# Rheological and Turbidity Study of Fibrin Hydrogels

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**Summary:** Fibrin hydrogels prepared from different fibrinogen concentrations (12.5, 25 and 50 mg/mL) were studied through oscillatory rheology (gelation kinetics, stress amplitude tests and frequency sweeps), static experiments (creep and recovery tests) and turbidity (gelation kinetics). The experimental parameters obtained from these measurements (including gel point, characteristic times for different kinetic phases, loss and storage moduli), were used to gain some insight on the processes concurring to form the network structure of fibrin hydrogels. Here, we show (turbidity measures) that lateral fibre aggregation appears to be significantly depressed with increasing fibrinogen concentration. Among other important findings, a caveat in using absolute data of mechanical properties from gelation kinetics: we have shown that at low fibrinogen concentration, the actual modulus values are significantly affected by the application of shear stress during gelation.

**Keywords:** fibrin; fibrinogen; gelation kinetics; mechanical properties

## Introduction

Artificial fibrin clots, commonly referred to as *fibrin hydrogels, fibrin glue, fibrin sealants* or *tissue sealants*, are obtained through the same four-step process that leads to blood coagulation: conversion of fibrinogen to fibrin through thrombin-mediated cleavage of the fibrinopeptides; self-assembly of fibrin; chemical cross-linking of fibrin through the enzymatic action of factor XIII, previously activated by thrombin and the co-factor $CaCl_2$; and fibre branching to form a three-dimensional network.[1,2] Fibrin hydrogels are commonly employed as biodegradable glues or sealants in a number of surgical setting,[3,4] and a number of sealant products based on exogenous (Tisseel, Crosseal, Evicel, FloSeal) or endogenous fibrinogen (Thrombin-JMI, Recothrom, Evithrom) are commercially available and often used as haemostatic agents during internal surgery. Fibrin materials are also widely used as provisional matrices in skin grafts,[5] tissue engineering[6] e.g. of cartilage,[7] cell encapsulation[8] comprising 3D migration studies[9] and controlled release of growth factors.[10] This wide use is predominantly due to a) their unique regulatory status: fibrin is a biological product and is naturally used as a provisional matrix; b) very favourable physico-chemical and biological characteristics, which include biocompatibility, biodegradability, high water content, *in situ* formation and promotion of tissue repair.[3,6,11]

The mechanical properties of a matrix play a major role in influencing morphology, metabolism, and phenotype of cells in contact with it.[12–18] Therefore, a reproducible and easy-to-rationalise mechanical behaviour is essential for a material to provide reliable biological results. On the other hand, a certain variability has always characterised the mechanical characterisation of fibrin gels. In this work we have focused on the dependency of the kinetics of fibrin gelation and on the values of the

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final mechanical properties on fibrinogen concentration.

The phenomenon of fibrin gelation has been studied since the 1940's, when Ferry and Morrison showed how the rigidity and turbidity of the matrices exhibited an initial exponential increase, eventually reaching a plateau when the gelation process completed.[19] It is now well-known that the final, thrombin-cross-linked gels are strain hardening,[20,21] and present a substantial independence of G′ on frequency in oscillatory measurements[22] and of compliance on time in creep measurements,[23] at least for small strains, whereas at high strains irreversible deformation may take place; this indicates that the hydrogels have an almost purely elastic behaviour with negligible relaxation phenomena.

