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Hydration of polymeric components of cartilage — an infrared spectroscopic study on hyaluronic acid and chondroitin sulfate

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Abstract

Hydrated polysaccharides are major constituents of cartilage and play an important role in its water-binding properties. Infrared (IR) spectroscopy and sorption isotherms have been used to investigate the hydration behavior of the glycosaminoglycans hyaluronic acid and chondroitin sulfate. IR-dichroism of the vibrational modes of the pyranose ring is found at relative humidities (RH) smaller than 84%. The IR-dichroism data for the vibrational modes of the pyranose ring have been analyzed with respect to the helical structure of these polysaccharides. The orientation vanishes at higher relative humidities (> 84%), because a strong increase in the water uptake occurs in the observed sorption isotherms. Differences in the IR-absorbance of the O–H stretching mode of sorbed water between hyaluronic acid and chondroitin sulfate are shown to be caused by the additional hydration of the sulfate groups. The corresponding H-bonds are weaker than those of the hydration shell of the pyranose rings.

Keywords: Hyaluronic acid; Chondroitin sulfate; Sorption isotherms; Hydration mechanism; IR spectroscopy; Molecular order

1. Introduction

Cartilage plays an important role as a shock absorber and space holder in living organisms [1]. These physical properties are closely connected with its molecular structure and intermolecular interactions between polymeric components, such as glycosaminoglycans (GAG). GAG constitute a major part of the proteoglycans of cartilage. They represent the main source of high water binding capacity of cartilage. The swelling of cartilage is based on the binding of water to polar groups of GAG (carboxylate, sulfate), on the electrostatic repulsion between GAG molecules and entropic contributions resulting from the mixing of water and counterions [12].

Hyaluronic acid (HA) and chondroitin sulfate (CS) belong to the most important GAG that occur in cartilage. It was shown that they provide the high swelling pressure necessary for cartilage function [1,2]. The swelling properties of polysaccharides are also of large technological interest [3–8]. A direct approach for studying the water binding in a definite way represents the investigation of the dependence of polymer properties on the degree of hydration ('swelling'). The continuous variation of polymer hydration can be attained by adjusting the relative humidity [9,10]. The sorption isotherms measured in this way provide the relationship between the water content of the polymeric system and the relative humidity that is directly related to the water activity and the osmotic pressure.

Several methods for studying the swelling behavior of cartilage are available [11]. In the present study, infrared (IR) spectroscopy is used to obtain information on the water binding properties and mobility of the above mentioned polysaccharides. Discrete frequency ranges in the spectra can be assigned to different moieties of the polymers and therefore, detailed informations on the hydrogen bonding environment 'seen' by water molecules near a polymer surface become available. Additional information on the influence of water on specific functional groups of the polymers can be expected from the evaluation of the bands of the acetamido-, carboxylate- and hydroxyl groups and the vibrations of the pyranose ring.

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In the past, IR spectroscopy has been used successfully to characterize glycosaminoglycans [12–18]. The IR experiments, discussed in this paper, were performed on hydrated films whose water contents were controlled by regulating the relative humidity (RH) in a range from 8 to 96% with a stepwidth of 4%. Additionally, IR spectra are also useful in dealing with structural questions. We have found that the polysaccharide films exhibit dichroism in vibrational modes. The clear implication from this finding is that some degree of alignment occurs spontaneously at lower water contents indicating the existence of attractive forces between these polymers. The importance of such forces for the formation of supramolecular structures has already been described by Scott et al. [11]. It could be shown by measuring the surface polarity of the films, that the surface is less polar than usually discussed in the literature [19,20].

2. Experimental

2.1. Materials

Sodium salts of chondroitin sulfate and hyaluronic acid from bovine trachea were purchased from Fluka Feinchemikalien GmbH, Neu-Ulm (Germany). They were used without further purification.

2.2. Sample preparation and hydration

A 3% stock solution of HA or CS in water was spread on one side of a ZnSe attenuated total reflection (ATR) crystal and evaporated under a stream of nitrogen. The ATR crystal (six active reflections, angle of incidence 45°) was mounted on a commercially available horizontal Benchmark unit (Specac, UK) modified in order to equilibrate the films at well-defined relative humidities (RH) and to allow temperature control [21]. The relative humidity of the N_2 inflow was adjusted using a humidity generator (HumiVar, Germany). The equipment has been used to adjust the relative humidity continuously between 8 and 96%, with an accuracy of $\approx 0.5\%$.

