

Article

Role of Different Pre-Treatments on Composition and Rheology of Synovial Fluids

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Abstract: Different pre-treatments applied to synovial fluids (SF) before their analyses are tested to characterize SF after storage under different conditions and to investigate their evolution along a viscosupplementation treatment. The main techniques proposed involve steric exclusion chromatography with triple detection (SEC) and viscometry; it is the first time that such a study is developed. SEC gives the molecular weight distribution and concentration of hyaluronan (HA) and proteins separately; the steady state viscosity is always non-Newtonian and not directly related to SF composition. Pre-treatment of SF (storage in cold, filtration, centrifugation) allows us to conclude that, in order to store SF, it is best to freeze it, even if in some cases, viscosity is modified but not the composition. All the data obtained (including protease pre-treatment) allow us to conclude that a small fraction of HA-protein complex forms a loose 3D-network and controls the rheology.

Keywords: hyaluronan; steric exclusion chromatography; rheology; centrifugation; freezing; filtration; protease treatment

1. Introduction

Synovial fluid (SF) is a viscoelastic system with a characteristic non-Newtonian behaviour, consisting of hyaluronan (HA) and proteins, at least partly involved in a loose physical 3D-network based on secondary interactions of electrostatic nature [1]. The existence of such a complex has been discussed at length in the literature [2-8] and it is still under debate: on one hand, non covalent protein-HA interactions seems to be admitted by many authors at neutral pH [3-8]; on the other hand, HA and proteins are claimed to be separated in the SF fluid [9] and other authors consider that the rheological behaviour depends essentially on the HA contribution [10,11]. In healthy adults, HA is claimed to have a molecular weight (MW) in the range of 4 to 5×10^6 with a concentration ranging from 2.5 to 4 mg/mL [12-16]. This means a total amount of HA ranging from 4 to 8 mg per human knee joint, since the volume of SF is assumed to be 1 to 2 mL in normal conditions [12,13]. This natural polysaccharide plays an important role in lubrication, shock absorption and viscoelastic behaviour of SF [3,4,13]. From literature, it is clear that there is a direct relationship between the severity of the disease and the rheological properties of SF, which are usually related to the characteristics of HA (both concentration and molecular weight). In inflammatory and degenerative joint diseases, mainly osteoarthritis (OA) and rheumatoid arthritis, the MW and concentration of native HA are reduced. At the same time, the viscosity of corresponding SF also decreases [8,12,17-19]. However one part of this decrease might be attributed to changes in the SF protein composition which is secondary to inflammation. Indeed HA is an anionic polyelectrolyte, able to interact with positively charged groups of proteins even at a very low level (it was estimated as 2% protein bound to HA) [8].

In fact, the local stiffness of HA (characterized by its ability to form H-bonds and a relatively high persistence length of approximately 8nm [20]) and its often high MW gives a large hydrodynamic volume and consequently a high viscosity in aqueous solution [21-23]. Its conformation and/or degree of expansion in solution is sensitive to ionic concentration, especially at a low level. In biological conditions, this effect can be neglected due to the screening of electrostatic intra-chain repulsions [21,22]. In salt excess (0.1 or 0.15 M NaCl), it was shown that concentration and high molecular weight of HA also impose the critical conditions for entanglements to occur and limit its viscoelastic character [23]. However, globular proteins have a very low contribution to the viscosity of aqueous solutions. Therefore it is clear that HA is an important polymer to study in relation to the behaviour of SF. HA concentration in SF is often determined by titration of hexuronic acid [10], but in the absence of direct determination of HA molecular weight, it has been suggested to determine the viscosity of synovial fluid as a function of HA concentration. One can in turn extrapolate to zero concentration in order to get the intrinsic viscosity, allowing us to compare the samples from different patients [10,12].

Viscosupplementation (VS) is a therapeutic concept involving intra-articular injections of a high MW HA [13]. VS is aimed to decrease OA pain and improve joint function by restoring SF physiological

properties [24]. It is widely used in the treatment of knee and other joints affected by OA, but very little is known about the real changes of SF after HA injections. For this type of investigation, it is necessary to perform a study on OA synovial fluid before and after exogenous HA injections. Such a study necessitates storing the samples in convenient conditions and over a long period of time.

In this paper, we will discuss the influence of different pre-treatments such as temperature of storage, filtration and centrifugation on linear free HA characteristics on one hand, and the composition and rheological behaviour of OA SF on the other hand. This study is important in order to follow the evolution of SF during treatment of viscosupplementation, as well as to monitor the turnover of HA over time.

2. Results and Discussion

Our objective is to relate the composition of SF in proteins and HA with its rheological behaviour. It is known that even at high concentration proteins contribute minimally to solution viscosity (due to their low intrinsic viscosity around 3 mL/g). As commonly seen in the literature, the rheological behaviour of SF is claimed to be related to HA characteristics (concentration and molecular weight). Nevertheless, as was previously shown, HA-protein complex (even if only small amount of polymers is engaged) exists in SF which causes a particular non-Newtonian behaviour [3,25]. We have recently studied the influence of fibrinogen as a model of protein on the rheology of HA, and demonstrated the physical interaction at neutral pH which should be based on electrostatic interactions [1]. The viscometric characterization of such systems does not allow the direct determination of the role of HA molecular weight and/or concentration on the SF behaviour if the proteins are not completely separated from HA as previously claimed by Ogston and Sherman [7]. To avoid artefact, it was discussed by Sundblad [12] that the intrinsic viscosity of HA obtained by dilution and extrapolation to zero concentration (when the HA concentration is determined separately) is a good information, allowing us to compare SF characteristics corresponding to different diseases. From our research to date, the estimation of the intrinsic viscosity from a Huggins-type relationship at a finite concentration with a Huggins constant around 0.16 [12] gives a rough value due to the fact that for free HA in salt excess, the Huggins constant equals 0.4 [23]. Thus, the relation between intrinsic viscosity and MW is needed and it introduces the difficulty of having an absolute determination of the HA molecular weight [23]. Light scattering is the more powerful and adapted method to get MW values on well purified and calibrated samples, and the values obtained can be used as standard, allowing us to interpret results from volume of elution in SEC or mobility in electrophoresis after pronase treatment [26,27]. It is important to have a more direct determination of the composition of synovial fluids. To determine the composition of SF, a relatively new technique was proposed [28]: steric exclusion chromatography (SEC) which separates the components following their hydrodynamic volume. Different conditions are indicated in the literature: direct injection after dilution [26,29,30] or dilution and incubation with pronase [26,31] before the SEC experiment, using a light scattering detector or calibration with standards. The influence of such protease pre-treatment before SEC analysis will be also examined in this paper.

Finally, to study the properties of synovial fluids and particularly their rheological properties, it is important to store the samples after isolation in clearly defined conditions which do not modify their

characteristics (composition and viscosity). Given this objective, the influences of storage at 4 °C, freezing at -21 °C and thawing were examined; the same treatments were also tested on pure HA, used as reference.

