FLUORESCENCE STUDY OF MICROCVISCOSITY OF HYALURONAN HYDROGELS CONTAINING HYDROPHOBIC NANODOMAINS DURING FOURTEEN DAYS OF AGEING

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Abstract

This paper deals with the fluorescence study of microviscosity changes of hydrogel composed by hyaluronan and cationic surfactant cetyltrimethylammonium bromide (CTAB) during fortnight-ageing. The steady state anisotropy values obtained in the experiments were used to estimate the microviscosity of the corresponding hydrogel regions. Two different fluorescent probes were used, perylene for hydrophobic domains and rhodamine 6G for hydrophilic region. It is observed that the microviscosity in the hydrophobic domains of hydrogel is roughly constant, indicating that these domains do not undergo significant structural changes during ageing. However, the microviscosity of hydrophilic parts gradually decreases, which indicates structural changes in the nearby area of rhodamine molecules.

Keywords: fluorescence anisotropy, microviscosity, hyaluronan, CTAB, hydrogels

1. INTRODUCTION

The hydrogel is three-dimensional cross-linked networks of any water soluble polymer, which comprises a wide range of chemical compositions and bulk physical properties [1]. Their structure is able to absorb large amount of water which makes them permeable to oxygen, nutrients and other water-soluble metabolites [2]. Hydrogels can be formulated in a variety of physical forms like nanoparticles or films, therefore hydrogels are commonly used in experimental medicine and clinical practise. For example, hydrogels can be used in regenerative medicine [3], for tissue engineering [4-5] or like a drug or cancer carrier [6].

The hydrogels could degrade or eventually disintegrate and dissolve or they may be chemically stable. They are called ‘reversible’, or ‘physical’ gels when theirs networks are held together by molecular entanglements, or secondary forces like ionic and hydrophobic forces or H-bonding [7-8].

We prepared the hydrogel which was composed of hyaluronan and cationic surfactant cetyltrimethylammonium bromide, wherein the molecules of surfactant provide physical crosslinking of hyaluronan chains and create the hydrophobic nanodomains, in which the hydrophobic drugs can be solubilized. Hyaluronan is a naturally occurring linear polysaccharide that is composed of two subunits, D-glucuronate and N-acetyl-D-glucosamine linked by β(1–3) and β(1–4) bonds which are connected to unbranched chain [9]. This substance is commonly in the connective tissues of vertebrates from an umbilical cord, vitreous to the synovial fluid in the joints [10]. As a component of the extracellular matrix, hyaluronan plays an important role in the migration and proliferation of cells and the immune response of the 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 in which it is synthesized [11].

In this paper, we report the results of study of fluorescence anisotropy of perylene and rhodamine 6G solubilized in different regions of this hydrogel. Experimental data are used to estimate microviscosity around the probes during fourteen days of ageing.

2. MAIN TEXT

2.1 Materials, preparation of samples

Hyaluronan of high molecular weight (750–1000 kDa) was purchased from Contipro Biotech s. r. o., Czech Republic. Cationic surfactant cetyltrimethylammonium bromide (CTAB) of the best available purity was purchased from Sigma-Aldrich and used as received. Fluorescence probes, perylene and rhodamine 6G were also used without further purification. Stock solution of hyaluronan in 0.15 M NaCl was prepared in concentration 5.0 $g \cdot l^{-1}$ by slowly adding solid hyaluronan into solution of sodium chloride under stirring, followed by 24 h stirring in closed vessel to ensure complete dissolution. Stock solution of CTAB in 0.15 M NaCl was prepared in concentration 200 $mmol \cdot l^{-1}$. Stock solutions of fluorescence probes were prepared in a volatile solvent - perylene was dissolved in acetone and rhodamine 6G was blended in ethanol. Water used to prepare all solutions was doubly destilled (Millipore).

Gelation was carried out in glass tubes, at first, the stock solution of perylene/rhodamine was added to the tubes and then the acetone/ethanol was evaporated. The final concentration of this probe in samples was 1×10^{-6} $mol \cdot l^{-1}$. Then a certain amount of solutions of CTAB were added to these tubes and mixed on vortex mixer. Then the samples were left on a shaker for 24 hours at room temperature. Next day, the same amount of solution of hyaluronan as solution of CTAB was added to tubes, samples were again mixed on a vortex mixer and left for a further 24 hours on a shaker at room temperature. Individual samples were blended three times. The fluorescence spectra, lifetime and steady-state anisotropy of fluorescence were measured after 1, 7 and 14 days after preparation of these samples, wherein they were stored complete with the supernatant at room temperature in the dark.

