TURBIDIMETRY AND FLUORESCENCE STUDY OF HYALURONAN-SURFACTANT NANOPARTICLES FORMATION

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Abstract

This paper deals with electrostatic association of cationic micelles (CTAB or Septonex) with hyaluronan. Hyaluronan as negatively charged polyelectrolyte can interact with positively charged surfactant micelles via electrostatic interactions to form nano-core-shell like aggregates. These self-assembly nanoparticles can solubilize hydrophobic active substances and therefore, they are potential carriers in drug delivery applications. The cationic micelle/hyaluronan complexes were studied using turbidimetry and fluorescence spectroscopy method. Turbidimetric titration was chosen as an indicator of component aggregation which is related to the loss of transmitted light intensity due to the scattering effect. Fluorescent probe pyrene was selected for spectroscopy experiments because of its unique sensitivity to polarity of the medium, so this fluorescence method was used as an indicator of presence of hydrophobic domains in the system. Effect of components concentration and molecular weight of hyaluronan were evaluated. Results of turbidimetry revealed that aggregates formation (turbidity increasing) depends especially on a charge ratio of surfactant molecules and hyaluronan carboxyl groups. It was found a difference in association of low molecular weight and high molecular weight of hyaluronan. Fluorescence results confirmed presence and stability of micellar aggregates in studied systems.

Keywords: Hyaluronan, surfactant, nanoparticles, turbidimetry, fluorescence, pyrene

1. INTRODUCTION

Hyaluronan is a naturally occurring linear polysaccharide found in the extracellular matrix, especially of soft connective tissues. It is built from repeating disaccharide units, each of which possesses one carboxylate group and is therefore a polyelectrolyte bearing a negative charge. Despite the simple primary structure, hyaluronan has substantial size heterogenity in different tissues. Hyaluronan molecules have very diverse biological effects depending on the molecule size and spatial arrangement. Extensive studies on the chemical and physicochemical properties of hyaluronan and its physiological role in humans, together with its versatile properties, such as its biocompatibility, nonimmunogenicity, biodegradability, and viscoelasticity, have proved that it is an ideal biomaterial for cosmetic, medical and pharmaceutical applications [1].

Due to high hydrophilicity of hyaluronan, application in combination with hydrophobic substances, i.e. drugs is not possible. Complexes formed by hyaluronan and surfactants may combine hydrophilic and hydrophobic domains and be used as transport systems for hydrophobic substances [2]. Hyaluronan-surfactant interactions and physicochemical properties of this system were studied in several previous papers [3-7]. One possibility to prepare hyaluronan-micellar colloid aggregates is hyaluronan binding to preformed micelles. In this case, “concentrated” hyaluronan solution is gradually added to surfactant micellar solution. The aim of our work was to prepare hyaluronan-surfactant nanoparticles and study properties and formation process of these nanoparticles using turbidity measurements, fluorescence spectroscopy and dynamic light scattering method.

2. MATERIALS AND METHODS

Hyaluronan (as sodium salt of hyaluronic acid; HyA) at molecular weights of 15, 100 and 1500 kDa was purchased from CPN, Ltd., Czech Republic. Cationic surfactants CTAB (Cetyltrimethylammonium bromide) and Septonex (Carbethopendeciniumbromide) of the best available purity was purchased from Sigma Aldrich and GBNchem, respectively. Stock solutions of hyaluronan and surfactants were prepared in aqueous solution. All stock solutions were prepared by slow dissolution of powdered substances upon stirring and left stirred for 24 hours to ensure complete dissolution.

Turbidimetric titrations were carried out by adding hyaluronan solution at the concentration 0.3 g / L to the surfactant solutions at concentration 3 mmol / L. Turbidity measurements, reported as absorbance A, were performed at 400 nm using Varian Cary 50 spectrometer equipped with a 1 cm path length fiber optics probe at 24 ± 1 °C.

Aggregates form and aggregation behaviour of components were investigated using fluorescence spectroscopy and dynamic light scattering methods. Fluorescent probe pyrene was selected for spectroscopy experiments because of its unique sensitivity to polarity of the medium. Fluorescence emission spectra were recorded on fluorimeter AMINCO Bowman Series. Excitation wavelength of pyrene was 336 nm. Intensity ratio of first and third vibronic peaks (EmPI - the pyrene polarity index or the ratio of fluorescence intensities at 373 and 383 nm) is a reflection of the micropolarity in the vicinity of pyrene and it is used to detect the localization of pyrene in the system. EmPI for polar environment is in the range of 1.25 - 2.0 and indicate pyrene in aqueous solution. When pyrene is in the micellar solvent, EmPI is about 1.0 - 1.5. This value indicates that pyrene molecule is inside micelle, in palisade layer of micelle. EmPI for absolutely nonpolar solvent is about 0.5 - 0.6 (for example hydrocarbon solvent or micelle inner core) [8, 9]. Particle size distributions of aggregates were obtained by dynamic light scattering measurements using Zetasizer Nano ZS (Malvern Instruments). All measurements were performed at laboratory temperature.

