

## Rheological Studies of Hyaluronan/Modified Hyaluronan Mixtures and the Structure of Hyaluronic Solutions

Martin Chytil<sup>1,2</sup>, Miloslav Pekař<sup>1</sup>

<sup>1</sup>Institute of Physical and Applied Chemistry, Faculty of Chemistry, Brno University of Technology, Purkyňova 118, 612 00, Brno

<sup>2</sup>CPN, Ltd., 561 02, Dolní Dobrouč 401

### ABSTRACT

The structure of solutions of hyaluronan (HA) and HA/HMHA (hydrophobically modified HA) mixtures were studied by means of shear viscosity measurements, oscillation, creep and relaxational tests. Influence of molar mass, concentration, the presence of salt and the HA/HMHA weight ratio is discussed.

### INTRODUCTION

Hyaluronic acid is a naturally abundant linear polysaccharide that consists of repeating disaccharide structure units of  $\beta(1\rightarrow3)$  linked *D*-glucuronic acid and *N*-acetyl-*D*-glucosamine. The disaccharide units are linked via  $\beta(1\rightarrow4)$  glycosidic bond. HA contains negatively charged carboxylate groups, thus it is a polyelectrolyte and forms a salt form of hyaluronate or hyaluronan<sup>1-3</sup> at physiological pH. HA occurs in extracellular matrix of vertebrates connective tissues with a wide range of molar masses from several hundreds to 10 millions g/mol. HA plays many pivotal functions in the organism such as a space-filler or a scaffold for other macromolecules, creating a network with proteoglycans and proteins<sup>4</sup>. HA, thus maintains viscoelasticity of the tissues due to its extraordinary rheological properties.

HA also participates in various receptor-mediated cell processes<sup>3, 5, 6</sup>, e.g. mitosis, cell migration, tumor development, inflammation, etc. HA has found numbers of important applications in medicine, e.g. ophthalmology, visco-supplementary, osteoarthritis treatment, drug delivery<sup>1, 2, 7, 8</sup>, pharmacy and cosmetics due to its rheological and other unique properties. During technology processes, HA undergoes various mechanical stresses, thus rheology properties play important role in the processes and many applications of HA. Therefore, knowledge of a relationship between rheological parameters and structure of hyaluronic solutions is very helpful during these operations and utilizations<sup>8</sup>. Previous studies on rheology of HA solutions were mostly based on rotational shear viscosity measurements and/or oscillatory measurements<sup>3, 9</sup>. In this paper we tried to investigate the structure of HA solutions not only using these techniques but also by submitting HA solutions to a sudden load of various intensities and duration observing rheological parameters during and after removing the load. Thus, we used relaxation, creep and recovery tests for probing breakdown and buildup the structures of HA solutions.

## MATERIALS AND METHODS

### Materials

Bacterially produced HA of two molar masses of 1.63,  $1.69 \times 10^6 \text{ g.mol}^{-1}$  and  $5.6 \times 10^5 \text{ g.mol}^{-1}$ , and octyl chain bearing hydrophobically modified HA (HMHA) marked as  $C_8\text{nhHA9}$  of molar mass of  $4.5 \times 10^5 \text{ g.mol}^{-1}$  and the degree of substitution ( $DS$ ) of 100% were studied. HA was modified with a chemical linking of the alkyl chain via carbamate bond as described here<sup>10</sup>.  $DS$  here corresponds to a number of the disaccharide units in percentage occupied with one alkyl group. The samples were produced in the CPN, ltd. Company.

### Methods

Measurements were performed on rheometer RS Haake 100 with the cone-and-plate (CP) geometry of the cone diameter of 60 mm and  $1^\circ$  cone angle, and on STRESSTECH Rheologica Instruments rheometer with the CP geometry of 40 mm/ $4^\circ$  (NTNU, Trondheim). The oscillatory measurements were performed mostly in the range of frequencies,  $f = 0.005 - 10 \text{ Hz}$ , at the strain value determined from the strain sweep measurements. Shear viscosity was measured using the up-and-down stress or shear rate scans. For investigation of the breakdown and buildup the solution structure a sudden impulse of shear rate or shear stress was applied to the sample and rheological respond was observed in time. All the measurements were performed at  $(25 \pm 0.2)^\circ\text{C}$ .