All samples were investigated by means of increasing (hydration scan) and decreasing (dehydration scan) RH at constant temperature (25°C). The RH was varied in steps of 4%. Before IR-measurement were completed the sample was allowed to equilibrate for at least 10 min after reaching the RH in each step.

For the measurement of the sorption isotherms, an analogous moisture regulating device was combined with a microbalance system (Sartorius), in order to measure gravimetrically the quantity of water adsorbed by the polymer as a function of RH.

2.3. Infrared measurements

Infrared spectra were measured using a BioRad FTS-60a Fourier transform infrared spectrometer (FT-IR) which was supplemented with a wire grid polarisator. Typically, 128 scans were accumulated for light that is polarized parallel ($\|$) and perpendicular ($\perp$) with respect to the plane of incidence. Polarized absorbance spectra of the sample, $A_{\|}(v)$ and $A_{\perp}(v)$, were calculated using the corresponding single-channel spectra of the empty ATR crystal (without sample) as background. In some cases, the band shapes were fitted by a superposition of Lorentzian lines yielding the maximum wavenumbers, bandwidth and integral absorbances of the individual components.

The assignment of the different infrared bands was performed as follows [12,15,22]: the broad band in the region 3600–3000 cm^{-1} is due to the v(O–H) stretching mode (water, polysaccharides) and partially to the v(N–H) stretching vibration of the N-acetyl side chain. The band at ≈ 1635 cm^{-1} is assigned to the amide I mode (mainly C=O stretching coupled with N–H bending) and the shoulder at ≈ 1611 cm^{-1} to the antisymmetrical stretching mode of the carboxylate groups, $v_{as}(COO^-)$. The shoulder at 1550 cm^{-1} can be assigned to the amide II band and the feature at 1413 cm^{-1} originates from the symmetrical COO^- vibration, $v_s(COO^-)$, respectively. The absorbance in the region 1100–950 cm^{-1} mainly results from different vibrations of the pyranose ring. Additional bands in the spectrum of CS can be attributed to vibrations of the C–O–SO_3^--fragment: The intense feature centred at $\approx$ 1226 cm^{-1} is due to the antisymmetrical stretching mode, $v_{as}(SO_3^-)$, and a shoulder at ≈ 1063 cm^{-1} originates from the symmetrical vibration, $v_s(SO_3^-)$. A peak at 1124 cm^{-1} is probably due to the antisymmetrical C–O–S stretching.

2.4. Determination of the IR order parameter

The dichroitic ratio R is defined as

$$R = \frac{A_{\|}}{A_{\perp}}. \tag{1}$$

where $A_{\|}$ and $A_{\perp}$ denote the integrated baseline corrected absorbance of an absorption band in the parallel and perpendicular polarized spectra, respectively. The IR order parameter is defined by [23]

$$S_{IR} = \frac{R - K_1}{R + K_2}. \tag{2}$$

where K_1 and K_2 depend on the geometry of the measurement, on the film thickness (> 3 μm) and on the refractive indices of the ATR-crystal (2.44) and the sample (1.42). In our experiments, we used values corresponding to the thick film approximation, $K_1 = 2$ and $K_2 = 2.55$ [24].

3. Results and discussion

3.1. Sorption isotherms

In Fig. 1, the isotherms of water sorption of hyaluronic acid and chondroitin sulfate are shown as a function of the relative humidity. Upon an increase of RH, the number of water molecules adsorbed per polymer repeat unit (PRU) increases continuously. An almost linear increase occurs and the water uptake remains small up to a relative humidity of $\approx 75\%$ and it is markedly enhanced at higher relative humidities. The water activity of a saturated NaCl solution $= 0.75$ at 25°C [25]. The chloride starts to dissociate at RH $=$ 75%, i.e. at the same RH value at which cartilage compounds progressively imbibe water. We conclude that onset of the dissociation of sodium counterions represents an important factor that determines the observed water uptake. The hydration capacity of CS is obviously higher in comparison to HA. This difference probably results from the additional sulfate group of CS. Our experimental values are in good agreement with data published very recently by Jouon et al. [7].

In this paper, the sorption isotherms are exclusively used for the discussion of the IR spectroscopical data in dependence on the water content of the films. The number of primary binding sites for water molecules has been calculated from the sorption isotherms, given in Fig. 1, using the Brunauer–Emmett–Teller (BET) theory [9] (not shown, see Ref. [26]). The result of this analysis was that 2.1 ± 0.1 water molecules per PRU are specifically bound to HA and 3.1 ± 0.2 to CS.