Although the above is generally valid, it is also known that fibrin mechanical behaviour is often difficult to reproduce in quantitative terms, since it depends in a complex fashion on the interplay between two morphological parameters: the lateral size (thickness) of fibrin fibres and the density of their branching points. The highest moduli are reportedly provided by a compromise of the two parameters,[24] since, as stated by Weisel, "conditions that favour lateral aggregation tend to produce clots made of thick fibres with few branch points, while conditions that inhibit lateral aggregation tend to yield clots made up of thin fibres with many branch points".[25] The factors affecting these two parameters and their mode of action are often a source of confusion. For example, it has been reported that low ionic strength would favour lateral aggregation,[26] while other (earlier) reports suggested a non-linear dependency.[27]

Regarding the role of lateral size and branching density with respect to the development of mechanical properties, it has been noted that while increasing the branching density always leads to an increase in stiffness, a larger lateral size has been shown to produce the opposite effect.[24] This point is confirmed by the more or less pronounced turbidity during hardening; turbidity is linked to the fibre lateral size.[28] Indeed, the square of the average size can be calculated as the ratio of the slope and intercept of a graph of $c/\lambda^3\tau$ vs. $1/\lambda^2$ (c being the concentration of fibrinogen, $\lambda$ the wavelength used and $\tau$ the turbidity). Turbidity can be used to follow the gelation kinetics: it is always inversely proportional to thrombin concentration and temperature, and higher pH and $Ca^{2+}$ concentrations ($Ca^{2+}$ acts as a co-factor in the thrombin-mediated activation of factor XIII) reduce plateau turbidity values, while also slowing the gelation rate.[19,29] However, the gel moduli increase significantly with increasing fibrinogen concentration, whereas turbidity diminishes correspondingly,[19] which fits with the lateral size being somehow inversely related to the modulus.

Importantly, branching and lateral growth may follow different kinetics, and the apparently double-exponential rheological behaviour during gelation has been ascribed to their different rate, with branching being the faster process.[30] However, the non-mono-exponential dependency of the kinetic data may also be explained with a (hyperbolic) saturation model,[31] possibly because of the presence of a controlling factor (e.g. thrombin), which is a key physical determinant of the fibrin polymerisation process (controlling both the conversion of fibrinogen to fibrin and activation of factor XIII,[32,33] an enzyme involved in the cross-linking reactions of neighbouring fibrin molecules).

Here, in a general characterisation of the mechanical behaviour of fibrin gels produced at different fibrinogen concentrations, we have compared fittings of turbidity and rheology gelation data with single- and double-exponential and hyperbolic models, and discussed the results in the light of possible morphological differences.

## Materials and Methods

### Materials

Bovine fibrinogen (F8630, Type I-S, $\alpha$-chain MW: 63.5 kDa, $\beta$-chain MW: 56 kDa,

 

$\gamma$-chain MW: 47 kDa; soluble dimer MW: 340 kDa), bovine thrombin (T9549, ≥1,500 NIH units/mg protein), HEPES buffer and dodecane were purchased from Sigma-Aldrich (Gillingham, UK). $CaCl_2$ and NaCl AnalR® were purchased from VWR International Ltd. (Leicester, UK).

For all preparations equal volumes (typically 500 μL each) of a bovine fibrinogen solution (25, 50 or 100 mg/mL fibrinogen) in HEPES-buffered saline (HBS: 20 mM HEPES; 150 mM NaCl, pH = 7.4, preconditioned at 37 °C) and a bovine thrombin/$CaCl_2$ solution (2 U/mL thrombin, 40 mM $CaCl_2$) in the same buffer were mixed by pipetting (final conc.: 12.5, 25.0 or 50.0 mg/mL fibrinogen; 1 U/mL thrombin; 20 mM $CaCl_2$).

### Rheology

All oscillatory (gelation kinetics; stress amplitude tests; frequency sweep tests) and static (creep and recovery tests) rheological experiments were carried out in a Bohlin Instruments Gemini rheometer (Malvern Instruments Ltd., Malvern, UK) at 37 °C in stress-controlled mode, employing a parallel plate geometry (40 mm Ø upper plate; 100 μm measuring gap); all samples were covered with a layer of dodecane after lowering the upper plate to minimise water evaporation.

