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# A rheological model for spheroids including extra-cellular matrix

CLAUDE VERDIER<sup>1 (a)</sup> and LIVIU I. PALADE<sup>2,3</sup>

<sup>1</sup> *Université Grenoble-Alpes, CNRS, LIPhy, Grenoble, 38000, France*

<sup>2</sup> *Université de Lyon, CNRS, Institut Camille Jordan, UMR 5208, INSA-Lyon, Villeurbanne, 69621, France*

<sup>3</sup> *Université de Lyon, CNRS, LaMCoS UMR 5259, Pôle de Mathématiques, INSA-Lyon, Villeurbanne, 69621, France*

**Abstract** – The rheology of spheroids has been studied intensively recently and it was shown that the presence of the extra-cellular matrix (ECM) can have significant effects on the overall behaviour of these biological systems. Collagen I can indeed be a proxy between cells and bring new intriguing effects, as its content increases. To investigate these effects further, a two-phase emulsion model is proposed including interactions between cells and the ECM. Starting with the single cell and collagen individual viscoelastic properties, the model can be tested against previously obtained data for spheroids. The model has interesting features and capabilities for it covers a variety of behaviours and uses fitting parameters such as collagen and cell concentration, as well as adhesion energy. It is shown that the final intercellular collagen content can be large as compared to the initial one, and that this increase in collagen content induces a larger packing of cells, together with a larger adhesion energy.

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**Introduction.** – Spheroids are very interesting 3D biological systems, and good candidates to describe tumours [1]. Although there has been a growing interest to investigate how they react to osmotic pressures or mechanical forces [2–6], their mechanical properties are not yet satisfactorily understood. Indeed, cells packed together correspond to a concentrated suspension with shear-dependent viscosity or viscoelastic behaviour [7], but the extra-cellular matrix embedded into the system, or secreted by cells, makes the picture harder to describe.

In addition cells are viscoelastic, in particular cancer cells are known to exhibit different mechanical signatures like a larger deformability [8–10] or a glass transition depending on invasiveness or grade [11–14]. Moreover their mechanical behaviour depends largely on the environment, in particular they rigidify when in contact with a stiffer substrate, i.e. they are mechanosensitive [6, 15]. They can also transform when the microenvironment is mechanically altered [16].

A few studies have focused on the effect of extra-cellular matrix (ECM) and its potential role to change the rheology of the spheroids. In particular, ECM can be considered as a proxy [17, 18] acting as a porous material drained by water or the culture medium present in the spheroid [19]. But the influence of a larger collagen content may allow the building up of a new microstructure made of

interconnected fibers responsible for the larger mechanical moduli observed [20]. It is definitely accepted that the whole spheroid exhibits a viscoelastic pattern, due to the presence of cells embedded within the ECM, a gel-like system. In addition spheroids could be explored in more detail at larger frequencies to investigate possible poroelastic behaviors like the ones found for tendons [21].

Probably the best way to model the behaviour of such spheroids is to consider the ECM–water surrounding medium as a gel with viscous and elastic properties. Such ECM properties have been studied extensively in the literature in the linear and nonlinear regimes, showing the role of cross-linking effects [22], negative normal stresses [23], concentration-dependent shear moduli [24], nonlinear rheology following pre-stress protocols [25], and the insensitivity of Magnetic Resonance (MR) based diffusion measurements to various collagen hydrogels [26]. In addition the gel biopolymers enable cells to bind or adhere within connective tissues to allow stress transfer [27].

In this work we propose to study spheroids consisting of two phases, i.e. the ensemble of cells and the collagen matrix. We use the matrix mechanical properties as well as individual cell properties in contact with a similar environment, that is to say when they are surrounded by other cells. Then we consider a viscoelastic emulsion model, valid for large concentrations in order to predict the whole spheroid rheology in a large range of frequencies and scales. This is carried out in detail by varying the

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<sup>(a)</sup>claude.verdier@univ-grenoble-alpes.fr (corresponding author)

model parameters. Finally we optimize these parameters to rationalize previous results [20] obtained for spheroids containing an excess of collagen.