3.2. IR spectroscopy

In Fig. 2, the IR spectra of HA (Fig. 2a) and CS (Fig. 2b) in the region 3750–2750 cm^{-1} are shown. The broad absorption band is mainly assigned to the O–H stretching ν_{13}(OH) of water. The band consists of (mainly unresolved) subbands assigned to the hydrogen-bonded hydroxyl groups and to the Fermi resonance (3280 cm^{-1}) that involves the first overtone of the bending vibration of the water molecules at ν_2(OH) ≈ 1650 cm^{-1} [22]. With increasing RH the absorbance of the ν_{13}(OH) mode increases. The course of the integrated absorbance between 3700 and 2700 cm^{-1} of this band versus RH is given in Fig. 2(c). Its behavior is very similar to the RH dependence of the sorption isotherm (Fig. 1). From this point of view, the ν_{13}(OH) band represents an approximate measure of the amount of water taken up by the polymers.

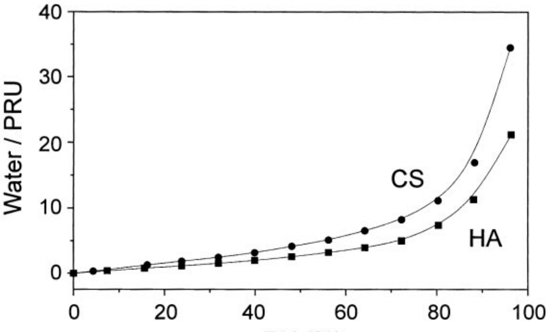

Fig. 1. Sorption isotherms of chondroitin sulfate (●) and hyaluronic acid (■). The number of adsorbed water molecules per polymer repeat unit (PRU) is given as a function of the relative humidity (RH). The fit of the absorption isotherms using the BET theory is represented by solid lines.

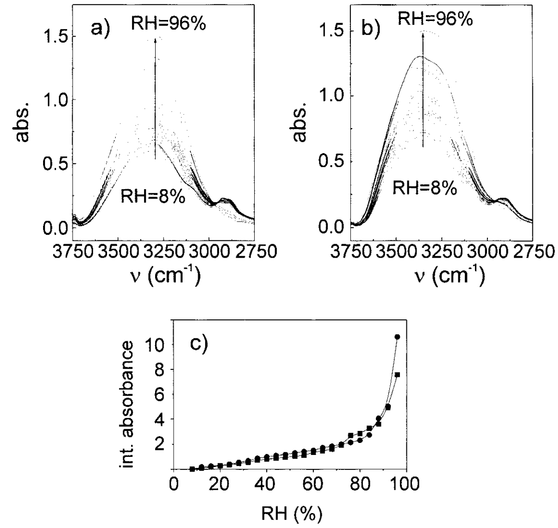

Fig. 2. Absorbance spectra of (a) hyaluronic acid and (b) chondroitin sulfate, in the range 3750–2750 cm^{-1} for relative humidities between 8 and 96% in steps of 4%. Vertical lines indicate the increase of humidity. In (c) the relative IR-absorbance of HA (■) and CS (●) is shown in dependence on the relative humidity.

The wavenumber at maximum absorbance (ν_{max}) of the OH-band is independent of RH, but it is different for HA and CS. Its centre of gravity, is displayed in Fig. 3. For HA and CS, ν was found at 3300 and 3370

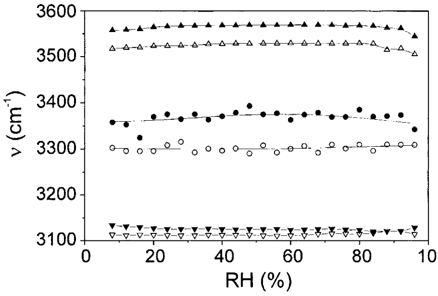

Fig. 3. Centre of gravity of the ν_{13}(OH) band at maximum absorbance (circles) and at half-maximum absorbance at the lower (triangle down) and higher (triangle up) frequency flanks for hyaluronic acid (open symbols) and chondroitin sulfate (filled symbols).

