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Viscoelastic properties in cancer: from cells to spheroids

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Abstract: AFM-based rheology methods enable the investigation of the viscoelastic properties of cancer cells. Such properties are known to be essential for cell functions, especially for malignant cells. Here, we investigate the relevance of these methods to characterize the viscoelasticity of bladder cancer cells of various invasiveness on soft substrates, and we find that rheology parameters are a signature of malignancy. We further investigate the viscoelastic moduli of spheroids composed of cancer cells and characterize the effect of the collagen microenvironment. Collagen is found to act as a proxy than can modify the global spheroid rheology, leading to an increase of moduli vs. frequency, predicted by a double power law model. Together, our results shed new light on how cancer cells and tissues adapt their viscoelastic properties depending on their malignancy and the microenvironment. This could be an attractive way to control their properties in the future, especially since spheroids constitute realistic *in vivo* tumor models.

Keywords: cancer cells; spheroids; AFM; viscoelastic; rheology; microenvironment

1. Introduction

Cell mechanical properties are essential in various processes like cell migration, embryogenesis, wound healing, the immune response, i.e. physiological processes but also in various pathological cases, in particular during cancer metastasis. Understanding how cell deformability can control the way cancer cells migrate or extravasate through blood vessels is essential. While it is well known that the elastic properties play a central role in such processes [1,2], the viscoelastic nature of the cell system is significant and has to be taken into account [3–5]. The many filaments (actin, microtubules and intermediate filaments) of the cell provide a rigid structure to the cell, thus an elastic response, and the surrounding fluid adds viscosity so that the overall properties become viscoelastic, similar to a physical gel, that can remodel constantly. This is indeed what is happening as the cell migrates, and F-actin filaments polymerize at the front to push the membrane forward, while the other actin monomers are recycled [6]. Several other studies have proposed a different behavior for the cell cytoplasm, where the fluid flow can affect the mechanical response and give rise to a poroelastic behavior [7,8]. It is therefore important to obtain experimental data regarding cell behavior under various mechanical solicitations such as constant force, prescribed deformation or rate of deformation. For this reason, new methods have been proposed to determine such properties, either locally using AFM [9,10], magnetocytometry [11], particle tracking measurements [12], or using global measurements thanks to microfluidics [13], micropipettes [14], optical tweezers [15] or optical coherence elastography [16].

While the mechanical properties of cancer at the cell scale has been extensively studied, less is known about the tissue level; therefore we aimed to understand how cellular properties combine and whether similar ones are retained at a higher scale. As could be expected, several features are preserved as classical tissues follow nonlinear elastic behaviors with a substantial role of the deformation rate [17]. Tissues are usually made of cells surrounded by the extra cellular matrix (ECM) inside a fluid and the

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40 concentrations of the different components depend on tissue type. Cell suspensions
41 have been described [18,19] using mixture models and exhibit a behavior close to that
42 of yield stress fluids, when the cell concentration is around 50% or more. On the other
43 end, with a large collagen content [20] and a small amount of cells (around 10%), the
44 behavior becomes viscoelastic but the effect of cells on the ECM is critical, since cells are
45 able to remodel the collagen network.

46 To investigate other biological tissues – in particular solid tumours – spheroids have
47 been developed over the years [21] and are considered to be good models. They are made
48 of cells closely packed together, surrounded by ECM in culture medium. Spheroids can
49 grow and make their own ECM, and cell adhesion molecules (cadherins in particular)
50 can form to bind cells within the spheroid afterwards. Generally tumours grow, but
51 this depends on the environment. Indeed, several earlier studies have focused on the
52 role of compressive stresses exerted from the outside onto the tumour [22,23], this effect
53 being to limit growth. Less results are devoted to the understanding of the mechanical
54 behavior of such spheroids, but recent models have focused on the flow of the interstitial
55 liquid within the spheroid [21] and reveal that poroelastic active models can describe
56 the spheroid [24]. These effects have also been observed on other tissues like cartilage
57 or tendons revealing notable high-frequency poroelastic responses [25,26], that can be
58 used to differentiate physiological and pathological tissues. Although these systems are
59 different, possible correlations can be made.

60 In this work, we choose to use AFM as an interesting device to probe both single
61 cancer cells and spheroids made of the same cells. We developed a microrheology
62 method based on initial indentation followed by oscillations at various frequencies [5,27],
63 to probe the viscoelastic properties of cells and spheroids. The method is presented
64 in § 3 together with modeling tools, and is used further on different cells in different
65 environments (§ 4). Then we adapt it to spheroids and obtain similar data (§ 5), but we
66 note that some differences do exist: viscoelastic properties of both cells and spheroids
67 depend on the microenvironment (substrate or collagen within the spheroid.) Therefore
68 we discuss these results (§ 6) and try to determine the key features that are relevant in
69 both cases (cells or spheroids), and how the differences obtained can be explained, based
70 on complementary confocal microscopy experiments.

