Colloid Polym Sci (2004) 282: 1067–1076
DOI 10.1007/s00396-003-1033-8

ORIGINAL CONTRIBUTION

M. Wloka
H. Rehage
H.-C. Flemming
J. Wingender

Rheological properties of viscoelastic biofilm extracellular polymeric substances and comparison to the behavior of calcium alginate gels

Received: 27 July 2003
Accepted: 24 November 2003
Published online: 15 January 2004

M. Wloka (✉) · H. Rehage
Institut für Umweltanalytik,
Universität Duisburg–Essen,
Universitätsstrasse 3–5,
45141 Essen, Germany
E-mail: markus.wloka@uni-essen.de
Tel.: +49-201-1834110
Fax: +49 201-1833951

H.-C. Flemming · J. Wingender
Institut für Grenzflächenbiotechnologie,
Universität Duisburg–Essen,
Geibelstr. 41, 47057 Duisburg, Germany

Abstract In this publication we present a detailed study of viscoelastic biofilms of *Pseudomonas aeruginosa*. Sample solutions were extracted from biofilm layers grown on Pseudomonas isolation agar. This aqueous solutions of extracellular polymeric substances exhibit weak elastic effects caused by entanglements and a small number of permanent junction points formed by calcium ions. The cross-linking mechanisms are confirmed by the Cox–Merz rule and dynamic frequency sweep tests, which result in an average lifetime of junction points of the order of 17 ms. The experimental data reveal 3.4×10^{17} elastically effective chains per liter of solution and no significant temperature effects in the regime between 2 and 24 °C. This result coincides pretty well with the concentration of dissolved polymer chains (2.9×10^{17} molecules/l). Upon addition of calcium ions, one observes the formation of stable supermolecular networks with permanent junction points. These cross-linking points did not show thermal fluctuations in time zones between 10 ms and several hours. The entanglement density of these gels is of the same order as observed in the non-cross-linked sol state (entrapped entanglements). In spite of the different molecular composition alginate gels show the same type of cross-linking mechanism as gels of extracellular polymeric substances.

Keywords Biofilm · *Pseudomonas aeruginosa* · Alginate · Rheological parameters · Gels

Introduction

Biofilms are the favored way of life for microorganisms [1, 2]. They can be found in almost every habitat on earth [2]. Typical components of biofilms are microorganisms, extracellular polymeric substances (EPS) and water up to 98% [3]. The gel-like EPS network is the metabolic product of bacteria and consists of different types of polysaccharides, but these compounds are often accompanied by nucleic acids and lipids [2]. In the case of mucoid strains of *Pseudomonas aeruginosa* the major exopolysaccharide is alginate, which consists of uronic acid residues, β-D-mannuronate and α-L-guloronate; thus, alginate is a polyelectrolyte [2]. It turns out that the exact structure of the biofilms depends on environmental factors, such as the age and type of microorganisms [2]. The EPS protect the biofilm from a hostile environment, support the cells with nutrients and allow communication between the cells [3]. The cavelike biofilm structures are responsible for good exchange of water and water-soluble molecules. The mechanical stability of these film structures strongly depends on the EPS. The forces responsible for adhesion of biofilms on surfaces and the stability of the biofilm matrix are caused by hydrogen bonds, dispersion forces and electrostatic interactions [4]. An extensive overview of different types of binding

mechanisms and their specific contributions to biofilm stability is given in Ref. [4]. Ions are of great importance because they may serve as cross-linking agents (junction points). In this way, supermolecular networks of charged polysaccharides can easily be formed. In the case of biofilm removal, cross-linking processes should be avoided. Cleaners have to weaken the matrix by means of chemical interactions. It is well known that biofilms have great influences on surface properties as they can turn hydrophobic areas into hydrophilic surroundings. This may influence friction resistance, heat transport and certain medical processes [4]. On account of different applications, it is interesting to study the basic mechanical properties of typical biofilms [5].

The EPS matrix can be considered as a physical gel, where chemical cross-linking mechanisms are excluded [6]. Basic theories about the gel-forming physical–chemical processes have recently been discussed [6]. In addition to hydrogen bonds and Coulomb interactions, there might also be an influence of mechanical interference caused by entanglements between polymer molecules. These processes can lead to gel-like properties of polysaccharides when the molar mass of the polymer exceeds a threshold value of 1.5×10^5 Da [7]. Physical forces, such as entanglements, van der Waals interactions or hydrogen bonds, generally lead to the formation of fluctuating junction points. In contrast to chemically induced cross-linking processes, which ultimately lead to the formation of permanent network structures, physical contacts are generally characterized by transient properties. For small perturbative forces and over short time periods these attachments act in a similar way as permanent linkages and confer elastic properties on the sample. For longer times, however, relaxation processes occur. The molecules can undergo diffusional motion, which leads to a certain stress decay. For longer loading durations, physical linkages can be broken, and this leads to a transition towards flow. In the ideal case, the behavior of the system after a long period is characteristic of a liquid. The fundamental properties of such systems show a marked dependence on the duration of the measurement or on the frequency in dynamic experiments. This type of behavior is well described by the expression ''transient or temporary'' network. Up to now very little is known about the rheological properties of biofilms. The first investigations of Charaklis [8] revealed a shear modulus in the range of 60 Pa. This value was measured with a Weissenberg rheogoniometer. ''In situ'' investigations of Stoodley et al. [9] were interpreted in terms of a Young modulus (17–40 Pa), and a shear modulus (27 Pa). Several tests were carried out in a flow-cell reactor, where structural deformations of filamentous streamers, caused by changes in hydrodynamic shear stress, were measured with microscopic methods. The resulting moduli could only be estimated (Young modulus 17–40 Pa, shear modulus 27 Pa). In these studies, a yield point was detected. Later Körstgens et al. [6] measured a value of 980 Pa for the yield point, and 6,500 Pa for the Young modulus. These data were obtained in uniaxial compression experiments using a special film rheometer. Ohashi et al. [10] studied the tensile strength of biofilms, which led to an estimated elastic modulus in the range 500–1,000 Pa.

