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Rheological study on lysozyme/tetramethylurea viscoelastic matrices

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Abstract

Rheological properties of lysozyme viscoelastic matrices resulting from a sol–gel transition taking place in organic/aqueous media at room temperature were investigated. Gel-like structures, of transparent appearance, developed out of lysozyme (5.0 mmol/dm^3) dispersed in tetramethylurea (TMU)/water binary mixtures, at TMU mass fraction (w) ranging from w_{TMU} 0.6 to 0.9. The wide linear viscoelastic region (LVR) observed, up to strains of 10%, was invariant throughout the TMU concentration range investigated, indicating that the 3D structures of protein matrices, although fragile, are quite flexible and able to withstand great deformation before rupture. Storage (G') and loss (G'') moduli continuously increased with increasing TMU concentration, the former at a greater rate, consequently leading systems to a decrease in the loss angle, tanδ. For gels developed out of binary systems at $w_{TMU}=0.9$, creep curves revealed behaviour that very nearly approaches that of a perfect elastic solid. Although gelification under the experimental conditions employed is macroscopically accomplished in a time interval that does not exceed 24 h (for the gel developed out of the solvent mixture of lowest TMU concentration, $w_{TMU}=0.6$), a slight decrease in loss angle can still be detected after that period. Such changes, however, have no effect on the LVR. Relaxation tests indicate that systems comprise at least two dynamically distinct contributions.

Keywords: Rheological properties; Lysozyme; Tetramethylurea; Solvent effects; Viscoelasticity; Protein networks; Sol–gel transitions

1. Introduction

It is well known that protein three-dimensional structure is dependent not only on the amino acid sequence in the polypeptide chain, but also on aspects related to the environment [1]. Under conditions different than native, proteins may undergo drastic changes in their tertiary structure, unfolding and exposing their hydrophobic interior to the medium. If conditions are adequate, such unfolded protein species may associate and form a reticulated three-dimensional network that extends throughout the entire medium, entrapping the solvent.

The comprehension of protein gelation mechanisms bears evident scientific importance, both as a means to unravel mechanisms involved in protein

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folding processes and from a practical viewpoint, e.g. as in the control of functional properties of proteins employed in food products [2–4].

Most studies on protein gelation approach sol–gel transitions taking place in protein aqueous solution as a consequence of heat treatment [2–5]. Whereas the importance of solvent on protein structural characteristics is usually acknowledged, as demonstrated by the wide interest in the mechanisms of protein denaturation mediated by polar co-solvents, such as urea or guanidinium chloride [6,7], studies on solvent effects, particularly organic solvents, on the protein capability of undergoing sol–gel transitions are less prominent in the literature. Considering that the biological milieu is organic–aqueous in nature, the study of protein folding and capacity of intermolecular association in such environments is certainly relevant within the framework of protein structural research.

In previous work [8], a peculiar protein sol–gel transition taking place in organic/aqueous media at room temperature was described for lysozyme dispersed in certain binary organic/aqueous solvents, above critical concentration conditions, both of the solvent binary mixture and the protein [8,9]. It was found that the sol–gel transition itself comprises two stages of distinct kinetics, the first leading the globular protein molecule to unfold as a consequence of the solvent transition, and the second involving protein reticulation through transient (non-covalent) contacts between unfolded protein species when a critical protein concentration is achieved. Geometrical characteristics of lysozyme, in both diluted and concentrated regimes, have been determined in these media through an X-ray small-angle scattering technique [10,11]. The fractal character of lysozyme in the solid-like matrices has also been verified [10].

In this work, we present a rheological characterisation of the protein viscoelastic matrices generated at room temperature when lysozyme, a globular protein displaying Newtonian behaviour in aqueous solution, is dispersed in tetramethylurea (TMU)/water at super-critical concentrations [8], both of the protein and of the binary solvent mixture. Tetramethylurea is a peculiar liquid that displays intriguing physico-chemical behaviour in its mixture with water [9,12–16], with which it is miscible in all proportions. Despite TMU being a derivative of urea, the universal protein denaturant, its interactions with the protein milieu, with which it shares the amide character and the apolar molecular domains, are of a strikingly distinct kind [8,10,11], as can also be appreciated is this work.

2. Materials and methods

Lysozyme was acquired from Pharmacia. Tetramethylurea used was analytical grade reagent from Sigma. Ultra-pure water (Elga System UHQ, 18 MΩ cm) was employed throughout. Lysozyme samples were prepared by careful dispersion of the protein in the binary mixtures. TMU mass fraction (w_{TMU}) in the organic/aqueous solvent ranged from 0.6 to 0.9, in 0.1-intervals, for a fixed lysozyme concentration of 0.50×10^{-2} mol/dm^3. After preparation and at intervals between tests, samples were stored under refrigeration. For experiments in which the dependence of rheological parameters on variables other than time was assessed, the time elapsed after sample preparation is indicated. Flow activation energy assays employed lysozyme concentration of 0.50×10^{-2} mol/dm^3 and TMU/water in the w_{TMU} range 0.1–0.5, and were run after ca. 3 min from sample preparation to assure Newtonian flow conditions. The temperature range in that experiment was 5.0–30.0 °C.

