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Inverse Spin Fractionation: a Tool to Fractionate Sodium Hyaluronate

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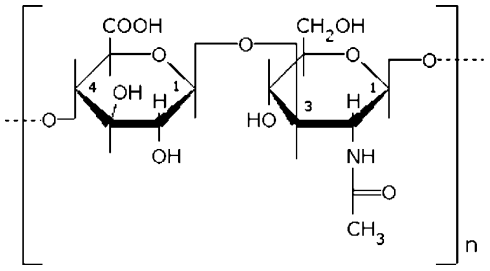
1. Introduction

In the past few years, there has been an increasing demand for polymer samples with reasonable molecular uniformity. For this reason, we recently developed a new large scale fractionation technique called continuous spin fractionation (CSF).[1,2] It overcomes some important limitations of continuous polymer fractionation (CPF),[3,4] a precursor technique representing a particular form of counter current extraction. CSF solves above all problems with the damming back of the source phase and with the efficiency of the separation process at higher overall polymer concentrations.

Both fractionation methods are based on the liquid−liquid phase separation of polymer-containing solutions.[5] Phase separation is induced by decreasing the solvent quality of the mixed solvent, for example by mixing a homogeneous polymer solution (feed, FD) with a liquid of sufficiently low solvent quality (extracting agent, EA). At the resulting overall composition (working point, WP), the system forms a polymer rich and a polymer lean phase. The former (gel phase) contains preferably the high molecular weight material of the initial polymer because of enthalpic reasons. The latter phase (sol phase) contains the shorter chain material because of entropic reasons. Due to the difference in their density, the two phases separate normally already upon standing. The resultant molecular weight distributions can be controlled via the process parameters (compositions of FD and EA, their mixing ratios, temperature). The main part of the CSF is a spinning nozzle, similar to those as used in the fiber industry. The FD is pressed through their holes (diameters on the order of 50−100 μm) into the EA. Because of the Rayleigh instability,[6,7] the initially formed threads of feed break up into tiny droplets immediately after leaving the nozzle. The fast transfer of the better soluble polymer species from the FD into the EA is facilitated by the small dimensions of the droplets. This enables the successful fractionation even with concentrated polymer solutions.

Although the CSF was meanwhile successfully applied to more than 10 different polymers (among others Novolak, cellulose acetate, pullulan, and dextran),[1,8] there are polymers where the usage of the spinning nozzle causes chain degradation because of the high shear rates (on the order of several hundreds s^{-1}) that are realized in the nozzle. Stiff molecules or polymers with very high molecular weight are particularly prone to chain scission. While passing the spinning nozzle, the mechanical stress is too high for the polymer, and the chains might break. This drawback can be eliminated by changing the procedure of the spin fractionation (i.e., by pressing the extracting agent through the spinning nozzle into the stirred polymer solution). In the following, we call this practice inverse spin fractionation (ISF).

Scheme 1. Structure of the Repetition Unit of Hyaluronic Acid



To investigate the advantages of ISF for the fractionation of easily degradable polymers, we have applied this new technique to sodium hyaluronate (NaHA). It is known from the literature that this polymer degrades upon standing in aqueous solution[9] and under mechanical stress (e.g., arising from filtration, stirring, pumping, drying, etc).[10−12] This makes NaHA the ideal test polymer for ISF. Furthermore, there is a practical incentive for the fractionation of this polymer, namely its usage in cataract surgeries.[13,14] For that purpose, the particular flow behavior of the aqueous solutions of NaHA plays a central role. To tailor the required rheological properties, it is necessary to select adequate values for the molecular weight and for the molecular weight distribution of the hyaluronic acid. Due to the fact that the necessary samples are normally not directly available, it is indispensable to produce them by fractionation. The high costs for sodium hyaluronate impeded us to perform the ISF in a large scale and continuous manner. Therefore, we demonstrate the fractionation only in batch experiments.