71 2. Materials and methods

72 2.1. Cancer cells

73 Three epithelial bladder cancer cell lines with increasing malignancy were used:
74 RT112 (luminal molecular subtype, Leibniz Institute DSMZ, Braunschweig, Germany),
75 T24 and J82 (American Type Culture Collection, Manassas, VA). RT112 is a moderately
76 differentiated cell line that exhibits low invasiveness, while T24 and J82 are both poorly
77 differentiated and very malignant cells, with J82 being the more invasive type. The
78 choice of such bladder cell lines came from earlier studies by the authors [28–31] and the
79 classification method was described previously [27]. These cells were cultured in RPMI
80 1640 (Gibco, Saint-Aubin, France) supplemented with 10% fetal bovine serum (FBS, Life
81 Technologies SAS, Villebon-sur-Yvette, France) and 1% penicillin–streptomycin (Life
82 Technologies SAS). One day before measurements, cells were seeded at low density
83 on polyacrylamide gels coated with 20 mg/mL fibronectin (PromoCell, Heidelberg,
84 Germany) overnight at 37°C in a humidified 5% CO₂ atmosphere. These cells are
85 considered to adhere firmly on such gels. AFM measurements were carried out on
86 isolated cells at 37°C. These cells were stably transfected with a plasmid expressing
87 LifeAct–GFP to stain F-actin according to previous work [32].

88 2.2. Polyacrylamide gels

89 Polyacrylamide gels of different rigidities were prepared prior to seeding of the
90 cells using a previous protocol [27]. Briefly, acrylamide, N-hydroxyethyl-acrylamide
91 and N,N-methylene-bisacrylamide (Sigma-Aldrich) in different amounts were mixed to

92 reach controlled rigidities of 5, 8 and 28 kPa, as measured by AFM. Functionalization
93 was achieved using fibronectin (20 mg/mL). Gels were kept under humid conditions
94 before cells were seeded.

95 2.3. Spheroids preparation

96 Spheroids can be made of various cells inside an extracellular matrix and are
97 reported to be good candidates for modelling tumors. This is why the same T24 cells
98 as above were selected to prepare them. As before, T24 cells were cultured in RPMI
99 1640 medium supplemented with 10% FBS and 1% penicillin–streptomycin. 96–well
100 plates were filled with a 1.5% sterile agarose solution (Abnova, Fischer Scientific, Illkirch,
101 France) which solidified quickly at room temperature. Then 10,000 cells were added
102 into the well. Finally, type I rat tail collagen (Corning, Bedford, MA, USA) at 0.01 or
103 0.03 mg/mL concentration (4°C and pH ~ 7.4) was added to the medium. The 96–well
104 plate was centrifugated for 10 min at 200 g. Cells remained at the bottom of the well and
105 were followed in time. After 3 days, spheroids usually presented a spherical compact
106 shape. Experiments were conducted then, with spheroid diameter D ranging between
107 270 and 400 μm . Spheroids were detached with a 100 μL –pipette (chopped nozzle) and
108 deposited into Petri dishes (TPP, to fit within the AFM setup) in culture medium. Up to
109 three spheroids could be measured at the same time. After sedimentation and proper
110 adhesion to the Petri dish bottom, the AFM cantilever was lowered to come into contact
111 with the top of the spheroid and measurements were carried out as explained below (§
112 3.2).

113 2.4. Confocal microscopy of spheroids

114 Confocal imaging of spheroids containing 0.01 mg/mL collagen was carried out
115 using a confocal Leica TCS SP8 (LIPhy platform). Cells with F–actin GFP labelling were
116 imaged in the green channel (argon laser, 488 nm) and collagen was imaged using the
117 reflectance technique in the red channel (HeNe laser, 633 nm) [20,33]. The spheroids
118 were prepared as described earlier, and let to sediment in a Petri dish (with a 170 μm –
119 glass coverslip bound to the bottom). Images were taken (X40 Leica oil–objective) from
120 the bottom to the median part, as it is difficult to penetrate deeper than 200 μm . Z–
121 stacks were acquired using steps of 0.35 μm . Then images were processed using the Fiji
122 software.

123 2.5. Statistical analysis

124 Statistical analysis was measured using a two-sample unpaired Student's t-test. Sta-
125 tistical significance was reached for * $p < 0.05$ and ** $p < 0.01$ while $p > 0.05$ was considered
126 non-significant. Means are presented with the SEM (standard error of the mean).

127 3. Microheology using AFM

128 3.1. Principles of viscoelasticity

129 The main idea behind viscoelasticity comes from the combined properties of soft
130 systems under applied deformations or forces. When the system is elastic, stresses (i.e.
131 forces per unit surface, in Pa) are proportional to deformations, whereas a viscous mate-
132 rial will flow under applied force, with stresses proportional to the rate of deformation.
133 In order to capture both viscous and elastic responses, one can apply a sinusoidal shear
134 deformation $\delta = \delta_0 \sin(\omega t)$, where δ_0 is the amplitude of the deformation, and $\omega = 2\pi f$
135 is the angular frequency (f is the frequency in Hz). At small enough deformations δ_0 ,
136 one can assume – and this needs to be checked – that the shear stress response is also
137 periodic in time $\tau(t) = \tau_0 \sin(\omega t + \phi)$ at the same angular frequency, where τ_0 is the
138 stress amplitude and where ϕ is a phase shift. Thus one can extract the components
139 of stress in phase ($\phi = 0$) and phase opposition ($\phi = \pi/2$), i.e. the moduli G' (elastic)
140 and G'' (viscous or loss) which appear as the coefficients in front of $\sin(\omega t)$ and $\cos(\omega t)$
141 terms. It is found that $G' = \tau_0 \cos\phi / \delta_0$ and $G'' = \tau_0 \sin\phi / \delta_0$. The resulting complex

142 $G^* = \frac{\sigma^*}{\delta^*} = G' + iG''$ is called the complex shear modulus and the loss angle ϕ is defined
 143 by $\tan\phi = \frac{G''}{G'}$, as the ratio of viscous to elastic stresses. Given these basic principles,
 144 let us now consider a real experiment based on the use of an Atomic Force Microscope
 145 (AFM).

