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FAKULTA CHEMICKÁ
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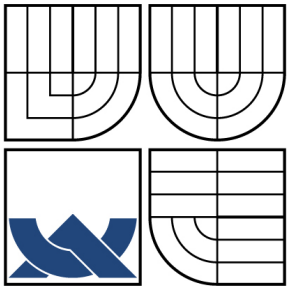
FACULTY OF CHEMISTRY
INSTITUTE OF PHYSICAL AND APPLIED CHEMISTRY

RHEOLOGY OF HYALURONANE SOLUTIONS

DIPLOMOVÁ PRÁCE
DIPLOMA THESIS

AUTOR PRÁCE — KRISTÝNA HLISNIKOVSKÁ
AUTHOR

BRNO 2008



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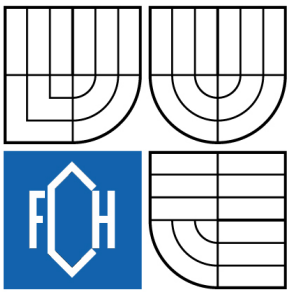

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SUPERVISOR

BRNO 2008

Vysoké učení technické v Brně
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Zadání diplomové práce

Číslo diplomové práce	**FCH-DIP0124/2007**	Akademický rok: **2007/2008**
Ústav	Ústav fyzikální a spotřební chemie	
Student(ka)	**Hlisnikovská Kristýna**	
Studijní program	Spotřební chemie (M2806)	
Studijní obor	Spotřební chemie (2806T002)	
Vedoucí diplomové práce	**doc. Ing. Miloslav Pekař, CSc.**	
Konzultanti diplomové práce	Ing. Martin Chytil	

Název diplomové práce:

Reologie roztoků hyaluronanů

Zadání diplomové práce:

1. Literární rešerše na téma reologie roztoků hyaluronanu, zaměřená zejména na dynamické testy.
2. Návrh relaxačních a krípových experimentů, zahrnujících i studium vlivu koncentrace a molekulové hmotnosti.
3. Provedení experimentů.
4. Vyhodnocení experimentů z hlediska reologického i fungování hylauronanu v lidském těle nebo faramaceutických přípravcích.

Termín odevzdání diplomové práce: 16.5.2008

Diplomová práce se odevzdává ve třech exemplářích na sekretariát ústavu a v elektronické formě vedoucímu diplomové práce. Toto zadání je přílohou diplomové práce.

Kristýna Hlisnikovská	doc. Ing. Miloslav Pekař, CSc.	
student(ka)	Vedoucí práce	Ředitel ústavu

V Brně, dne 1.9.2007

doc. Ing. Jaromír Havlica, CSc.
Děkan fakulty

Abstrakt

Předmětem tohoto studia bylo prozkoumat reologické chování vodných roztoků vysokomolekulárního hyaluronanu. Byl studován vliv zvyšující se koncentrace biopolymeru v roztoku, která se pohybovala v rozmezí 1–3 hm.%, a také vliv vzrůstající iontové síly rozpouštědla, způsobené přídavkem NaCl, na viskoelasticitu a stabilitu těchto roztoků.

Pro obsáhlejší popis viskoelasických vlastností roztoků byla použita, vedle běžných oscilačních měření, také metoda ceepových testů, ze které bylo možno určit důležité veličiny, jako je procentuální poměr viskózní χ_v a elastické χ_e složky vzorku, rovnovážná poddajnost J_e a viskozita při nulovém smykovém napětí η_0. Ty byly následně porovnávány s výstupy z jiných typů měření, jako jsou právě oscilační a tokové křivky, nebo nesly doplňující informace důležité pro detailnější popis viskoelastických vlastností těchto roztoků.

Ke studiu stability vzorků během namáhání pak byla použita metoda „peak hold“, která ukázala na velmi dobou mechanickou i časovou odolnost roztoků hyaluronanu a naznačila hranice, za kterými už dochází k trvalému poškození struktury a degradaci řetězců hyaluronanu a je s nimi proto potřeba při manipulaci s roztoky tohoto biopolymeru pro jejich další použití v aplikacích počítat.

Abstract

The objective of this work was to investigate the rheological behavior of the high-molecular weight hyaluronan solutions. The influence of increasing biopolymer concentration within the range of 1–3% wt and the influence of ionic strength, caused by the addition of NaCl into the solvent, on viscoelasticity and stability of the samples have been studied.

For further description of viscoelastic characteristics of the solutions, besides common oscillation measurements, we have also used the creep test method, from which we obtained other important characteristics such as percentual ratio of viscous, χ_v, and elastic, χ_e, portions of the sample, equilibrium compliance, J_e and zero shear viscosity, η_0. They were compared with the results from the other types of measurements, such as oscillation and flow curves measurements. The creep measurements results contain also some complementary information, important for more detailed description of viscoelastic properties of these solutions.

For the study of the sample stability during constant mechanical strain we have used the “peak hold” method. These measurements proved very good mechanical and time resistance of the HA solutions and specified the limits, beyond which we observed permanent damage of the structure and degradation of the hyaluronan chains, and which have to be taken that into account when manipulating with solutions of this biopolymer.

Klíčová slova

hyaluronan, tok, oscilace, creep&recovery, peak hold

Keywords

hyaluronan, flow, oscillations, creep&recovery, peak hold

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Prohlášení

Prohlašuji, že jsem diplomovou práci vypracovala samostatně, a že všechny použité literární zdroje jsem správně citovala. Diplomová práce je z hlediska obsahu majetkem Fakulty chemické VUT v Brně a může být využita ke komerčním účelům jen se souhlasem vedoucího diplomové práce a děkana FCH VUT.

Declaration

I declare that this diploma thesis has been worked out by myself and I cited all used information sources correctly and completely. The diploma thesis is in terms of its content a property of BUT Faculty of Chemistry and it can be used for commercial proposes only under permission of the diploma thesis supervisor and the dean of BUT Faculty of Chemistry.

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podpis diplomanta

Poděkování

Ráda bych poděkovala svému vedoucímu práce doc. Ing. Miloslavu Pekařovi, CSc za motivaci a inspiraci, svému konzultantovi Ing. Martinovi Chytilovi za cenné rady, trpělivost a dlouhé diskuze a hlavně své rodině za jejich podporu a lásku.

Table of content

1. Introduction

High molecular weight polysaccharides isolated from connective tissues have demonstrated unique rheological properties that are functions of molecular weight and concentration. These polymers have been evaluated for medical applications because of their superior biocompatibility and rapid bioresorption.

One of the most widely used polymers is hyaluronic acid (HYA). It is obtained from animal sources, mainly rooster combs, or from certain strains of streptococcus bacteria. For some applications, it is crosslinked to increase its molecular weight and alter its physical–chemical properties and resorption rate. Because of its presence in the vitreous humour, cartilage, and synovial fluid, it has been useful as a viscoelastic polymer during eye surgery and has been useful in supplementing synovial fluid in knees of patients suffering by osteoarthritis. In ophthalmologic surgery HYA is used to protect the corneal endothelium. In osteoarthritis, HYA injected into the knee joint has been shown to provide temporary pain relief. The importance of HYA in medical applications has been assumed to include both mechanical cushioning and lubricating properties.

Rheology, a branch of mechanics, is the study of those properties of materials which determine their response to mechanical force. It is defined as the flow of fluids and deformation of solids under stress or strain. The instruments used to measure a material's rheological properties are called Rheometers.

Rheological measurements can be operated in different modes. Steady shear mode, known as flow experiments, shows correlation between shear stress and shear rate. Flow curves describe viscosity behaviour of the material and it is most useful in obtaining data required in process engineering calculations. Dynamic mode (oscillation measurements) is a commonly used method for investigation of the structure of a material, for the processing and final applications of studied materials. Creep&recovery test is the most direct measurement of materials elasticity and as compared with the oscillations it is more sensitive for case when we need measurements to be performed at long lasting timescale.

All these test methods were used to investigate rheological behaviour of aqueous hyaluronan solutions. Flow and oscillation experiments belong to conventional tests in hyaluronan research, whereas creep&recovery, and one special type of flow curve – peak hold, have not been performed in HYA research yet. Thus we tried to use them to enlarge the knowledge about hyaluronan physicochemical properties (from the point of rheology).

2. Theoretical background

2.1. Rheology

2.1.1. Introduction to rheology

Rheology has been properly defined as the study of the flow and deformation of materials and inter-relates various properties of a system defined below (Fig. 2.1).

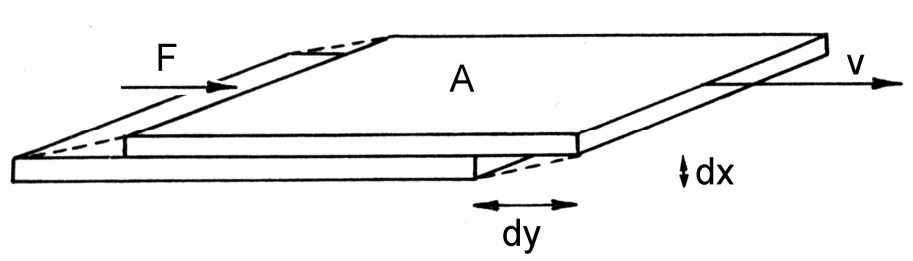

Fig. 2.1 Model of material deformation

If the force, *F*, is applied longitudinally to the top face (area *A* m^2) of the cube, then the stress σ (Nm^{-2}) applied to the system may be defined as

$$\sigma = \frac{F}{A} \tag{2.1}$$

The application of such a stress is seen to cause a lateral displacement rate of *v* (ms^{-1}) in the position of the upper face. If we consider the deformation of the top layers at any particular instant in time where the lateral displacement in the position of the upper face is d*y*. The strain, γ, present in the system as result of this deformation is given by

$$\gamma = \frac{\mathrm{d}y}{\mathrm{d}x}. \tag{2.2}$$

The ratio of applied stress to resultant strain is termed the shear modulus *G*.

$$G = \frac{\sigma}{\gamma} \tag{2.3}$$

The rate of strain (shear rate), $\dot{\gamma}$ (s^{-1}), may be also defined as

$$\dot{\gamma} = \frac{\mathrm{d}v}{\mathrm{d}x}. \tag{2.4}$$

The dynamic viscosity, η (Nsm^{-2} or Pas), of element is given by

$$\eta = \frac{\sigma}{\dot{\gamma}} \tag{2.5}$$

The definitive aims of rheology may be considered to be the prediction of the force system necessary to cause a given deformation of flow in a body, or conversely, the prediction of the deformation or flow resulting from the application of given force system to a body.

Rheological techniques may be used to study the structure of systems ranging in consistency from fluids to solids. Application of a force to a fluid produces a flow. When this force is removed the fluid does not return to its original state, and thus can be said to have exhibited irreversible deformation. When a force is applied to a perfectly elastic solid, the solid undergoes deformation but it dos not flow. When the force is removed the solid returns to its original state and thus can be said to have exhibited reversible deformation.

In reality, many systems show properties intermediate between ideal solid-like and fluid-like behaviour. Such materials are termed viscoelastic or pseudoplastic. When a force is applied to a viscoelastic material, it behaves in a similar manner to a perfectly elastic solid provided the applied force does not exceed a critical value (yield stress). If this critical value is exceeded, however, the material flows as a fluid and when the applied force is removed it does not return completely to its original state.

Rheological investigations can be sub-divided onto three main areas: flow, oscillatory and creep measurements.

2.1.2. Flow measurements

From a practical point of view the shear stress together with associated apparent viscosity are the most important rheological flow properties. Some typical shear stress – shear rate variations are presented in Fig. 2.2. The corresponding apparent viscosities would be as in Fig. 2.3.

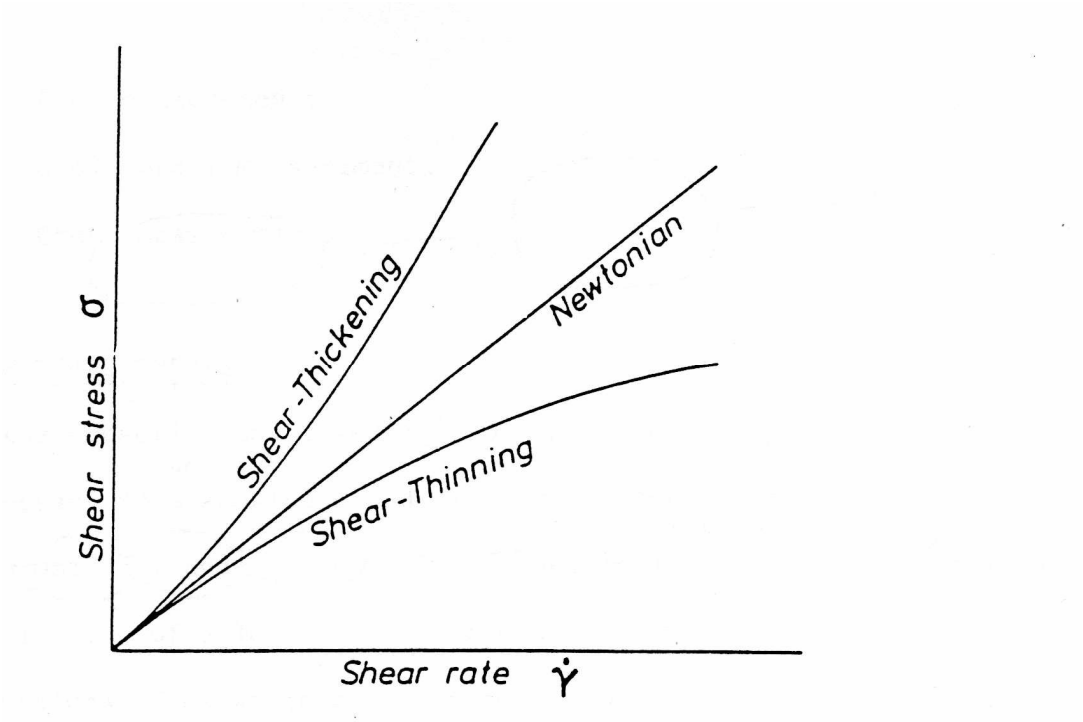

Fig. 2.2 Some typical shear stress – shear rate behaviour

From the pictures (Fig. 2.2, Fig. 2.3) it can be seen that the simplest form of flow behaviour is that of a Newtonian liquid. For such a fluid there is a linear relationship between stress and shear rate which in turn corresponds to a Newtonian fluid having a constant viscosity (i.e. independent of shear rate and time of shear). Whilst the definitive viscosity of a Newtonian liquid may be determined by a single measurement at any shear rate value, most materials possess more complex flow characteristics and are said to exhibit non-Newtonian behaviour. For such fluids it is insufficient to base rheological predictions solely on the measurement of viscosity at one particular shear rates.

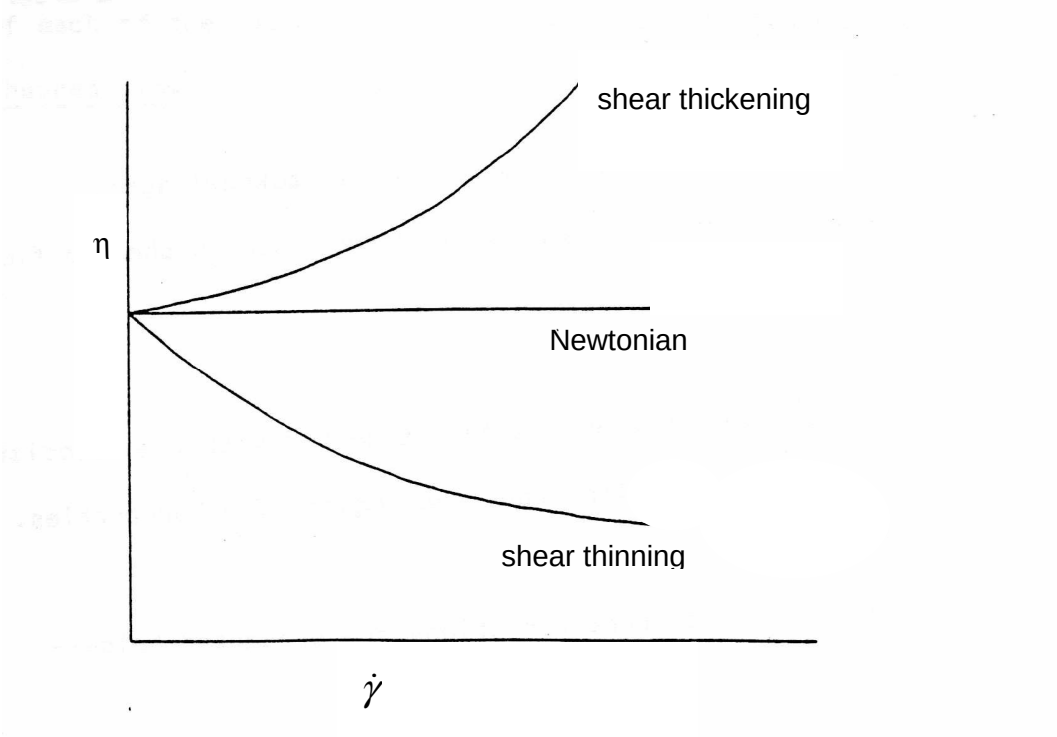

Fig. 2.3 Viscosity curves for different material behaviour

If a material exhibits a reduction in viscosity with increasing rate of shear in steady flow then it is described as shear thinning and said to undergo pseudoplastic flow.

