Stress relaxation and creep on living cells with the atomic force microscope: a means to calculate elastic moduli and viscosities of cell components

Susana Moreno-Flores¹, Rafael Benitez², María dM Vivanco³ and José Luis Toca-Herrera¹, ⁴

¹ Biosurfaces Unit, CIC BiomaGUNE, Paseo Miramón 182, E-20009 San Sebastián-Donostia, Spain
² Departamento Matematicas, Centro Universitario de Plasencia, Universidad de Extremadura, Avenida Virgen del Puerto 2, E-10600 Plasencia, Spain
³ Cell Biology and Stem Cells Unit, CIC BioGUNE, Parque tecnológico de Bizkaia, Ed. 801A, E-48160 Derio, Spain

E-mail: jltocaherrera@cicbiomagune.es and jose.toca-herrera@boku.ac.at

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Abstract

In this work we present a unified method to study the mechanical properties of cells using the atomic force microscope. Stress relaxation and creep compliance measurements permitted us to determine, the relaxation times, the Young moduli and the viscosity of breast cancer cells (MCF-7). The results show that the mechanical behaviour of MCF-7 cells responds to a two-layered model of similar elasticity but differing viscosity. Treatment of MCF-7 cells with an actin-depolymerising agent results in an overall decrease in both cell elasticity and viscosity, however to a different extent for each layer. The layer that undergoes the smaller decrease (36–38%) is assigned to the cell membrane/cortex while the layer that experiences the larger decrease (70–80%) is attributed to the cell cytoplasm. The combination of the method presented in this work, together with the approach based on stress relaxation microscopy (Moreno-Flores et al 2010 J. Biomech. 43 349–54), constitutes a unique AFM-based experimental framework to study cell mechanics. This methodology can also be extended to study the mechanical properties of biomaterials in general.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Physical forces largely influence cell development, cell function and cell fate, but they also can induce pathological transformations [1]. Likewise cells react to such forces and their responsive behaviour tends to preserve their homeostasis.

Among other techniques [2–5], atomic force microscopy (AFM) has been profusely used to characterize the mechanical properties of adherent living cells [6]. The most extended non-rheological approach consists in locally applying a normal load on a defined position within the cell surface [7, 8]. The load is exerted by a cantilever that is brought into contact with the surface of the cell through the cantilever tip, usually a pyramid, a cone or a bead attached to its free end. The force is monitored together with the sample’s indentation, and the resulting curve is analysed in terms of elastic contact mechanics to obtain the Young’s moduli. Sneddon’s modelization of a conical tip impinging on an elastic semi-infinite space [9] together with the Hertz model for spherical tips [10] are the most widely used models. To comply with the demanding
assumptions, tip–cell adhesion is assumed to be negligible, the experiments are made under small deformations (not larger than 10%) or the models are conveniently modified to account for finite thickness samples [11]. However, the models do not contemplate the fact that in living cells the force–indentation curves may indeed vary with the loading rate and the extent of the indentation or applied load [12–14]. The analysis according to an elastic contact mechanics model would lead to Young’s moduli that may depend on loading rate and applied load, which is in contradiction to the definition of Young’s modulus as a material’s intrinsic parameter.

A more rigorous approach considers the cell’s viscoelastic nature. Typical experiments of stress relaxation [15, 16], on the one hand, and creep compliance [17–19] on the other hand, show that single cells such as chondrocytes and bacteria exhibit both elastic and viscous behaviour. In all reported cases, the single modal behaviour of the cells was analysed according to a linear viscoelastic model characterized by a single apparent viscosity and two elastic moduli. The elastic moduli account for the instantaneous and the relaxed elastic responses of the cell. However, there are certain cell types whose viscoelastic behaviour is not single modal and thus this model proved to be insufficient [20, 21].

In this work we report on the obtaining of viscoelastic parameters of breast cancer living cells that exhibit a complex behaviour. To this aim, we combine both stress relaxation and creep compliance single-cell studies with the atomic force microscope and we analysed the results according to a viscoelastic model defined by a common set of parameters. Breast carcinoma cells, MCF-7, behave as a complex linear viscoelastic model characterized by a single apparent viscosity and two elastic moduli. The elastic moduli account for the instantaneus and the relaxed elastic responses of the cell. However, there are certain cell types whose viscoelastic behaviour is not single modal and thus this model proved to be insufficient [20, 21].