2.2 Instrumentation

The steady-state measurements of anisotropy were carried out on a luminescence spectrophotometer Fluorolog (HORIBA Jobin Yvon) with 150-W xenon lamp as the excitation. Measurements were realized in L-format instrumental configuration and an automatic interchangeable wheel with Glan-Thompson polarizers.

The value of fluorescence anisotropy was determined as

$$r = \frac{I_V - GI_H}{I_V + GI_H}, \tag{1}$$

where the subscripts of the fluorescence intensity values (I) refer to horizontal (H) and vertical (V) 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 2 s. The anisotropy measurement on the one sample was carried out five times and the result was average value of anisotropy. Individual samples were blended three times, so all anisotropy values of the probes in the hydrogels presented in this work are the mean values of evaluated anisotropy values for individual samples. For samples with perylene, the excitation wavelength was set to 389 nm and emission was elected to 475 nm, for samples containing rhodamine, the excitation wavelength was set to 455 nm and emission was set to 560 nm. Temperature of the sample chamber was set on 25 °C. The gels were applied between the two quartz glasses, which has been placed diagonally into a standard cuvette.

The lifetime was measured on TCSPC lifetime spectrofluorometer Fluorocube from HORIBA Jobin Yvon. The samples containing perylene were excited by the NanoLED diode 389 nm (pulse width 1.2 ns) and the emission was observed at 475 nm. The samples with rhodamine 6G were excited by the NanoLED diode 455 nm with pulse width 1.2 ns and the emission monochromator was set on 560 nm. The lifetime was measured at orientation of polarizers in magic angle and peak preset was set to 10 000 counts. Temperature of the sample chamber was set on 25 °C. The gels were applied to a special cuvette for solid samples.

Average lifetime was calculated according to equation

$$\tau = \sum_{i=1}^{n} \alpha_i \tau_i \quad (2)$$

where α_i is pre-exponential factor (0–1) and τ_i is corresponding lifetime.

2.3 Methods

The microviscosity of environment was evaluated by determining the equivalent viscosity, i.e. viscosity of homogeneous environment from which the fluorescent probe is given the same response as in the measurement of the microenvironment. For this purpose the calibration curves were measured - the dependence of the fluorescence anisotropy to the viscosity of homogeneous environment. This homogenous environment was consisted of a mixture of glycerol and water. The values of viscosity of this mixture were determined from article [12].

Following equation, which was obtained by fitting the calibration data with a linear curve, were used to calculate microviscosity of different parts of hyaluronan gels.

Hydrophobic domains (perylene): $y = 0.000578\,x + 0.017584$

Hydrophilic parts (rhodamine): $y = 0.001433\,x + 0.103345$

2.4 Results and discussion

Hydrophobic nanodomains during fortnight-ageing

Important property of the carrier system is the ability to incorporate hydrophobic molecules of drugs. Therefore, it is important the presence of hydrophobic domains in its structure. In the studied gel composed by hyaluronan and CTAB hydrophobic domains are formed aggregates of entrapped molecules of surfactant, which also serves as a physical crosslinking hyaluronan chains.

In hydrogels, the perylene has two lifetimes – short-time fraction (2%) has lifetime $\tau_1 = (3.38 \pm 0.15)$ ns and long-time fraction (98%), which correspond to molecules of perylene incorporated to hydrophobic environment, has lifetime $\tau_2 = (5.66 \pm 0.01)$ ns. The average lifetime has been calculated to $\tau = (5.61 \pm 0.02)$ ns. The value of these lifetimes during the fortnight-ageing gel has not changed.

The following table 1 shows the obtained value of steady-state anisotropy of fluorescence of perylene and calculated values of microviscosity. As we can see, the values are not changed during ageing, which indicates that hydrophobic domains do not undergo significant structural changes during this time.

Table 1 Steady-state anisotropy of fluorescence of perylene, incorporated in hydrogel, during fortnight-ageing and calculated values of microviscosity of perylene surroundings.