The present article is an attempt to increase our knowledge on biofilm stability. Instead of native biofilms we used defined solutions of EPS macromolecules in order to solve problems of heterogeneity and ageing. All experiments were performed using a Rheometrics Fluids Spectrometer II, which allowed us to measure elastic and viscous properties as a function of time or frequency.

Experimental

Biofilm and EPS solution preparation

Biofilms were obtained by cultivating the environmental mucoid strain *P. aeruginosa* SG 81 on the surface of Pseudomonas isolation agar (PIA, Difco) in Petri dishes for 24 h at 36 °C. These microorganisms were originally isolated from a technical water system. Single colonies were suspended in sterile 0.14 M NaCl solutions to reach an approximate cell density of 6×10^8 cells/ml. Some (0.1 ml) of the suspension was subsequently spread-plated on PIA. After incubation for 24 h at 36 °C, the confluent slime layers were carefully removed from the agar surface with a spatula and were suspended in 0.14 M NaCl solution. The mass ratio of bacterial films with respect to the solvent water was about 1:16. Homogenizing was carried out with a magnetic stirrer for 30 min. After separation of the EPS from the bacteria by centrifugation at 40,000*g* at 10 °C for 2 h the solutions were filtered through cellulose acetate membranes having an average pore diameter of 0.2 μm [11, 12]. The resulting clear filtrate formed the EPS stock solution for all the measurements. Experiments were performed at ambient (22 ± 2 °C) and low (2 ± 2 °C) temperatures. We investigated four separate EPS stock solutions which were prepared within 9 weeks of each other. In this context it is interesting to note that even the dilute EPS solutions contained a small number of calcium ions. These ions were accumulated from the agar medium during growth of the biofilm and remained after preparation in the EPS solution. This special calcium concentration could be determined with inductively coupled plasma optical emission spectroscopy. The sample was mixed with water and sprayed into the plasma. Atoms in the plasma emitted photons with characteristic wavelengths for each element. The emitted light was recorded by an optical spectrometer and compared to standards for the use in quantitative analysis. It turned out that the calcium concentration was lower than 0.125 mmol/l. This low calcium ion concentration did not lead to significant cross-linking processes. Distinct effects were only observed at ion concentrations 10 times higher.

EPS and calcium alginate gel preparation

It is well known that divalent cations like calcium can be responsible for most of the binding energy of EPS in biofilms [4]. The Coulomb interactions between negatively charged polysaccharides and positive ions are relatively strong and can be compared to junctions induced by chemical reactions. For systematic comparison of EPS solutions with relevant calcium alginate gels, we used a

special method to prepare homogeneous gel structures with algal alginates [13, 14]. Without this procedure anisotropic networks were obtained, which are often denoted as ionotropic gels [15]. Microscopic experiments with crossed polarizers did not show any birefringence effects of our alginate gels. We can, hence, conclude that these structures have isotropic character. In order to form these gels, we first dissolved the cross-linking compounds in water. Finely dispersed $CaCO_3$ and slowly hydrolyzing acid glucono-δ-lactone (GDL), both obtained from Fluka, were used for homogeneous and slow release of calcium ions. In these experiments, the molar ratio between $CaCO_3$ and GDL was fixed at 1:2. Calcium alginate gels were prepared by adding sodium alginate from Fluka and Manucol LB obtained from ISP Alginate to a dispersion of different concentrations of $CaCO_3$ in water. Ultrasound energy was applied to form small calcium carbonate crystals. We finally obtained a 1% w/v sodium-alginate–$CaCO_3$ solution. Freshly prepared GDL was finally added to this mixture. According to different contents of calcium ions, homogeneous gel formation could be observed within a time scale of 2–12 h.

The 1% sodium alginate solutions had a measured zero-shear viscosity of $\eta_0 = 0.04 \pm 0.01$ Pa s. We investigated this special solution because the viscous resistance was not too different from the viscosity of the EPS solutions prepared from *P. aeruginosa* ($\eta_0 = 0.07 \pm 0.02$ Pa s). In this context, it is interesting to note that bacterial and algal alginates have different molecular weights and properties [16]. It is, therefore, only possible to compare these data qualitatively. EPS gels were prepared by adding $CaCO_3$ to an EPS solution under stirring. This mixture was sonicated for 2 min to homogenize it. Freshly prepared GDL solution was added afterwards. The molar ratio between $CaCO_3$ and GDL was 1:2.

Rheometer

Rheological experiments were performed using a strain-controlled Rheometrics RFS II spectrometer with a 0.02 rad cone–plate (50-mm diameter). A Couette geometry (cup diameter 34.0 mm, bob diameter 32.0 mm, bob length 33.4 mm) was used for measurements of low viscosity. To avoid water evaporation during the experiments, a special solvent trap was used. The angular frequency range investigated was 0.001–100 rad/s, and steady-state shear rates were applied in the range between 0.1 and 1,500 1/s.