Rheological measurements employed a Paar Physica MCR 300 rheometer equipped with cone-plate geometry (25 mm radius and 1° cone angle). Oscillatory, transient and static experiments were run. All experiments, except those leading to the determination of flow activation energy, were performed at 20.00 ± 0.05 °C (Peltier system). Flow activation energy values were determined from Arrhenius plots employing data obtained from Newtonian flow curves in the region $w_{TMU}\leq0.5$, recorded immediately (ca. 3 min) after sample preparation. Experimental conditions for the experiments comprised shear rates in the range 1000–3000 s^{-1}, with 50 points (3-s acquisition time) collected in that interval. Considering that the temperature range employed was 5.0–30.0 °C in 5.0 °C steps, the total time for the experiment with each solvent concentration was lower than 20 min,

which is too short a time for the observation of any deviation from Newtonian behaviour for samples in the conditions indicated. Oscillatory tests comprised strain amplitude sweep tests, which were carried in the range 0.1–1000% strain at 5 Hz, and frequency sweep tests, carried in the range 0.1–40 Hz at 5% strain. Transient experiments included relaxation tests, run at a constant strain, within the linear viscoelastic region, and creep tests, also carried out within the LVR. Relaxation tests consisted of applying a constant strain to systems, while measuring the variation in internal shear stress as a function of time. Creep tests comprised the application of a constant shear stress onto systems for a given time interval, followed by removal of stress and monitoring of system strain response as a function of time. Creep curves for lysozyme viscoelastic networks at $w_{TMU}=0.6$ (for 31-day-old samples) and at $w_{TMU}=0.6$ and 0.7 (for 3-day-old samples) are not presented due to torque values required in these cases being too close to the minimum limit of instrument torque. Frequency sweep tests were also run at 20 °C, which is within the limited temperature range (5.0–30.0 °C) in which the matrices could be manipulated (above 30 °C, gel structure starts to be degraded, with protein precipitating at approx. 40 °C; below 5 °C, sample condensation effects within the measuring device employed impaired further measurements). Thus, considering the very narrow temperature range, no use was made of the time–temperature superposition principle, consequently restricting the frequency range investigated [17]. Linear viscoelastic conditions were employed throughout the rheological tests.

3. Results and discussion

Strain amplitude sweep tests are presented in Figs. 1 and 2 for lysozyme matrices evolved out of binary TMU/water mixtures, at $w_{TMU}=0.6$, 0.7, 0.8 and 0.9, for different sample ages. At $w_{TMU}<0.5$, lysozyme does not undergo the sol–gel transition within the same time scale, as previously shown [8]. The wide linear viscoelastic region (LVR) observed, up to strains of 10%, indicates that the 3D structure of protein matrices, although fragile, is quite flexible and able to withstand great deformation before rupture. The LVR was found to be invariant throughout the TMU concentration and time ranges investigated (compare Figs. 1 and 2). Despite maintenance of the LVR, storage (G') and loss (G'') moduli continuously increased with increasing TMU concentration, the former at a greater rate, consequently leading systems to a decrease in the loss angle, tanδ (Fig. 3). It can also be observed in Fig. 3 that increase in G' values is more pronounced at $w_{TMU}>0.8$. The matrices show a solid-like character throughout the solvent concentration range investigated, as indicated by tanδ values consistently lower than unity.

We examine the events described above with reference to the phenomenology of gelification processes in aqueous solutions of proteins. In those systems, the sol–gel transition, different from what is observed here, is most often brought about by heat. The process involves partial unfolding of the molecule, followed by the formation of covalent and non-covalent linkages, which generate a 3D spatial structure entrapping the solvent. Different from this, the phase transition observed with the lysozyme systems described here does not involve the establishment of any covalent intra- or intermolecular cross-linkages, but rather multiple transient contacts that co-operatively sustain the 3D spatial skeleton of the gel. Partial unfolding here is brought about by a microconfigurational transition taking place at the solvent level [8,9]. The transient character of the viscoelastic matrices formed has been previously characterised in reversibility studies [8].

Although derived from a solvent effect rather than a temperature effect, with very distinct characteristics as described above, the transition observed for systems studied in the present work share certain common characteristics to the heat-mediated sol–gel transition in hydrocolloids, such as protein gels [2]. For the lysozyme/TMU/water systems, the sol–gel transition similarly comprises a first phase, of very fast kinetics, related to partial unfolding of the protein in response to a complex solvent microconfigurational transition [8,9,16] and a second one, of intermediate kinetics (occurring in less than a few min for the highest TMU concentration, $w_{TMU}=0.9$), related to the forma-

