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Mechanosensitivity of cancer cells in contact with soft substrates using AFM

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Abstract

Cancer cells are usually found to be softer than usual cells, but their stiffness changes when in contact with different environments, i.e. they are mechanosensitive. Usually, they adhere to a substrate by adapting their cytoskeleton, thus affecting their rheological properties. This feature could become an efficient mechanism used by cancer cells when invading the surrounding tissues, i.e. they could remodel their cytoskeleton in order to achieve particular deformations. Here we study how local rheological properties of cancer cells – measured in AFM force modulation mode – are affected by a change of the environment. Cancer cells are plated on functionalized PolyAcrylAmide (PAA) substrates of different elasticities, as well as on an endothelium substrate. The analysis requires to account for thin viscoelastic substrate corrections, so a new three-layer model has been developed. The main results show that cell rheology is locally different (nucleus, perinuclear, edge locations) and changes with invasiveness. A general mechanosensitive trend is found where the cell elastic modulus G_N^0 and transition frequency f_T increase with substrate elasticity, but this trend fails with a real endothelium substrate. These effects are investigated further during cell transmigration, where the actin cytoskeleton undergoes a reorganization process necessary to push through the endothelial gap, in agreement with local viscoelasticity changes measured with the AFM. Taken altogether, these results introduce a new possible extravasation mechanism.

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Keywords: AFM, cancer cells, rheology, viscoelasticity, substrate correction, trans-endothelial migration

INTRODUCTION

The role of cell mechanics has been investigated a lot in recent years, and is essential in many biological phenomena which depend on the cell ability to modify its shape and cytoskeleton, and therefore its rheology. These changes are important during embryogenesis, cell division, cell migration, metastasis or the epithelial–mesenchymal transition, to mention but a few such phenomena. Therefore the investigation of cell mechanics has become a major issue, and has led to the development of specific tools to study cell (visco)elasticity, such as optical tweezers, optical stretchers, microplates, micropipettes, magnetic tweezers, AFM, microbead tracking with laser or light, to investigate cell internal microrheological properties (1).

Due to the variety of possible ways to investigate cell mechanics, different cell types have been tested, in particular metastatic cancer cells are of great concern (2) and possibly might become softer as they invade new tissues (3), in particular as they pass from the blood through the endothelium (4). More precisely, E , the elastic Young modulus of cells, seems to decrease when cells become invasive as compared to normal ones (5–7). This concept is not always admitted, as cells may require more stiffness to break through a biological tissue, as shown recently by combining AFM and confocal microscopy

(8). One way to answer this question is to study the cytoskeleton, a complex dynamic system, involving acto–myosin contractile elements (9), and other sub–structures like the intermediate filaments (10) or microtubules (11). The cytoskeleton is both locally and mechanically heterogeneous (12). In particular, the region on top of the nucleus of adherent cells – easily accessible with AFM for instance – or the perinuclear part, can exhibit significantly different mechanical properties, as compared to protrusions or filopodia (13, 14). Finally the nucleus plays a role in cell elasticity (15), as its deformability is a prerequisite to squeeze through complex confined networks (16). Another important parameter is viscosity, which can be quite relevant (17) because of the viscous components in the cell cytoplasm and could be a potential marker in cancer cells (18).

Recently, studies focusing on the environment revealed important strategies used by cells to remodel their cytoskeleton, a process known as mechanosensing. It is known that cells can adapt their response when the substrate has changing stiffness (19), or cells migrate towards the more rigid substrate (20), where they spread and reinforce their focal adhesions (21). Cell spreading also increases for thinner gels, since they feel the substrate below (22). Cells also adapt their stiffness depending on substrate elasticity (23). When in contact with plates of rapidly changing stiffness, cells develop larger forces via remodeling of the actomyosin cortex (24). Regarding the particular case of cancer cells, it has been shown that human breast cancer invasion correlates with a stiffening of the surrounding ECM (25) depending on cell–type, matrix, integrins, or collective effects (26). Metastatic cancer cells also interact and indent elastic gels as a possible way to measure their invasiveness (27). Further works showed that the heterogeneity of complex matrigels mimicking the microenvironment can promote cell invasion (28). In tumors, cells remodel the Extra Cellular Matrix (ECM) around them to force progression (29, 30). This change in force generation has been studied using traction force microscopy in 2D (31) and 3D (32, 33) but is not yet related so clearly to the cytoskeleton or cancer cells.

To investigate such features, a technique allowing to sense local properties is thus well adapted. AFM allows to obtain microrheological properties in force modulation mode (14, 17, 34, 35). Despite the commonly available AFM softwares or home–made systems, few results have focused on the role of the environment on cell viscoelastic properties. Thus, in this work, we consider the effect of an elastic substrate on the mechanical properties of adhering cancer cells, and evaluate their mechanosensitivity. This is carried out for three different elastic gels (E ranging from 5 kPa to 28 kPa), and for an endothelium substrate. Microrheological properties are obtained in a large frequency range [1–500 Hz] allowing to consider both elastic and viscous components (36). Since substrates are thin, a three–layer model, inspired from previous work (37), is used to account for substrate corrections, in the context of dynamic rheology. Then, using a viscoelastic model (14), two main parameters are identified, corresponding to the signature of cancer cells, namely G_N^0 , the elastic plateau modulus and f_T , the transition frequency from elastic to glassy state (38). The evolution of these parameters shows surprising changes when the endothelium substrate comes into play. This is confirmed by separate transmigration experiments, showing that cytoskeleton changes are associated with cell shapes in order to cross the endothelial barrier.

MATERIALS AND METHODS

Cell culture

Cancer cells – Three epithelial bladder cancer cell lines, representing increasing malignancy states of metastasis progression, were used, RT112, T24 and J82 (ATCC, Rockville, MD). RT112 cancer cells are moderately differentiated while T24 and J82 cancer cells are poorly differentiated and have a higher malignancy potential (especially J82 cells). The choice of these three bladder cell lines comes from earlier studies by the authors (39–41). Cell lines are classified according to the corresponding tumor they were taken from, using the Tumor–Node–Metastasis system (TNM system – UICC 2009). T stands for the stage and describes how far the primary tumor has grown into the bladder (T_0 – T_4). The majority of bladder cancers are non-invasive papillary tumors of lower grade (T_a). N is the spread to lymph nodes near the bladder (N_0 – N_3) and M is the spread of the tumor to other parts of the body (M_0 , M_1). This information is combined to give the overall stage (G_0 – G_4). According to these standards, RT112 (German tumorbank, luminal molecular subtype) is a T_a - G_2 cell type. T24 (ATCC-HTB-4, luminal/basal molecular subtype) is a more invasive cell line (T_2 - G_3). J82 (ATCC-HTB-1, luminal/basal molecular subtype) is a very invasive cell line (T_3 - G_3). Taken together, this gives a wide variety of invasiveness. Cells were cultured in RPMI 1640 (Gibco, Saint-Aubin, France) supplemented with 10% fetal calf serum and 1% penicillin streptomycin. One day before measurements, cells were seeded at a density of $3.0 \cdot 10^4$ cells per well on gels coated with 20 $\mu\text{g/mL}$ fibronectin (Promocell, Heidelberg, Germany) overnight at 37°C in humidified 5% CO_2 atmosphere. Such cells are considered to be in a low migrating state since the fibronectin concentration is quite high. AFM measurements were carried out on isolated cells at 37°C. Cancer cell lines were transfected with the LifeAct plasmid expressing actin-GFP (Green Fluorescent Protein-pEGFP).

