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Paper:

Curtis, D., Holder, A., Badiei, N., Claypole, J., Walters, M., Thomas, B., Barrow, M., Deganello, D., Brown, M., Williams, P. & Hawkins, K. (2015). Validation of Optimal Fourier Rheometry for rapidly gelling materials and its application in the study of Collagen gelation. *Journal of non-Newtonian Fluid Mechanics*

http://dx.doi.org/doi:10.1016/j.jnnfm.2015.01.003

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Journal of Non-Newtonian Fluid Mechanics 222 (2015) 253–259

Contents lists available at ScienceDirect

Journal of Non-Newtonian Fluid Mechanics

journal homepage: http://www.elsevier.com/locate/jnnfm

Validation of Optimal Fourier Rheometry for rapidly gelling materials and its application in the study of collagen gelation ☆

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ARTICLE INFO

Article history:
Received 5 September 2014
Received in revised form 12 January 2015
Accepted 13 January 2015
Available online 24 January 2015

Keywords:
Gel Point
Mutation number
OFR
FTMS
Collagen

ABSTRACT

Rheological Gel Point measurements may incur errors in the case of rapid gelling systems due to the limitations of multiple frequency oscillatory shear techniques such as frequency sweeps and Fourier Transform Mechanical Spectroscopy, FTMS. These limitations are associated with sample mutation and data interpolation. In the present paper we consider how an alternative rapid characterisation technique known as Optimal Fourier Rheometry, OFR, can be used to study a rapidly gelling material, namely collagen at near physiological temperatures. The OFR technique is validated using a model reference gelling system whose GP characteristics have been widely reported. An analysis of the susceptibility of OFR measurements to rheometrical artefacts is made prior to its use in the study of rapid gelling collagen gels formed over a range of physiologically relevant collagen concentrations. The results of this OFR study are the first measurements of the stress relaxation characteristics of collagen gels performed in a single rheological experiment.

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1. Introduction

The gelation characteristics/properties of biopolymer systems are of significant scientific interest and are a fundamentally important aspect of many biological processes. Salient examples include the formation of collagen gels, the microstructure of which depends on polymerisation conditions [1,2] and which are widely used as scaffolds in tissue engineering and 3D cell culture applications [3,4]. Other examples include the formation of fibrin gels which form the principal microstructural component of blood clots and provide the requisite mechanical properties for haemostatic functionality [5,6]. Such systems undergo a sol–gel transition which can be identified by rheological Gel Point, GP, measurements [7–11]. In many biopolymer systems such as fibrin–thrombin gels (or clots formed in whole blood) this can occur rapidly due, for example, to underlying prothrombotic conditions. The inherent rapidity of collagen gel formation at physiologically relevant conditions has restricted previous rheological studies of the GP in collagen based systems to sub physiological temperatures [12] or have necessitated the use of pepsin-solubilised collagen [13]. The ability to accurately measure the viscoelastic properties at the GP has particular relevance in characterising the fractal microstructure of biological systems such as blood clots [7,8,11,14].

A convenient and widely reported technique for detection of the GP involves measurements of the complex shear modulus, G^*, over a range of frequencies, ω, in oscillatory shear. At the GP the elastic and viscous components of the complex modulus, G' and G'', respectively scale in oscillatory frequency, ω, as $G'(\omega) \sim G''(\omega) \sim \omega^{\alpha}$ where α is termed the stress relaxation exponent [15]. Thus, the GP may be identified as the instant where the G' and G'' scale in frequency according to identical power laws [15], behaviour corresponding to attainment of a frequency *independent* phase angle, $\delta(= \text{atan}(G''/G'))$. GP measurements may involve 'frequency sweeps' with repeated consecutive application of a set of small amplitude oscillatory shear, SAOS, waveforms [15,16], or by Fourier Transform Mechanical Spectroscopy, FTMS, in which $G^*(\omega)$ is found by simultaneous application of several harmonic frequencies in a composite waveform and its subsequent Fourier analysis [17,18]. Frequency sweeps are limited to relatively slow gelation processes due to sample mutation and interpolation errors [9,19,20]. FTMS may overcome these limitations, but is unsuitable for markedly strain sensitive materials, such as fibrin gels, due to the strain amplitude of the composite waveform exceeding the linear viscoelastic range (LVR) [9].