**Materials and methods.** – Collagen was prepared according to the manufacturer’s protocol (Corning). Rat tail collagen I was mixed with Phosphate Buffer Saline (PBS, 1X) and NaOH (1M) was added at 4°C until the correct pH was obtained (7.4). Then it was let to polymerise at 37°C for 30 minutes. Collagen properties were measured as described in previous work [28]. Briefly, classical rheometry tests at 37°C using parallel plate geometry (20mm-diameter, gap size 500-800  $\mu\text{m}$ ) were carried out in oscillatory shear mode in the [0.05Hz-10Hz] range, using a small deformation of 1%, within the linear range. Then experiments using Atomic Force Microscopy (AFM) in force modulation mode were done following a previous method [29, 30] using indentation of a flat collagen layer (in PBS) with a pyramidal AFM tip (Bruker, MLCT, half angle  $\theta \sim 20^\circ$ ). This geometry was preferred as compared to colloidal probes, since it avoids adhesion to such soft gels. An initial indentation  $\delta_0$  was applied, followed by small oscillations in the linear regime ( $\delta \ll \delta_0$ ), thus allowing to measure the complex shear moduli  $G^*(\omega) = \frac{1-\nu}{3\delta_0 \tan \theta} \left\{ \frac{F^*}{\delta^*} - i\omega b(0) \right\}$ , where  $G^*=G'+iG''$ ,  $G'$  and  $G''$  are respectively the elastic and loss shear moduli,  $\nu$  is the Poisson ratio ( $\nu \sim 0.5$ ),  $F^*$  and  $\delta^*$  are the complex force and indentation, and the last term is the drag on the surrounding liquid, with  $b(0)$  being a coefficient containing the geometry of the system as well as the fluid viscosity [29]. Using these complementary experiments, the range [0.05Hz-1kHz] was covered. Note that the hypothesis  $\nu \sim 0.5$  involves the fact that poroelastic effects are neglected. However, this could become important for some hydrogels, in particular when analyzing relaxation curves at longer times [31]. In the present study, since fast sollicitation frequencies are used, it is known that the fluid does not move relative to the microstructure if  $f > 10$  Hz [32, 33], which is mainly true in our AFM experiments. This argument is further enhanced by checking the necessary agreement between classical rheology and AFM data.

T24 cells (epithelial bladder cancer line) were cultured in RPMI medium with 10% FBS and 1% penicillin-streptomycin. These cells have been described before and their properties were previously found to depend on the environment, i.e. the substrate. In some cases, corrections depending on substrate stiffness need to be made [12]. Here we chose to measure T24 cell properties as if they were in contact with other cells, like in a real spheroid. The best possible way to do so is to form a spheroid for 3 days (see description below) and approach a small AFM tip close to the surface, where cells are located. In such a case, no substrate corrections were needed since the environment consists of cells themselves. Such AFM measurements were carried out with a pyramidal tip (Bruker, MLCT, half angle  $\theta \sim 20^\circ$ ) for their viscoelastic shear mod-

uli  $G'$  and  $G''$  at 37°C (same method as above). It was indeed important to find a relevant micro-environment for these cells, like in a real spheroid.

Spheroids were prepared in 15 $\mu\text{L}$  hanging droplets containing 5,000 T24 cells in culture medium including small amounts of collagen (initial concentration  $c_0 = 0.01$  mg/mL or 0.03 mg/mL) as described previously [20]. This method allows cells to accumulate ECM without having too much collagen around the spheroids, which would render AFM measurements more difficult. A spheroid was formed after 3 days. Spheroids were transferred into a Petri dish for AFM measurements at 37°C. A large tipless cantilever (Nanosensors, TL-NCL model, length 225  $\mu\text{m}$ , width 38  $\mu\text{m}$  [6]) was chosen for these measurements in force modulation mode, in order to make a plane-spherical contact. Viscoelastic data was obtained in the range [1Hz-200Hz]. The other spheroids were kept for confocal microscopy imaging.

**Properties of the collagen matrices.** – After collecting data from classical rheology and AFM, results were plotted on the same graph, as a function of frequency. Matching of the two types of experimental results was found to work nicely as previously shown for PolyAcrylamide samples [30]. Such results are shown in Fig. 1. A typical power-law behaviour [34] was found for both  $G'$  and  $G''$  at different collagen concentrations ( $c=1$  mg/mL, 2 mg/mL, 4 mg/mL) in the range [0.05Hz-10Hz] and both  $G'$  and  $G''$  increased faster in the high frequency range. Note that the measured values of the moduli were usually smaller as compared to cell’s values [6]. Finally fitting of the data was achieved using simple power-laws as described below.