146 3.2. Oscillations using an AFM

147 AFM experiments were carried out using a Nanowizard II AFM (JPK Instruments)
 148 mounted on a Zeiss microscope (Observer D1). AFM is based on the use of a tip placed
 149 on a cantilever that can be set in contact with a surface to investigate the forces due to
 150 the interactions between the tip and the substrate. The laser reflected by the cantilever
 151 onto a photodiode gives the tip displacement and therefore the force thanks to proper
 152 calibration. Calibration was performed here for the sensibility parameter ($s \sim 30\text{--}80$
 153 nm/Volt) then using the thermal fluctuation method [34] to finally obtain the cantilever
 154 stiffness (k in N/m). In this study, we used soft cantilevers (MLCT, lever C, Bruker,
 155 Billerica, CA) with stiffness on the order $k \sim 0.01$ N/m for cells, whereas for spheroids
 156 stiffer cantilevers (Nanosensors, TL-NCL model) were favored, with $k \sim 30$ N/m. The
 157 basic idea is to perform an initial indentation δ_0 then to superimpose small sinusoidal
 158 deformations δ at a given frequency f . For cells, we start from the Sneddon relationship
 159 [35] for pyramidal tips, where the applied force F_0 is related to the indentation δ_0 by:
 160 $F_0 = \frac{3}{4} \frac{E \tan\theta \delta_0^2}{1-\nu^2}$, where E is the elastic Young modulus, ν is the Poisson ratio ($\nu \sim 0.5$),
 161 and θ is the half-pyramid angle. This relationship is then linearized (assuming $\delta \ll \delta_0$),
 162 to obtain:

$$G^*(\omega) = G' + iG'' = \frac{1-\nu}{3 \tan\theta \delta_0} \left\{ \frac{F^*(\omega)}{\delta^*(\omega)} - i\omega b(0) \right\} \quad (1)$$

163 The final calibration regards the hydrodynamic drag created by oscillations of the
 164 cantilever in a fluid: the drag is proportional to viscosity and velocity but also depends
 165 on geometry. Here the geometry of the cantilever is different each time, so the best way
 166 to calibrate it is to oscillate far from the substrate (given distance h) then come closer to
 167 it ($h \sim 0$). The drag force is a pure imaginary number $F^*/\delta^* = i\omega b(h)$, where $b(h)$ is to
 168 be found. Using the method suggested by Alcaraz *et al.* [36], we found that $b(0)=6.95$
 169 10^{-6} N.s/m for the MLCT cantilevers that we used in a typical culture medium.

170 In the case of spheroids, another method was proposed using large tipless can-
 171 tilevers [37] indenting the spherical aggregate. The previous formula is then adapted
 172 from Hertz: $F_0 = \frac{4}{3} \frac{E \sqrt{R} \delta_0^{3/2}}{1-\nu^2}$ and one finds, after linearization:

$$G^*(\omega) = G' + iG'' = \frac{1-\nu}{4(R\delta_0)^{1/2}} \left\{ \frac{F^*(\omega)}{\delta^*(\omega)} - i\omega b(0) \right\} \quad (2)$$

173 where R is the spheroid radius, and the other parameters remain unchanged. In
 174 that case the hydrodynamic drag calibration gave $b(0)=3.45 \cdot 10^{-5}$ N.s/m.

175 Therefore, using this method, it is possible to obtain the viscoelastic data (G', G'')
 176 over a large range of frequency ranging from 1 Hz to 1 kHz.

177 3.3. Substrate effects

178 Investigating cells in a complex environment demands to evaluate the mechanical
 179 properties of the surrounding media or tissues. Typically values of soft tissues (excluding
 180 bones) range from 50 Pa to 20 kPa. Therefore, our studies focused on such media. When
 181 using AFM on cells plated on a soft/rigid substrate, it is important to account for its
 182 rigidity. Indeed, the cell apparent modulus is overestimated on a rigid substrate but
 183 can be estimated for different cantilever geometries [38,39]. On the other hand, less is
 184 known on soft substrates where the apparent modulus can be underestimated as shown
 185 recently [27,40,41]. This is precisely what has been done prior to this study. The details
 186 of the calculations can be found in our previous work [27]. Corrections make use of an

187 equivalent medium made of the glass substrate covered by a thick gel on top of which
 188 cells are deposited. This medium is described by a simple apparent modulus [42], and
 189 equation (1) is modified as shown previously [27] to obtain the corrected moduli (G' ,
 190 G'').

