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Research paper

Hyper-frequency viscoelastic spectroscopy of biomaterials

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ABSTRACT

With the emergence of new biomaterials and elastography imaging techniques, there is a need for innovative instruments dedicated to viscoelasticity measurements. In this work, we introduce a novel hyper-frequency viscoelastic spectroscopy (HFVS) technique dedicated to characterize soft media subjected to mid-to-very-high frequency stress ranges (or, equivalently, to probe short-to-very-short relaxation times). HFVS, which has been implemented in an analytical instrument performing non-contact measurements in less than 1 s between 10 and 1000 Hz, is a suitable tool to study viscoelasticity for bio-applications. In this context, HFVS has been compared to classical oscillatory rheometry on several classes of soft materials currently encountered in tissue repair, bioengineering and elastography imaging on a frequency range between 10 and 100 Hz. After having demonstrated the good correspondence between HFVS and rheometry, this study has been completed by exploring the sensitivity of HFVS to physicochemically induced variations of viscoelasticity. HFVS opens promising perspectives in the challenging field of biomaterial science and for viscoelasticity-based quality control of materials.

1. Introduction

Emerging research fields such as tissue replacement and engineering, injected biomaterials in tissue regeneration context, cellular mechanical transduction and elastography imaging require a good understanding and mastering of soft material mechanical properties. In most cases, viscoelastic properties play a major role in the relevance of elastography imaging for diagnostic purposes, as well as in the safety and the efficiency of biomaterial-based therapies and repair techniques.

Existing technologies for rheological characterization of soft solid materials (Ferry, 1980), like rotational rheometers and dynamic mechanical analysis (DMA), proved their

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efficiency. However, these technologies present several limitations in the context of biomaterial characterization and biomedical applications. Indeed, since measurements require mechanical contact with the sample, the classical tests can be destructive, which could lead to loss of costly and rare material samples. In addition, long measurement times to explore viscoelasticity over wide frequency ranges make spectroscopic observations impossible for rapid chemically or physiologically induced effects, like material polymerization or blood coagulation (Schmitt et al., 2011). Finally, the fact that only low frequencies (typically below 300 Hz) can be assessed by classical techniques limits a great extent to explore high-frequency behavior of biomaterials and living tissues.

In the context of biomechanics of soft tissues, a practical case is given by dynamic elastography imaging. Indeed, with the recent emergence of dynamic elastography based on ultrasound and magnetic resonance imaging (MRI), reliable tools to dynamically characterize soft tissues and materials over wide frequency ranges are needed to provide gold standard measurements. Dynamic elastography (Greenleaf et al., 2003; Mariappan et al., 2010) is a medical imaging modality which aims, by means of relatively high-frequency propagating shear waves, to image *in vivo* viscoelasticity of soft tissues and organs for diagnosis purpose. In this context, viscoelastic characterization is fundamental for two reasons. First, it is relevant for laboratory developments and investigations where a mechanical characterization over a broad range of frequencies allows calibrating and validating new elastography imaging models and techniques (e.g., see Hadj Henni et al., 2008; Vappou et al., 2009). To circumvent the frequency limits of classical rheometers and dynamic mechanical analyzers, and to provide high-frequency viscoelastic measurements, some approaches were developed. In the context of dynamic elastography, some authors proposed to use the empirical principle of time–temperature superposition to artificially increase the frequency range (Doyley et al., 2010) but this technique does not provide direct measurements. Others developed in-house measurement techniques based on the study of plane shear wave propagation to assess viscoelastic properties of soft materials (Catheline et al., 2004; Oudry et al., 2009a,b; Hadj Henni et al., 2010; Schmitt et al., 2011). In addition to the need of large material samples, the elastography method has the disadvantage to impose the use of an imaging modality (ultrasound or MRI) to scan the material. One can thus see the advantage of a dedicated viscoelastic spectroscopy instrument performing direct measurements and handling of small samples. The second major motivation for assessing high-frequency viscoelastic properties of biomaterials is related to its relevance for diagnosis and therapy monitoring. Indeed, most biological tissues like breast (Tanter et al., 2008), liver (Klatt et al., 2007; Kiss et al., 2009) or brain (Sack et al., 2008) exhibit complex frequency-dependent viscoelastic behaviors, which makes comparison between studies difficult. Such a fact could strongly complicate future standardization efforts in the field of elastography imaging. Consequently, to build a reliable database on viscoelastic properties of healthy and pathological tissues, and to construct optimal clinical diagnosis strategies based on such mechanical information, a dedicated reference instrument covering a broad range of frequencies currently unattainable would be highly beneficial.