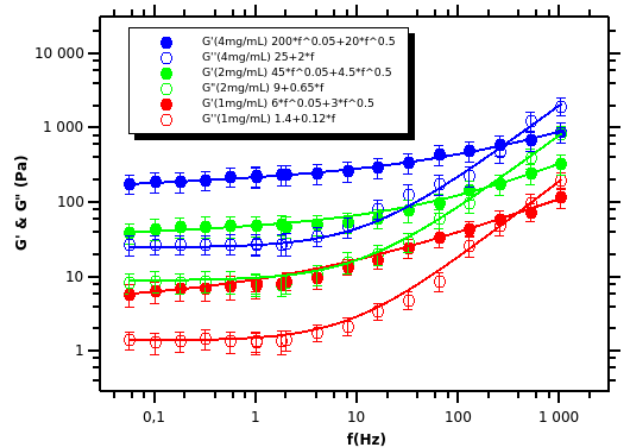


Fig. 1: Rheological properties of collagen matrices ( $c = 1\text{mg/mL}$ ,  $2\text{ mg/mL}$  and  $4\text{mg/mL}$ ). Data collected from classical rheology [0.05Hz-10Hz] and AFM [1Hz-1kHz]. Average of  $N=3$  measurements, error bars are Standard Errors of the Mean (SEM).

**Cell properties.** — To investigate cell rheology, it was necessary to consider cells in the proper environment as it is known that the micro-environment plays a role on their cytoskeleton organization, therefore changes cellular mechanical properties [6, 15]. Cells within a spheroid are surrounded by other cells and the ECM therefore it is convenient to use such a micro-environment [13]. Unfortunately, using AFM, it is hard to have access to the cell mechanical properties within the tissue, unlike when using other techniques such as bead tracking microrheology [35]. Here we preferred to use cell properties (T24 epithelial bladder cancer cells) obtained when in contact with similar cells, i.e. in the real spheroid. Fig. 2 below represents the  $G'$  and  $G''$  moduli obtained for cells located on the spheroid's periphery, as suggested before [13]. This is the closest representation of the micro-environment experienced by cells in a spheroid. The frequency range was chosen from 1 Hz to 600 Hz using three points per decade. The cell elastic modulus  $G'$  exhibits a frequency-dependent modulus with a slope  $\sim 0.17$  at small frequencies (see modeling below), increasing faster at larger frequencies. As for the  $G''$  modulus, it is similar to  $G'$  but showing different slopes [12].

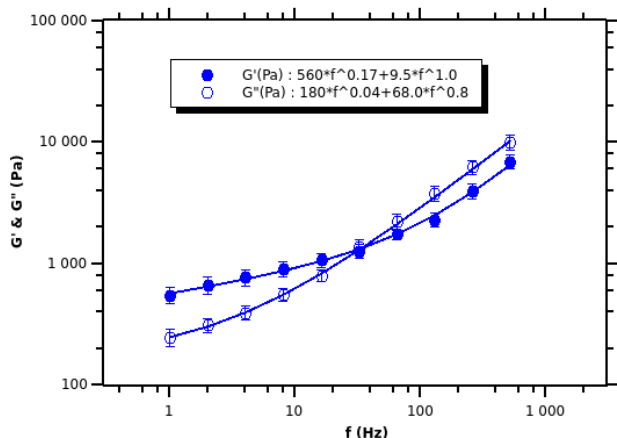


Fig. 2: Rheological data of a T24 cell, measured at the spheroid surface, as a close representation of the cell's environment. Average of  $N=20$  measurements, error bars are Standard Errors of the Mean (SEM),  $c_0=0.01\text{mg/mL}$ .

**Two-phase model with interfacial tension.** — Our starting point is Palierne's seminal work published in the early 90s [36, 37]. This two-phase model designed for polymeric or viscoelastic systems requires the knowledge of complex shear moduli obtained for small deformations, i.e. in the linear regime. The model has been used mainly for two-phase polymeric systems (see for example the works on polymer blends [38]). Nevertheless it may be used for materials such as gels or cellular media exhibiting known viscoelastic properties.

**Emulsion model.** Here the medium, i.e. the collagen gel, has a characteristic complex modulus  $G_m^*(\omega)$  whereas the inclusions (cells) have modulus  $G_i^*(\omega)$ . The volume fraction of the inclusions is  $\phi$  but different inclusions may be considered with different sizes and concentrations (in case of heterogeneous sizes). Here we assumed that only cells with a typical radius  $R$  coexist, which is usually the case since cell size is roughly constant. In this case,  $G^*(\omega)$ , the average modulus of the spheroid (i.e. cells embedded in the collagen matrix) in the case of non-diluted emulsions [36] is written as:

$$G^*(\omega) = G_m^*(\omega) \frac{1 + 3\phi H^*(\omega)}{1 - 2\phi H^*(\omega)} \quad (1)$$