191 3.4. Rheological model

192 Different authors have tried to model viscoelastic data. Previous studies report that
 193 polymers or complex materials [43,44] exhibiting wide relaxation spectra have power
 194 law dependence, i.e. moduli G' and G'' vary with a certain power of the frequency
 195 (f). For example, it was reported that cells have moduli (G', G'') varying with the same
 196 small exponents $\sim 0.1 - 0.3$, and behave as Newtonian fluids at higher frequencies
 197 [10]. Nevertheless a single exponent is usually not enough to cover the whole frequency
 198 range, therefore it is better to define different frequency domains with different exponents
 199 [5,27,45]. This is what is done here. Assuming that both moduli exhibit two different
 200 behaviors according to the frequency range, as shown in Figure 1, a double power-law
 201 behavior can be assumed, of the following form:

$$G' = G_0 \left(\frac{f}{f_0}\right)^a + G_1 \left(\frac{f}{f_0}\right)^b, \quad G'' = G_2 \left(\frac{f}{f_0}\right)^c + G_3 \left(\frac{f}{f_0}\right)^d \quad (3)$$

202 Note that we used $f_0 = 1$ Hz and f is expressed in Hz. By making this dimensionless
 203 reduction, we can get rid of complex units. Indeed G_0, G_1, G_2, G_3 are simply expressed
 204 in Pascals (Pa), whereas exponents a, b, c, d are dimensionless. We note further that:

- 205 • A plateau for G' is sometimes obtained at low frequencies (see Figure 3A below) so
 206 that $a = 0$, and G_0 is the so-called elastic plateau modulus [5].
- 207 • A single power law for G' can be enough to describe the data (see Figure 3B for
 208 instance), then $G_1 = 0$.
- 209 • Note that for low frequencies, when the second term is negligible, $G' \sim G_0 \left(\frac{f}{f_0}\right)^a$
 210 and $G'' \sim G_2 \left(\frac{f}{f_0}\right)^c$ so that $G_0 \sim G'(1\text{Hz})$ and similarly $G_2 \sim G''(1\text{Hz})$
- 211 • We also recover most of the cases studied before, in particular Alcaraz *et al.*[10]
 212 found $G_1 = 0, a = c$ and $d = 1$ for single cells.

213 4. Results on live cancer cells

214 4.1. Cell viscoelastic properties

215 The viscoelastic properties of the three different cell lines with increasing inva-
 216 siveness (RT112 < T24 < J82) were measured using AFM as described in § 3.2. The
 217 measurements were located above the cell nucleus with a maximum indentation of the
 218 AFM tip of 500 nm. Cells were adherent on gel substrates of different rigidities and the
 219 contribution of the substrate to the viscoelastic properties was corrected as described in
 220 § 3.3 ensuring that only the microrheology of the cells is characterized. This correction
 221 is essential as it allows the comparison between different cell lines. Figure 1 shows the
 222 elastic modulus G' (black) and the loss modulus G'' (red) of cells that were adherent on
 223 a soft gel of 8 kPa (which is similar to the stiffness of the endothelial monolayer [27]).
 224 First, one can observe that the viscoelastic properties follow a common behavior with
 225 G' and G'' increasing with frequency with the ratio of G''/G' when the invasiveness
 226 increases, suggesting an effect of the invasiveness on the microrheology of cancer cells.
 227 Moreover, the elastic modulus of the less invasive cells RT112 reveals a plateau modulus
 228 at the lower frequencies (Figure 1A), which is not present for T24 and J82 (Figure 1B-C),
 229 indicating that invasiveness has the effect of making cells less elastic.

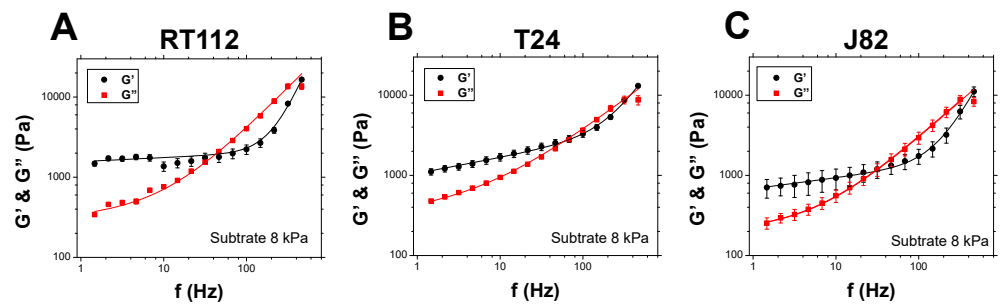


Figure 1. Viscoelastic moduli of RT112, T24 and J82 cells on a **8kPa substrate**. The elastic modulus G' (black) and the loss modulus G'' (red) are shown as a function of frequency f . These measurements were done above the cell nucleus. The lines correspond to the model in Eq. (3), using (A) $G_0=1567$ Pa, $a=0.05$, $G_1=0.005$ Pa, $b=2.4$, $G_2=321$ Pa, $c=0.07$, $G_3=29.4$ Pa, $d=1.05$, (B) $G_0=1060$ Pa, $a=0.20$, $G_1=0.24$ Pa, $b=1.7$, $G_2=351$ Pa, $c=0.08$, $G_3=84$ Pa, $d=0.8$ and (C) $G_0=682$ Pa, $a=0.14$, $G_1=0.04$ Pa, $b=2$, $G_2=205$ Pa, $c=0.01$, $G_3=40$ Pa, $d=0.92$. $N = 5$, error bars represent the mean \pm SEM (standard error of the mean).

230 Quantitative parameters of cancer cell microrheology were then found using the
 231 model in Equation (3) and summarized in Figure 2 and Figure S1 (supplementary
 232 material). First, the elastic modulus G_0 decreases significantly between RT112 and J82
 233 (Figure 2A) while the slope a of G' increases (Figure 2B). At high frequency, G_1 of RT112
 234 increases significantly as compared to T24 and J82 (Figure S1A) while the slope b of
 235 G' decreases (Figure 2C). These results indicate that the less invasive cells are more
 236 elastic. In addition, the loss modulus G'' exhibits a similar dependency on the cancer
 237 cell invasiveness. Indeed, the slope c (Figure 2D, 8kPa) of the more invasive cells J82 is
 238 lower than RT112 and T24 and close to zero, revealing that the loss modulus follows a
 239 single power law.