Endothelial cells – Human Umbilical Vascular Endothelial Cells (HUVECs) purchased from Promocell (Heidelberg, Germany) were plated in complete culture medium (Promocell) on glass coverslips coated with fibronectin (10 $\mu\text{g/mL}$) and left 3 days at 37°C in humidified 5% CO_2 atmosphere to spread and achieve confluence.

Gel preparation

PolyAcrylAmide (PAA) gels were prepared using a previous protocol (42). Three gels containing OH bonds – enabling easy functionalization using fibronectin (20 $\mu\text{g/mL}$) – were prepared by mixing acrylamide (30% w/w), N-Hydroxyethylacrylamide (N-HEA, 5.85% w/w) and N,N-methylene-bisacrylamide (2% w/w) in different amounts (Sigma-Aldrich, Saint Quentin-Fallavier, France). Three concentrations of bisacrylamide were used (0.1–0.3–0.6 %) whereas the acrylamide content was fixed at 3.2%, and N-HEA was at 1.25% concentration in the final 50mM HEPES solution. Polymerization was initiated by incorporating N,N,N,N-tetramethylethylene-diamine (TEMED, Sigma) and ammonium persulfate 10% solution (APS, Sigma-Aldrich). Gels of thickness 70 μm were prepared on a pre-treated glass Petri dish for better adhesion. They were kept in humid conditions, so that they did not dry. After polymerization, samples were tested using AFM in

contact mode by simple extend–retraction indentation, or were characterized using a rheometer measuring the plateau modulus G_0 (30). Since viscous effects were negligible, one could assume that $E \sim 3G_0$. Both measurements agreed and gel moduli were $E \sim 5 \pm 1$ kPa, 8 ± 1.5 kPa and 28 ± 3 kPa.

AFM measurements

Experiments were carried out using a Nanowizard II AFM (JPK Instruments, Berlin, Germany) mounted on a Zeiss microscope (Observer D1, Carl Zeiss, Jena, Germany). All measurements were conducted in Force Modulation Mode at 37°C using the Petri Dish Heater (JPK Instruments, Berlin, Germany). We used triangular silicon nitride cantilevers with four–sided pyramidal tips (MLCT, lever C, Bruker, Camarillo, CA, USA) with a nominal spring constant $k \sim 0.01$ N m^{−1} calibrated using the thermal noise method (43). Sharp tips are better suited to reach a higher spatial resolution. We kept the applied force lower than 2 nN to remain in the linear elastic regime where the Hertz model can be used. When the cantilever tip gets into contact with the sample, the force acting on the cantilever increases to a previously chosen setpoint F , corresponding to an initial indentation δ . The relationship is given by Sneddon’s modification of the Hertzian contact mechanics model (44):

$$F = \frac{3E \tan \theta}{4(1 - \nu^2)} \delta^2 = \frac{3}{4} \bar{E} \tan \theta \delta^2 \quad (1)$$

where E is the cell’s Young modulus, ν its Poisson ratio ($\nu \sim 0.5$), $\bar{E} = E/(1 - \nu^2)$ is the apparent modulus and $\theta = 20^\circ$ is the half pyramid angle. δ is chosen so that the tip penetration depth into the sample is large enough to have a sufficient contact area and not too large to remain within the linear elasticity regime.

AFM measurements were carried out at different geometrical locations, namely N, P and E (14). N designates a location on top of the nucleus, this does not mean that the nucleus is measured, since it is known that actin cytoskeleton may be present in the thin layer between the nucleus and the cell membrane. P is usually located 10 μ m away from the nucleus geometric center. Finally, E represents the edge of the cell, typically 5 μ m from the edge.

Correction for indentation of a cell on a thin soft substrate: three–layer model

The precise analysis of the indentation experiment of a cell is highly dependent of its environment consisting of soft (gel or HUVEC monolayer) and hard substrates (glass dish). The Hertz formula, i.e. Eq. (1), is only valid as long as the indentation depth δ is small compared to sample thickness. The influence of the substrate grows with increasing indentation depth and starts to be non-negligible at about one tenth of the thickness h of the substrate. In order to overcome this difficulty, corrections are necessary. This effect was discussed earlier for different indenters (bead (45), cone (13) or pyramid (36)) by introducing the dimensionless parameter $\chi = \delta/h$. In the case of indentation on a *hard substrate*, the cell elastic modulus appears much higher than expected whereas, on a *soft gel*, the cell modulus may appear smaller than its actual value.

The analysis presented here is a generalization of corrections of the Hertz formula for indentation of a single layer on a substrate treated as a multi-scaling power law functional (46). This correction has been verified on various hard and soft combinations of a layer and substrates with various axially symmetric indenter shapes. The numerical model employed is based on a Papkovitch-Neuber Potential representation of the displacement field (37). The problem of a thin substrate has been discussed previously (37, 46). In the present experimental configuration, cells are spread on a soft gel (or cell monolayer) in contact with a hard layer, i.e. glass, therefore one has to consider two layers on top of a hard substrate. Let us call (E_i, ν_i, h_i) the Young modulus, the Poisson ratio and the thickness respectively. The indices i denote the layers for $i = 1, 2$ and $i = 3$ for the glass substrate. Let us further remark that the substrate thickness is infinite, i.e. $h_3 \rightarrow \infty$. The three-layer system is first decomposed by considering a two-layer system (layers 1 and 2). The power law functional (37, 46) provides an apparent effective modulus \bar{E}_{12} fitting all the data independent of the indenter shape in the form $\bar{E}_{12} = \bar{E}_2 + \frac{\bar{E}_1 - \bar{E}_2}{1 + (\frac{1}{\beta_0} \frac{\delta}{h_1})^{\eta_0}}$, where β_0 and η_0 are fitting parameters. The power law functional is then generalized to the three-layer system, and the apparent effective modulus is:

$$\bar{E}_{(\delta)} = \bar{E}_3 + \frac{\bar{E}_2 + \frac{\bar{E}_1 - \bar{E}_2}{1 + (\frac{1}{\beta_0} \frac{\delta}{h_1})^{\eta_0}} - \bar{E}_3}{1 + (\frac{1}{\beta_1} \frac{\delta}{h_{12}})^{\eta_1}} \quad (2)$$

where β_0, η_0, β_1 and η_1 denote a series of shape parameters, and $h_{12} = h_1 + h_2$ is used in what follows. This formula has been verified using the numerical model for indentation of the multilayer system (47) and is detailed in Appendix 1.