☆ Dedicated to Prof Ken Walters FRS on the occasion of his 80th Birthday.

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http://dx.doi.org/10.1016/j.jnnfm.2015.01.003
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Due to the rapidity of gelation, GP measurements on acid-solubilised rat tail (type I) collagen are not viable at physiological (37 °C) temperatures and remain challenging at near-physiological temperatures. Yet the potential use of such biopolymer materials for *in vivo* tissue engineering applications for example [3], prompts the need to better understand their rheological properties. In the present paper we consider how recent developments in a rapid oscillatory shear characterisation technique, known as Optimal Fourier Rheometry, OFR [21] can be successfully applied to the study of such systems at near physiological temperatures.

2. Theoretical

2.1. Fourier Transform Mechanical Spectroscopy (FTMS)

In FTMS the input waveform, i.e. that applied to the test material, is generated by combining a sinusoidal waveform with several of its harmonics. The dynamic viscoelastic parameters at each of the discrete component frequencies are obtained by comparing the Fourier Transform, FT, of the input and response waveforms [17,18]. Whilst FTMS significantly reduces the time required to obtain G' and G'' over a range of frequencies, the amplitude of the applied waveform increases as more harmonics are included and may exceed the LVR for strain sensitive biopolymer systems even where a modest number of harmonics are employed [9]. Reducing the amplitude of the harmonics in an attempt to maintain linearity generally leads to a loss of resolution in the pre-GP data. This can cause inaccurate GP identification in a sample of low initial viscosity.

2.2. Optimal Fourier Rheometry (OFR)

Optimal Fourier Rheometry (OFR) is a 'multi-frequency' technique involving frequency modulated (chirp) waveforms of the following form [21].

$$\gamma(t) = \gamma_0 \sin(2\pi K(e^{t/L} - 1)) \quad (1)$$

where

$$K = \frac{Tf_1}{\ln(f_2/f_1)} \quad (2)$$

and

$$L = \frac{T}{\ln(f_2/f_1)} \quad (3)$$

where γ_0 denotes the strain amplitude, T denotes the waveform duration (herein set to $1/f_1$) and f_1 and f_2 denote the initial (lowest)

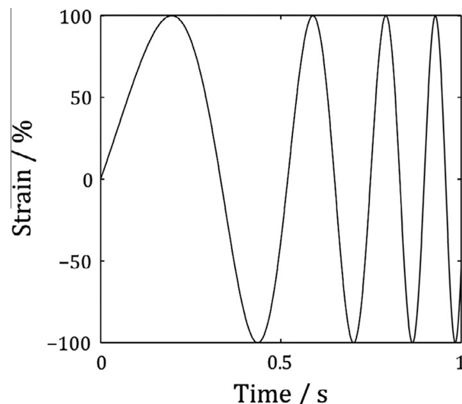

Fig. 1. OFR waveform characterised by the parameters $f_1 = 1$ Hz, $f_2 = 10$ Hz and T = 1 s with a peak strain amplitude, γ_0, of 100%.

and final (highest) frequency (Hz) of the waveform's frequency range, respectively. A typical OFR waveform is shown in Fig. 1. The dynamic rheological parameters $G'(f)$ and $G''(f)$ are calculated by comparing the FT of the strain input and stress response signals (see Section 3.3.2).

In contrast to FTMS, in which the perturbation signal consists of discrete frequencies, the OFR waveform undergoes a continuous frequency modulation between two predefined limits. Hence, Fourier analysis of OFR waveforms identifies a number of frequency components limited only by the sampling rate of the original perturbation and response waveforms. As a result, OFR offers two significant advantages over FTMS, namely (i) the ability to obtain very high densities of data ($G'(f)$ and $G''(f)$) over a finite frequency window, and (ii) the strain amplitude is independent of the number of component frequencies sampled.

As the OFR technique has been relatively little reported, preliminary experiments were conducted to assess its validity and examine any potential restrictions on its application to biopolymer systems undergoing gelation. The test systems chosen for this aspect of the work were gels formed from aqueous solutions of gelatin. The GP characteristics of these systems have been widely reported and they have been used as model reference systems in previous rheometric studies invoking GP measurements [18].