where  $H^*(\omega) =$

$$\frac{\frac{4\alpha}{R} [2G_m^*(\omega) + 5G_i^*(\omega)] + [G_i^*(\omega) - G_m^*(\omega)]D^*(\omega)}{\frac{40\alpha}{R} [G_m^*(\omega) + G_i^*(\omega)] + [2G_i^*(\omega) + 3G_m^*(\omega)]D^*(\omega)} \quad (2)$$

and we introduced the complex modulus  $D^*$ :

$$D^*(\omega) = 16G_m^*(\omega) + 19G_i^*(\omega). \quad (3)$$

In this formula,  $\alpha$  is the interfacial tension and may be considered here as an interaction energy per unit area between cells and the matrix. It will be significant when cells adhere a lot to the matrix or are able to make a sufficiently large number of bonds. This may be the case for high collagen content. Note that for the case of dilute emulsions, Eq.(1) is different and should be replaced by  $G^*(\omega) = G_m^*(\omega)(1 + \frac{5}{2}\phi H^*(\omega))$ . This allows to recover the usual Einstein's formula [39] for two Newtonian fluids.

To summarize, we could use this model based on the cell properties  $G_i^*(\omega)$  determined previously and the gel properties  $G_m^*(\omega)$ , known for different collagen I concentrations. Note that the collagen concentration  $c$  (in mg/mL) is unknown within the spheroid, and similarly  $\phi$ , the cell volume concentration is to be determined.  $\phi$  should be large since most of the spheroid is made of cells, and one could expect the ECM content to be possibly in the range 1-15% [5], assuming that cells also make their own matrix. The other adjustable parameters are  $\alpha$ , the adhesion energy per unit area (in N/m), and  $R$  the cell radius, but the latter one is known since such T24 cells usually have a radius of  $10 \pm 1 \mu\text{m}$ .

The set of Eqs (1)-(2)-(3) being rather hard to read, we first attempted to study a simplified problem by noticing that collagen moduli  $G_m'$  and  $G_m''$  were much smaller (an order of magnitude or more) than the corresponding cell moduli  $G_i'$  and  $G_i''$ . Therefore, we could neglect the viscoelastic part in Eq (1). This would imply that  $D^*(\omega) \sim 19G_i^*(\omega)$  and  $H^*(\omega) \sim \frac{1}{2}$  therefore  $G^*(\omega) \sim G_m^*(\omega) \frac{1+1.5\phi}{1-\phi}$ . So  $G^*(\omega)$  scales with the matrix complex modulus  $G_m^*(\omega)$ , with the prefactor  $\frac{1+1.5\phi}{1-\phi}$ . Note that a small collagen content like 5% gives  $\phi = 0.95$  therefore a prefactor of 48.5. Values of the prefactor are

shown in Table 1 below. This shows that even though the collagen modulus  $G_m^*(\omega)$  is rather small (Plateau of  $G'$  around 200 Pa), as compared to cell's moduli (plateau of  $G'$  at 500 Pa), the amplification due to spherical inclusions such as cells could lead to the high values obtained in spheroids [20]. The simplified formula also originates from analysis of the effect of the adhesion energy  $\alpha$ . If  $\alpha/R$  becomes very small as compared to the other moduli (and collagen moduli are neglected vs. inclusions moduli) then the function  $H^*(\omega)$  again becomes independent of  $\alpha$  and close to  $1/2$ .

$\phi$	0.05	0.1	0.3	0.5	0.7	0.9	0.95
$\frac{1+1.5\phi}{1-\phi}$	1.13	1.28	2.07	3.5	6.83	23.5	48.5

Table 1: Values of the prefactor

In what follows, calculations are made by accounting for all terms in the set of Eqs (1)-(2)-(3) without simplification. We will therefore consider the effect of all parameters and consequently check the influence of  $G_m^*(\omega)$ ,  $\phi$ ,  $\alpha/R$  upon the numerical results, while  $G_i^*(\omega)$  remains fixed (Fig. 2).

