Amphiphilic derivatives of sodium alginate and hyaluronate for cartilage repair: Rheological properties

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Abstract: Various amphiphilic derivatives of sodium alginate and hyaluronate were prepared by covalent fixation of long alkyl chains (dodecyl and octadecyl) with various ratios on the polysaccharide backbones via ester functions. In the semidilute regime, aqueous solutions of the resulting compounds exhibited the typical rheological properties of hydrophobically associating polymers: tremendous enhancement of zero shear rate Newtonian viscosity, steep shear-thinning behavior, and formation of physically cross-linked gel-like networks. The influence of the alkyl chain length, its content on the polysaccharide and of the polymer concentration in the solution was well identified. All obtained results are discussed with respect to the schedule of conditions related to materials, which could be used for cartilage repair, such as in synovial fluid viscosupplementation as well as in cartilage replacement. In particular, it is seen that HA-C_{12}-5 (hyaluronate substituted with 5% of dodecyl chains) and HA-C_{18}-1 (hyaluronate substituted with 1% of octadecyl chains) in a 0.15*N* NaCl solution at 8 g/L have rheological properties quite similar to those of healthy synovial fluid. On the other hand, the rheological parameters of solutions at 8 g/L in 0.15*N* NaCl of some of derivatives, such as, for example, AA-C_{12}-8 (alginate substituted with 8% of dodecyl chains) or HA-C_{18}-2, are well fitted for a use in cartilage repair. © 2000 John Wiley & Sons, Inc.

Key words: associative alginate and hyaluronate; rheological properties; viscosupplementation; cartilage replacement

INTRODUCTION

Articular cartilage is mainly composed of a highly hydrated collagen network, in which chains of hyaluronate (HA), associated to proteoglycans—proteins carrying various glycosaminoglycans—are entrapped.[1,2] HA is an anionic polysaccharide that plays a central role in monitoring the mechanical properties of cartilage, as it maintains the structure of the glycosaminoglycans' aggregates, thus conferring resilience and elastic strength to the cartilaginous matrix.[3] HA is also one of the major components of synovial fluid. The presence of high molar mass HA ($\overline{M_w} = 10^6$ to 10^7 g/mol) at high concentrations (2 to 4 mg/mL) in this fluid, results in a highly visco-elastic solution with optimum lubricating functions at low shear rate, and excellent shock-absorbing properties at high shear rate.[4]

In joint diseases such as osteoarthritis, HA is degraded, which results both in the loss of the viscoelastic properties of synovial fluid and in the deterioration of the mechanical functions of the cartilage tissue. Cartilaginous wounds, generated in the course of the disease and evidenced by pain and stiffness of the joints, stem from these degradations.[5] Owing to the avascular nature of cartilage, such lesions do not self-repair as a normal hyaline cartilage, but as a fibrous tissue of poor mechanical quality.

At the present time, numerous studies are undertaken to design a suitable replacement material of the damaged cartilage.[6–9] Such a material must display physical and morphological properties as similar as possible to those of healthy cartilage; for instance, high tensile and compression strengths, low coefficient of friction, and appropriate pore size and distribution to allow cellular rehabitation. The ideal material should, in addition, promote cell attachment and proliferation,

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thus leading to the reconstruction of the natural tissue.[10]

Our work aims at developing new replacement biomaterials capable of mimicking as closely as possible most of the mechanical, structural, and biological properties of synovial fluid and cartilage. To face this challenge, the preparation of hydrophobically associating water-soluble derivatives of sodium HA and alginate (AA) was envisaged, based on our previous experience in this domain with other polysaccharides, i.e., propyleneglycol AA and pectin.[11–14]

The covalent anchoring of hydrophobic side-chains on water-soluble polysaccharides affords amphiphilic derivatives, that usually present associative properties in water and thus lead to solutions with high viscosity and, under certain conditions, to physically cross-linked gel-like networks. Highly viscous solutions may be regarded as substitutes for synovial fluid, to protect cartilage in the earlier stages of the disease. In the case in which cartilaginous wounds are already installed, hydrogels could then be used as replacement biomaterials.

