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Effect of Low Concentrations of Hyaluronan and Chondroitin Sulfate on Flow Rates

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The effect of high concentrations of hyaluronan (HA) and chondroitin sulfate (CS) on viscoelastic properties is well documented; while, the effect of low concentrations on flow is still unclear, and thus, investigated in this study. We prepared columns filled with EAH Sepharose 4B agarose gel coupled with HA or CS. A control column was filled with just EAH Sepharose 4B agarose gel. We measured the flow volume of 0, 1/243, 1/81, 1/27, 1/9, 1/3, and 1 mg/mL HA and CS in PBS through these columns in 5 min. The flow ratio was calculated by dividing each flow volume by that of PBS alone. We also measured the maximum-fluid velocity of 0, 1/9, and 1 mg/mL CS solutions using fast fluorescence scanning. As the concentration of HA increased, the flow ratio significantly decreased in all columns. Compared with the control column, the flow ratio of HA solutions through HA- and CS-columns significantly decreased at higher HA concentrations. The flow ratio of 1/9 mg/mL CS was significantly increased compared with PBS; yet, as the CS concentration further increased, the flow ratio then decreased in all columns. The flow ratio of CS solution through CS-columns was significantly higher at higher CS concentrations, compared with the control column; while, it was significantly lower through HA-columns. The observed maximum velocities by fast fluorescence scanning supported the peaked flow ratio at 1/9 mg/mL CS. In low concentration solutions, HA and CS have interactions that decrease flow velocity, with the exception of specific concentrations of CS (1/243–1/9 mg/mL) that contribute to increase flow velocity.

Key words: hyaluronan, chondroitin sulfate, flow

INTRODUCTION

A flow of fluid is a basic event in cells, tissues, and organs to survive and function, and one of the major factors that contributes to this essential event is the viscous property of the vital fluid. Hyaluronan (HA), chondroitin sulfate (CS), and other glycosaminoglycans (GAGs) are involved in this property, as a lot of water can be potentially contained within the molecules. Furthermore, most CS proteoglycans, such as aggrecan, versican, brevican, neurocan, and CD44 can bind to HA[1)2)]. The rheological properties of each GAG have been well documented[3)~5)], but the interactions of each GAG, especially those between HA and CS, are still mostly unclear.

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Turley and Roth have shown that HA-derivatized beads spontaneously agglutinate with CS-derivatized beads although neither bead type shows any self-agglutination, suggesting the interaction between HA and CS is specific and appears to occur between their carbohydrate chains[6]. Nishimura et al. examined the role of HA-CS interactions on viscoelastic properties using capillary and cone-on-plate viscometers[7]. The CS markedly increased the viscosity of HA solutions, although the viscosities of CS solutions themselves were very low. They concluded that CS controls the viscoelastic properties of HA solutions. Scott et al. calculated that HA was able to form stable heteroduplexes with CS using computer molecular dynamics[8]. However, the effect of low concentrations of HA and CS on flow has not been investigated.

The more investigation of the property of HA and CS may enable to realize more ideal drug in the field of clinical ophthalmology. HA and CS are used for the treatment of corneal epithelium[9)~12)]. HA and CS as ophthalmic viscoelastic devices are also used for intraocular surgery. Briefly, the purpose of the ophthalmic viscoelastic device is maintaining or creating space by cohesive property, and sealing capsular tear and coating corneal endothelium by dispersive property[13)14)], respectively. On the other hand, residual ophthalmic viscoelastic device often become a cause of an intraocular pressure spike after cataract surgery[15)~17)]. Further, the precise mechanism of these favorable and unfavorable events is still unclear.

In the present study, we focused on the contributions of low concentration HA and CS solutions on flow rates, and investigated the flow ratio of each solution using columns of HA- and CS-beads and fast fluorescence scanning. We demonstrate that HA and CS interactions lead to decreased flow velocity, except for specific concentrations of CS that increase the flow velocity. This unique property may be important for fluid flow in our biological and physiological reactions.

MATERIALS AND METHODS

Materials

The following materials were used in this study: HA (MW; 1,300–2,000 kDa, sodium salt, from rooster comb) from Sigma (St Louis, MO); CS (chondroitin sulfate A, MW; 50 kDa, sodium salt, from whale cartilage) from Seikagaku Corp. (Tokyo, Japan); EAH Sepharose 4B from GE Healthcare (Buckinghamshire, England); glass Econo-Column columns (1.0×10 cm) from Bio-Rad (Hercules, CA); acetic acid, sodium chloride, and sodium phosphate from Nacalai Tesque, Inc. (Kyoto, Japan); carbodiimide from Sigma (St Louis, MO). Here we defined as low concentrations of hyaluronan and chondroitin sulfate less than physiological concentrations for HA (3–5 mg/ml) in the vitreous humor of the human eye and in joints[18] and CS (0.55 mg/g) in synovial fluid[19], respectively.