Another rational supporting this study is given by the vocal folds, which are a typical example of soft tissues vibrating *in vivo* at frequencies around 100 Hz for male and 1600 Hz for female soprano voices (Švec et al., 2008). The rheology of these tissues, which is well understood at low frequencies but remains unexplored in higher ranges (Chan and Rodriguez, 2008; Goodyer et al., 2009), is a fundamental aspect to consider for developing efficient and functional repair strategies of vocal folds or their replacement by biomaterials (Titze et al., 2004; Duflo et al., 2006; Thibeault et al., 2011).

In the present study, hyper-frequency viscoelastic spectroscopy (HFVS) implemented in a prototype instrument (manufactured by RheoSpectris) is introduced and compared to classical oscillatory rheometry for different classes of soft materials used in bioengineering applications. The sensitivity of HFVS was tested by investigating the viscoelasticity of agar–gelatin gels as a function of their constituent concentrations.

2. Materials

2.1. *Silicone gel*

Silicone materials are widely used in biomedical applications as, for example, implementable heart valves (Ghanbari et al., 2009; Kidane et al., 2009). For this study, we used a standard commercial blend containing 75 wt% of a platinum-catalyzed silicone rubber (polyorganosiloxane and amorphous silica mixture, Eco-Flex® 0030, Smooth-On Inc., Easton, PA, USA, lots A #109181 and B #109178) and 25 wt% of a polyorganosiloxane softener (Slacker®, Smooth-On Inc., lot #119144). The mixture was disposed into various molds (25 mm diameter Petri dishes for classical rheometry and 14.5 mm diameter test tubes for RheoSpectris) and maintained at ambient temperature (25 °C) for 4 h to enhance gelation before measurements.

2.2. *Polyvinyl chloride (PVC) plastisol*

Plastisol is a polyvinyl chloride (PVC) thermoplastic polymer proposed to manufacture mimicking phantoms for imaging applications (Samani et al., 2003; Baghani et al., 2009). We mixed a *plastic* basis solution (M-F Manufacturing Co., Fort Worth, TX, USA, lot #22282 LP) with a *plastic softener* (M-F Manufacturing Co., lot #4228 S) in a 75%/25% v/v proportion, respectively. The softener solution permitted to modulate the plastisol viscoelasticity. Once mixed, the solution was heated in a microwave oven to 180 °C, poured into containers (25 mm diameter plates for the rheometer and 14.5 mm diameter test tubes for RheoSpectris), and then kept at ambient temperature for cooling and to initiate the polymerization process. Measurements were conducted after 45 min of polymerization.

2.3. *Polyvinyl alcohol cryogel*

Polyvinyl alcohol cryogel (PVA-C) is a synthetic polymer soluble in water. Its viscoelastic properties can be modulated

by freeze–thaw cycles (Park et al., 2001; Fromageau et al., 2007). PVA-C is currently used for tissue repair or replacement (Swięszkowski et al., 2006; Ghanbari et al., 2009) and for the fabrication of elastography imaging phantoms (Dumont et al., 2009; Le Floc'h et al., 2010). The precise mechanical characterization of PVA-C is a major requirement for its functionality and durability *in vivo*. We used commercial PVA-C (Vassar Brothers Medical Center, Poughkeepsie, NY, USA, lot #02070082) dissolved in pure water at 80 °C at a concentration of 10% w/w. The solution was degassed into a vacuum chamber for 10 min and then poured into a plastic flat mold (to form thin membranes) and test tubes (8.6 mm diameter) for rheometer and RheoSpectris measurements, respectively. Samples were submitted to a unique freeze–thaw cycle (12 h at −20 °C followed by 12 h at +20 °C, with freeze–thaw rates of ±0.2 °C/min) to initiate cross-linking and obtain a viscoelastic material. Circular samples for rheometry testing were cut from the cross-linked PVA-C membranes obtained after freezing and thawing cycles.