The model parameters β_0, η_0, β_1 and η_1 were fitted for relevant values of the cell modulus E_1 , ranging from 1 kPa to 20 kPa, with gel modulus E_2 taking values of 5, 8 and 28 kPa, as in the experiments, and the stiffness of the glass substrate E_3 was considered very large ~ 70 MPa. h_1 , the cell height, was measured experimentally on the nucleus, perinuclear region and cell edge. The thickness $h_2 = 70 \mu\text{m}$ in the case of a cell deposited on gels, or $h_2 \sim 8 \mu\text{m}$ in the case of endothelial cells.

Dynamic AFM measurements: G' and G''

In order to carry out microrheology measurements, an initial indentation δ_0 was made, corresponding to the applied force $F_0 = \frac{3}{4} \bar{E}_{(\delta_0)} \tan \theta \delta_0^2$ in Eq. (1). A small perturbation $\delta(\omega)$ at angular frequency ω ($\omega = 2\pi f$ and the frequency f varies from 1 Hz to 500 Hz) was superposed to the initial indentation δ_0 . During this procedure the tip remained in contact with the cell. We imposed the indentation $\delta(\omega)$ and measured the force response $F(\omega)$ (17, 36). The perturbation being small, Eq. (1) was linearized around the equilibrium $F_0 + F = \frac{3}{4} \bar{E}_{(\delta_0 + \delta)} \tan \theta (\delta_0 + \delta)^2$ as shown in Appendix 2. At first order:

$$\frac{F}{\delta} \frac{2}{3 \tan \theta \delta_0} = \bar{E}_1 k_1(\chi_0; \chi_1) + \bar{E}_2 k_2(\chi_0; \chi_1) + \bar{E}_3 k_3(\chi_1) \quad (3)$$

where k_1, k_2 , and k_3 are functions of the reduced indentations $\chi_0 = \frac{\delta_0}{\beta_0 h_1}$ and $\chi_1 = \frac{\delta_0}{\beta_1 h_{12}}$. By the correspondence principle of linear viscoelasticity, one uses a complex indentation δ^* and force F^* . The hydrodynamic viscous drag $i\omega b(0)$ is

subtracted from $\frac{F^*(\omega)}{\delta^*(\omega)}$ as explained previously (14, 17). $b(0)$ is estimated from the extrapolation of $b(h)$ as a function of the tip-sample separation h to $h = 0$. For the sake of simplicity, it is not written in Eq. (4) which now reads, in complex form:

$$G^*(\omega) = G_1^*(\omega) = \frac{F^*(\omega)}{\delta^*(\omega)} \frac{1 - \nu_1}{3 \tan \theta \delta_0} \frac{1}{k_1} - E_2^* \frac{k_2}{k_1} \frac{1 - \nu_1}{2(1 - \nu_2^2)} - E_3^* \frac{k_3}{k_1} \frac{1 - \nu_1}{2(1 - \nu_3^2)} \quad (4)$$

where $G^*(\omega) = G' + iG''$ is the required cell complex viscoelastic modulus. Note that E_2^* and E_3^* can be complex as well. This is the case with endothelial cells (E_2^*). For the third layer (glass), $E_3^* = E_3$ is real. The initial indentation δ_0 and heights h_1 and h_{12} are known experimentally. We impose $\delta_0 = 500$ nm, $h_1 \sim 10$ μ m on the nucleus (N), $\delta_0 = 400$ nm, $h_1 \sim 1$ μ m on the perinuclear region (P) and $\delta_0 = 100$ nm, $h_1 \sim 200$ nm on the edge (E). h_2 was given above. Functions $k_1(\chi_0; \chi_1)$, $k_2(\chi_0; \chi_1)$ and $k_3(\chi_1)$ are calculated using parameters β_0 , η_0 , β_1 and η_1 from Appendix 1. The procedure requires an initial value of $G_1^*(\omega)$ to start iterating (since k_1 in Eq. (4) depends on G_1^*) to find the new $G^* = G_1^*$. Convergence is verified when the relative error between two iteration moduli is smaller than 1%, this happens after around twenty iterations.

Modeling G' and G''

After obtaining experimental rheology data using correction formulae, it was found that in most cases G' is flat at low frequencies, then increases to reach a power law with slope a , in terms of ω . On the other hand, G'' decreases with slope $-n_f$ or is flat at low frequencies, then increases as a power law with the same exponent a . A simple model (14) was preferred:

$$G'(\omega) = G_N^0 + g_1 \omega^a \quad (5)$$

$$G''(\omega) = k_0 \omega^{-n_f} + b g_1 \omega^a \quad (6)$$

where G_N^0 is the so-called plateau modulus (Pa), a is the slope of G' and G'' at high frequencies, b is the ratio $\frac{G''}{G'}$ when power laws dominate at high frequencies, and k_0 and g_1 are constants. Note that there exists a transition frequency f_T corresponding to crossing of G' and G'' at the higher frequencies, given by $f_T = \frac{1}{2\pi} \left(\frac{G_N^0}{(b-1)g_1} \right)^{1/a}$. This model is slightly different from previous ones (17, 38) but agrees with several authors (48) showing that universal power law behavior is not always met. In particular, at low frequency, we simply obtain an elastic plateau modulus G_N^0 . Parameters G_N^0 and f_T will be used in what follows to quantify differences in cancer cell microrheological properties.

Cell transendothelial migration

To verify the results postulated with the AFM, and understand how an increase in stiffness or change in viscoelasticity can affect the cytoskeleton of cancer cells during transmigration, an experiment was carried out, using cancer cells in contact with a HUVEC monolayer on a gel of elasticity $E = 8$ kPa. A stamping procedure was used with a patterned PDMS block functionalized with fibronectin (25 μ g/mL). Stamps were put in contact with gels for one hour, then were gently detached

(42). Endothelial cells (HUVECs) were seeded in complete culture medium (Promocell) and left to adhere overnight and form a monolayer on the circular 80 μm -patterns. Finally invasive cancer cells (J82) were put in solution and left to sediment until in contact with the patterns. As many patterns in parallel were used, cases with one single cancer cell adhering and transmigrating through the HUVEC monolayer could be followed. J82 cells were transfected for actin-GFP so that the actin cytoskeleton could be visualized on the confocal microscope (Zeiss, LSM, Germany). HUVECs were stained with CellTrace Far Red DDAO-SE (Life technologies) to visualize their shape. The microscope was equipped with multiwave acquisition (green and red) and a chamber to maintain 37°C and supply 5% CO₂ for the cells. After cancer cells sedimented, they came in contact with the endothelial cells, found their way through the cell-cell junctions then passed through and spread below. Fluorescence was used to visualize cancer cells and endothelial cells at the same time.

Statistical analysis

Data were analyzed by a two-sample unpaired Student's t-test. n is the number of cells per condition. Values in Fig. 1–3–4–5 correspond to $p < 0.001$ (***), $p < 0.01$ (**), $p < 0.05$ (*) and $p > 0.05$ (n.s.). Values were considered statistically significant when $p < 0.01$. Error bars in all figures represent the standard error of the mean (SEM).