3. Methods

3.1. Sample preparation

3.1.1. Gelatin gels

The required mass of general purpose gelatin powder (Fisher G015053) was added to deionised (type I) water (dH_2O) (heated to 60 °C) and agitated vigorously for 5 min to give a final gelatin concentration of 30 wt% (this high concentration allowing sufficient stress resolution in the pre-GP state). The gelatin solution was maintained at 60 °C for 45 min with further agitation every 10 min to ensure complete dissolution of the gelatin powder. The solution was then aliquoted and refrigerated until use. Aliquots were melted in a 60 °C water bath for 45 min before being immediately transferred to the temperature controlled stage of the rheometer which was maintained at 60 °C, see below.

3.1.2. Collagen gels

High concentration type I rat tail collagen (RTC) (10 mg/ml, BD Bioscience), dH_2O and 1M NaOH (Fluka) was placed on ice. The required amounts of dH_2O, 10x Phosphate Buffered Saline (Fluka) and RTC were then mixed well using a pipette tip before NaOH was added to initiate gelation (as per the manufacturer's instructions). The collagen gelation process is temperature dependent [1] with the rate of gelation being significantly reduced at low temperatures, hence the rheometer's Peltier plate temperature (see below) was lowered to 5 °C immediately prior to sample loading.

3.2. Rheometry

All experiments were performed using a TA-Instruments ARES-G2 controlled strain type rheometer fitted with a 50 mm Titanium parallel plate geometry. Temperature control was achieved through the use of a Peltier plate system, the gap zero setting being performed at the test temperature. A shearing gap, h, of 200 μm was employed throughout to minimise sample inertia effects. The free surface of the sample was coated with a thin layer of silicone oil (10 mPa s) to prevent evaporation. A preliminary study employing a range of gaps confirmed the absence of wall slip for gelatin samples while for collagen (in which sample inertia constraints require the use of small gaps) insignificant 3rd and 5th

Table 1
Details of FTMS waveforms for the study of 30 wt% gelatin.

LF-FTMS (Peak strain: 21%)		HF-FTMS (Peak strain: 24%)	
f/Hz	γ_o/ %	f/Hz	γ_o/ %
0.2	10	1	10
0.8	5	4	5
1.6	5	7	5
3.2	5	10	5

harmonic contributions in the response of the material to single frequency strain waves confirmed the absence of slip [22,23].

3.2.1. Fourier Transform Mechanical Spectroscopy, FTMS

The arbitrary waveform generation capability of the ARES-G2 was employed to acquire FTMS data (i.e. unprocessed stress and strain signals) which were processed using an appropriate Matlab [24] routine (see Section 3.3.1). The rheometers arbitrary wave utility allowed successive data points to be acquired more rapidly than in standard FTMS tests (which involve a delay between successive measurements). Two FTMS waveforms were used in this study, namely (i) 'high frequency' (HF-FTMS) and (ii) 'low frequency' (LF-FTMS) waveforms, respectively. The details of each waveform are given in Table 1. The peak strain of each waveform was confirmed as being within the LVR by performing separate strain amplitude sweeps in the pre- and post-GP regimes. The data sampling rate employed was 1000 Hz throughout.

3.2.2. Optimal Fourier Rheometry, OFR

OFR was implemented using the arbitrary wave function of the TA Instruments ARES-G2 rheometer. Two OFR waveforms were used, these being termed 'low frequency' and 'high frequency' (LF-OFR and HF-OFR), respectively. The details of each waveform are presented in Table 2. A conditioning time, t_c, was added between waveforms in which the strain amplitude was set to 0%, the inclusion of the conditioning time allowing adequate stress relaxation between successive waveforms and ensuring that the waveform was suitably periodic for subsequent FFT analysis. All data was obtained using a sampling rate of 1000 Hz and was analysed using an appropriate Matlab [24] routine (see Section 3.3.2).