2.4. *Chitosan hydrogel*

The polysaccharide-based chitosan hydrogel, which is extracted from the exoskeleton of crustaceans, is a biomaterial used in tissue engineering and repair (Suh and Matthew, 2000; Kim et al., 2008). The viscoelastic characterization of chitosan is important to ensure its *in vivo* function and to control its rheological evolution in pre- and post-gel states (Cho et al., 2007). In the present study, a commercial chitosan powder (Marinard Biotech, Rivière-au-Renard, QC, Canada), with an average molecular weight of 2×10^6 g/mol and a polydispersity of 3.9, was dissolved at 80 °C in an oxalic acid aqueous solution (1 M and purity of 98% from Laboratoire MAT, Québec, QC, Canada) at a proportion of 5% (w/v). The preparation was centrifuged at 3000 RPM for 5 min to remove air bubbles. The solution was then poured into Petri dishes and test tubes, and stored at ambient temperature for 96 h to complete the gelation before measurements.

2.5. *Agar–gelatin gel*

Agar–gelatin mixtures, widely used as phantom materials for medical imaging applications (Madsen et al., 2005), were prepared by dissolving porcine skin gelatin powder (Sigma Chemical, Saint-Louis, MO, USA, G2500-1 kg, batch #058K010) into distilled water at 75 °C and concentrations of 1.5, 3, 7 and 10% (w/w). Agar powder (Sigma Chemical, A9799-1 kg, batch # 019K0011) was added when the solution reached a temperature of 70 °C, at a concentration of 1% (w/w) for all preparations, except for the 1.5% w/w gelatin one for which no agar was used. Agar–gelatin gelation was initiated by storing samples at a steady temperature of 5 °C for 12 h and all viscoelastic measurements were performed after samples reached room temperature (25 °C).

3. Methods

3.1. *Rheological spectroscopy measurements*

Hyper-frequency characterization was performed by the RheoSpectris viscoelastic spectroscope (Rheolution Inc., Montreal, QC, Canada). This new instrument allows characterizing viscoelasticity of soft solid materials in a frequency range between 10 and 1000 Hz. The basic principle of RheoSpectris consists in mechanically exciting a confined sample over a wide frequency range by applying a high-frequency transient vibration. The resonant dynamical response of the material, contained in a cylindrical tube, is measured without contact by means of a high sensitivity optical sensor (see Fig. 1). Physically, as detailed both experimentally and theoretically in Hadj Henni et al. (2010), the sample resonance eigenmodes depend on its viscoelasticity and are induced by the propagation and the constructive superposition of shear waves into the confined volume. This instrument performs rapid measurements (in less than 1 s) on small material volumes (as less as 1 ml) contained in sample holders. It is worth specifying that no filtering or averaging treatments were applied to the measured raw signals.

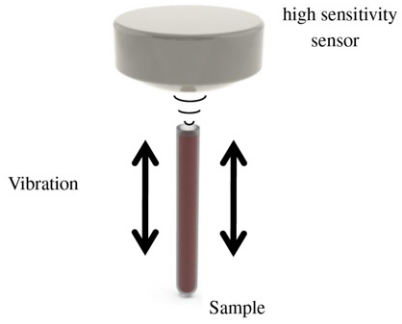

Fig. 1 – Schematic view of the sample mechanical excitation and dynamical response measurement of RheoSpectris for hyper-frequency viscoelastic spectroscopy.

For this study, material samples were contained in cylindrical tubes of volumes that ranged between 5 and 14 ml; material characteristics and the fabrication process dictated the choice of the sample volume. All measurements were performed at room temperature (25 °C). For all materials, HFVS measurements were realized in the linear viscoelastic (LVE) range. This ensured that materials vibrated at small amplitudes and, as a consequence, prevented slipping effects.

Classical rotational rheometry measurements were performed using a Physica MCR 501 rheometer (Anton Paar GmbH, Graz, Austria). Viscoelastic samples shaped into discs were confined between two plates of 25 mm diameter with a gap (sample thickness) of 1–1.5 mm. The surface of the plates was rough to prevent slippage during rotation. Tests were performed in dynamic mode, running first strain sweeps between 0.01 and 1 to evaluate the linear viscoelastic response of the material at low and moderate frequencies (0.1 and 10 Hz, respectively). Second, a frequency sweep between 0.01 and 100 Hz following 25 logarithmically spaced measurement points was carried out in the LVE regime. The dynamic storage and loss moduli G' and G'' were measured as functions of strain and frequency. The upper limit of the LVE was determined as the shear strain for which G' and G'' curves presented a maximum deviation of 5% with respect to values corresponding to a 0.01 strain. All measurements were performed at a constant temperature of 25 °C, maintained by means of a Pelletier heating system.