2. Experimental Section

2.1. Materials. For the present study, we have used a cosmetic grade sodium hyaluronate supplied by Synopharm (Charge Nr. 0405A185) with an unspecified average molecular weight of 1550 kg/mol, indicated by the supplier. According to our capillary viscometric measurements, the molecular weight is 1100 kg/mol using the Kuhn−Mark−Houwink parameters $K = 3.46 \times 10^{-2}$ mL/g and $a = 0.779$ for aqueous solutions containing 0.15 M NaCl at 25 °C.[15] Before further usage, the polymer was dried under oil pump vacuum at 50 °C until it had reached constant weight. The structure of the repetition unit of hyaluronic acid is given in Scheme 1.

The low molecular weight materials were bidistilled water and 2-propanol; 2-POH was purchased from Riedle-de-Haen in p.a. quality and used as a nonsolvent without further purification. The different salts were either supplied by Fluka (NaCl, $NaNO_3$, and $NaHCO_3$) or Carl Roth (NaH_2PO_4 and Na_2HPO_4) in p.a. quality.

2.2. Methods. ISF Apparatus. The extracting agent was pressed with a syringe through a spinning nozzle consisting of a gold/platinum alloy with 1035 holes of 60 μm diameter each into a stirred polymer solution. The two phase system arising from this procedure in the mixing vessel was stirred for 2 days and then separated by means of a centrifuge. The polymer was recovered by drying the polymer solutions at 50 °C gradually reducing the pressure down to oil pump vacuum.

Cloud Points. The cloud points were obtained by titrating homogeneous polymer solutions with the nonsolvent until the clear solution

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becomes turbid. The cloud points were either observed visually or by means of an apparatus as described before.[16]

Gel Permeation Chromatography (GPC). These experiments were carried out in aqueous solutions (containing 8.5 g of $NaNO_3$ and 4.2 g of $NaHCO_3$ per liter) using the columns HEMA BIO 40, HEMA BIO 1000, and SUPREMA 300, supplied by PSS for GPC measurements at room temperature. The differential refractometer Gynkotek RI-71 was employed as the detector, and dextran standards (PSS) served for calibration. Therefore, the molecular weights for sodium hyaluronate resulting from GPC are apparent values only and are indicated by a star.

Viscometry. The experiments were performed at 25 °C using Ubbelohde capillary viscometers for dilution sequences of type I with a capillary diameter of 0.63 mm, in combination with AVS 310 (Schott, Mainz, Germany). Hagenbach corrections were applied. The viscometric and rheological measurements were carried out in aqueous buffer solution consisting of 0.146 M NaCl, 0.002 M NaH_2PO_4, and 0.0003 M Na_2HPO_4. To calculate the viscosity average molecular weight the Kuhn−Mark−Houwink parameters, $K = 3.46 \times 10^{-2}$ mL/g and $a = 0.779$ for aqueous solutions containing 0.15 M NaCl at 25 °C were used.[15] The experimental error for $[\eta]$ is typically ±1%, leading also to an error of around ±1% for M_η.

Rheometer. A rotational rheometer TA AR-1000 (TA Instruments, Inc., USA) was used applying a steel cone-plate geometry (diameter, 20 mm; angle, 1°) and a solvent trap. The frequency sweeps were performed from 0.1 to 100 rad/s at a constant oscillatory stress of 4.775 Pa. All measurements were conducted in the linear viscoelastic region, as ensured by independent strain sweep tests. In these experiments, the strain ranged from 1 to 500% at a constant frequency of 62.5 rad/s.

3. Results and Discussion

3.1. Orienting Experiments. Water is the most commonly used solvent for sodium hyaluronate. For this reason and for the potential use in the pharmaceutical field, we chose water as the solvent component for the fractionation of NaHA. No additional salts were utilized for that purpose to keep the number of components low and the recovery of the polymer simple. Preliminary experiments (e.g., phase separation behavior and location of cloud point curve) were also performed for a number of nonsolvents (among others 2-POH, ethanol, THF, and acetone). 2-POH was finally chosen because of its adequate solvent power.

A general problem with high molecular weight NaHA is the manageability of its aqueous solutions due to the extremely high viscosity even at rather low polymer concentrations. This limits the experimentally manageable region to solutions with less than 2wt-% NaHA. For this reason, the representation of ternary phase diagrams in terms of the usual Gibbs phase triangle is unsuitable; all cloud points would be located very close to its baseline. The information shown in Figure 1 is therefore presented in Cartesian coordinates. The phase diagram serves as the basis for fractionation because the homogeneity of feed and extracting agent must be guaranteed; furthermore, the working point (composition of the total mixture produced in the course of fractionation) must lie within the miscibility gap as indicated in this graph.