2.1. Composition of synovial fluid by SEC

The conditions adopted in this work for SEC experiments allow us to separately determine the characteristics of HA and proteins in a very dilute synovial fluid [25] (Figure 1). Identification was completed using a ultra-violet detector (UV) permitting us to conclude that proteins are (in most cases) completely separated from linear HA (Figure 2). These results confirm the data from literature obtained with only one different column (Superose 6 from Pharmacia [29] or TSK 6000PW from Toyo Soda [28,30]).

Figure 1. SEC chromatogram of a synovial fluid allowing the determination of soluble proteins and HA molecular weights and their concentrations. T = 30 °C, eluent 0.1 M NaNO₃. LS is the light scattering signal (red); AUX 1 is the refractive index signal (blue); AUX 2 is the viscometric signal (green).

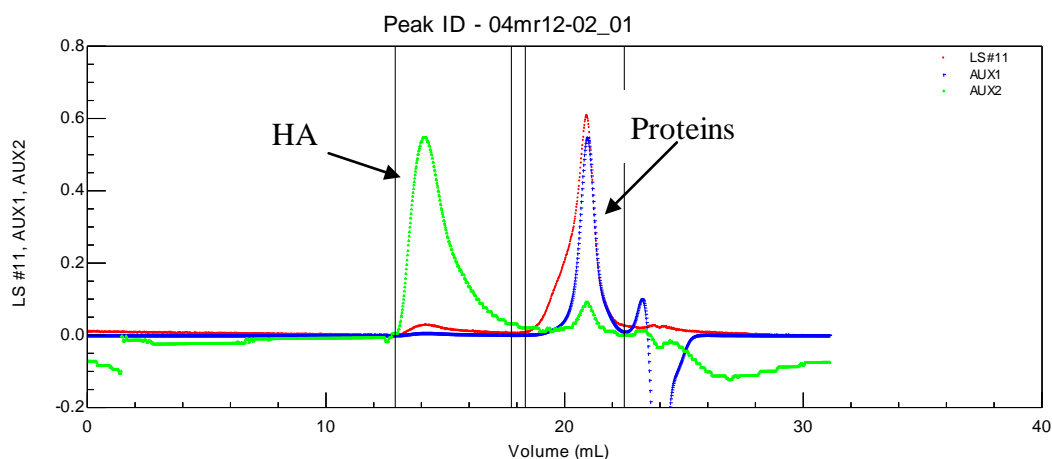
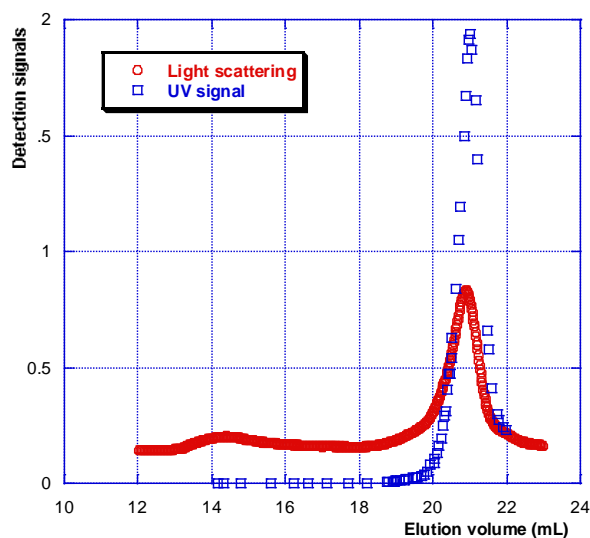


Figure 2. Specific UV identification of protein elution in SEC experiment on a synovial fluid.



HA is eluted first with a large hydrodynamic volume and a high molecular weight as shown by the large light scattering and viscosity signals under the eluted peak. Secondly, the protein pool is eluted, with a large signal for weight concentration (refractive index signal) and very small signals for viscosity and light scattering. Some of the data obtained for the composition and viscosity of different synovial fluids (named LS_n) are given in Table 1. Values of the molecular weight characterizing HA and proteins at their respective concentration peak are also given in Table 2. Due to the low concentration of HA in SEC analysis, the integrals of refractive index and light scattering signals for the weight-average molecular weight calculation remain small. Better precision to compare SF is obtained at the concentration peaks of HA and proteins. Nevertheless, a good agreement is found between the different series of data (see Tables 1 and 2).

Table 1. Characteristics of different synovial fluids obtained by SEC after different pre-treatments.

	[HA] g/L in SF	Mw HA × 10 ⁻⁶	*Average C*Mw × 10 ⁻⁶	[Proteins] g/L in SF	Mw Proteins × 10 ⁻⁵	Viscosity (Pa.s) at 1 s ⁻¹
LS 1						
Initial	1.57	1.6	2.35	29	1.03	0.737
Frozen	1.9	1.08	2.05	30	1.18	0.978
Filtrated on 0.8 μm	1.8	1.2	2.00	30.5	1.13	0.630
Initial after centrifugation	1.9	1.26	2.28	28.6	1.23	0.830
LS2						
Initial	1.13	1.25	1.41	29.5	1.42	0.059
Frozen	1.18	1.2	1.21	29.3	1.23	0.061
Filtrated on 0.8 μm	1.2	1.07	1.12	30.5	1.41	0.043
LS3						
Initial	0.72	0.95	0.75	28.2	1.12	1.792
Frozen	1.25	1.12	1.51	30.1	1.33	1.677
Filtrated on 3 μm	0.82	1.08	0.78	21.2	1.24	0.364
LS4						
Initial	0.68	1.12	0.78	28.3	1.12	1.601
Frozen	1.2	1.10	1.43	30.2	1.31	1.492
Filtrated on 3 μm	0.71	1.20	0.77	26.0	1.30	0.458
Initial after centrifugation	1.5	1.06	1.78	31.1	1.39	1.342
LS12						
Initial	0.51	1.65	0.79	29.5	1.28	0.066
Frozen	0.45	1.07	0.48	28.0	1.27	0.031

C*Mw is given with C in g/L and Mw en g/mol.

In all cases, the intrinsic viscosity of proteins is found equal to 2.9–3.1 mL/g (data not shown) in agreement with a globular conformation and a weight-average molecular weight around 100,000 (Table 1). M value at peak is around 85,000 (Table 2). The effective separation on the SEC columns is justified when the product M [η] representing the hydrodynamic volume of the different polymers is estimated: the molecular weight of hyaluronan is in the range of 1 × 10⁶ with an intrinsic viscosity in the

range of 2,000 mL/g. In conclusion, the product $M[\eta] \sim 2 \times 10^9$ (mol.mL) is very high compared to that of proteins ($M[\eta] \sim 3 \times 10^5$ (mol.mL)) allowing the effective separation in SEC.

Table 2. Molecular weight of HA and proteins at the maximum of concentration peak signal in SEC.