3.3. Data analysis

3.3.1. FTMS data analysis

Arbitrary wave FTMS data in the form of unprocessed stress and strain waveforms were processed by applying a fast Fourier transform (FFT) to consecutively recorded periods of stress and strain data with length $1/f_1$ seconds, these being zero padded to contain 2^n data points (where n refers to the smallest integer greater than the original signal length). A Bartlett type windowing function was employed to minimise discontinuity errors. The FTMS test frequencies were then identified by FFT analysis and the values of $G'(f)$ and $G''(f)$ were calculated as follows:

$$G'(f) = \mathrm{Re}\left[\frac{\mathrm{FFT}(\sigma(t))}{\mathrm{FFT}(\gamma(t))}\right] \tag{4}$$

Table 2
Details of OFR waveforms.

	LF-OFR	HF-OFR
f_1/Hz	0.2	1.0
f_2/Hz	3.2	10.0
T/s	5.0	1.0
γ_o	10.0	10.0
t_c/s	1.0	1.0

and

$$G''(f) = \mathrm{Im}\left[\frac{\mathrm{FFT}(\sigma(t))}{\mathrm{FFT}(\gamma(t))}\right] \tag{5}$$

where Re and Im denote the real and imaginary parts of the complex FFT outputs, respectively [21]. All FTMS analysis was performed using an appropriate Matlab [24] routine with the resulting values of $G'(f)$ and $G''(f)$ being recorded and passed to the GP identification routine described in Section 3.3.3. For the samples studied herein an initial assessment of sample inertia based on the relation $\rho \ll G'/h^2f^2$, where ρ denotes the sample density [25] indicated that sample inertia corrections were not required.

3.3.2. OFR analysis

OFR waveforms were analysed by applying an FFT to the stress and strain waveforms (each of which was zero padded to contain 2^n data points) with $G'(f)$ and $G''(f)$ being calculated from Eqs. (4) and (5), respectively. Windowing functions were not employed in the OFR analysis as preliminary experiments established that the correct value of α was not recovered when any of the common windowing functions used in digital signal processing (Hamming, Hanning and Bartlett) were used to analyse gelatin data – even in the absence of sample mutation. This was deemed to be a consequence of disproportionate amplitude attenuation of the waveform frequency components due to their temporal variation, i.e. the OFR frequency cycles continuously between the limits of f_1 and f_2 during the data sampling period. The inclusion of a predetermined conditioning time ensured that the OFR waveform was periodic and hence no discontinuity errors (which would usually require the use of a windowing function) were present. As in the FTMS analysis described above, the values of $G'(f)$ and $G''(f)$ were recorded and passed to the GP identification routine described in Section 3.3.3. OFR analysis was performed using an appropriate Matlab [24] routine and no inertia correction was deemed necessary.

3.3.3. Gel point identification

The FTMS or OFR measurements of $G'(f,t)$ and $G''(f,t)$ were linearised using the relationships $\ln G' = \alpha' \ln f + k'$ and $\ln G'' = \alpha'' \ln f + k''$. The scaling exponents, α' and α'' were subsequently determined by linear regression with their corresponding residuals being summed to provide a single parameter, SSE, which characterises the quality of the power-law fit. The GP was determined by identifying the intersection of cubic fits to $\alpha'(t)$ and $\alpha''(t)$ such that $\alpha' = \alpha'' = \alpha$ (see Fig. 2), i.e. the point at which $G'(f)$ and $G''(f)$ scale with identical stress relaxation exponents (see Fig. 4B and E). For all GP data reported herein the parameter SSE was less than 10^{-2}, indicating the expected power-law scaling of $G'(f)$ and $G''(f)$ at the GP. A measure of the uncertainty of the value of α (i.e. $\Delta\alpha$) was obtained using

$$\Delta\alpha = \alpha\sqrt{\left(\frac{\Delta\alpha'}{\alpha'}\right)^2 + \left(\frac{\Delta\alpha''}{\alpha''}\right)^2} \tag{6}$$

where $\Delta\alpha'$ and $\Delta\alpha''$ denote to the standard error of the linear regressions of $\ln G'(\ln f)$ and $\ln G''(\ln f)$ at the GP, respectively.

