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PROPERTIES OF NEW MATERIALS

RHEOLOGICAL STUDY ON HYALURONIC ACID AND ITS DERIVATIVE SOLUTIONS

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Key Words: Hyaluronic Acid, Polysaccharides, Rheology, Viscosity, Viscoelasticity, Molecular Weight, Concentration

ABSTRACT

Rheological measurements were performed on Hyaluronic acid (HA) and its derivative solutions to evaluate steady flow viscosity and dynamics response with the aim to correlate the materials properties to the concentration, molecular weight and chemical structure. At low molecular weight and concentration, the HA solutions behaved as viscous liquid, whereas a soft-gel response was evident at higher molecular weight and concentration due to chains entanglement. Increasing the molecular weight was more effective than increasing concentration in promoting entanglement of molecular chains of HA. Comparing the behavior of HA solutions with that of Hyaluronic acid derivatives, it is showed that it is possible to modulate the rheological properties of HA based solutions by chemical modification preserving the biocompatibility of the materials. The results of the rheological analysis provide a valuable tool to properly design optimal substitutes for specific biomedical application.

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INTRODUCTION

Hyaluronic acid (HA) or hyaluronan is a naturally occurring, biocompatible, and biodegradable linear polysaccharide composed of repeating disaccharide units of glucoronic acid and N-acetyl glucosamine linked by β 1-3 and β 1-4 glicosidic bonds. HA is present in all soft tissues of higher organism, and in particular concentrations in the synovial fluids and vitreous humor of the eyes with molecular weight in the range of $3\text{-}6 \times 10^6$ [1].

Commercially available HA is obtained from different sources, mainly extracted from umbilical cord, rooster comb, synovial fluid, or vitreous humor. Recently, there is also biotechnological production of HA from Streptococcus. Hyaluronic acid is already used in several biomedical applications and an increasing number of potential applications are currently being explored. Hyaluronic acid solutions are widely used as an aid in ophtalmological surgery. In cataract surgery, for example, the role of HA solutions is to facilitate the procedures and to protect the corneal endothelium [2]. Another main application of HA is in the treatment of inflammatory and degenerative joint diseases [3]. The physiological function of synovial fluids is to lubricate the joint at low strain frequency (e.g., resting or walking) and to prevent mechanical damage at high strain frequency (e.g., running). Therefore, it behaves as viscous liquid at low frequency and as an elastic 'shock absorber' at high frequency [4]. During an inflammatory processes such as rheumatoid arthritis, the rheological properties of synovial fluids are compromised by a reduction of Hyaluronic molecular weight and concentration [5].

Along with fulfilling structural roles related to its lubricating and water retaining properties; HA plays an important role in a number of biological processes such as cell mobility and cell-cell interactions [1].

Following an injection of HA in human body, a fast degradation process often occurs. The short residence time of HA strongly limits the possibility to broadening its range of biomedical applications. Therefore, a mounting research effort has recently been devoted to explore ways of chemically modifying HA by crosslink or coupling reactions. The aim of this study was to analyze the effects of molecular weight, concentration and chemical modifications on the rheological properties of HA and its derivatives solutions.

Materials

This study was carried out on Hyaluronic acid, on the benzyl esters of Hyaluronic acid (Hyaff 11) partially esterified and on ACP (Auto Crosslinked

Polysaccharides). All the materials were supplied by FAB (Fidia Advanced Biopolymers S.p.A., Abano Terme, Italy).

Hyaluronic Acid

Hyaluronic acid at two different molecular weights (150 kDa and 1.200 kDa) were used as received.

Hyaluronic Acid Esters

HA esters are prepared by treating a quaternary ammonium salt of HA with an esterifying agent in a suitable aprotic solvent at a controlled temperature [6]. The degree of esterification achieved is determined only by the quantity of alkylating agent added. In this study we analyzed Hyaff11 at 50% of degree of esterification and 150 kDa of molecular weight (Hyaff11p50).

ACP

ACP polymers are inter- and intra-molecular esters of HA in which part of the carboxyl groups are esterified with hydroxyl groups belonging to the same molecule and/or different molecules of the polysaccharide thus forming a mixture of lactones and intermolecular ester bonds. The level of crosslinking can be varied by modulating the reaction conditions. The crosslinking reaction can be carried out on HA of the desired molecular weight. The unique feature of this technology with respect to alternative approaches to crosslink HA is that no bridge molecules are present between the crosslinked HA chains [7]. This ensures that only the natural component of HA are released upon degradation of ACP. We used ACP synthesized from HA of 200 kDa with two different level of crosslink 5% (ACP-5%) and 15% (ACP-15%). The percentage indicates the nominal level of crosslinking although it is not a true measure of the percentage of carboxyl groups bound to hydroxyl residues.