In the gelation experiments fibrinogen and thrombin/$CaCl_2$ solutions were rapidly mixed and then applied onto the bottom rheometer plate, starting the shear (5 Pa, 1 Hz) immediately thereafter; the evolution of storage and loss moduli (G′ and G″, respectively) was monitored as a function of time. The asymptotic behaviour of G′ with time was fitted with a hyperbolic function

$$G'(t) = \frac{G'_{plateau} * t}{t + \tau_{rheo}} \tag{1}$$

and with a double exponential function

$$G'(t) = G'_0 + G'_1\left(1 - \exp\left(-\frac{t}{\tau_1^{rheo}}\right)\right) + G'_2\left(1 - \exp\left(-\frac{t}{\tau_2^{rheo}}\right)\right) \tag{2}$$

where $G'_0$ is the storage modulus of the initial solutions (that can generally be neglected) and $G'^{kin}_{plateau} = G'_1 + G'_2$, while $1/\tau_1^{rheo}$ and $1/\tau_2^{rheo}$ are the characteristic rates of the two processes (supposedly branching and lateral growth).

In other oscillatory experiments samples were allowed to gel for 3 hours at 37 °C in between the parallel plates (always with a 100 μm gap and covered with dodecane to avoid water evaporation) prior to the measurements. G′ values were then recorded as a function of shear stress (stress amplitude tests: 1–1000 Pa; 1 Hz) or of oscillatory frequency (frequency sweep tests: 0.01–100 Hz; 5 Pa). The stress amplitude tests were then reported as a function of recorded strain.

Creep and recovery tests (5 Pa stress, 10 min creep time, 20 min recovery time) were carried out to measure gel compliance (J) in response to a prolonged static shear stress; the viscoelastic behaviour of the 12.5 mg/mL fibrin hydrogels allowed calculating different J values for those gels by fitting the creep curve with a 3-element standard linear solid (SLS) viscoelastic model

$$J = J_0 + (J_m - J_0)\left(1 - \exp\left(1 - \frac{t}{\tau^{creep}}\right)\right) \tag{3}$$

where $J_0$ is the purely elastic ("glassy") compliance, $J_m$ is the maximum compliance, which is inversely related to the overall shear modulus ($G = \frac{1}{J_m}$), and $\tau^{creep}$ is the relaxation time of the viscoelastic component.

### Turbidity

The precursor solutions were mixed in a 96-well plate and the time evolution of the optical density (OD) at 550 nm of the mixtures was recorded for 100 min in a Synergy 2 multi-mode BIOTek® microplate reader (NorthStar Scientific Ltd., Leeds, UK). Individual turbidity plots were fitted with a double exponential function similar to that used in the gelation kinetics

 

experiments

$$OD(t) = OD_0 + \Delta OD_1\left(1 - \exp\left(-\frac{1}{\tau_1^{turb}}\right)\right) + \Delta OD_2\left(1 - \exp\left(-\frac{t}{\tau_2^{turb}}\right)\right) \quad (4)$$

## Results and Discussion

### Elastic Character of Fibrin Gels

All fibrin gels showed a stress-hardening behaviour (Figure 1, left), which was more pronounced at higher concentrations and is generally observed in these materials.[34]

We have also investigated the gel behaviour as a function of oscillatory frequency, paying specific attention to the low frequency end of the spectrum, which is more sensitive to relaxation processes. In literature, factor XIII or factor XIII-containing medium is typically added to ensure chemical cross-linking.[35] Here, we have only used a HEPES buffer, but yet, the hydrogels showed a flat behaviour of G′ throughout a very extended frequency range (Figure 1, right), which is typical of true, cross-linked gels.[36] This point is further backed by the low values of tanδ recorded for all these materials (typically below 0.1, see Table 1). The reason for this eminently elastic behaviour in the absence of added Factor XIII is possibly the presence of this transaminase in the fibrinogen preparation, which is then activated by thrombin.