## RESULTS AND DISCUSSIONS

### Rheology of HA and HMHA solutions

Previous rheology studies on HA have reported about non-Newtonian shear-thinning behavior of HA aqueous solutions<sup>1, 3, 8</sup> which is very advantageous in many applications. HA also displays extraordinary rheology behavior under various conditions<sup>3</sup> and forms associated structures resulting

e.g. in high viscoelasticity of the HA solutions, particularly of high molecular HA, by increasing the storage modulus,  $G'$ , (Fig.1).  $G'$  increases with the concentration and molar mass of HA with the shift of the cross point ( $G' = G''$ ) to lower frequencies.

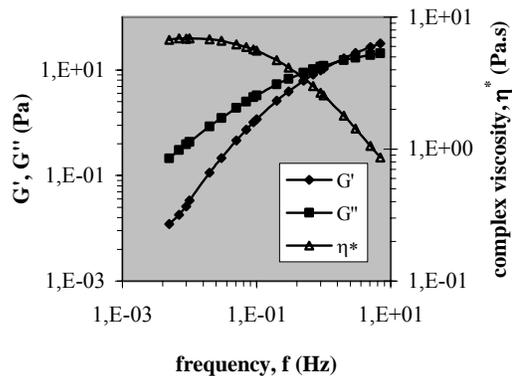


Figure 1. Oscillatory measurements of 0.75% w/w HA ( $1,690 \text{ kg.mol}^{-1}$ )

On the other hand, HMHA marked as  $C_8\text{nhHA9}$  behaves like a viscous liquid with  $G'$  much lower than  $G''$  in the whole range of the frequencies studied (Fig. 2). It displays weaker shear-thinning, however, with the similar shear viscosities to the HA of the similar molar mass. Thus, the hydrophobic modification of HA leads to the disruption of the HA associative structure depending on the length of the alkyl and  $DS$ .

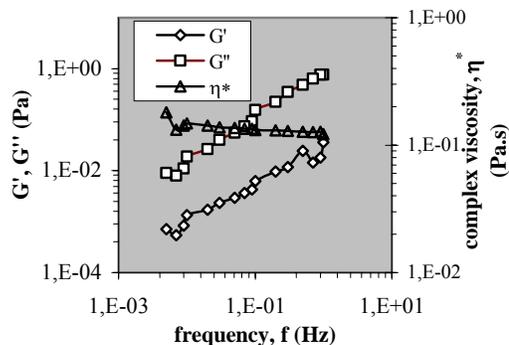


Figure 2. Oscillatory measurements of 1% w/w solution of octyl bearing HMHA marked as C<sub>8</sub>nhHA9

### Influence of NaCl

HA displays polyelectrolyte character due to the presence of charged carboxylate groups on the backbone, therefore numbers of the HA properties will be influenced by the presence of any counterions.

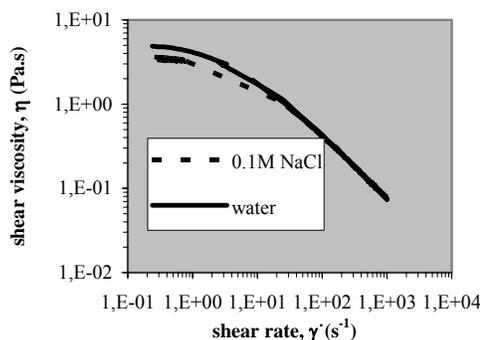


Figure 3. Influence of added NaCl on rheology behavior of 1% w/w HA (1,630 kg.mol<sup>-1</sup>)

Figure 3 illustrates the influence of added NaCl into the solution of HA that induces a decrease of shear viscosities at mainly lower shear rates, and thus *zero shear viscosity*,  $\eta_0$ , ( $\dot{\gamma} \rightarrow 0$ ) as well. Addition of salt also decreases storage modulus,  $G'$ , and from certain amount of NaCl, to the values below  $G''$  (data not shown). Presence of salt, thus, disturbs the

associative structure of HA which is mediated particularly by electrostatic repulsion forces between the charged HA polymer chains.