Results

EPS solutions

For the rheological tests we used four different EPS solutions. All figures show the average of at least four different measurements per EPS solution prepared. In periodic experiments the shear strain is varied sinusoidally at an angular frequency ω. The response of the material to the periodic change consists of a sinusoidal shear stress which is out of phase with the strain. The shear stress is made up of two different components which are in phase with the deformation and out of phase with the strain. From the phase angle, δ, the shear stress, σ, and the shear strain, γ, it is possible to calculate the storage modulus, $G'(\omega)$. This parameter describes the elastic properties of the sample. The loss modulus, $G''(\omega)$, is proportional to the viscous resistance [17, 18].

$$G'(\omega) = \left(\frac{\sigma}{\gamma}\right) \cos\delta \tag{1}$$

$$G''(\omega) = \left(\frac{\sigma}{\gamma}\right) \sin\delta \tag{2}$$

The periodically varying functions can be expressed as a complex quantity according to Eq. (3). The magnitude of the complex viscosity, $|\eta^*|(\omega)$, can be calculated from Eq. (5) [19].

$$G^*(\omega,\gamma) = G'(\omega,\gamma) + iG''(\omega,\gamma) \tag{3}$$

$$\eta^*(\omega,\gamma) = \frac{G^*(\omega,\gamma)}{i\omega} \tag{4}$$

$$|\eta^*|(\omega) = \frac{\sqrt{G'^2(\omega) + G''^2(\omega)}}{\omega} \tag{5}$$

A typical strain–sweep experiment is shown in Fig. 1. We measured the storage modulus and the loss modulus as a function of the shear strain. The linear viscoelastic regime is characterized by constant rheological properties (plateau regime). It is easy to recognize that the supermolecular network structure can be stretched up to a limit of about 70% before nonlinear properties occur. This is typical for rubber–elastic materials, which can support rather large deformations without rupture. No substance otherwise constituted will sustain the high deformations that are typical of rubberlike materials. A maximum elongation of 5–10 times the unstretched length is commonplace among typical gels. And when the stress is removed, the material recovers its original dimensions. This phenomenon is not restricted to a certain class of materials, but can be observed in many different systems. Macromolecules, inorganic polymers like sulfur or polyphosphazenes, biopolymers such as elastin and collagen display the previously mentioned characteristics. All these molecules are capable of building infinitely long, supermolecular network structures.

In dynamic experiments the flow behavior was investigated as a function of the angular frequency (Fig. 2). It is evident that the EPS solutions exhibit viscoelastic properties. The experimental data can be qualitatively described by the relations for a Maxwell material. This element consists of a spring and a dashpot connected in series. The elastic spring corresponds to a shear modulus G_0 and the dashpot represents the constant viscosity η_0. The dynamic properties of the Maxwell element can be represented by linear differential equations, the solutions of which give the desired material functions. The Maxwell model describes the phenomenon of stress relaxation and can, therefore, be used to illustrate the dynamic properties

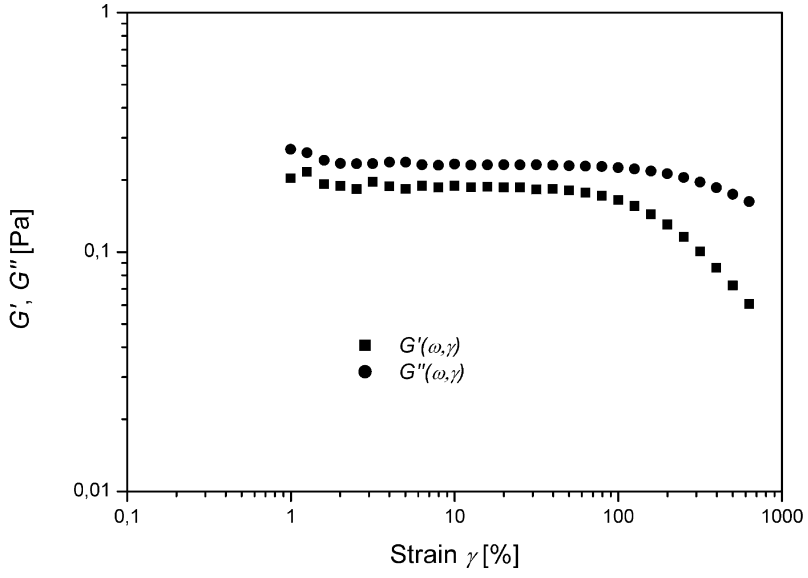

Fig. 1 Test of the Cox–Merz rule. Comparison of the steady-state shear viscosity $\eta(\dot{\gamma})$ and the magnitude of the complex viscosity $|\eta^*|(\omega)$ at equal values of the frequency and shear rate (EPS solution)

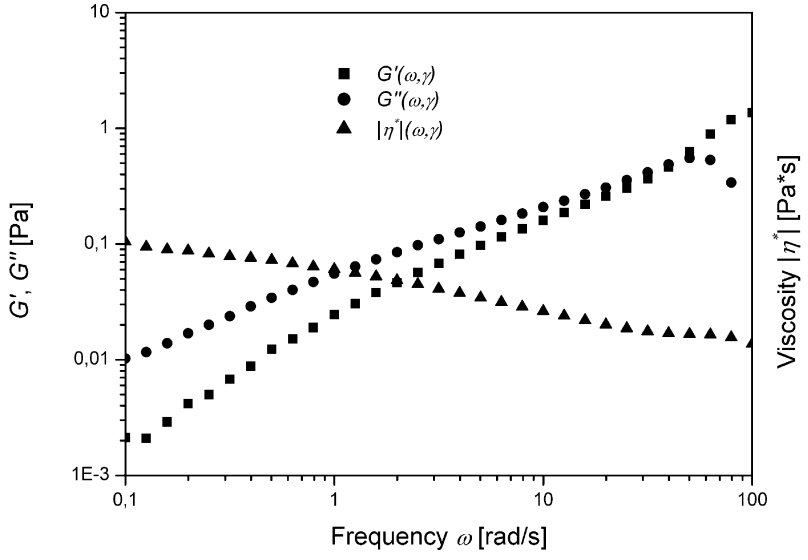

Fig. 2 Gel formation of a 1% sodium-alginate-$CaCO_3$ solution with added glucono-δ-lactone. $G'(\omega)$ and $G''(\omega)$ are plotted as a function of time at $\omega = 10$ rad/s and $\gamma = 1\%$

of viscoelastic liquids. The behavior under harmonic oscillations can be obtained from the following formulas [20, 21]:

$$G'(\omega) = G_0 \frac{\omega^2\lambda^2}{1+\omega^2\lambda^2}, \tag{6}$$

$$G''(\omega) - \eta_s\omega = G_0 \frac{\omega\lambda}{1+\omega^2\lambda^2}. \tag{7}$$

At low values of shear strain the storage modulus and the loss modulus are only functions of the angular frequency. Above a certain threshold value, these functions also depend on the strain amplitude. η_s describes the solvent viscosity and λ denotes the stress relaxation time of the Maxwell material.