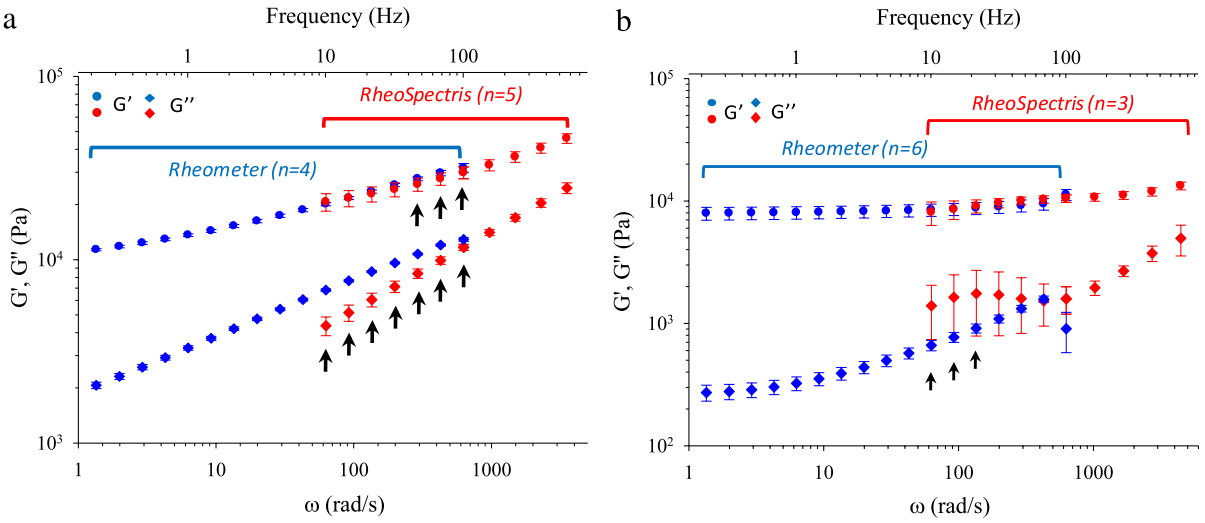

Fig. 2 – Superposition of low- and high-frequency dynamic moduli (*G′* and *G″* represented by filled circles and diamonds, respectively) obtained by classical rheometry (blue) and RheoSpectris (red) for (a) the silicone gel and (b) the polyvinyl chloride (PVC) plastisol. Arrows designate measurement points presenting a statistically significant difference ($p < 0.05$) between the two measurement methods. "*n*" signifies the number of samples investigated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. *Statistical analysis*

For each tested material, results are presented in this article by comparing the viscoelastic measurements obtained by classical dynamic rheometry and HFVS. Upper limits of hyper-frequency results have been confined below 1000 Hz to focus on the comparison of these measurements with classical rheometry. Two-way analyses of variance (ANOVA, SigmaStat Ver. 3.11, SYSTAT Software Inc., Chicago, IL, USA) with the Tukey test for pairwise multiple comparisons were performed to evaluate if viscoelasticity differences existed between measurement methods for overlapping frequencies. The statistical significance was fixed at 5%. Measurement points presenting statistically significant differences are represented by arrows, as reported in Figs. 2 and 3.

4. Results

Table 1 summarizes experimental dynamic strains and frequency ranges used to characterize each material tested with the MCR 501 and RheoSpectris instruments. Selected strain values ensured to the materials to be mechanically tested in the linear viscoelastic regime over the selected excitation frequencies. Table 1 also summarizes variabilities (*i.e.* ratios of standard deviations to mean values) obtained on the elasticity (*G′*) and lost (*G″*) moduli for each material and testing instrument. One can see that the mean variability of storage and loss modulus measurements depended on the tested material. Globally, mean variability values on storage moduli for both instruments were comparable. On the other hand, variability measurements on loss moduli measured by RheoSpectris were relatively larger compared with the MCR 501 instrument.

Fig. 2 presents dynamic moduli measured with the Physica MCR 501 rheometer and RheoSpectris for the silicone gel and the PVC plastisol (Fig. 2(a) and (b), respectively). Fig. 2(a) shows, for the silicone gel, that measurements with both instruments of storage moduli were in good agreement. Nevertheless, differences were noted between instruments at 46–100 Hz ($p < 0.05$). Panel (a) also reveals a strong dependence on frequency of *G′* that varied by 510%, i.e. from 10.9 kPa at 0.14 Hz to 45.8 kPa at 562 Hz. On the other hand, loss modulus data presented statistically significant differences between the two instruments for overlapping frequencies ($p < 0.001$).