We have employed creep tests to confirm the overwhelmingly elastic performance of these gels prepared without adding exogenous Factor XIII (Figure 2). 25.0 and 50 mg/mL fibrin hydrogels showed a purely elastic response: instantaneous change in the shear compliance ($J$) at the beginning and at the end of the creep phase, negligible variation of compliance during the application of the stress ($J_m = J_0$) and recovery to zero compliance at the end of the experiment. The 12.5 mg/mL fibrin hydrogels showed a significant time-dependent increase in $J$ during creep; the overall variation of $J$ was rather small, about 10% of the "glassy" compliance $J_0$ ($(J_m - J_0)/J_0 < 0.13$) and was not associated to any irreversible deformation, probably due to the low stress used.

Due to the presence of this minor viscoelastic component, the creep behaviour of the 12.5 mg/mL samples was fitted with classical 3-element standard linear solid (SLS) viscoelastic model, which provided a value for the relaxation time ($\tau^{creep}$) of $1.6 \pm 0.2$ min.

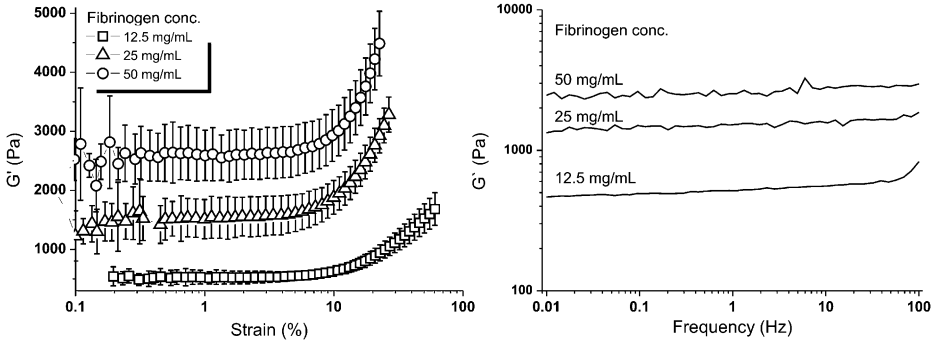


***Figure 1.***

*Left:* Stress amplitude tests (G′ vs. strain) for hydrogels prepared with different fibrinogen concentrations (frequency = 1 Hz; shear stress = 1–1000 Pa; temp. = 37 °C); please note that the experiments were run in a stress controlled mode ($n = 3$). *Right:* Frequency sweep tests (frequency = 0.01–100 Hz; shear stress = 5 Pa; temp = 37 °C) for the same hydrogels. No significant change in G′ could be recorded for any of the fibrin hydrogels as a function of frequency. All curves are averages over three samples.

 

***Table 1.***
Summary of the rheological (oscillatory gelation kinetics and frequency sweep measurements) and turbidity data ($n = 3$).

| | | | Fibrinogen concentration | | |
|---|---|---|---|---|---|
| | | | 12.5 mg/mL | 25 mg/mL | 50 mg/mL |
| Kinetics | Rheology | $\tau_1^{rheo}$ (min) [a] | $10 \pm 11$ | $20 \pm 18$ | $10 \pm 6$ |
| | | $G'_1$ (Pa) [a] | $120 \pm 100$ | $240 \pm 90$ | $1830 \pm 1310$ |
| | | $\tau_2^{rheo}$ (min) [a] | $190 \pm 260$ | $40 \pm 30$ | $180 \pm 40$ |
| | | $G'_2$ (Pa) [a] | $50 \pm 10$ | $260 \pm 80$ | $1510 \pm 400$ |
| | | *Gel point* (min) [b] | $3.0 \pm 0.5$ | $3.3 \pm 0.8$ | $6.8 \pm 2.3$ |
| | Turbidity | $\tau_1^{turb}$ (min) [c] | $1.6 \pm 0.1$ | $3.3 \pm 0.1$ | = |
| | | $\frac{\Delta(OD)_1}{OD_0}$ (%) [c] | $760 \pm 92\%$ | $156 \pm 21\%$ | = |
| | | $\tau_2^{turb}$ (min) [c] | $9.5 \pm 0.5$ | $22.0 \pm 5.2$ | $9.7 \pm 0.8$ |
| | | $\frac{\Delta(OD)_2}{OD_0}$ (%) [c] | $233 \pm 29\%$ | $48 \pm 7\%$ | $107 \pm 22\%$ |
| Gel properties | | $\tan\delta^{kin}$ [d] | $0.081 \pm 0.005$ | $0.063 \pm 0.006$ | $0.065 \pm 0.008$ |
| | | $\tan\delta^{freq}$ [d] | $0.059 \pm 0.022$ | $0.046 \pm 0.037$ | $0.119 \pm 0.137$ |
| | | $G'^{kin}_{plateau}$ (Pa) [e] | $170 \pm 110$ | $500 \pm 170$ | $3340 \pm 1710$ |
| | | $G'^{freq}_{plateau}$ (Pa) [f] | $490 \pm 90$ | $1450 \pm 280$ | $2860 \pm 680$ |
| | | $G$ (Pa) [g] | $460 \pm 120$ | $1240 \pm 90$ | $2370 \pm 290$ |