3. RESULTS AND DISCUSSION

3.1. Turbidimetry

First, aggregation process of cationic micelles and hyaluronan was studied using turbidimetric titration. Turbidimetric titration was chosen as an indicator of the loss of intensity of transmitted light due to the scattering effect of particles associated from hyaluronan and surfactant in it. During the titration experiments components ratio was changed and a process of component interactions was studied. Turbidimetry results are displayed as the dependency of absorbance on the surfactant/hyaluronan ratio (T/D). The T/D ratio represents moles of surfactant per moles of binding sites on hyaluronan chain (carboxylic groups of hyaluronan).

In **Figure 1** are shown absorbance values depending on T/D ratio of components in the system. We can see

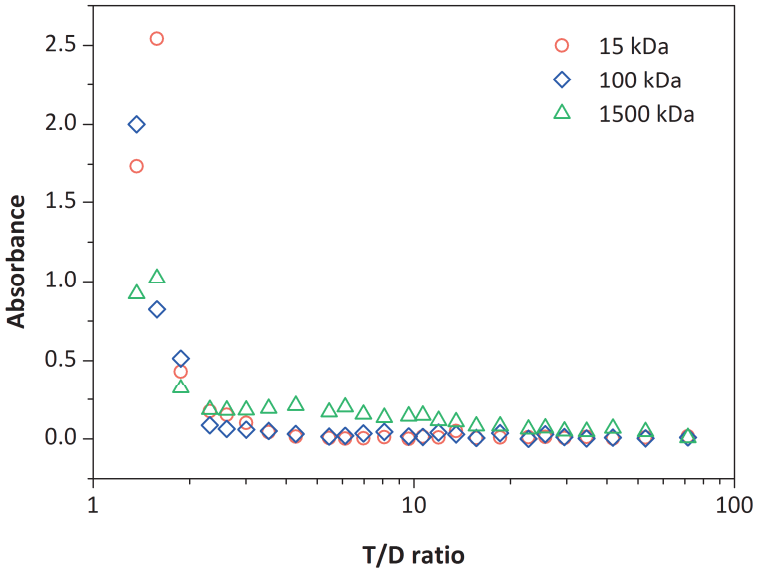

Figure 1 Turbidimetric titration graph for adding hyaluronan 15, 100 and 1500 kDa (0.3 g / L) to CTAB at initial concentration 3 mmol / L

that hyaluronan molecular weight affects aggregates formation (turbidity increasing) slightly. In the case of hyaluronan molecular weight 1500 kDa, absorbance increases gradually from start of titration (T/D = 70). Opalescence and slight turbidity of sample were noticed. A steep increase of absorbance occurs at T/D = 2.3 indicates intense aggregation for all studied molecular weights of hyaluronan. Phase separation (precipitation) was observed in the system after this T/D ratio also for 15 and 100 kDa. Acquired absorbance data shows a very similar trend for both surfactants (CTAB and Septonex).

3.2. Fluorescence spectroscopy

Presence of hydrophobic micellar domains during titration was study by measurement emission spectra of hydrophobic pyrene. EmPI values are very good parameter for evaluation of pyrene localization in the system. However, it is necessary to complete the results with other techniques for compact view of the system.

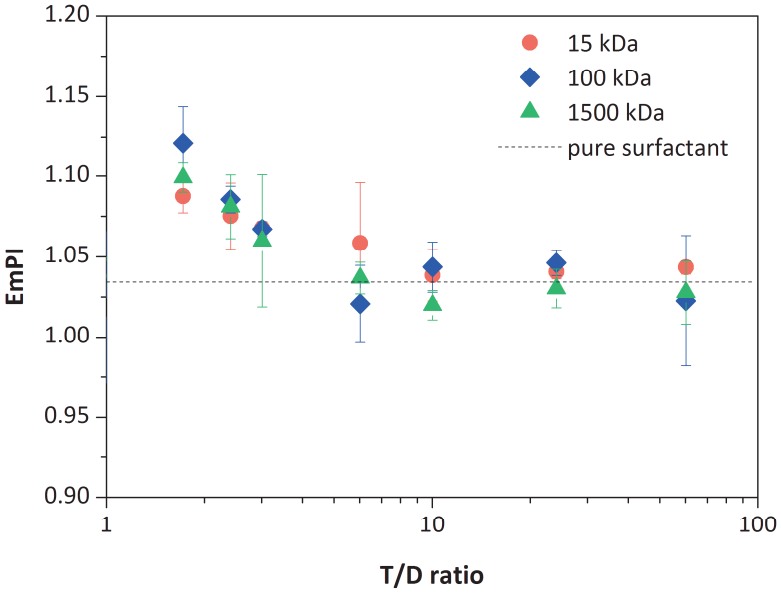

Figure 2 EmPI depending on charge ratio of CTAB and hyaluronan carboxyl groups hyaluronan molecular weights 15, 100 and 1500 kDa

EmPI values (1.0 - 1.2) in **Figure 2** indicate presence of micellar aggregates in the system during the whole of titration experiments. On the other hand, it is impossible to determine exact appearance of aggregates. Slight increase of EmPI values is caused by rearrangement of aggregates relating to reorganization of fluorescent probe in the system. Opalescence and turbidity of the sample were observed with decreasing T/D ratio under limit T/D = 6. A degree of turbidity depends especially on molecular weight of hyaluronan. Turbidity and phase separation of the system increase with increasing hyaluronan molecular weight. In **Figure 3** are shown samples containing hyaluronan 1500 kDa with all degree of turbidity and phase separation.

