5 Fingerprinting the nonlinear rheology of fibrin clots

Viscoelastic materials such as colloidal suspensions and polymer gels generally exhibit a nonlinear stress response to an applied shear strain and various methods have been devised to probe this nonlinearity. A popular method is the large amplitude oscillatory shear technique (LAOS), where a sinusoidal strain is applied and the stress response is measured. Nonlinearities in the stress can be "fingerprinted" by Fourier transforming, decomposition, or Lissajous plots. In biopolymer networks it is, however, difficult to interpret the physical basis of these fingerprints, since their nonlinear behavior is still incompletely understood. In this chapter, we explore different methods to probe the nonlinearity in the strain response of fibrin gels to an applied large amplitude oscillatory stress. We find that the sensitivity of Fourier Transform strain spectra and Lissajous stress/strain curves to the onset of strain-stiffening is comparable to the sensitivity of differential measurements, where a small oscillatory stress is superposed on a steady prestress. In the nonlinear regime, the Lissajous curves show complex shapes, whose interpretation is uncertain.

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5.1 Introduction

Soft matter is a class of materials that is built of mesoscopic building blocks such as colloids, polymers, or bubbles. These building blocks are much larger than molecules, yet small enough to be susceptible to thermal fluctuations. Soft matter tends to exhibit viscoelastic behavior, which is time-dependent and changes under applied load. Biological materials such as cells and tissues can also be categorized as soft matter, since they are built of protein biopolymers such as actin, collagen, and fibrin. Gels reconstituted from purified protein biopolymers are viscoelastic and remarkably sensitive to applied loads. Typically, the gels already start to exhibit a nonlinear elastic response at shear or tensile strains of a few percent [285]. Crosslinked biopolymer networks generally strain-stiffen strongly, sometimes by up to factors of 100-1000, before rupture [246, 267, 307, 94, 325, 39]. Fibrin gels offer a particularly striking example of nonlinearity. They can withstand shear strains of up to 300% and can stiffen up to 1000-fold [191]. This rheological response provides resilience, which likely contributes to the efficacy of fibrin blood clots in stemming blood loss and promoting wound healing [169].

Several methods have been devised to characterize the nonlinear response of soft materials to an applied shear. A traditional method is large amplitude oscillatory shear (LAOS). In a strain-controlled experiment (which is most common), a sinusoidal strain, $\gamma(t)$, is applied, and the oscillatory stress response, $\sigma(t)$, is measured (Fig. 5.1 A):

$$\gamma(t) = \gamma_0 \sin(t)$$
$$\sigma(t) = \sigma_0 \sin(\omega t + \delta),$$

where $\gamma_0$ is strain amplitude and the phase difference $\delta = G'' / G'$. Here, $G''$ is the viscous (out-of-phase) component, and $G'$ is the elastic (in-phase) component of the complex shear modulus, $G^*$:

$$G^*(\omega) = G'(\omega) + iG''(\omega)$$

Oscillatory measurements on fibrin clots show an increase in the elastic modulus above critical strain amplitudes of around 10% [149, 13]. However, it is difficult to interpret the modulus at strains above the critical strain, since the stress response in the nonlinear regime is highly non-sinusoidal [155]. $G^*$ is commonly obtained in LAOS from the first harmonic in the response, and does not take into account higher-order terms, which do store energy [93]. For strain-stiffening materials such as fibrin gels, $G^*$ underestimates the modulus in the nonlinear regime, since its correspond to the ratio of stress over strain, $\sigma/\gamma$, which is smaller than the local derivative, or slope, $\delta\sigma/\delta\gamma$ (see Fig. 5.1 D). Several methods have been proposed to probe the differential modulus, $K^* = \delta\sigma/\delta\gamma$. One method is known as the prestress or differential technique, where a small amplitude oscillatory stress is superposed on a large steady stress (Fig. 5.1 B). This method works particularly well for materials that are predominantly elastic.
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and show minimal creep, and have already been applied to fibrin gels previously [39, 232]. An alternative method is the strain-ramp protocol, where the material is subjected to a steady shear with strain rate, \( \dot{\gamma} \) [39, 265] (Fig. 5.1 C). This method returns a rate-dependent elastic modulus, which tends to agree with the prestress method at small rates of strain. In case of fibrin gels, the differential moduli obtained from the strain-ramp and prestress methods agree well, since these gels are nearly perfect elastic solids [39].