2. Materials and methods

2.1. Sample preparation

MCF-7 cells were grown at 37 °C and 5% CO2 in Dulbecco’s modified eagle medium (DMEM, Sigma) supplemented with 8% foetal bovine serum (FBS, Sigma), 2% 200 mM L-glutamine and 0.4% penicillin/streptomycin (PEN/STREP, Sigma). For force measurements, the cells were subcultured on borosilicate glass cover slips (diameter 24 mm and 0.16 thickness) for one day. Prior to force measurements, the cells were washed in CO2-independent cell medium (Leibowitz medium, L15, Sigma) and measured in the same medium at 37 °C. Cytochalasin D from Zygosporium masonii (≥99%, Fluka), an inhibitor of the actin polymerization [22], was added to the cells at a concentration of 5 μM. Force measurements in cytochalasin D were performed after an incubation time of 15 min, whereupon cells undergo morphological changes.

2.2. Force and height–time curves

Measurements were carried out on different cell clusters for the same sample with a Nanowizard II (JPK Instruments, Germany) coupled with the Cell Hesion® module (JPK Instruments, Germany) that allows large vertical piezoelement displacements (≤100 μm) and a transmission optical microscope (Axio Observer D1 Zeiss, Germany). Uncoated SiN cantilevers of a nominal spring constant of 0.01 N m−1 (MLCT, Veeco Instr., USA) were used. The cantilevers were previously cleaned in acetone and ethanol or alternatively plasma-cleaned (Argon plasma, Harrick, USA) to remove impurities and their spring constants evaluated by the thermal method [23]. The cell-coated glass substrates were then mounted in a low-volume cell incubator (Biocell, JPK Instruments, Germany) with 400 μL L15 cell medium and thermalized at 37 °C. Individual force–time curves were recorded on three different cell clusters at a speed of 5 μm s−1 and at maximum loads of 0.5, 1, 2, 3 and 4 nN. The speed was chosen to be sufficiently high to expect a reliable performance of the set-up and assumed to be sudden for the model to apply. Theoretical treatments of creep [24] and stress relaxation [25] that consider the effect of loading rates predict changes in the amplitude of the signal, but not in the time constants or the relaxed values (the signal at long times). The AFM cantilever was kept in contact with the cells for 2 s at constant force and at constant height to obtain the stress relaxation and creep curves on the same cell, respectively.

Cell deformations (Δl0) in experiments at constant height are calculated from a set of displacement–time curves at different loads (Li) [26]. The constant height attained by the piezoelement at each load L (ZL) is registered and subtracted from the height reached at the lowest load L0 (in this case 0.5 nN). The resulting quantity (ZL − ZL0) is thus a relative displacement, which is not only influenced by the applied load, but also by sample deformation and cantilever deflection (ZL − ZL0 = Δl0 + δrel). Relative sample deformation at load L0 is thus obtained by subtracting the relative cantilever deflection (δrel = (L0 − L0)/k, k being the spring constant) to the relative piezoelement deflection. The absolute sample deformation or, in other words, the cell deformation at each load is then calculated by adding an offset, Δl(Δl0 = Δl0 + Δl0 ref). This offset is obtained by fitting the relative displacements to a power law of the form Δl0 ref = Δl0 ref + K L0.5, K being a constant that relates to the material’s properties. This approach assumes that the force is directly proportional to the square of the deformation, which agrees with the mechanistic model of an elastic half-space being impinged by a conical [9] and a pyramidal tip [27], and the reported efforts in assessing the mechanical properties of living cells as such [28]. In this case, we use this approach to calculate cell deformations as shown in figure 1.

3. Results and discussion

The atomic force microscope not only allows us to monitor the time evolution of the cantilever’s force but also the cantilever’s vertical position at all stages. When the AFM tip is brought
Figure 1. Cell deformations ($\Delta l_0$) in experiments of constant force for 13 different MCF-7 cells. The black points are the experimental data and the blue points are the averages. The averages have been fitted to a function of the form $KL^{0.5}$ (dashed blue line). The optical micrograph shows two of the cells studied.

into contact with the surface of the sample for a definite time period, two types of experiments are possible. Figure 2 shows the typical outputs of these two experiments on a living MCF-7 cell. In the so-called constant height mode (figure 2(a)), the vertical position of the cantilever is set constant while the cantilever’s force varies with time; analogously, in the constant force mode (figure 2(b)) the vertical position of the cantilever changes with time while the cantilever’s force is kept constant. The first case deals with force relaxation tests while the second case resembles creep compliance tests, since variations of the vertical position of the cantilever can be directly related to the deformation of the cell ($\Delta l(t)$) at constant force. Both force and deformation are proportional to the stress and strain, respectively, through the contact area and the thickness of the cell (figure 2).