4. Results

4.1. Mutation artefacts in OFR measurements

A fundamental assumption underlying oscillatory shear experiments is that the rheological properties of the sample remain 'stable' (time-invariant) throughout the period t_{exp} of deformation. A criterion for this time-invariance has been established in terms

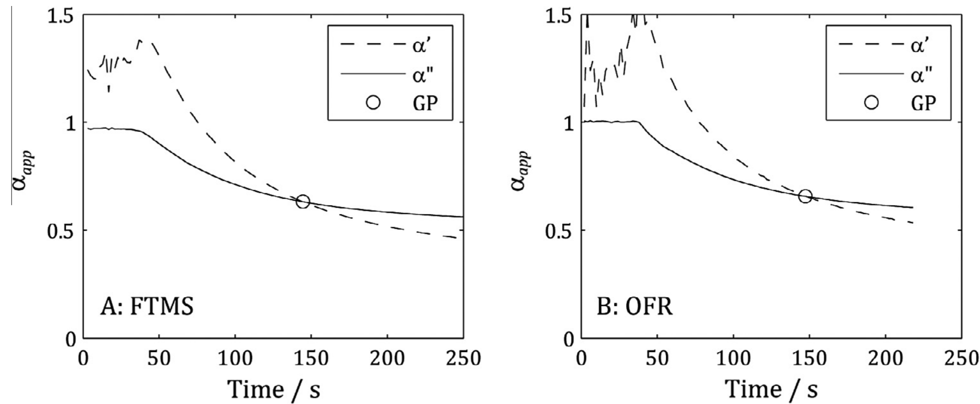

Fig. 2. Gel Point identification for gelatin using data obtained by HF-FTMS (A) and HF-OFR (B).

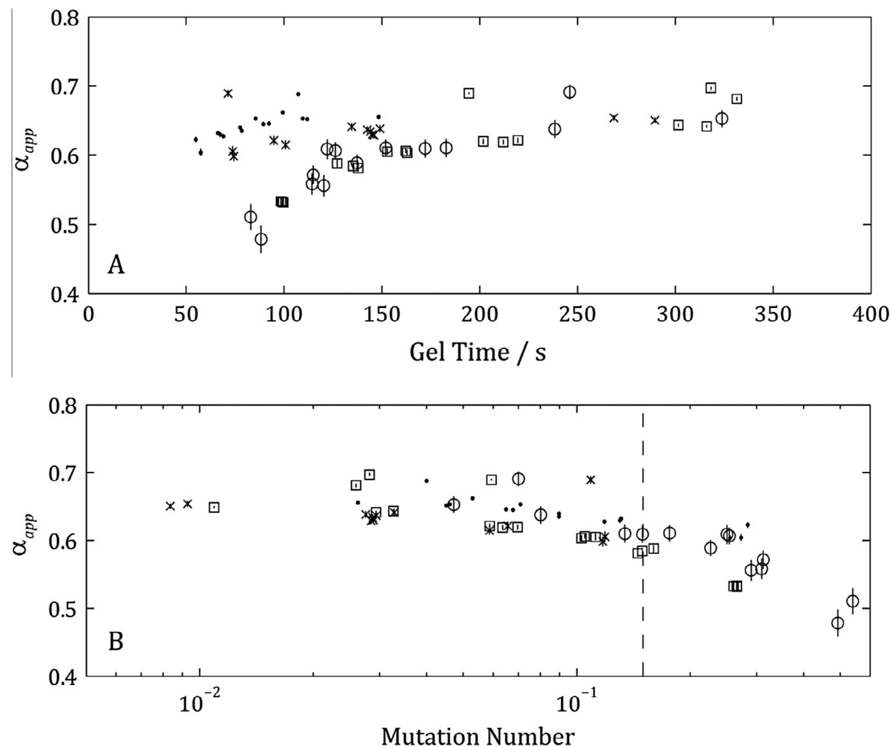

Fig. 3. Comparison of gelatin GP data obtained by (1) HF-FTMS [x] (2) LF-FTMS [o] (3) LF-OFR [□] and (4) HF-OFR (·). The dashed line in B shows the critical mutation criterion of $N_{mu} = (T/G')(dG'/dt) = 0.15$ as reported by Winter et al. [16, ER1]. Vertical lines overlayed on each data point indicate the uncertainty (as defined through Eq. (6)) associated with each measurement.