The advantage of the differential moduli is that they can be directly and quantitatively compared with theoretical models, which has helped to elucidate the mechanistic origin of the nonlinear rheology of actin gels [94], intermediate filament networks [325, 180, 182], and fibrin [232]. In contrast, the moduli obtained from LAOS suffer from a non-sinusoidal signal in the nonlinear regime and cannot be directly compared with models. However, it has been proposed that LAOS can be a useful and unique technique to fingerprint the nonlinear response of soft materials provided that the full spectrum of the stress (strain) response, including higher harmonics, is analyzed [81]. Several fingerprinting methods have been proposed and applied to nonbiological soft matter such as foams [256], and polymers [79], and to a few selected biological materials, such as native pedal muscus [78, 79]. However, these methods have not yet been applied to fibrin gels.

The most common method to analyze LAOS data in the nonlinear regime is known as Fourier transform (FT) rheology. The stress response to an oscillatory strain input is decomposed in terms of a Fourier series [312]:

\[
\sigma(t; \omega, \gamma_0) = \gamma_0 \sum_{n \text{ odd}} G'_n(\omega; \gamma_0) + G''_n(\omega; \gamma_0) \cos n\omega t, \tag{5.3}
\]

where the mode number \( n \) includes only odd harmonics, since the stress is a
function of shear direction and its sign changes as the sign of shearing changes. $G'_1$ and $G''_1$ are the first-harmonic viscoelastic coefficients that are generally reported by commercial rheometer software. At small strain amplitudes and low frequencies, the Fourier transformed stress shows a single response peak with intensity $I(\omega_1)$ at the excitation frequency, $\omega$. This dominant intensity contributes in 99% to the linear response. The contribution of 3rd and 5th harmonic, $I_3(\omega_1)$ and $I_5(\omega_1)$, is less than 1% and it is usually not included into data analysis. As the strain amplitude increases, higher harmonics appear, and the ratio of their intensities relative to the first harmonic (n=1) has been proposed to provide a characteristic fingerprint of the material [313]. Furthermore, it has been proposed that the appearance of higher harmonics is a more sensitive indicator of nonlinearity than the appearance of nonlinearity in the first-harmonic modulus, $G'_1$ [245]. Since the intensity of the higher harmonics grows with an odd power of strain amplitude [212], the intensity of the third harmonic scaled by the first, $I_3/I_1$, is expected to show a quadratic dependence on strain. Based on this scaling, two new nonlinear coefficients to characterize the relaxation dynamics of polymer chains were recently introduced [146]. However, the physical meaning of these coefficients, and in fact the interpretation of the higher harmonics in the Fourier spectrum themselves, is not fully understood yet and remains a matter of debate [159]. It is particularly problematic to deconvolve the elastic (stored energy) and viscous (dissipated energy) response, since the FT analysis only considers the nonlinearity of the total stress. To overcome this limitation, an alternative method was recently proposed, where the total LAOS stress is decomposed into an elastic stress, $\sigma'(\gamma(t))$, which is a function of strain, and a viscous stress, $\sigma''(\dot{\gamma}(t))$, which is a function of strain rate [59]. This decomposition provides generalized storage and loss moduli that remain meaningful at large deformations:

$$\sigma' \equiv \gamma_0 \sum_{n \text{ odd}} G'_n(\omega, \gamma_0) \sin n\omega t$$

$$\sigma'' \equiv \gamma_0 \sum_{n \text{ odd}} G''_n(\omega, \gamma_0) \cos n\omega t$$

(5.4)

Further orthogonal decomposition of the elastic and viscous stress using Chebyshev polynomials of the first kind gives harmonic components, or Chebyshev coefficients [79]. These coefficients provide a quantification of the elastic and viscous nonlinearities:

$$\sigma'(x) = \gamma_0 \sum_{n \text{ odd}} e_n(\omega, \gamma_0) T_n(x)$$

$$\sigma''(x) = \gamma_0 \sum_{n \text{ odd}} \nu_n(\omega, \gamma_0) T_n(y),$$

(5.5)

where $T_n(x)$ is the $n^{th}$-order Chebyshev polynomial of the first kind, and $e_n(\omega, \gamma_0)$ and $\nu_n(\omega, \gamma_0)$ are the elastic and viscous Chebyshev coefficients, respectively. The latter coefficients can be directly related to the time-domain Fourier coefficients [79]:
\[ e_n = G'_n (-1)^{(n-1)/2}, \text{n odd} \]
\[ \nu_n = G''_n / \omega = \eta'_n, \text{n odd} \]

The sign of the third-order Fourier coefficient can now be attributed to the stiffening/softening and thickening/thinning nature of the probed material.