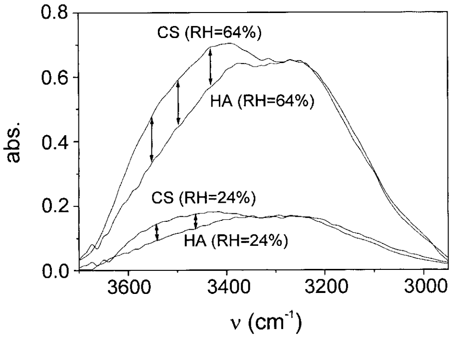

Fig. 4. Difference absorbance spectra of hyaluronic acid (HA) and chondroitin sulfate (CS) at relative humidities between 24 and 64%. The difference spectra are calculated by subtraction of the absorbance spectra recorded at RH = 8%.

cm^{-1}, respectively. Note that the centre of gravity of the broad O–H stretching band of bulk water is located near 3400 cm^{-1} (not shown). Additionally, in Fig. 3 the wavenumbers at half-maximum absorbance are given as a function of relative humidity. It can be seen that the absorption at the lower frequency side is almost equal for HA and CS, whereas that at the high-frequency flank is different causing the shift of ≈ 70 cm^{-1} in the centre of gravity of the band. On the other hand, the left-hand flank of the ν_{13}(OH) band of CS roughly agrees to that of bulk water (not shown). These results give rise to the conclusion that the right part of the O–H stretching band originates mainly from the hydration of the pyranose rings present in CS as well as in HA, whereas the left-hand flank of the ν_{13}(O–H) band is dominated by the hydration shell of the sulfate groups of CS.

In Fig. 4, spectra of water adsorbed onto HA and CS are plotted. They were calculated as the difference between a spectrum of hydrated polymer (RH = 24 and 64%) minus the corresponding spectrum of the nearly dry polymer (RH = 8%). In that way, the OH-band of water is separated from the residual groups contributing to the broad feature of the spectrum. At the lower frequency side, the difference spectra of HA and CS recorded at equal relative humidities are nearly equal. At the high frequency side, however, the water sorbed to CS absorbs considerably stronger than the hydration shell of HA. The difference is increasing with relative humidity.

It is generally accepted that the ν_{13}(OH) bandwidth qualitatively reflects the distribution of hydrogen bonds of variable strength over a variety of binding sites. In the light of this interpretation, the centre of gravity can be related to the major fraction of hydrogen bonds and the extra absorbance of CS on its high-frequency side can be assigned to populations of water representing weaker hydrogen bonds in the hydration shell of the sulfate groups (see below) [21,27].

The observed spectral differences between HA and CS have to be related to the sole structural difference, i.e. the presence of the –O–SO_3^--side group in CS. The maximum IR absorbance of this group occurs at ≈ 1240 cm^{-1} due to the antisymmetric SO_3^- stretching vibration (Fig. 5b). In the absorbance spectrum of HA this band is not observed (Fig. 5a). Hence, the weakening of the H-bond structure of the sorbed water is obviously caused by the presence of the –O–SO_3^--moieties. The position of the SO_3^--stretching band does not change with varying hydration. This group is either screened from the water to some degree, or its stretching mode is generally less sensitive to the progressive increase of hydration as it was found, for example, upon hydration of sodium bis(2-ethylhexyl) sulfosuccinate (AOT) reversed micelles [28]. The broad $\nu_{as}(SO_3^-)$ band shows a stronger subpeak at ≈ 1225 cm^{-1} and a weaker subpeak at ≈ 1258 cm^{-1}. Such a doublet might indicate that the degeneracy of this vibration is lifted by nonsymmetric water-mediated interactions between the counterions and the sulfate group. For example, in AOT reversed micelles the water molecules are assumed to form bridges between the SO_3^- group and the sodium cations [29]. The perturbation of the water structure in the coordination shell of the sulfate group is suggested to cause the weakening of its interactions and thus, the left-hand shift of the respective ν(OH) frequency when compared with the water interacting with the pyranose rings.