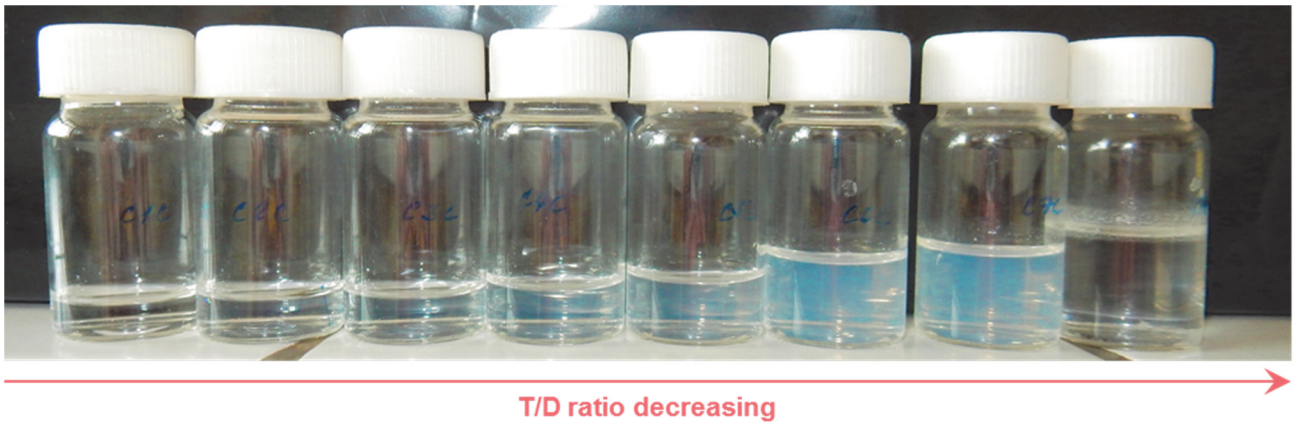

Figure 3 Samples prepared for fluorescence/DLS measurements based on titration of hyaluronan 0.3 g / L 1500 kDa to CTAB 3 mmol / L

Increase of EmPI values indicates movement of pyrene molecules into more polar environment due to disintegration of aggregates leading to release of the probe molecules into bulk solvent or creation of opener structure of micellar aggregates to polar environment.

It is important to note that pyrene concentration in the system decrease with decreasing T/D ratio from 3.3×10^{-6} to 1.0×10^{-6} mol / L through a similar method of the samples preparation as in the case of turbidimetric titration. This can also influence localization of pyrene in the system although only slightly.

3.3. Dynamic light scattering

Using DLS method it was found that molecular weight of hyaluronan has important effect on polydispersity of the system. Polydispersity decreased from 15 kDa to 1500 kDa High polydispersity index of sample (greater than 0.7) indicates a very broad size distribution unsuitable for dynamic light scattering technique. In the case of hyaluronan molecular weight 15 kDa, samples were very polydisperse until T/D ratio = 3. After this limit, aggregates in the system were bigger and almost monodisperse. T/D ratio limit for molecular weight 100 kDa was 6 and in the case of 1500 kDa, all of samples containing hyaluronan were monodisperse and suitable for dynamic light scattering measurements.

Effect of hyaluronan molecular weight on aggregates sizes is not significant. Monodisperse systems with T/D = 3 - 1.8 contain aggregates of sizes in the range 100 - 200 nm, while samples with T/D = 1.7 contain bigger aggregates (200 - 600 nm).

4. CONCLUSIONS

This study deals with interactions between hyaluronan and surfactant leading to formation of their nanoparticles that may be used as transport systems for hydrophobic substances. Turbidimetric titration provides information about hyaluronan-surfactant interactions and aggregates formation. Pyrene fluorescence spectroscopy is high sensitive method for determination of fluorescent probe pyrene localization in the system relating to characterization of aggregates form.

Results of turbidimetry revealed that aggregates formation (turbidity increasing) depends especially on a charge ratio of surfactant molecules and hyaluronan carboxyl groups. It was found a difference in association of low molecular weight and high molecular weight of hyaluronan. Fluorescence results confirmed presence and stability of micellar aggregates in studied systems. Dynamic light scattering measurements provided information about aggregates sizes and polydispersity of the system in dependence on molecular weight of hyaluronan.

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