240 These results demonstrate that the cell microrheology parameters can provide
 241 the signature of malignancy, with more invasive cell lines being less elastic and more
 242 deformable, which gives them the advantage to rapidly change their shape and migrate
 243 through endothelial barriers.

244 4.2. Comparison on various substrates

245 While the use of 8 kPa soft gels is biologically relevant in the context of metastasis,
 246 when cancer cells are in contact with the endothelium monolayer (having a similar
 247 rigidity [27]), it is also important to investigate the effect of the substrate rigidity on
 248 the viscoelastic properties. This is relevant to understand how cancer cells modify their
 249 micro-structure depending on the substrate. Here, we use polyacrylamide gels with
 250 calibrated stiffnesses of 5–8–28 kPa. The viscoelastic moduli were measured above the
 251 cell nucleus and corrected for the effect of the substrate, as mentioned above, and the
 252 microrheological parameters were determined using Equation (3) and are summarized
 253 in Figure 2.

254 These results reveal that invasive cells are sensitive to the substrate rigidity. Indeed,
 255 the rheology parameters of T24 and J82 exhibit significant changes with gel rigidity
 256 increase. In particular, G_0 increases while a and b (exponents at low and high frequencies
 257 respectively) decrease when the substrate becomes more rigid (Figure 2A-B-C). This
 258 suggests that the cells become more rigid when the substrate is stiffer. Interestingly,
 259 the less invasive cell line RT112 does not show a similar behavior, indicating that the
 260 more invasive cells are more sensitive to the substrate rigidity. However, the effect of the
 261 substrate is less pronounced on the loss modulus. Indeed, the model reveals that the
 262 exponent c of G'' (low frequencies) increases significantly for RT112 and J82 with the gel
 263 rigidity (Figure 2D).

264 Taken together, these results reveal that the mechanosensitivity of cancer cells to
 265 the substrate depends on their invasiveness.

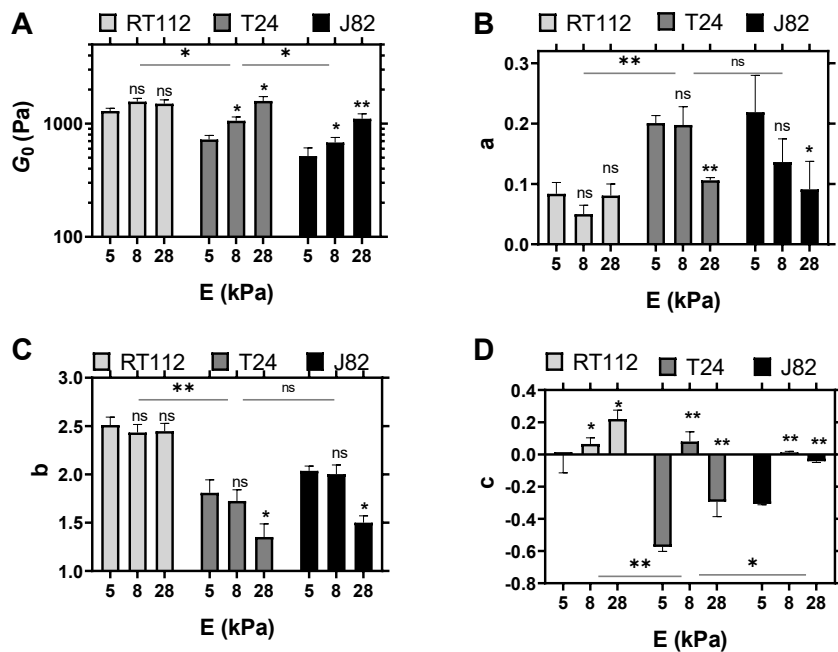


Figure 2. Parameters G_0 (A), exponent a (B), exponent b (C), exponent c (D) for RT112, T24 and J82 cancer cells on different gels ($E=5-8-28$ kPa). The parameters were extracted from fitting averaged G' and G'' for each condition with Eq. (3). Three different adjustments were performed and the means of the fitted parameters were obtained. Errors bars represent the mean \pm SEM (standard error of the mean). Asterisks represent a significant difference using student t-test: ns=not significant, * $p<0.05$ and ** $p<0.01$. For each cell line, the statistical asterisk is versus the condition 5kPa. A bar is added under the asterisk when a statistical difference is found between RT112–T24 and T24–J82 on 8 kPa gels.

266 In summary, we have shown here that the microrheology of cancer cells is depen-
 267 dent on both (i) the invasiveness of the cells and (ii) the substrate rigidity. These findings
 268 shed light on the adaptative power of the more invasive cell lines that can deform and
 269 pass through barriers but can also change their mechanical properties depending on the
 270 type of substrate.

271 5. Results on spheroids

272 5.1. Spheroid viscoelastic properties

273 Experimental data on such spheroids are interesting and already reveal features
 274 associated with cell mechanical properties as well as spheroid content. Figure 3 shows
 275 two sets of measurements conducted on T24 spheroids: without collagen (diameter of
 276 $320 \mu\text{m}$) in Figure 3A and with collagen at 0.03 mg/mL concentration (diameter of 410
 277 μm) in Figure 3B.