Additionally, in Fig. 5 (see insert) absorbance spectra in the region 1700–1500 cm^{-1} for HA and CS are shown for different relative humidities. The absorbance in this spectral region is gradually decreasing at higher relative humidities as a result of the dilution of the polysaccharide. The enlarged region shows that the band position for the $\nu_{as}(COO^-)$ mode shifts from 1611 to 1603 cm^{-1} for HA as well as for CS while increasing

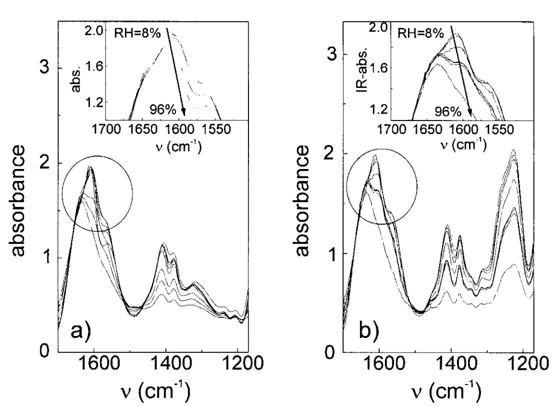

Fig. 5. Absorbance spectra of hyaluronic acid (a) and chondroitin sulfate (b) in the range 1700–1170 cm^{-1}, for different degrees of hydration. The insert enlarges the maximum of the $\nu_{as}(COO^-)$-band. The arrow indicates spectral changes after progressive hydration (RH = 8–96%).

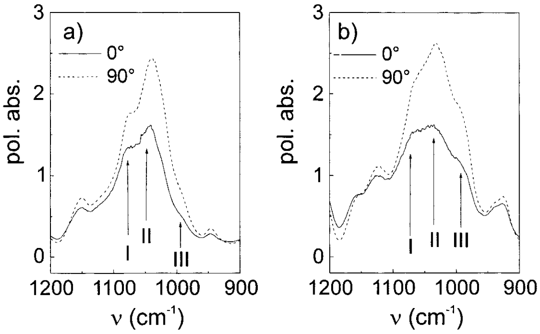

Fig. 6. Absorbance of polarized spectra for hyaluronic acid (a) and chondroitin sulfate (b), in the range 1200–900 cm^{-1} at RH = 8%. The absorbance of the ⊥-polarized (dotted line) spectra; $A_{\perp}(\nu)$, are multiplied by a factor of 2 for direct comparison with $A_{\|}(\nu)$ (see text).

the hydration. In agreement with Zundel [27] and Siesler and Holland-Moritz [30] stretching frequencies are lowered as a result of the formation of hydrogen bonds. This indicates that the observed frequency shift is caused by the association of water molecules to the carboxylate group via hydrogen bonds. Further changes of band positions were not observed in this spectral region. As shown by Sheehan et al. [31], the existence of this band in the observed spectral region provides strong evidence that the effective pH in the film is in the range of ≈ 4–7 giving rise to the deprotonated state (COO$^-$). This result is confirmed by the absence of IR absorption in the range of the C=O stretching mode at 1750–1700 cm^{-1}. The position of the symmetrical stretching vibration ν_s(COO$^-$) at 1413 cm^{-1} is independent of the hydration state. This spectral region is less sensitive towards band shifts in comparison to the antisymmetrical stretching vibration.

3.3. IR linear dichroism

Polarized IR spectra of polymer films in a nearly dry atmosphere (RH = 8%) in the spectral region between 1200 and 900 cm^{-1} are shown in Fig. 6 for HA (Fig. 6a) and CS (Fig. 6b). Note that the relation $A_{\|} = 2A_{\perp}$ holds in the absence of macroscopic molecular order due to the dependence of the strength of the electric field on the polarization direction [24]. Significant differences in the spectra between the parallel and the perpendicular polarization are observed for the bands assigned as I–III. These bands correspond mainly to the vibrations of the pyranose ring.

In Fig. 7, the IR order parameter S_{IR} calculated from Eqs. (1) and (2) is given as a function of relative humidity of HA (Fig. 7a) and CS (Fig. 7b). For isotropically distributed transition moments S_{IR} must be very close to zero. At low hydration, negative values of the IR order parameter were found, which indicate a preferential orientation of the sample. The transition moments are not isotropically distributed in this case. S_{IR} changes only very weakly up to relative humidities of RH ≈ 80%, but at RH ≥ 84% the orientation of the sample decreases considerably. At relative humidities higher than RH ≈ 92% the orientation becomes virtually isotropic. Both effects are reversible with respect to the relative humidity of the films.