*Application to the rheology of spheroids.* We have at our disposal the data from a previous study where collagen was added when making spheroids [20]. Two initial concentrations  $c_0$  were used: 0.01 mg/mL and 0.03 mg/mL. This does not mean that the local collagen content ( $c$ ) is the same when the spheroids are formed. But it seems that cells are able to aggregate collagen around them in a more concentrated form, as already observed [28]. Indeed spheroids were prepared in hanging droplets containing collagen; cells used it to adhere and spheroids exhibited a round shape. The same T24 cells were used for this study. Spheroids radii were  $R=140.6 \mu\text{m} \pm 17.6 \mu\text{m}$  ( $c_0=0.01$  mg/mL) and  $R=144.8 \mu\text{m} \pm 23.3 \mu\text{m}$  ( $c_0=0.03$  mg/mL) as compared to  $R=120.2 \mu\text{m} \pm 24 \mu\text{m}$  when no collagen was used. Fig. 3 shows typical confocal microscopy images of such spheroids obtained using the two initial collagen contents:  $c_0 = 0.01$  mg/mL and 0.03 mg/mL. As shown in the red channel, the reflectance of collagen was enhanced at higher collagen content, thus allowing to check the presence of collagen inside the spheroid. Thus we postulated that the collagen content can be high within the spheroid, probably between 1 and 4 mg/mL. Cells (green channel, transfected with the LifeAct plasmid expressing actin-GFP) seemed to form nice round spheroids except for the first case where no extra collagen was added.

We will now model the different phases of the spheroid. The first one is collagen. Using the previous data in Fig. 1, it is found that the collagen behaviour versus frequency  $f$  varies according to power laws. Fits of our previous data give the respective formulae for the three contents, as also shown in Fig. 1. For  $c = 4$  mg/mL, we find  $G'(f) = 200 * f^{0.05} + 20 * f^{0.5}$  and  $G''(f) = 25 + 2 * f$ . For  $c = 2$  mg/mL,  $G'(f) = 45 * f^{0.05} + 4.5 * f^{0.5}$  and  $G''(f) = 9 + 0.65 * f$ , and finally the lowest collagen content,  $c = 1$

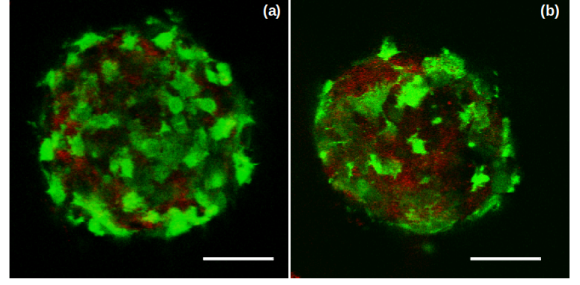


Fig. 3: Confocal microscopy images of spheroids prepared using T24 cells, at different initial collagen contents (a)  $c_0 = 0.01$  mg/mL, (b)  $c_0 = 0.03$  mg/mL. Scale bar 100  $\mu\text{m}$ .

mg/mL, gives  $G'(f) = 6 * f^{0.05} + 3 * f^{0.5}$  and  $G''(f) = 1.4 + 0.12 * f$ , where  $f$  is in Hz, and  $G'$ ,  $G''$  are in Pascals (Pa). Remarkably, these power law exponents are independent of the concentration, so only the prefactors are different.

Similarly, we fitted the viscoelastic response of T24 cells, as shown in Fig. 2. The moduli behave as  $G'(f) = 560 * f^{0.17} + 9.5 * f^{1.0}$  and  $G''(f) = 180 * f^{0.04} + 68 * f^{0.8}$ . T24 cells moduli exhibit a small slope at the lowest frequencies then the slope increases at higher ones.

Let us consider the effects of the main parameters. Regarding modulus  $G_i^*$ , cells are assumed to have the behaviour depicted in Fig. 2 so this modulus is fixed. Then the values of the collagen modulus  $G_m^*$  can be changed depending on its content ( $c$  in mg/mL) within the spheroid (see Fig. 1). Finally, the roles of the cell content ( $\phi$ ) and adhesion energy per unit area ( $\alpha/R$ ) will be studied.

*Effect of collagen matrix within the spheroid.* Here we consider the case  $\phi = 0.8$  which corresponds to typical cell volume concentration, as seen for example in Fig. 3. For this case, we used  $\alpha = 10.0$  mN/m with  $R = 10 \mu\text{m}$ . Plots of  $G'$  and  $G''$  are shown in Fig. 4 for collagen matrix concentrations 1, 2 and 4 mg/mL.