As figure 2 shows, the force in stress relaxation experiments decays with time while the cell deformation increases with time. Both decays are related and should be analysed according to a common mechanical model. The model, depicted in figure 3, has been previously proved successful in describing force relaxation experiments on the same type of cells [21] and consists of a parallel arrangement of two Maxwell elements and a spring [29].

Indeed, if the cell is subjected to a sudden and constant deformation and providing that the contact area does not change with time, the force decays biexponentially:

$$F(t) = a_0 + a_1 \exp\left(-\frac{t - t_0}{\tau_1}\right) + a_2 \exp\left(-\frac{t - t_0}{\tau_2}\right)$$

with $\tau_1 = \eta_1/E_1$ and $\tau_2 = \eta_2/E_2$, $E_1$ and $E_2$ being the compressive moduli and $\eta_1$, $\eta_2$ the compressive viscosities of the first and the second Maxwell element, respectively. Likewise, if a sudden and constant force is applied on the cell’s surface, the cell’s deformation should also follow a biexponential decay:

$$Z - Z_0 = c_0 + c_1 \exp(x_1t) + c_2 \exp(x_2t).$$

With $x_1$ and $x_2$ being both negative and nonlinear functions of the compressive moduli and viscosities (derivation

Figure 2. Typical force relaxation (a) and creep (b) experiments on the nuclear region of a living MCF-7 cell. During the time the AFM tip is in contact with the cell (starting at $t_0$) either the force is set to vary with time (constant height mode) or the vertical position (constant force mode). Force $F$ and deformation $\Delta l$ are related to the stress and strain through the contact area ($a_c$) and the cell height ($l_0$).
the applied load range, which is one of the prerequisites to dispersive at lower loads, the parameters are constant within as a function of the applied load. Although the data are rather available in the appendix):  

\[ x_1 = \frac{-r_1 + \sqrt{r_1^2 - 4r_0r_2}}{2r_2}, \quad x_2 = \frac{-r_1 - \sqrt{r_1^2 - 4r_0r_2}}{2r_2}, \]

\[ r_0 = \frac{E_0E_1E_2}{\eta_1\eta_2}, \quad r_1 = \frac{E_1}{\eta_1}(E_0 + E_2) + \frac{E_2}{\eta_2}(E_0 + E_1), \]

\[ r_2 = E_0 + E_1 + E_2. \]  

Figure 4(a) shows a set of force relaxation curves obtained at different loads (black) and their respective fittings to a biexponential decay (red). The measurements were performed on the apical zone of living MCF-7 cells. Figure 4(b) shows the corresponding creep curves and fittings obtained on the same cell’s position. Correlation coefficients range from 0.959 to 0.999 in both experiments, which indicates that the quality of the fitting is fairly good. Figures 4(c) and (d) show the time constants for the force relaxation (\( \tau_1 \) and \( \tau_2 \)) and the creep experiments (-\( x_1^{-1} \), -\( x_2^{-1} \)) on different MCF-7 cells \((n = 13)\) as a function of the applied load. Although the data are rather dispersive at lower loads, the parameters are constant within the applied load range, which is one of the prerequisites to obtain a unique set of compressive moduli and viscosities.

To calculate \( E_0, \ E_1, \ E_2, \ \eta_1 \) and \( \eta_2 \) we assume that the cell’s compression is sudden enough so that the shape of the perturbation does not influence the cell response and the contact area in the force relaxation experiments does not change with time. According to that, the proposed model predicts the following expressions for the compressive moduli and viscosities (see the appendix for a detailed derivation):

\[ E_0 = \frac{A_0}{\epsilon_0} = \frac{a_0l_0}{\Delta l_0 a_c} \]

\[ E_1 = \frac{a_0l_0}{\Delta l_0 a_c} \left[ \frac{1}{(1 - \frac{\Delta l_0}{\Delta l})} \left( 1 + \frac{1}{x_1\tau_1} + \frac{1}{x_2\tau_2} + \frac{1}{x_1x_2\tau_2} \right) - 1 \right] \]

\[ E_2 = \frac{a_0l_0}{\Delta l_0 a_c} \left( 1 - \Delta l_0 \right) \left( \frac{1}{x_1\tau_1} - \frac{1}{x_2\tau_2} - \frac{1}{x_1x_2\tau_2} \right) \]