In the regime of low frequencies, the loss modulus is larger than the storage modulus. At these conditions, stress relaxation occurs and flow properties dominate. With increasing angular frequency, the elastic response becomes more evident. The intersection point between G' and G'' describes the average relaxation time $\lambda = 1/\omega_{int}$. We obtained a characteristic value of the order of 17 ms. This time constant can be characterized as the average lifetime of the junction points. Under experimental conditions, where the average lifetime of the cross-linking points is much smaller than the time span of observation, there are numerous breaking and reformation processes of junctions points. As a consequence, an applied shear stress relaxes through chemical pathways. This phenomenon can be compared with a

situation sometimes observed in daily life. Imagine two persons are pulling on the opposite ends of a rope. As long as the rope is still intact, the tensile stress is homogeneously distributed along the connection line. But as soon as breakage occurs, the mechanical forces decay to zero (stress relaxation). The rate constant of this phenomenon is correlated to the dynamics of fracture. And in a similar manner, stress decay may occur by rupture processes of junction points. Under conditions of short breaking times, we observe stress decay when investigating a large ensemble of network stands. If, however, the experiment is performed at short times, there is no fluctuation of junction points and we will observe the behavior of a permanently cross-linked network. In this regime, the storage modulus attains a plateau level and $G'(\omega) \gg G''(\omega)$. For the EPS solution investigated we obtained an equilibrium shear modulus of $G_0 = 1.4 \pm 0.2$ Pa. The intermediate frequency range is characterized by ambivalent viscoelastic behavior.

It is easy to recognize that the experimental data show multiexponential relaxation processes. Further insight can be obtained by calculating the relaxation time spectrum, $H(\lambda)$, which is accessible from experimental dynamic data like the storage modulus and the loss modulus. For the conversion of the relaxation time spectrum $H(\lambda)$ into $G(t)$, $G'(\omega,\gamma)$ and $G''(\omega,\gamma)$ you have to solve one of the integral equations (Eq. 8, 9, 10) [22]:

$$G(t) = \int_{-\infty}^{+\infty} H(\lambda) e^{-t/\lambda} \mathrm{d}ln\lambda, \tag{8}$$

$$G'(\omega) = \int_{-\infty}^{+\infty} H(\lambda) \frac{\omega^2\lambda^2}{1+\omega^2\lambda^2} \mathrm{d}\ln\lambda, \tag{9}$$

$$G''(\omega) = \int_{-\infty}^{+\infty} H(\lambda) \frac{\omega\lambda}{1+\omega^2\lambda^2} \mathrm{d}\ln\lambda. \tag{10}$$

The relaxation time spectrum (Fig. 3) describes the distribution of relaxation times and moduli. The relaxation time generally denotes the conversion of reversibly stored energy into irreversibly dissipated energy. Calculation of the relaxation spectrum was carried out using rheoscience rheocalc software with algorithms based on the NLREG program developed at the University of Freiburg, Germany [23].

The course of the curve is similar to amorphous non-cross-linked polymers of high molecular weight with long side groups as described in Ref. [24]. Such systems are viscoelastic liquids and exhibit viscous flow at sufficiently long times. The maxima represent concentrations of relaxation processes in certain regimes of the time scale. At long times, when steady-state flow is reached, $H(\lambda)$ becomes very small on logarithmic scales. The gradual drop of the curve is due to the large molecular weight distribution. In solutions of macromolecules it is often observed that the steady-state shear viscosity, $\eta(\dot{\gamma})$,corresponds with the magnitude of the complex viscosity, $|\eta^*|(\omega)$. This holds for equal values of the shear rate $\dot{\gamma}$ and the angular frequency ω (Eq. 11). This phenomenon is known as the empirical Cox–Merz-rule [16].

$$\eta(\dot{\gamma}) = |\eta^*|(\omega) \qquad \text{for} \quad \dot{\gamma} = \omega \tag{11}$$

This special law describes a simple relationship between linear and nonlinear viscoelastic properties [16]. Relevant data are summarized in Fig. 4. It turns out that the shear viscosity coincides pretty well with the magnitude of the complex viscous resistance. It is well known that such good agreement is often obtained for