Biological aspects (biocompatibility, nontoxicity, nonimmunogenicity, biodegradability, etc.) are imperious as well. In this respect, HA is an obvious choice because it is the most important natural polysaccharide found both in cartilage and synovial fluid. AA sodium salt, a polysaccharide extracted from brown seaweed algae, is another candidate to achieve our present goal. The use of a mixed HA/AA gel has been recently suggested, owing to the potential suitability of the resulting matrix for articular surgery applications.[15,16] Moreover, calcium alginate hydrogel has already been proposed as cell carrier for cartilage engineering.[7,17]

In the present work, the rheological properties of aqueous solutions of amphiphilic sodium AA and HA derivatives were investigated. The results obtained are described and discussed with regard to the schedule of conditions corresponding to a use as a synovial fluid substitute or a cartilage replacement biomaterial.

MATERIALS AND METHODS

Synthesis of the amphiphilic derivatives

The synthesis is derived from the procedure previously described by Della Valle et al.[18] Briefly, it consists of the reaction, in a homogeneous medium (dimethylsulfoxide), of an alkyl halide (here dodecyl or octadecyl bromide), with the carboxylic groups of the considered polysaccharides, preliminarily transformed into their tetrabutylammonium salts. The long alkyl chains are thus linked to the polysaccharide backbone via ester functions. The detailed synthesis procedure is described elsewhere.[19]

The nomenclature used is: P-C_n-x, where P is the polysaccharide of concern (AA for sodium alginate and HA for sodium hyaluronate), C_n the covalently bound long alkyl chain (n = 12 or 18), and x the substitution ratio (mol alkyl chain/100 mol monosaccharide unit). AA ($\overline{M_w}$ #250,000 g/mol; Fig. 1) from *Macrocystis pyrifera* was purchased from Sigma (France). HA ($\overline{M_w}$ #480,000 g/mol; Fig. 1) was obtained from Acros Organics (France).

Rheological experiments

Polymer aqueous solutions were prepared at various concentrations (from 3 to 20 g/L, depending on the polysaccharide, the alkyl chain length, and the substitution ratio) by gentle stirring in 0.15*N* NaCl for 24 h. Centrifugation (GR 2022 Centrifuge, JOUAN, Saint-Herblain, France) was subsequently performed (twice 20 min, 3000*g*) and samples, thus devoid of entrapped air bubbles, were then stored at 4°C overnight before rheological measurements were performed.

Experiments at imposed shear stress were performed on an SR 200 dynamic stress rheometer (Rheometrics Scientific, USA), fitted with a parallel plate geometry (25-mm diameter for hydrophobically modified derivative solutions; 40-mm diameter for the low viscosity parent polysaccharide solutions). The quantity of fluid on the plate was 2 mL (associative derivatives) or 5 mL (parent polymers). Temperature control (37° ± 0.1°C) was achieved with a Peltier system in the bottom plate.

RESULTS

The rheological characterization of a material generally comprises both studies in the flow and oscillatory modes. In the flow mode, the rheological behavior of non-Newtonian polymer solutions is classically evaluated by the variation of the viscosity, η, with the shear rate, $\dot{\gamma}$.

Special attention is given to the following parameters: 1. η_0, the viscosity at the first Newtonian plateau at zero shear rate; 2. $\dot{\gamma}_c$, the critical shear rate at which the first deviation from Newtonian flow occurs; 3. the

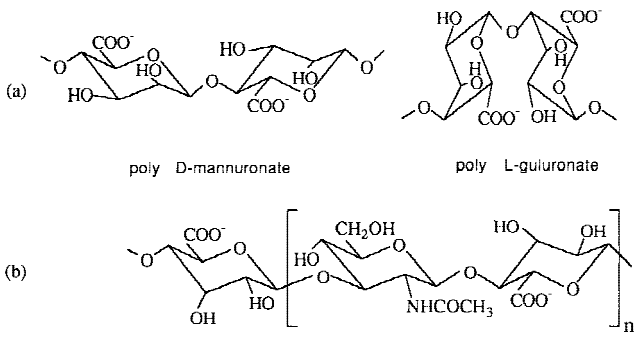

Figure 1. Chemical structures of mannuronate and guluronate units found in AA (a) and of HA (b).

slope in the region where viscosity decreases, giving an indication of the material tendency to pseudoplasticity; and 4. $\eta\infty$, the viscosity at the second Newtonian plateau at high shear rates (rarely observed, as such shear rates are usually out of the rheometer shear range).