A simple way to investigate the degradation of a polymer is to measure its intrinsic viscosity $[\eta]$. To investigate the effect caused by the spinning nozzle on the degradation of NaHA, the feed solution was treated in two different ways. On one hand, the polymer was dissolved, agitated for 1 day by means of tumble mixer at room temperature, and then recovered at 50 °C in a vacuum oven. Alternatively, the polymer solution was additionally pressed through the spinning nozzle after the stirring and before recovery.

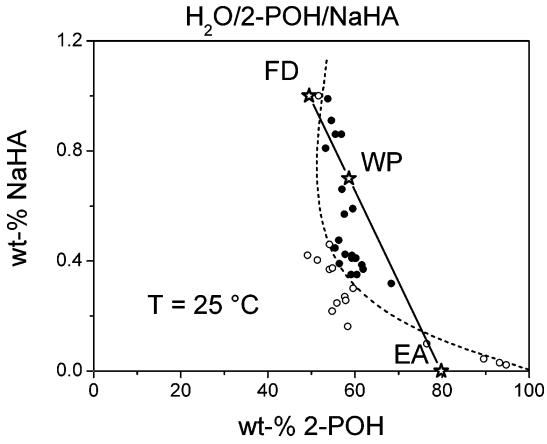

Figure 1. Solubility map of ternary system water/2-POH/NaHA at 25 °C. The dotted cloud point curve separates the composition range of homogeneous mixtures (open circles) from the composition range of two phase mixtures (full circles). Also shown are the compositions of feed (FD), extracting agent (EA), and the working point (WP) used for the fractionation.

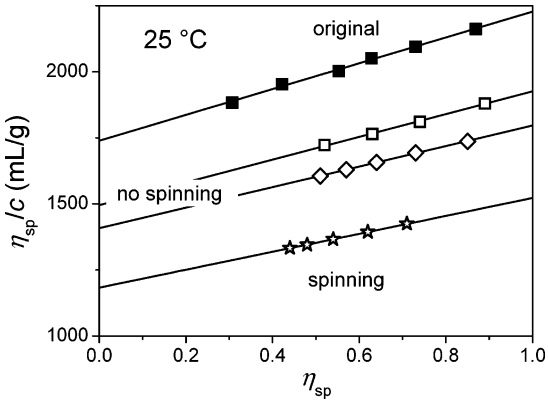

Figure 2. Schulz−Blaschke plots for aqueous solutions of the virgin material (full square) and for the polymers recovered from 1 wt % solutions in water (open squares) or from the mixed solvent (2-POH content 50 wt %, open diamonds). The data referring to the polymer recovered after pressing a 1 wt % solution in the mixed solvent (2-POH content 50 wt %) through the spinning nozzle are represented by the open asterisks.

Figure 2 shows the Schulz−Blaschke plots for the original polymer and for the polymer recovered after the different treatments of the feed solutions. The first sample was recovered from a binary aqueous solution, the second one was solved in a mixture consisting of solvent and nonsolvent, and the last one was like the second but additionally pressed through the spinning nozzle. It is easy to recognize that the intrinsic viscosities of all recovered samples are smaller than the one of the initial polymer (1740 mL/g), indicating degradation during the dissolution and the drying process. The degradation of the polymer is stronger if nonsolvent is present in the mixture ($[\eta]_{binary} = 1500$ mL/g, $[\eta]_{ternary} = 1410$ mL/g). The reason is the influence of the solvent quality on the degradation: the presence of nonsolvent leads to dry friction of the polymer strands, which reduces the molecular mobility and increases the energy the system can store. If the energy becomes too high, the polymer chains break.[17,18]

In the case of the ternary polymer solution that was additionally pressed through the spinning nozzle, the intrinsic viscosity is 1180 mL/g, indicating a stronger degradation. For this reason, the normal spin fractionation is not advisable but rather the inverse spin fractionation.