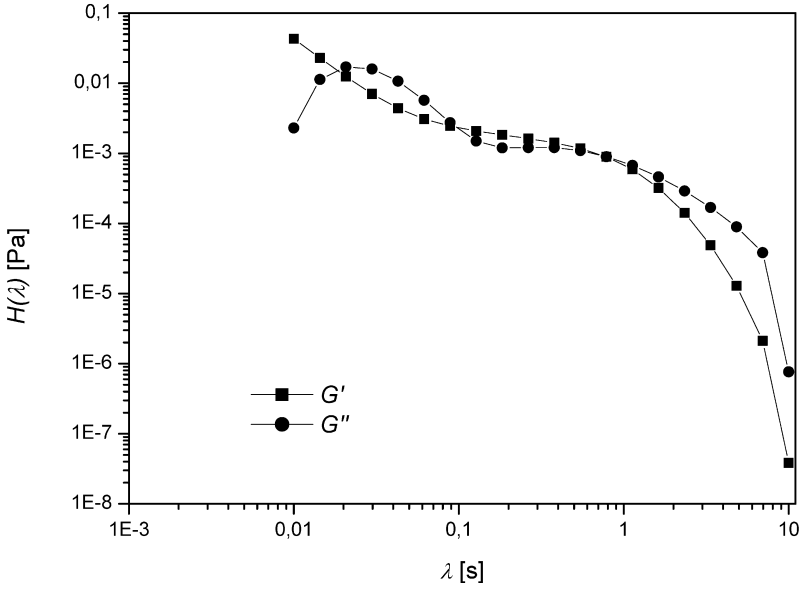

Fig. 3 Dynamic properties of the storage modulus $G'(\omega)$ and the loss modulus $G''(\omega)$ as a function of the applied strain γ ($\omega = 10$ rad/s, $T = 20$ °C) for an extracellular polymeric substance (*EPS*) solution

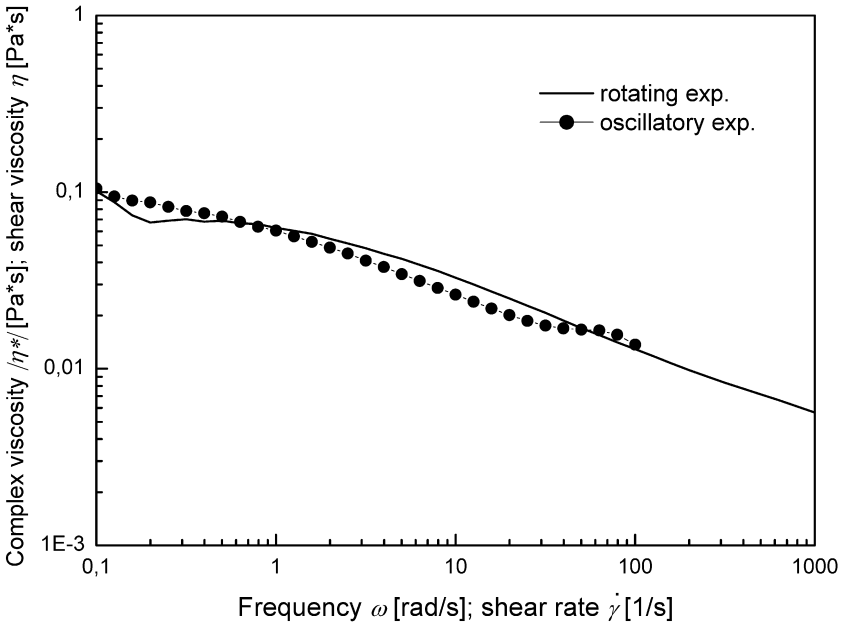

Fig. 4 Dynamic properties of $G'(\omega)$, $G''(\omega)$ and the complex viscosity $|\eta^*|(\omega)$ as a function of the applied frequency ω ($\gamma = 50\%$, $T = 21$ °C). The course of the curves is equivalent to a generalized Maxwell model

solutions of entangled macromolecules [25]. If other types of cross-linked mechanisms, such as Coulomb forces, complex formations or hydrogen bonds, are included, the magnitude of the complex viscosity is generally larger than the steady-state values of the shear viscosity [25]. This can, for instance, be observed in alginate gels, at conditions where junction points are formed by Coulomb forces. Discrepancies between the magnitude of the complex viscosity and the steady-state shear viscosity can be explained by rupture processes of junction points which occur during flow. In analogy to dilute polymer solutions we can conclude that the EPS solutions exhibit typical properties of entangled macromolecules. In this context it is interesting to note that the-EPS solutions contained a small number of calcium ions, originating from the agar growth medium, which are capable of bridging polysaccharide chains via Coulomb forces. But the low amounts measured have no significant effects on three-dimensional cross-linking processes (see the next subsection).

From the theory of rubberlike elasticity, it is possible to calculate the number of elastically effective network strands per unit volume [24, 26]. The theory predicts

$$G_0 = AvkT. \tag{12}$$

Here, G_0 denotes the equilibrium shear modulus which coincides with the plateau regime of $G'(\omega)$. A is a prefactor of order unity, v describes the concentration of elastically effective chains, k denotes the Boltzmann constant and T is the absolute temperature. Inserting the experimental values from Fig. 2 leads to an average number of 3.4×10^{17} elastically effective network strands per liter of solution. As the exact value of A is not known and the plateau value of the storage modulus is not very well pronounced, v will only describe the right order of magnitude. The concentration of polymer chains in solution (2.9×10^{17} 1/l) was calculated from the concentration of uronic acids. A value of 1.12 g/l was determined by spectroscopy [12, 27]. The average molecular weight of alginate was determined from intrinsic viscosities (2,370,000 g/mol) [28]. It is evident that the concentration of macromolecules is of the same order as the density of elastically effective chains determined by rheological measurements. This means that each macromolecule is temporarily linked to two other chains. As the functionality of the junction points is not known, we can only give information on average concentrations of network stands, which act as entropy-elastic springs. By varying the temperature and investigating fresh and 9-week-old EPS solutions we found deviations of the order of 6%. Such small discrepancies can be regarded as systematic errors which are caused by inaccuracy of data measuring and heterogeneity problems of the biological material. No hints for thixotropy or rheopexy were found.

Solutions of cross-linked EPS and calcium alginate gels

The previously mentioned experiments show that dilute solutions of EPS and sodium alginate tend to form weakly cross-linked temporary network structures which are stabilized by entanglements. Upon addition of calcium ions, we obtain more stable junction points which are formed by Coulomb interactions.