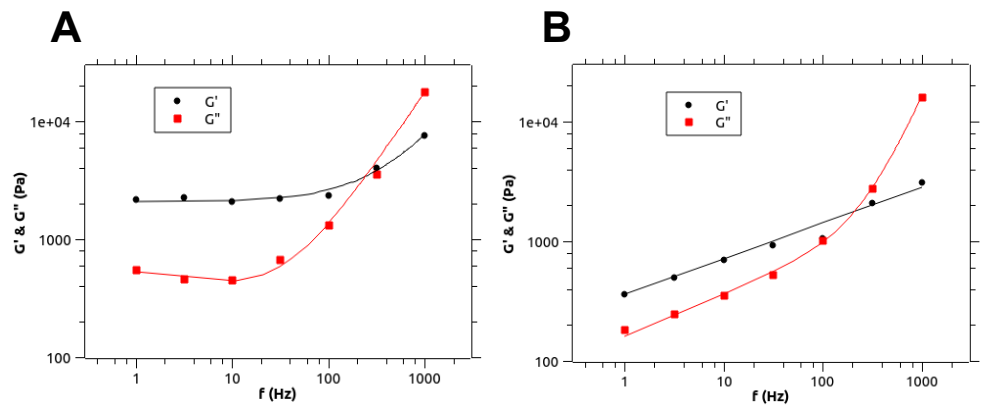


Figure 3. (A) Viscoelastic properties (G' , G'') of T24 spheroid with **no collagen**. $D \sim 320 \mu\text{m}$. Initial number of cells=10,000. The lines correspond to the model in Eq.(3), using $G_0=2100 \text{ Pa}$, $a=0$, $G_1=4.8 \text{ Pa}$, $b=1.02$, $G_2=530 \text{ Pa}$, $c=-0.15$, $G_3=4.4 \text{ Pa}$, $d=1.2$. (B) Viscoelastic properties (G' , G'') of T24 spheroid containing collagen at **0.03 mg/mL**. $D \sim 410 \mu\text{m}$. Initial number of cells=10,000. The same model gives $G_0=358 \text{ Pa}$, $a=0.30$, $G_1=0 \text{ Pa}$, $b=0$, $G_2=161 \text{ Pa}$, $c=0.35$, $G_3=0.026 \text{ Pa}$, $d=1.92$.

278 There is indeed a difference with respect to the low frequency behavior with a
 279 plateau modulus for G' and G'' in the case without collagen (Figure 3A) whereas in-
 280 terestingly, when collagen is added (Figure 3B), moduli G' and G'' exhibit power law
 281 behavior with no plateau at low frequencies. Modelling of this behavior has been carried
 282 out using Equation (3).

283 5.2. Role of collagen

284 The role of collagen has been further characterized using spheroids cultured at three
 285 different concentrations of collagen: 0 - 0.01 mg/mL and 0.03 mg/mL. The spheroids
 286 all have a similar number of initial cells (10,000 cells) and have diameters ranging
 287 between $300 \mu\text{m}$ and $410 \mu\text{m}$. The viscoelastic properties (G' , G'') were acquired as
 288 described previously and fitted using the model in Eq.(3). The fitting parameters are
 289 summarized in Figure 4 and Table 1. First, one can observe that the exponent a of G' (at
 290 low frequency) increases with the collagen concentration (Figure 4B). In particular, a is
 291 null when there is no collagen. Collagen also has an effect on G' at the high frequencies,
 292 where both G_1 and b decrease for the highest concentration of collagen (Figure 4A-B),
 293 suggesting that the spheroid modifies its elastic properties when collagen is added in
 294 the microenvironment. Finally, we also show the effect of collagen on the loss modulus
 295 G'' , where both exponents c and d (low and high frequencies) increase when collagen is
 296 present (Figure 4B).

297 In summary, these results demonstrate that the collagen changes the viscoelastic
 298 properties of the spheroid, in particular the low frequency plateau modulus disappears
 299 and gives rise to a small frequency-dependence, typical of a soft glassy rheological
 300 system [44].

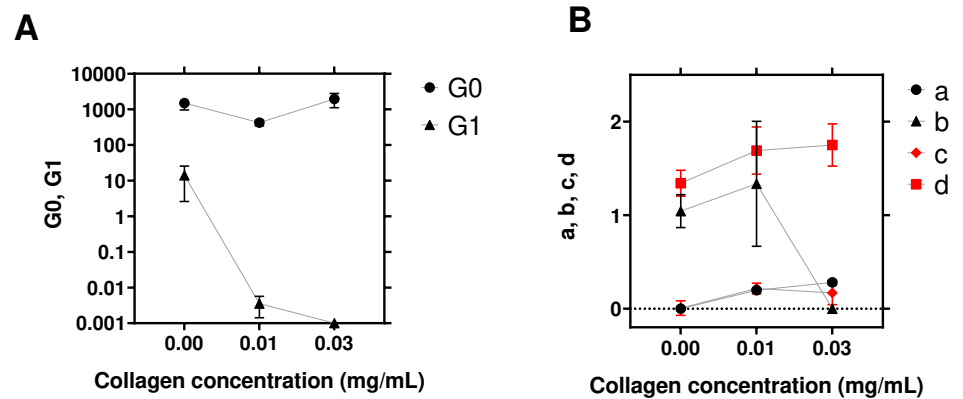


Figure 4. Role of collagen (concentration: 0 – 0.01 – 0.03 mg/mL) on parameters G_0 , G_1 (A) and exponents a , b , c , d (B). The values of all parameters are reported in Table 1. For each collagen concentration, all spheroids with a size ranging from 300 μm to 410 μm were considered. The parameters were then extracted by fitting the averaged G' and G'' with the model in Eq. (3). For each condition, three fits were performed to obtain means of the parameters. The error bars represent the standard error of the mean (SEM).