\( \eta_1 = E_1\tau_1 \quad \eta_2 = E_2\tau_2 \]  

where \( a_c \) is the projection of the actual contact area along the normal direction (i.e. the direction along which the force is applied and measured), \( \Delta l_0 \) is the cell deformation in stress relaxation experiments (which is assumed to be constant with time) and \( l_0 \) is the cell height at the nuclear region, where the mechanical texts were made (5.8±0.1 μm, \( n = 13 \) as obtained from AFM). Values for \( a_c \) cannot be directly obtained from AFM experiments and assumptions are frequently made that are valid for perfectly elastic samples and small indentations (Hertz model and derived approaches [6]). In this case we
assume that $a_e$ is the projected area defined by the cell surface being in contact with the pyramidal tip [30]; the deformed surface follows the tip’s shape and it extends along the normal direction as far as the cell’s deformation (figure 5). In this case, the projected area is $a_e = 2\Delta l^2 \tan^2 \alpha$, where $\alpha$ is the half-opening angle of the pyramid. Since the tip is a truncated pyramid with a sphere-like apex, the expression is valid as long as the deformation is higher than $R(1 - \sin \alpha)$, where $R$ is the curvature radius of the tip apex. The computed cell deformations (see figure 1) are in all cases higher than that limiting value, which is of the order of $10^{-2} \mu m$.

Figure 6 shows the calculated compressive moduli and viscosities according to equations (4) as a function of the applied load. The magnitudes do not exhibit a clear tendency towards higher or lower values within the range of loads studied, and they are considered to be fairly constant. Weighted averages of the calculated magnitudes thus give $E_0 = 2.4 \pm 0.2$ kPa, $E_1 = 1.1 \pm 0.2$ kPa and $E_2 = 0.8 \pm 0.2$ kPa for the compressive moduli and $\eta_1 = 81 \pm 18$ Pa s and $\eta_2 = 720 \pm 161$ Pa s for the compressive viscosities.

The multimodal response of both force and creep and the various mechanical parameters reflect the cell heterogeneity across the basal and apical surfaces. In our experiments, the local deformations induced to the cell were about 17–43% of the cell height. Thus it is not unlikely that the tip may perturb various cellular substructures that respond differently to the mechanical stimulus. Cell membrane and cell cytoplasm are thought the most likely assignments for the $E_1$, $\eta_1$ and $E_2$, $\eta_2$ pairs, respectively, since the corresponding time constants ($\tau_i = \eta_i/E_i$) are similar to those reported for cell membrane and cell cytoplasm [31]. Cytoplasmic shear viscosities have been reported to be within a wide range of values, which seem to depend mainly on cell type and on the measurement technique: values in the range 250–2800 Pa s have been calculated for lung macrophages using twisting magnetometry [32], 210±143 Pa s for J774 macrophages using magnetic tweezers [33], but also 4000 Pa s for mouse 3T3 fibroblasts through magnetic bead rheometry [34] or within 100–10^5 Pa s for chick fibroblasts using microplates [3]. In the case of breast cancer, MCF-7, cells, no values of viscosities have been previously reported to our knowledge. Recent studies report Young’s moduli as high as 15–30 kPa [35], but also as low as 0.3–0.6 kPa [3] for the same type of cell and measuring technique. In this work, the instantaneous compressive modulus, $E_\infty = E_0 + E_1 + E_2$ could be compared with the literature values assuming perfect elastic behaviour as reported [3, 35]. Our work gives an intermediate value of 4.7 ± 0.4 kPa.