A different way to decompose the LAOS stress response into elastic and viscous components is to use a geometrical interpretation of the stress in the form of Lissajous-Bowditch plots [231]. These are parametric plots of the total shear stress versus strain whose shape provides another way to fingerprint the nonlinear rheological response of the material. In the linear regime, Lissajous curves are straight lines for a purely elastic solid, circles for a purely viscous fluid, and ellipses for a viscoelastic material. The area enclosed by the Lissajous curve can be physically interpreted as the total energy dissipated per unit volume, \( E_d = \pi \gamma^2 G'_1 \), where \( G'_1 \) is the loss modulus of the first harmonic [93]. As the strain amplitude increases, the Lissajous curve shape for viscoelastic materials deviates from an elliptical shape in a manner that reflects the type of nonlinear response. The frequency dependence of this response can be probed by performing LAOS tests at different frequencies, \( \omega \), and arranging the resulting Lissajous curves in the form of a Pipkin diagram [233]. This diagram visualizes how the elastic and viscous response depend on frequency under increased levels of deformation. The onset of nonlinearity can be estimated from the Lissajous curves through the minimum-strain modulus, \( G'_M \) (the slope of the Lissajous curve at zero strain), and the large-strain modulus, \( G'_L \) (the ratio of stress and strain at maximum strain), which capture the local elastic response of the material at small and large strains, respectively [79]. These moduli can be related to the FT description and the Chebyshev stress decomposition, providing a geometrical interpretation of the elastic and viscous moduli in the nonlinear regime:

\[ G'_M \equiv \frac{d\sigma}{d\gamma} = \sum_{n \text{ odd}} nG'_n = e_1 - 3e_3 + ... \]
\[ G'_L \equiv \frac{\sigma}{\gamma} = \sum_{n \text{ odd}} G'_n (-1)^{(n-1)/2} = e_1 + e_3 + ... \]

The strain-stiffening index, \( S_{\gamma_0} \equiv (G_L(\gamma_0) - G_M(\gamma_0))/G_L(\gamma_0) \), represents a measure of the elastic nonlinearity [79]. Similarly, the minimum-strain and large-strain dynamic viscosity (\( \eta'_M \) and \( \eta'_L \)) can be used to estimate a shear-thickening index, \( T_{\gamma_0} \equiv (\eta_L(\gamma_0) - \eta_M(\gamma_0))/\eta_L(\gamma_0) \). Lissajous curves like higher harmonics in FT rheology, have been proposed to give a more precise estimate of the onset of nonlinearity than \( G'_1(\omega) \) [77]. Moreover, analysis of the Lissajous curves can provide evidence of strain-stiffening in biopolymer networks for which stiffening is so weak that the first harmonic does not show it [78].

In this chapter, we explore various methods to analyze the full nonlinear response of fibrin gels to a large amplitude oscillatory stress. In particular, we test the performance of nonlinearity indices obtained from FT rheology and Lissajous curves to probe the onset of strain-stiffening, and we compare their
sensitivity to that of both the first harmonic of the LAOS response and differential measurements.

5.2 Experiments

Large amplitude oscillatory shear measurements and prestress tests (see Chapter 2 for measurement techniques) were performed on fibrin coarse clots (see Chapter 3 for sample preparation) with a wide range of protein concentrations (0.1-8 mg/ml). Samples were polymerized for periods of 1 to 4 hours, depending on fibrin content, to ensure that the network structure and the modulus reached steady-state. We performed LAOS experiments on a stress-controlled rheometer, where the oscillatory strain response to a sinusoidal stress was measured. Due to stress input the mathematical formalism of the various fingerprinting methods described in section II is not directly applicable to our data, since the stress decomposition assumes a strain-controlled experiment. In future, we plan to make a direct comparison of LAOS in stress and strain control. LAOS data were collected using a constant oscillation frequency of 0.5 Hz (except for the data shown in Fig. 5.3 C) and a stress amplitude that was increased logarithmically until sample breakage. The time-dependent sinusoidal stress and strain response were measured using RheoPlus software (Anton Paar, version 3.31). Lissajous curves (normalized by the maximum stress and maximum strain) were constructed based on a single oscillation. At the beginning of each sinusoidal cycle, several oscillations were applied to ensure a steady-state strain response.