Table 1. Values of the fitting parameters of G_0 , a , G_1 , b , G_2 , c , G_3 , d for different collagen concentrations (0, 0.01 and 0.03 mg/mL).

parameters	c=0	c=0.01 mg/mL	c=0.03 mg/mL
G_0 (Pa)	1492	423	1946
a	0	0.2	0.3
G_1 (Pa)	14	0.004	0
b	1	1.3	0
G_2 (Pa)	364	197	1152
c	0.0005	0.22	0.17
G_3 (Pa)	3.6	0.6	2.8
d	1.34	1.69	1.75

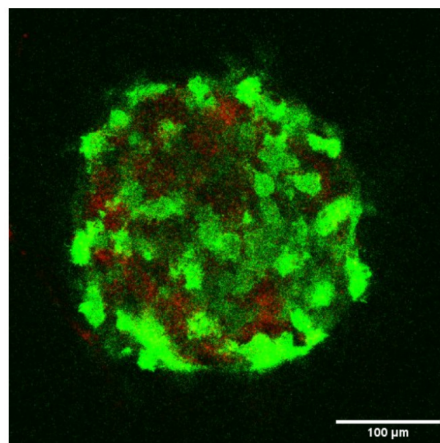


Figure 5. Confocal image of T24 spheroid with 0.01 mg/mL collagen concentration (GFP cells in green, collagen in red). $D \sim 350 \mu\text{m}$.

301 Finally, we further verified the effect of collagen on the spheroids by confocal
302 microscopy, where it is possible to visualize both labeled cells (using transfection with
303 LifeAct GFP) and the collagen (reflectance technique [20] at 650 nm). This is shown in
304 Figure 5, where cells are visible and collagen appears in between cells. Therefore, the
305 microstructure of the spheroid relies on the ability of cells to bind to the extracellular
306 matrix and construct a particular network. In the first case, there is no collagen to be
307 seen (data not shown) but when using collagen, this provides a network to build on, and
308 this affects dramatically the mechanical properties as seen above in Figure 3.

309 6. Discussion and conclusion

310 The importance of mechanical properties of cells and tissues in relation with dif-
311 ferent physiological or pathological conditions is of utmost importance [46]. Here we
312 propose to determine cell and spheroids properties using dynamic rheology thanks to
313 AFM. The technique has already been applied successfully for cells [5,10,27]. However,
314 less is known about the rheology of tissues, in particular only a few biological systems
315 were tested for viscoelasticity so far [26,47–49], and there was no data available for
316 spheroids, as proposed in this new study. AFM was found to be a powerful tool for
317 measuring elastic and loss moduli (G' , G'') and revealed important new features. To
318 analyze the data, a new model was used in Eq.(3), based on the fact that the usual power
319 law behaviors fail to describe single cell or spheroid properties [27,45,50]. Indeed it is
320 common to use a single power law exponent describing the elastic modulus G' in the
321 low frequency regime [10,51]. But the loss modulus G'' has a different behavior at low
322 and high frequencies with respective small and high slopes. This can also be the case for
323 the storage modulus G' .

324 The investigation of the microrheology of single cancer cells revealed that dynamic
325 mechanical properties are clearly dependent on *cell type* (i.e. invasiveness) and *substrate*
326 *stiffness* (Figures 1–2). New fitting parameters using the new model suggest that possible
327 differences between invasive or non-invasive cells can be clearly identified through the
328 new parameters G_0 and a , to name just these two (see Figure 2). With respect to different
329 substrates, cancer cells also revealed their ability to behave differently, in relation with
330 their cytoskeleton [27,52]. The above parameters G_0 and a , as well as c and d , were also
331 used successfully to exhibit this microrheology evolution when the substrate stiffness
332 increased from 5 to 28 kPa (see Figure 2). Finally, invasive cancer cells were found to be
333 more mechanosensitive with respect to an increase of substrate stiffness.

334 When treating the case of spheroids, which are known to be good tumor models
335 [21,24,37,53], interesting data was found, regarding the role of collagen or the ECM
336 [54]. Indeed it is known that collagen can be synthesized by cells or can be recruited
337 from the extracellular matrix. Using small amounts of collagen (here ranging from 0
338 to 0.03 mg/mL), it was found that cells re-arrange efficiently in order to form a more
339 compact structure (see Figure 5) using collagen as a proxy [24,55]. This resulted in
340 various behaviors due to the viscoelastic nature of collagen [20,56]. Indeed, collagen has
341 enhanced viscous properties at low frequency and exhibits very small moduli (typically
342 a few tens or hundreds of Pa). This effect modified quite significantly the low-frequency
343 behavior of the spheroids as shown by comparing Figure 3A and Figure 3B. Initial
344 slopes $\sim 0.3 - 0.35$ were found for G' and G'' in Figure 3B, and this was quite different
345 from the case without collagen (Figure 3A). In the presence of collagen, the spheroid
346 behavior becomes that of a glassy system, probably because cells rearrange constantly
347 in a disordered way to try to find a stable configuration, that could occur if adhesion
348 proteins are expressed and enhance elastic effects at longer times (as compared to the
349 ones used in this study). Finally, the analysis of rheological parameters (Figure 4) showed
350 that G_0 and a are quite relevant, as with single cells, but we further noticed that G_1 , b , c
351 and d can also predict such changes. Altogether these parameters may be quite essential
352 for investigating the microstructural evolution of spheroids in time, when considering
353 the main interactive components such as cells, ECM, fluid and adhesion proteins [20].