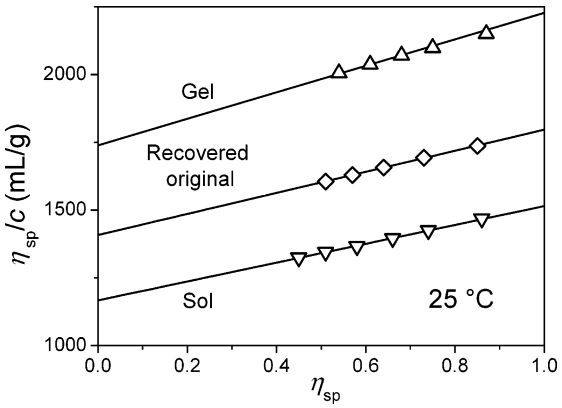

Figure 3. Schulz−Blaschke plots for aqueous buffer solutions of the polymer recovered from the mixed solvent in the absence of spinning (cf. Figure 2) and for the two fractions obtained in the course of ISF.

3.2. Inverse Spin Fractionation. A mixture consisting of 80 wt % of 2-POH and 20 wt % of water was chosen as the extracting agent. It turned out that pure 2-POH is too strong as a nonsolvent. If it is added to the feed solution, it causes a precipitation of the polymer. By adding water to the extracting agent, the solvent power is increased, and someone obtains the liquid−liquid phase separation that is advantageous for the fractionation. The degradation of the polymer due to the presence of the nonsolvent in the mixture can anyway not be avoided as the spinning process requires the usage of nonsolvent in order to achieve the phase separation.

To reduce the amount of needed solvent, the composition of the feed should be as close as possible to the cloud point curve. Due to the high viscosity of the polymer solution, the NaHA concentration for the ternary feed cannot be chosen higher than about 1 wt %. The working point also has to be close to the cloud point curve. If the working point lies too deep inside the miscibility gap, all polymer is found in the gel phase. In addition, the polymer concentration of the working point should not be too low, because all polymeric material would be found in the sol phase. In both cases, no fractionation would take place. On the basis of the orienting experiments, the compositions of FD, EA, and WP (see Figure 1) were chosen for the fractionation in such a manner that about half of the starting material is found in each of the arising phases.

Figure 3 shows the Schulz−Blaschke plots of the recovered feed, and the two obtained fractions. Deviating from the goal that 50 wt % of the starting material should be found in each phase, the up scaled fractionation yielded 61 wt % in the sol-phase.

An easy way to find out whether degradation of the polymer takes place during the fractionation or not is the comparison of the intrinsic viscosity of the initial polymer and that calculated from the intrinsic viscosities of the fractions according to eq 1 (where w_x is the weight fraction of the initial polymer contained in phase x)

$$[\eta]_{\text{original}} = w_{\text{sol}}[\eta]_{\text{sol}} + w_{\text{gel}}[\eta]_{\text{gel}} \qquad (1)$$

In the case that no degradation takes place, eq 1 must be fulfilled, while degradation leads to lower values for the right-hand side of this relation. In our case, the intrinsic viscosity of the original polymer is 1740 mL/g, whereas the right side yields 1410 mL/g, proving once more the degradation of NaHA. On the other hand, the calculated value matches with the intrinsic viscosity of the recovered feed. This indicates that the degradation takes place almost exclusively during the dissolution and

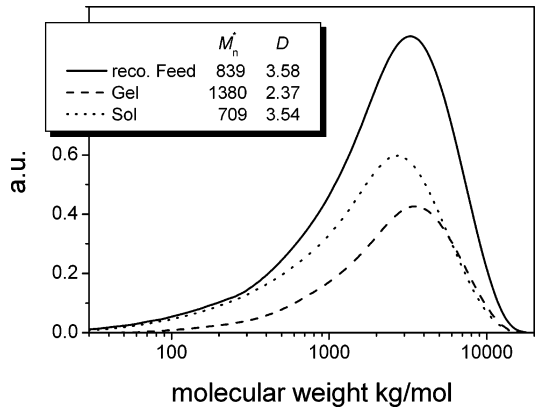

Figure 4. Differential molecular weight distributions of the recovered sodium hyaluronate sample (normalized to one) and of the two fractions normalized to one and multiplied by their fraction with respect to the starting material. Number average molecular weights in kg/mol and polydispersities are indicated in the graph.