[a] From double exponential fittings of the dependency of G′ on time during gelation experiments.
[b] Calculated as the time when $G' = G''$.
[c] From double exponential fittings of the dependency of turbidity on time during gelation experiments.
[d] Calculated as $G''/G'$, where the values of G″ and G′ were averaged over the last 10 minutes of the gelation experiments ($\tan\delta^{kin}$) or over the entire frequency spectrum (0.01–100 Hz, $\tan\delta^{freq}$ [d]). The samples identified as "*kin*" were subjected to 5Pa oscillatory stress during gelation, while the "*freq*" samples were left undisturbed for 3 hours.
[e] Averaged over the last 10 minutes of the gelation (1 Hz).
[f] Averaged over the entire frequency spectrum (0.01–100 Hz).
[g] From creep experiments.

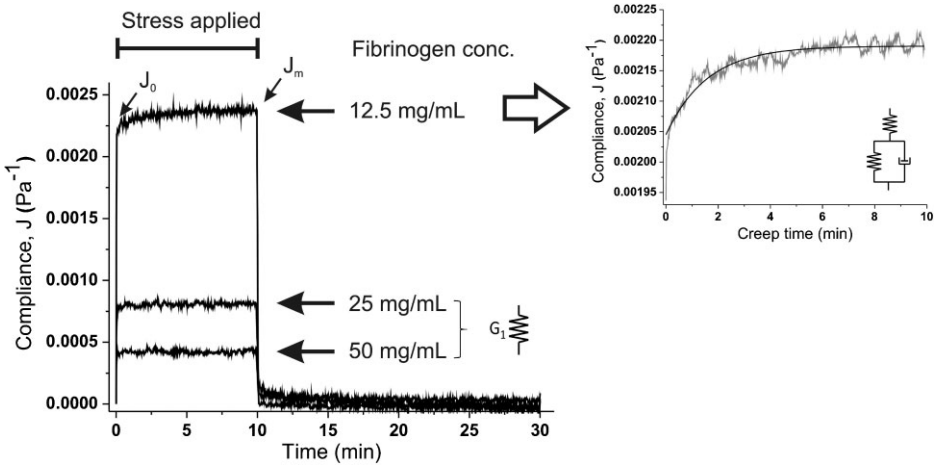


***Figure 2.***
Typical creep and recovery curves for hydrogels prepared from different fibrinogen concentrations (steady shear stress = 5 Pa; creep time = 10 min; recovery time = 20 min). $J_o$ = "glassy" or elastic compliance; $J_m$ = maximum compliance reached at the end of the creep phase (n = 3). ***Inset***: the viscoelastic behaviour of the 12.5 mg/mL sample could be easily fitted with a 3-element SLS model.