354 The final question relies on the ability to describe a macroscopic system (i.e. tissue
 355 level) with a constitutive equation taking into account all the components. Obviously the
 356 signature of single cells with (G' , G'') varying like power law models is persistent at the
 357 macroscopic level (spheroid), and this could be a starting point for future modeling. This
 358 is an interesting question raised by homogenisation of poroelastic media containing
 359 inclusions/cells embedded in a matrix [57]. Such studies could be very useful since
 360 both microscopic and macroscopic properties have been measured in this study. Addi-
 361 tionally the active behavior of cells [24] could be considered. But this requires further
 362 investigations.

363 Therefore these results confirm the interest of AFM rheology to investigate cells
 364 and tissues; it provides mechanical parameters (or cues) to test biological samples in the
 365 time or frequency domains, possibly leading to therapeutical investigations.

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 367 experiments, Y.A., A.G., J.R.; data analysis, Y.A., A.G.; writing—review and editing, Y.A., A.G.,
 368 C.V. All authors have read and agreed to the published version of the manuscript.”

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Supplementary materials

Viscoelastic properties of cancer systems: from cells to spheroids

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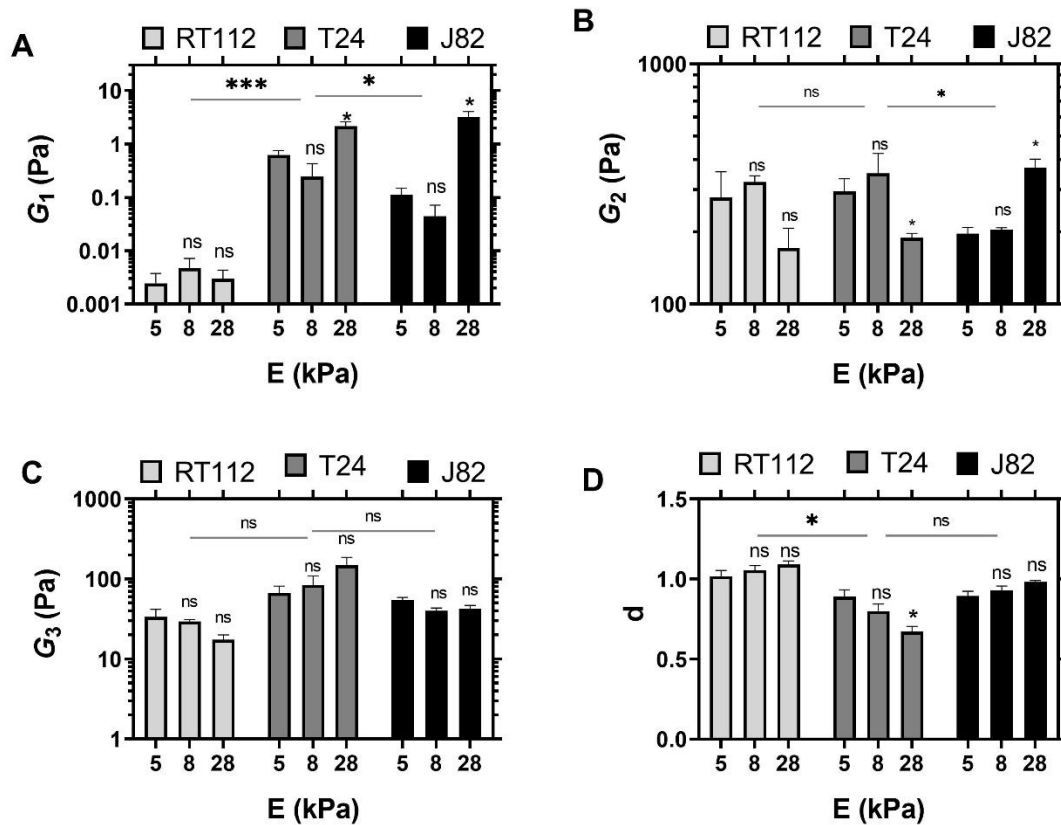


Figure S1. Parameters G_1 (A), G_2 (B), G_3 (C) and exponent d (D) for RT112, T24 and J82 cancer cells on different gels ($E= 5-8-28$ kPa). These parameters are a complement of the fitted parameters shown in Figure 2. The parameters were extracted from fitting G' and G'' with Eq. (3). Three different adjustments were performed, and the means of the parameters were obtained. Error bars represent the mean and SEM (standard error of the mean). Asterisks denote a significant difference by student t-test: ns=not significant, * $p < 0.05$, ** $p < 0.01$ relative to condition 5 kPa. A bar is added under the asterisk when a difference is found between RT112-T24 and T24-J82 on 8 kPa gels.