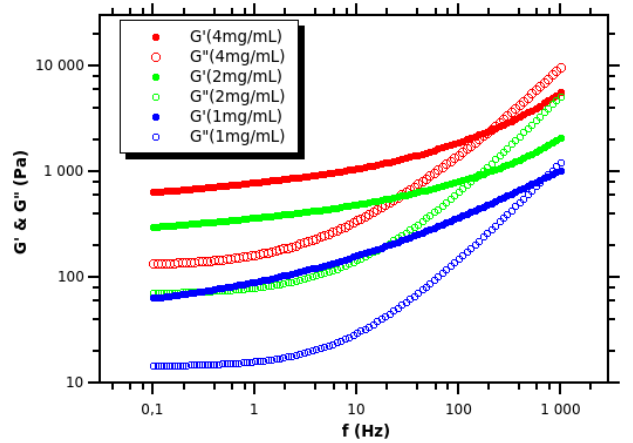


Fig. 4: Predictions of the two-phase emulsion model for 80% cell volume concentration,  $\alpha/R=10^3$  Pa, at collagen contents  $c = 1$  mg/mL, 2 mg/mL and 4 mg/mL.

As expected, spheroids moduli increase with collagen content, from 1 to 4 mg/mL. It is found that the resulting spheroid properties are in the range of the data previously reported [20], and this will be studied further below.

*Role of cell concentration.* Next we studied the role of cell concentration. Usually, in spheroids, cells are closely packed but the presence of collagen, as seen in confocal microscopy (Fig. 3) suggests that the intercellular spacing can be modified. It has been estimated that the ECM content can sometimes reach  $\sim 15\%$  in cases where cells make their own ECM [5]. Therefore, we selected typical cell concentrations between  $\phi = 0.8$  and  $\phi = 1$ . For this case we used the intermediate collagen content ( $c = 2\text{mg/mL}$ ) and  $\alpha/R=10^3$  Pa. Simulations are shown in Fig.5. It can be concluded that cell concentration has an important effect on the results, especially due to the large difference between matrix and cell's moduli.

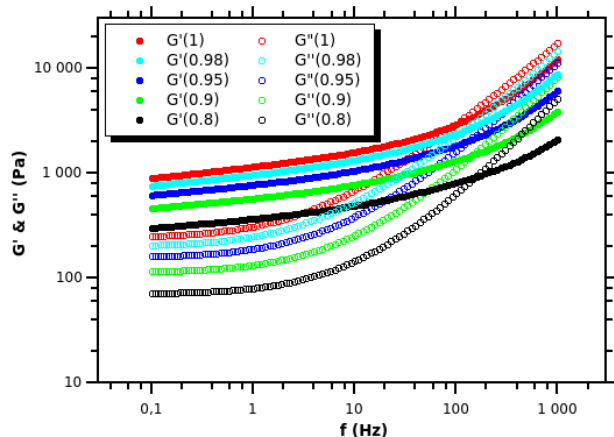


Fig. 5: Predictions of the two-phase emulsion model for various cell concentrations  $\phi = 0.8, 0.9, 0.95, 0.98, 1$ , using  $\alpha/R=10^3$  Pa, and collagen content  $c = 2$  mg/mL.

*Effect of adhesion energy between cells and the matrix.*

Regarding the effect of the adhesion energy, we used a large range of  $\alpha$ -values from 0.3, 3, 30, and 300 mN/m at a cell density  $\phi = 0.9$ , and medium collagen content properties ( $c = 2\text{mg/mL}$ ). This is shown in Fig. 6. The choice of  $\alpha$  was justified by previous AFM experiments [40] investigating the work of de-adhesion  $\sim 10^{-15}\text{J}$  between cells and their receptors, on corresponding surface areas  $\sim 1\mu\text{m}^2$ , leading to adhesion energies around 1 mN/m, depending on area, number of bonds, loading rate, etc.

To summarize results from Fig. 6, it appears that the role of  $\alpha/R$  is important, for the chosen parameters and the values of the current viscoelastic parameters. This role becomes more important at lower frequencies where it was found that higher values of the adhesion energy increased the values of  $G'$  and  $G''$ , in particular enhancing the formation of a plateau (in  $G'$ ) at low frequencies. Increasing the adhesion energy also increased the transition frequency

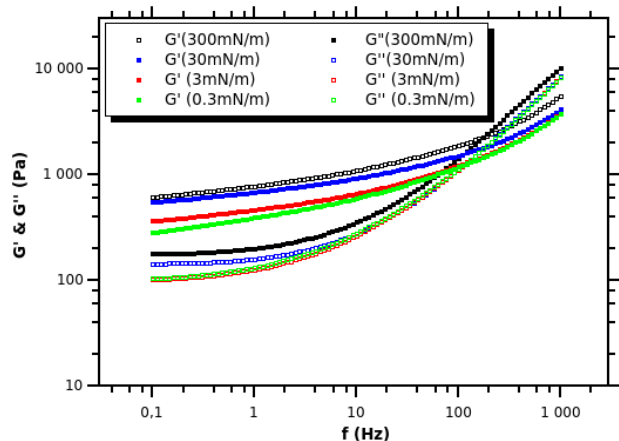


Fig. 6: Predictions of the two-phase emulsion model for various adhesion energies  $\alpha = 0.3\text{-}3\text{-}30\text{-}300$  mN/m, with  $R = 10$   $\mu\text{m}$ , collagen content  $c = 2$  mg/mL, and  $\phi = 0.9$ .