Solutions of HA and its derivatives were obtained by adding bidistilled water to the powders supplied by FAB in order to obtain different weight concentrations.

Methods

The rheological properties of Hyaluronic Acid and its derivatives were evaluated on a Bohlin VOR Rheometer (Bohlin Reologi A B, Lund, Sweden) at controlled temperature of 37°C and/or 25°C. The geometry was cone and plate (CP 5/30 cell) and coaxial cylinders geometry (Bohlin "small sample cell"). The outer cylinder or the lower plate was forced to rotate or oscillate, whereas the stress transferred from the fluid to the inner cylinder or upper plate was measured by means of a Linear Variable Displacement Transducer (LVDT) system.

The non-linear flow properties of the investigated materials were evaluated through steady shear measurements to determine the viscosity η as function of shear rate, while the small-amplitude oscillatory shear experiments were performed to measure the unsteady response of the samples and hence the determination of their linear viscoelastic properties. This technique has been successfully used to determine the structure-mechanical properties relationships of biological tissues [8, 9].

In dynamic experiment the material is subjected to a sinusoidal shear strain:

$$\gamma = \gamma_0 \sin(\omega t)$$

where γ_0 is the shear strain amplitude, ω is the oscillation frequency (which can be also expressed as 2π f where f is the frequency in Hz) and t the time. The mechanical response, expressed as shear stress τ of viscoelastic materials, is intermediate between an ideal pure elastic solid (obeying to the Hooke's law) and an ideal pure viscous fluid (obeying to the Newton's law) and therefore is out of phase respect to the imposed deformation as expressed by:

$$\tau = G'(\omega)\,\gamma_0 \sin(\omega t) + G''(\omega)\,\gamma_0 \cos(\omega t)$$

where $G'(\omega)$ is the shear storage modulus and $G''(\omega)$ is the shear loss modulus. G' gives information about the elasticity or the energy stored in the material during deformation, whereas G'' describes the viscous character or the energy dissipated as heat.

The combined viscous and elastic behavior is given by the absolute value of complex shear modulus G^*:

$$G^*(\omega) = \sqrt{G'^2 + G''^2}$$

or by the absolute value of complex viscosity η^* defined as:

$$\eta^*(\omega) = \frac{\sqrt{G'^2 + G''^2}}{\omega}$$

which is usually compared with the steady shear viscosity in order to evaluate the effect of large deformations and shear rates on the material structure.

The ratio between the viscous modulus and the elastic modulus is expressed by the loss tangent:

$$\tan\delta = \frac{G'}{G''}$$

where δ is the phase angle.

To determine the region of linearity, strain sweep tests at fixed oscillation frequency were performed. Specifically, the dynamic moduli were monitored while logarithmically varying the strain amplitude γ_0.

RESULTS AND DISCUSSION

Steady Shear Viscosity

Steady shear experiments were performed in order to evaluate the effect of molecular weight and concentration on the shear viscosity of Hyaluronic acid and its chemically modified derivatives.

Figure 1 shows the rheological flow behavior of HA low and high molecular weight and Hyaff11p50 (HAp50) at a concentration of 20 mg/ml. Low molecular weight Hyaluronic acid (150 KDa) exhibited essential Newtonian characteristics throughout all the shear rate range analyzed (10^0-10^3 s^{-1}). Pseudoplastic behavior (shear thinning) were observed for high molecular weight HA (1.2 MDa). The shear viscosity decreased with the shear rate in a S-shaped fashion. At low shear rate (<1 s^{-1}) the viscosity is almost constant decreasing slightly with the shear rate (Newtonian plateau). At shear rate greater than 1 s^{-1} the viscosity sharply drops with the shear rate (thinning). At higher shear rate, a second plateau should be expected. The rheological behavior of these solutions is typical of entangled networks. In these networks, the rheological response is controlled by the rate of entanglement formation and disruption. At low shear rate, the two rates are comparable and the total number of active entanglement is almost constant. As the shear rate increases, the rate of disruption becomes predominant leading to the thinning. Similarly to low molecular weight HA, the benzyl ester exhibited pseudo-Newtonian characteristics in the shear rate range 10^{-1}-10^3 s^{-1}. The viscosity of Hyaff11p50 was found to be lower than low molecular weight HA. This result is probably due to a reduced hydrophillicity of the