RESULTS

Local microrheology depends on cell location and substrate stiffness

We first present results of the measurements made using T24 cancer cells on a soft gel. The Young modulus is $E_2 = 5$ kPa in this case and different locations are tested in Fig. 1 A–D. We calculated the contribution of the substrate as explained above. Both measurements can be seen, with and without corrections. It is clear that the shear moduli G' and G'' are affected by the presence of the substrate. One can also note that the measurements depend on the tip position. To clarify this, Fig. 1 D shows the modulus of $|G^*|$ at 1.4 Hz ($|G^*| = \sqrt{G'^2 + G''^2}$), which increases as the point of measurement moves from the center (nucleus N) to the perinuclear region (P) and towards the lamellipodium (or edge E).

We next turn to the evolution of the dynamic moduli when substrate stiffness E_2 is increasing. We used three gels with $E_2 = 5, 8, 28$ kPa. This is presented in Fig. 1 E–H. $|G^*|$ at low frequencies (1.4 Hz) shows an increase of cell elasticity when E_2 increases, as seen in Fig. 1 H. This is due to the cell actin microstructure (14). As an illustration, the shape and microstructure of T24 cells adhering on the PAA gels (5, 8, 28 kPa) are shown in Fig. 2 A–C using confocal microscopy. T24 cells spread more and develop more actin fibres on rigid gels as expected.

Cell microrheological properties are linked to cell invasiveness

The influence of invasiveness was studied next. Three epithelial bladder cancer cells of increasing invasiveness (RT112 < T24 < J82) were plated on the 8 kPa PAA gel. Their viscoelastic properties are shown in Fig. 3 A–C, and display a common

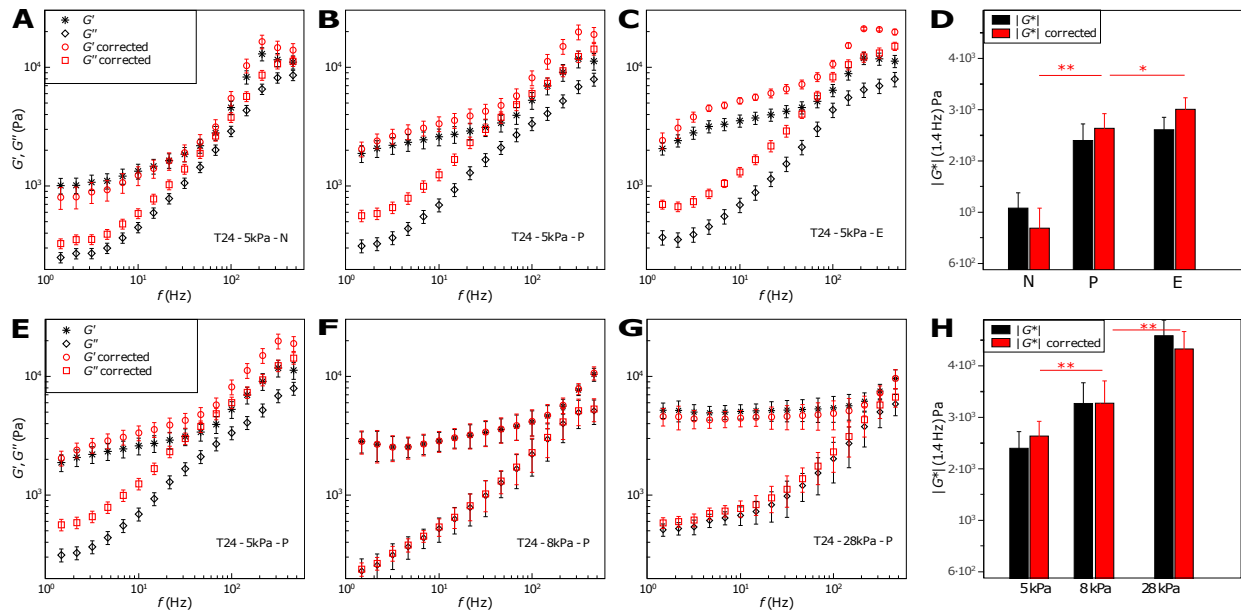


Figure 1: Correction for indentation with T24 cells. (A–C) Original and corrected viscoelastic moduli on a 5 kPa-gel at three locations N = Nucleus, P = Perinuclear region, E = Edge. (D) Moduli $|G^*|$ at 1.4 Hz at the three locations N, P, E. (E–G) Viscoelastic moduli measured on the perinuclear region (P) on three gels $E_2 = 5, 8, 28$ kPa. $n = 5$, error bars represent SEM. (H) Moduli $|G^*|$ at 1.4 Hz for three gels $E_2 = 5, 8, 28$ kPa. Statistical relevance is shown for corrected values of $|G^*|$.

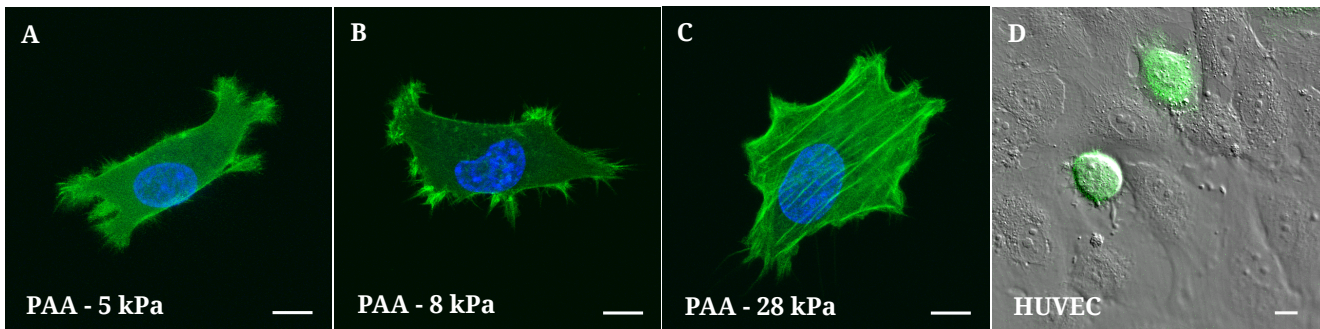


Figure 2: (A–C) Fluorescence of a GFP-actin T24 cell on three PAA substrates ($E_2 = 5, 8, 28$ kPa) with respective areas 863 μm^2 , 1025 μm^2 and 1541 μm^2 (D) DIC image of a HUVEC monolayer with green fluorescent T24 cells superimposed. Due to the round shape of the cells, it is not possible to define a lamellipodium or edge (E). Scale bar = 10 μ m.

trend, with lower moduli G' and G'' as the invasiveness increases. Substrate corrections are shown together with raw data. One can conclude that the microrheological properties of cancer cells depend on their invasiveness, i.e. moduli are lower for the more invasive cells. This is clearly shown for $|G^*|$ (at 1.4 Hz) in Fig. 3 D. Such results were previously reported for the global elastic moduli E of cancer cells (5), their local elasticity (6, 7, 49) or viscoelasticity (14, 35). Note here that accounting for substrate effects has a significant effect on the results.