References for SF	M of HA at peak	M of Proteins at peak
LS1 initial	1,560,000	83,000
after freezing	1,277,000	83,400
filtrated	1,547,000	
after centrifugation	1,397,000	
LS2 initial	1,450,000	86,750
after freezing	1,035,000	85,800
filtrated	1,235,000	
LS3 initial	1,276,000	87,100
after freezing	1,218,000	88,500
filtrated	1,228,000	
LS4 initial	1,611,000	88,300
after freezing	1,680,000	89,600
filtrated	1,298,000	
after centrifugation	1,217,000	

From Table 1, it is concluded that there is no significant difference neither between the values of concentration and molecular weight for HA in the different SF tested, nor for proteins characteristics. The molecular weight and concentration of HA in initial OA SF are between 1 to 1.6×10^6 (g/mol) and 0.6 to 1.6 g/L respectively. These results are in agreement with those obtained previously on a wide range of SF, showing that the concentration of HA varies from 0.2 to 2.5 g/L and soluble proteins from 20 to 35g/L [25]. The values of Mw found for the different HA are also in good agreement with the data from the literature (1 to 3×10^6 g/mol), especially when the authors use the SEC analysis [28-30]. In addition, the products C^*M_w obtained for the different SF tested can be related to the viscosity measured at a given shear rate (here 1 s^{-1}) (Table 1). It is clear that these values are not following a parallel trend: for example, LS1 and LS2 with higher C^*M_w have lower viscosity compared with LS3 and LS4. This indicates that different interactions with proteins exist in the different SF which control rheology, even if the yield and average Mw of these proteins are of the same order of magnitude and are probably engaged in the complex at a low rate. As suggested before, the interaction is based on random electrostatic interaction between positive sites in proteins and anionic sites of HA.

It is interesting to discuss the relation between composition and viscosity of SF: the larger viscosities are obtained with initial LS3 and LS4 which have lower contents in HA compared with LS1 and LS2 (even if the Mw is of the same order of magnitude) as mentioned previously (Table 1). This confirms that some interactions between HA and proteins may cause the increase of the effective viscosities. Considering the values of Mw and HA concentration given in Table 1 (from 0.6 to 1.6 g/L and $M_w = 1$ to 1.6×10^6), it is possible to estimate the viscosity of a HA solution (at same concentration and molecular weight) in the absence of proteins, from the relation between viscosity and the product ($M_w \cdot C$) as given in the literature (but only valid in the Newtonian plateau) [21,23]. We note that the calculated values are much lower than the measured ones, confirming the existence of

hyaluronan-protein interactions to form a loose physical network. For LS3, the calculated viscosity equals 2×10^{-3} Pa.s, while the experimental value is 1.79 Pa.s; for LS12, it is found 2.2×10^{-3} Pa.s when the measured value is 6.6×10^{-2} Pa.s. Some other values are given in Table 3, confirming the large difference between calculated and experimental viscosity values. These values also confirm that the product $C \cdot Mw$ is not in direct relation with the viscosity of SF. It is the first time that this type of experiments (SEC associated with rheology) is developed on a large series of synovial fluids allowing us to draw a clear conclusion.

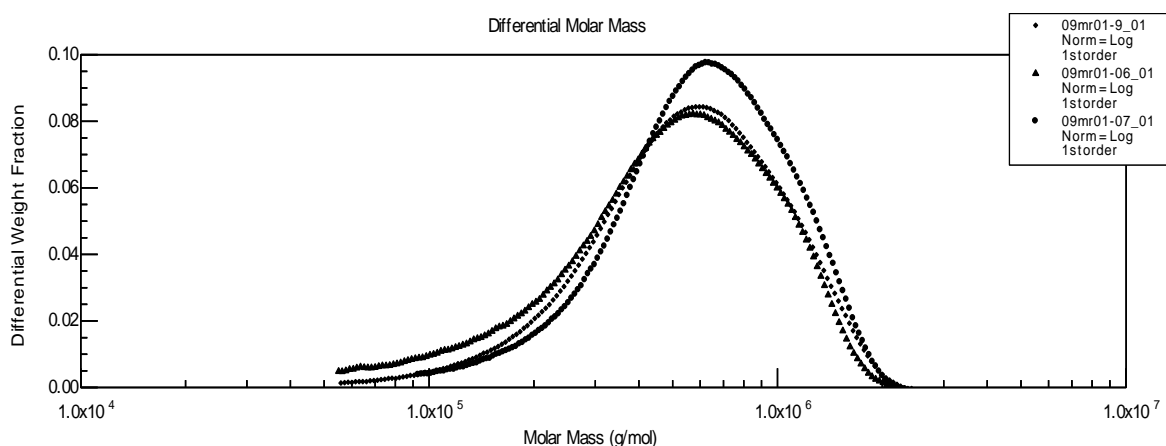
Table 3. Characteristics of different synovial fluids obtained by SEC analysis and viscometry at 25 °C. Comparison with calculated viscosity values.

	Mw HA $\times 10^{-6}$	C HA g/L	Mw Proteins $\times 10^{-5}$	C Proteins g/L	C*Mw $\times 10^{-6}$ for HA	η Pa.s (1 s^{-1}) at 25 °C
LS5	3.36	2.3	0.94	18.8	7.7	2.98 (calc: 0.176)
LS6	1.53	2.1	1.23	24.7	3.2	1.66 (calc:0.024)
LS7	3.44	1.4	1.35	33.5	4.8	0.49 (calc:0.043)
LS8	1.14	1.9	2.20	35	2.1	0.22 (calc:0.003)
LS9	1.45	1.1	2.90	32	1.6	0.08 (calc:0.002)

2.2. Stability of linear HA and SF at 4 and -21 °C.

We were interested in testing the stability of SF stored at 4 °C or frozen (-21 °C) after collection from a patient, so as to be able in the future to investigate large series of SF along viscosupplementation treatments. These two storage techniques are mentioned in the literature [28-30]. As a model, a linear HA was tested in the same conditions and the results obtained for molecular weight distribution are given in Figure 3.

Figure 3. Influence of pre-treatment on molecular weight distribution of pure hyaluronan. \blacktriangle Initial linear HA; \bullet linear HA stored at 4 °C; \blacklozenge HA after freezing at -21 °C.