 

### Gelation Kinetics

The fibrin gelation kinetics may offer additional information about possible structural differences between gels; we have followed this process by measuring both G′ as a measure of gel rigidity, which is supposedly dominated, or at least heavily affected by, branching/cross-linking developed in the early stages of the process, and the gel turbidity, which is predominantly related to the fibre lateral size.

All samples gelled (when $G' = G''$) within a few minutes after mixing fibrinogen and thrombin (Figure 3A-C). Interestingly, the most concentrated sample gelled at a slightly slower rate than the others (Figure 3D). This is possibly due to the higher fibrinogen-to-thrombin ratio (thrombin concentration is constant), which may slow the overall efficiency of fibrinopeptide cleavage, resulting in slower self-assembly and cross-linking processes.

Both for rheology and turbidity curves (Figure 4A and 4B), it is possible to recognise an asymptotic behaviour, with plateau values reached within 2–3 hours for G′ and G″, and within 30 mins for the optical density. In addition to this different time behaviour, moduli and opacity also showed an opposite dependency on fibrinogen concentration, respectively increasing and decreasing with increasing concentration. If we consider the interpretation that branching is the major contributor to mechanical properties, and lateral size to turbidity, these data support the general model that increasing fibrinogen concentration determines an increase in branching and a reduction in lateral size. Single and double exponential and hyperbolic models

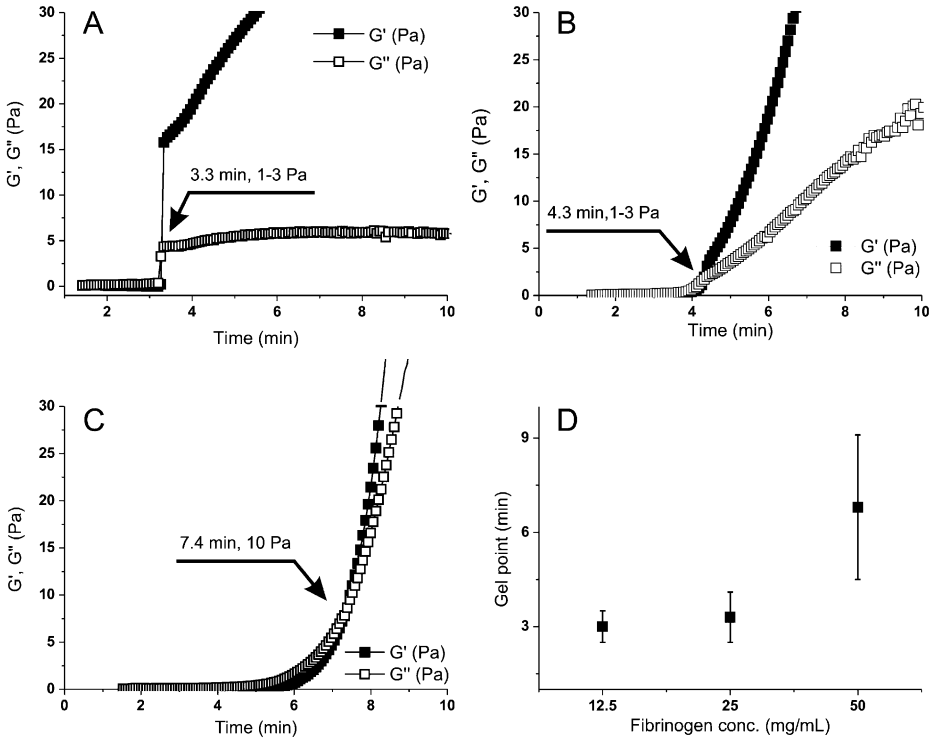


***Figure 3.***

*A to C:* Examples of the time evolution of G′ and G″ for fibrin gels at different concentrations (frequency = 1 Hz; shear stress = 5 Pa; temperature = 37 °C. *D:* The gel points (time points where $G' = G''$) of the 12.5 and 25 mg/mL samples are virtually indistinguishable, while at the highest fibrinogen concentration they occur at later times (and higher G′/G″ values). (n = 3).