of a mutation number, N_{mu}, which quantifies the change of a dynamic rheological property during an experiment. In a study of the gelation of model polyurethanes, Winter et al [19,20] identified $N_{mu} < 0.15$ as a cut-off point below which the sample could be assumed to be 'quasi-stable'. Fig. 3 shows the apparent value of the stress relaxation exponent, α_{app} for gelatin systems undergoing increasingly rapid gelation as a function of (a) gel time and (b) mutation number, N_{mu}. Rapid gelation appears to be associated with reduced values of α_{app} for LF-FTMS and LF-OFR, however, the high frequency variants of these tests show no such decrease in the value of α_{app}. Fig. 3b confirms that this apparent decrease in α_{app} is a consequence of mutation artefacts apparent in LF-FTMS and LF-OFR procedures. At such (relatively) low frequencies the material may not remain time-invariant throughout the (relatively) long period of deformation. Hence, OFR is subject to the same mutation artefacts as FTMS. Further, the data presented in Fig. 3 shows that OFR and FTMS provide the same estimate of α under valid rheometrical conditions.

4.2. Comparison of FTMS and OFR techniques

Fig. 4 shows $G'(f)$ and $G''(f)$ for pre-GP (i.e. $t = 0.5 \times t_g$), GP (i.e. $t = t_g$) and post-GP ($t = 2 \times t_g$) gelatin gels as measured by HF-FTMS and HF-OFR with excellent agreement between the two techniques being observed and confirming the validity of OFR for gelatin systems throughout gelation. One significant