Information on the kinetics of cross-linking formation is given in Fig. 5. Immediately after inserting the solution into the measuring system, the solution is still in the sol state. At these conditions, the viscous properties are dominant and $G''(\omega)$ is larger than $G'(\omega)$. As a function of time, the number of junction points steadily increases. The intersection point between $G'(\omega)$ and $G''(\omega)$ is often described as the sol–gel transition [29]. At this threshold value, a three-dimensional network is first detected, spanning the whole sample. With increasing reaction time, more and more junction points are formed and the elastic response in this regime is larger than the dissipated energy. Finally, a plateau level is reached, where all cross-linking reactions are terminated.

In order to investigate these three-dimensional network structures, we prepared homogenous calcium gels. In a series of experiments, sodium alginate and EPS gels were prepared with calcium concentrations of 1, 3, 5, 7 and 10 mmol/l (Fluka sodium alginate) (Fig. 6). It is worthwhile mentioning that EPS gels prepared from older solutions showed syneresis effects. These gels were not stable for times longer than 24 h in contrast to gels prepared with fresh solution, which showed no time-dependent effects.

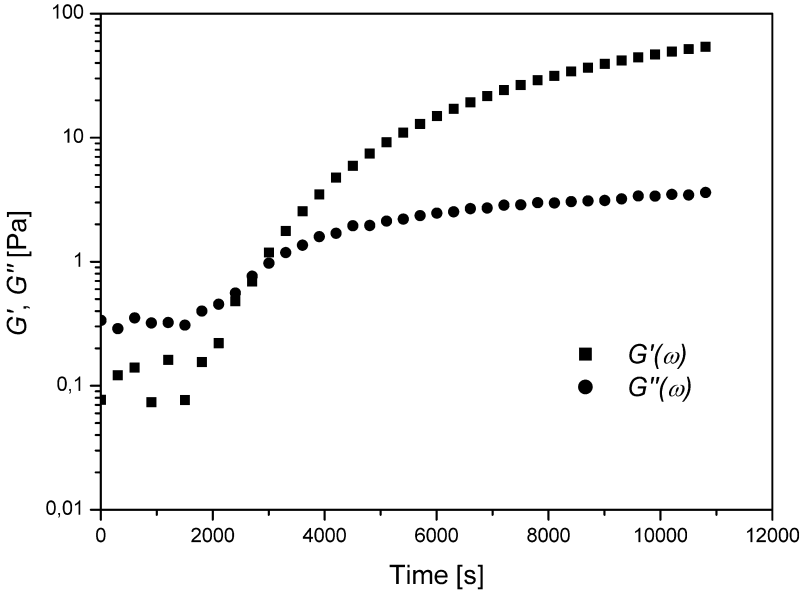

Fig. 5 The relaxation time spectrum $H(\lambda)$ of an EPS solution calculated from values of $G'(\omega)$ and $G''(\omega)$

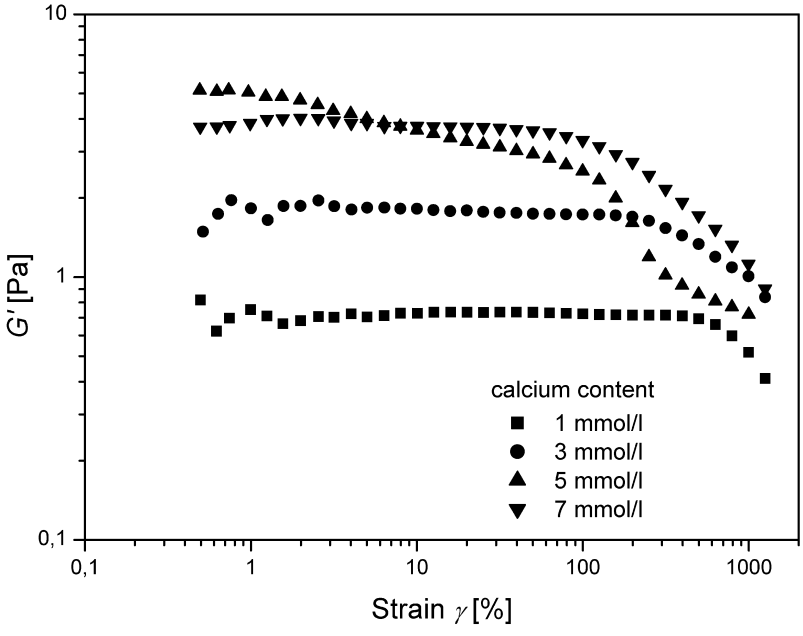

Fig. 6 Dynamic properties of $G'(\omega)$ as a function of γ ($\omega = 10$ rad/s) for EPS-gels with different concentrations of calcium ions

Inspection of the data reveals that with increasing calcium concentration the gel becomes more cross-linked. This leads to the observed plateau values of G'. Binding is provided by electrostatic interactions between divalent calcium ions and carboxylic groups of the polysaccharides. Additionally there is also the possibility for London dispersion forces on account of hydrophobic pockets formed by acetylated groups of the alginate as described in Ref. [4]. In order to get information on the number of hydrophobic domains, we used fluorescence spectroscopy. It is well known that nonpolar fluorescence markers such as pyrene specifically adsorb on hydrophobic groups [30]. As the fluorescence spectrum of pyrene depends on the polarity of the surrounding liquid of this probe, it is easy to recognize hydrophobic domains, because in this case the heights of the peaks are changed [30]. In alginate gels, however, we obtained the typical spectrum of water surrounded pyrene; indicating the absence of hydrophobic domains (Fig. 7).

The strain sweep experiment of Fig. 6 shows that the EPS gels can be deformed for several hundred percent, before nonlinear properties occur. This can again be explained by the rubber–elastic properties of those materials.