 

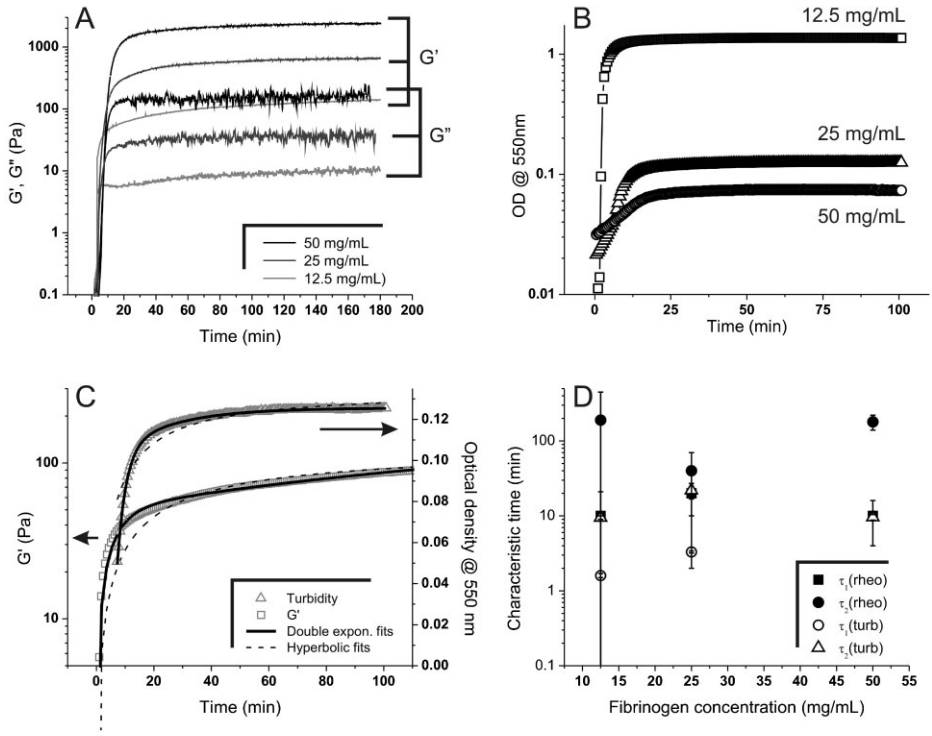


***Figure 4.***

*A*. Evolution of storage and loss moduli as a function of time during the gelation of fibrin at three different fibrinogen concentrations (frequency = 1 Hz; shear stress = 5 Pa; temperature = 37 °C). *B*. Evolution of turbidity (optical density at 550 nm) for the same systems (average over three samples). *C*. Comparison of double exponential and hyperbolic fittings for the time dependency of G′ and turbidity of 25 mg/mL fibrinogen gels: double exponentials always provided better results than hyperbolic models. *D*. Comparison of characteristic times for the different processes identified via turbidity and rheology.

were used to fit the kinetic data: hyperbolic models may take into account any enzyme-related saturatable behaviour, while single or double exponentials may allow to emphasise the presence or absence of different phases in the gelation process. Double exponentials always provided a better adherence to the experimental results (Figure 4C), except for the turbidity of the 50 mg/mL samples (single exponential fits best), suggesting a biphasic behaviour both in rheology and turbidity.

As mentioned in the introduction, a biphasic G′ behaviour has already been ascribed to fast fibre branching/cross-linking (characteristic time: 10 min or less) followed by a slower fibre aggregation (characteristic time: tens of minutes).[30] This is confirmed by our data and in Table 1 (first four rows); these two processes are respectively identified as 1 and 2. Both processes seem to contribute in a similar fashion to the plateau G′ value, but process 1 (branching/cross-linking) essentially coincides with the macroscopic gelation (see the similarity between $\tau_1^{rheo}$ and gel point, while $\tau_2^{rheo}$ is significantly longer).