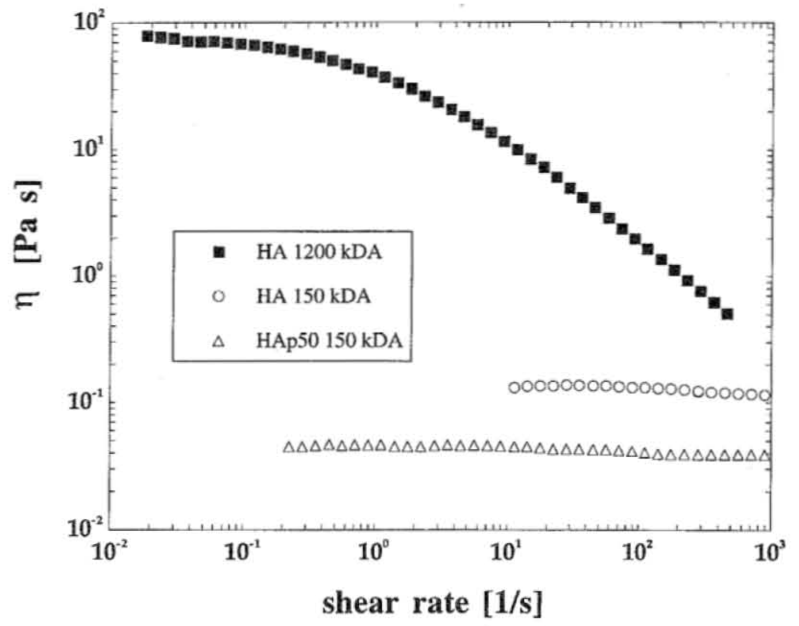

Figure 1. Flow curves of Hyaluronic acid low molecular weight (150 KDa), high molecular weight (1.200 kDa) and Hyaff11p50 (Hap50) at a concentration of 20 mg/ml (T=37°C).

polymer induced by the presence of the benzyl groups. This may lead to a reduction of the radius of gyration of the solvated molecules and therefore to a reduced viscosity. Figure 2 shows the flow curve of ACP at 10 mg/ml. Although low molecular weight HA was used for synthesis, the rheological behavior is comparable to the high molecular weight HA. This is due to the presence of inter- and intramolecular crosslinks which lead to a factual increase of molecular weight. The possibility to reproduce the rheological behavior of high molecular weight HA by crosslinking low molecular weight HA is of particular industrial interest. Indeed, the extraction of hyaluronic acid of low molecular weight requires less expensive sources and procedures.

Viscoelastic Properties

The rheological tests were carried out to evaluate the viscous (G″) and elastic response (G′) of these materials and their dependence upon molecular weight, concentration and chemical structure.

According to the viscosimetric data, low molecular weight HA and its derivatives ester solutions behaved as viscous liquid (G″>G′) (Figure 3). The

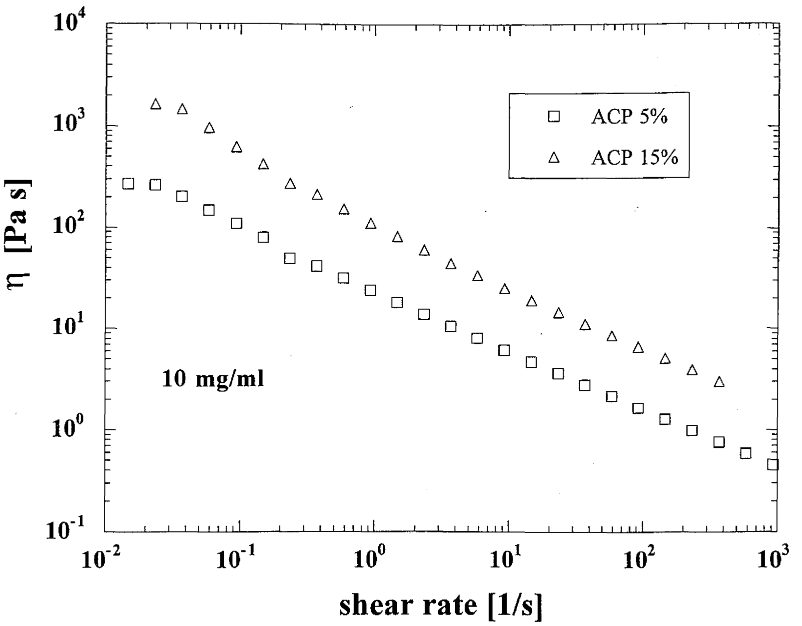

Figure 2. Flow curves of ACP at different degree of crosslinking and at fixed concentration of 10 mg/ml (T=25°C).