Typical dynamic properties of EPS gels are summarized in Fig. 8. The data were obtained for an EPS stock solution upon addition of 7 mmol/l calcium. The storage and loss moduli are independent of the frequency in the measured range between 10^{-4} and 100 rad/s. Experimental data for other calcium concentrations showed

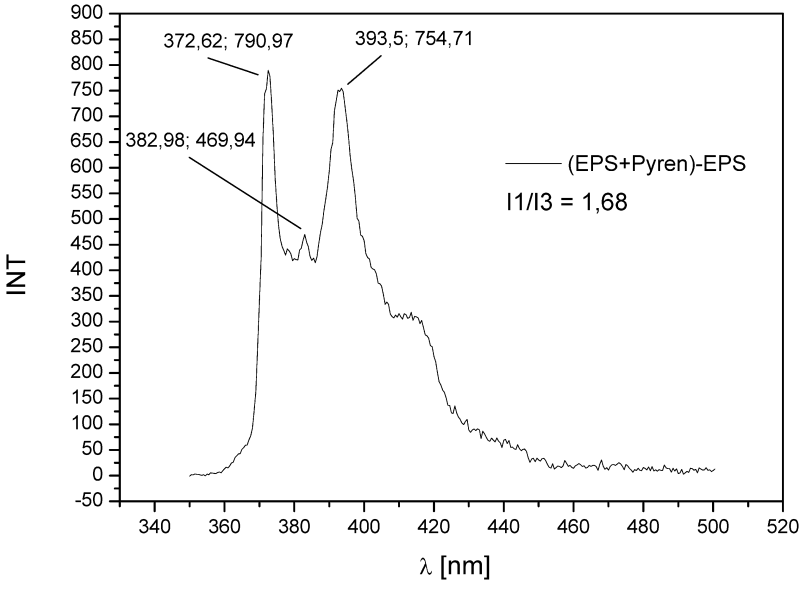

Fig. 7 Fluorescence spectrum of an EPS solution with 10^{-6} mol/l pyrene as a marker. The weak fluorescence signal of the EPS without pyrene was substracted from the spectrum

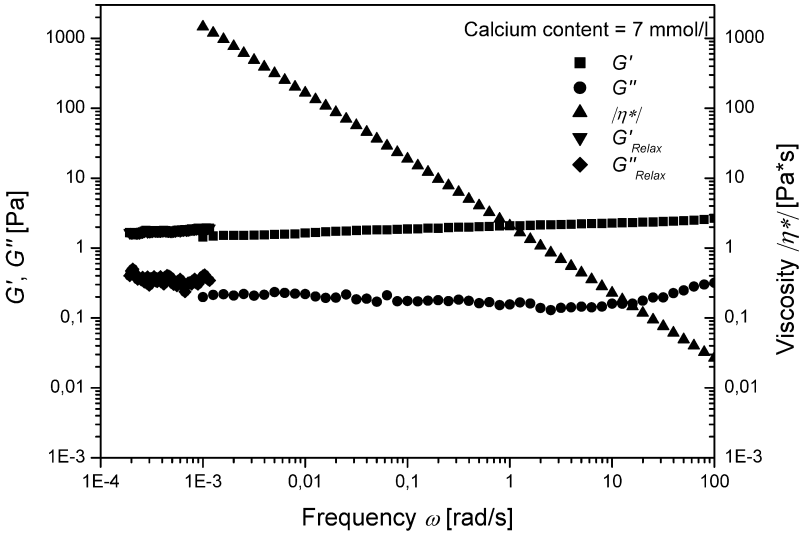

Fig. 8 Dynamic properties of $G'(\omega)$, $G''(\omega)$ and $|\eta^*|(\omega)$ as a function of ω ($\gamma = 10\%$, $T = 24$ °C) for an EPS solution after addition of 7 mmol/l calcium

Table 1 Values of the equilibrium shear modulus, G_0, for gels of extracellular polymeric substances (*EPS*), sodium alginate (Fluka) and Manucol LB (ISP Alginate) with different amounts of calcium. $T = 24$ °C

Ca gel system	G_0 (Pa)						
	1 mmol/l Ca	3 mmol/l Ca	5 mmol/l Ca	7 mmol/l Ca	10 mmol/l Ca	14 mmol/l Ca	20 mmol/l Ca
EPS	0.6	2.7	6.4	4.2	–	–	–
Fluka alginate	0.04	2.9	33	80	75	–	–
Manucol	–	–	–	5.6	14	30	122

similar properties, representing the same type of binding mechanism. The extended plateau values indicate the absence of stress relaxation processes. We can, therefore, conclude that the junction points formed in these EPS gels are stable at least in intervals between 10 ms and 2.5 h. In contrast to entanglements, these junction points do not open with time. The cross-linking process, induced by Coulomb forces, evidently leads to the formation of very stable gels, which are reminiscent of network structures formed by chemical reactions. Experimental data lower than 10^{-3} rad/s were obtained from additional stress relaxation tests. The measured time relaxation modulus, $G(t)$, was converted into dynamic moduli $G'(\omega)$ and $G''(\omega)$ [24]:

$$G'(\omega) = \omega \int_0^\infty G(t) \cdot \sin \omega t \cdot \mathrm{d}t, \tag{13}$$

$$G''(\omega) = \omega \int_0^\infty G(t) \cdot \cos \omega t \cdot \mathrm{d}t. \tag{14}$$

The EPS gels show lower values of the shear modulus compared with sodium alginate gels measured at the same calcium concentration (Table 1). The acetyl groups of bacterial alginate could act as spacers between polysaccharides, preventing more junction points and resulting in different behavior, as described in Ref. [2].