Conversely, if a material exhibits an increase in viscosity with increasing shear rate it is described as shear thickening and said to undergo dilatant flow.

The next curve (Fig. 2.4) may be subdivided as follows: at very low shear rates, the material exhibits a Newtonian plateau (A). The forces acting on the particles in this region are the colloidal origin. In region B the forces are both colloidal and hydrodynamic in origin, the net effect being shear thinning. At higher shear rates the hydrodynamic forces overcome the colloidal forces and a second Newtonian region (C) is exhibited.

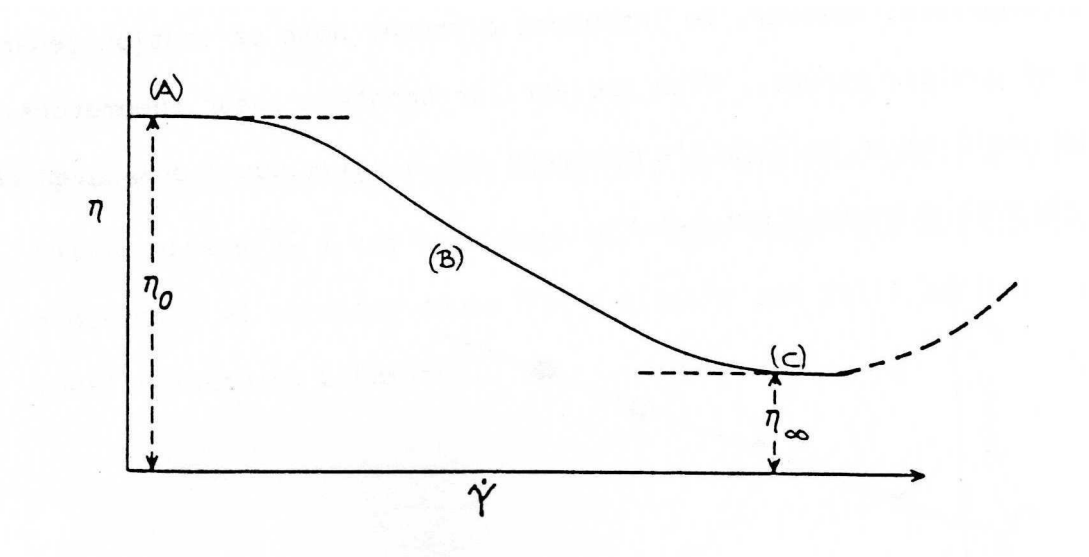

Fig. 2.4 Typical flow curve for a shear thinning material

2.1.3. Oscillatory measurements

The basic principle of an oscillatory rheometer is to induce a sinusoidal shear deformation in the sample and measure the resultant stress response, the time scale probed is determined by the frequency of oscillation, ω, of the shear deformation. Measuring this time dependent stress response at a single frequency immediately reveals key differences between materials. If the material is an ideal elastic solid, then the sample stress is proportional to the strain deformation. The stress is always exactly in phase with the applied sinusoidal strain deformation. In contrast, if the material is purely viscous fluid, the stress in the sample is proportional to the rate of strain deformation. The applied strain and the measures stress are out of phase, with a phase angle, $\delta = \pi/2$. Viscoelastic materials show a response that contains both in-phase, and out-of-phase contributions as shown in Fig. 2.5. [1]

Many material parameters can be obtained from the measured strain and stress. The ratio of the elastic (in-phase) stress and the strain is defined as the elastic or storage modulus, G', and it relates to the materials ability to store energy. Similarly, the loss modulus, G'', of the material is the ratio of the viscous (out-of-phase) component and the stress, and it is related to the materials ability to dissipate stress through heat. The sum of loss and storage modulus is the complex modulus $G^* = G' + iG''$. It means a measure of the resistance to deformation of the sample. [2]

The complex viscosity η^* is a most useful parameter and can be calculated directly from the complex modulus. This viscosity can be related to the viscosity measured in a steady shear test by a relation known as the Cox-Mertz rule. Note, that the application of the Cox-Mertz rule is limited to neat polymer resins. The complex viscosity approaches a finite value at low frequencies. This value is the zero shear viscosity of the material.

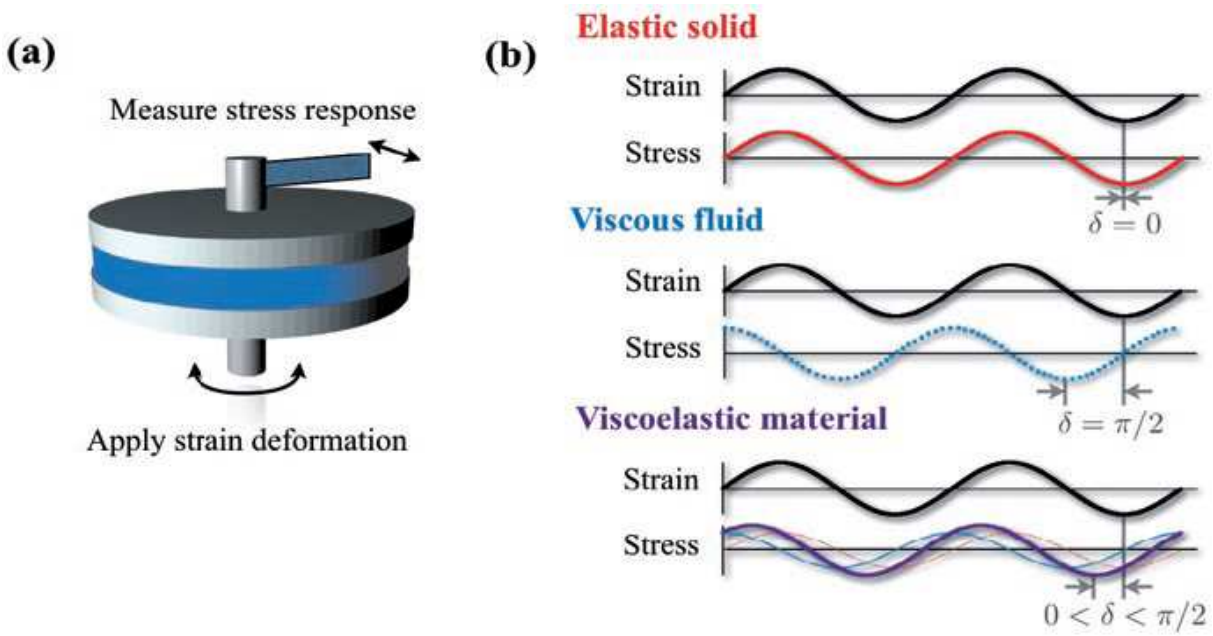

Fig. 2.5 Schematic representation of a typical rheometry setup, with the sample placed between two plates (a); schematic stress response to oscillatory strain deformation for an elastic solid, a viscous fluid and the viscoelastic material (b)

The phase δ is a measure of the presence of elastic structure within the material, a low value indicating greater elastic behaviour. A material is equally elastic and viscous if the phase is 45° or tan $\delta = 1$. The frequency at this point is characteristic for a material and therefore used to define a characteristic material time, the relaxation time, $\tau_{relax} = 1/\omega$, where ω is the angular frequency.

Tan δ is defined as the ratio of the modules G''/G' and indicates the relative degree of energy dissipation or damping of the material. [3]

2.1.4. Creep and recovery measurements

The creep test is a simple and quick test for obtaining initial information on the viscoelastic properties of a sample from viscosity-relevant (as opposed to oscillation measurements) data. In this experiment (Fig. 2.6) a constant force (shear stress) is applied to the sample at time t_0 and removed again a time t_1. The recovery up to time t_2 is recorded.

The sample responds initially to the force applied at t_0 with deformation. In other words, it starts to creep. At t_1 (after removal of the force), the sample recovers again. There are three different types of creep and creep&recovery curves.

The first case we will consider in an ideal elastic body as exemplified by a steel spring (Fig. 2.6). If a force is applied to the spring it responds with a deformation but returns to its original state after the force is removed. If the force σ is doubled, the deformation γ is also doubles.

Ideally, the energy will be recovered 100%. The body with these properties is also known as a Hooken body.

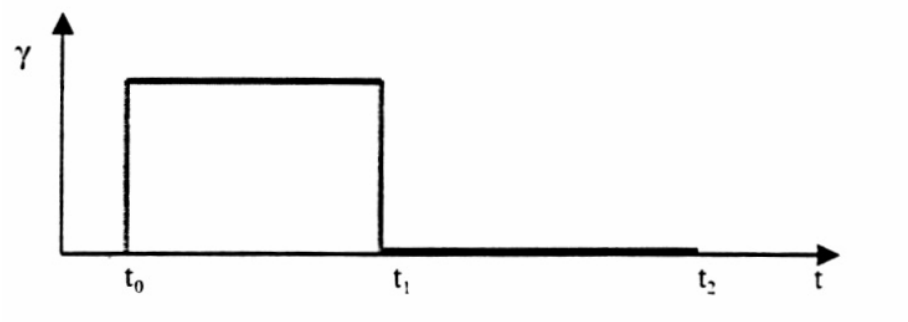

Fig. 2.6 Ideal elastic body

The second case we will consider is water as an example of an ideal fluid (Fig. 2.7). The force σ is applied to the fluid causes a linear deformation γ over time. In other words, the sample begins to flow. If the force is removed from the sample, the deformation attained at this time (in our case t_1) is fully retained. The model in this case accords to Newton.

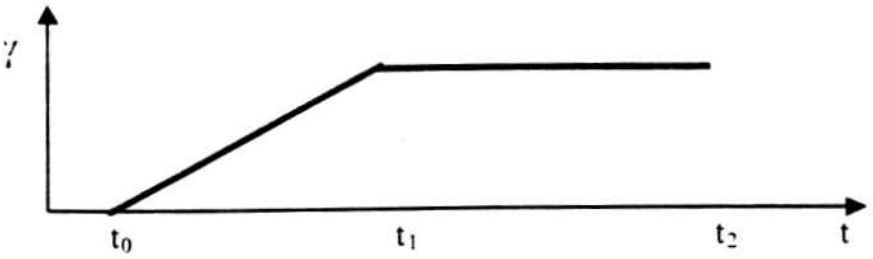

Fig. 2.7 Ideal viscous body

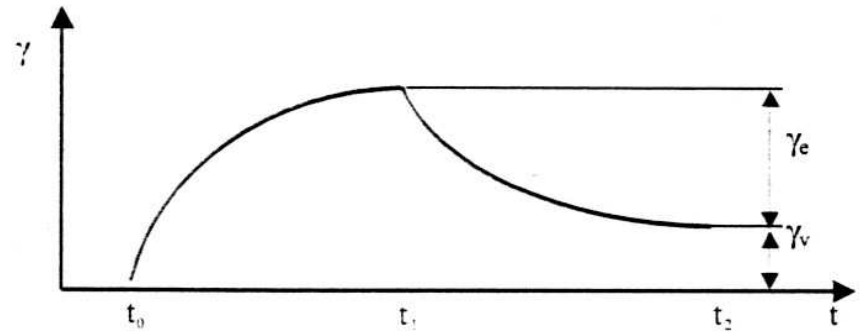

Fig. 2.8 Real viscoelastic body

A real body is both viscous and elastic (

Fig. 2.8). This means that when a force is applied at time t_0 deformation begins to take place much more slowly and, if we wait long enough (until t_1), the curve will approach a constant slope. When the force is removed, part of the energy stored in the body will be released. The

result is a recovery of elastic part γ_e and a permanent deformation of the viscous part γ_v. A viscoelastic solid will therefore recover after a time lag but it will do so almost completely [4].

Creep data may be described in terms of creep compliance function:

$$J = \mathrm{f}(t) = \frac{\gamma}{\sigma_{\text{constant}}}. \tag{2.6}$$

Compliance curves generated at different stress levels overlap when data are collected in the range of linear viscoelastic behaviour. With a perfectly elastic solid, $J = 1/G$, the reciprocal of the shear modulus; however, different time patterns in experimental testing mean that $J(t) = 1/G(t)$. Equation 2.6 is presented in terms of shear deformation. Similar material functions can be determined from creep data generated in tension (D(f)) and bulk compression (B(t)) studies.

To develop a mechanical analogue describing creep behaviour, the starting point is the Kelvin model which contains a spring connected in parallel with a dashpot. When this system is subjected to shear strain, the spring and dashpot are strained equally:

$$\gamma = (\gamma)_{\text{spring}} = (\gamma)_{\text{dashpot}} \tag{2.7}$$

The total shear stress (σ) caused by the deformation is the sum of the individual shear stresses which can be written as

$$\sigma = G\gamma + \mu\dot{\gamma} \tag{2.8}$$

Differentiating this equation with respect to time yields

$$\frac{1}{G}\frac{\mathrm{d}\sigma}{\mathrm{d}t} = \dot{\gamma} + (\lambda_{\text{ret}})\frac{\mathrm{d}\dot{\gamma}}{\mathrm{d}t} \tag{2.9}$$

where the retardation time ($\lambda_{\text{ret}} = \mu/G$) is unique for any substance. If a material was a Hooken solid, the retardation time would be zero and the maximum strain would be obtained immediately with the application of stress: time to achieve maximum strain in viscoelastic materials is delayed (or retarded). The retardation time can be though of in terms of extensional viscosity (η_E) and Young´s modulus (E) if testing is conducted in uniaxial tension or compression.

In creep, where the material is allowed to flow after being subjected to a constant shear stress (σ_0), the change in stress with time is zero ($\mathrm{d}\sigma/\mathrm{d}t = 0$) and the solution to eq. 2.9 is

$$\gamma = \mathrm{f}(t) = \frac{\sigma_0}{G}\left(1 - \exp\left(\frac{-t}{\lambda_{\text{ret}}}\right)\right) \tag{2.10}$$

showing that the initial strain is zero ($\gamma = 0$ at $t = 0$). Equation 2.10 predicts a strain that asymptotically approaches the maximum strain (σ/G) associated with the spring. λ_{ret} is the time taken for the delayed strain to reach approximately 63,2% (1-1/e) of the final value. Materials with a large retardation time reach full deformation slowly.

The Kelvin model shows excellent elastic retardation but is not general enough to model creep in many biological materials. The solution to this problem is to use a Burger model, which is a Kelvin and a Maxwell model placed in series. Data following this mechanical analogue show an initial elastic response due to the free spring, retarded elastic behaviour

related to the parallel spring-dashpot combination and Newtonian type flow after long periods of time due to the free dashpot:

$$\gamma = \mathrm{f}(t) = \frac{\sigma_0}{G_0} + \frac{\sigma_0}{G_1}\left(1 - \exp\left(\frac{-t}{\lambda_{\mathrm{ret}}}\right)\right) + \frac{\sigma_0 t}{\mu_0} \tag{2.11}$$

where $\lambda_{\mathrm{ret}} = \mu_1/G_1$, the retardation time of Kelvin portion of the model.