Preparation of HA- and CS-columns

HA and CS were cross-linked to activated EAH Sepharose 4B gel according to a manufacturer's protocol using 2 mg of HA or CS per gel for the coupling reaction. The concentration of HA or CS was in excess of the concentration of the coupling group. The control gel for the preparation of control column was obtained by blocking the activated groups with acetic acid. All gels were equilibrated in phosphate-buffer saline (PBS).

Measurement of flow volume through columns

Freshly prepared HA- and CS-Sepharose 4B gels were packed with constant pressure in a glass Econo-Column to the same height

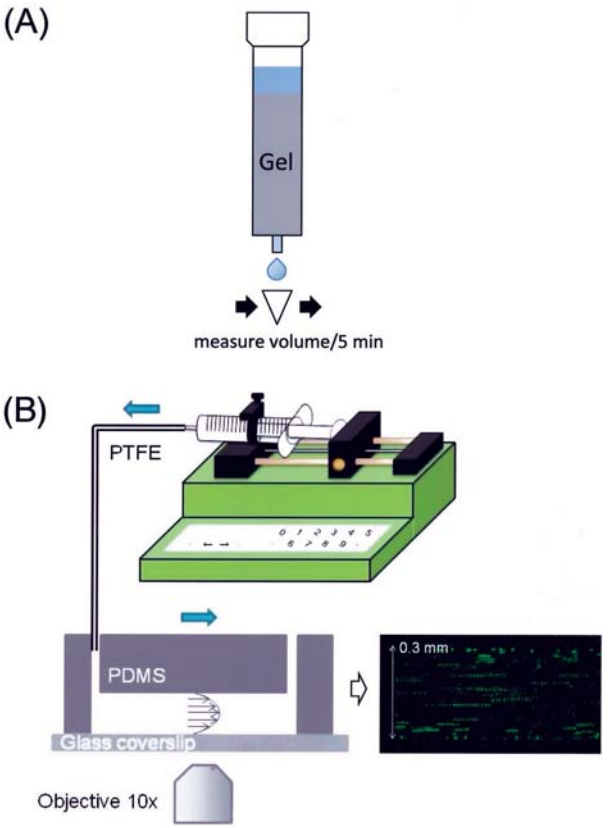

Figure 1. Schematic presentation of the measurement system for columns (a) and for PIV in the PDMS microfluidic channel (b). The objective lens was connected to a confocal fluorescent microscopic system in (b). Green objects are moving particles in the PDMS microfluidic channel. PIV images of solutions of PBS, 1/9 mg/mL CS, and 1 mg/mL CS in a frame were analyzed and summarized in Table 1.

precisely in PBS. A control column was prepared by a similar method. HA or CS was dissolved at a concentration of 1.0 mg/mL in PBS and diluted to the appropriate concentrations (1/243, 1/81, 1/27, 1/9, and 1/3 mg/mL) with PBS. Solutions were applied to columns at 25 ℃. We collected the solution that flowed out from the column by gravity-drop for 5 min twenty times and measured the weight. We then repeated these experiments 5 times on a different day (Figure 1a).

Fast fluorescence scanning for PIV using microfluidic channels

To explain the dependence of flow rate on CS concentration, we investigated the maximum-fluid velocity of the CS solutions using fast fluorescence scanning. The measurement system for particle image velocimetry (PIV) using fluorescence microscopy and the observed fluorescence image are shown in Figure 1b. A syringe pump (CellPoint Scientific Inc., Gaithersburg, MD) was connected to a polydimethylsiloxane (PDMS) microfluidic channel of 0.3 mm internal diameter with a polytetrafluoroethylene (PTFE) tube of 0.7 mm internal diameter (GL science, Tokyo, Japan). The PDMS microfluidic channel was fabricated by a reported method[20]. We used fluorescent beads (Peakflow™ 515 nm, Invitrogen, Carlsbad, CA) for the PIV in the PDMS microfluidic channel. The velocity of fluid was measured at 25 ℃ using a fluorescence microscope (Olympus, IX70) with a 10× (Olympus, Plan N) objective attached to a CSU10 confocal scanner (Yokogawa Electric Co., Tokyo, Japan)[21]. The excitation wavelength of the fluorescent beads was 488 nm and the emission was detected at 510 nm. The time resolution of each frame was approximately 30 ms. The confocal plane was fixed during recording. The flow rate of the syringe pump for scanning was 10 μm/s.

