HYDROPHOBIC NANODOMAINS IN HYALURONAN-SURFACTANT SYSTEM: A FLUORESCENCE ANISOTROPY STUDY

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Abstract

This paper deals with the fluorescence anisotropy study of hydrophobic nanodomains formed by interaction between hyaluronan (Hya) and cationic surfactant cetyltrimethylammonium bromide (CTAB) below critical micelle concentration in aqueous solution and the effect of sodium chloride addition to this system. Hyaluronan as negatively charged polyelectrolyte can interact with cationic surfactants via electrostatic interactions to form aggregates, which could be potential used for targeted drug delivery. Two fluorescent probes, which are solubilized in hydrophobic region, were used - perylene and diphenylhexatrien (DPH). The emission spectra, total intensity of fluorescence and steady-state anisotropy of fluorescence in the samples were measured. It was observed that initial addition of CTAB to hyaluronan aqueous solution leads to forming some small hydrophobic domains linked to hyaluronan chains. Then an increasing concentration of CTAB causes phase separation and formation of a condensed phase. Addition of sodium chloride to the samples leads to reorganization of this system - the condensed phase is dissolved and probably free micelles (possibly mixture of micelle linked to hyaluronan chain with free micelle) are formed in the solution.

Keywords: Hyaluronan, surfactants, fluorescence spectroscopy, anisotropy

1. INTRODUCTION

Hyaluronan is a naturally occurring linear polysaccharide which is composed of two sub-units: D-glucuronate and N-acetyl-D-glucosamine linked by β(1-3) and β(1-4) bonds to unbranched chain [1]. This substance is common in connective tissues of vertebrates [2]. As a component of extracellular matrix, hyaluronan plays an important role in migration and proliferation of cells and immune response of organism, facilitates wound healing and acts as a lubricant and shock absorber in the synovial fluid. Its specific biological activity is influenced by its molecular weight, length and circumstances of its synthesis. Hyaluronan was found to be implicated in tumor cell behavior [3]. A high content of intracellular hyaluronan and its accumulation in the extracellular matrix was shown to be able to create a microenvironment appropriate for migration, proliferation and invasiveness of malignant cells [4, 5]. This fact contributes to the idea of using hyaluronan as a part of targeted delivery agent. Unfortunately, hydrophilic hyaluronan with massive hydration shell is not able to carry nonpolar substances. Because many molecules of drugs are hydrophobic, hyaluronan has been chemically modified to induce micelle-like properties or directly conjugated with hydrophobic drugs. But this process could change its biological activity and compatibility. Next way could be the physical interaction with suitable substance which allows solubilisation of hydrophobic molecules in presence of hyaluronan. For example, interactions between hyaluronan and surfactant may lead to formation aggregates in which the surfactant hydrophobic nanodomains can solubilize hydrophobic substances and hyaluronan has a role biocompatible carrier and targeted agent.

The study of interaction between hyaluronan and surfactant were subject of several previous studies. Due to negative hyaluronan charge almost all of them used cationic surfactants. Phase behaviour of systems containing alkyltrimethylammonium bromides (8-16 carbons), hyaluronan, water and salt (mostly NaBr) were investigated by Thalberg et al [6-10]. The NMR, methods of phase separation, conductivity, solubilisation of dye was used. Thalberg found that there is a certain cationic surfactant concentration above it marked

formation of hyaluronan-surfactant complexes can be observed. Increasing salt concentration prefers free micelles in comparison than formation of hyaluronan-linked micelles. Fluorescence probes (pyrene, nile red) and tensiometry were used for determination of critical aggregation concentration of hyaluronan-surfactants system in physiological solution [11]. Yin et al. used pyrene fluorescence to investigate interactions of high molecular weight hyaluronan with anionic sodium dodecyl sulphate, nonionic Cremophor EL and Tween 80 in water [12].

In this paper, we report the results of fluorescence study of aggregates formed by interaction between hyaluronan (Hya) and cationic surfactant cetyltrimethylammonium bromide (CTAB) below its critical micelle concentration. We used two hydrophobic probes, which simulated behaviour of hydrophobic drugs. These aggregates were studied by fluorescence intensity and fluorescence anisotropy measurement which can be used for determining microviscosity and structural changes of surrounding of fluorescent probes.

2. MAIN TEXT

2.1. Materials, preparation of samples

Hyaluronan (300 kDa) was purchased from Contipro Biotech s. r. o., Czech Republic. Cationic surfactant cetyltrimethylammonium bromide of the best available purity was purchased from Sigma-Aldrich and used as received. Fluorescence probes, perylene (Fluka) and DPH (Fluka) were also used without further purification.