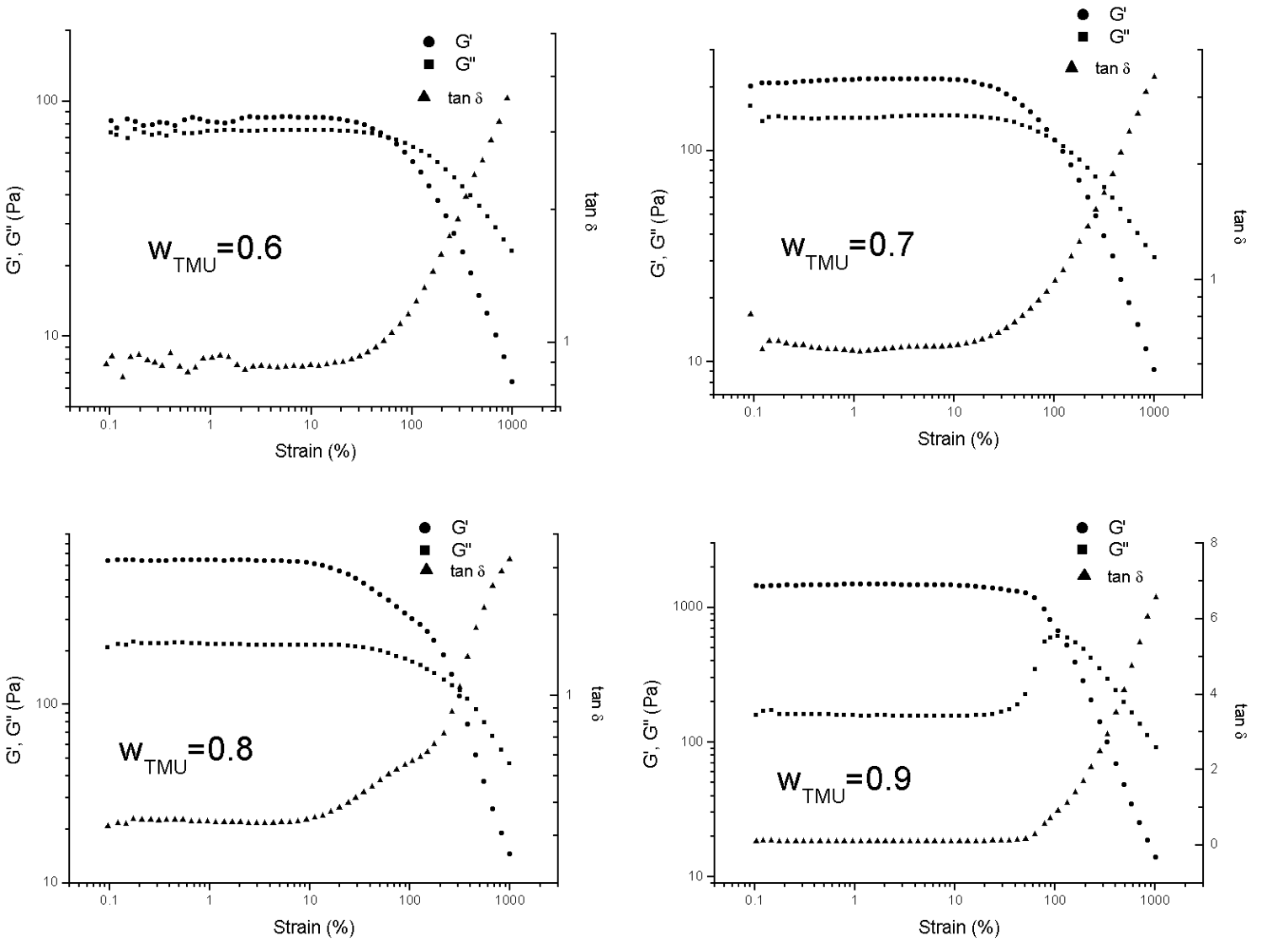

Fig. 1. Strain amplitude sweep for lysozyme viscoelastic matrices produced in different TMU/water solvent compositions (31-day-old samples).