Conclusions

The results of the rheological measurements show that dilute aqueous solutions of EPS exhibit weak elastic effects. This phenomenon is caused by entanglements and a small number of permanent junction points formed by impurities of calcium ions, which are naturally present in EPS solutions. Cross-linking mechanisms mainly caused by mechanical forces are further confirmed by the Cox–Merz-rule, which approximately holds for EPS solutions. The entangled network solutions have temporary character, and the average lifetime of the junction points is of the order of 17 ms. The network density can be calculated from the zero-shear modulus. Analysis of the experimental data reveals values of about 3.4×10^{17} elastically effective chains per liter of solution, which coincides with the number of polymer chains per liter of solution (2.9×10^{17}), determined by spectroscopy as described in Ref. [27]. This agreement can be understood by assuming that each polymer chain is temporarily linked to two other macromolecules. The EPS solutions did not show significant temperature effects in the regime between 2 and 24 °C. Upon addition of calcium ions, the polysaccharide chains were bridged and formed stable, supermolecular networks. Dynamic experiments showed that the junction points did not fluctuate in time intervals between 10 ms and several hours. With increasing numbers of calcium ions the network structures became stabler, resulting in higher values for the zero-shear modulus. We can conclude that besides entanglements, Coulomb forces give a second significant contribution to the elastic response of the EPS gels. As we did not observe any relaxation processes in the gel state, mechanical junctions seem to be stable in these networks. This is often observed for entrapped entanglements, which cannot open because the ends of the macromolecules are fixed by Coulomb interactions [24]. In analogy to calcium alginate gels, networks of EPS macromolecules show the same type of rheological behavior. Close inspection, however, reveals that changes occur on grounds of varying molecular weights and different amounts of guluronate and mannuronate monomers. Calcium-induced junction mechanisms, however, seem to be similar in both systems, in spite of the different monomer sequences and residues [31]. Transferring these data to a natural system, one can assume that natural calcium concentrations over a certain threshold value tend to form stable biofilm networks, which can immobilize bacteria in such a system.

Acknowledgements Financial support by grants of the "Deutsche Forschungsgemeinschaft" (DFG Re 681/12-1) and the "Fonds der Chemischen Industrie" are gratefully acknowledged. The authors are particularly grateful to W. Borchard for his valuable suggestions and comments.

References

1. Costerton JW, Irvin RT (1981) Annu Rev Microbiol 35:299–324
2. Sutherland IW (2001) Water Sci Techol 43: 77–86
3. Wingender J, Neu TR, Flemming H-C, (1999) Microbial extracellular polymeric substances. Springer, Berlin Heidelberg New York
4. Mayer C, Moritz R, Kirschner C, Borchard W, Maibaum R, Wingender J, Flemming H-C (1999) Int J Biol Macromol 26:3–16
5. Costerton JW, Stewart PS (2001) Spektrum Wiss 11:58–65
6. Körstgens V, Flemming H-C, Wingender J, Borchard W (2001) J Microbiol Methods 46:9–17
7. Flemming H-C, Wingender J, Mayer C, Körstgens V, Borchard W (2000) Community structure and co-operation in biofilms. SGM symposium 59. Cambridge University Press, Cambridge, pp 87–105
8. Charaklis WG (1979) Biofilm development and destruction. Electric Power Institute, Palo Alto
9. Stoodley P, Lewandowski Z, Boyle JD, Lappin-Scott HM (1999) Biotechnol Bioeng 65:83–92
10. Ohashi A, Koyama T, Syutsubo K, Harada H (1999) Water SciTechnol 39:261–268
11. Nielsen PH, Jahn A (1999) In: Wingender J, Neu T, Flemming H-C(eds) Microbial extracellular polymeric substances. Springer, Berlin Heidelberg New York, pp 49–72
12. Wingender J et al (2001) Methods Enzymol 336:302–314
13. Draget KI, Ostgaard K, Smidsrod O (1989) Appl Microbiol Biotechnol 31:79–83
14. Draget KI, Ostgaard K, Smidsrod O (1991) Carbohydr Polym 14:159–178
15. Thiele H (1967) Histolyse und Histogenese. Akademische Verlagsgesellschaft, Frankfurt am Main
16. Fischer P, Rehage H (1997) Rheol Acta 36:13–27
17. Gehm L(1998) Rheologie—praxisorientierte Grundlagen und Glossar, Vincentz, Hanover
18. Mezger T (2000) Das Rheologie-Handbuch. Vincentz, Hanover
19. Rehage H, Hoffmann H (1991) Mol Phys 74:933–973
20. Löbl M, Thurn H, Hoffmann H (1984) Ber Bunsenges Phys Chem 88:1102
21. Thurn H, Löbl M, Hoffmann H (1985) J Phys Chem 89:517
22. Tschoegl NW (1989) The phenomenological theory of linear viscoelastic behavior. Springer, Berlin Heidelberg New York
23. Honerkamp J, Weese J (1993) Rheol Acta 32:65–73
24. Ferry JD (1980) Viscoelastic properties of polymers. Wiley, New York
25. Kulicke W-M (1986) Fliessverhalten von Stoffen und Stoffgemischen. Hüthig and Wepf, Basel
26. Rehage H (1982) Rheologische Untersuchungen an viskoelastischen Tensidlösungen. Dissertation. University of Bayreuth, Germany
27. Filisetti-Cozzi TMCC, Carpita NC (1991) Anal Biochem 197:157–162
28. Grobe S, Wingender J, Trüper HG (1995) J Appl Bacteriol 79:94–102
29. Winter HH (1987) Polym Eng Sci 27,22:1698–1702
30. Dominguez A, Fernandez A, Gonzales N, Iglesias E, Montenegro L (1997) J Chem Educ 74:1227–1231
31. Geddie JL, Sutherland IW (1994) Biotechnol Appl Biochem 20:117–129