Process 2 in the G′ time evolution may be related to (irregular) lateral association of cross-linked fibres, leading to an increase in the number of elastically active cross-linked centres.

Turbidity data (Table 1, rows 6–10; see also Figure 4D, compare white triangles with black squares) offer a clear indication of a process occurring in the same time scale as the branching/cross-linking, with a characteristic time ($\tau_2^{turb}$) again in the region of

 

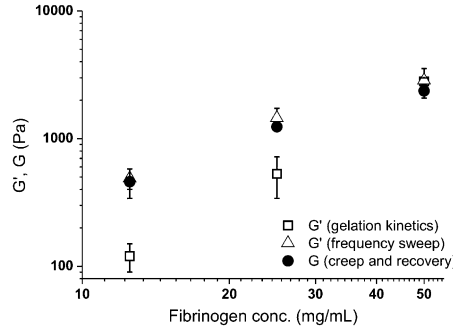


***Figure 5.***
Comparison of G′ (oscillatory measurements) or G (creep measurements) for gels obtained from different fibrinogen concentrations. At lower concentrations, the samples used to follow the gelation kinetics showed significantly lower modulus values than those left undisturbed during gelation.

10 min. Therefore, it is reasonable to identify process 2 in turbidity and process 1 in rheology with early branching/cross-linking leading to gelation.

However, it is also possible to identify an earlier phenomenon (process 1 in turbidity): the associated optical density decreases sharply with increasing fibrinogen concentration, and becomes negligible at 50 mg/mL. A similar trend has already been widely recorded[7] and has been associated to the formation of thinner fibres (fine gels in the Ferry and Morrison terminology[19]) at higher fibrinogen concentration and thicker, more laterally associated (coarse, less transparent gels) at lower ones, as also convincingly explained by Kaibara.[30] Process 1 in turbidity data could, therefore, be interpreted as early lateral fibre aggregation.

It can be concluded that the two analytical techniques offer a rather complementary overview of the kinetics of fibrin production: both allow to follow "gelation", i.e. the phenomenon of fibre branching and cross-linking, but while turbidity also monitors the early aggregation of fibres in a colloidal state (process 1 in turbidity), G′ permits to follow the process of lateral association of cross-linked fibres.

### Dependency of Fibrin Mechanical Properties on Gelling Conditions

The values of shear modulus (G′ in oscillatory measurements, G in creep experiments) of the final gels recorded for samples that were not subjected to any shear stress during gelation are substantially identical (Figure 5). On the contrary, whenever the hardening kinetics was followed rheologically and the samples were subjected to a continuous, oscillating shear stress throughout the process of gelation, a significantly lower G′ could be recorded for lower fibrinogen concentrations. Thus, it appears that the fibre cross-linking was remarkably less effective in these samples, possibly because of their lower viscosity (easier re-orientation), although the different lateral size of the fibres may have an effect too. This different behaviour may also affect the values of tanδ: although not statistically significant, tanδ for 12.5 and 25 mg/mL gels is higher for samples left undisturbed for 3 hours.

## Conclusion

This study has showed that

a) strain-hardening, cross-linked fibrin gels can be obtained even in the absence of exogenous Factor XIII, probably due to the presence of the enzyme in fibrinogen preparations;
b) fibrin gelation can be followed both via oscillatory rheology and via turbidity. Both appear to be sensitive to the fibre branching process leading to macroscopic gelation; turbidity also

 

allows to follow the fibre lateral growth, which is specifically important at low fibrinogen concentrations, while rheology also measures a post-curing phase, which is possibly related to the association of cross-linked fibres;

c) the rheological data from cross-linking kinetics should not be taken blindly, since they may significantly underestimate the (storage) modulus values.

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