(crossing between  $G'$  and  $G''$ ). Thus, the more important the adhesion, the more elastic the spheroids became.

*Optimizing emulsion model parameters for real spheroids.* Finally, real spheroid properties were considered within the [1Hz-200Hz] range as previously described using AFM microrheology in plane-sphere contact [20]. Measurements were carried out in a manner similar to the one proposed on tissues [21]. Measurements display typical slopes as a function of frequency. To fit the data, parameters were optimized as follows. We chose the best collagen concentration between available values 1, 2 or 4 mg/mL, optimized  $\phi$  and  $\alpha/R$  using the least squares method. Results are shown in Fig. 7. Fits were in very good agreement with experimental data.

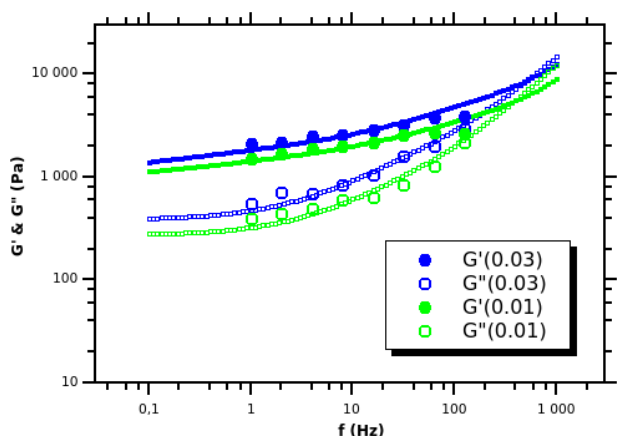


Fig. 7: Spheroids properties at  $c_0 = 0.01$  mg/mL and 0.03 mg/mL [20]. Optimized fits with parameters from Table 2.

Table 2 summarizes these results, at a fixed value  $R = 10$   $\mu\text{m}$ . For both cases  $c_0 = 0.01$  and 0.03 mg/mL,

$c_0$ (mg/mL)	0.01	0.03
$c$ (mg/mL)	4	4
$\phi$	$0.88 \pm 0.01$	$0.94 \pm 0.01$
$\alpha$ (mN/m)	$59 \pm 3$	$81 \pm 3$

Table 2: Optimal parameters ( $R = 10\mu\text{m}$ ).  $\phi$  and  $\alpha$  optimized by least squares curve fitting with errors.

the optimal collagen concentration  $c = 4$  mg/mL seemed to be relevant, and the cell volume fraction was in the range of that measured experimentally using confocal microscopy  $\phi \sim 0.82$  and  $0.90$  respectively (data not shown). Finally cells seemed to be more densely packed in the second case ( $c_0 = 0.03$  mg/mL), meaning that collagen plays the role of an interstitial layer enhancing the microstructure as well as the viscoelastic properties. This is confirmed by a higher value of the adhesion energy  $\alpha = 81 \pm 3$  mN/m ( $c_0 = 0.03$  mg/L) whereas it is only  $59 \pm 3$  mN/m at  $c_0 = 0.01$  mg/L. Further calculations are provided in the Supplementary Information (SI) on the sensitivity of the method with respect to parameters  $c$ ,  $\phi$  and  $\alpha$ .

**Discussion.** – The rheological properties of spheroids are of great importance in order to understand how microstructural changes can affect mechanics, possibly giving rise to tumour invasion and the formation of metastases. Furthermore, their growth is also a topic of major interest. Tumours grow and exert pressure on the environment through a process limited by the surrounding medium [41]. Earlier models considered spherical growth depending on the nutrients, but more sophisticated ones now use numerical tools to evaluate hoop stresses inside and at the periphery of the growing spheroid [5, 42, 43]. Interesting studies have shown the effect of collagen on such processes, in particular the environment microstructure can become the signature of cancer progression and prognosis [44–46]. In this work, we investigated the role of the ECM on the viscoelastic properties of spheroids grown during 3 days [20], in culture medium containing an excess of collagen I (initial concentrations  $c_0 = 0.01$  or  $0.03$  mg/mL). Using a model particularly well adapted for viscoelastic behaviors, we studied the possible effects of ECM density, cell concentration and adhesion. This was assessed by modeling frequency-dependent individual cell and collagen properties (Figures 1-2). Based on these properties, we used the two-phase emulsion model [36, 37] able to recover the spheroid viscoelastic data.