The Burgers model (Fig. 2.9) can also be expressed in terms of creep compliance dividing by eq. 2.11 by a constant stress:

$$\frac{\gamma}{\sigma_0} = \mathrm{f}(t) = \frac{1}{G_0} + \frac{1}{G_1}\left(1 - \exp\left(\frac{-t}{\lambda_{\mathrm{ret}}}\right)\right) + \frac{t}{\mu_0} \tag{2.12}$$

Writing the result as a creep compliance function yields

$$J = \mathrm{f}(t) = J_0 + J_1\left(1 - \exp\left(\frac{-t}{\lambda_{\mathrm{ret}}}\right)\right) + \frac{t}{\mu_0} \tag{2.13}$$

where J_0 is the instantaneous compliance, J_1 is the retarded compliance, λ_{ret} is the retardation time (μ_1/G_1) of the Kelvin component and μ_0 is the Newtonian viscosity of the free dashpot. The sum of J_0 and J_1 is called the steady state compliance. Using same procedure could be also expressed in term of the creep compliance function. The Burgers model, less the free spring (G_0), is sometimes called the Jeffreys model.

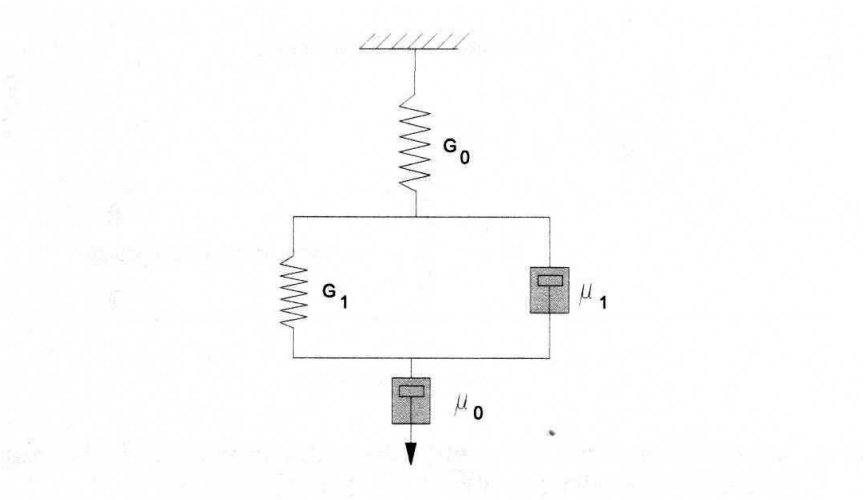

Fig. 2.9 Four element Burgers model

When conducting creep experiments, controlled stress rheometers allow one to measure the strain recovery when the constant stress in removed. The complete creep and recovery curve may be expressed using the Burgers model. When calculated as compliance, the creep is given by equation 2.13 for $0 < t < t_1$, where t_1 is the time when the constant stress is removed. At the beginning of creep, there is an instantaneous change in compliance (J_0) due to the spring in the Maxwel portion of the model. Then, the Kelvin component produces an exponential change in compliance related to the retardation time. After sufficient time has passed, the independent dashpot generates a purely viscous

response. Data from the linear portion of the creep curve are related to two parameters: the slope equal to $1/\mu_0$; and the intercept sometimes called the steady state compliance, is equal to $J_0 + J_1$.

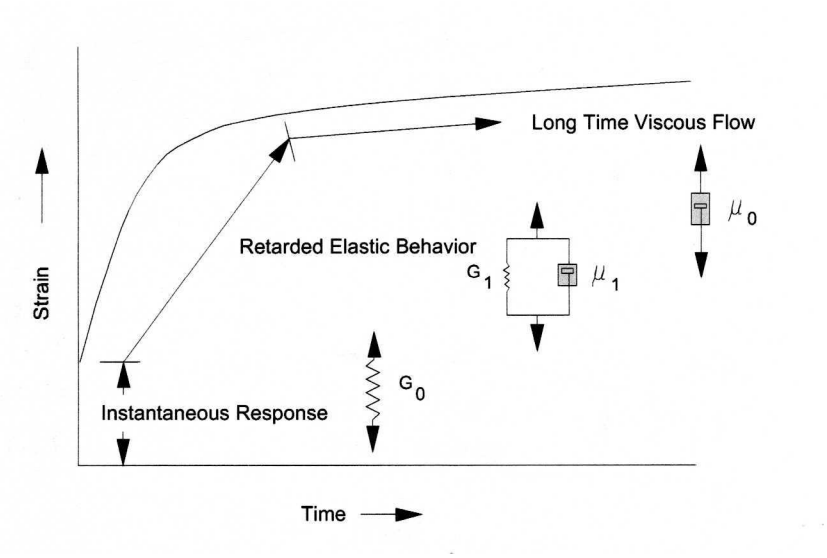

Fig. 2.10 Typical creep curve showing where various elements of the Burgers model describe flow behaviour

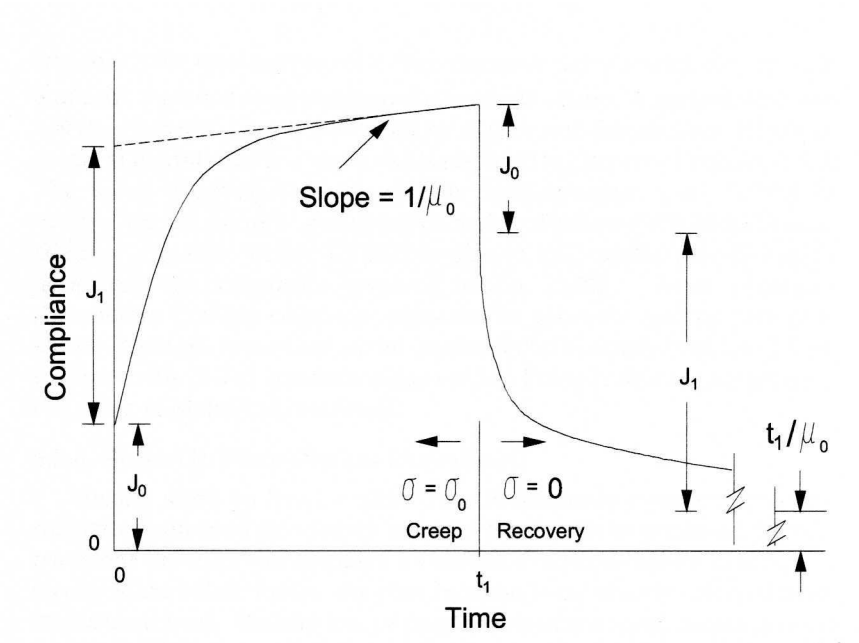

Fig. 2.11 Compliance and recovery curves showing compliance parameters for Burgers model

At $t = t_1$, the stress is removed ($\sigma = 0$) and there is an instantaneous change in compliance equal to J_0. The free dashpot causes permanent deformation in the material related to compliance of t_1/μ_0. This factor is directly related to the non-recoverable sample strain of $\sigma_0 t_1/\mu_0$. If a substance obeying the Burgers model is tested in the linear viscoelastic region of material behaviour, then the values of J_0 and J_1 determined from the creep curve will be equal to the values of J_0 and J_1 determined from the recovery curve.

If necessary, additional Kelvin elements can be added to the Burgers model to better represent experimental data. Mathematically, this idea can be described with the following equation

$$J = \mathrm{f}(t) = J_0 + \sum_{i=1}^{m} J_i \left(1 - \exp\left(\frac{-t}{(\lambda_{\mathrm{ret}})_i} \right) \right) + \frac{t}{\mu_0} \tag{2.14}$$

where m is the total number of Kelvin elements in the model, each having a unique retarded compliance and retardation time.

2.1.5. Peak hold

Peak hold measurement is special type of flow curve. Ordinarily, the flow curves are provided as the dependence of continuous increase in shear on the timescale. During the peak hold test, shear holds constant at defined intervals and the time dependence of viscosity at the constant applied shear stress or rate can be obtained (Fig. 2.12).

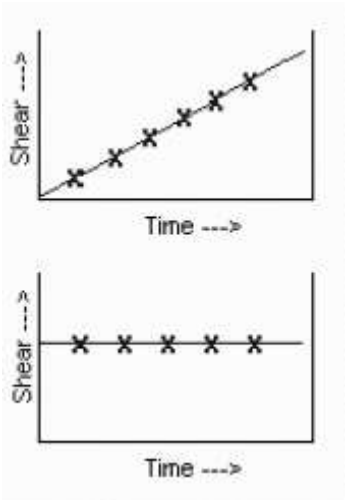

Fig. 2.12 Differences between continuous and peak hold flow

2.2. Hyaluronic acid

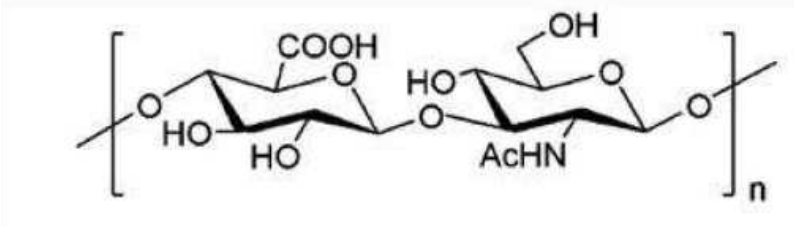

Fig. 2.13 Structure of hyaluronic acid monomer

2.2.1. Structure

Hyaluronan synthase enzymes synthesize large, linear polymers of the repeating disaccharide structure of hyaluronan by alternate addition of glucuronic acid and N-acetylglucosamine to the growing chain using their activated nucleotide sugars (UDP–glucuronic acid and UDP-N-acetylglucosamine) as substrates. The number of repeat disaccharides, *n*, in a completed hyaluronan molecule can reach 10 000 or more, a molecular mass of ~ 4 million Daltons (each disaccharide is ~ 400 Daltons). The average length of a disaccharide is ~ 1 nm. Thus, a hyaluronan molecule of 10 000 repeats could extend 10 µm if stretched from end to end, a length approximately equal to the diameter of a human erythrocyte.

2.2.2. Hyaluronan Synthases

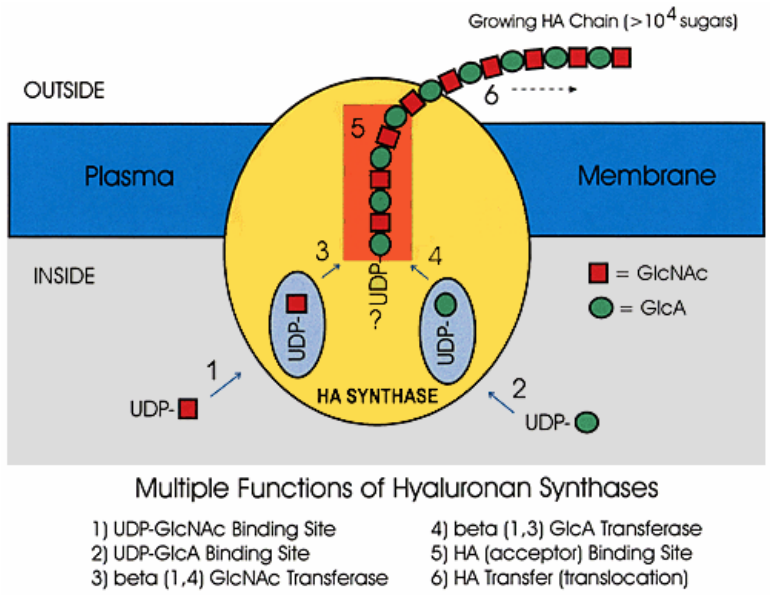

Fig. 2.14 Hyaluronan biosynthesis

Hyaluronan is produced by many organisms and plays roles essential or very important for life. The enzyme catalysts that create the hyaluronan polysaccharide chain are called hyaluronan synthases [HASs]. HASs are efficient, dual-action glycosyltransferases that catalyze the reaction:

n UDP–GlcNAc + n UDP–GlcUA $\rightarrow$ [GlcNAc–GlcUA]$_n$ + 2n UDP

In contrast to the widely held belief that “one enzyme transfers one sugar,” the HASs co-polymerize both N–acetylglucosamine and glucuronic acid into the hyaluronan chain. Elongation rates of 10 to 100 sugars per second have been measured in vitro. In all known organisms, the hyaluronan polymer is secreted or transported out of the cell upon synthesis, and the HAS activity has been found associated with the membrane fraction.

2.2.3. The role of hyaluronan in organism

Hyaluronan is present in all vertebrates, perhaps arising in animals with notochords. It is also present in the capsule of some strains of Streptococci that quite likely pirated the enzymatic machinery for its synthesis from vertebrate hosts. Hyaluronan is a major constituent of the extracellular matrices in which most tissues differentiate. It is also an essential component of many extracellular matrices in mature tissues. In some cases, hyaluronan is a major constituent; as, for example, in the vitreous of the human eye (0,1–0,4 mg/g wet weight), or in synovial joint fluid (3–4 mg/ml), or in the matrix produced by the cumulus cells around the oocyte prior to ovulation (~0.5 mg/ml), or in the pathological matrix that occludes the artery in coronary restenosis. In others, while representing less of the mass of the tissue, hyaluronan serves as an essential structural element in the matrix. For example, hyaluronan is present at ~1 mg/g wet weight in hyaline cartilages, enough to fill the tissue volume in the absence of other constituents. However, aggrecan, the large chondroitin sulfate proteoglycan, is present at a much higher concentration (25–50 mg/g wet weight), and hyaluronan retains aggrecan molecules in the matrix through specific protein-hyaluronan interactions which mask the hyaluronan backbone. Hyaluronan is less concentrated in the matrix of other connective tissues, such as those surrounding smooth muscle cells in the aorta and fibroblasts in the dermis of skin. Like cartilage however, hyaluronan forms a scaffold for binding large chondroitin sulfate proteoglycans in the matrices of these tissues.

2.2.4. Solution structure

In a physiological solution, the backbone of a hyaluronan molecule is stiffened by a combination of the chemical structure of the disaccharide, internal hydrogen bonds, and interactions with solvent. The axial hydrogen atoms form a non-polar, relatively hydrophobic face while the equatorial side chains form a more polar, hydrophilic face, thereby creating a twisting ribbon structure. Consequently, a hyaluronan molecule assumes an expanded random coil structure in physiological solutions which occupies a very large domain. The actual mass of hyaluronan within this domain is very low, ~ 0,1% (wt/vol) or less when the macromolecule is present at a very dilute concentration in saline. This means that the

domains of individual molecules would overlap each other at concentrations of 1 mg hyaluronan per ml or higher.

The domain structure of hyaluronan has interesting and important consequences. Small molecules such as water, electrolytes and nutrients can freely diffuse through the solvent within the domain. However, large molecules such as proteins will be partially excluded from the domain because of their hydrodynamic sizes in solution. The hyaluronan network in the domain allows less and less space for other molecules the larger they are. This leads both to slower diffusion of macromolecules through the network and to their lower concentration in the network compared to the surrounding hyaluronan free compartments. Interestingly, the hyaluronan chains are constantly moving in the solution, and the effective “pores“ in the network continuously change in size. Statistically, all sizes of pores can exist, but with different probabilities. This means that in principle, all molecules can pass through a hyaluronan network, but with different degrees of retardation depending on their hydrodynamic volumes.

2.2.5. Viscoelastic properties

The concentration of hyaluronan in tissues is often higher than would be expected if individual molecules maintained their expanded domain structures. In many cases the hyaluronan is organized into the extracellular matrix by specific interactions with other matrix macromolecules. However, high molecular weight hyaluronan at high concentration in solution (for example, 5 million Daltons at concentrations above 0,1 mg/ml) can also form entangled molecular networks through steric interactions and self association between and within individual molecules. The latter can occur when a stretch of the hydrophobic face of the ribbon structure of the backbone interacts reversibly with the hydrophobic face on a comparable stretch of hyaluronan on another molecule or in a different region of the same molecule. Such networks exhibit different properties than would isolated hyaluronan molecules. They can resist rapid, short-duration fluid flow through the network, thereby exhibiting elastic properties which can distribute load or shear forces within the network. On the other hand, slow fluid flow of longer duration can partially separate and align the molecules, allowing their movement and exhibiting viscous properties. Procedures for introducing covalent cross-links in hyaluronan matrices have been developed to create stable networks and semi-solid materials exhibiting pronounced viscoelastic properties [5].