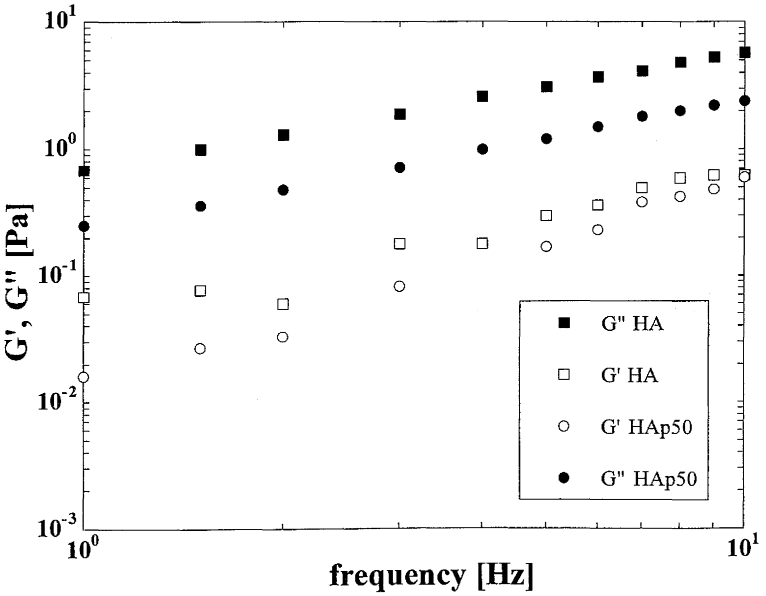

Figure 3. Mechanical spectra of Hyaluronic acid low molecular weight (150 KDa) and Hyaff11p50 (Hap50) at a concentration of 20 mg/ml (T=37°C).

loss modulus of the benzyl ester was lower than that of HA solution in all frequency range. These results indicate that at this value of molecular weight of HA no entanglement occurs among polymers chains. At higher molecular weight, HA molecules start to entangle with each other forming a network structure as indicated by the mechanical spectra of Figure 4. At low frequency, the solution presented viscous behavior (G″>G′) while at high frequency an elastic behavior (G′>G″) was observed. This transition, indicated by the crossing of the G′ and G″curves, occurred at a given value of the frequency (f*) corresponding to the intrinsic rate of disentanglement of polymer chains. At frequency lower than crossover frequency (f*), molecular chains can releasing stress by disentanglement and molecular rearrangement during the period of oscillation and hence G″ predominates over G′. On the other side, when the frequency is greater than f*, molecular chains cannot disentangle during the short period of oscillation and therefore they behave as a temporary cross-linked network (G′>G″). These results are in agreement with the observation reported the literature [11]. Since the disentanglement rate depends upon the mobility of the polymer chains, the crossover frequency (f*) will depend on both molecular weight and concentration of the solution. Increasing the polymer concentration, the disentanglement