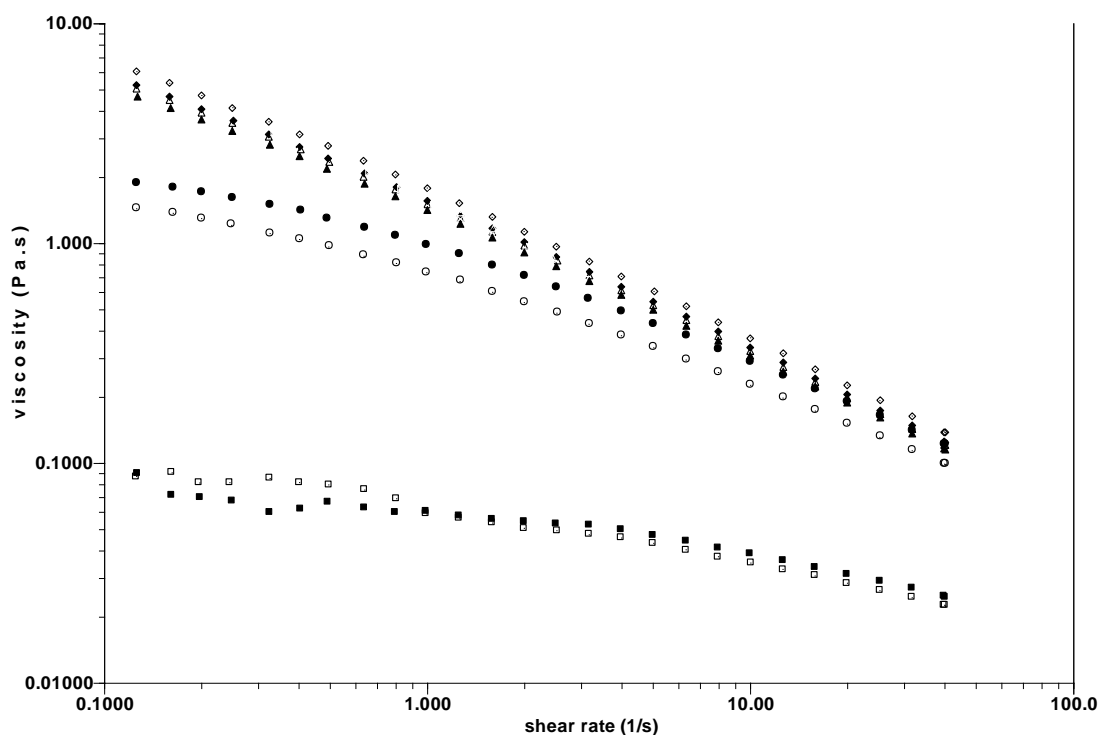
There is a near overlapping of the molecular weight distributions as well as of viscosity data: Mw of the initial polysaccharide after direct dissolution equals 644,000, after one month at 4 °C it gives

Mw = 633,000, and after freezing and thawing Mw = 600,000. From these results, our conclusion is that for a pure HA, the solution properties and molecular weights are quite independent from the conditions adopted to store the samples. Nevertheless, for longer storage times, it seems advisable to recommend freezing HA solutions.

From our previous results on SF stability [25], it was shown that SF kept at 4 °C is stable over a long period of time (2 months). This agrees with recommendations from Balazs [10,16,32]. But to preserve samples, it is recommended to freeze them for longer storage time; this technique is adopted by other authors [28-30]. It is important to demonstrate that the pre-treatment used has no significant influence on the properties and composition of SF. For that purpose, the rheological behaviour of different SF tested before and after freezing is shown in Figure 4, and some viscometric experimental values are given at 1 s⁻¹ in Table 1.

The steady state viscosities of the initial SF studied at low shear rate vary in a ratio of around 100 and are always non-Newtonian even at lower shear rates than represented in Figure 4. The influence of freezing on the viscosity is shown clearly dependant on the nature of the synovial fluid considered. In one case (LS1), the viscosity after freezing increases in relation to a reinforcement of the network junctions, as observed previously in some polysaccharide physical gels. Self association of HA was mentioned in NaCl aqueous solution [33] but also at low temperatures [34,35]. For the SF named LS2, there is no modification whatsoever. For LS3 and LS4, a very small decrease of the viscosity corresponds to a small modification of the physical network.

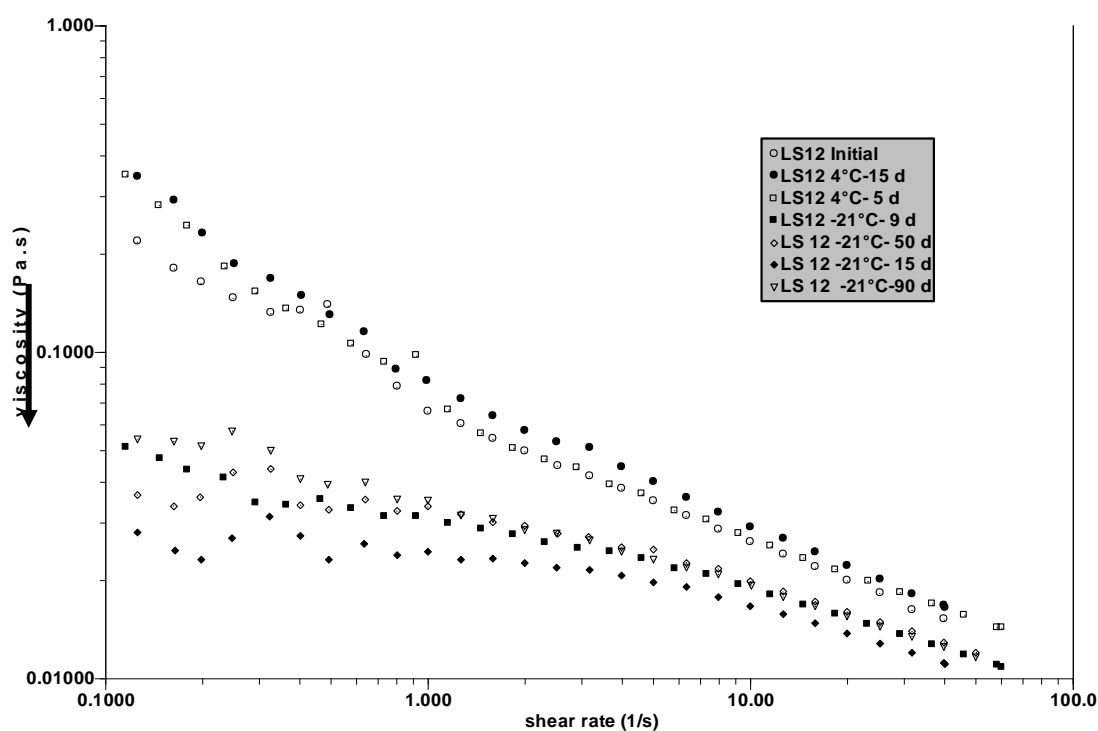
Figure 4. Influence of freezing at -21 °C on rheological behaviour of 4 different SF measured at 25 °C. Open signs correspond to the initial SF; the corresponding filled signs are the frozen SF. ○●LS1; □■LS2; ◇◆LS3; △▲LS4.



In a separated series of experiments, initial SF is divided in two parts: one is kept at 4 °C and the other is frozen at −21 °C. Measurements are performed after different periods of time to control the stability at 4 °C and to observe the role of successive freezing and thawing. The results are given in Figure 5. It is confirmed that the stability of SF is good at 4 °C after 15 days but, in this specific case, the first cycle of freezing and thawing causes a large decrease in the viscosity of the SF examined (named LS12).