2.3. Background research

2.3.1. Rheology of hyaluronic acid

Hyaluronic acid HYA is a naturally abundant linear polysacharide that consist of repeating disacharide structure units. It contains negatively charged carboxylate groups, thus it is a polyelectrolyte and forms a salt of hyaluronate or hyaluronan at physiological pH [6]–[8]. HYA occurs in extracellular matrix of vertebrates' connective tissues with a wide range of molar masses from several hundreds to 10 millions Da. HYA plays many pivotal functions in the organism such as space-filler or scaffold for other macromolecules, creating a network wit proteoglycans and proteins [9]. HA, thus maintains viscoelasticity of the tissues due to its extraordinary rheological properties. It also participates in various receptor-mediated cell processes [8], [10], [11], e.g. mitosis, cell migration, tumor developent, inflammation, etc. HA has found number of important applications in medicine, e.g. opthalmology, visco-suplementary, osteoartgrititis treatment, drug delivery [6], [7], [12], [13], pharmacy and cosmetics due to its rheolgical and other unique properties. Therefore, knowledge of a relationship between rheological parameters and structure is very helpful during these operations and utilizations [13].

The previous studies on rheology of HA solution were mostly based on rotational shear viscosity measurements and oscillatory measurements [8], [14]. The first rheological measurements of hyaluronic acid were done by Gibbs et al [15] who measured the dynamic viscoelastic properties of its sodium salt over the frequency range. The effects of varying temperature, hyaluronic acid concentration, pH and ionic strength on the dynamic shear moduli were studied. It was shown that hyaluronic acid behaves as non-Newtonian liquid.

Haluronate solutions at neutral pH and physiological ionic strength show high viscosity at relatively low concentrations and also display substantial solid-like character, which is called viscoelastic behaviour [16]. The influence of salt addition was studied and it was shown that according to increasing ionic strength, the electrostatic repulsions are suppressed and it may cause enhanced coupling. Increase in viscosity, at sufficiently high polymer concentration, was observed [16].

Rheological properties of hyaluronan solutions are related not only to the molecular weight or concentration, but also to the origin of the sample [17].

2.3.2. Creep&recovery measurements

It hasn't been found any reference where creep and recovery tests on hyaluronic acid were performed. This type of measurement is usually used to study viscoelastic properties of more solid-like materials than hyaluronan solutions are.

At the University of Pittsburgh, the physical aging behaviour of amorphous selenium has been investigated using conventional and interrupted creep experiments by research team of Donald J. Plazek [18].

Another field where creep and recovery tests are one of the common methods is rheology of food and cosmetics gels and emulsions. The study of low oil content food emulsions by M. Dolz [19] can be mentioned as typical example of the combination both of the dynamic methods (oscillations together with creep and recovery analyses), which are used to obtain information about internal structure of complex systems.

3. The aim of the work

The object of our work was to investigate the rheological properties of hyaluronan in aqueous solution by using two less common types of rheological measurement: creep&recovery test and peak hold test. We focused on optimalization of the set-up and compared the results with the conventional used types of rheological experiments, such as oscillations and flow measurements, which we also measured. The main reason was to study hyaluronan physicochemical properties more deeply and with the different point of view. From these methods we can obtain more complex information about HYA behaviour and stability during mechanical processes, such as mixing and filtration and try to improve its properties due to the requirements of pharmaceutical industry.

4. Experimental part

4.1. Materials

Hyaluronan (hyaluronic acid)

ID: 170706, M_w ~ 1,69 MDa, matter content: 93,8%, proteins: 0,072%, uronic acids: 46,3%, sodium hyaluronate: 95,7%, produced by Contipro Group Holding

Sterile water for injection

ID: UL 3312, produced by Fresenius Kabi

Sodium Chloride

ID: 30423, EINECS: 321-598-3, M_w = 58,44 g/mol, produced by Lach-Ner s.r.o., Neratovice

4.2. Instrumentation

Rotational rheometer AR G2, TA Instruments

- geometry: cone-plate (60 mm, 1°), solvent trap (Fig. 4.1)
- sample volume: 1 ml
- temperature: 25°C
- software: Rheology Advantage Instrument Control AR, product version V5.2.0

Rheology Advantage Data Analysis, product version V5.2.18

4.3. Usage of solvent trap

Solvent trap covers should be used if the sample is likely to dry out during a measurement or if solvent evaporation will occur. The relevant solvent is put into the solvent reservoir, once the cover is in place the free volume between the cover and the edge of the sample is saturated with vapour.

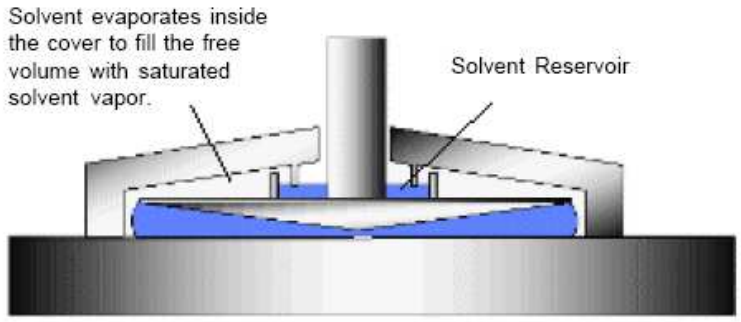

Fig. 4.1 Solvent trap cover and geometry

The efficiency of the solvent trap is shown in Fig. 4.2, where it is obvious that just in few minutes viscosity increase of the sample occurs, because of its dehydration and therefore there is a need to use solvent trap also in short-time measurements. Even a decrease of the viscosity is observed at the curve with used solvent trap. The polymer chains in the hyaluronan solution react to applied stress by releasing their structure, even low stress was used.

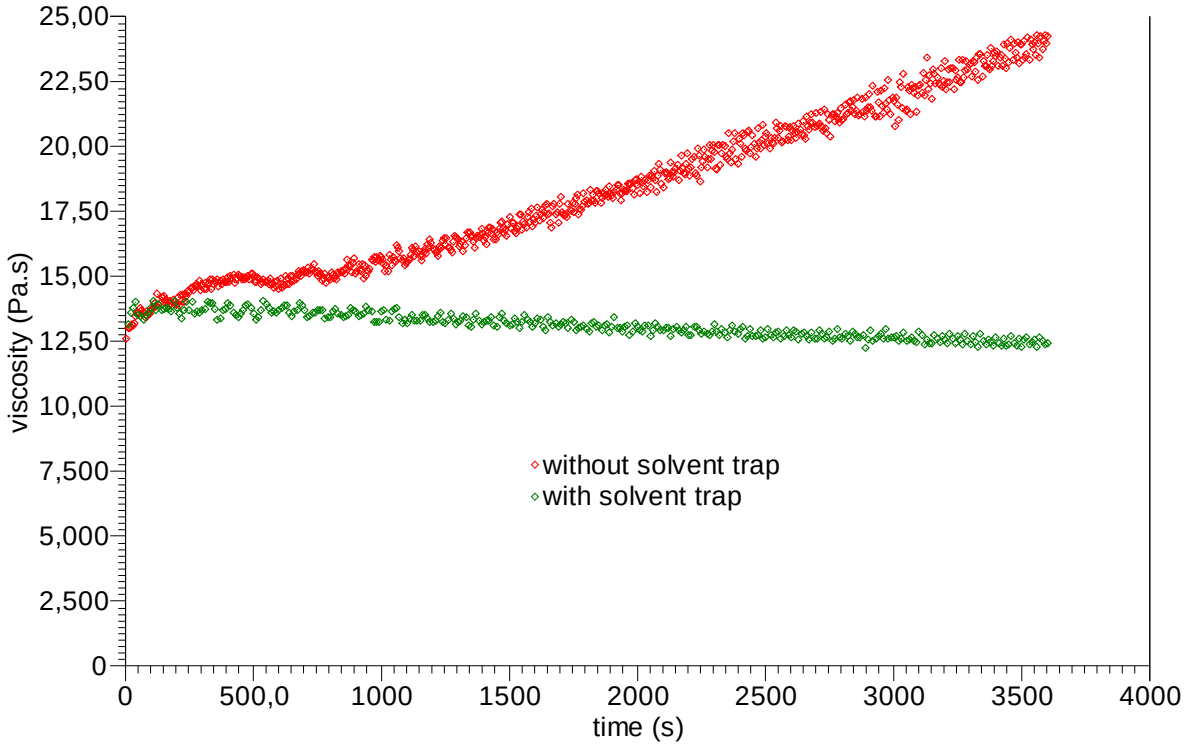

Fig. 4.2 The example of measurement with and without solvent trap by constant strain 0,5 Pa and temperature 25℃

4.4. Samples and their preparation

4.4.1. Series

There were prepared two series of hyaluronan solutions. In the first one there were water solutions of this polymer of concentration 1; 1,5; 2 and 3 weight percent. One-percent solution should illustrate concentration of the hyaluronan in synuvial fluid which is present in joints. The sample containing three percent of HYA was selected as the highest possible concentration that we were able to prepare, thus the solution was still homogenous, because in more concentrated solutions the viscoelasticity can be better observed. The samples of concentration 1,5 and 2% were used complete the series.

In the second series there were used only solutions of the highest hyaluronan concentrations that differed in ionic strength of solvent (0,01; 0,15 and 1 M NaCl). The ionic strength was

modifying by the addition of sodium chloride into the injection water before preparation of the sample.

In the first sample the solution didn't contain any chloride. Into the second one there was added minimum amount of salt, so that we could find out, whether the environment of very weak ionic strength is able to influence the properties of polysaccharide. The other sample should simulate physiological solution and from the view-point of HYA utilization research and its modifications in health department, is the most important sample. In the last case there was used 1M NaCl environment, it is the relatively high concentration of salt so we were curious how the HYA would react in this case.

4.4.2. Preparation

As the base material was used dehydrated HYA in form of white powder, which we solved in the injection water either clean or with addition of chloride. The solutions with lower concentration (1; 1,5%) were prepared by stirring in magnetic stirrer by as lowest rotational speed as was possible for about 24 hours to prevent degradation of the sample structure. Afterward we let the samples relax for another 24 hours in refrigerator. In case of 2% solution the stirrer stiffened in sample after few hours and than it was taken out and from that point we stirred sample just manually by metal stick for short time in few hours. This procedure had been done for two days and before the first measurement the sample was put in the refrigerator, similarly as other samples with lower concentrations.

In the case of 3% solutions the magnetic stirrer could not be used at all and sample was firstly let to relax for couple of hours until it started to swell and than it was also stirred for period of at least two days manually and it was kept in refrigerator for another two days, so that all bubbles that got into the sample by stirring went out.

4.5. Types of measurements and settings

Totally there were used four types of measurement: flow tests, oscillations, creep&recovery and peak hold. The setting of flow and oscillations was relatively easy with regard to long-time usage of these measurements not only in our workplace. In case of the other two methods the adjustment of settings was significant part of our work, because these measurements were not used in HYA research so far and therefore we will provide detailed description later on.

4.5.1. Flow settings

Flow experiments were measured to obtain a region, where the viscosity is independent on increasing stress, known as linear or Newtonian region (or viscoelastic region). The retrieved values helped us to set up creep and peak hold measurements. Steady state flow means, that stress doesn't increase continuously but step by step and the response of the sample should be more accurately.

1 and 1,5% hyaluronan solution

- conditioning step: 0 Pa; 5 min
- steady state flow step: shear stress 0,1–150 Pa; 10 points per decade; log mode

2 and 3% hyaluronan solution

- conditioning step: 0 Pa; 5 min
- steady state flow step: shear stress 0,5–250 Pa; 10 points per decade; log mode

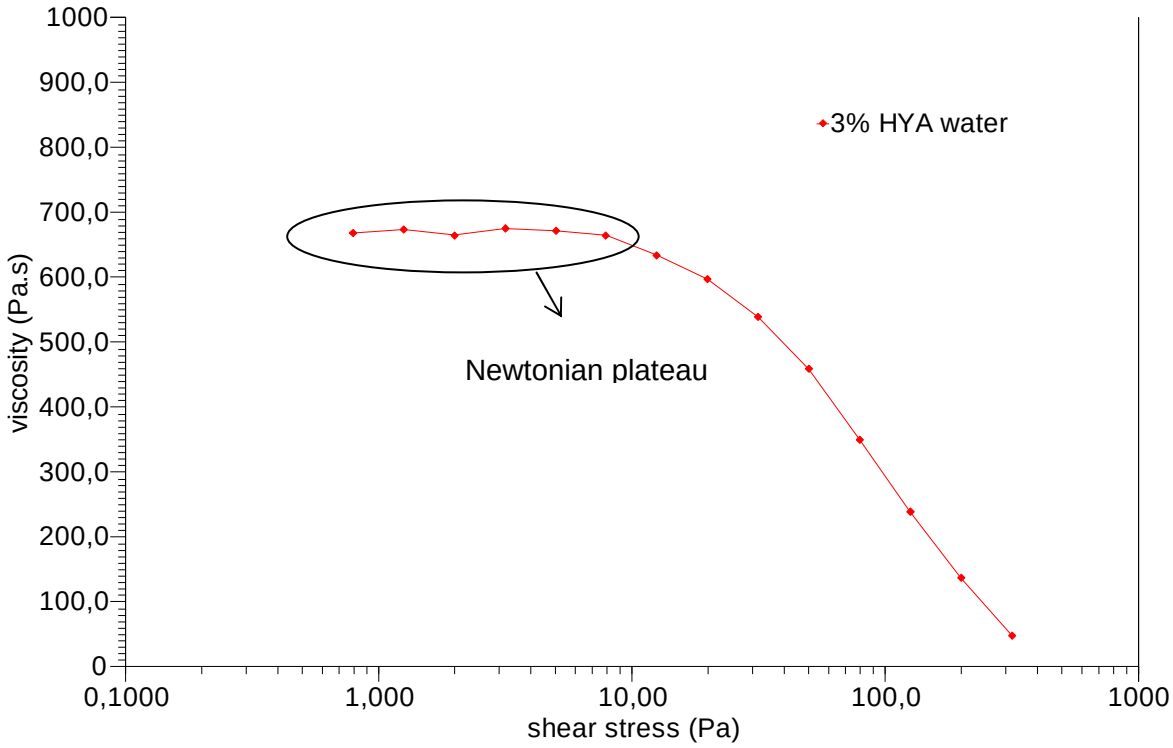

Fig. 4.3 Newtonian plateau for 3 % hyaluronan aqueous solution

4.5.2. Oscillations

All oscillatory experiments have to be carried out at small strain or stress amplitude in order to remain within the so-called linear viscoelastic region. Inside this region, which is limited by the critical stress (or strain), the material's structure is in an equilibrium state and the relation between the applied stress and the measured strain is linear; this means that all material functions are dependent of time or frequency only at constant temperature. The linear viscoelastic region of a material was determined in an oscillatory measurement at a constant frequency 1 Hz with increasing strain amplitude 0,001–1. The measured moduli remain constant as long as the critical strain has not been reached. The end of the linear region

is given by a decrease in elastic modulus and an increase of the phase, shown in Fig. 4.4. The strain equal to 0,01 has been chosen for frequency sweep measurements.

frequency sweep test

- conditioning step: 0 Hz; 5 min

- frequency sweep step: frequency 20–2,5.10^{-3}; strain 0,01; 5 points per decade; log mode

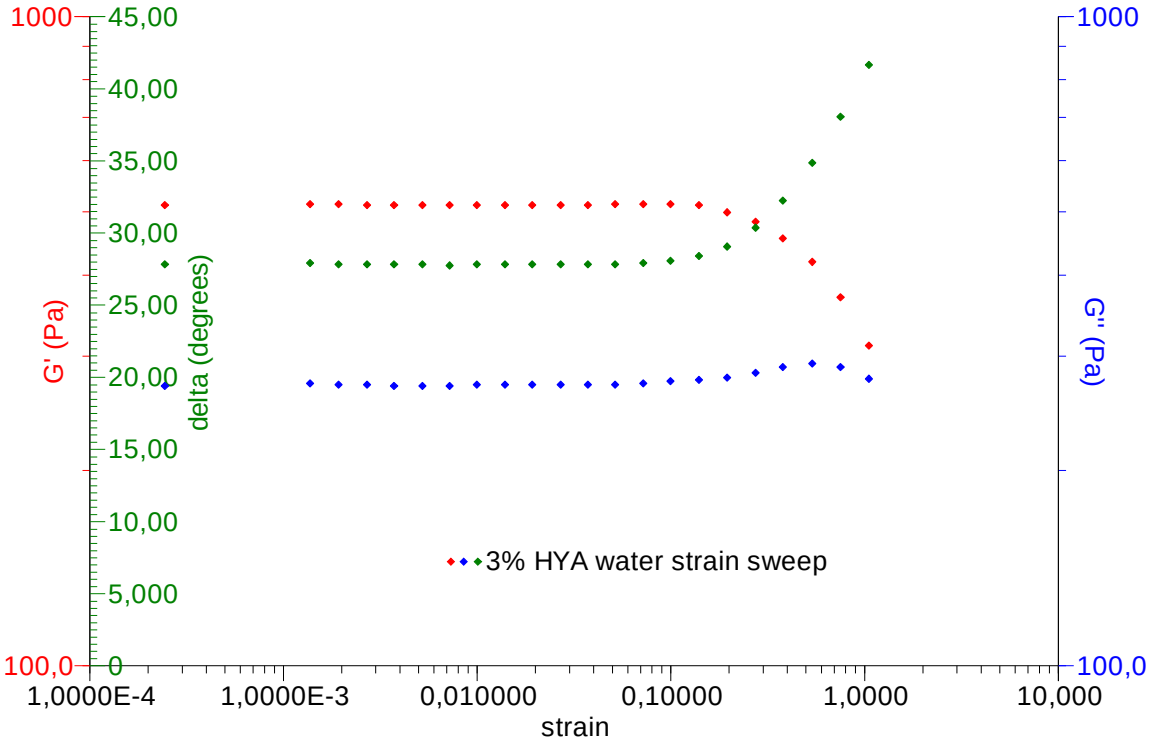

Fig. 4.4 Strain sweep measurement for 3 % hyaluronan aqueous solution

4.5.3. Creep&recovery

In creep part, the sample should be exposed to the shear stress chosen from the viscoelastic region (4.5.1). Three different values of shear stress were used for repeated measurements.