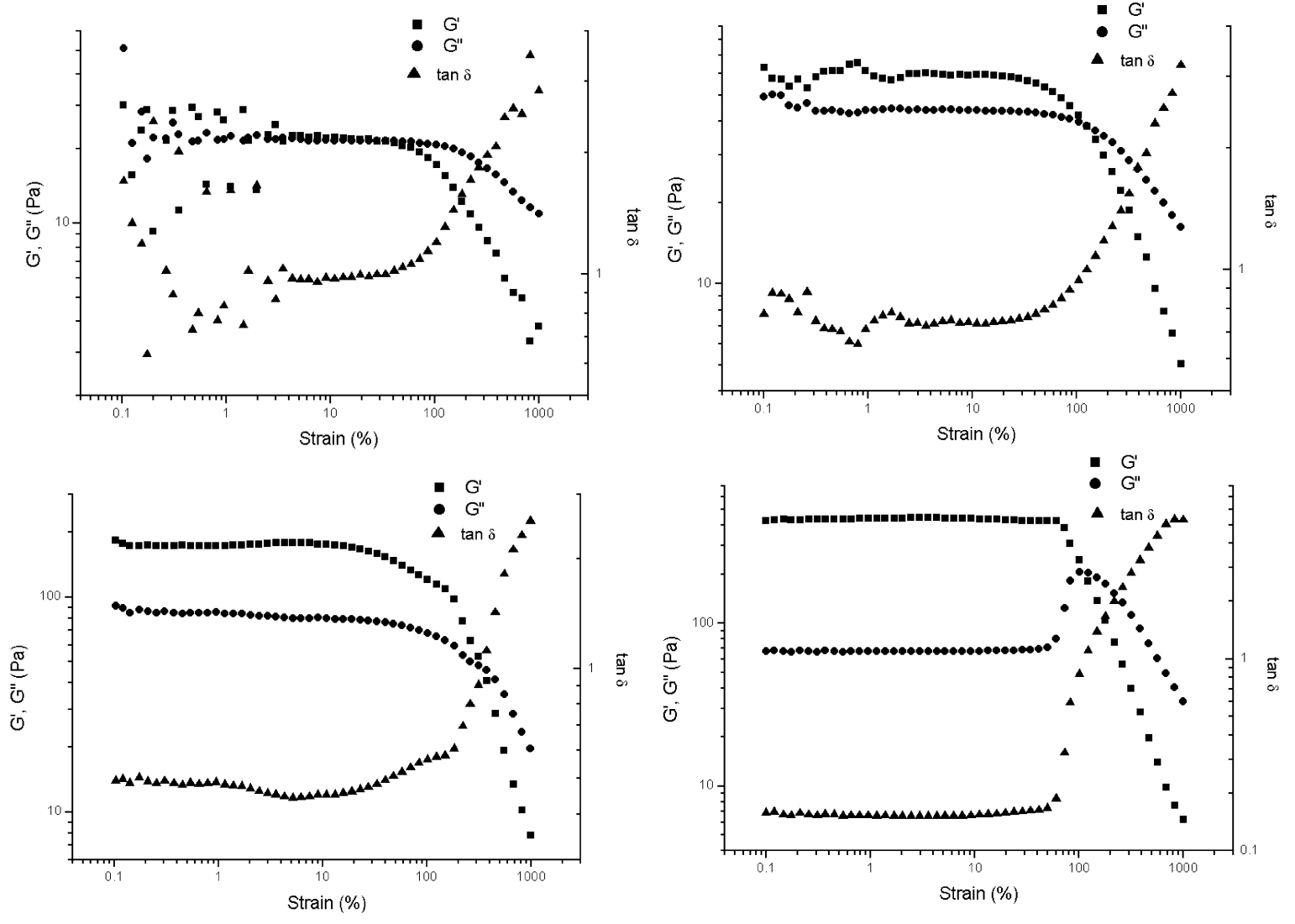

Fig. 2. Strain amplitude sweep for lysozyme viscoelastic matrices produced at different TMU/water solvent compositions (3-day-old samples).

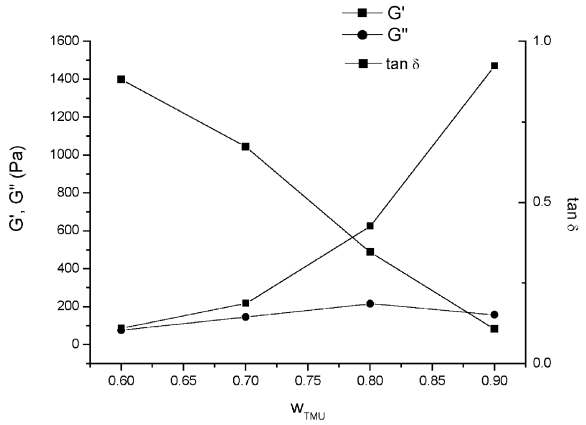

Fig. 3. Variation of the loss angle (tanδ), storage (G') and loss (G'') moduli for lysozyme viscoelastic matrices produced at different TMU/water solvent compositions (31-day-old samples).

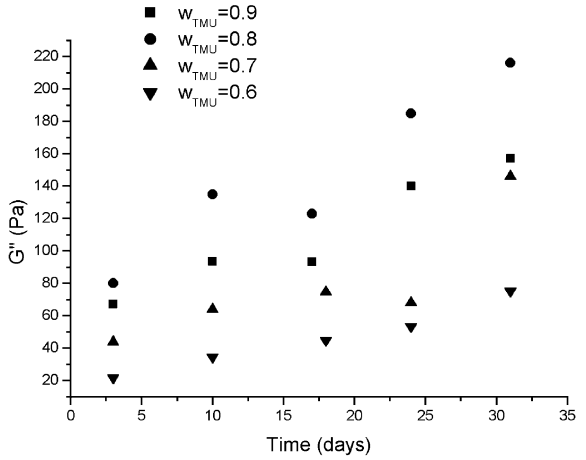

Fig. 5. Variation of loss modulus (G'') with time for lysozyme viscoelastic matrices for distinct TMU contents in the organic/aqueous solvent mixture.

tion of the primary macromolecular spatial structure or matrix skeleton. A belated stage (ageing), of considerably slower kinetics (systems may take weeks to reach a steady state), also takes place. In this work, G' and G'' varied as a function of time, as shown in Figs. 4 and 5. It can be observed that the resulting tanδ variation (Fig. 6), indicative of enhanced elasticity, is not in fact very intense and does not seem to affect the LVR, which was found to be nearly constant for samples with very distinct ages (Figs. 1 and 2).

Relaxation tests were performed and the resulting curves are presented in Figs. 7 and 8. Curves adequately fitted a second-order exponential decay, indicative of two dynamically distinct contributions in the matrices, which differ from each other by one order of magnitude (Table 1). Relaxation

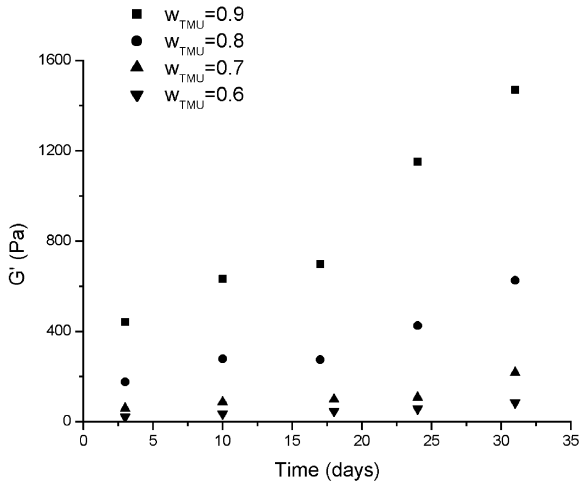

Fig. 4. Variation of storage modulus (G') with time for lysozyme viscoelastic matrices for distinct TMU contents in the organic/aqueous solvent mixture.

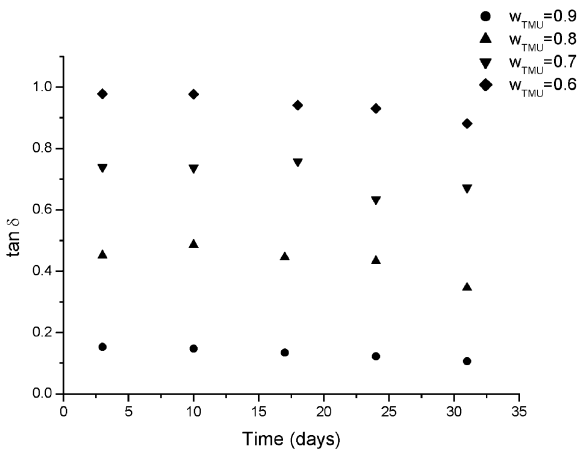

Fig. 6. Variation of tanδ with time for lysozyme viscoelastic matrices for distinct TMU contents in the organic/aqueous solvent mixture.