Hyaluronan (1 g / l) and CTAB (2mmol / l) stock solutions were prepared by slow addition of powdered substances into water under stirring, followed by 24 h stirring in closed vessel to ensure complete dissolution. Stock solutions of fluorescent probes were prepared in volatile solvent - acetone (10^{-4} mol / l). Further saturated solution of sodium chloride was prepared.

Two series of samples were prepared - with hyaluronan concentration 0.01 g / l and 0.02 g / l, respectively. In the series, concentration of CTAB increased from 0.01 to 0.2 mmol / l. At first, stock solution of fluorescent probe was added to glass vials to obtain final concentration of perylen or DPH in samples equal to 10^{-6} mol / l and acetone was evaporated under reduce pressure. Then the stock solutions of hyaluronan and CTAB were added to vials according to the required quantity. The samples were supplemented with mili-Q water to a total volume of 5 ml. Then they were left on a shaker for 12 hours at room temperature. Individual samples were blended three times. The emission spectra and steady-state anisotropy of the samples were measured. Fluorescence measurements were followed by addition of NaCl solution to vials to obtain sodium chloride concentration equal to 0.15 mol / l. The samples were left on shaker for next 24 hours and then fluorescence spectra and anisotropy were measured again.

2.2. Methods and instrumentation

All fluorescence measurements were recorded on Aminco BOWMAN Series 2 fluorescence spectrometer. Temperature of the sample chamber was set on 25 °C. In the case of perylene the excitation wavelength was 410 nm, emission scan was acquired in the range from 420 to 520 nm. Anisotropy and total fluorescence intensity values were obtain at emission wavelength 442 nm. DPH was excited at 385 nm and emission spectra were recorded from 395 to 495 nm. Anisotropy and total intensity values were measured at emission wavelength 441 nm.

The value of fluorescence anisotropy was determined as

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}}, \qquad (1)$$

where the subscripts of the fluorescence intensity values (I) refer to horizontal (VH) and vertical (VV) orientation of polarizers. The G factor (instrumental correcting factor) was automatically determined by the software

supplied by the manufacturer. The integration time was chosen to 8 s. The anisotropy measurement on the one sample was carried out five times and the result was average value of anisotropy.

Total fluorescence intensity of the sample was evaluated according to equation

$$I_T = I_{VV} + 2GI_{VH}. \quad (2)$$

2.3. Results and discussions

Interactions in aqueous solution

Because perylene and DPH are both very hydrophobic probes, increase in total fluorescence intensity indicates formation of hydrophobic domains, in which hydrophobic molecules can be solubilized. Acquired fluorescence intensity data shows a very similar trend for both probes. Differences between obtained anisotropy data is caused by different shape and size of molecules of DPH and perylene and their dissimilar rotational dynamics.

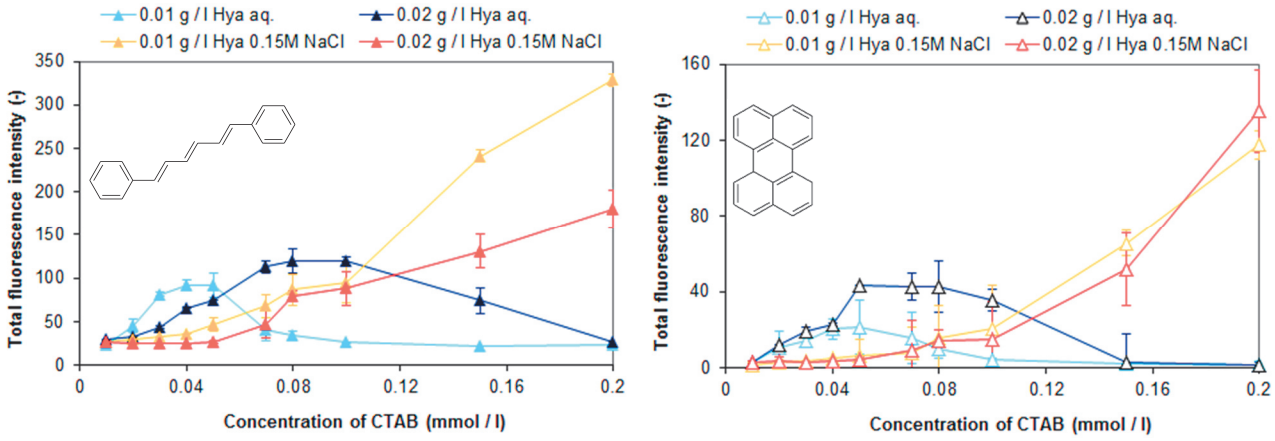

Figure 1 Total fluorescence intensity of DPH (left) and perylene (right) as a function of CTAB concentration in the samples