The viscoelastic behavior of the polyvinyl chloride (PVC) plastisol is reported in Fig. 2(b). This material exhibited a smooth dispersion, i.e. a moderate dependence of the storage modulus on frequency. Indeed, the minimum of *G′* was 7.7 kPa at 0.14 Hz and its maximum was 13.3 kPa at 707 Hz. The rheological characterization of *G′* was in agreement with both instruments over overlapping frequencies ($p = 0.457$). However, the assessment of the loss modulus with the Physica MCR 501 and RheoSpectris varied between 10 Hz and 22 Hz ($p < 0.05$).

Fig. 3 shows the dynamic moduli for the *Polyvinyl alcohol* cryogel (PVA-C) and the chitosan hydrogel. Concerning the PVA-C, reported in Fig. 3(a), a moderate dispersion of the material elasticity (8.5 kPa at 1000 Hz compared to 4.8 kPa at 0.14 Hz) and a strong variability particularly for loss moduli were noted. This variance is explained by the strong dependence of the PVA polymerization process on the thermal history during freeze–thaw cycles, inducing large variations of the material viscoelasticity from sample to sample. No statistical differences between results obtained by both instruments over examined frequencies were noted ($p > 0.15$ for the storage modulus and $p > 0.05$ for the loss modulus), confirming the good agreement between the rotational rheometry and HFVS results.

Fig. 3(b) shows results for the chitosan hydrogel obtained for each measurement technique. It is clearly observable that

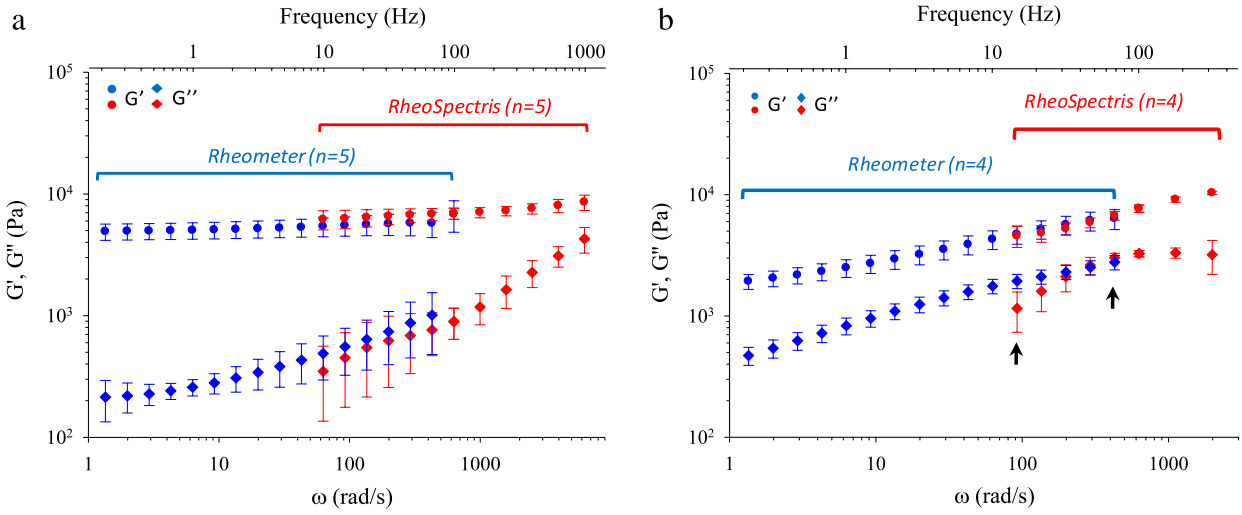

Fig. 3 – Superposition of low- and high-frequency dynamic moduli (G′ and G″ represented with filled circles and diamonds, respectively) obtained by classical rheometry (blue) and RheoSpectris (red) for (a) the polyvinyl alcohol cryogel (PVA-C) and (b) the chitosan hydrogel. Arrows designate measurement points presenting a statistically significant difference ($p < 0.01$) between both measurement methods. "n" designates the number of samples investigated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1 – Experimental values of dynamic strains (ε), frequency ranges, elasticity modulus (G′) and lost modulus (G″) variability values for the materials tested on the MCR 501 and the RheoSpectris instruments.