Ageing gel [day]	Steady-state anisotropy of fluorescence	Microviscosity [mPa·s]
1	**0.029** ± 0.002	19.8 ± 1.4
7	**0.0293** ± 0.0017	20.3 ± 1.2
14	**0.0291** ± 0.0013	19.9 ± 0.9

Hydrophilic region during fortnight-ageing

In the samples, the rhodamine has two lifetimes, which both increased during ageing of gel. The average lifetime increased from $\tau = (3{,}71 \pm 0{,}08)$ ns to $\tau = (3{,}91 \pm 0{,}07)$ ns, which signalized the some structural changes at the hydrophilic part of hydrogel.

The following table 2 shows the obtained value of steady-state fluorescence anisotropy of rhodamine and calculated values of microviscosity of its surrounding. Relatively high anisotropy values were measured. The values of anisotropy/microviscosity decreased during ageing, suggesting that the hydrophilic parts around the molecules of rhodamine are subject to changes – its surrounding has been more and more fluid.

Table 2 Steady-state anisotropy of fluorescence of rhodamine 6G during fortnight-ageing of hydrogel and calculated values of microviscosity of rhodamine surroundings.

Ageing gel [day]	Steady-state anisotropy of fluorescence	Microviscosity [mPa·s]
1	**0,216** ± 0,011	79 ± 5
7	**0,202** ± 0,019	69 ± 4
14	**0,190** ± 0,011	60 ± 3

Time development of values of microviscosity of individual hydrogel parts is shown on Fig. 1. The roughly constant values of microviscosity of hydrophobic domains do not indicate structural changes of perylene surroundings, against it the values of microviscosity of hydrophilic region nearby molecules of rhodamine decreased by about 25 percent during ageing.

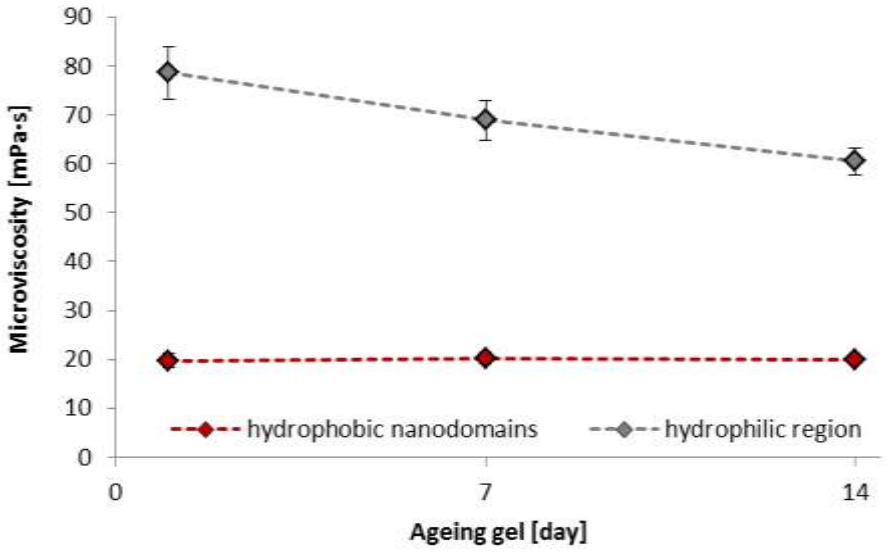

Fig. 1 Microviscosity of hydrophobic nanodomains and hydrophilic region of hyaluronan hydrogel determined first, seventh and fourteenth day of ageing.

3. CONCLUSION

Using analysis of photophysical response of two different fluorescence probes dissolved in different region of hydrogel composed of hyaluronan and cationic surfactant CTAB, we have obtained information about its microstructural changes during fortnight-ageing. From steady-state measurement of fluorescence anisotropy the microviscosity of different region of hydrogel has been estimated as a function of the days of ageing. The microviscosity of hydrophobic nanodomains has not changed during ageing, so we can these domains describe as a structural stable. Whereas the microviscosity of hydrophilic region, in terms surrounding of rhodamine molecules, decreased by about 25 percent, which probably indicates some microstructural changes in this area of hyaluronan hydrogel.

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