Duration of creep part is dependent on time when the linear response of the sample occurs. Recovery part should be performed three times longer at zero stress.

1 and 1,5% hyaluronan solution

- conditioning step: 0 Pa; 10 min

- creep step: shear stress 0,5; 0,75; and 1 Pa; 4 min, sample period 30 s

- recovery step: shear stress 0 Pa; 12 min, sample period 30 s

2 and 3 % hyaluronan solution

- conditioning step: 0 Pa; 10 min
- creep step: shear stress 1; 2; and 5 Pa; 4 min, sample period 30 s
- recovery step: shear stress 0 Pa; 12 min, sample period 30 s

4.5.4. Peak hold

Peak hold is a special case of flow viscosity curve. We could either obtain the viscosity dependence on the increasing shear stress from this measurement. But in contrast to common viscosity curve shear stress didn't increase continuously during the peak hold, it was applied to the sample in steps. The stress was reduced to the small value (from linear viscoelastic region) between the peaks to observe the relaxation of the sample. The zero value of applied stress couldn't be used because of inability to measure shear viscosity. We set up two tests with different duration of peaks and relaxation parts, long and short, to obtain the time dependence of hyaluronan solution behaviour.

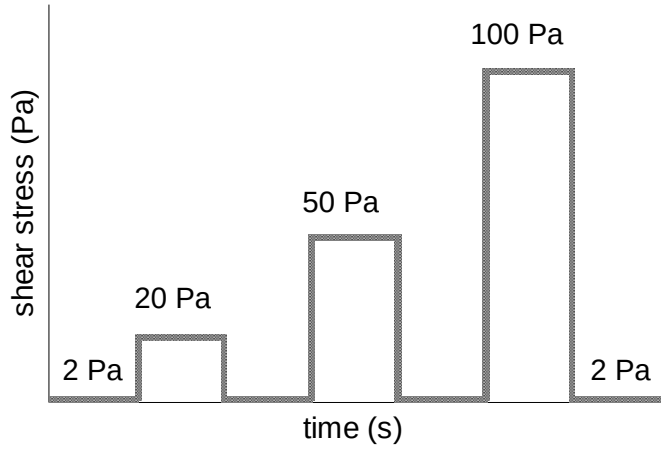

Fig. 4.5 Demonstration of the peak hold step for 2 and 3 % HYA solutions

1 and 1,5 % hyaluronan solution, short

- conditioning step: 0 Pa; 10 min
- peak hold step: shear stress 20; 50; and 100 Pa; 30 s, sample points 5
- relaxation step: shear stress 1 Pa; 10 min, sample points 20

1 and 1,5 % hyaluronan solution, long

- conditioning step: 0 Pa; 10 min
- peak hold step: shear stress 20; 50; and 100 Pa; 5 min, sample points 20
- relaxation step: shear stress 1 Pa; 25 min, sample points 25

2 and 3 % hyaluronan solution, short

- conditioning step: 0 Pa; 10 min
- peak hold step: shear stress 50; 100; and 200 Pa; 30 s, sample points 5
- relaxation step: shear stress 2 Pa; 10 min, sample points 20

2 and 3 % hyaluronan solution, long

- conditioning step: 0 Pa; 10 min
- peak hold step: shear stress 50; 100; and 200 Pa; 5 min, sample points 20
- relaxation step: shear stress 2 Pa; 25 min, sample points 25

5. Results and discussion

In the literature the information about rheological properties of hyaluronic acid, its salts and derivates in aqueous solutions is usually obtained from the flow curves or oscillation measurements. But there is a number of other different types of rheological tests such as creep&recovery or peak hold, thus we decided to use these less common methods as well.

Creep&recovery experiment is easy and relatively fast tool, how to get information about viscoelastic properties of the material. Peak hold test, a special type of rotational test at constant shear conditions, describes stability, time and mechanical resistance of the solution.

All of these four different methods were applied to the two series of hyaluronan solutions. One series varied in the weight concentration of hyaluronan in the aqueous solution within the range of 1–3%. The other contained hyaluronan solution only with the one concentration equal to 3 wt.% and differed in the ionic strength of the solvent that was achieved by the addition of various concentrations NaCl (0; 0,01; 0,15; 1 M).

5.1. Flow curves

First of all, flow curves were measured for each sample, i.e. the dependence of shear viscosity on shear rate or shear stress, respectively. By these measurements the correct preparation of the samples was checked if no degradation of sample during the storage occurs. Figure 2 shows the flow curves of HYA solutions of different concentrations. There is a remarkable decrease of viscosity of sample with increasing of shear rate and this type of behaviour is called shear thinning behaviour (or pseudoplastic). The curve has Newtonian character (Newtonian plateau) in the first part of the dependence thus the viscosity in this part is independent on the current shear conditions. This part of Newtonian behaviour was very important for setting the shearing conditions at creep&recovery test and peak hold. The rheological properties were observed as function of HYA concentration and ionic strength of the solution (Fig. 5.1). In the former case we can clearly see how the viscosity of HYA increases with its concentration in the solution. This is due to larger amount of polymers chains in the solution and their entanglements. This contributes to hydrogen bonds creation and electrostatic interactions between fibres are stronger.

The influence of solvent ionic strength on the 3 wt. % HYA solution was more interesting to be observed (Fig. 5.2). In the case when solvent contains 0,15 M of NaCl the decrease in viscosity is caused by shielding the electrostatic repulsions between the carboxyl groups by NaCl ions. HYA molecules become more flexible and thanks to the intermolecular interactions polymer domains loose their volume thus they can move easier in the solution.

By the samples where ionic strength is equal to 1, reverse effect occurs, the viscosity grow substantially. This might be due to overlap some critical concentration of salt, over which the

domains can't shrink any more. Insight domain the osmotic pressure increases and this could lead to the viscosity growth.

Almost no differences in rheological behaviour were observed by the sample with the lowest addition of salt (0,01 M NaCl) as compared with pure 3 % HYA aqueous solution. Thus we decided to not to deal with this concentration any more.

To analyze the flow curves the rheological model (Cross) was applied by using the software TA Data Analysis. We got the characteristic values of sample such as zero shear viscosity η_0, critical shear rate $\dot{\gamma}$ and relaxation time τ_{relax}. There is one curve inserted by rheological model on Fig. 5.2. Its equation is

$$\left(\frac{\eta-\eta_\infty}{\eta_0-\eta_\infty}\right)=\frac{1}{\left(1+(\tau\cdot\dot{\gamma})^m\right)} \tag{5.1}$$

where η_0 (a) is the zero shear viscosity, the magnitude of the viscosity at the lower Newtonian plateau. Newtonian behaviour means the independence of the viscosity from the shear rate. Zero shear viscosity is a critical material property and can prove valuable in making assessments of suspension and emulsion stability, estimates of comparative polymer molecular weight and tracking changes due to process or formulation variables etc. η_∞ (b) is the infinite shear viscosity. This tells us how our product is likely to behave in very high shear processing situations such as blade, knife and roller coating. The parameter "*m*" (d) is known as the (Cross) rate constant. It is dimensionless and is a measure of the degree of dependence of viscosity on shear rate in the shear thinning region. A value of zero for *m* indicates Newtonian behaviour with *m* tending to unity for increasingly shear thinning behaviour. "τ" (c) is known as the Cross time constant (or relaxation time) and it describes the rate of relaxation of stresses in a material (e.g. a viscoelastic fluid) that has been deformed to a defined strain. The reciprocal, $1/\tau$, gives us a critical shear rate that proves a useful indicator of the onset shear rate for shear thinning. *m* and $1/\tau$ can be related to texture, application properties, pumping, mixing and pouring characteristics and many other everyday flow processes which often occur in the shear thinning region of the fluid's flow behaviour [2].

The analysis of all curves is written to Tab. 5.1. All values are average value from analysis of more than three measurements. In Fig. 5.3 and Fig. 5.4, there is shown the dependence of zero shear viscosity for both series of samples.

Tab. 5.1 Data obtained from the flow curves after cross analysis, zero shear viscosity and relaxation time

	water				ionic strenght	
	1% HYA	1,5% HYA	2% HYA	3% HYA	0,15 M	1 M
$\eta_{0\ flow}$ (Pa.s)	14,73	63,93	159,5	669,6	615,8	893,3
$\lambda_{relax\ flow}$ (s)	0,515	1,045	1,637	3,871	3,234	6,048

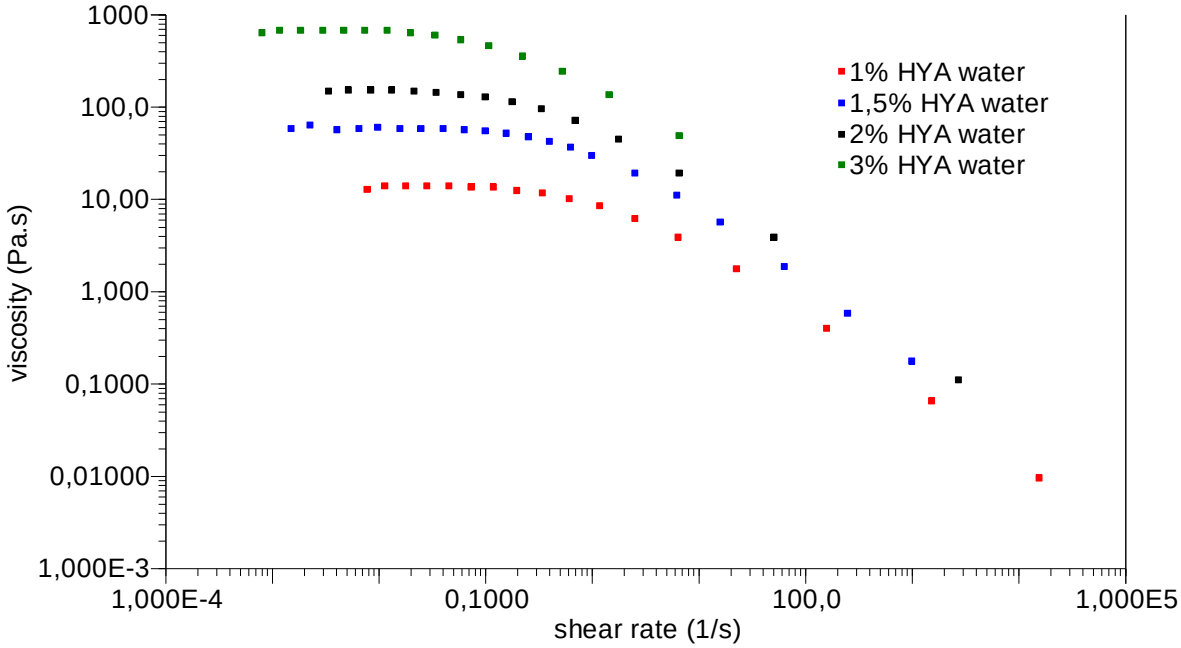

Fig. 5.1 The dependence of viscosity as a function of increasing HYA aqueous solution, shear stress 0,5–200 Pa, temperature 25℃

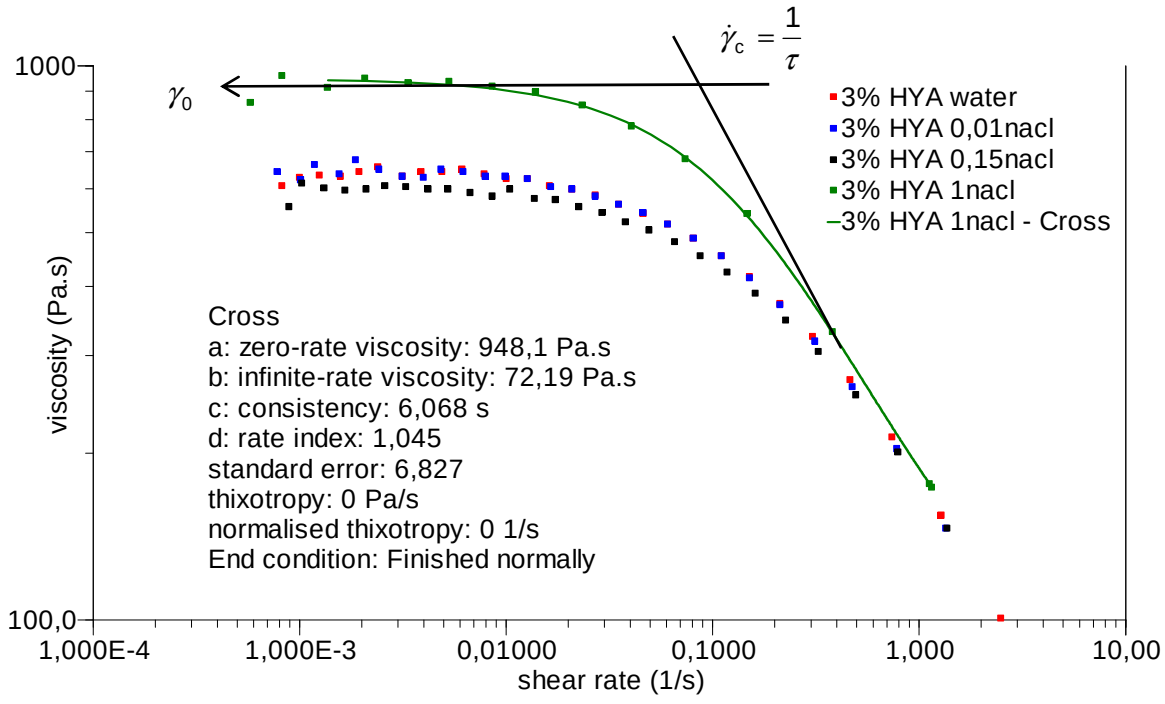

Fig. 5.2 Increase and decrease of the viscosity dependent on ionic strength of 3 wt % HYA solutions, illustration of the relaxation time determination and Cross analysis