### Creep and Relaxation Tests

The purpose of the experiments in this section was to monitor breakdown and buildup the structure of HA solutions upon adding perturbing shear and observing rheological parameters during the shear and after removing the shear either at low probing shear stress or rate, or zero shear stress (creep). These experiments can also simulate the processes in synovial joints, cartilage and other tissues being exposed to the mechanical stress during walk or move.

Figures 4, 5 illustrate the breakdown and buildup the structures by means of monitoring shear viscosity as a function of time. All the HA solutions exhibited an increase of the shear viscosity in time after removing the shear. This increase corresponds to a structure formation and depends on concentration and molar mass of the sample and also on the strength and duration of the load.

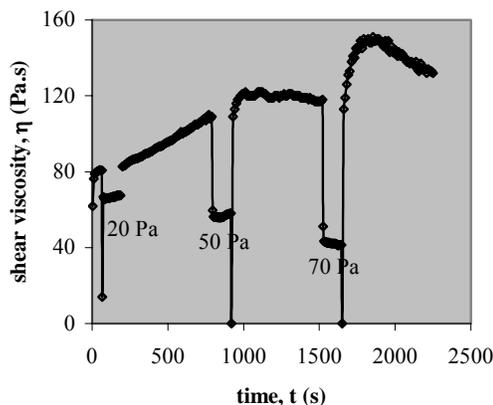


Figure 4. Viscosity as a function of shear time under a continuous shear stress of 5 Pa interrupted periodically with shear stress impulses of 2 min. duration for 2% w/w HA (1,630 kg.mol<sup>-1</sup>)

Highly concentrated high molecular HA displays firstly moderate viscosity increase followed then with a remarkable thickening of the structure after higher perturbing shear (Fig. 4). In comparison, the low molecular HA (Fig. 5) exhibits steeper increase of the shear viscosity in time even after low shear stresses. At such shear stresses the chains of less concentrated HA of lower molar mass are already and more oriented and stretched than those of high molecular HA which seems to be more efficient in associating the structure<sup>11</sup> after the load has been removed. However, too high shear leads to irreversible disruption of the structure (Fig. 5).

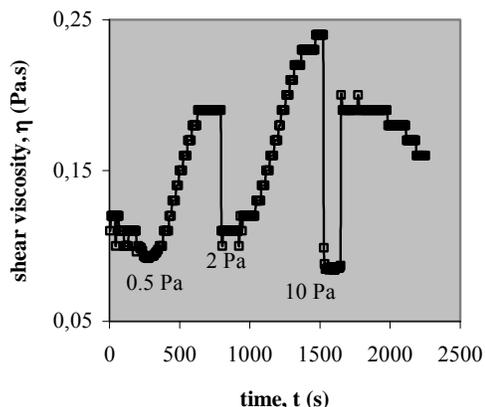


Figure 5. Viscosity as a function of shear time under a continuous shear stress of 0.05 Pa interrupted periodically with shear stress impulses of 2 min. duration for 1% w/w HA ( $560 \text{ kg}\cdot\text{mol}^{-1}$ )

#### Study of HA/HMHA mixtures

HA unique rheological properties are in addition used in ophthalmology. A HMHA with amphiphilic character due to the presence of hydrophobic groups formed by the alkyl chains can form associative aggregates in aqueous solutions which are able to solubilize a hydrophobic compound, e. g. a drug. The mixture of HA and such HMHA can provide the properties of both.

Therefore, rheology of such mixtures has been investigated.