5.3 Results

5.3.1 Nonlinear elasticity probed by LAOS and prestress tests

To quantify the nonlinear behavior of fibrin networks, we used the LAOS technique and compared the results with data obtained by the prestress method. All fibrin networks were homogenous and isotropic, and their mesh size decreased with increasing protein content (image panel in Fig. 5.2). In oscillatory measurements with increasing stress amplitude, two viscoelastic regimes were visible, as shown in the inset of Fig. 5.2 A. In the linear regime, where the applied stress is sufficiently small, the elastic and viscous moduli are independent of stress amplitude, \( \sigma \). Above a critical stress value, \( \sigma_{crit} \), the network stiffness increases with stress. This nonlinear stress-stiffening response is followed by sample failure at a maximum stress value, \( \sigma_{max} \). The corresponding maximum strains were rather large, being of order 250%. This is typical of fibrin gels, and reflects their extraordinary extensibility [232, 44, 239, 43]. Stress-stiffening occurred over the entire range of fibrin concentrations, but the extent of stiffening depended on protein content. Dilute networks stiffened more (relative to the initial, linear modulus) than concentrated networks Fig. 5.2 A.

Prestress measurements also revealed a linear and nonlinear elastic regime (Fig. 5.2 B). However, the stress dependence of the differential elastic modu-
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Figure 5.2. Nonlinear elastic response of fibrin networks subjected to either oscillatory or steady shear. Elastic moduli are normalized by the linear modulus and the stress is normalized by the critical stress value at the onset of nonlinearity. Symbols correspond to 0.5 mg/ml (squares), 2 mg/ml (circles) and 5 mg/ml (triangles) fibrin networks. (A) Elastic response to an oscillatory stress (LAOS test). (Inset): Non-normalized storage (solid symbols) and loss (open symbols) moduli as a function of non-normalized stress amplitude for a 2 mg/ml fibrin network. The moduli are constant at small stresses up to a critical value, $\sigma_{\text{crit}}$, and increase thereafter. (B) Differential elastic modulus as a function of applied prestress. Image panel shows maximum intensity projections of z-stacks of the corresponding fibrin networks over a 10 $\mu$m depth with 0.1 $\mu$m steps between confocal planes. Scale bars, 10 $\mu$m.

lus in the nonlinear regime was more complex than for $G'$, and concentration dependent. The stiffness showed an initial fast rise above the critical stress, followed by a slower rise or even a distinct plateau (for 5 mg/ml), and yet another fast rise till sample ruptured. Similar to the LAOS tests, the degree of stiffening was higher for dilute gels than for dense gels. Strikingly, the prestress tests show a 10-fold larger degree of stiffening than the LAOS tests. Also, the maximum stress normalized by the critical stress was somewhat larger in prestress tests. This difference may be caused by an underestimation of the LAOS analysis, which does not account for higher harmonic terms in the strain response in the calculation of the viscoelastic moduli.
5.3.2 Decomposition of the Time-Dependent Oscillatory Strain Response

The calculation of the viscoelastic shear moduli, $G'$ and $G''$, from a LAOS test assumes that the strain response to the applied sinusoidal stress is also sinusoidal. However, inspection of the strain response in the nonlinear regime shows deviation from sinusoidal shape (Fig. 5.3 A). The nonlinear distortion in the response becomes more evident when $\sigma$ is plotted versus $\gamma$ in a Lissajous plot (Fig. 5.3 B). For a viscoelastic material, the Lissajous plot in the linear regime has an ellipsoidal shape whose enclosed area is a measure of the viscously dissipated energy in an oscillation cycle. In the nonlinear regime, the ellipsoidal shape is generally distorted for soft materials, and the enclosed area often increases with increasing stress [93]. The nonlinearity in the strain response is also evident in the Fourier transformed strain response. In the linear regime, only one harmonic at the fundamental frequency is visible (inset of Fig. 5.3 C), whereas in the nonlinear regime numerous peaks corresponding to higher order harmonics appear (Fig. 5.3 C).