Parent polymer solutions, respectively at 10 g/L for AA and 8 g/L for HA, display the typical behavior of Newtonain fluids. Their viscosity is quite low, respectively 0.02 and 0.07 Pa.s, and does not depend on the shear rate in the range 1–100 s^{-1} (curves not shown). Such a behavior is generally observed for solutions of polysaccharides of low molecular weight or at low concentration (C_p). However, it becomes non-Newtonian with a shear-thinning tendency, as C_p or $\overline{M_w}$ is increased,[20,21] owing to a more entangled organization of the macromolecular chains.

Figure 2 displays the variation of η versus $\dot{\gamma}$ and versus σ (stress) for aqueous solutions of AA-C_{18}-1.3 at various concentrations. The main rheological parameters thus obtained in the flow mode for solutions of this derivative, as well as for AA-C_{12}-8 (curves not shown), are presented in Table I. Similarly, the main rheological parameters obtained for the solutions of sodium HA derivatives, in the flow mode are shown in Table II. For the HA derivatives, a slipping effect on the rheometer bottom plate with shear rate increase, hampered the determination of $\dot{\gamma}_c$.

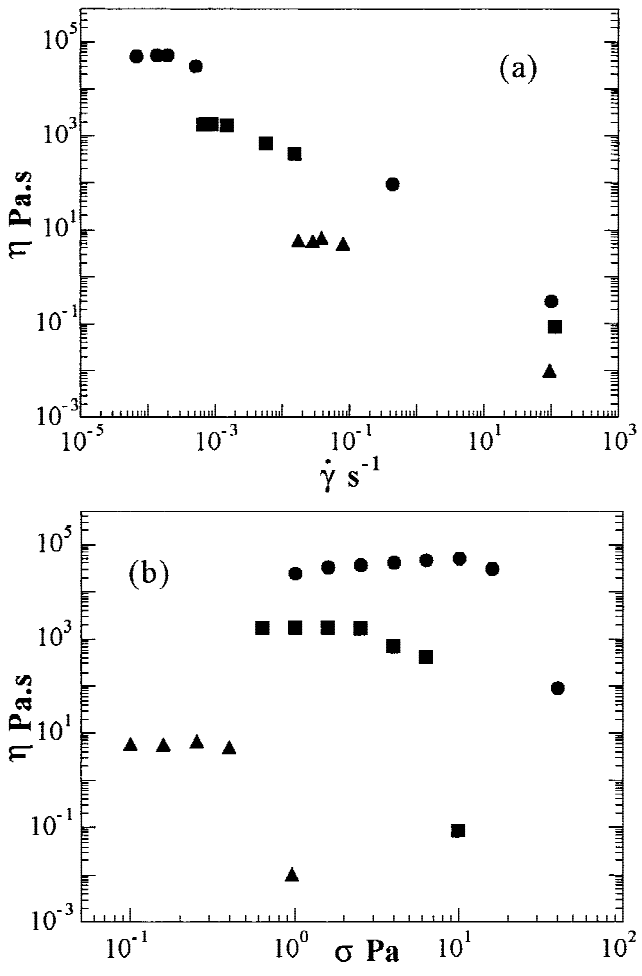

Figure 2. Viscometric behavior of AA-C_{18}-1.3 in 0.15*N* NaCl, 37°C, at three concentrations: ●, 10 g/L; ■, 5 g/L; ▲, 3 g/L. η, viscosity; $\dot{\gamma}$, shear rate (a); σ, stress (b).

As expected, in the concentration range under investigation, all solutions of AA or HA substituted by C_{12} or C_{18} alkyl chains exhibited tremendously enhanced viscosity at zero shear rate (η_0), i.e., 10^2- to 10^5-fold higher than those of the solutions of the corresponding parent polysaccharides at the same concentrations. Such an increase in η_0 is related to the superposition of two networks: one corresponding to the entangled polymer chains and the other one attributed to the presence of hydrophobic intermolecular associations, resulting in the formation of a three-dimensional network.[22]

These junctions are labile, as the hydrophobic association energy is low.[23] Thus, on further increasing the applied stress and above a critical value (σ_c, related to $\dot{\gamma}_c$), the onset of shear-thinning is observed. This phenomenon corresponds to the disentanglement of the polymer chains together with the breakdown of the intermolecular hydrophobic interactions.[24] Thus, for AA derivatives, viscosity was found to decrease approximately as $-0.9\ \dot{\gamma}$, thus evidencing the pseudoplastic character of the solutions.