Treatment of the MCF-7 cells with cytochalasin was thus used to verify the assignment of the observed bimodal response to cell membrane and cell cytoplasm. Cytochalasin is a drug that disrupts the F-actin cytoskeleton and therefore greatly affects the cytoplasm structure. Figures 7(a) and (b) shows the force relaxation and creep compliance of the same MCF-7 cells before and after drug treatment, respectively. Indeed, force relaxes to a larger extent and the deformation is larger when the drug is present in the medium. This qualitatively indicates that the cell turns more viscous and less elastic, which quantitatively corresponds to a noticeable decrease of the compressive moduli and viscosities within (figure 7(c)). Since the drug is expected to perturb the cytoskeleton, the elastic and viscous parameters of the cell cytoplasm, $E_2$ and $\eta_2$, should be affected to a greater extent than the mechanical parameters of the cell membrane, $E_1$ and $\eta_1$. The weighted averages of these quantities are plotted in figure 8, which shows that upon cytochalasin treatment $E_1$ and $\eta_1$ undergo a decrease of 36% and 38%, respectively, while likewise for $E_2$ and $\eta_2$ the decrease amounts to 70 and 80%. Consequently, cytochalasin treatment does not only decrease cell elasticity but it also decreases cell viscosity, mainly fluidizing the cell cytoplasm. On the other hand, it is clear the effect of the drug on both viscoelastic processes; it is thus reasonable to consider the term ‘cell membrane’ as a layer composite of the membrane itself and a cortical actin cytoskeleton (the so-called cortex, see [31]), while the term ‘cytoplasm’ can be attributed to the layer underneath, composed mainly of cytoskeleton and cell organelles. The bimodal response is clearly observable within a load range of 1.5–4 nN, which means that cell deformations of 1–2 μm are sufficient to probe both membrane/cortex and...
the underlying cytoplasm. Consequently, the thickness of the membrane/cortex for the MCF-7 cells should be no larger than 1 μm.

4. Conclusion

In this work we have shown that it is possible to apply the atomic force microscope to combine stress relaxation and creep compliance measurements on living cells and determine their viscoelastic parameters. The methodology, together with the approach based on stress relaxation microscopy (STREM), encompasses a robust AFM-based experimental framework that allows a fairly rigorous description of the cell mechanics. The mechanical behaviour of MCF-7 cells responds to a two-layered model. The addition of an actin-depolymerising agent such as cytochalasin D allows the assignment of the observed viscoelastic processes to cell membrane/cortex and the cell cytoplasm, respectively. Drug treatment results in a remarkable decrease in both cell elasticity and viscosity, of which those attributed to cell cytoplasm are most affected. The experimental results estimate an upper limit for the size of the membrane/cortex layer of 1 μm.

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Figure 7. Force relaxation (a) and creep compliance (b) tests on the same cell before and after cytochalasin treatment (black and grey lines, respectively). The cell is depicted in the optical micrographs: ‘control’ refers to the cells before the drug treatment, ‘cytch D’ refers to the cells after the drug treatment. (c) Compressive elastic moduli and viscosities as a function of the applied load for the ‘control’ and ‘cytch D’ cases.

Figure 8. Weighted averages of the compressive elastic moduli (a) and viscosities (b) before and after drug treatment. The parameters $E_0$, $E_2$ and $\eta_2$ decrease to a larger extent than the parameters $E_1$ and $\eta_1$. 
Appendix. Calculation of elastic moduli and viscosities from stress relaxation and creep experiments: Zener’s model

Figure 3 shows the viscoelastic model used to represent the cell’s behaviour. In this model the stress can be split into the sum of three terms:

\[ \sigma = \sigma_0 + \sigma_i + \sigma_2. \]  

(A.1)

Also we have the following equations:

\[ \sigma_0 = E_0 \varepsilon \]

\[ \dot{\varepsilon} = \frac{\dot{\sigma}_i}{E_i} + \frac{\sigma_i}{\eta_i} \quad \text{for } i = 1, 2. \]  

(A.2)

Differentiating twice equation (A.2), and taking into account equation (A.1), we obtain the following three equations:

\[ \sigma = E_0 \varepsilon + \sigma_1 + \sigma_2 \]  

(A.3)

\[ \dot{\sigma} = \dot{\varepsilon}(E_0 + E_1 + E_2) - \frac{E_1}{\eta_1} \sigma_1 - \frac{E_2}{\eta_2} \sigma_2 \]  

(A.4)

\[ \ddot{\sigma} = \ddot{\varepsilon}(E_0 + E_1 + E_2) - \dot{\varepsilon} \left( \frac{E_1^2}{\eta_1} + \frac{E_2^2}{\eta_2} \right) + \frac{E_1}{\eta_1} \sigma_1 + \frac{E_2}{\eta_2} \sigma_2. \]  