![Figure 5.3](image_url)

**Figure 5.3.** Oscillatory strain response upon application of a sinusoidal stress in the linear regime (thick line, strain amplitude $\gamma_0 = 1\%$) and in the nonlinear regime (thin line, $\gamma_0 = 177\%$). Plots show data in the time domain (A), corresponding Lissajous plots (B), and FT spectra recorded with an oscillation frequency of 0.1 Hz (C). Curves are normalized by the maximum amplitude of the oscillatory signal.
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It has been shown that the shape of the Lissajous curves can provide additional information about the material properties in the nonlinear regime. For fibrin networks, the shape of the Lissajous curves depends on strain amplitude as well as on protein concentration. At a strain amplitude of 1%, the Lissajous curves for all fibrin gels have an elliptical shape (Fig. 5.4, A-0.2 mg/ml, B-1 mg/ml and C-5 mg/ml). However, the area enclosed by the Lissajous curve decreases with increasing protein concentration. Fibrin networks of 5 mg/ml are nearly perfect elastic solids, with a Lissajous curve that is almost a straight line. As the strain amplitude increases, the Lissajous curves start to deviate from an elliptical shape, indicating a crossover from linear to nonlinear behavior. Following the Lissajous curve clockwise and starting at zero strain (Fig. 5.4 A, $\gamma=67\%$), stress increases with strain. The slope of the Lissajous curve increases however more rapidly when the strain approaches its maximum value in the cycle. When the strain starts to decrease back to zero, the stress decreases to zero as well, after which the other half of the cycle starts, with negative stress and strain. The critical strain amplitude at the onset of stress-stiffening behavior depends on fibrin concentration. The denser the network, the lower is the strain required to enter the nonlinear regime. The Lissajous curves show a marked inflection for gels of 1 and 5 mg/ml fibrin, where the stress rises rapidly at a constant strain (arrow in Fig. 5.4 B, $\gamma=68\%$). This feature is seen for all fibrin concentrations above 1 mg/ml in the high strain regime.

The area enclosed by the Lissajous curve (calculated with stress and strain in dimensional form) is a measure of the energy per unit volume, $E_d$, that is dissipated by viscous flow. This energy can be directly related to the loss modulus, $E_d = \pi \gamma^2 G''$. An increase in applied stress causes an increase in $E_d$ as evident from the increase in the area enclosed by the Lissajous curves (Fig. 5.5). For all fibrin networks, $E_d$ increases as $\gamma^2$ up to a strain amplitude of 10%, after which it increases more rapidly with strain (with a power-law exponent of 4.4) until sample breakage at the maximum strain (inset in Fig. 5.5). This strain dependence of the $E_d$ is in line with the stress dependence of $G''$, which is constant at small stresses and increases nonlinearly with stress above $\sigma_{crit}$ (inset of Fig. 5.2 A). The increase in dissipated energy relative to the linear regime varies between $10^5$-$10^6$ and does not show a clear concentration dependence, in contrast to the relative degree of elastic stiffening, which decreases with concentration (Fig. 5.2 A).
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5.3

A

\[ \text{raw } \tau \]

\[ \gamma = 1\% \]

\[ \gamma = 10\% \]

\[ \gamma = 20\% \]

\[ \gamma = 33\% \]

\[ \gamma = 67\% \]

\[ \gamma = 81\% \]

\[ \gamma = 91\% \]

\[ \gamma = 108\% \]

\[ \gamma = 120\% \]

B

\[ \text{raw } \phi \]

\[ \gamma = 1\% \]

\[ \gamma = 10\% \]

\[ \gamma = 20\% \]

\[ \gamma = 40\% \]

\[ \gamma = 68\% \]

\[ \gamma = 86\% \]

\[ \gamma = 110\% \]

\[ \gamma = 140\% \]

\[ \gamma = 160\% \]
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Figure 5.4. Changes in the shape of Lissajous curves with increasing strain amplitude for 0.2 (A), 1 (B) and 5 mg/ml (C) fibrin networks.