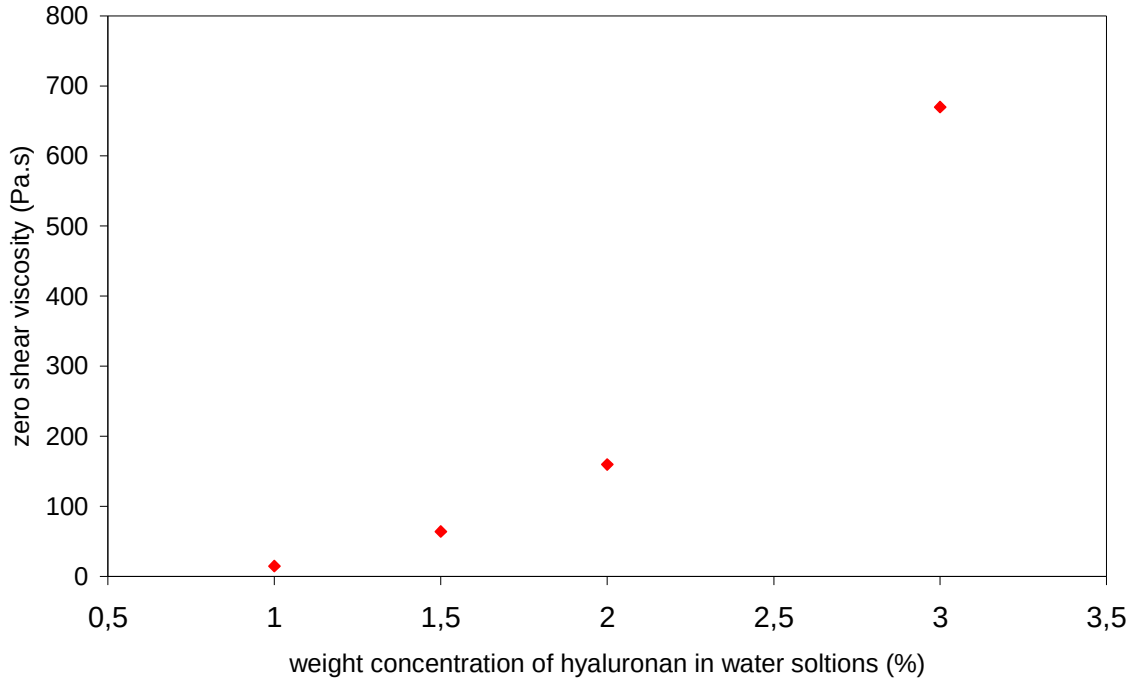

Fig. 5.3 Increase of zero shear viscosity obtained from Cross analysis as a function of HYA weight concentration in aqueous solutions

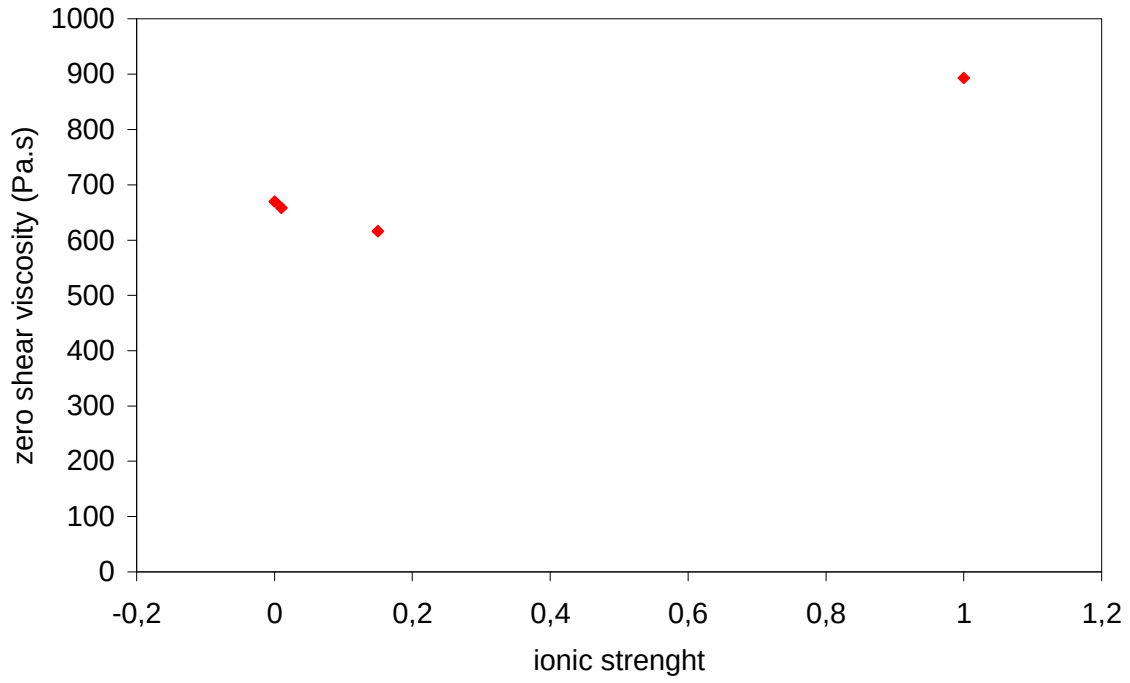

Fig. 5.4 The viscosity behaviour as a function of ionic strength, small decrease with weak and significant increase for high ionic strength solutions

5.2. Oscillation measurements

Dynamic mechanical analysis (oscillation measurements) is a commonly used method for investigation of the structure of a material, for the processing and final applications of studied materials. It enables characterization of the structural differences between materials and provides information about how the materials will behave during various processes. Such information is important both for product development as well as process design and optimization. In fact, all real materials display more or less viscoelastic behaviour depending on timescale of a process, and therefore, full characterization of a material's rheology requires elasticity information in addition to viscosity information. Dynamic mechanical analysis is unique powerful method because it measures both properties simultaneously.

In order to obtain oscillation measurement data, the frequency sweep test was performed. In this experiment the testing oscillation frequency is varied to receive the frequency response of a material such as storage modulus, G', loss modulus, G'', complex viscosity, η^*, and phase angle δ.

For solutions of high-molecular weight hyaluronan is typical that storage modulus G' and the loss modulus G'' intersect each other and this is known as crossover point (Fig. 5.5). At frequencies lower than the frequency of the crossover, the viscous part, represented by G'', is greater than the elastic part, which means that the sample behaves like a liquid. With increasing frequency both the loss and the storage modulus increase, but G' increases more steeply. Above the crossover frequency, characteristic for this material, the storage modulus G' is larger than the loss modulus G''. This detects the internal structure of material caused by the mutual interactions between the polymer chains. For the easier assessment of whether the sample behaves more like a solid or more like a liquid, the phase angle δ is used and is given by

$$\delta = \arctan\left(\frac{G'}{G''}\right) \text{ or } \tan\delta = \frac{G''}{G'} \tag{5.2}$$

In Fig. 5.6 is evident that phase angle decreases with the angular frequency and from the above frequency is clear that when $G' = G''$ (crossover point), $\tan\delta = 1$. At lower frequencies $\tan\delta$ is higher than 1 and sample behaves like liquid and at higher frequencies $\tan\delta$ is lower than 1, thus the sample displays solid–like behaviour.

The inverse value of the crossover angular frequency is defined as a relaxation time $\tau_{relax} = 1/\omega_{crossover}$. For higher concentrations of hyaluronan and the highest ionic strength (1 M NaCl), the transition from a predominately viscous region to elastic region occurs at longer relaxation times. It means that it takes more time for heavily entangled polymer chains to disentangle. Furthermore, the value of crossover point, $(G' = G'')_{crossover}$ increases with the HYA concentration and increasing ionic strength, and the crossover frequency decreases. The quantities determined from the crosspoint are displayed in Tab. 5.2.

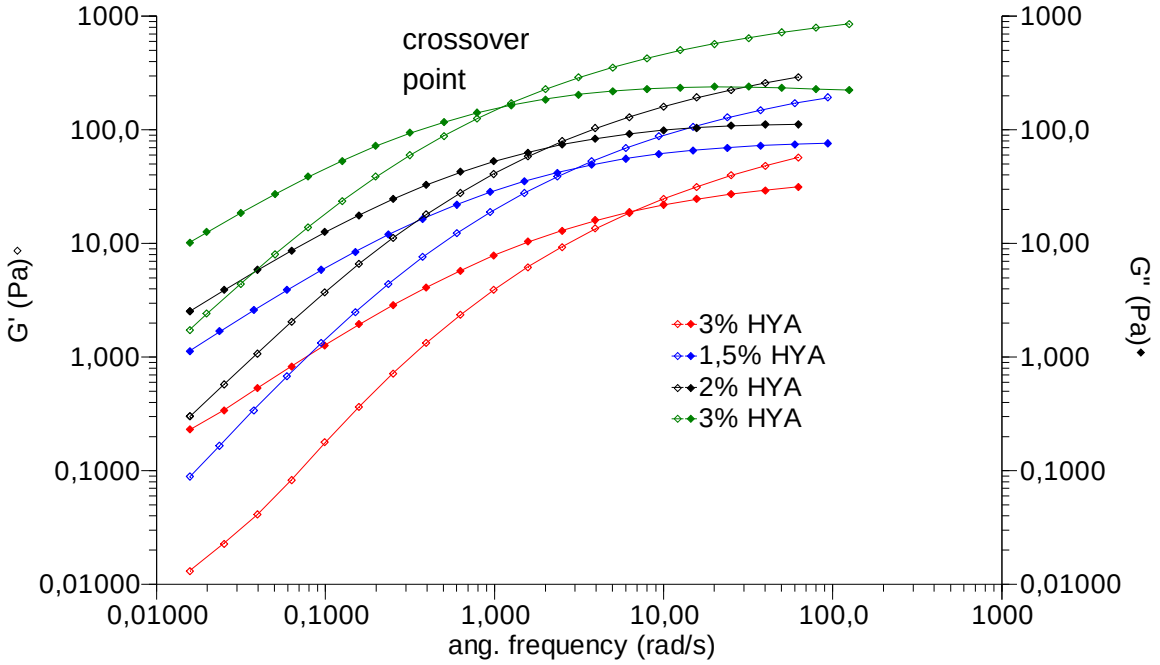

Fig. 5.5 Loss G'' and storage G' modulus behaviour for increasing of angular frequency for the solutions of the different HYA concentration

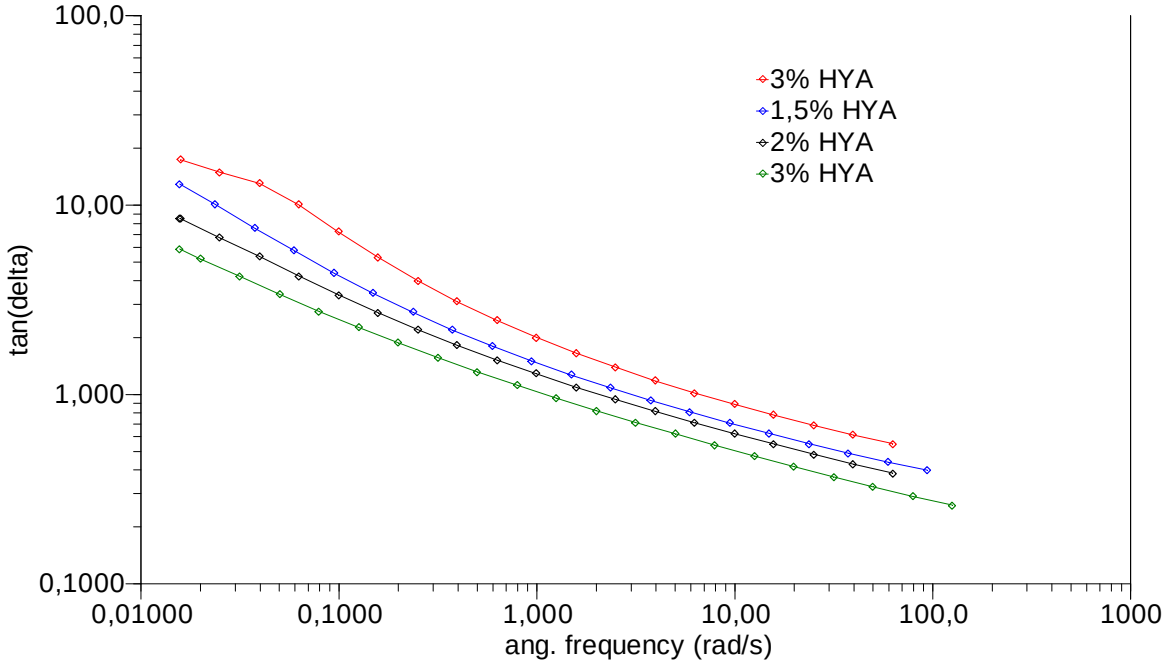

Fig. 5.6 Decrease in delta degree dependence with the increasing angular frequency corresponds to more elastic-like behaviour

Tab. 5.2 Data obtained from crossover point analysis, value of the loss and storage modulus, crossover angular frequency and relaxation time of the hyaluronic acid water solution with and without the ionic strength influence

	water				ionic strenght	
	1% HYA	1,5% HYA	2% HYA	3% HYA	0,15	1
G´ = G´´ (Pa)	19,28	45,75	69,45	147,6	158,8	167,7
ω_{cross} (rad/s)	6,683	3,026	2,072	1,147	1,283	0,9983
$\tau_{relax\ osc}$ (s)	0,1496	0,3305	0,4826	0,8718	0,7794	1,0017
$\tau_{relax\ flow}$ (s)	0,515	1,045	1,637	3,871	3,234	6,048

The relaxation times from flow curves are obviously longer, that from oscillations. They both increase with the HYA concentration and ionic strength, but the difference is in the way of their determination. Oscillations are much more sensitive method performed at very low shear stress, thus the relaxation time represents material in the rest conditions. Flow curves simulate a movement and the relaxation time is obtained from the inflexion of the curve between linear vicsoelastic region and shear thinning part. Thus, the real material time occurs somewhere within this interval.

For many polymer solutions the frequency dependence of complex viscosity η^* and the shear rate dependence of η are observed to be closely superimposable when the same numerical values of ω and $\dot{\gamma}$ are compared in Fig. 5.7. This empirical correlation is known as Cox-Mertz rule and it is also important to note, that in such an experiment we can measure η^* as an oscillatory experiment at small strain, for a sample such as gel, whose structure would be totally disrupted by a steady shear experiment (flow measurement).

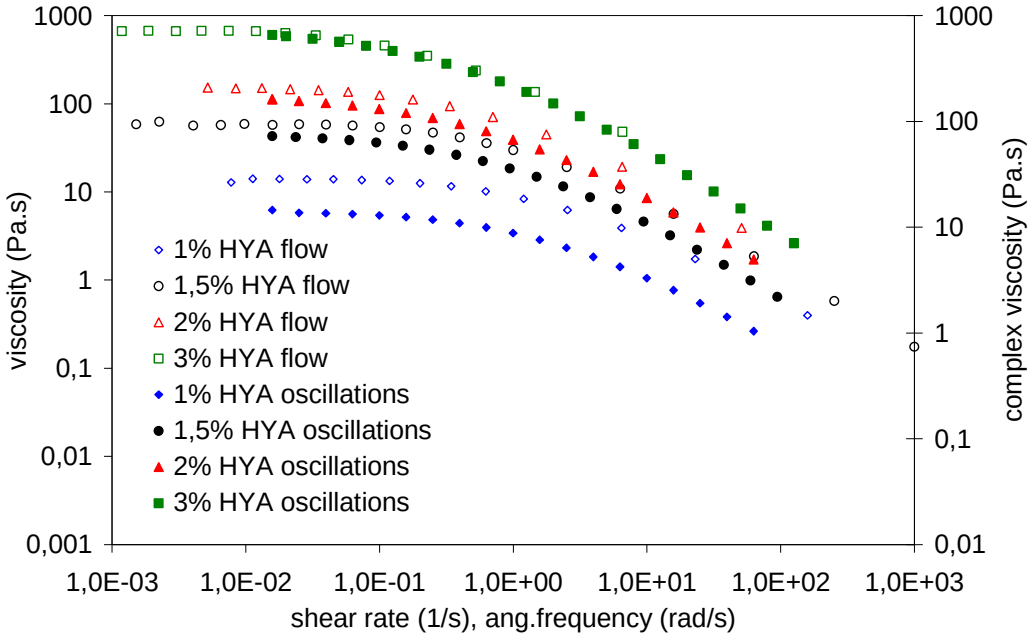

Fig. 5.7 Comparation of complex viscosity obtained from oscillatory measurements and shear viscosity obtained from flow measurements, Cox-Mertz rule was applied

5.3. Creep&recovery

The method described in the previous subchapter is based upon measuring the oscillatory response of a material at certain frequencies, but important information about the viscoelastic response of the materials can be also obtained by measuring the response of materials as function of time, i.e. creep&recovery experiments. In this test a sample is subjected to a constant stress and the amount of deformation is monitored as a function of the time.

All experiments were performed without exceeding a maximum stress of 5 Pa, which is within the Newtonian plateau of the flow curve, and a recovery time lasted triple of the creep time (12 minutes). The Fig. 5.8 shows the results for the 3% hyaluronan aqueous solution with the applied stresses ranging from 1 to 5 Pa. The output of creep&recovery test is dependence of strain γ or compliance J on time. Strain means the measurement of deformation relative to a reference configuration of length, area or volume and compliance is the strain divided by the corresponding stress. The compliance is a material property similar to the viscosity in steady state flow and it defines how compliant a sample is: the higher the compliance the easier the sample can be deformed by a given stress. The creep and recoverable compliance are independent of the stress within the testing range Fig. 5.8.