When a polymer solution is subjected to an oscillatory stress, two parameters can be determined: G', the storage modulus, and G'', the loss modulus. G' characterizes the elastic response of the material and quantifies the energy stored by the material after the sinusoidal solicitation. G'' is related to the viscous response of the material and represents the energy dissipated during the process.

Experiments in the oscillatory regime were performed with the amphiphilic derivatives and the results were compared with those corresponding to the parent polymer. The concentrations were 10 g/L for AA and AA derivatives, and 8 g/L for HA and HA derivatives, and the frequency range under investigation was 0.1–10 rad/s.

As expected, for both parent polymers (AA and HA), the slopes of G' and G'' versus ω (log/log coordinates) were close to respectively 2 and 1 (results not shown) in agreement with theoretical predictions. These polymer solutions behave as viscous fluids, and under the effect of Brownian motion, a complete relaxation of the chains can be obtained within the time scale of the experiment.[24,25]

Figures 3 and 4 show the variation of G' and G'' versus ω for solutions of AA-C_{18}-1.3 (Fig. 3), HA-C_{12}-5 [Fig. 4(a)], and HA-C_{18}-1 and HA-C_{18}-2 [Fig. 4(b)]. These curves illustrate the rheological behavior of amphiphilic polysaccharides. The main rheological parameters determined from the entire study in the os-

TABLE I
Rheological Parameters of Amphiphilic Derivatives of Sodium AA (0.15*N* NaCl, 37°C)

Polymer	AA	AA-C_{12}-8			AA-C_{18}-1.3		
C_p (g/L)	10	10	15	20	3	5	10
η_0 (Pa.s)	0.07	4500	20,000	50,000	6	1700	50,000
$\dot{\gamma}_c$ (s^{-1})	—	3×10^{-4}	3×10^{-4}	10^{-4}	8×10^{-2}	1.5×10^{-3}	2×10^{-4}
Slope	—	−0.7	−0.95	−0.85	−0.9	−0.95	−0.9
G' (Pa)	—	8	50	200	0.3	4	50
G'' (Pa)	—	2	15	50	0.1	0.9	8

C_p, polymer concentration; η_0, viscosity at the first Newtonian plateau; $\dot{\gamma}_c$, critical shear rate; slope, in the linear shear-thinning domain (η vs. $\dot{\gamma}_c$); G' and G'', storage and loss moduli, respectively, corresponding to the linear visco-elasticity domain.

cillatory mode are presented in Table I for AA and in Table II for HA.

For the solutions of AA-C_{18}-1.3 at 3, 5, and 10 g/L (Fig. 3 and Table I), in the investigated frequency range 0.1–10 rad/s, no crossing point (i.e., for which $G' = G''$) was observed. Even at the lowest (3 g/L) of the three concentrations studied, G' was higher than G'' and both moduli were almost constant in the investigated frequency domain. Aqueous solutions of HA-C_{18}-2 at 4 and 7 g/L followed the same trends [Fig. 4(b) and Table II]. All these rheological characteristics are typical of elastic Hookean solids and the samples visually appear like gels.

Another behavior was observed for the solutions of HA-C_{12}-5 [Fig. 4(a) and Table II] at 6, 8, and 12 g/L and for HA-C_{18}-1 at 7 and 8 g/L [Fig. 4(b) and Table II]. In the studied frequency range, G' and G'' increased with frequency (with $G'' > G'$) and crossed at ω_c. These solutions typically have visco-elastic behavior. However, in the terminal zone, G' and G'' do not vary as ω^2 and ω, as classically observed for a Maxwell fluid. For instance, the slopes of G' and G'' versus ω (log/log coordinates) are 0.8 and 0.45, respectively, for the HA-C_{12}-5 solution at 8 g/L [Fig. 4(a)]. This phenomenon is generally attributed to the superimposition of both the disentanglement of the polymer chains and the disengagement of the apolar segments from hydrophobic associations.[25]

Various parameters can modify the rheological properties of associative polymer solutions in the flow and oscillatory modes. These parameters can be intrinsic, such as the alkyl chain length or the substitution level, or extrinsic, such as the polymer concentration or the ionic strength in case of polyelectrolytes.

As can be seen in Figure 2, η_0 and σ_c increase with the polymer concentration. As the polymer concentration is increased, the polymer chains become closer and closer, thus promoting the setting of intermolecular associations between the apolar segments. This leads to the formation of a more and more compact network, which provokes the increase in η_0 and σ_c.[26] The mechanical spectra of the AA derivative solutions (Fig. 3) confirmed the network densification. It is furthermore observed that, when concentration increased, the difference between G' and G'' increased, indicating that the hydrogels are more and more elastic.