Figure 5.5. Dissipated energy calculated from the area enclosed by Lissajous curves for fibrin networks of different concentrations: 0.2 (circles), 0.4 (squares), 0.6 (triangles), 4 (diamonds) and 7 mg/ml (stars). (Inset): Maximum strain amplitude where networks rupture as a function of protein concentration.
A different way to analyze nonlinearities in the material response is to quantify the intensities of higher harmonics in the Fourier spectrum of the strain response. The FT spectra for all fibrin concentrations show an increasing number of higher harmonics peaks and the increase in peaks intensities with increasing strain amplitude (Fig. 5.6). Since the stress has odd symmetry with respect to the strain, only odd harmonics are visible (inset in Fig. 5.6). The intensity of the higher harmonics has been predicted to decrease as $1/n^2$ for strain-hardening materials [159]. For the fibrin networks, we observe a somewhat weaker scaling of $I_n/I_1$ with $n$ (power-law exponent -1.7) for the first 10 harmonics, and a stronger $n$-dependence for higher order harmonics.

![Figure 5.6.](image)

**Figure 5.6.** Contribution of higher harmonics to the nonlinear LAOS response of a 2 mg/ml fibrin network. With increasing strain amplitude (vertical arrow), the intensity $I_n$ of the higher harmonic contributions (normalized by the first harmonic, $I_1$) increases, and more and more higher harmonic peaks are detected. The gray solid line corresponds to the predicted $1/n^2$ scaling for strain-stiffening materials [159]. *(Inset):* The number of higher harmonics detected as a function of strain amplitude.

Both FT spectra and Lissajous plots can be used to identify the strain amplitude where nonlinearity first sets in [312]. For the FT spectra, one can define a nonlinearity parameter, $S$, either as the amplitude of the third harmonic relative to the first, $\sum_{n=3}^{\infty} I_n/\omega_1$ or as the sum of amplitudes of all higher harmonics normalized by the first, $S = \sum_{n=3}^{\infty} I_n/\omega_1$. As shown in Fig. 5.7 A, both nonlinearity parameters (up and down triangles) show onset of nonlinearity at the same strain amplitude, which is 1% for a 2 mg/ml fibrin network. For the Lissajous curves, the strain-stiffening index, $S(\gamma_0) = (G_L(\gamma_0) - G_M(\gamma_0))/G_L(\gamma_0)$, where $G_M$ is the slope of the Lissajous curve at zero strain and $G'_L$ is the ratio of stress and strain at maximum strain. The Lissajous index (circles) is less sensitive to the onset of nonlinearity than the FT spectra, and it is even somewhat less sensitive than the first harmonic modulus, $S_{LAOS} = G'_L(\gamma)/G_0(\gamma)$ (squares).
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Figure 5.7. Nonlinearity parameters for a 2 mg/ml fibrin network plotted against strain amplitude, calculated from three different methods: the first harmonic elastic modulus extracted directly from the stress-stiffening curve, $S_{LAOS} = G'(\gamma)/G_0(\gamma)$ (squares), the elastic stiffening ratio calculated from Lissajous curves, $S_{Lissajous} = L(\gamma_0)/M(\gamma_0)$ (circles), the sum of the amplitudes of higher harmonics in the FT spectrum, $S_{FT\Sigma} = \sum_{n=3}^{\infty} I_{2n}/I_1$ (up triangles) and the amplitude of the third harmonic in the FT spectrum, $S_{FT3} = I_{3\omega}/I_{1\omega}$ (down triangles), respectively, each normalized by the first harmonic. (B) Concentration dependence of the critical strain obtained from LAOS strain-stiffening curves (black squares), prestress measurements (yellow diamonds), and from $S_{Lissajous}$ (red circles) and $S_{FT}$ (green up triangles).