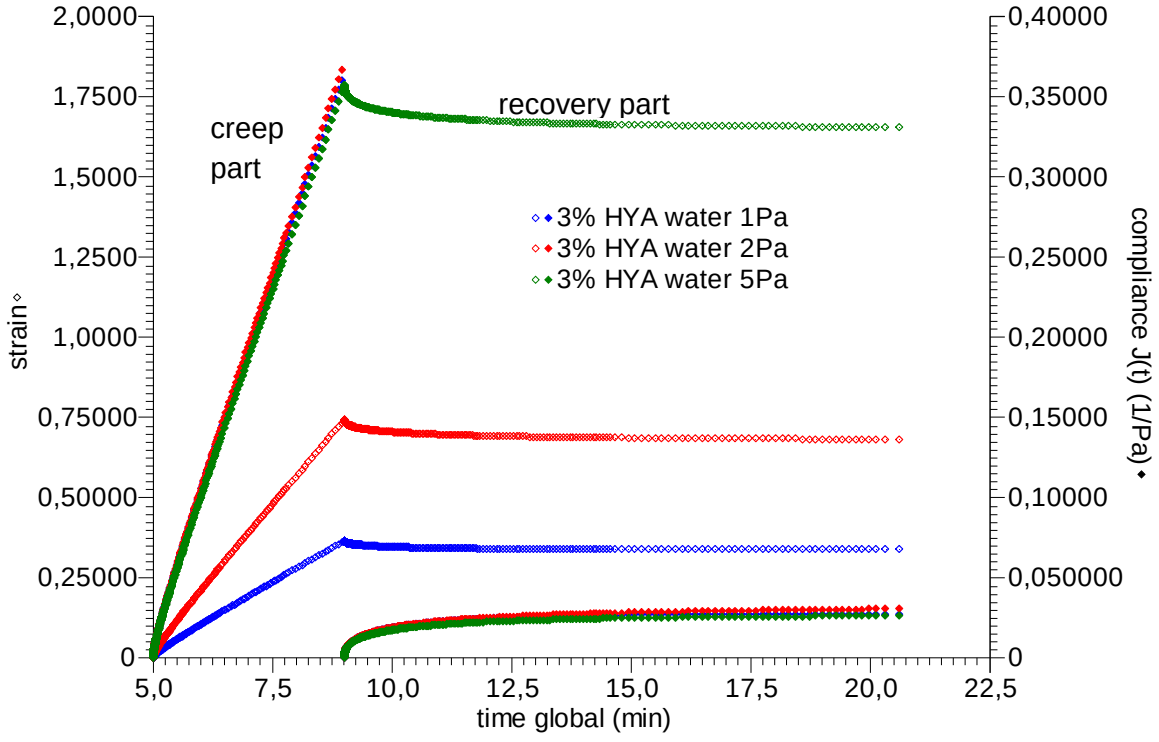

Fig. 5.8 The plot of strain ◆ and compliance ◇ against time for 3% hyaluronan water solutions at three different stress values. All the values were selected from the linear Newtonian region, thus the compliance curves are identical.

From the strain dependence of recovery portion we obtained the ratio of viscous γ_v and elastic γ_e segments as a percentage share of strain decrease. The ratio was calculated as a difference of the first point and final part of recovery curve, where strain reach constant values (Fig. 5.9).

Creep part of compliance dependence were fit, using TA Analysis software, by the rheological model named "Discrete retardation spectrum". It responds to the Burger model containing more than one Kelvin element (2.14). Utilization of more elements in parallel leads to the better curve description, but involves to "retardation time spectrum", not only to one value of retardation time λ_{ret}, thus we can't compare the value with other test methods. The rest of model parameters give us values of the zero shear viscosity η_0, the compliance $J_{1\text{-}6}$ and set of the retardation times λ_{ret} ($t_{1\text{-}6}$) for each of the Kelvin elements.

Discrete retardation spectrum applied to recovery part results to the value of compliance at infinite time called equilibrium compliance J_e (Fig. 5.10). This quantity means the rheological parameter for elasticity.

Percentual values of viscous and elastic portions are together with the equilibrium compliance are noted in Tab. 5.3. These are the average values from several measurements.

With increasing concentration and ionic strength could be observed obvious growth in elastic response. As well as in the flow measurements m it is due to larger amount of polymers chains in the solution and their entanglements. High concentrated samples looks like and behaves more like a solid, than liquid. This leads to lower resistivity during flow, but better mechanical properties like fortress and elasticity.

Tab. 5.3 Percentage values of viscous and elastic forces in HYA solutions

	water				ionic strenght	
	1% HYA	1,5% HYA	2% HYA	3% HYA	0,15	1
γ_e (%)	1,047	1,847	3,97	6,985	7,454	8,985
γ_v (%)	98,95	98,15	96,03	93,01	92,55	91,02
J_e (1/Pa)	0,1874	0,0754	0,0609	0,0275	0,0274	0,0255
J_e (%)	0,997	1,756	3,795	6,905	7,213	8,632

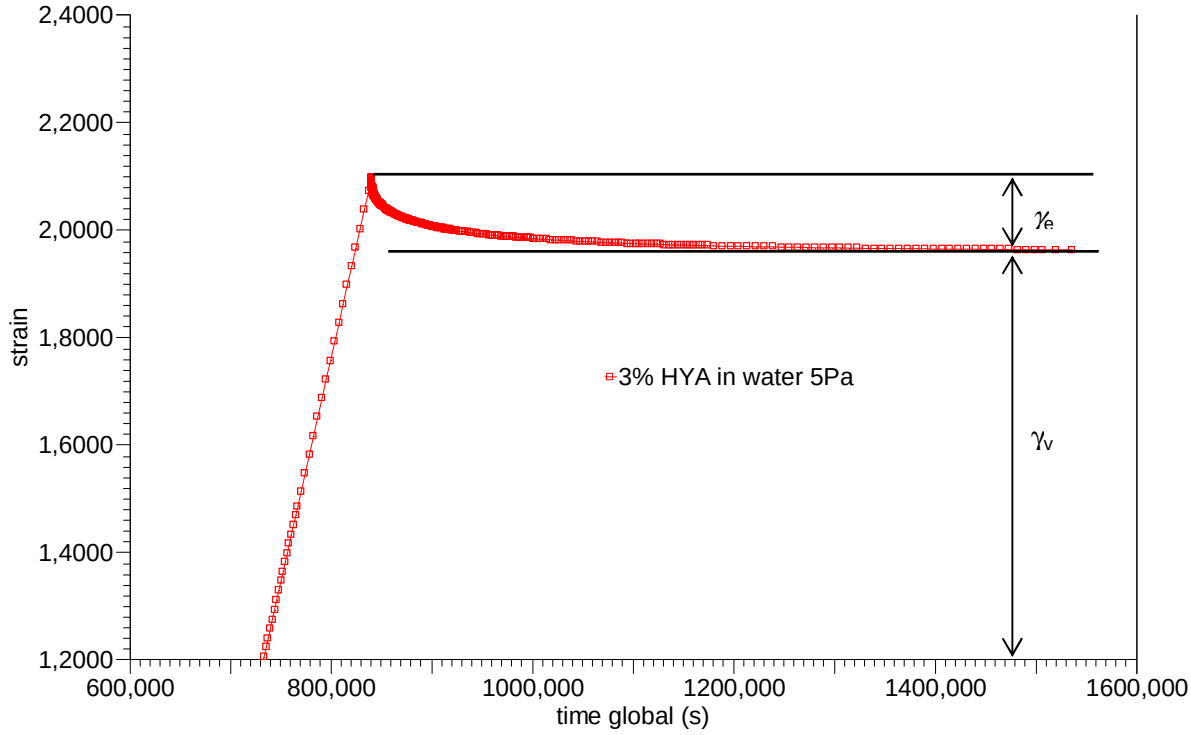

Fig. 5.9 The illustration of determination γ_v viscous and elastic γ_e forces from recovery portion

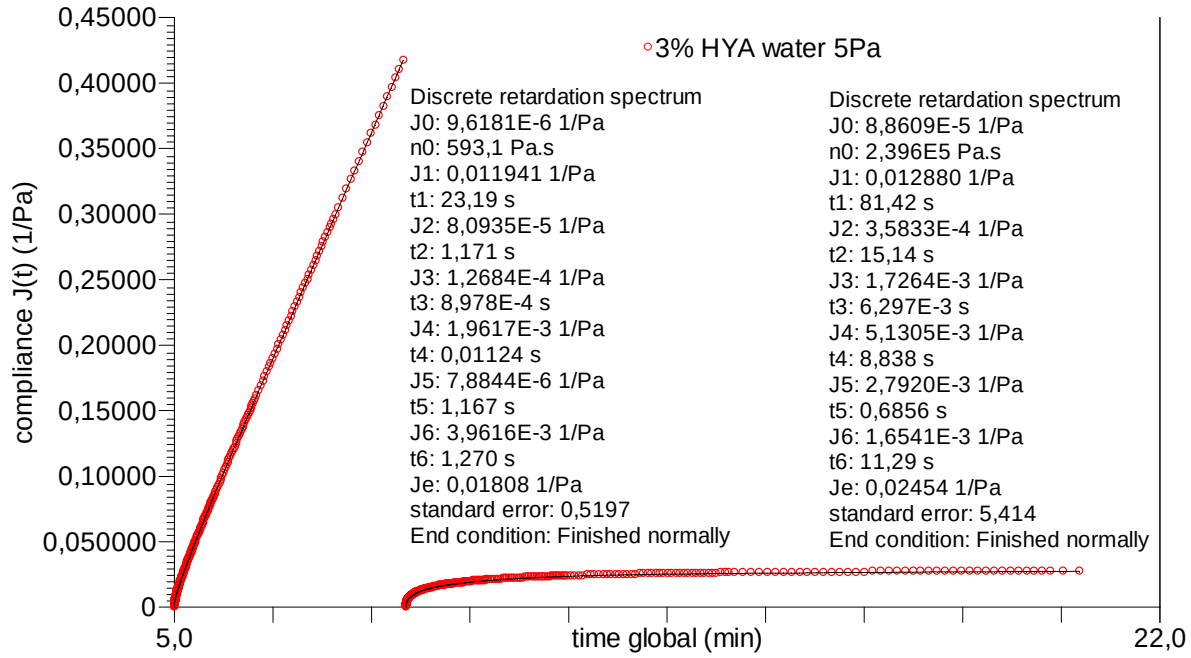

Fig. 5.10 Analysis of the creep&recovery curve using TA Analysis software

5.3.1. Comparing creep&recovery results with flow and oscillations

Results from flow and creep measurements are presented in Tab. 5.4. The value of flow and creep viscosity are not absolutely identical, but on the other hand the difference is rather imponderable. It could be said, that the usage of creep&recovery measurement to get η_0 is possible and obtained values are comparable with those from flow experiments.

In the case of creep and oscillation experiments, they are not so much comparable, they more complete each other. The oscillation measurement provides the short time response of the material and the creep recovery test the long time response. Nevertheless, the equilibrium compliance extracted from the recovery experiment could be compared to the elastic component determined from the ratio $G'/G''2$,, obtained from oscillatory measurements, as a function of frequency with $\omega \rightarrow 0$ (Fig. 5.11), [3], [20]. Even the extrapolation isn't very precise method, the results fits to J_e values as shown in Fig. 5.12.

Tab. 5.4 Confrontation of results obtained from different test methods

	water				ionic strength	
	1% HYA	1,5% HYA	2% HYA	3% HYA	0,15	1
$\eta_{0\ flow}$ (Pa.s)	14,73	63,93	159,5	669,6	615,8	893,3
$\eta_{0\ creep}$ (Pa.s)	13,75	62,39	162,4	680,75	611,8	855,5
$J_{e\ creep}$ (1/Pa)	0,1874	0,0754	0,0609	0,0275	0,0274	0,0255
G'/G''^2_{osc} (1/Pa)	0,1618	0,1786	0,1556	0,0225	0,0213	0,0194

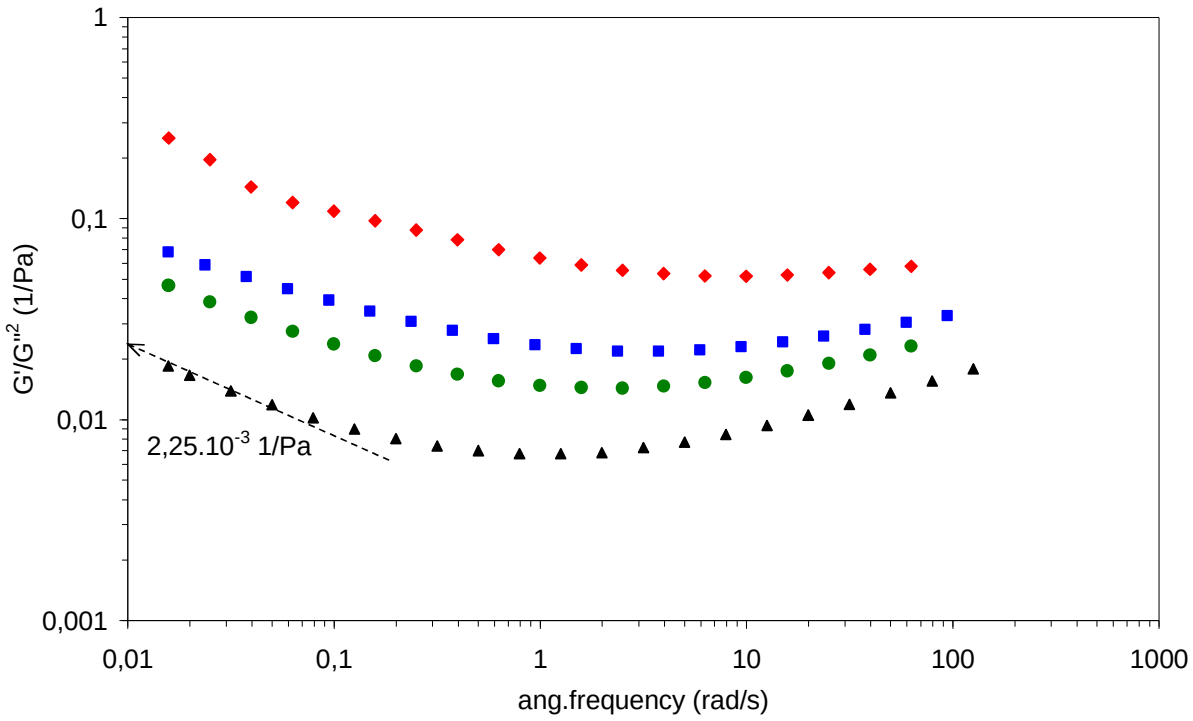

Fig. 5.11 Dynamic moduli ratio G'/G''^2 limited to the zero angular frequency could be compared with the equilibrium compliance J_e

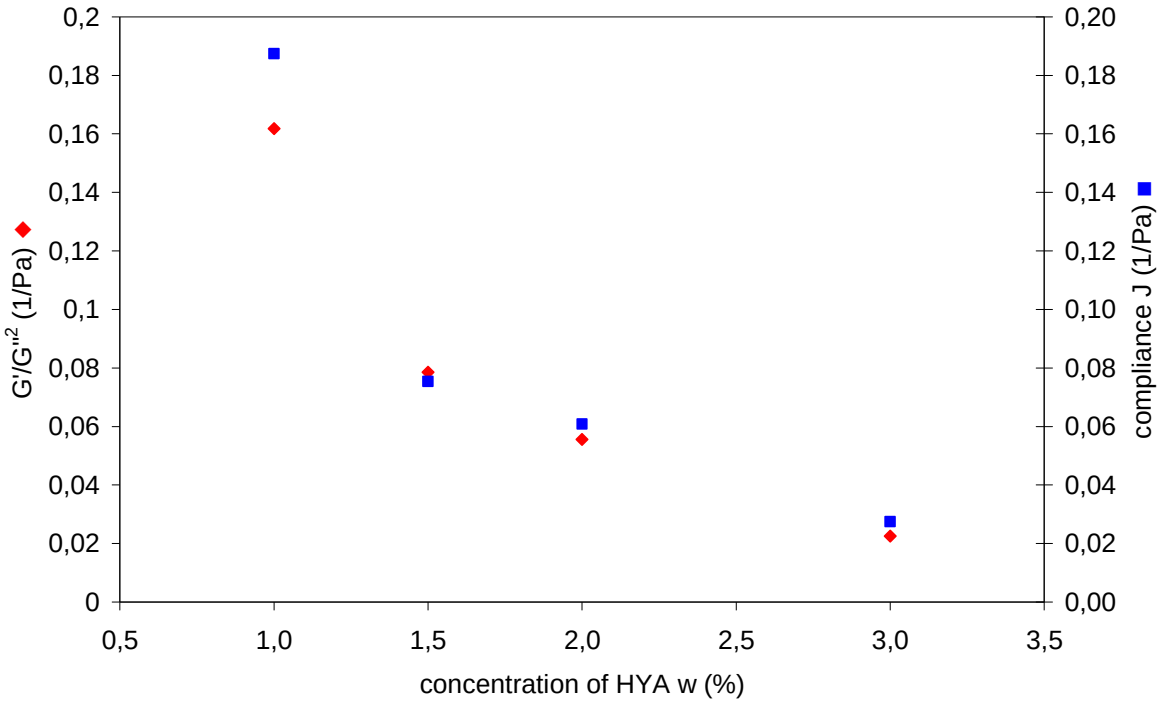

Fig. 5.12 Comparison of extrapolation of dynamic moduli ratio G´/G´´² with J_e

5.4. Peak hold

The creep&recovery test is not only one possibility how to measure stress analysis of sample and its response. The other option can be peak hold method. Peak hold is a special case of flow curve when the increase of the shear stress is not continuous like it was in case of viscous curves. The pulses of constant shear stress are applied to the sample. Material could relax between these pulses by low values of stress within Newtonian region. Two types of this test with the different pulse duration were performed. During shorter experiment only 30 seconds pulses were carried out and by longer experiment the pulse duration was increased to 5 minutes. Details are mentioned in the chapter 4.5.4.