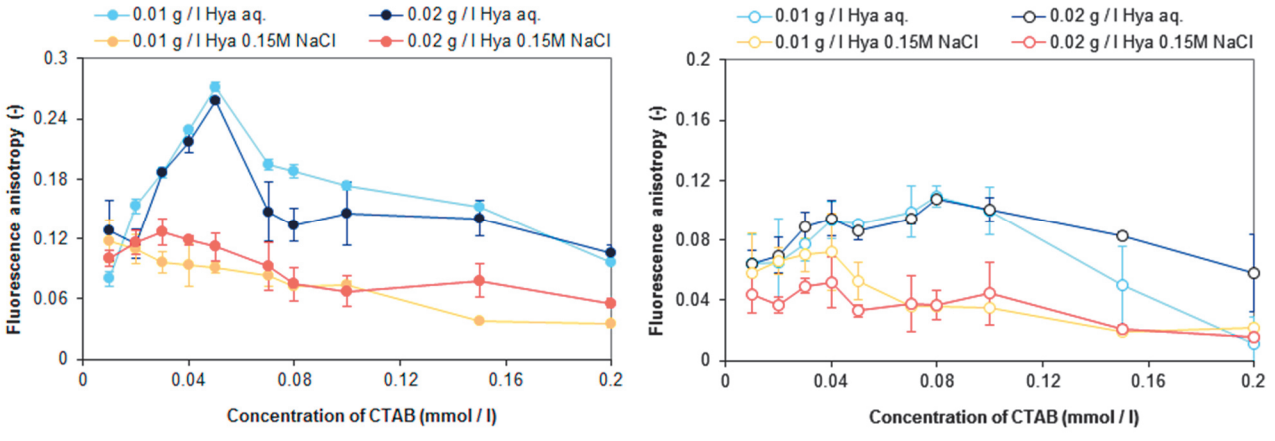

Figure 2 Steady-state fluorescence anisotropy of DPH (left) and perylene (right) as a function of CTAB concentration in the samples

Figure 1 shows the results obtained for the total fluorescence intensity of DPH and perylene as a function of the concentration of CTAB in the system. As can be seen, the initial addition of CTAB to hyaluronan aqueous solution leads to forming some small hydrophobic domains immediately. In series with higher content of Hya,

the aggregates are formed in larger scale of CTAB concentration. The reduction of fluorescence intensity is caused by phase separation and formation of condensed phase on the wall of the vials. Saturation of critical number of binding sites on hyaluronan chain by surfactant molecules is probably origin of this condensation. The condensed phase exhibits fluorescence in UV chamber, so it contains a large amount of hydrophobic domains (**Figure 3**). This phenomenon clearly shows to present interaction between hyaluronan and CTAB. Because condensed phase wasn't poured to cuvette together with liquid portion of samples, the fluorescence in these cases is lower than samples with a lower concentration of CTAB.

Initial expand in steady-state fluorescence anisotropy of DPH indicates the beginning of aggregation and rises organization of the system. Perylene has lower values of anisotropy due to its smaller size. Where molecules of DPH stuck, molecules of perylene are allowed to rotate. In comparison to samples with NaCl, anisotropy values are higher in aqueous solution for both probes. Thus surroundings around perylene and DPH are more viscous which signalized smaller size of domains and formation of small hydrophobic domains linked to hyaluronan chains.

Figure 3 The condensed phase on the wall of the vial. Left - natural light, right - UV

Interactions in 0.15M NaCl

Addition of sodium chloride (adequate to concentration of physiological solution) to samples causes dissolution of condensed phase, which signalized reorganization of this system and reduction of interaction between Hya and CTAB. Fluorescence intensity of both fluorescent probes in the samples with sodium chloride constantly increases which indicates the formation more or larger hydrophobic domains. Simultaneously values of fluorescence anisotropy are lower than samples with aqueous solution. Therefore we can deduce formation of free micelles in the solution.

3. CONCLUSION

One of the essential properties of drug delivery carrier is the ability to incorporate molecules of hydrophobic substances; therefore the presence of hydrophobic domains is important. For this reason the interactions between hyaluronan and CTAB were studied through the analysis of the photophysical response of two hydrophobic fluorescent probes. It was observed that the initial addition of CTAB to hyaluronan aqueous solution leads to forming some small hydrophobic domains linked to hyaluronan chains. Then an increasing concentration of CTAB causes phase separation and formation of a condensed phase with large amount of hydrophobic domains.

Addition of sodium chloride to the samples leads to reorganization of this system and reduction of electrostatic interactions between Hya and CTAB. The condensed phase is dissolved and probably free micelles, possibly mixture of micelle linked to hyaluronan chain with free micelle, are formed in the solution. This reorganization is not desirable for use Hya-CTAB aggregates like a targeted drug carrier.

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