Statistical analysis

Experiments were repeated 5 times. The results are presented as the mean ± standard deviation (SD). The data were analyzed by one sample t test and a two-tailed *P*-value compared to the calculated control value.

RESULTS

Flow ratio of HA solution through control, HA-, and CS-columns

As the HA concentration in PBS increased,

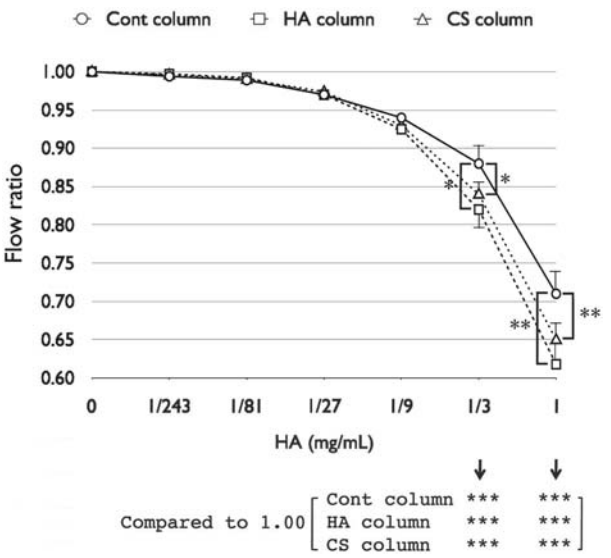

Figure 2. Flow ratio of hyaluronan (HA) solution through columns filled with EAH Sepharose 4B agarose gel coupled with HA or chondroitin sulfate (CS). A control column was filled with just EAH Sepharose 4B agarose gel. As the concentration of HA in PBS increased, the flow ratio significantly decreased in all columns. Compared with the control column, the flow ratio through HA- and CS-columns significantly decreased at high HA concentrations. The *P*-values for the flow ratio (compared with 1 or the control column) of each column at each HA concentration are shown as asterisks under the *arrows* and beside the *brackets*. Data are the mean±SD (n=5). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

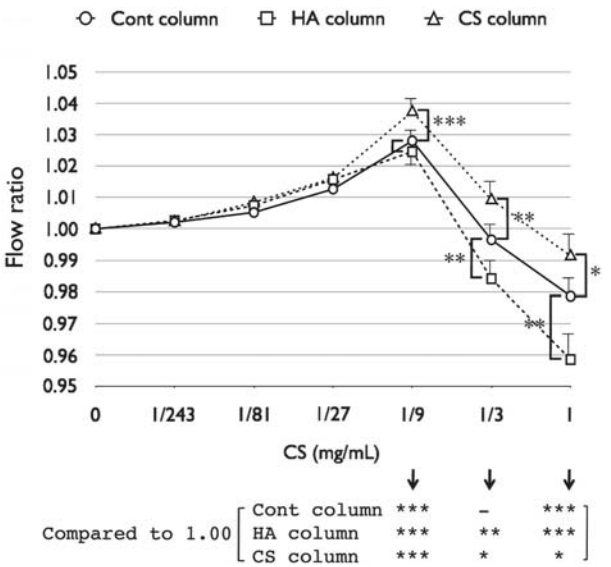

Figure 3. Flow ratio of CS solution through control, HA-, or CS-columns. As the CS concentration in PBS increased, the flow ratio significantly increased, peaking at 1/9 mg/mL CS, and then significantly decreased until 1 mg/mL CS in all columns. Compared with the control column, the flow ratios of CS solution through CS-columns were significantly higher, while, the ratios through HA-columns were significantly lower at high CS concentrations. The *P*-values for the flow ratio (compared with 1 or the control column) of each column at each CS concentration are shown as asterisks under the *arrows* and beside the *brackets*. Data are the mean±SD (n=5). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

the flow ratio decreased in all columns, and significantly decreased at higher concentrations of HA (Figure 2). Compared with the control-column, the flow ratio of higher concentrations of HA solution through HA- and CS-columns significantly decreased and it decreased more through the HA-column than the CS-column.

Flow ratio of CS solution through control, HA-, and CS-columns

As the concentration of CS in PBS increased, the flow ratio of CS solution increased until 1/9 mg/mL CS. It was significantly increased at 1/9 mg/mL CS and then significantly decreased at 1 mg/mL CS in all columns (Figure 3). The flow ratios of higher concentrations of CS solution were significantly higher through the CS-column compared with the control-column; while, the ratios were significantly lower for the HA-column.