The same phenomenon was observed for HA-C_{12}-5 solutions [Fig. 4(a)]. Their mechanical behavior remained typically visco-elastic in the frequency range, but G' and G'' increased with concentration whereas the crossing point ω_c was shifted to a lower frequency.

It is obvious that the rheological properties of AA and HA derivative solutions can be easily modulated by varying the level of substitution. For instance, by increasing the content in C_{18} chains from 1 to 2% on HA backbone, the typical visco-elastic solution was

TABLE II
Rheological Parameters of Amphiphilic Derivatives of Sodium HA (0.15*N* NaCl, 37°C)

Polymer	HA	HA-C_{12}-5			HA-C_{18}-1		HA-C_{18}-2	
C_p (g/L)	10	6	8	12	7	8	4	7
η_0 (Pa.s)	0.02	15	40	140	7	30	450	5000
G' (Pa)	—	4	9	30	1.5	5	6	20
G'' (Pa)	—	4	7	27	2.2	5	2	4
ω_c (rad/s)	—	1.6	1	0.6	>2	1.2	—	—

See Table I for symbols. ω_c, crossing point frequency ($G' = G''$).

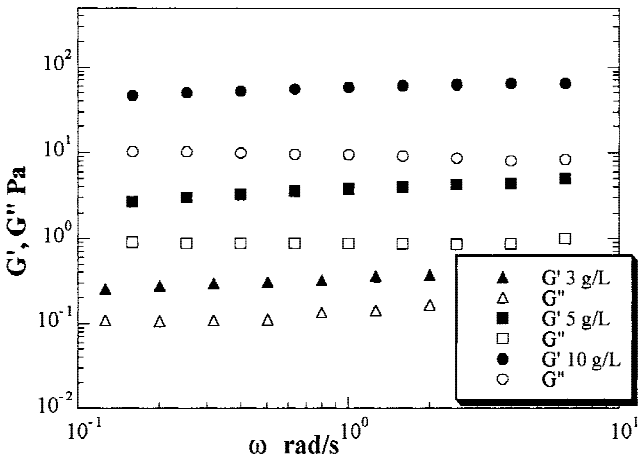

Figure 3. Mechanical spectra of AA-C_{18}-1.3 in 0.15*N* NaCl, 37°C. G', G'', elastic and viscous moduli, respectively; ω, frequency.

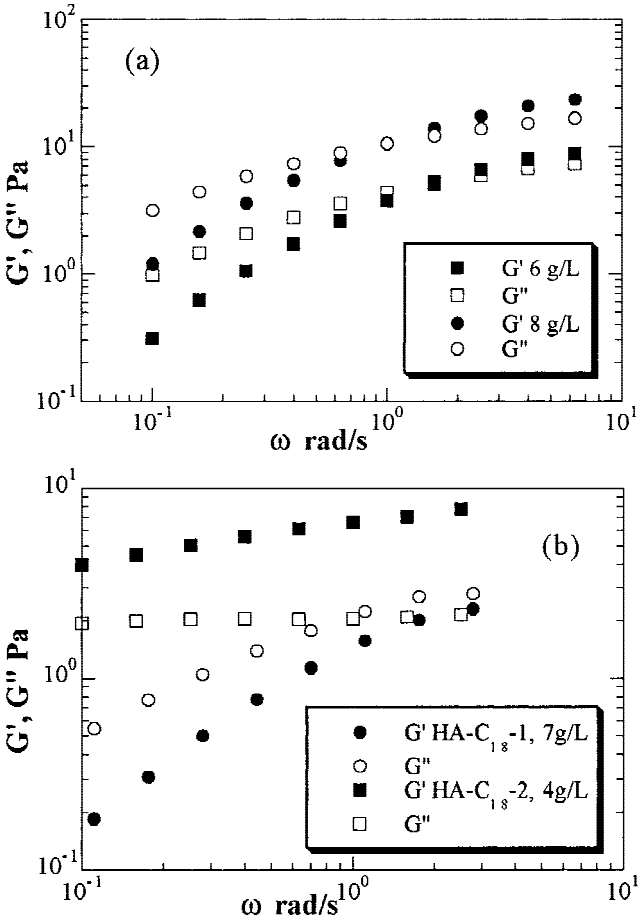

Figure 4. Mechanical spectra of HA-C_{12}-5 (a) and of HA-C_{18}-1 and HA-C_{18}-2 (b) in 0.15*N* NaCl, 37°C.