(A.5)

Multiplying equation (A.3) by \( \frac{E_1}{\eta_1} + \frac{E_2}{\eta_2} \) and equation (A.4) by \( \frac{E_1}{\eta_1} \) and after adding these equations to (A.5) we get

\[ \ddot{\sigma} + A \dot{\sigma} + B \sigma = r_2 \dot{\varepsilon} + r_1 \dot{\sigma} + r_0 \varepsilon \]  

(A.6)

where \( A = \frac{E_1}{\eta_1} + \frac{E_2}{\eta_2}, B = \frac{E_1 \dot{E}_2 + E_2 \dot{E}_1}{\eta_1 \eta_2}, r_0 = \frac{E_0 E_1 E_2}{\eta_1 \eta_2 \eta_3}, r_1 = \frac{E_0 (E_0 + E_1)}{\eta_1 \eta_2}, r_2 = E_0 + E_1 + E_2. \)

A.1. Experiments at constant strain: stress relaxation

Let us consider the particular case of a constant strain \( \varepsilon(t) = \varepsilon_0. \) Then equation (A.6) reduces to

\[ \ddot{\sigma} + A \dot{\sigma} + B \sigma = r_2 \dot{\varepsilon} + r_1 \dot{\sigma} + r_0 \varepsilon_0. \]  

(A.7)

The general solution to equation (A.7) has the form \( \sigma(t) = \sigma_p(t) + \sigma_0(t), \) where \( \sigma_0(t) \) is the general solution of the homogeneous equation \( \ddot{\sigma} + A \dot{\sigma} + B \sigma = 0 \) and \( \sigma_p(t) \) is a particular solution of (A.7). Since the right-hand side of equation (A.7) is constant we may find a constant particular solution \( \sigma_p(t) = E_0 \varepsilon_0. \) On the other hand, the general solution of the homogeneous equation takes the form \( \sigma_0(t) = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2}, \) with \( \tau_i = \frac{\eta_i}{E_i} \) for \( i = 1, 2. \) Therefore the general solution of equation (A.7) is

\[ \sigma(t) = E_0 \varepsilon_0 + A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2}. \]  

(A.8)

A.2. Experiments at constant height: creep

Considering now a constant stress \( \sigma(t) = \sigma_0, \) equation (A.6) takes the form

\[ r_2 \dot{\varepsilon} + r_1 \dot{\sigma} + r_0 \varepsilon = B \sigma_0. \]  

(A.9)

The particular solution to this equation is given by the constant function \( \varepsilon_p(t) = \frac{r_0}{\tau_0} = \frac{r_1}{\tau_1}. \) The general solution to the homogeneous equation \( r_2 \ddot{\varepsilon} + r_1 \dot{\varepsilon} + r_0 \varepsilon = 0 \) is given by \( \varepsilon_0(t) = C_1 e^{r_1 t/\tau_1} + C_2 e^{r_2 t/\tau_2}; \) \( x_1 \) and \( x_2 \) are the roots of the characteristic polynomial \( r_2 x^2 + r_1 x + r_2 \) which are given by

\[ x_1 = -\frac{r_1 + \sqrt{r_1^2 - 4 r_0 r_2}}{2 r_2}, \quad x_2 = -\frac{r_1 - \sqrt{r_1^2 - 4 r_0 r_2}}{2 r_2}. \]  

(A.10)

Both are real and negative, because \( r_1^2 - 4 r_0 r_2 = \left( \frac{E_0}{\eta_1} (E_0 + E_1) - \frac{E_2}{\eta_2} (E_0 + E_1)^2 + \frac{4 E_1 E_2}{\eta_1 \eta_2} \right) > 0. \) Thus the general solution to equation (A.5) is

\[ \varepsilon(t) = \sigma_0 E_0 + C_1 e^{x_1 t} + C_2 e^{x_2 t}. \]  

(A.11)

A.3. Obtaining parameters

We assume we have experimentally obtained two signals that follow Zener’s model. Then we have

\[ \sigma(t) = A_0 + A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} \]  

(A.12)

\[ \varepsilon(t) = C_0 + C_1 e^{x_1 t} + C_2 e^{x_2 t}. \]  

(A.13)