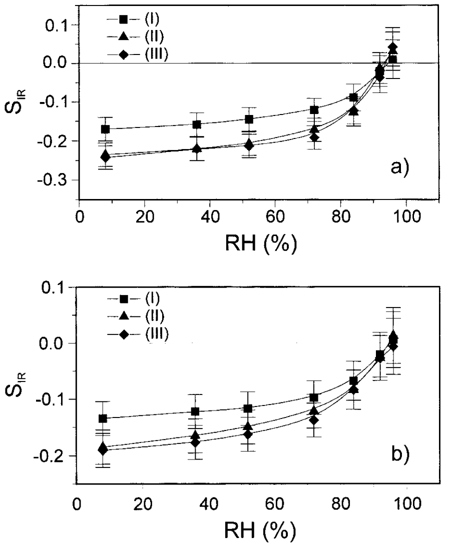

Fig. 7. IR order parameter, S_{IR}, of hyaluronic acid (a) and chondroitin sulfate (b) in dependence on the relative humidity.

The absorption bands II and III (as described in Fig. 6) shift to higher wavenumbers upon increasing the relative humidity. The respective graphs closely resemble the behavior of the corresponding IR order parameters (compare Figs. 7 and 8). This result might be

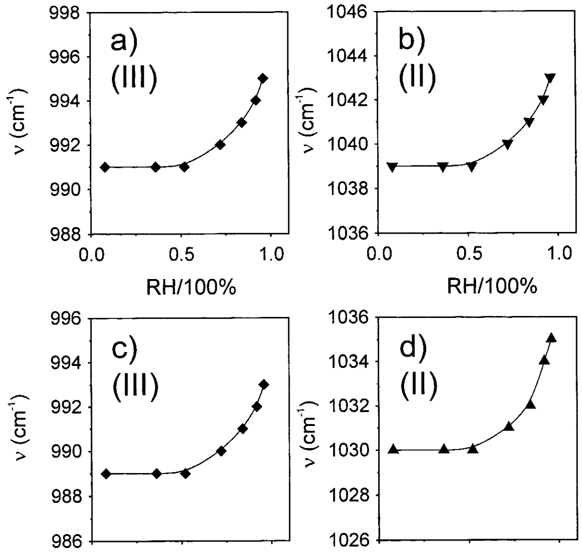

Fig. 8. Position of the absorption maximum of band II and III (cf. Fig. 6) as a function of the relative humidity for hyaluronic acid (a,b) and chondroitin sulfate (c,d).

important for the interpretation of the IR linear dichroism.

The reduction of the order parameters of the films starts at about RH 84%, corresponding to a water content of about ten water molecules per PRU, as calculated from the sorption isotherm. By using NMR relaxation times a number of four to nine tightly bound water molecules per PRU was obtained for GAG solutions [32]. Using MD simulations, Kaufmann et al. [33] have calculated, that on average about ten hydrogen bonds are formed between water and a single repeating unit of HA. When the RH is increased beyond this critical value of hydration, free volume necessary for the motion and reorientation of polymer chains is obviously formed giving rise to the increased disorder of the polymer chains accompanied by an increased uptake of water as observed in the sorption isotherms.

It is remarkable that the observed IR-dichroism appeared for samples without carrying out any previous macroscopic orientation (for instance upon stretching the polymer film). Lee et al. found for such previously oriented HA-films a phase transition [5,6]. A discontinuity in the speed of sound was found in the range of relative humidities between 84 and 88%. Using different techniques, Jouon et al. [7] have confirmed these results. It was found that HA films show a transition of their mechanical properties around RH = 84%, which corresponds to a plastification of the material. DSC studies have shown that freezable water appears at this humidity. The critical value of RH at which the change of the IR-dichroism indicates the lubrication of the polymeric chains is in agreement with these results.

The IR-dichroism reflects the mean orientation of the transition moments of the corresponding vibrational modes. IR order parameters $S_{\mathrm{IR}} \neq 0$ indicate the macroscopic orientation of the polymer helices of HA and CS with respect to the ATR crystal. Note that this ordering appears spontaneously, i.e. without special manipulation as applied for example to crystalline samples [15]. Independent preparations yield identical results. That means that the molecular orientations are symmetric with respect to the normal of the ATR crystal. Strains of HA potentially arrange into helical structures with hydrophobic and hydrophilic pockets between adjacent helices [31]. We suggest that the polymeric chains spontaneously align parallel with the ATR surface. At low RH, the ordering is driven by attractive interactions between neighboring chains and by intermolecular forces which possibly cause a helical conformation. Upon increasing the relative humidity, water imbibes into the hydrophilic pockets giving rise to the swelling of the system. The hydration weakens the stabilizing forces between the polymer chains and between their units and, as a result, the macroscopic order decreases and finally disappears. This process starts already at about RH 50%, i.e. at about four water molecules per PRU, indicated by a shift of the vibrational bands of the polymer rings. At RH higher than 84% the increased water content of more than ten water molecules per PRU is sufficient to hydrate all polar groups. This leads to an enhancement of the mobility resulting in a decrease of the degree of molecular ordering. At nearly full hydration (RH $\geq 92\%$), the orientation of the polymer chains becomes isotropic and the IR-dichroism vanishes completely.