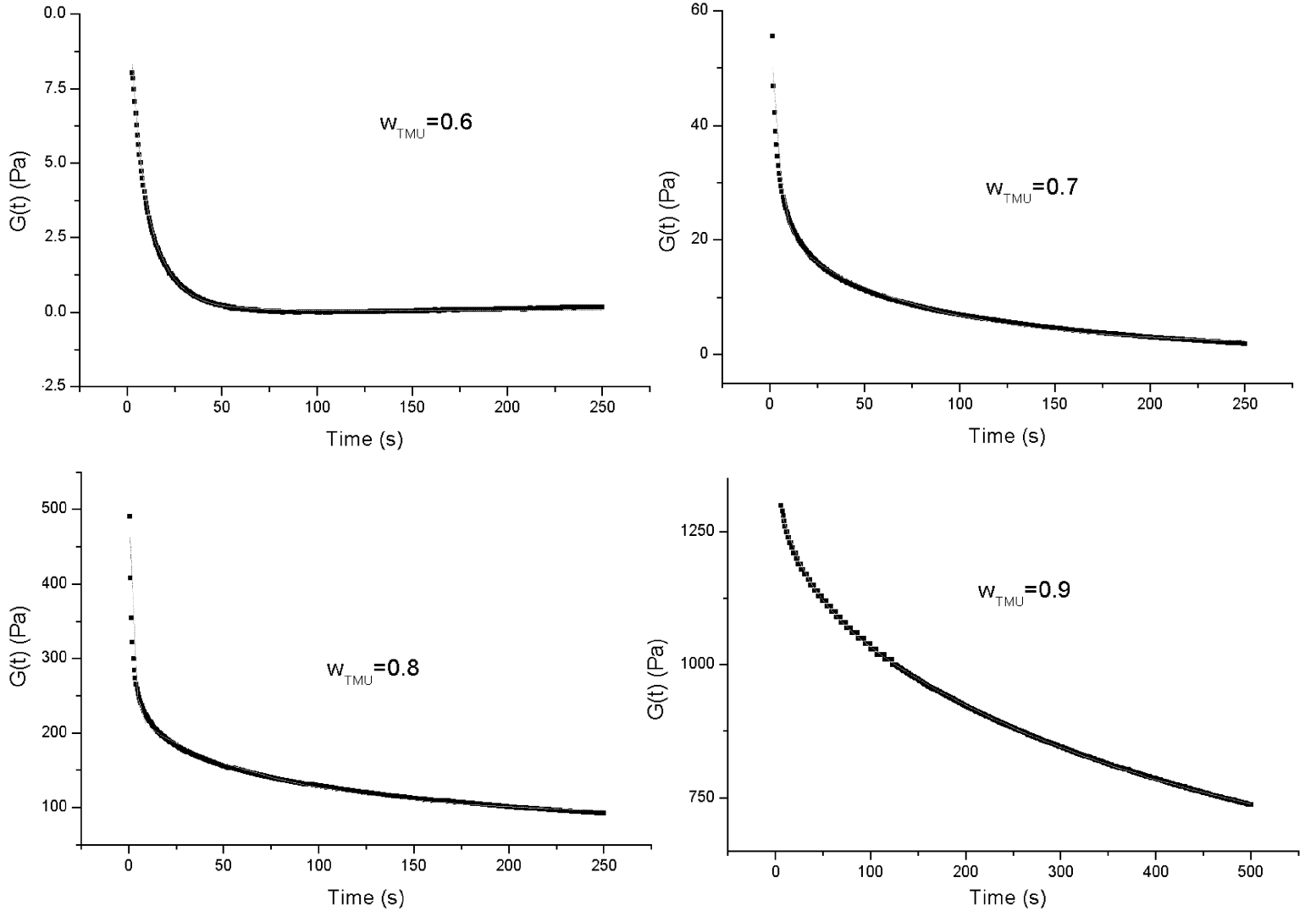

Fig. 7. Relaxation and fitting curves (second-order exponential decay) for lysozyme viscoelastic matrices produced at different TMU/water solvent compositions (31-day-old samples).