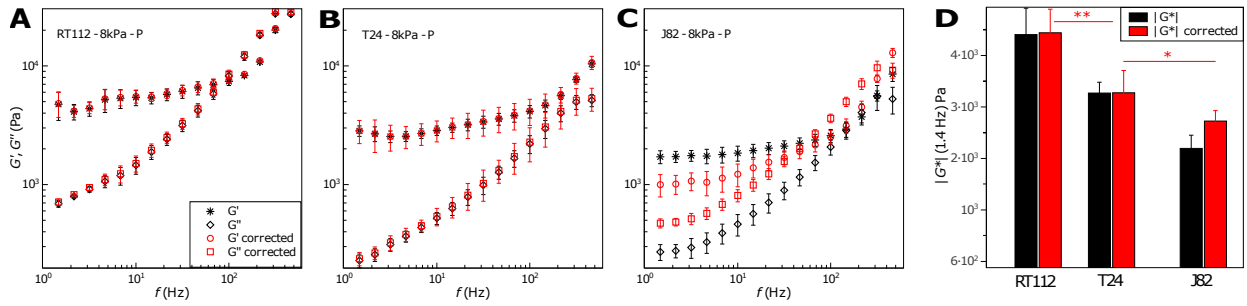


Figure 3: (A–C) Viscoelastic moduli – raw and corrected values – of three cancer cells (RT112 – T24 – J82) on the 8 kPa gel. $n=5$, error bars represent SEM. (D) Moduli $|G^*|$ at 1.4 Hz for the three cell types RT112, T24, J82. Statistical relevance is shown for corrected values of $|G^*|$.

Invasive cells have an inverse behaviour on the HUVEC substrate

We next report the behaviour of the same cancer cells in contact with the endothelial monolayer. Fig. 4 A–D shows these measurements on the perinuclear region (P). Indeed, on a HUVEC monolayer, cells do not spread much and remain round (see Fig. 2 D) so it is hard to distinguish a lamellipodium (or edge E). Only measurements on the nucleus (N) and perinuclear region (P) could therefore be made. Note that corrections in Fig. 4 A–C use the real viscoelastic properties of endothelial cells – measured separately on a glass substrate (14) – and again modify the results. In this case the viscoelastic properties of cancer cells vary differently as before. Fig. 4 D summarizes the evolution of $|G^*|$ at 1.4 Hz showing that more invasive cells are stiffer. Finally, the crossover frequency f_T varies significantly. For T24 cells, f_T cannot be reported because this crossover is out of the range of frequencies used. In the next part we will compare quantitative numbers, such as G_N^0 and f_T .

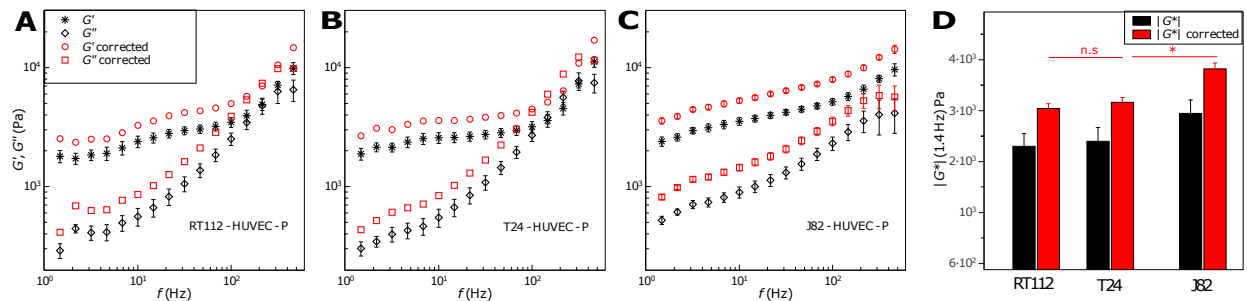


Figure 4: (A–C) Viscoelastic moduli – raw and corrected values – of three cancer cells (RT112 – T24 – J82), measured in the perinuclear region (P), on the HUVEC monolayer. $n = 5$, error bars represent SEM. (D) Corresponding values of $|G^*|$ at 1.4 Hz for the three cell types. Statistical relevance is shown for corrected values of $|G^*|$.

Parameters G_N^0 and f_T increase with substrate elasticity

In order to quantify the changes highlighted in the previous part, two parameters were studied, namely the plateau modulus G_N^0 (Fig. 5 A–C) and the transition frequency f_T (Fig. 5 D–F). These parameters were defined earlier in Eqs (5). Their evolution is shown at locations N and P for all cell types. To compare results on HUVECs and gels, we assume that $E_2 \sim 3 G_N^0 \sim$

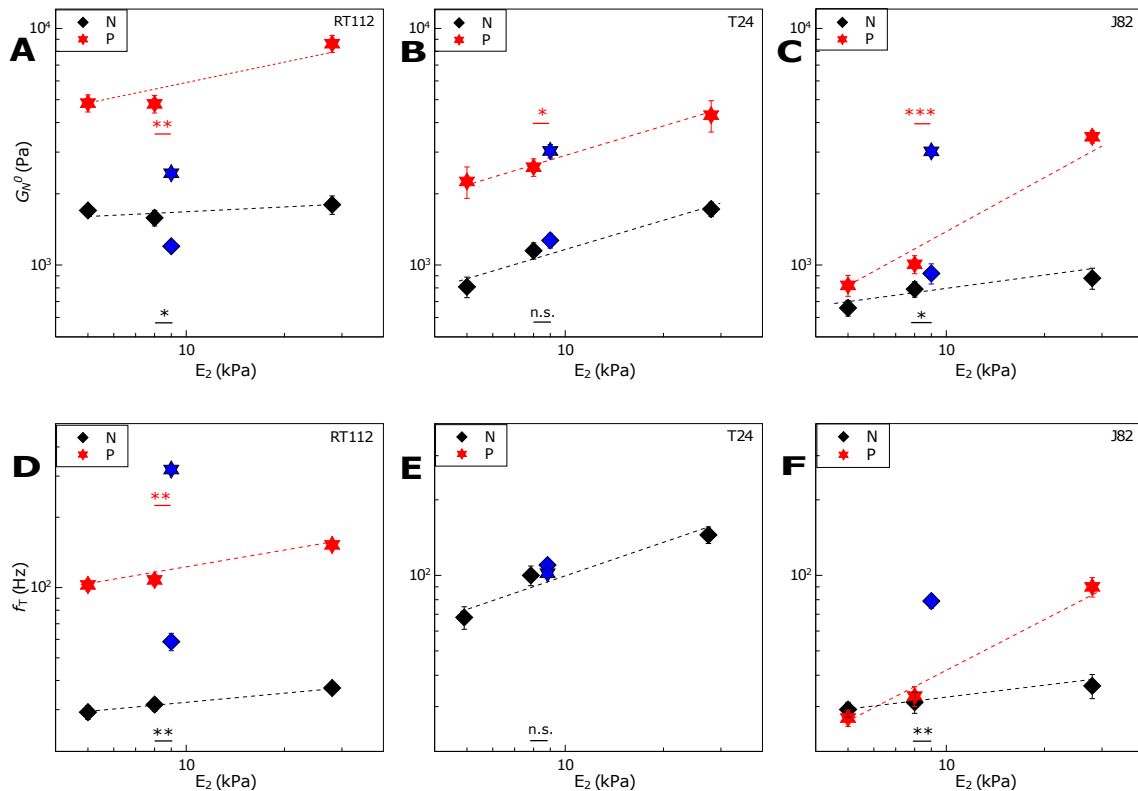


Figure 5: G_N^0 (A–C) and f_T (D–F) for RT112 – T24 – J82 cancer cells, both on rigid substrates ($E_2 = 5, 8, 28$ kPa) and HUVEC monolayer ($E_2 = 9$ kPa). P = Perinuclear region (red stars), N = Nucleus (black diamonds). $n = 5$, error bars represent SEM. The blue points represent data on the HUVEC substrate. Lines are just a guide for the eye. Statistical relevances of G_N^0 and f_T are shown when comparing the HUVEC substrate with the gel of rigidity $E_2 = 8$ kPa.