Peak hold measurement is resulting in the dependence of viscosity on time for different shear stress peaks. It was observed if viscosity is decreasing due to the applied stress on a sample. Such as decreases could mean non-reversible changes in structure of sample as a response to applied stress amplitudes or duration of stress pulse length. Preview of the peak hold measurement could be observed in Fig. 5.13.

Our attention was focused on the relaxation parts between single stress pulses. Average values of viscosity from relaxation parts (between single stress pulses) are set in the Tab. 5.5.

It was found out that viscosity values are getting back after stress peak removal very fast to reach the initial of even higher viscosity values in despite of very strong applied stresses, close to the critical stress over non-reversible deformation of HYA solution structure occurs.

So that, the manipulation with the hyaluronan purely aqueous solutions could be done and is really safe till some critical shear stress value. Similar measurement was performed with the carboxymethylcellulose and is mentioned to compare how big influence the increasing shear stress could have.

Further it could be discussed the influence of ionic strength. The increasing content of NaCl in solution leads to lower relaxation ability of sample as Tab. 5.5 reflects. It was observed that if higher stress is applied on the sample, the emphatic decline of viscosity value and non-reversible structure changes were occurred.

The differences between both types of the flow measurements were plotted in Fig. 5.15.

Tab. 5.5 Viscosity values obtained from the relaxation parts after shear stress was removed and their dependence on increasing HYA concentration, ionic strength, applied stress and experiment duration

	1 Pa		after 20 Pa		after 50 Pa		after 100 Pa		after 200 Pa	
	short	long	short	long	short	long	short	long	short	long
1% HYA	13,72	13,40	13,75	13,56	13,69	13,58	13,65	14,10		
1,5% HYA	61,63	63,11	61,89	63,50	62,90	63,07	62,96	63,37		
2% HYA	169,7	158,5			168,9	158,7	169,2	159,8	169,5	159,6
3% HYA	629,2	620,8			637,0	628,6	633,9	640,4	623,8	630,3
0,15 M NaCl	599,4	620,8			620,9	635,0	611,0	629,7	611,4	616,4
1 M NaCl	920,2	1099,3			923,2	1005,2	894,9	971,1	896,3	914,9

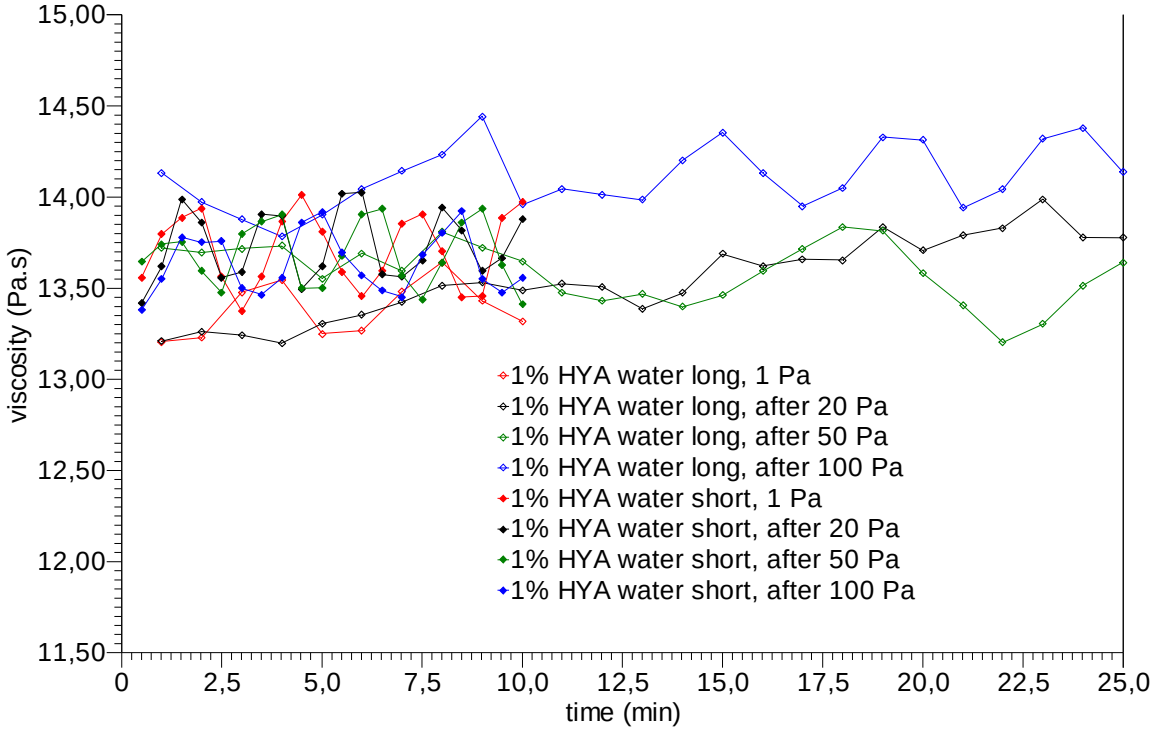

Fig. 5.13 The comparison of short ◆ and long ◇ peak hold test

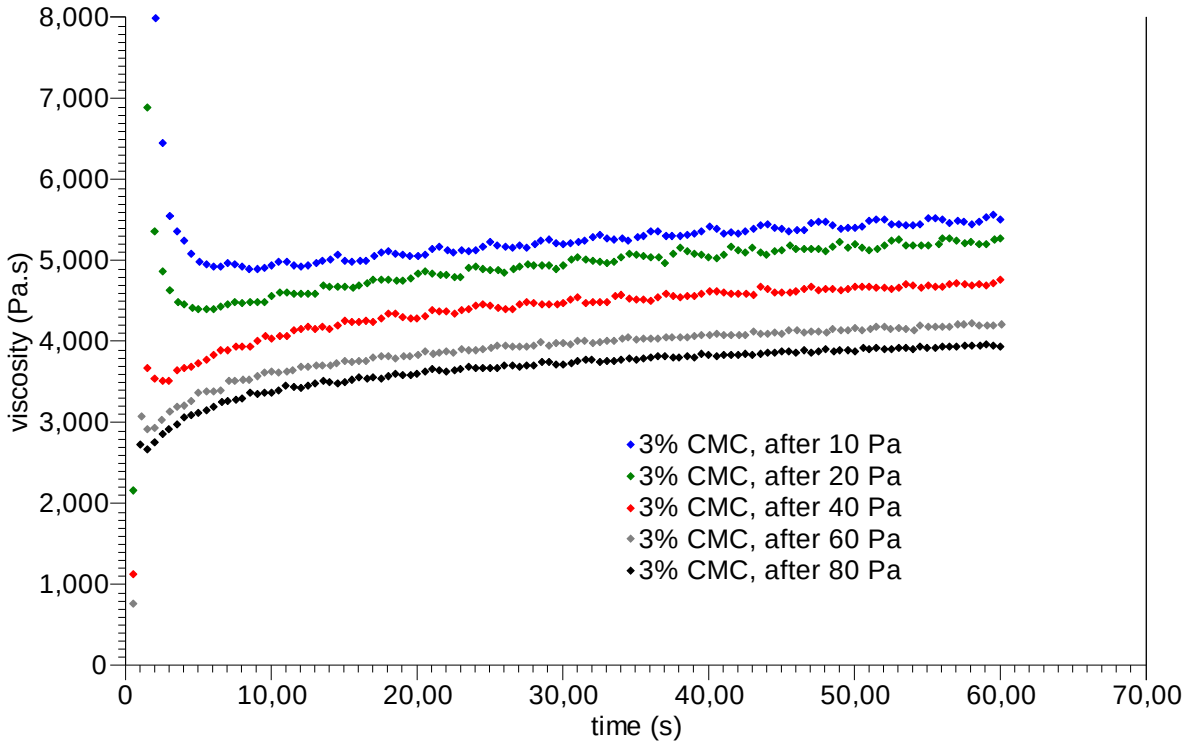

Fig. 5.14 The demonstration of 3% wt. CMC destruction by the applied stresses

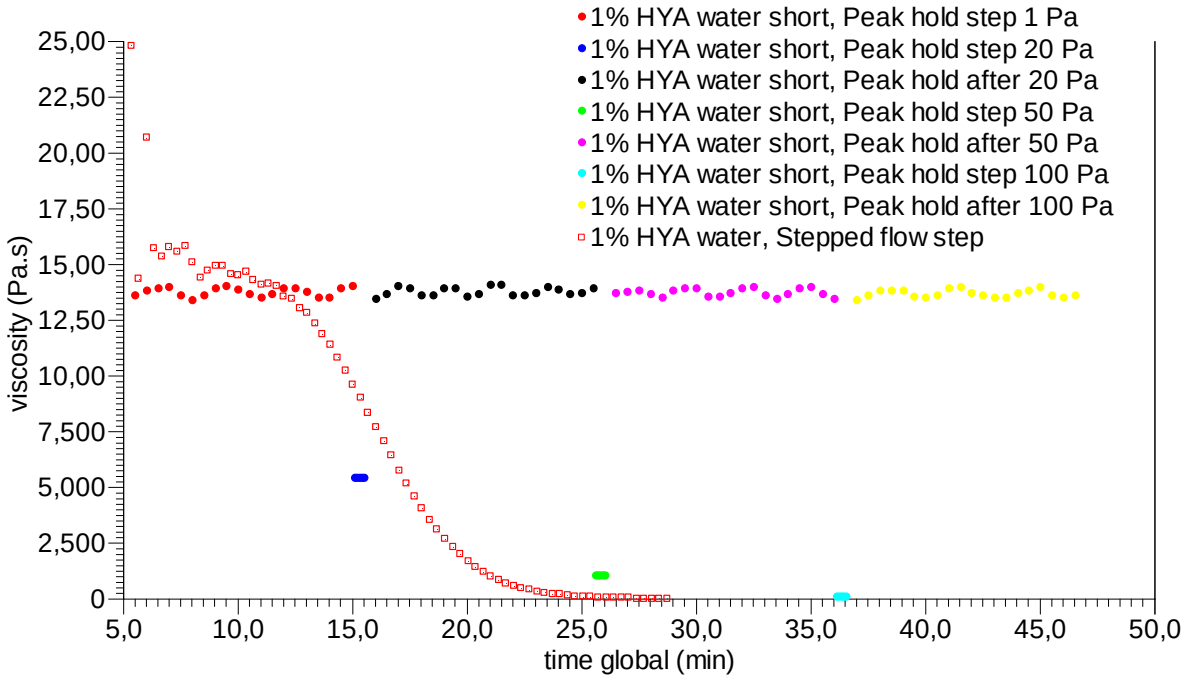

Fig. 5.15 Difference between viscous flow and peak hold test

6. Conclusions

In this work, the rheology of hyaluronan solutions was investigated using different types of experiments, such as flow tests – viscosity curves and peak hold tests, oscillations, and creep&recovery test. We tried to compare the commonly used methods, viscosity curves and oscillations, with peak hold and creep&recovery, which have not been performed in hyaluronan research.

Flow curves give us information about the dependence of sample viscosity on increasing shear stress or rate. We observed remarkable shear-thinning behaviour (of our solutions of HYA). The values of zero shear viscosity η_0 increase with HYA concentration and with the high NaCl content, while it decreases with the weak ionic strength of the solvent. The relaxation time, τ, behaves similarly η_0.

Peak hold tests were performed to enlarge the knowledge about time and mechanical stability of HYA in aqueous solution. It was very interesting to examine, how quickly HYA solutions, after removal of applied stresses, could turn back towards their original state. Moreover, there is not significant difference in the relaxation of the samples' viscosities after different stress values, till some critical stress, over which the solutions undergo irreversible degradation of their structure. By the addition of NaCl, the samples could not reach the same values of shear viscosity, after stress removal, thus they were negatively influenced by an ionic strength.

During the oscillation measurements we also investigated the frequency dependence of the storage, G', and loss modulus, G'', mostly their crosspoint properties, G_{cross}. With the increasing concentration of HYA in solution and in high ionic strength solution G_{cross} shifts to the lower frequencies, thus the sample behaves more elastic. The reverse value of crosspoint angular frequency gives us a relaxation time, $\tau_{relax\ osc}$. It is an important parameter describing viscoelasticity of the material. We have compared it with the flow relaxation time, $\tau_{relax\ flow}$. The former one is somewhat larger, because it is obtained from the flow curve inflex point, which occurs out of Newtonian region, where shear is much larger than in oscillations. Thus the real material relaxation time occurs somewhere between these two values.

Another quantity obtained from the oscillations is the complex (or dynamic) shear viscosity, η^*. The comparison of the dynamic shear viscosity η^* (ω) with the steady shear viscosity $\eta(\dot{\gamma})$ at comparable angular frequency and shear rate values shows shear-thinning rheology at all frequencies and shear rates studied. A characteristic failure of the Cox-Mertz relation is observed at low ω and $\dot{\gamma}$, where $\eta^*(\omega) > \eta(\dot{\gamma})$. The viscosity curves from both measurements fit well for all the concentrations of hyaluronan.

We report the first studies of the creep response of HYA solutions, which provide more definite information on whether the material behaves as a viscoelastic solid or a viscoelastic fluid at very low levels of applied shear. From our results is obvious that HYA behaves much more viscous than elastic; for 1 wt. % HYA solution only 1 % of behaviour is formed by elastic portion. Elasticity of the samples increases with the HYA concentration as well as with the ionic strength. Great advantage of this test lies in the possibility to obtain the information

about sample flow properties – zero shear viscosity as well as the viscoelastic response in the one relatively fast experiment instead of measuring flow curves and oscillations that are rather time-consuming. Results from creep&recovery were compared with just mentioned experiments to verify their mutual interchange ability. Zero shear viscosity, $\eta_{0\ flow}$, obtained from flow curves and ratio of the oscillations moduli, G'/G''^2, were confronted with zero shear viscosity, $\eta_{0\ creep}$, and equilibrium compliance, J_e, obtained from creep&recovery. The results are nearly identical, thus the experiments can be substituted with each other.

7. References

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8. List of symbols

HYA	Hyaluronan, Hyaluronic acid
CMC	Carboxymethylcellulose
σ	shear stress
γ	strain
$\dot{\gamma}$	shear rate
τ_{relax}	relaxation time
η	viscosity
η_0	zero shear viscosity
G'	storage moduli
G''	loss moduli
ω	angular frequency
δ	delta (loss) degree
λ_{ret}	retardation time
J	compliance
J_e	equilibrium compliance
γ_e	elastic portion of sample behaviour
γ_v	viscous portion of sample behaviour