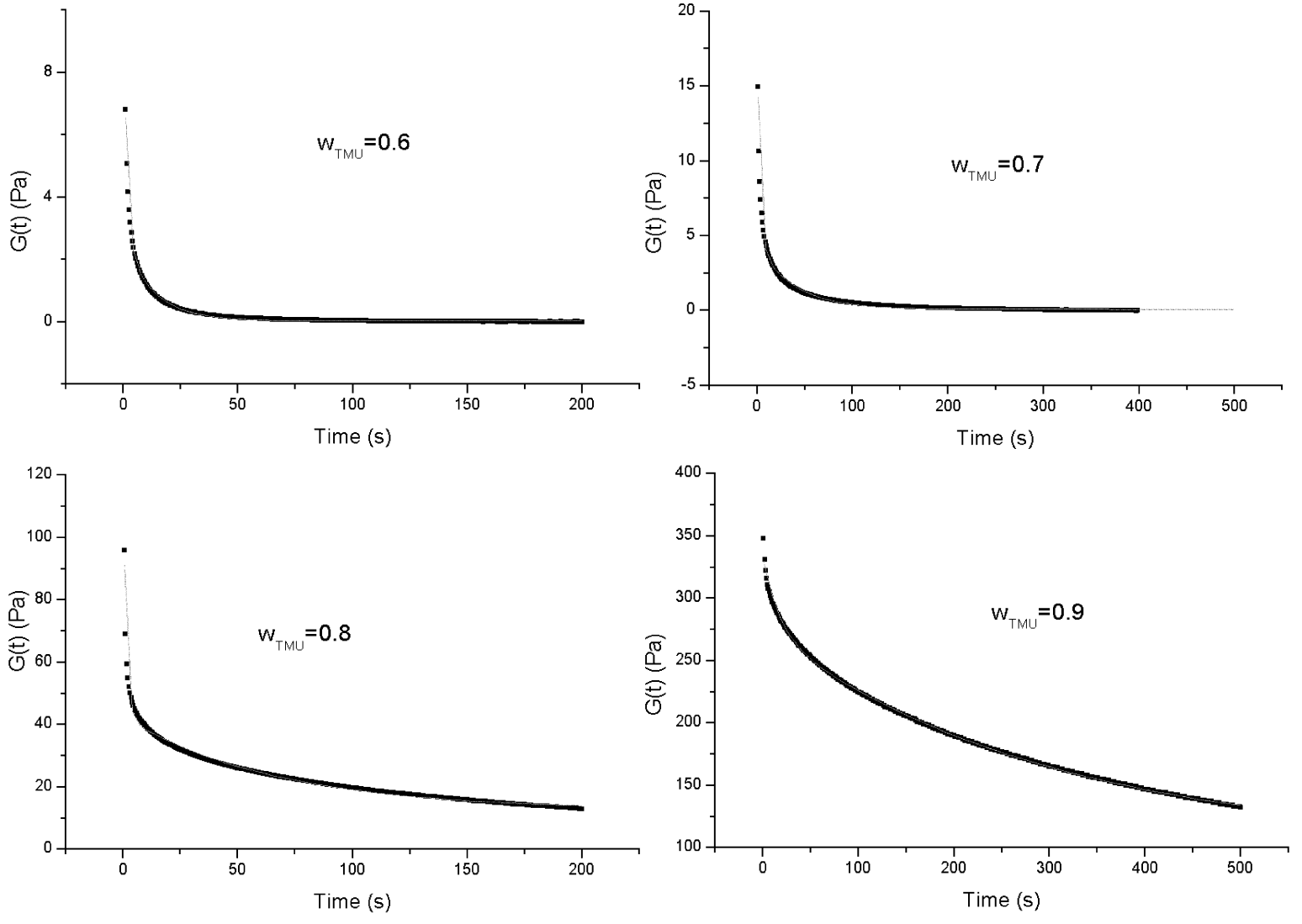

Fig. 8. Relaxation and fitting curves (second-order exponential decay) for lysozyme viscoelastic matrices produced at different TMU/water solvent compositions (3-day-old samples).

Table 1
Relaxation times for the two distinct dynamical populations present in 3-day-old TMU/lysozyme networks

TMU/H_2O solvent composition	$\tau_{population\ 1}$ (s)	$\tau_{population\ 2}$ (s)
$w_{TMU}=0.6$	1.5	13
$w_{TMU}=0.7$	3.0	38
$w_{TMU}=0.8$	1.2	65
$w_{TMU}=0.9$	16	294

times increase with TMU concentration, as would be expected for systems in which flow becomes more restricted due to the network entanglement postulated to occur within the matrices. The analysis of relaxation curves for 3- and 31-day-old samples did not reveal notable changes in curve shapes, although the $G(t)$ range is distinct due to sample age, as expected.

Creep test curves displayed the profiles shown in Fig. 9. Their analysis allowed us to determine the elastic and viscous contributions in the lysozyme matrices, shown in Table 2. As is well known, the immediate system response during the application of stress in a creep curve corresponds to the elastic components of sample, while the deformation that follows, with sample still under stress, is due to the viscous part. When stress is removed, the restoration response observed is again due to the elastic portion, with the strain plateau that the sample eventually reaches being ascribed to the non-restorable, viscous contribution. Observation of Table 2 shows that the elastic component is the major contribution for the matrix evolved from the binary solvent at $w_{TMU}=0.9$. In fact, the creep curve profile for that sample (Fig. 9, $w_{TMU}=0.9$) very closely approaches that of a perfectly elastic solid. For the other solvent compositions, the creep curves in Fig. 9 show very characteristic viscoelastic profiles, with the elastic character increasing with increasing w_{TMU}.

The two relaxation times that have been determined from a second-order exponential fit of G as a function of time cannot be a priori coupled to the elastic and viscous contributions separately, since they are rather likely to reflect the result of an interplay between them. From an examination of Table 2, it is possible to infer that these two contributions reach approximately similar values at circa $w_{TMU}=0.6$. That is, the solvent composition identified by previous work [8] as the critical composition for onset of the viscoelastic transition, and in which similar concentrations of folded and unfolded protein species were found to be present when dispersed in these media in the diluted regime [11]. At that same solvent composition, the C=O stretching band in TMU/water Raman spectra [9] was found to display a peculiar splitting that revealed two components of similar intensity, which have been interpreted as suggestive of the presence of two microenvironments to which the carbonyls would be exposed in the binary mixture.

In Fig. 10, it is evident that the dependence of the elastic modulus G' on frequency tends to be less intense as the TMU content increases in the binary mixture. Accordingly, in the region spanning from $w_{TMU}=0.6$ to 0.8, the curve profile is characteristic of polydispersed systems. At $w_{TMU}=0.9$, however, the profile displayed approaches that of a monodispersed system. This solvent composition corresponds to matrices for which nearly perfect elastic behaviour was found in the creep tests (Fig. 9). Despite the possible correlations suggested by results presented in this work, we refrain from ascribing them to any morphological aspects of the protein networks investigated, since that would require more substantial experimental data.