9 kPa, for endothelial cells (50). Fig. 5 represents the evolution of G_N^0 and f_T in terms of elasticity E_2 , with HUVECs (9 kPa) being close to the elastic substrate $E_2 = 8$ kPa. Justification of the model for the endothelial monolayer as a thin substrate can be found in the Supplemental Information. G_N^0 and f_T both increase linearly (log–log scale) with substrate rigidity E_2 , at a higher rate for location P, as compared to N. One could expect the HUVEC data to lie along the evolution curve. On the contrary, the values found for G_N^0 and f_T are quite different from the ones expected for an elastic substrate. G_N^0 is higher in the perinucleus region (P) than on the nucleus (N). With the HUVEC monolayer, G_N^0 is higher than expected for J82 cells, equal for T24 and lower for RT112 cells. Finally, regarding f_T (when defined), differences are also found on the HUVECs: RT112 exhibits larger f_T , T24 value exists only on HUVECs at the perinucleus (P) and f_T for invasive J82 cells shows a large change on the nucleus (N) but is not defined on the perinucleus (P). The presence of the endothelium (HUVEC) shows indeed differences that could have been expected, due to the different nature of the substrates, both in terms of cytoskeleton, as well as adhesion molecules. Thus the next section investigates the actual behaviour of J82 cancer cells in contact with the endothelium, until transmigration is achieved. The actin cytoskeleton organisation (51) will be followed, and linked with microrheological changes observed above.

Transendothelial migration requires actin reorganization of cancer cells

When cancer cells are in contact with the endothelium substrate, they may possibly transmigrate (4), so it is important to understand how they adapt their actin cytoskeleton. This effect could be related to the mechanical features shown previously, and to mechanosensitivity. One example of these features is shown in Fig. 6. Cancer cells are made to adhere to endothelial cells patterns. An isolated cancer cell was followed during its interactions with the HUVEC monolayer. One can follow the cell shape and actin organisation during the course of transmigration. Initially, the cell is round ($t = 0$, Fig. 6 A) and the actin seems to be concentrated on the sides. This local concentration can be linked to the cell mechanical properties, as measured above. Then the cell becomes ovoid ($t = 15$ min, Fig. 6 B) and actin seems to relocate close to the junction, as if the cell was pushing harder to penetrate. Finally the cell squeezes through the junction and is found below the endothelial monolayer ($t = 20$ min, Fig. 6 C) where it has spread. Fig. 6 D also shows the polar distribution of actin along the cell edge. At time $t = 15$ min, there is indeed a very large actin concentration in the $[0 - 70^\circ]$ angular range, corresponding to the endothelial gap, whereas the other two times only show a few actin focal zones necessary to bind with the substrate.

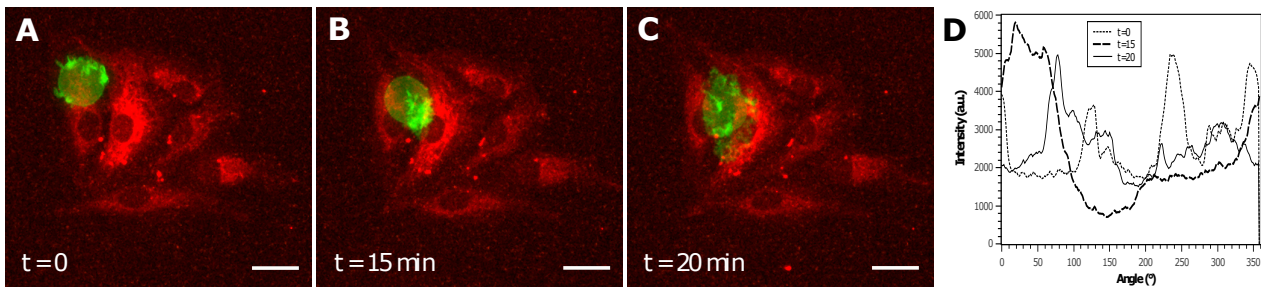


Figure 6: Transmigration of a J82 cancer cell (green) in contact with the endothelium (red). (A) The cell is round initially ($t = 0$) with actin concentrated at the periphery, (B) then takes an ovoid shape with actin relocation close to the endothelial gap ($t = 15$ min), (C) then transmigrates and spreads below the endothelium ($t = 20$ min). Scale bar = $25 \mu\text{m}$. (D) Polar representations of the actin intensity at the cell edge measured from the center of geometry, at the different times. Note in particular the large actin area for $t = 15$ min.

DISCUSSION

The choice of bladder cancer cells comes from previous studies by the authors (39–41), where various properties associated with invasiveness – such as adhesion and rheology – were reported. RT112 cancer cells are moderately differentiated while T24 and J82 cancer cells are poorly differentiated and have a higher malignancy potential (especially J82 cells). In addition, these cancer cells were used before as models for molecular classification (52). They exhibited various expression degrees of mTOR-associated genes, relevant for chemotherapy. Adhesion molecules or ligands (CD43, MUC-1) present at the cancer cell surface have been identified (40, 41, 53) as well as receptors (ICAM-1, VCAM-1) on the HUVEC side (39). Note that CD43 (Leukosialin) and MUC-1 (Mucin 1) are expressed more on invasive cells especially T24 and J82 cell lines. When

plated on gels functionalized with fibronectin, cancer cell receptors are more likely to be $\alpha_v\beta_3$ or $\alpha_5\beta_1$ integrins (54) interacting with the extra-cellular matrix (fibronectin). In addition, the expressions of α_5 and β_1 are particularly relevant for cancer cells such as T24 (55). Finally, during the process of metastasis, it was shown that the NF κ -B pathway can be involved in adhesion molecules overexpression (39).