Table 1. Observed maximum velocities pf PBS and CS solutions by fast fluorescence scanning

	PBS	CS 1/9 mg/mL	CS 1 mg/mL
V_{max} (mm/s)	23.3	26.8	23.4
Ratio to V_{max} In PBS	1	1.15	1.00

Maximum flow velocities of fluorescent beads in a confocal plane

The observed maximum velocities in solutions of PBS, 1/9 mg/mL CS, and 1 mg/mL CS are summarized in Table 1. The ratios of maximum velocity and flow rate (V_{max}, Q) versus

those of PBS were 1.15, 1.04 and 1.00, 0.99 in 1/9 mg/mL CS and 1 mg/mL CS, respectively. These velocities corresponded to the flow ratios at each CS concentration.

DISCUSSION

Here, we demonstrated that the flow ratios of both HA- and CS-columns decreased with the increase in HA concentration, and the flow ratio of the HA-column decreased more than CS-column, as shown in Figure 2. These results may simply indicate that HA increases viscosity more than CS. As shown in Figure 3, with the increase of CS concentration, flow ratios increased and peaked at 1/9 mg/mL CS, and then decreased in all columns. These results may indicate that an appropriate concentration of CS, corresponding to 1/9 mg/mL in our analysis, maximized flow velocity. These data were supported by the maximum-fluid velocities determined by fast fluorescence scanning, as shown in Table 1. Furthermore, as shown in Figure 3, the flow ratios of CS solutions through the CS-column were significantly higher compared with the control column at higher CS concentrations; while, the ratios were significantly lower through the HA-column. These results may indicate that HA cross-linked to the gel interacted with the CS in fluid to decrease flow velocity; while, CS cross-linked to the gel interacted with the CS in fluid to increase flow velocity. To our knowledge, this unique property has not been described elsewhere.

HA and CS showed interactions that decrease flow velocity, with the exception of specific concentrations of CS (1/243–1/9 mg/mL) that contribute to increase flow velocity. Precise mechanism of this phenomenon is unclear, but we speculate as follows. Mesoporous of Sepharose gel filled with CS may contribute to increase flow velocity through the columns within the range of specific concentrations of CS. HA mutually anneals with its chain end which forms longer helices, and finally it demonstrates increased viscosity as shown with high molecular weight. Once CS, a low molecular weight having sulfate group, anneals with HA, it may interfere with these HAs' assemblings within the range of specific concentrations of CS.

CS proteoglycans may be utilized instead of CS in physiological processes. Keller et al. demonstrated that versican, a large proteoglycan with numerous CS side chains attached, appears to be a central component of outflow resistance *in vivo*, where it may control open flow channels in the trabecular meshwork[22]. Nishimura et al. demonstrated that CS, as well as aggrecan, a large proteoglycan with numerous CS side chains attached, increased the viscosity of hyaluronan solutions *in vitro*[7]. CS is also capable of affecting flow resistance when coexisting with HA, as shown in our present study, and both versican and aggrecan, CS proteoglycans, have potential to bind with HA due to a G1 domain at the amino-terminal within the molecules[1].

The rheological performance of GAGs is sensitively influenced by many factors, such as molecular size and concentration of GAGs, temperature of the analysis, dissolved buffer, pH, and osmotic pressure[4)23)~27)]. Therefore, these factors were rigorously controlled to obtain to accurate data. Column conditions easily change during a series of measurements, which is unavoidable. In the present study, we strictly set the temperature for each experiment at 25℃. To avoid an error due to the column condition, we calculated the flow ratio by dividing by the flow rate of PBS, which was determined just before each measurement 5 times and used as a control. Furthermore, we repeated each

experiment 5 times on another day to confirm the reproducibility of the data.

When solutions are Newtonian fluids, the velocity distribution in the circular tube is parabolic (Hagen-Poiseuille flow). Both V_{max} and Q depend on the pressure drop (ΔP) and viscosity (μ) as shown in eq. [1] and [2], respectively[28].

$$\nu_{max} = \frac{\Delta P R^2}{4\mu L} \qquad [1]$$

$$Q = \frac{\pi\rho\Delta P R^4}{8\mu L} \qquad [2]$$

R, L, and ρ are the radius of the circular tube, length of the tube, and the density of the solution, respectively. When $\Delta P/\mu$ in the 1/9 mg/mL CS solution becomes larger than that in PBS and the value in 1 mg/mL CS solution is the same as that in PBS, we can explain these experimental results. The CS concentration dependence of the flow ratio can be explained by the effect of non-Newtonian fluids, which induce non-linearity of viscosity (μ) and pressure drop (ΔP) on the flow rate[29)30)].

We concluded that low concentrations of HA and CS have interactions that decrease flow velocity, with the exception of specific concentrations of CS (1/243–1/9 mg/mL) that contribute to an increased flow velocity. The role of CS in flow velocity varies depending on the accompanying glycosaminoglycan and its concentration. This unique property may have an effective role in our biological and physiological reactions in life.

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