A Newtonian flow condition for lysozyme/TMU/water systems below $w_{TMU}=0.5$, for a lysozyme concentration of 0.50×10^{-2} mol/dm^3 and 3 min after sample preparation, has been investigated in the temperature range 5.0–30.0 °C, with the aim of obtaining information relative to activation flow energy values (E_a) as a function of the organic component concentration in the binary mixture (w_{TMU}). The curve profile obtained for the variation of E_a as a function of w_{TMU}, shown in Fig. 11, reveals that incipient structural transitions are already under way before the transition point at $w_{TMU}=0.6$ is achieved.

The observed decrease in tanδ as a function of time verified in this work (Fig. 6) reveals that a very slow increase in the elastic character of the matrices takes place with time. The ageing of the gel possibly involves subtle alterations in the

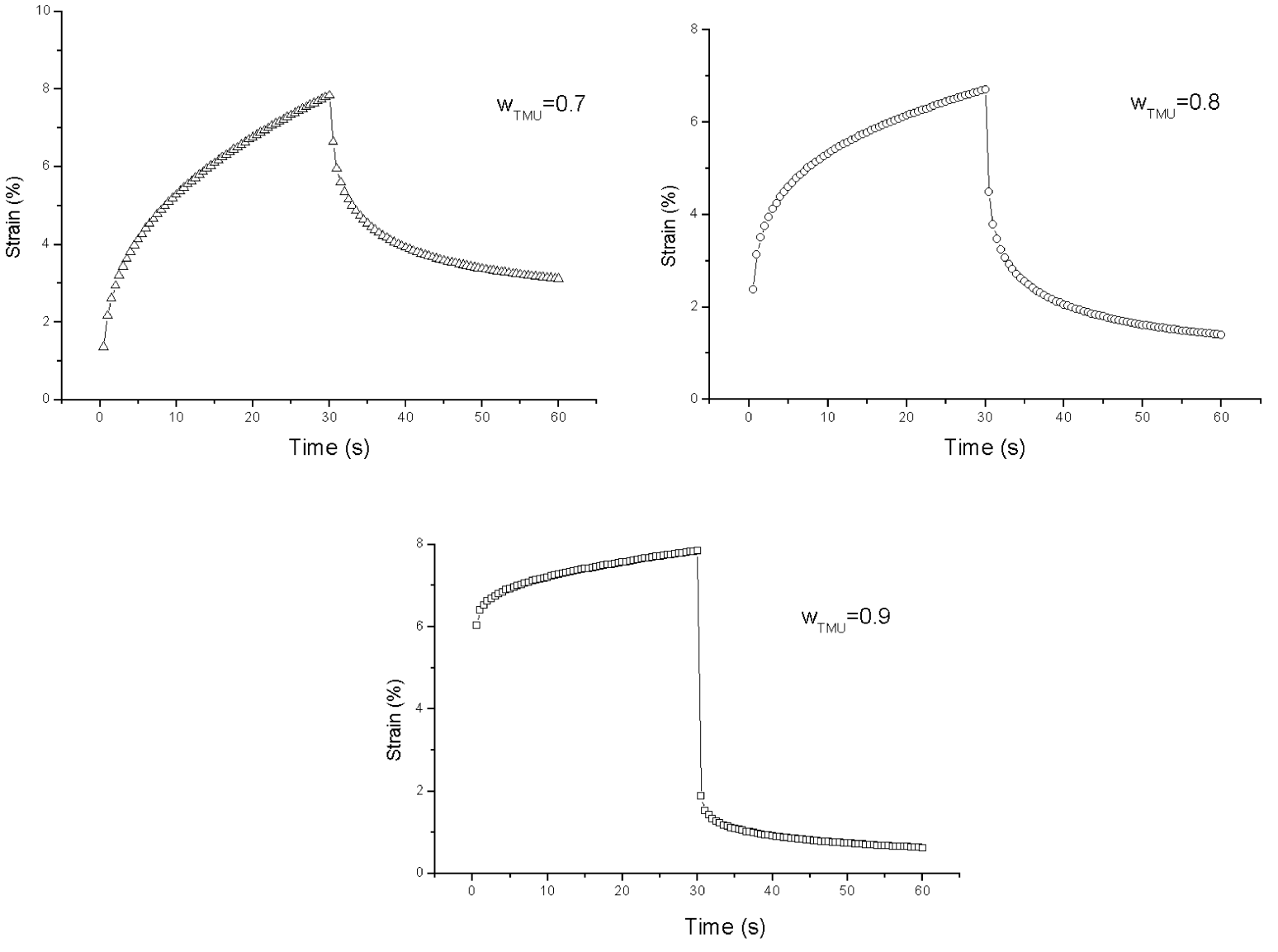

Fig. 9. Creep curves for lysozyme viscoelastic matrices produced at different TMU/water solvent compositions (31-day-old samples).