Rheological data corrections

The effect of a cell adhering on a rigid substrate has been investigated previously and corrections have been proposed (13, 14, 45). But softer substrates – with rigidity close to that of the cell – can also play a role, therefore it is necessary to account for the effect of both substrates, the soft one with elasticity E_2 and the rigid one with elasticity E_3 (glass for instance) to determine E_1 , the cell elastic modulus, when the environment has different stiffnesses or thicknesses. Here we used a previous model (47) to investigate these properties numerically, taking into account the exact tip shape – a pyramid finishing with a cone – as considered by other authors (56). An important result is that, depending on substrate stiffness, cell modulus E_1 can be overestimated (when $E_1 < E_2$) or underestimated (when $E_1 > E_2$), this being clearly illustrated in Fig. 7 of Appendix 1: the effective modulus (due to three layers) can be an increasing or decreasing function of the reduced indentation. The other important parameters are the indentation δ , cell height h_1 and substrate thickness h_2 . Note that the spreading of cells on soft gels was studied earlier (22) when gel thickness was varied, in the particular case of mesenchymal stem cells. Cells spread more when the thickness h_2 decreased, and cell area leveled out for high thicknesses. This latter statement is in agreement with our model at small indentations (or large heights) as the apparent stiffness also shows a plateau in Fig. 7, corresponding to the expected modulus. Finally, our model was applied to dynamic rheology and linearized in Eq. (4) to obtain the complex dynamic viscoelastic modulus G^* , accounting for substrate effects, including both rigidity and thickness. Validation of the model on simple cases can be found in the Supplemental Information.

Cell stiffens away from nucleus

Next, rheological measurements were made at different locations. Fig. 1 A–D shows the raw data obtained at positions N, P, E, indicating respectively the center, perinuclear region and cell edge. These locations have been chosen based on geometry as well as microstructure considerations. The data is corrected, as explained above, due to the presence of the gel (5 kPa), and moduli increase when going from the nucleus to the edge. Corrections are not negligible as shown by differences in the G' and G'' moduli up to 40%. The final corrected value of the cell shear elastic modulus $|G^*|$ is around 1 kPa at low frequency on the nucleus (N), thus $E_1 \sim 3$ kPa (assuming $E_1 \sim 3 |G^*|$). This means that cell rigidity is lower than substrate rigidity (5 kPa), so we expect an overestimated value of E_1 in the experiments, which is confirmed here, since G' decreases after correction. Note that this effect is reversed at higher frequencies when $|G^*| > 1.7$ kPa ($E_1 > 5$ kPa). In that case, G' increases after correction. Regarding G'' , the effects are more subtle and harder to explain, but G'' increases after correction (see Suppl. Information). On the perinuclear region (P) or the edge (E), one finds $|G^*| > 2$ kPa, thus $E_1 > 6$ kPa > 5 kPa, therefore the underlying substrate now becomes softer so the experimental data are underestimated, and the correction predicts higher values of G' (and G''). After making corrections, we note (Fig. 1 D) that the cell is stiffer when going from the center (3 kPa) to the edge

(9 kPa). It is not so surprising that the center is less rigid, because the AFM tip feels the cytoskeleton as well as the nucleus at position N. Isolated nuclei are known to have a Young modulus $E \sim 5\text{--}8$ kPa (15), so 3 kPa is the average of nucleus, cytoplasm and membrane effects. The edge (E) or perinucleus (P), on the other hand, contain actin bundles, which are quite rigid. This result was shown earlier (13) and confirmed later using AFM combined with confocal microscopy (14). Disruption of actin filaments using Latrunculin A fluidifies the cytoskeleton whereas Y27632 (an actomyosin-inhibiting drug) rigidifies the cell edge (14).

Substrate stiffness increases cell rheology modulus $|G^*|$

Fig. 1 *E–H* also shows the influence of substrate rigidity, with $E_2 = 5\text{--}8\text{--}28$ kPa. These results are of interest. Corrections show an increase of the modulus $|G^*|$ after correction when $E_2 = 5$ kPa, hardly no change for $E_2 = 8$ kPa, and an increase for $E_2 = 28$ kPa (Fig. 1 *H*). We find respectively $|G^*| \sim 2.5\text{--}3.2\text{--}4.3$ kPa thus $E_1 \sim 7.5\text{--}9.6\text{--}13$ kPa to be compared respectively to $5\text{--}8\text{--}28$ kPa. Thus the measurements underestimate the values of $|G^*|$ in the first case, are unchanged in the second case, and overestimate $|G^*|$ in the last case. We can conclude that cells adapt their rheology, i.e. are mechanosensitive. A global trend is a cell elasticity increase with substrate stiffness. This is in agreement with the spreading of cells on more rigid substrates (19, 20, 22, 57) and the development of stress fibres, in connection with focal adhesions. This result can be found only when substrate corrections are made.

Invasiveness decreases cell rheology modulus $|G^*|$ as cells become glassy

The role of invasiveness on cell microrheological properties is shown in Fig. 3, using three cell types RT112, T24 and J82, by increasing order of invasiveness. When comparing measurements on the perinuclear area (P), a clear decrease in viscoelastic moduli is observed with cell invasiveness, both for G' and G'' , persistent after corrections. This confirms common trends found in the literature (5–7, 49), but more complete data is found, as G' and G'' (Fig. 3 *A–C*) vs. frequency, as well as $|G^*|$ (Fig. 3 *D*) are exhibited. In this respect, the more invasive J82 cell shows a lower f_T (crossing of G' and G'') around 40 Hz, revealing an earlier transition to the disordered glassy state. This was also observed for other malign cells, like MCF-7 and MDA-MB-231 (35).

Most invasive cells stiffen on HUVEC monolayer

The next graphs (Fig. 4) correspond to measurements carried out with the three cell types on a HUVEC monolayer, at position P. This position was chosen in order to compare with PAA substrates. Only two locations (N and P) are common between gels and HUVECs, as seen in Fig. 2. Indeed, cancer cells do not spread when in contact with the HUVEC monolayer. One could argue that the proteins in presence are different in both cases. Here we can assume that bonds are all strong ones, because they are receptor–ligand bonds involving integrins and fibronectin on gels, and ICAM-1 vs. other ligands (CD43 and MUC1) in the case of the HUVEC substrate (41). So the comparison can hold. To correct the data, we assume that the HUVEC monolayer behaves like a thin viscoelastic layer as explained in the Supplements. Indeed, in the range of parameters studied, the small indentation ($\sim 500\text{nm}$) insures no differences whatever the height of the soft substrate, so the uneven HUVEC monolayer (2–10 μm) is only affecting results through its viscoelastic properties, but not its height. The corrected

data – using a viscoelastic HUVEC substrate – shows increasing values of $|G^*|$ with invasiveness, which is the opposite of the results found previously on gels. $|G^*|$ increases from 3 kPa for RT112 cells up to 3.8 kPa for J82 invasive cells (Fig. 4 D). To investigate these changes more accurately, we make use of the model described previously.