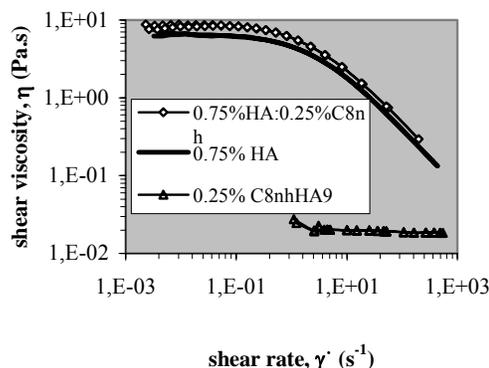


Figure 6. Shear viscosity study of HA/C<sub>8</sub>nhHA9 mixtures; concentration of the samples are expressed in weight %.

The solutions of high molecular HA and HMHA marked as C<sub>8</sub>nhHA9 were mixed in various weight ratios upon gentle stirring. Firstly, HA concentration was kept constant and the HMHA was being added, secondly the overall mixture concentration was kept at 1% w/w and the HA/C<sub>8</sub>nhHA9 ratio was altered.

The increasing amount of 1% w/w C<sub>8</sub>nhHA9 solution in the mixture induces decrease in shear viscosities comparing to 1% w/w HA. However, from a certain HA/C<sub>8</sub>nhHA9 ratio, the values of the mixture shear viscosities are significantly higher than simple sum of the shear viscosities of the single HA and C<sub>8</sub>nhHA9 solutions of the same weight concentration as in the mixture (Fig. 6). This observation indicates some kind of interactions between the components upon mixing. At such ratios C<sub>8</sub>nhHA9 is still well above its *critical micellization concentration*, CMC (determined elsewhere) which is necessary for potential solubilization purposes.

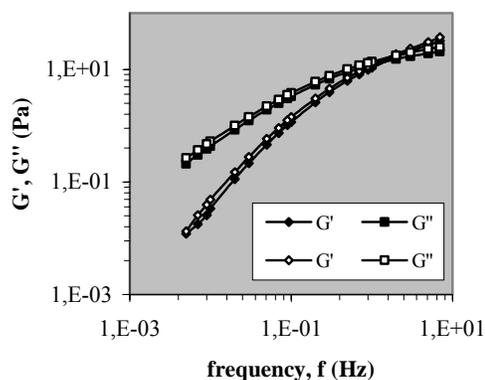


Figure 7. Oscillatory study of HA/C<sub>8</sub>nhHA<sub>9</sub> mixtures; full symbols correspond to 0.75% w/w HA (1,690 kg.mol<sup>-1</sup>), empty symbols correspond to HA/C<sub>8</sub>nhHA<sub>9</sub> mixture of weight ratio 3:1 (0.75% w/w: 0.25% w/w)

The storage modulus,  $G'$ , also increases after adding the HMHA solution, nevertheless, loss modulus,  $G''$ , increases as well due to increase of the overall viscosity from the increase in concentration. Thus the phase angle indicating liquid or solid like behavior remains practically the same or at higher concentration of C<sub>8</sub>nhHA<sub>9</sub> in the mixture increases above 45° ( $G' < G''$ ) in the frequencies studied, which indicates liquid like behavior.

## CONCLUSIONS

This paper reports about basic experiments on the structure of hyaluronic solutions with the regard to concentration, molar mass, presence of salt, and also brings a comparison between HA and its hydrophobically modified analogue. Experiments show that HA forms associative structures, resulting e. g. in the increase of  $G'$  modulus above  $G''$  modulus, that are sensitive to the presence of salt and also to hydrophobic modification of HA. Both adding of salt and the modification leads to rather liquid like behavior.

HA solutions exhibit the breakdown and buildup their structure upon subjecting to

shear followed by the remove of the perturbing force with the result in an increase of the solution viscosity. The increase depends on concentration, molar mass of the sample and also on the strength and duration of the perturbing shear.

Study of HA/HMHA mixtures showed that from a certain weight ratio between HA and HMHA the solution viscosity significantly increases as a result of interactions between the mixing components. Nevertheless, additional increasing of the HMHA content leads to a decrease of  $G'$ , and thus the overall elasticity, and the mixture becomes more liquid.

## ACKNOWLEDGEMENT

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