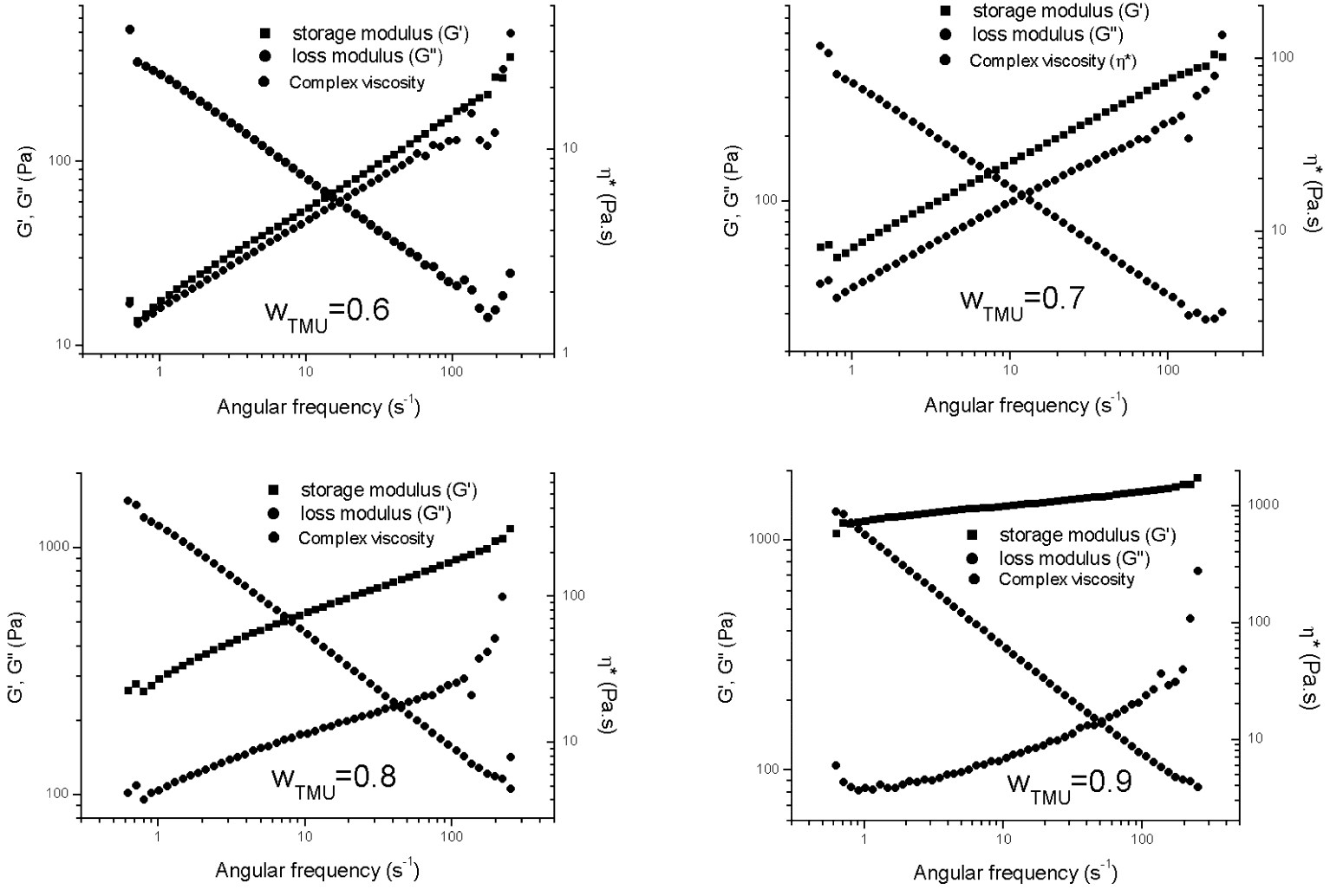

Fig. 10. Frequency dependence of dynamic moduli (G' and G'') and complex viscosity for lysozyme viscoelastic matrices.

Table 2
Elastic and viscous contributions in 31-day-old TMU/lysozyme networks

TMU/H_2O solvent composition	Component (%)	
	Viscous	Elastic
$w_{TMU}=0.6$	–	–
$w_{TMU}=0.7$	40	60
$w_{TMU}=0.8$	21	79
$w_{TMU}=0.9$	8	92

matrix architecture, which are unable, however, to significantly affect gel resistance to rupture, as verified from the maintenance of the linear viscoelastic region in the time interval investigated (Figs. 1 and 2).

4. Summary

Lysozyme viscoelastic matrices, developed from tetramethylurea/water environments, were rheologically characterised in this work. The matrices are solid-like, as indicated by $\tan\delta < 1.0$ throughout the solvent concentration range investigated, and optically transparent. Storage (G') and loss (G'') moduli increase with increasing TMU concentration in the binary solvent, with the elastic component taking the lead, resulting in systems reaching nearly perfect elastic behaviour when the water concentration is lowest ($w_{TMU}=0.9$).

The matrices, sustained through transient (non-covalent) multiple contacts, display a spatial network structure of great flexibility, able to stand considerable deformation before rupture, as indicated by the wide extent of the linear viscoelastic region observed. A significant variation in elasticity was observed as a function of TMU concentration in the binary mixture, as well as a slight tendency towards increased elasticity as a function of gel ageing.

Relaxation times (τ) increased with TMU content in the binary solvent, with two τ values being identified, one order of magnitude distinct from each other.

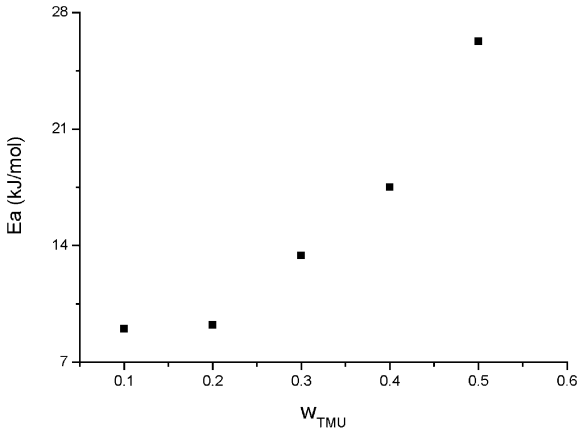

Fig. 11. Variation of flow activation energy for lysozyme/TMU/water systems (in the Newtonian region) as a function of TMU/water solvent composition.

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