		Silicone gel	PVC plastisol	PVA-C	Chitosan
MCR 501	ε	0.01	0.08	0.05	0.03
	$[f_{min}–f_{max}]$ Hz	[0.14–100]	[0.14–100]	[0.14–100]	[0.14–68]
	G′ variability	2%	11%	18%	16%
	G″ variability	2%	12%	34%	15%
RheoSpectris	ε	0.002	0.006	0.018	0.025
	$[f_{min}–f_{max}]$ Hz	[10–562]	[10–707]	[10–1000]	[14–316]
	G′ variability	8%	10%	13%	9%
	G″ variability	6%	34%	39%	19%

the material presents a strong dispersion since the elastic modulus at 316 Hz is seven times greater than it appears at 0.14 Hz. Concerning G′, both rheometry and HFVS results were concordant ($p > 0.536$) and the statistical comparison of the loss modulus revealed differences only for the minimum and maximum compared frequencies ($p < 0.01$).

4.1. *Sensitivity of the HFVS instrument*

The sensitivity of the HFVS technique was examined by characterizing four agar–gelatin materials with various concentrations of the constituents. The first gel was composed of 1.5 wt% of gelatin only, while the other preparations contained 3, 7 and 10 wt% of gelatin along with 1 wt% of agar. The storage and loss moduli, and phase angle (δ) frequency dependence of each mixture, are represented in Fig. 4 between 10 Hz and 1000 Hz. From Fig. 4(a), it appears that the gelatin concentration had a strong effect on the viscoelastic behavior of the mixtures since both elastic and loss moduli increased with gelatin content. These gels behave as solid-like materials with elastic modulus values much larger than the loss ones. The maximum measured variability (with $n = 4$ for each mixture), which are not represented in panel (a) for clarity, were 4% and 17% for storage and loss moduli, respectively. The solid-like behavior of the agar–gelatin gel can also be appreciated in panel (b) that shows the phase angle (δ) as a function of frequency for different compositions of the mixtures. One can see that viscous effects were inversely proportional to the gelatin concentration. In addition, it is interesting to note that the frequency at which δ shows a maximum remained constant (600 Hz) for a fixed concentration of agar (1%).

Since studies on the mixed agar–gelatin gel system showed that the two polymers form individual networks (Watase and Nishinari, 1980; Clark et al., 1983), the modulus increases and the solid-like character observed here are most probably a unique contribution of the gelatin chains to the macromolecular network that gets stronger at high gelatin content. However, the frequency for maximum phase angle seems mostly related to the presence of the agar network that makes the overall network more fluid-like at very high frequencies, as seen in Fig. 4(b). These measurements show that HFVS allows one to characterize very low elasticity gels (with $G' \approx 200$ Pa) presenting relatively high loss moduli. These results also demonstrate that hyper-frequency