After this first step, successive freezing does not significantly change the viscosity. The stability of SF at 4 °C is confirmed, but freezing destabilized the network in LS12 which has in fact the lowest initial viscosity (compared to the previous samples tested) and consequently the less stable 3 D-network. If the steady state viscosity as a function of the shear rate for LS2 (Figure 4) and LS12 (Figure 5) are compared, it is clear that they are quite different: LS12 has a large non-Newtonian behaviour, whereas for LS2 there is nearly no shear rate influence at low shear rate. This behaviour indicates more efficient protein-HA interaction (or a larger amount of complex formed) in LS12 forming a loose network which is destabilized by freezing (when LS2 is nearly not perturbed).

Figure 5. Influence of successive cycles of freezing (at −21 °C) and thawing after different periods of time (in days d) on the SF named LS12. Experiments for storage at 4 °C are joined. Measurements are performed at 25 °C. The arrow indicates the drop in viscosity after the first freezing.



The compositions of the SF after freezing are not clearly modified (Table 1). In some cases, molecular weight of HA appears to decrease (LS1 and LS12) and its concentration increases slightly (LS1, LS3, LS4). In these SF, proteins concentration also increases in relation to the disruption of HA- protein association, with only a small influence on the resulting viscosity. An exception is shown regarding LS12, where concentration, molecular weight of HA and viscosity decrease. In the literature,

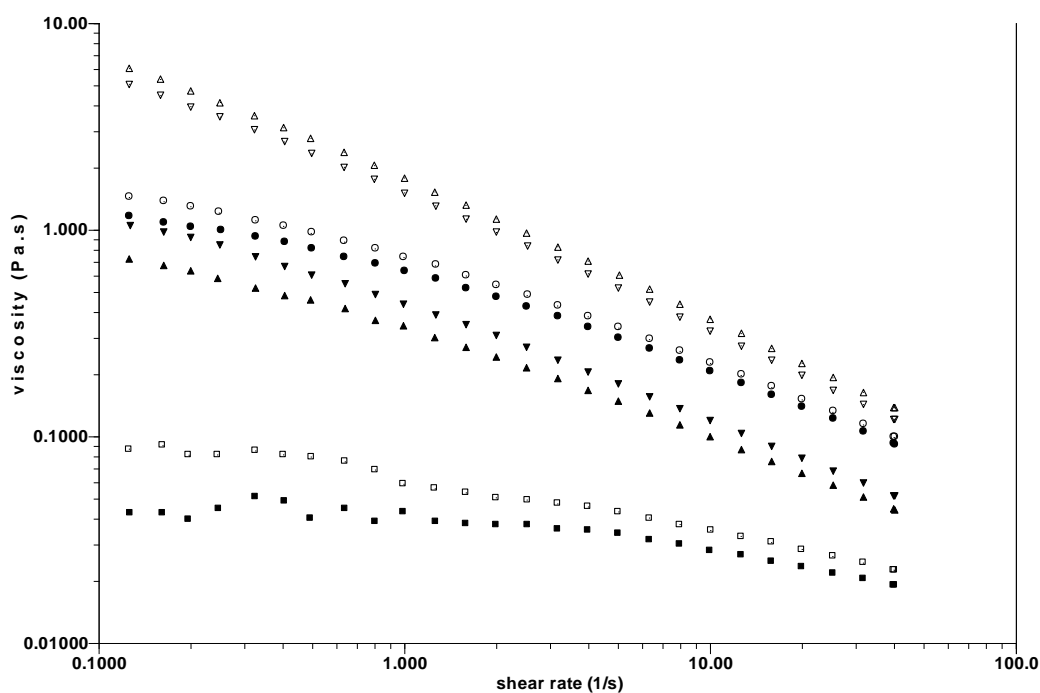
it was also previously found that freezing causes a small decrease in MW, passing from 1.3 to 1×10^6 (Table 5 in reference 29).

In conclusion, it is clear that the influence of freezing pre-treatment depends on the synovial fluid to be studied. But in the majority of cases, when the viscosity is not too low, freezing can be applied to store SF without significant modification.

2.3. Role of filtration and centrifugation on the composition and rheology of SF

Synovial fluid is constituted of HA and a small portion of proteins which form a physical network, playing an important role in the global viscosity as previously discussed. For the purpose of characterization, it is important to be able to separate these components to simplify the analysis. Centrifugation to separate cells followed by chemical analysis was proposed by Balazs [14,32], but it does not allow us to separate protein contribution. Otherwise, different pre-treatments were adopted to separate HA from proteins, from which an enzymatic hydrolysis allowed us to eliminate proteins [5,6,31,36]. In our analysis, we intend to compare treatments of filtration and centrifugation used before characterizing the SF especially by SEC. As a first step, SF were filtrated through porous membranes with pore diameters of $0.8 \mu\text{m}$ for LS2 and LS1, and with $3 \mu\text{m}$ for LS3 and LS4. Due to their much larger viscosity, meaning also a more cross-linked system, it was not possible to pass LS3 and LS4 through a porous $0.8 \mu\text{m}$ membrane in absence of dilution. Their viscosity was determined before and after filtration; all the values are given in Table 1 and Figure 6.

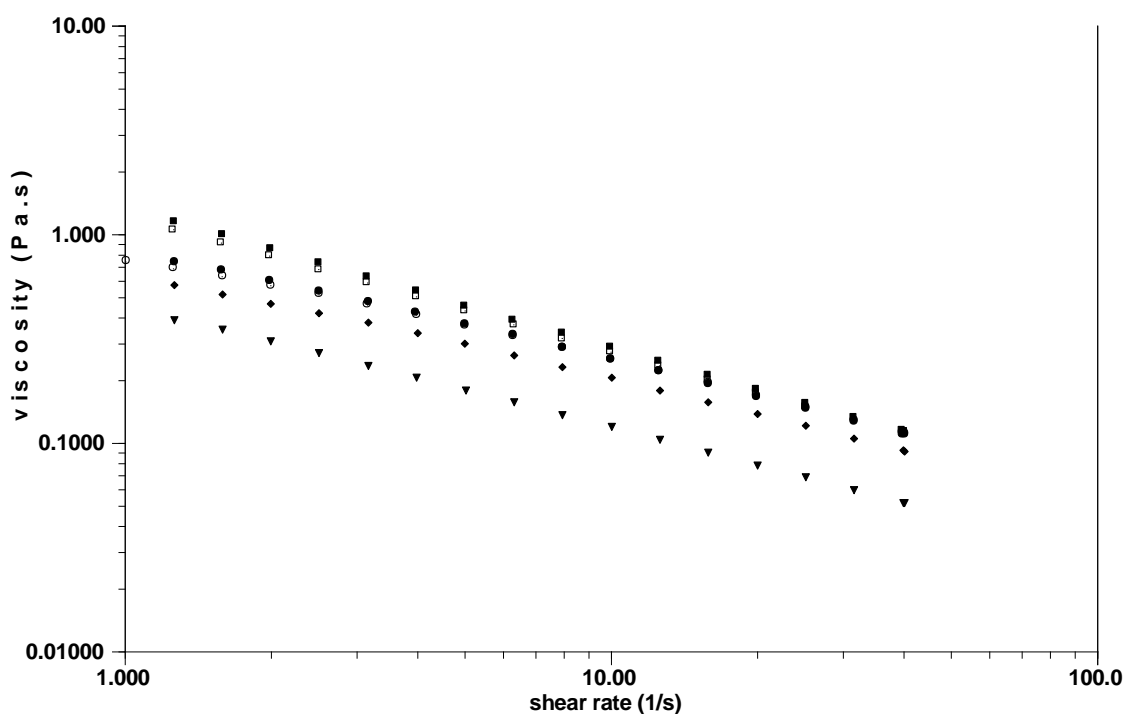
Figure 6. Influence of filtration on rheological behaviour. Open signs for the initial SF; corresponding filled signs correspond to the filtrated samples. \circ \bullet LS1; \square \blacksquare LS2; \triangle \blacktriangle LS3; ∇ \blacktriangledown LS4.