transformed into a gel [Fig. 4(b)]. Theoretically, the high-frequency maximum of the *G*″ versus ω curve is related to a characteristic time T_d, called the relaxation time, which corresponds to the time required for a polymer chain to escape from its tube by curvilinear diffusion.[22] This maximum being rarely observed, the value of T_d is usually calculated as the reciprocal of ω_c. From Figure 4(b), T_d is found to be 0.3 s for HA-C_{18}-1 solution at 7 g/L. For HA-C_{18}-2 at the same concentration, it is observed that T_d is greater than 10 s as this sample displays an elastic behavior in the frequency range 0.1–2 rad/s, which means that the crossing point is below 0.1 rad/s. This proves that an increase in the substitution level provokes the formation in solution of a more compact network, in which the polymer chains cannot freely relax. The result is a high increase in the value of the relaxation time.

DISCUSSION AND CONCLUSION

All the above results confirm that it is possible to tailor AA or HA derivatives, so that their solutions can have controlled rheological properties. Two kinds of potential applications can thus be considered: 1. use as a visco-supplementation fluid (substitute for synovial fluid); and 2. use as a biomaterial for cartilage replacement.

Viscosupplementation

Synovial liquid exhibits different rheological properties depending on whether it is healthy or degenerated. These properties are illustrated in Figure 5(a,b), for the flow and oscillatory modes, respectively. Some of the main rheological parameters of human synovial fluid, healthy or pathologically deteriorated, are presented in Table III.

Synovial fluid is a highly visco-elastic fluid responsible for lubrication and damping in joints. Because of the flexible molecular network formed by entangled high molecular mass HA chains, healthy synovial fluid exhibits a high viscosity at the first Newtonian plateau and a pronounced shear-thinning character.[5] In the frequency range 0.1–10 rad/s, healthy synovial

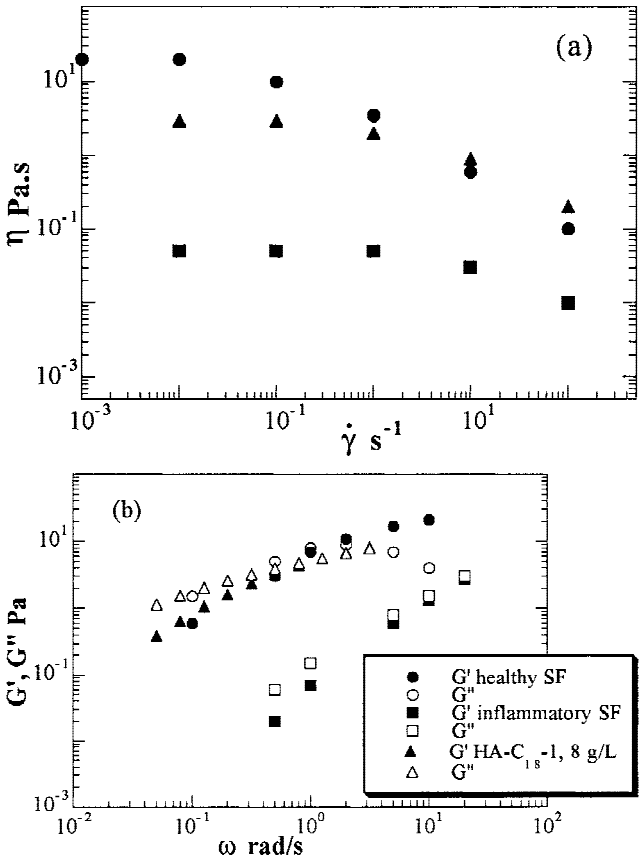

Figure 5. Rheological properties of synovial fluid (SF) at 37°C. (a) Viscometric behavior, ●, healthy; ▲, degenerated; ■, inflammatory. From Balasz and Gibbs.[27] (b) SF mechanical spectra (from Schurtz and Ribitsch[5]) compared with that of HA-C_{18}-1, in 0.15*N* NaCl.