To obtain the coefficients \( E_0, E_2, \eta_1, \eta_2 \) and \( \eta_3 \) from the experimental coefficients \( A_0, \tau_1, \tau_2, C_0, x_1 \) and \( x_2, \) we assume we know \( \sigma_0 \) and \( \varepsilon_0. \) \( E_0 \) is thus easily obtained as

\[ E_0 = \frac{A_0}{\varepsilon_0}. \]  

(A.14)

We can in turn get the value of \( r_0 \) from its definition and \( E_0, r_0 = \frac{E_0}{\eta_1} \frac{E_0}{\eta_2} = \frac{E_0}{\tau_1 \tau_2}. \) Knowing \( r_0, \) we can obtain \( r_1 \) and \( r_2 \) in terms of \( x_1, x_2 \) and \( r_0 \) by multiplying the expressions of \( x_1 \) and \( x_2, x_1 x_2 = \frac{r_1^2 - \sqrt{r_1^2 - 4 r_0 r_2}}{4 r_2}, \) \( \frac{4 A_0 E_2}{4 r_2} = \frac{A_0}{\tau_2}. \) We thus obtain \( r_2 \) as

\[ r_2 = \frac{r_0}{x_1 x_2}. \]  

(A.15)

and \( r_1 \) as

\[ r_1 = -r_2 x_1 - r_0 \frac{1}{x_1} = -r_0 \left( \frac{1}{x_1} + \frac{1}{x_2} \right). \]  

(A.16)

We can then rewrite the expressions of \( r_2 \) and \( r_1 \) in terms of \( E_0, \tau_1, \tau_2, r_1 \) and \( r_2 \) as follows:

\[ E_1 + E_2 = r_2 - E_0 \]

\[ \frac{E_1}{\tau_2} + \frac{E_2}{\tau_1} = r_1 - E_0 \left( \frac{1}{\tau_1} + \frac{1}{\tau_2} \right). \]  

(A.17)

Equations (A.17) are a system of two linear equations with two unknowns \( (E_1 \) and \( E_2). \) The solution gives

\[ E_1 = r_2 - \left[ E_0 + \frac{1}{(\eta_1 - \frac{E_0}{\tau_2})} \left( r_2 - \frac{E_0}{\tau_2} - \frac{E_0}{\tau_1} \right) \right] \]

\[ E_2 = \frac{1}{(\tau_2 - \eta_2)} \left( r_2 - \frac{E_0}{\tau_2} - \frac{E_0}{\tau_1} \right) \]  

(A.18)
which turns to
\[
E_1 = \frac{A_0}{\varnothing_0} \left( 1 - \frac{\tau_1}{\tau} \right) \left( 1 + \frac{1}{x_1 \tau_1} + \frac{1}{x_2 \tau_2} + \frac{1}{x_1 x_2 \tau_1 \tau_2} \right)
\]
\[
\quad + \frac{1}{x_1 x_2 \tau_1 \tau_2} - 1 \right) \right] \quad \text{(A.19)}
\]
and
\[
E_2 = \frac{A_0}{\varnothing_0 (1 - \frac{\tau_1}{\tau})} \left( \frac{1}{x_1 x_2 \tau_1 \tau_2} - \frac{1}{x_1 \tau_1} - \frac{1}{x_2 \tau_2} - 1 \right)
\]
as a function of the experimental parameters. Once \( E_1 \) and \( E_2 \) are known, it is possible to calculate the viscosities \( \eta_1 \) and \( \eta_2 \) by substitution into their respective expressions:
\[
\eta_1 = E_1 \tau_1 = \frac{A_0 \tau_1}{\varnothing_0} \left( 1 - \frac{\tau_1}{\tau} \right) \left( 1 + \frac{1}{x_1 \tau_1} + \frac{1}{x_2 \tau_2} + \frac{1}{x_1 x_2 \tau_1 \tau_2} \right)
\]
\[
\quad + \frac{1}{x_1 x_2 \tau_1 \tau_2} - 1 \right) \right) \right] \quad \text{(A.20)}
\]
\[
\eta_2 = E_2 \tau_2 = \frac{A_0 \tau_2}{\varnothing_0 (1 - \frac{\tau_1}{\tau})} \left( \frac{1}{x_1 x_2 \tau_1 \tau_2} - \frac{1}{x_1 \tau_1} - \frac{1}{x_2 \tau_2} - 1 \right) \]

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