It is clear that filtration of SF directly after aspiration from the knee (in absence of dilution) has a very important role in the rheology of synovial fluid. Nevertheless, it again appears to depend on the nature of the SF tested: the decrease in viscosity is very large for LS3 and LS4, the more viscous samples, even if they were filtrated on larger pores membranes, but their protein content also decreases. On the other hand, much lower variation is obtained for LS1 and LS2 (values are given in Table 1). For all the samples, the concentration and molecular weight of HA on filtrated samples are in the same range of magnitude as for the initial SF.

Two of these SF were centrifuged at 50,000 g during 20 minutes at 4 °C. The small sediment was then isolated and weighted. The sediments from LS1 and LS4 represent around 3 w%. The rheological behaviour is barely modified compared to the initial state, corresponding to a stable network. It remains larger than that of the filtrated state, especially for LS4 (Figure7). Our conclusion is that centrifugation does not appear to modify the rheological properties of SF especially on highly viscous fluids in which the 3D- network looks stable.

Figure 7. Comparison of the influence of filtration and centrifugation on viscosity, as a function of shear rate for two synovial fluids at 25 °C. Respectively: initial, centrifuged and filtrated SF ○ ● ◆ LS1; □ ■ ▼ LS4.



The composition of SF after centrifugation was analyzed: the molecular weight distribution is not significantly modified but it is more sensitive when compared with the molecular weight at the peak signal of SEC: very small decreases in M are obtained (Table 2). As a conclusion, it is shown that only small modifications are observed between the content and molecular weight of HA obtained after filtration or after centrifugation, in comparison with their initial characteristics. From these results, it is concluded that there is no separation between proteins and HA isolated and/or engaged in the

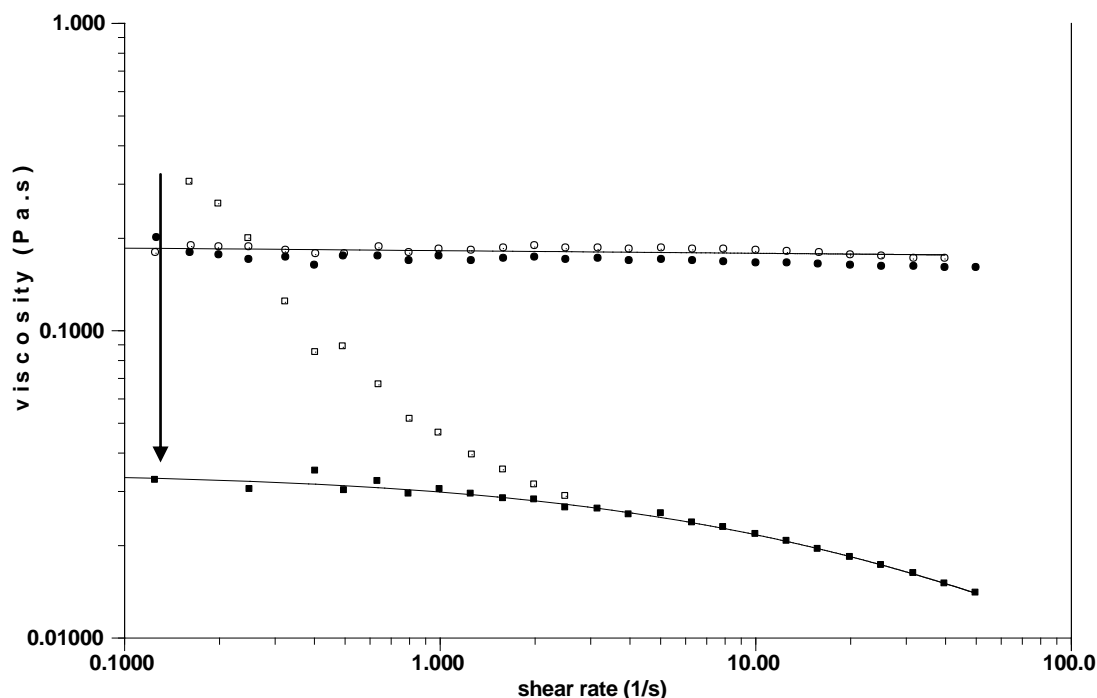
3D- network during these pre-treatments. We also conclude that the structure of the SF is not perturbed definitively, which would prevent us from interpreting the viscosity data in terms of HA composition.

Our conclusion is that, as soon as recovered, the synovial fluids may be frozen and then be stored over a long period of time without significant modification in their composition nor in their rheological behaviour. A 3D-network is clearly responsive to the rheological behaviour of SF. It can be at least partially separated (on some SF), by filtration on porous membrane, with no major modification in its composition but exerting a major influence on rheology. Large dilution applied on initial SF before filtration (on 0.2 μm pores membrane able to eliminate, if still stable, the majority of protein-HA complex) and injection in SEC experiment as proposed in our work, permits a good separation between HA and proteins which are then characterized individually.