TABLE III
Rheological Parameters of Different Synovial Fluids (SF) and of Amphiphilic HA Solutions

	η_0 (Pa.s)	$\dot{\gamma}_c$ (s^{-1})	Gel Point ($G' = G''$) (Pa)	ω_c (rad/s)
Healthy SF	1–40[a]	0.01–0.025[a]	1–8[b]	1–2[b]
Degenerated SF	0.1–1[a]	0.05–0.125[a]	—	—
Chronic inflammatory SF	0.004–0.01[a]	1–50[a]	0.2–0.5b	>20[b]
HA-C_{12}-5[c]	40	—	10	1
HA-C_{18}-1[c]	30	—	6	1.2

See Tables I and II for symbols.
[a]Schurtz and Ribitsch.[5]
[b]Balazs and Gibbs.[27]
[c]8 g/L, 0.15*N* NaCl, 37°C.

fluid behaves as a visco-elastic fluid and, in the frequency range corresponding to the motion of the knee joint in walking and in running (approximately 2–8 rad/s), G' is higher than G''.[27] In contrast, in pathologically deteriorated synovial fluids, the molecular weight of HA is at least 10-fold lower than in healthy fluid; consequently, the polymeric network is altered, and all the fluid rheological properties are strongly modified: viscosity is greatly reduced, the critical shear rate is enhanced, and pseudo-plasticity is lost. In the frequency range 2–8 rad/s, G' is lower than G'', and there is no gel point.

For the solutions of amphiphilic AA, a shear-thinning character was observed (slope ranging from −0.85 to −0.95), similar to that of healthy synovial fluid (slope # −1). Although this phenomenon could not be observed with HA derivatives because of the slipping effect occurring during the rheological analysis, it is assumed that, because the properties of the HA compounds were quite comparable to those of AA derivatives, the shear thinning character would be similar. Moreover, considering the rheological properties of certain amphiphilic derivatives of HA (Table III), it is clear that, at 8 g/L in 0.15*N* NaCl, 37°C, they can be regarded as potential substitutes for synovial fluid.

Cartilage replacement

The aim of this work was to propose materials with rheological properties suited to a use in cartilage wounds and which could promote chondrocyte colonization and proliferation, together with the synthesis of a healthy neomatrix.

Tables I and II and Figures 2, 3, and 4, show that the rheological properties of amphiphilic polysaccharide solutions can be modulated by means of various parameters such as the length of the immobilized alkyl chain, the substitution ratio, and the solution polymer concentration. Moreover, experiments on the association–dissociation dynamics of these derivatives were performed: they consisted in subjecting a viscous polymer solution to a high shear rate to strongly decrease viscosity. Then a lower shear rate was applied, and the time of initial viscosity recovery was determined. Thus, for instance, the viscosity of an AA-C_{12}-8 solution (15 g/L, 0.15*N* NaCl, 37°C) was dropped from 12,000 to 3 Pa.s by applying a high stress (30 Pa) for 600 s. Then the stress was lowered to 3 Pa and the solution recovered 70% of its initial value after about 300 s and 100% after 600 s. Under similar conditions, the time for 70% viscosity recovery of an HA-C_{18}-2 solution (4 g/L), was 80 s. These characteristics are quite attractive for the envisaged applications because: 1. the viscous solutions or hydrogels can be administered or implanted, simply using a syringe, owing to their steep shear-thinning behavior; 2. accordingly, wound outlines are perfectly filled; and 3. after injection, the fluid recovers its initial consistency within a short period of time.

From the rheological point of view, it is clear that some of the described products can fulfill numerous points of the schedule of conditions related to materials useful for cartilage repair, either for viscosupplementation or for cartilage replacement.

The biological behavior of such materials is another point still more important to evaluate. This topic has been investigated in the perspective of using the hydrogels as cartilage replacement materials. Some amphiphilic HA derivatives have been extensively tested, after sterilization, in *in vitro* experiments. It was found that sterilization (dry form, 121°C, 1 bar pressure, water-saturated atmosphere) caused a slight modification of the rheological properties because of the chemical degradation of the polysaccharidic backbone. In contrast, after 6-month storage (hydrogel form, 4°C), no degradation was observed. It was also proved that the derivatives were not toxic toward chondrocytes, and that they favored the cellular proliferation and the synthesis of glycosaminoglycans. Moreover, the corresponding hydrogels were implanted in rat knees according to a described model of osteochondral defect. The microscopic analysis of the repaired tissue showed that it was similar to hyaline cartilage. Moreover, a good integration of the hydrogels in the defect was observed. The results of these various biological experiments will be reported in detail in forthcoming articles.

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