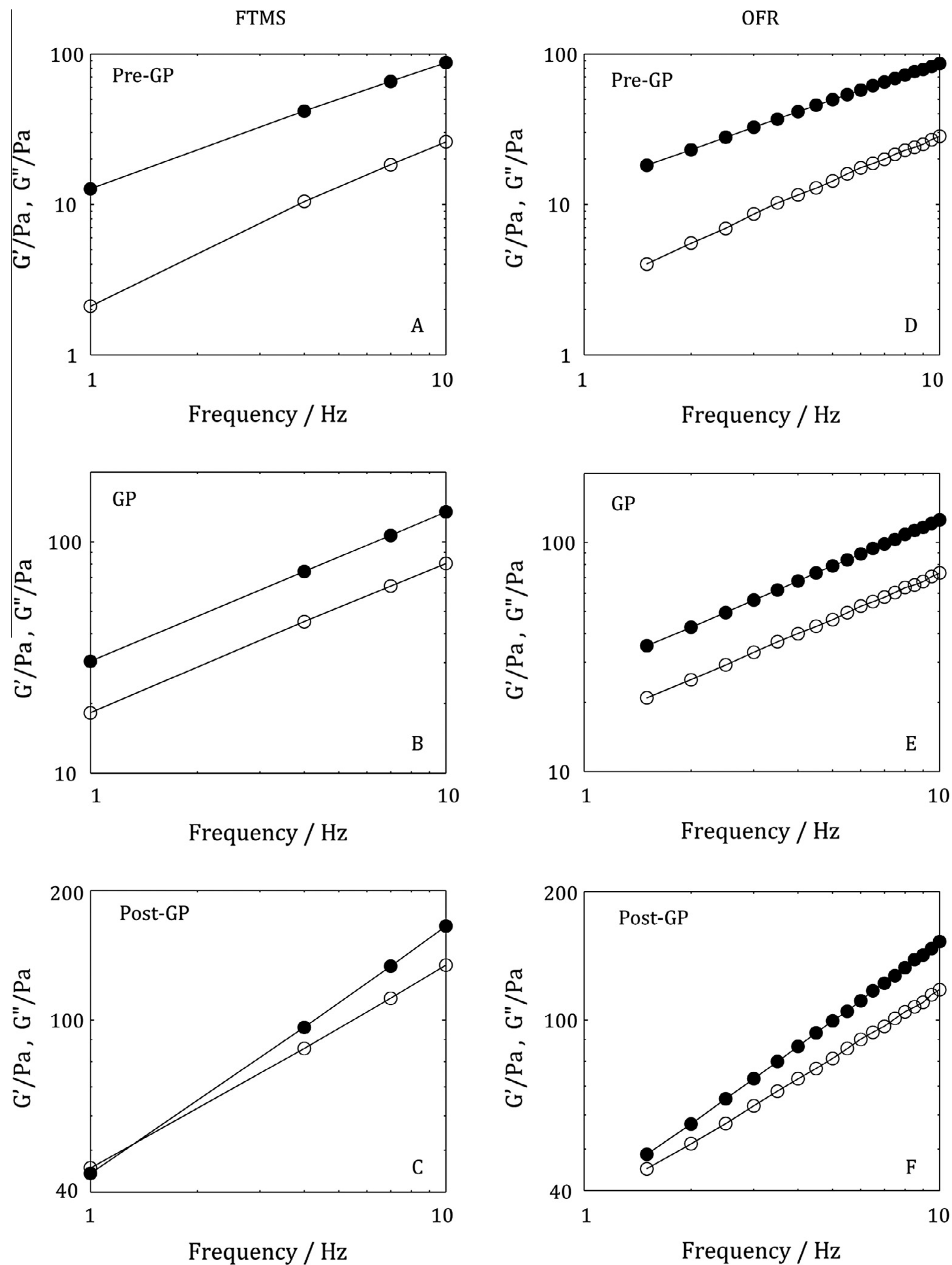

Fig. 4. Comparison of pre-GP, GP and post-GP rheological data for 30 wt% gelatin obtained using FTMS and OFR. Closed and open symbols refer to G' and G'', respectively.

argument in favour of OFR over FTMS concerns the increased number of frequency data points that can be obtained (see Fig. 4). In the context of GP measurement this allows the GP to be determined with a greater degree of precision. Fig. 5 shows the uncertainty (as calculated using Eq. (6)) as a function of sample mutation number for 30 wt% gelatin and shows that, in all cases, OFR outperforms FTMS in terms of the uncertainty in the value of α.

4.3. Application of OFR to collagen gels

The results for 30 wt% gelatin presented in Sections 4.1 and 4.2 validate the use of OFR for the study of rapidly gelling materials. The next system to be examined was acid solubilised type I collagen at temperatures approaching physiological temperatures. Fig. 6 shows the variation of α as a function of collagen concentration, increasing values of concentration resulting in lower values of α. Fig. 6 shows that the stress relaxation characteristics (α) of collagen gels are dependent on collagen concentration and significantly are not limited to values of $\alpha = 0.5$ or 0.7 as suggested in the literature [12,13]. These results also offer an explanation for some of the variation found in the literature regarding the value of α. Fig. 7 shows a plot of δ as a function of time for a sample prepared at 8 mg/ml collagen at 28 °C obtained using the HF-OFR technique. The results for 18 oscillatory frequencies in the range 1.5 Hz to 10 Hz show the transition between a viscoelastic liquid like pre-GP response (decreasing δ with increasing frequency) and viscoelastic solid like post-GP response (increasing δ with increasing frequency) at the GP.

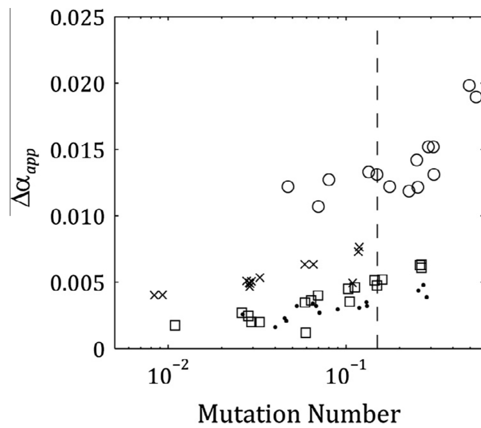

Fig. 5. Uncertainty in α^a as a function of mutation number for gelatin samples (1) HF-FTMS [x] (2) LF-FTMS [o] (3) LF-FR [□] and (4) HF-OFR (·).

