) was purchased from GBNchem, Czech Republic. The surfactants were of the best available quality and used as received without further purification.

Stock solutions of hyaluronan and surfactants were prepared in deionized water, in a phosphate buffer solution or in a model physiological solution (0.15 M NaCl).

The fluorescence probe pyrene (Fluka, purity $\geq 99.0\%$; Sigma Aldrich, Czech Republic) was used for the fluorescence measurements. The stock solution of the fluorescence probe was prepared in a volatile solvent (acetone). An appropriate amount of the stock solution was added to a glass vial and acetone was evaporated under reduced pressure to obtain the final concentration of pyrene in the samples equal to 10^{-7} M. Subsequently, samples of the required composition and solvent were prepared in these vials by mixing corresponding volumes of hyaluronan and surfactant solutions. All samples were stirred for 24 h, at least, at laboratory temperature before the acquisition of their fluorescence spectra. In this way, series of samples were prepared with constant hyaluronan and increasing surfactant concentrations.

Samples with varying surfactant concentration and no hyaluronan were prepared by simple dilution of the surfactant stock solution of the concentration about ten times higher than the critical micelle concentration.

2.2. Methods

The aggregation and solubilization properties were studied by means of pyrene as the fluorescence probe. The fluorescence emission spectra were recorded on a FLUOROLOG (Horiba Scientific, France) fluorescence spectrometer. For the emission spectra, recording the excitation wavelength was set at 336 nm and the emission scan was acquired in the range from 360 to 530 nm. In the excitation measurements, the emission wavelength was set at 392 nm and the excitation scan was acquired in the range from 310 to 340 nm.

Pyrene experiments were evaluated by plotting the fluorescence intensity ratio of the first and third vibronic peaks from the emission scan at 373 and 383 nm, respectively (EmPI – the pyrene emission polarity index), against the surfactant concentration. The pyrene polarity index is the reflection of the polarity in the vicinity of the pyrene environment and it is used to detect the localization of pyrene in the system. A typical sigmoidal curve was obtained indicating the formation of nonpolar domains (micelles with hydrophobic cores) solubilizing pyrene molecules. Ideally, a sharp decrease would be observed at the critical aggregation or micellar concentration. Curves were fitted by the Boltzmann model and the concentration at the inflex point can be considered as the critical micelle (or critical aggregation) concentration.

The Boltzmann model equation is given by:

$$y = \frac{A_1 - A_2}{1 + e^{\frac{x-x_0}{\Delta x}}} + A_2,$$

where the variable y corresponds to the EmPI value, the independent variable (x) is the total concentration of the surfactant, A_1 and A_2 are the upper and lower limits of the sigmoid, respectively, x_0 is the center of the sigmoid, and Δx is directly related to the independent variable range, where the abrupt change of the dependent variable occurs.

Thus, from the Boltzmann equation, the width of the concentration interval in which the sharp intensity decrease occurred can be obtained. This interval spreads from the onset up to the offset of the aggregates (micelles) formation, i.e., to the concentration range in which aggregates capable of pyrene solubilization are progressively formed.

The pyrene experiments were also evaluated by the ratio of fluorescence intensities at 470 nm, which corresponds to the excimer emission maximum, and the first vibronic peak (at 373 nm). This ratio is referred to as the Ex:Mo. It is an indicator of the probability of the excimer formation in the system and reflects the accumulation of pyrene molecules in hydrophobic domains.

In addition, the pyrene experiments were evaluated by the ratio of fluorescence intensities at 333 and 338 nm of the excitation spectra also. This ratio is called the pyrene excitation polarity index (ExPI) and

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