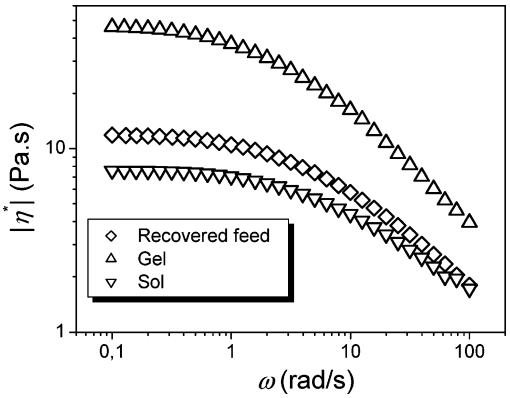

Figure 5. Rheological measurement for different fractions at $T = 20$ °C, polymer concentration: 2 wt % in aqueous buffer solution.

the recovering of the polymer but not during the fractionation process if one presses the extracting agent through the nozzle instead of the feed. This means that by applying the inverse spin fractionation method additional degradation can be avoided.

Figure 4 shows the molecular weight distributions of the recovered polymer and of the two fractions. Most of the lower molecular weight homologues of the original material below approximately 100 kg/mol are by the fractionation accumulated in the sol fraction; correspondingly, the molecular uniformity of the gel fraction increases considerably.

For the sol fraction, the amount of high molecular weight components is decreased, and the amount of low molecular weight is increased. In the case of the gel fraction, the shortest chains were removed completely. Therefore, the very high polydispersity $D = (M_w^*/M_n^*)$ of the starting is retrieved in the sol fraction, whereas the polydispersity of the gel fraction was reduced because of the separation of the very low molecular weight material.

To obtain first information on effects of fractionation on the rheological properties of the solutions of NaHA, we have performed some orienting oscillatory measurements. From the results shown in Figure 5, one can calculate the longest relaxation time τ for this polymer, assuming that the Cox−Merz rule[19] holds true. The intersection point of a line through the plateau region (parallel to the abscissa, indicating the complex zero shear viscosity) with another line through the linear descending region of the shear rate dependence of the

Table 1

sample	$[\eta]$, mL/g	M_η, kg/mol	D	$\lvert\eta^*\rvert_0$, Pa s	τ, s
original	1740	1100	3.58	44 ± 1	2.29 ± 0.2
recovered feed	1410	840	3.63	12 ± 0.5	1.69 ± 0.2
gel	1780	1130	2.37	47 ± 2	3.25 ± 0.3
sol	1170	660	3.54	7.6 ± 0.5	1.24 ± 0.1

complex viscosities allows an estimation (with an error of about 10%) of the longest relaxation time of the system. The values are collected in Table 1. As expected, the relaxation becomes slower with increasing molecular weight. The extraordinarily large values, lying in the range of seconds, reflect the uncommonly high molecular weight of NaHA. These data are in agreement with that reported for synovial fluids.[20] Although for normal synovial fluids, which contain (from rheological point of view) hyaluronic acid as the main component but additionally also a large variety of other inorganic and organic materials (e.g. glucose, phospholepids, etc.), the relaxation times lie in the range of 40−125 s, for synovial fluids found in samples of patients with chronicle polyarthritis, they are in the range of seconds. This decrease in relaxation time is explained by degradation of the hyaluronic acid that takes place because of the inflammation process. The NaHA used for our study has a molecular weight comparable with the hyaluronic acid found in synovial fluids of patients with chronicle polyarthritis.

Table 1 summarizes the characteristic values for the initial polymer, the recovered feed, and the two obtained fractions.

4. Outlook

The described modification of CSF (i.e., the deployment of the spinning nozzles for the "atomization" of the extracting agent instead of the feed) minimizes the degradation hampering the fractionation of "fragile" polymers. By means of the ISF, it was possible to separate the homologues of sodium hyaluronate (one example for the class of such materials) according to their molar mass. This means that samples of sodium hyaluronate with desired molecular weights or/and molecular weight distributions become accessible by tailoring the fractionation parameters (e.g., temperature and composition of WP, EA, and FD). Such samples would be of great importance for basic research (taking sodium hyaluronate as a model substance) and to improve the properties of viscoelastica used in the field of cataract surgeries.

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