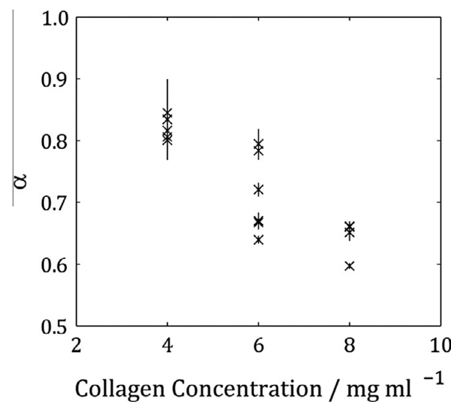

Fig. 6. Variation of stress relaxation characteristics of collagen gels over a range of concentration, measurements were made using the HF-OFR routine at near physiological temperature (28 °C). Vertical lines superposed on each data point indicate uncertainty in the measurement as defined by Eq. (6).

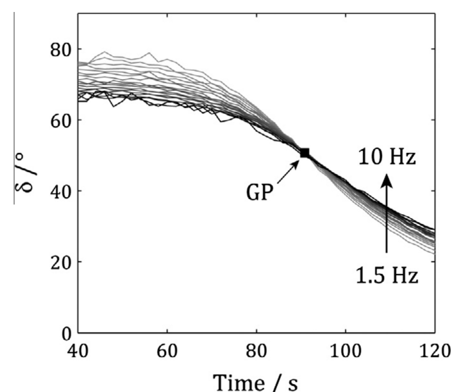

Fig. 7. Collagen GP data in terms of frequency independence of $\delta(t)$ for an 8 mg/ml collagen gel. Data was obtained using HF-OFR at 28 °C, results shown for 18 oscillatory test frequencies. The GP is identified with '■' and was determined as described in Section 3.3.3.

5. Discussion

The present study investigated the validity of OFR based GP measurements in rapidly forming gelling systems. GP measurements on gelatin (see Fig. 3) show that OFR suffers from the same mutation artefacts as FTMS tests, with values of α_{app} deviating from an average value of 0.68 at $N_{mu} > 0.15$. This critical value of N_{mu} (= 0.15) is in agreement with that found by Winter et al. [19,20]. Shifting the frequency content to higher values allows accurate measurements of α at relatively low gel times (ca 60 s) in the case of both OFR and FTMS. The advantage of the OFR technique in GP measurements lies in its ability to produce data at a much greater number of frequencies compared to FTMS (see Fig. 4). Thus the level of uncertainty in the deduced values of α is reduced (Fig. 5).

Previous rheological studies of collagen gelation have utilised a different method of GP detection which involved overlaying time courses of tan δ obtained at various frequencies on several samples in separate consecutive tests [12,13]. Collagen microstructure and gelation kinetics are extremely sensitive to polymerisation conditions such as collagen concentration, sample pH and temperature [1,2]. Consequently, no frequency independent point was observed in these previous studies and the authors estimated the GP to be at the intersection of tan $\delta(t)$ at the highest and lowest frequency employed in the experiments [12,13]. Further, no assessment of mutation artefacts has previously been performed in studies of collagen gelation [12,13]. The study reported herein measured the GP in collagen gels in a *single* experiment and as such no variation between the frequency responses as a consequence of sample variation could occur. Further, an assessment of OFR showed that the technique employed in the study of collagen gelation herein (HF-OFR) was not subject to mutation artefacts.

Contrary to previous reports in which no systematic variation in α was observed upon variation of the collagen concentration [13], OFR measurements of the GP at different concentrations of collagen show that α is dependent on the collagen concentration with α decreasing with increasing concentration (see Fig. 6). This is an important finding insofar that the gel network at the GP provides a template for further microstructural growth [26] and suggests that the measurement of α at the GP is potentially important in the field of tissue engineering, for control and early evaluation of microstructure. The study presented herein also demonstrates the utility of OFR in the study of rapidly gelling systems such as those associated with collagen gelation at near-physiological temperatures. However, in order to measure the GP in even more rapidly forming gels, such as acid-solubilised collagen at physiological temperature, a further shift to higher frequencies is likely to be necessary.

Acknowledgements

This work was supported by EPSRC (UK) Grants EP/C513037/1, EP/G061882 and EP/I019405/1.

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