The critical strain, $\gamma_{crit}$, extracted from the dependence of all different stiffening indexes on the strain amplitude decreases about 10-fold as the fibrin concentration increases from 0.2 to 10 $\mu$M, and saturating to a constant level above 10 $\mu$M (which corresponds to 3 mg/ml) (Fig. 5.7 B). This implies an earlier onset of strain-stiffening with increasing fibrin concentration, in accordance with the earlier onset of non-ellipsoidal Lissajous curves (Fig. 5.4). The critical strain extracted from LAOS tests and the strain-stiffening index for the Lissajous curves decreases from 20 to 2%. The $\gamma_{crit}$ extracted from the higher harmonics in FT spectra is 2-fold smaller, indicating a higher sensitivity to the onset of nonlinearity. The FT spectra yield critical strain values which are in rather good agreement with the critical strain measured by the prestress method (yellow diamonds in Fig. 5.7 B). Interestingly, the sum of the amplitudes of higher harmonics also shows a similar strain-dependence as the differential elastic modulus, $K'(\gamma)$, on fibrin concentration (Fig. 5.8). $S_{FT\Sigma}$ is constant, rises above the critical strain, and then shows a slower rise for more dilute gels (squares: 0.5 mg/ml, circles: 2 mg/ml) or even a distinct plateau for denser gels (triangles: 5 mg/ml) followed by a further rise in stiffness. This complex strain dependence may have the same origin as the analogous strain-dependence of $K'(\gamma)$ shown in Fig. 5.2 B, which was ascribed to nonlinear stretching of the fiber backbones. However, $S_{FT\Sigma}$ has no clear physical interpretation, unlike $K'(\gamma)$. 
Figure 5.8. Dependence of the sum of the amplitudes of higher harmonics, $S_{FT\Sigma} = \sum_{n=3}^{\infty} I_{\Sigma\omega} / I_{1\omega}$ on strain amplitude for 0.5 (squares), 2 (circles) and 5 mg/ml (triangles) fibrin networks.

5.4 Discussion and concluding remarks

Large amplitude oscillatory shear is a popular method to probe the response of soft materials to large shear deformations. Soft materials tend to exhibit a highly nonlinear viscoelastic response, which is reflected in a highly non-sinusoidal stress (or strain) response to an applied sinusoidal strain (or stress).

In this chapter we examined the information stored in the non-sinusoidal strain response of fibrin gels to an applied oscillatory stress. We first looked at the nonlinear response of the elastic shear modulus, $G'$, which represents the first harmonic in the strain response. We found that the first harmonic strongly underestimates the nonlinearity of fibrin gels. This is especially obvious when comparing the stress-stiffening behavior of fibrin networks obtained from LAOS tests with the stiffening measured by the prestress method (Fig. 5.2). Pre-stressing the sample has the advantage of allowing locally linear measurements even at high stress, thus providing more accurate measurements of the moduli in the nonlinear regime. Moreover, we observed that the differential elastic modulus, $K'$, shows a richer dependence on stress than $G'$, revealing three distinct regimes in the nonlinear response. As explained in Chapter 3, these regimes can be ascribed to distinct structural levels at which fibrin gels deform. This richness is lost in the LAOS data, where a continuous increase in $G'$ with strain is observed till the network ruptures. However, the full nonlinear response in LAOS captured in the form of a FT spectrum of the strain response does show a similar, complex stress-dependence as the differential modulus (Fig. 5.8). Moreover, the full nonlinear response provides a more sensitive measure of the onset of nonlinearity than the first harmonic modulus. The FT spectra show comparable values for the critical strain as prestress measurements (Fig. 5.7 B). These observations indicate that a full characterization of the nonlinearity of
fibrin networks with LAOS requires a consideration of the higher harmonic contributions in the rheological response.

The Lissajous curves for fibrin networks in the nonlinear regime show complex shapes, which strongly deviate from the ellipsoidal shape in the linear regime. The curves show clear evidence for strain-stiffening in the form of a steep rise in stress at high strain. Similar behavior was previously reported for strain-stiffening actin networks [320, 293, 265]. However, in fibrin networks Lissajous curves show unique for this biopolymer stress shape at high protein concentrations with the characteristic inflections, which, to the best of our knowledge, have not been reported for other soft materials. It is unclear whether these inflections reflect some intrinsic material property related to the hierarchical structure of fibrin, or perhaps some form of elastic coupling, similar to creep ringing [326]. For polymer solutions, changes in the shape of Lissajous curves were previously shown to be indicative of shear-induced changes in the microstructure [145]. To resolve this question for fibrin, it will be interesting to check the influence of bundle size of the fibrin fibers on the shape of the Lissajous curves. If the inflections are related to the internal structure of protofibril bundles, then a reduced bundle size should affect the Lissajous curve shape.

The FT rheology and Lissajous curves provided in this chapter consider the total stress, which is a sum of elastic and viscous stresses. In future, it will be interesting to decompose the strain response into elastic and viscous contributions, to achieve a more physically meaningful interpretation of the data [79]. Moreover, a comparison needs to be made between LAOS performed in stress and strain control. In this way, LAOS analysis may eventually provide a fingerprint of the nonlinear rheology of fibrin gels (in the form Chebyshev coefficient) that is complementary to prestress experiments.

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