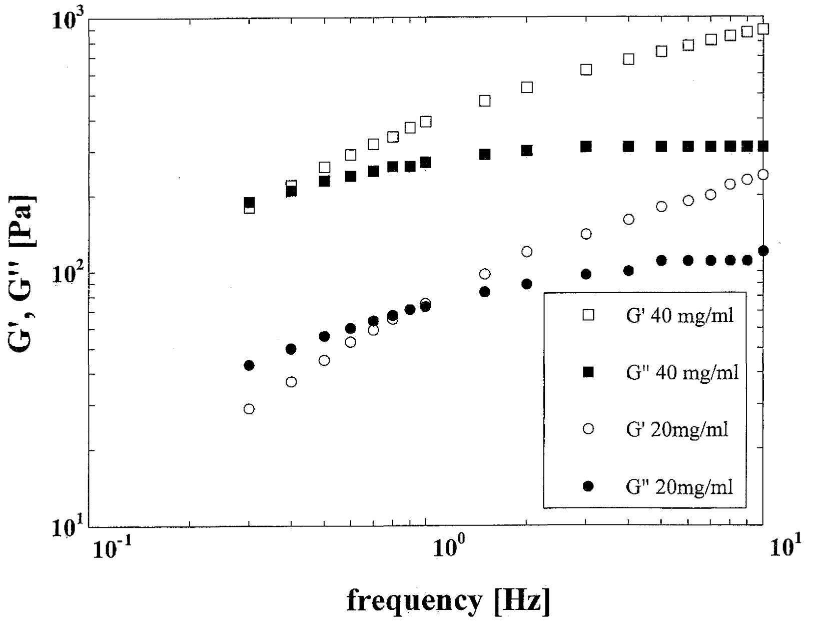

Figure 4. Mechanical spectra of Hyaluronic acid high molecular weight (1200 KDa) at 20 mg/ml and 40 mg/ml of concentration (T=37°C).

rate decreases resulting in a decrease of f*. Indeed, Figure 4 shows that f* decreases from 1 to 0.15 Hz by increasing the concentration from 20 to 40 mg/ml. In low molecular weight solutions, f* is higher than 10 Hz and therefore G″>G′ in all frequency range (Figure 3).

An alternative way to change the disentanglement rate of low molecular weight HA solution is by covalently bridging HA molecules. Figure 5 shows the mechanical spectra of ACP solutions. The rheological behavior of ACP differed substantially from that of HA solutions. G′ and G″ are almost constant with frequency and are parallel to each other within all frequency range. The elastic and viscous moduli increased with crosslink percentage. Similar behavior has been observed for many biological gels (e.g., collagen, agarose) and it has been classified by Murphy as "weak gel" behavior [8, 9]. In our case, the overall rheological response is due to the contributions of permanent covalent crosslinks and to entanglements. The inter- and intramolecular crosslinks bring about a reduction of intrinsic mobility of the polymer chains leading to a decreases in f*. Furthermore, since the molecular weight of pendant chains is widely dispersed, there will not be a unique value of disentanglement rate but rather a distribution. This would explain the weak dependence of G″ upon frequency.

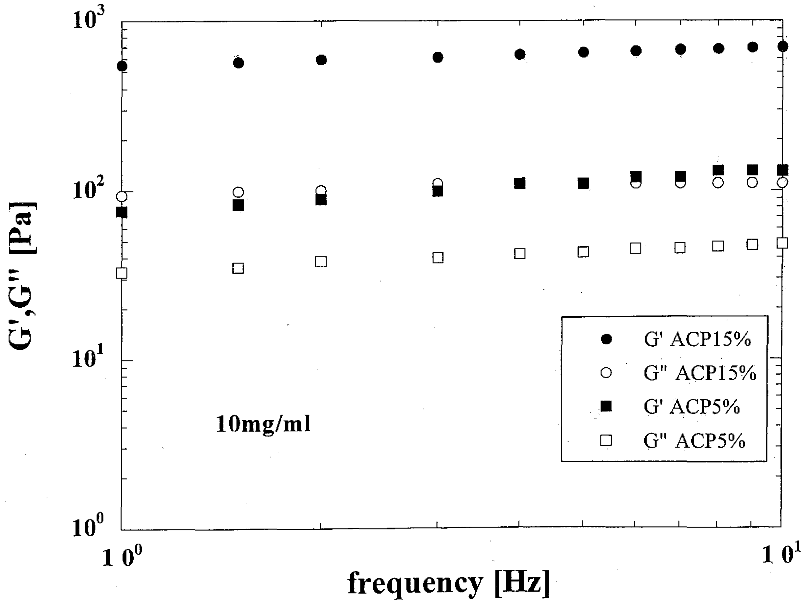

Figure 5. Mechanical spectra of ACP's at fixed concentration of 10 mg/ml (T=25°C).

CONCLUSION

The rheological analysis is a useful tool to explore relationships between mechanical behavior and structure, concentration and molecular weight of biopolymers solutions. It provides valuable indications to better design proper substitutes for specific biomedical application.

By using rheological analysis, we observed that HA solutions behave as viscous liquid or entanglement network as function of molecular weight and concentration. Entanglement formation is more affected by an increase of molecular weight than by an increase of concentration. Moreover, the results showed the possibility to modulate the rheological properties of HA based solutions by chemical modification without altering the biocompatibility of the HA molecules. By autocrosslinking HA at low molecular weight it is possible to obtain the all range of rheological behaviors spanning from dilute solutions to "weak gels".

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