2.4. Influence of protease pretreatment on composition and rheology of SF

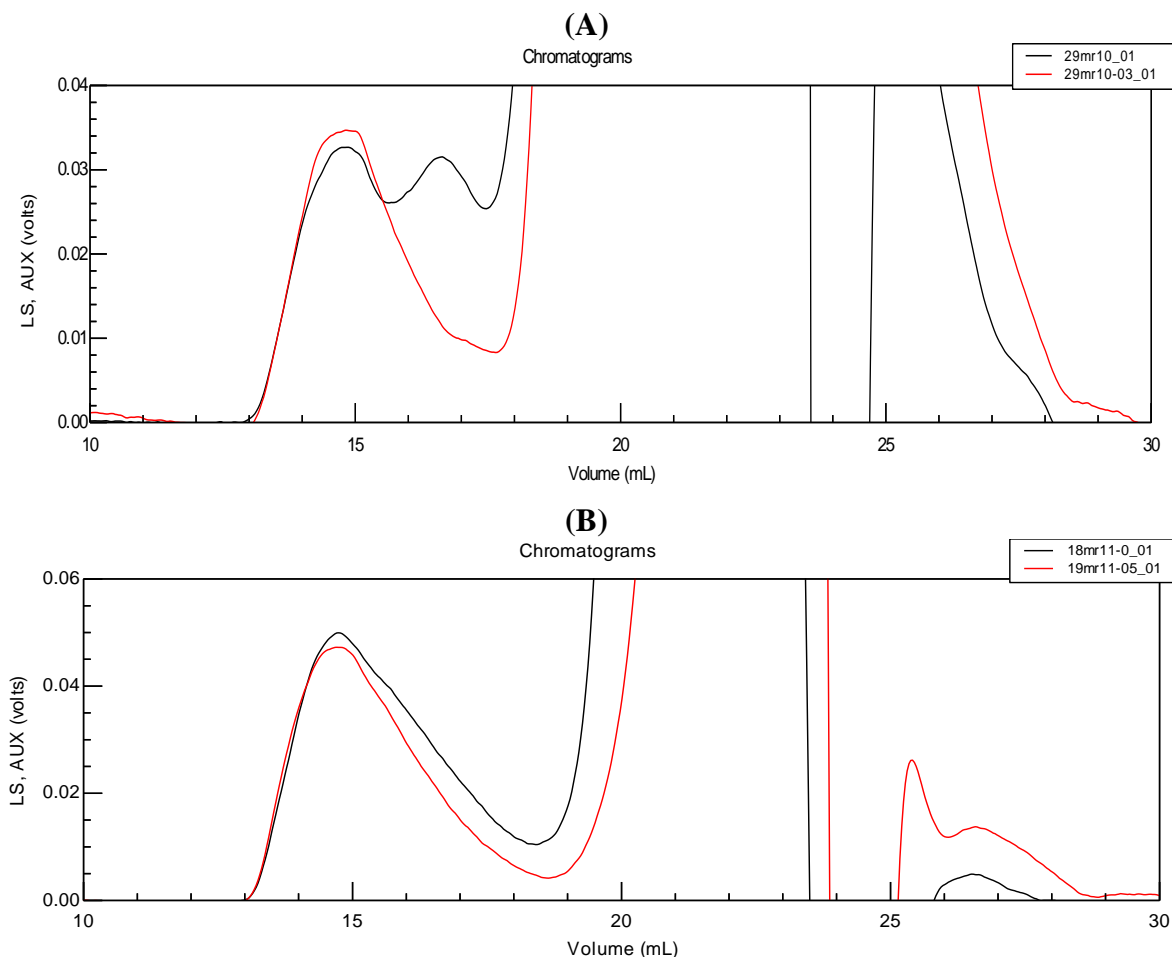
A 30 g/L solution of bacterial HA was tested as reference, and the same addition of protease was performed on HA and on SF at room temperature. It is shown that there is no change in the Newtonian viscosity for pure HA after protease treatment (Figure 8). It is shown that the viscosity even at high C^*M_w remains Newtonian whereas SF was usually non-Newtonian. With SF, a large change in viscosity, especially at low shear rate, is observed in the presence of protease and the behaviour becomes Newtonian with a viscosity at low shear rate of around 0.03 Pa.s. In the same time, the SF becomes clearer corresponding to the disruption of HA/protein aggregates.

Figure 8. Steady state viscosity as a function of the shear rate before (open symbols) and after (closed symbols) protease addition. HA at 30 g/L in 0.15 M NaCl (\circ , \bullet); SF (\square , \blacksquare). Continuous lines are the Williamson fits used to determine the zero shear rate viscosities. The arrow represents the drop in viscosity after enzymatic treatment.



It is thus important to compare SEC results and control the SF composition after protease action. In Figure 9, the two chromatograms (A and B) of the enlarged part of eluted materials for two types SF are shown before and after protease action. Chromatogram A described SF composition as applied in the rheological test (Figure 8).

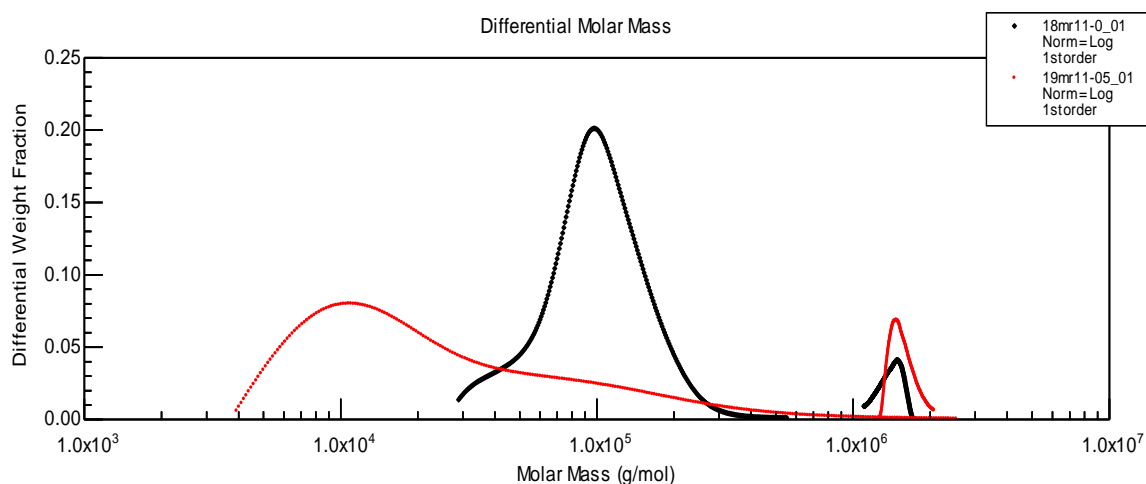
Figure 9. Chromatograms of two different synovial fluids (A and B) before (black) and after protease treatment (red). The traces are those for the polymer concentration; enlargement of the HA signal is shown at a elution volume of around 15 mL.



The analysis of HA molecular weight given at maximum concentration (first peak) shows the same values before and after enzymatic treatment. It is clear that after enzymatic treatment, the first peak (corresponding to HA elution) is better separated from that of proteins in Figure 9A. Nevertheless, the difference between chromatograms before and after enzyme treatment again depends on the state of proteins (degree of aggregation) or HA-protein association: the second small peak in elution may exist or not, depending on the particular SF tested (compare chromatograms (A) and (B) in Figure 9). From this data, we conclude that the action of protease may be recommended to make the SEC analysis easier (but preventing protein analysis). Nevertheless, the differences observed in the analyses remain small and do not fundamentally change our previous conclusions. In Figure 10, the molecular weight distribution for the two components of SF given in Figure 9 B is shown. After enzymatic treatment, the

initial protein peak (corresponding to $M_w \sim 100,000$) is shifted to a much lower molecular weight due to partial hydrolysis. However HA molecular weight distribution remains located at the same position.