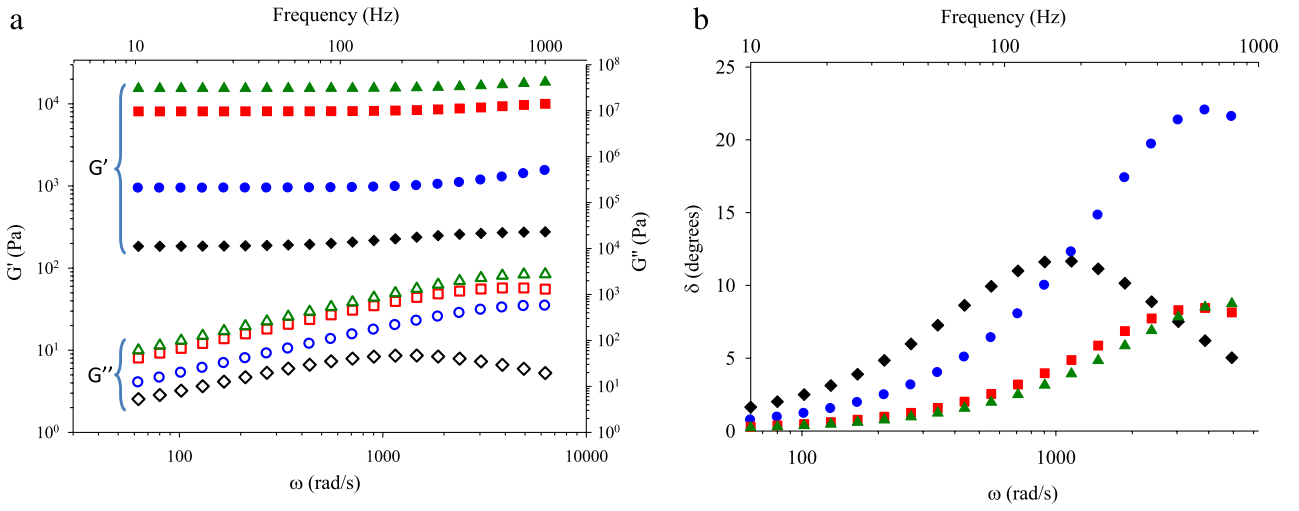

Fig. 4 – Comparison of mean ($n = 4$) values of (a) storage and loss moduli and (b) phase angle (δ) of gels with different concentrations of gelatin and agar: 1.5% and 0% (G' and δ represented by ♦ and G'' by ◊), 3% and 1% (G' and δ represented by ● and G''○), 7% and 1% (G' and δ represented by ■ and G'' by □) and 10% and 1% (G' and δ represented by and ▲ and G'' by △).

measurements are sensitive enough to measure viscoelastic variations due to physicochemical differences. However, it is important to note here that the instrument used for HFVS cannot measure the properties of fluids. Tests performed on a Newtonian fluid (polybutene with a viscosity of 1600 Pa s) and on a viscoelastic Boger fluid with a dominant viscous behavior (Sepehr et al., 2004) did not succeed. HFVS is hence clearly suitable to characterize viscoelastic materials exhibiting a dominant elastic behavior.

5. Discussion

Different biomaterials were characterized in viscoelastic spectroscopy using classical rheometry and a novel hyper-frequency measurement technology. Differences at specific frequencies in assessing the loss modulus between standard rheometry and RheoSpectris were noted for the silicone gel, polyvinyl chloride plastisol and chitosan hydrogel. On the other hand, a quite good agreement was observed for the elastic modulus. Some differences were nevertheless observed between the two measurement techniques for the silicone gel. Globally, observed differences, notably noted at both ends of overlapping frequencies covered by both instruments, can be explained by two factors. First, the random nature of gelation (for the silicone gel and chitosan hydrogel) and polymerization (for PVA-C and PVC plastisol) processes can induce inter-sample variability on the final viscoelastic properties. This effect also explains the intra-measurement technique variability observed in Figs. 2 and 3. Second, differences may also be due to current limits of both technologies on dynamic scanning since rheometry is more adapted to low-frequency characterization while the RheoSpectris is dedicated to medium and hyper-frequency measurements. Globally, since the comparison of viscoelastic spectrograms revealed that the two methods were generally in good agreement and presented a comparable inter-sample variability, particularly for the storage modulus, one can conclude on the validity of the HFVS RheoSpectris technique. In addition, the capacity of HFVS to characterize the viscoelastic behavior of gels as a function of their chemical composition was demonstrated, along with the sensitivity of the technique to finely analyze physical and chemical effects.

It would be interesting in a future work to compare the HFVS approach with the time–temperature equivalence principle traditionally used in rheometry to study dispersion effects over larger frequency ranges. Fundamentally, these dispersion phenomena are directly related to the intrinsic response of the tested materials since the increase of the storage modulus (or dispersion) intensifies with relaxation time.

For biomedical applications, HFVS is particularly well adapted to support the development of new biomaterials with specific rheological properties and to monitor their behavior in interaction with the physiological environment. The rapidity of measurements (less than 1 s) and the fact that they are contactless make HFVS particularly suitable to analyze time-varying bioprocesses and to characterize biomaterials under sterile conditions. In the context of dynamic elastography imaging, hyper-frequency measurements can serve to establish, *ex vivo*, statistically significant databases on viscoelastic properties of healthy and pathological soft tissues. For this purpose, tissue samples can be confined into cylindrical extractors comparable to those used in biopsy. These data would greatly help to calibrate emerging elastography imaging techniques. For industrial applications, HFVS may serve to precisely monitor, in real time during the fabrication (by on-line measurements) or in quality control laboratories, rheological properties of biomaterials and, more generally, industrial products.

Conflict of interest statement

Two of the authors are the founders of Rheolution Inc., a private company that develops and commercializes the RheoSpectris™ instrument.

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