The IR order parameter of an ensemble of polymer chains on the ATR crystal may be written as

$$S_{\mathrm{IR}} = S_{\mathrm{d}} \cdot S_{\mu} \qquad (3)$$

where the order parameter of helix orientation, $S_{\mathrm{d}} \equiv \langle P_2(\theta) \rangle$ and the segmential order parameter of the respective transition moment, $S_{\mu} \equiv \langle P_2(\theta_{\mu}) \rangle$ are defined as the mean second order Legendre polynomial, $P_2(x) \equiv 0.5(3\cos^2 x - 1)$, $(x = \theta, \theta_{\mu})$. The angle θ encloses the fibre axis of the polymer and the normal of the ATR surface. For polymers tending to align with the surface one obtains $0 > S_{\mathrm{d}} > -0.5$ [34]. The angle θ_{μ} encloses the transition moment of the IR mode and the fibre axis of the polymer. Hence, for transition moments pointing predominantly along the fibre axis $(0 \leq \theta_{\mu} \leq 54°)$, one obtains $S_{\mu} > 0$ and thus, $S_{\mathrm{IR}} < 0$. A negative IR order parameter is also expected in the special case if the transition moments are cylindrically distributed about the fibre axis $(S_{\mu} = 0.25)$, $S_{\mathrm{IR}} = -0.125$. This qualitative estimation shows that the assumed structure is compatible with the observed negative IR order parameters of the pyranose rings. Moreover, in the helical structure given by Sheehan, the pyranose rings wind about the helix axis [31]. Their planes orient nearly parallel to the surface of a cylinder the axis of which coincides with that of the helix. In this structure, the transition moments of the ring vibrations are expected to point preferentially along the chain axis and/or parallel with respect to the cylinder surface yielding $S_{\mathrm{IR}} < 0$ in agreement with our observation.

4. Conclusions

Sorption isotherms of water were measured for hyaluronic acid and chondroitin sulfate in the range of relative humidities (RH) between 0 and 96%. A low-expressed increase of sorption of water molecules occurs up to about RH = 75%. At higher relative humidities, the water uptake increases strongly. The water binding capacity is higher for CS compared to HA due to the sulfate group.

The investigation of hydration-dependent changes of the IR spectra gives a view of the water binding and the structural changes of HA and CS upon gradual hydration. The integral intensity of the ν(OH) stretching mode increases in parallel to the water sorption of the films. Differences of the shape of the broad ν_{13}(OH) band between CS and HA suggest different energetic characteristics of the primary hydration shell. It is concluded that the water is less strongly bound to the sulfate group of CS compared to the water that interacts with the pyranose ring. Shifts of the antisymmetric vibrations of the carboxylate groups to lower frequencies occur over the entire range of water concentrations, indicating the formation of hydrogen bonds between water molecules and those groups.

At a water content of about four water molecules per PRU, the absorption band assigned to the pyranose ring starts to shift to higher wavenumbers. Obviously, this water content decreases efficiently the packing of the rings, thus enabling a higher mobility of the ring groups.

An interesting result is that linear IR dichroism of the pyranose ring vibrations was observed without carrying out any previous orientation. This IR dichroism hardly changes for $\mathrm{RH} < 80\%$. At $\approx 84\%$ it drops strongly and vanishes for $\mathrm{RH} > 92\%$. Other authors have also found this hydration transition using previously oriented HA films. This behavior can be understood in terms of critical water contents. At the critical RH of $\approx 80\%$, primary hydration sites of the polymer such as the pyranose rings, the carboxylate and sulfate (only CS) groups, are saturated by water molecules via hydrogen bonds. The free volume formed by this water layer is correlated with a higher reorientational mobility resulting in a diminished polymer orientation. This may also explain the strong increase of the water uptake driven by the entropic gain due to the configurational fluctuations and the mixing process of polymer and water.

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