Collagen has been shown to be a linker within spheroids using confocal microscopy (Fig. 3). Furthermore, we could also analyze its role on the overall spheroid’s rheology as its concentration increases, using the two-phase emulsion model. It was shown that a high collagen content ( $c$  varying from 1 to 4 mg/mL) enhanced the viscoelastic properties (Fig. 4). Another parameter, cell concentration ( $\phi$ ), was seen to have a quite significant contribution to reinforce viscoelastic properties as well (Fig.5). Indeed, the simplified Eq. (1) already showed the importance of cell

packing. With the complete model, this effect still remains. Finally the role of the adhesion energy  $\alpha$  (between cells and the matrix) was found to be significant, in particular when  $\alpha/R$  becomes comparable with the other moduli, i.e. cell ( $G_i^*$ ) and collagen ones ( $G_m^*$ ). This was particularly noticeable at the lowest frequencies used in the experiments (Fig.6). A more elastic behavior (higher elastic plateau and higher transition frequency) was observed for high values of the adhesion energy  $\alpha$ . This was also found in works dealing with polymer emulsions [38], reporting the relevance of  $\alpha$  at lower frequencies, when the terminal relaxation time scales with interfacial tension.

After analyzing two different spheroids [20], prepared with various initial collagen contents ( $c_0 = 0.01$  mg/mL or  $0.03$  mg/mL) we used the emulsion model to optimize the parameters and came up with the best possible fits (Fig. 7 and Table 2). The results of the model were found to be in very good agreement with the experiments. This motivates further discussion in view of the parameters found. Indeed the packing of cells within the spheroid seems to be linked with the presence of collagen forming a dense meshwork, enabling cells to connect with each others, using adhesion molecules like cadherins [47]. The optimal adhesion energy increased from 59 to 81 mN/m, when increasing  $c_0$  (see Table 2). In addition, an increase in the initial collagen content was found to enhance cell packing (higher value of  $\phi$ ) – which was rather unexpected – this giving rise to higher viscoelastic moduli (see Figs 1-2). Altogether, the model, when used as an inverse method, provides good estimations of parameters  $\phi$ ,  $\alpha$  and  $c$ . These parameters could not be measured independently, but  $\phi$  is in reasonable agreement with confocal images,  $c$  takes on realistic values, and similarly for  $\alpha$ .

The model presented here predicts a rich variety of viscoelastic properties and power-law dependencies. Hence it would be interesting to use it to study spheroids in details in the future, for example with various cell types or ECM leading to different microstructures [48]. As it is general enough [36], the model could also apply to multi-component spheroids, i.e. containing cells of different nature, size or viscoelastic properties. Regarding stiffness, such invasive T24 cells are known to be soft as previously described [8, 9], but applications to spheroids containing stiffer cells (muscle cells, cartilages) should be also possible since no further hypotheses are made. Finally it could be useful to monitor rheological properties during tumour growth [41–43]. To investigate this further, the associated microstructures will have to be minutely analyzed using confocal microscopy, in order to extract the basic relationships or forces involved in such interactions, in relation with the adhesion energy  $\alpha$ , depending on bond strength, number of bonds and loading rate [40]. Finally cells can pull on collagen to generate forces or remodel it [28]. This could lead to matrix stiffening at larger deformations, and could eventually modify spheroids properties.

**Conclusions.** – The rheology of spheroids has insufficiently been studied so far. Here we used previous viscoelastic data on spheroids measured with an AFM, as well as the individual response of the other components (i.e. cells and collagen). Results were combined in the framework of an emulsion model including interfacial energy. The results were in very good agreement with the experiments. They showed that the final spheroid microstructure contains dense collagen regions within the inter-cellular spacing. Finally an increased initial collagen concentration led to an enhanced compactness of the spheroids with a smaller inter-cellular spacing, and a larger adhesion energy. Thus collagen plays the role of a connecting adhesive layer between the cells and improves spheroid stability. With this model, future predictions should be possible regarding time-dependent spheroid growth.

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