Figure 10. Molecular weight distribution of proteins and HA before (black) and after (red) protease treatment, corresponding to chromatograms shown in Figure 9B.



3. Experimental Section

Different SF (named LSn with $n = 1$ to 12) were obtained during a therapeutic procedure by sterile aspiration of the affected joint in patients suffering from knee OA. In all patients, arthrocentesis was carefully performed by an experienced senior rheumatologist, using a needle of 50 mm/20 G in order to remove as much SF as possible before HA viscosupplement injection. SF was then collected in sterile tubes to study the role of pretreatment. As soon as possible after the aspiration, the samples were separated in different aliquots. The samples were either stored at $4\text{ }^{\circ}\text{C}$ for different periods of time, frozen at $-21\text{ }^{\circ}\text{C}$ or centrifuged at $50,000\text{ g}$ and $4\text{ }^{\circ}\text{C}$ for 20 minutes. After centrifugation, the supernatant was analyzed and a small amount of sediment was isolated, dried and weighted.

A bacterial protease Type XIV, from *Spreptomyces griseus* was used to hydrolyze proteins and highlight their role both in SEC analysis and in the rheological behaviour of SF. An enzymatic solution was prepared by direct dissolution of 1mg protease in 2 mL of a 0.15 M phosphate buffer at $\text{pH} = 7$. A few μL were added to 5 mL of synovial fluid, and then left for 24 hours at ambient temperature. A sample of initial SF was stored in the same conditions as a blank.

A bacterial linear hyaluronan ($M_w \sim 650,000$) at 10 g/L in 0.15 M NaCl was chosen as the model to test the role of storage and freezing on characteristics of pure HA. A linear HA ($M_w = 195,000$) at 30 g/L in 0.15 M NaCl was used for the test, with protease added in the same conditions as for synovial fluid, to control its role on HA. These HA samples obtained by fermentation were delivered by ARD cy (Pomacle, France).

Synovial fluids and linear HA were characterized by size exclusion chromatography (SEC) using a Waters Alliance GPCV2000 (USA) equipped with three detectors on line: a differential refractometer, a viscometric detector, and a multi angle laser light scattering (MALLS) detector from Wyatt (USA) [21,37,38]. The concentration of HA in the sample that was injected was lower than 0.5 g/L

(synovial fluids are usually diluted in the eluent in a ratio 1/5 before injection) with an injection volume of 100 μL using two columns in series (Shodex OH-pack 805 and 806). These columns allow the separation of high hydrodynamic volume HA molecules from proteins of lower values. Knowing the volume injected and the dn/dc , it is easy to determine the eluted polymer concentration. In this treatment, it is supposed that no low MW HA are eluted in the same time as proteins, which would suppose a molecular weight for HA as low as $M_w < 10,000$ (calculated for the same hydrodynamic volume as proteins). Before injection, all the samples were filtrated on a 0.2 μm pore membrane ("Sartorius AG" cellulose acetate filter) in order to retain large aggregates (or eventually flocculated proteins). The eluent used was a 0.1 M NaNO_3 aqueous solution (which remains of the same order as ionic concentration in physiological conditions), at 30 $^\circ\text{C}$ as elution temperature and a flow rate of 0.5 mL/min. The MW distribution and weight-average molecular weight (M_w) of the eluted polymers were obtained as characteristics of the biopolymers. The refractive index increment was $dn/dc = 0.153$ for HA, and 0.190 for protein pool [39]. Only soluble proteins were detected in SEC experiments. Few experiments were performed with an additional detector, a Waters 2487 Dual Absorbance detector allowing us to locate the proteins in the elution profile at a fixed wavelength of 280 nm. Advantage of SEC technique is that the direct determination of the molecular weight of HA and proteins (completely separated in the majority of cases) is obtained without calibration, even if the concentrations determined are slightly underestimated due to the eventual retention of a small amount of HA/proteins complex during filtration. The protease pre-treatment used to hydrolyze proteins gives a better SEC separation, but it is not necessary in the majority of cases. The large ratio in weight fraction between proteins and HA involves a delicate determination of the HA characteristics, but on the average, all of the values obtained in successive analysis consistently give the same type of values. Better precision is obtained when the molecular weight values at maximum of the concentration peak in chromatograms are compared.

The total amount of soluble proteins was also determined using the Bradford titration following dilution of the SF (ratio 1/20). The interference with HA was taken into account in a calibration curve performed with BSA. The HA content of SF is much lower than that of proteins and its contribution does not exceed 2% in the protein determination. The content of soluble proteins determined by SEC was confirmed by the direct Bradford titration.

The rheological behaviour of SF was determined with an AR 1000 rheometer from TA Instruments (USA) at 25 $^\circ\text{C}$ using a cone and plate geometry (4 cm diameter plate with 3.59 $^\circ$ cone). Steady state viscosity η was determined as a function of the shear rate $\dot{\gamma}$ in flow experiments.

4. Conclusions

Firstly, this work was performed to investigate the role of pre-treatments of SF on its rheological properties and composition determined by SEC. Our primary objective was to establish the best conditions possible for storage of SF samples after their collection from knees. They would later be used for further investigation, particularly to study the role of viscosupplementation treatment on resulting SF composition and viscometric behaviour. Unfortunately, it is difficult to draw one unique conclusion: the results depend essentially upon the structure of the considered SF (mainly proteins and

HA contents) especially when the viscosity is low in relation with a looser and instable network established between HA and proteins. The influence of different pre-treatments (freezing, filtration, and centrifugation) on viscosity measured as a function of the shear rate and on the composition obtained by SEC on different SF has been discussed. Given this analysis and results, we conclude that freezing has a relatively weak influence on rheology and composition in the majority of cases, especially given SF with large initial viscosity. Freezing is thus recommended for storage over a long period. In the absence of dilution, centrifugation (even at high rate) does not modify the 3D-network nor the rheology, the structure and the composition of the SF. Filtration on porous membranes, in the absence of dilution, causes a dramatic decrease in viscosity, especially for SF with higher viscosity, due to retention by the filter of the highly cross-linked fraction of polymers. Concerning the compositions of the different SF samples, they are not particularly perturbed by the different pre-treatments, especially when compared to the supposed low amount of loosely cross-linked material which is clearly evidenced.

Secondly, concerning the techniques used for characterization (composition analysis) of synovial fluids, the use of SEC with triple detection after filtration on 0.2 μm membrane of the diluted SF is demonstrated as important. Measurement of steady state viscosity performed directly or after freezing the initial SF is also convenient to obtain information on the behaviour of synovial fluids. Our results also show that viscosity cannot be used to approach the molecular weight due to shear rate effect in viscometers (in relation with the non-Newtonian behaviour), but also due to the influence of interactions between HA and proteins (even after partial elimination). Such influence leads an increase in viscosity, due to formation of a loose 3D-network.

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