$|G^*|$ increases with substrate stiffness, not on HUVECs

After fitting the different graphs in Fig. 1–3–4, the rheological model (14) allows to identify G_N^0 , the plateau modulus, and f_T , the transition frequency. The other parameters (a , b , k_0 and g_1) can also be obtained (14) but will not be discussed further. To include the effect of the endothelial substrate, we used previous data on HUVECs (50) where the elasticity was found ($E_2 \sim 9$ kPa). This value is also included to exhibit G_N^0 (Fig. 4 A–C) and f_T (Fig. 4 D–F) at positions N and P. The substrates have been chosen to cover a large range of elasticities (5–28 kPa), and the evolution of G_N^0 in log–log scale is a power law, with a positive slope for position (P), as observed before when averaging different locations (23). At position (N), G_N^0 seems to increase less with substrate stiffness, this is in agreement with indentation on the nucleus (18) where no differences were found. Thus our results reconcile these two studies (18, 23) by enabling comparison of measurements at different locations. When the cells become more invasive (excluding HUVECs), the slope of G_N^0 vs. E_2 at position (P) seems to increase, suggesting that the more invasive cell type is more sensitive to gel stiffness. f_T generally increases with E_2 , therefore cells become more elastic, i.e. they do not enter the glassy state. Note that in some cases, f_T could not be determined since there is no crossing of G' and G'' . Finally we note that RT112 cells become less rigid on HUVECs whereas J82 invasive cells become more rigid, especially on the perinucleus (P). This could be the signature of invasive J82 cells which start to rigidify (P) to break through the endothelium, so that the softer inner part (N) follows and collapses through the endothelial gap. This process is not explained yet, although morphology changes may come from the Rho–family of GTPases, which control the cytoskeleton and acto–myosin contractility (51).

Invasive J82 cell stiffens in perinuclear area (P) on HUVECs

Looking now closely at the HUVEC substrate, important changes are found on the perinuclear region (P). For less invasive cells, G_N^0 is much lower for RT112, equal for T24 cells, and much higher for J82 cells than expected for a 9 kPa substrate (Fig. 5 A–C). These results are also observed for data on the nucleus (N) but less clearly. This suggests a strong reorganization of the cytoskeleton in the case of invasive cancer cells. Furthermore f_T changes a lot on the perinucleus (P): Less invasive cells (RT112) remain elastic with a high $f_T = 300$ Hz, while invasive T24 cells become glassy: $f_T = 100$ Hz, and J82 do not show any transition frequency (Fig. 5 D–F). This latter case is an indication of new organization of the cytoskeleton, corresponding to a glassy state, giving rise to the same slopes of G' and G'' (17, 35, 38), and could be a signature of invasiveness. On the nucleus (N), f_T increases for RT112 on HUVECs (while G_N^0 decreases) therefore such cells adapt to the substrate. There is no change for T24 cells. In the case of J82 cells, f_T also increases (while G_N^0 does not change) i.e. the nucleus remains elastic. Thus we show for the first time the importance of the transition frequency f_T . This parameter, together with G_N^0 , shows that few changes occur around the nucleus, as opposed to the perinuclear region, highlighting possible mechanotransduction pathways between cytoskeleton and nucleus, associated with linker proteins (58).

J82 stiffening correlates with actin reorganization during transmigration

Finally, to illustrate the change in cancer cell mechanics when in contact with the HUVEC monolayer, transendothelial migration was followed, in order to study the relationship with microrheology measured above. Fig. 6 exhibits a J82 cancer cell transmigrating through a small pattern of HUVECs (6-8 cells). At the onset of transmigration ($t = 0$, Fig. 6 A), the GFP-actin J82 cell shows reinforcement of its cytoskeleton at the periphery, as shown by high fluorescence intensity areas. This seems to go along with our above results and Fig. 5 showing a higher G_N^0 on the perinuclear region (as compared to the nuclear region). This suggests that the cell edges are required to maintain stress at the periphery. Next the actin cortex seems to relocalize below the nucleus ($t = 15$ min, Fig. 6 B) to push through the gap. It is not yet possible to measure this with the AFM from below. Finally the cell is able to go through the gap and spread on the gel ($t = 20$ min, Fig. 6 C) to complete transmigration (see also Supplemental movie S1). This actin reorganization is also notable in Fig. 6 D, where the actin intensity indicates a very localized c, corresponding to the precise location of the endothelial gap. Such results of course need to be confirmed further. In any case, this explanation provides an answer to the controversy on 'softer' cells, as opposed to more 'rigid' ones, indicating that the local rheology is important to understand this pathological mechanism: higher rigidity on the edge is required first, followed by actin reorganization to push through the gap, until the nucleus follows.

CONCLUSION

In this work, we first explained the need for correcting rheology measurements in order to obtain reliable cell viscoelastic data, which are very sensitive with respect to substrate rigidity and thickness. Our results demonstrate that cancer cell properties are local, vary with invasiveness, and that cells adapt their rheology depending on substrate elasticity. In the case of an endothelial substrate, mechanosensitive effects were observed. With respect to this behaviour and possible transendothelial migration leading to metastasis, invasive cancer cells exhibit locally tuned dynamic mechanical properties. Further experiments are now possible to obtain real-time *in vitro* cell mechanical properties during transmigration.

SUPPORTING MATERIAL

A discussion on the effects of rheology (elasticity, viscoelasticity) and thickness of the substrate is presented in relation with the substrate correction model and contains three figures S1, S2 and S3. One movie S1 presents confocal microscopy of transmigrating J82 cancer cell across the HUVEC monolayer.

AUTHOR CONTRIBUTIONS

Y.A. performed all experiments and analysed data. A.C. developed indentation models. V.M.L., V.S.R., R.M., V.L. contributed analytical and experimental tools. A.D and C.V. designed research. Y.A., A.D. and C.V. wrote the paper.

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APPENDIX 1: NUMERICAL SIMULATIONS OF INDENTATION WITH A THREE-LAYER MODEL

The corrected Hertz formula for the apparent elastic modulus obtained by the indentation of a double layered substrate proposed in this paper in Eq. (2) was assessed using results obtained by the numerical model for the indentation of a multilayered substrate proposed in (47). As for the single layer model, the computations are based on (i) the Papkovitch-Neuber potential description of the displacements field in each layer, (ii) displacement and traction continuity across the interfaces and (iii) a frictionless contact condition using the exact shape of a rigid indenter. However several algorithmic details have been adapted to the precise shape of the AFM indenter, i.e. a rounded cone with a sharp angle of 19 degrees. The sharp angle imposed a novel estimation of the apparent contact modulus based on the precise geometry of the rounded cone, i.e. combining the spherical shape with a vertically shifted cone, whereas in (37, 47) only the cone was used. Moreover the starting point and the algorithm for the computation of the contact area have also been modified to insure a fast and robust convergence.

A typical example of indentation of a double layered substrate using the AFM tip with elastic moduli and layer thicknesses of the discussed experiment is displayed in Fig. 7. The picture displays several characteristic features of the indentation of a layered medium and is coherent with experimental observations and theoretical expectations.

One can remark that values of the apparent contact modulus at small indentation depth converge to the value of the apparent contact modulus of the first layer. Note that the apparent contact modulus of a material also depends on the Poisson coefficient and is not equal to the Young modulus of the material. This convergence to the apparent contact modulus was obtained after using corrections based on the exact shape of the AFM indenter as explained before.

Furthermore, the model predicts correctly the apparent contact modulus of the first layer even when the second layer is softer. In addition, the apparent contact modulus presents in this case a minimum located in the second layer zone as expected.

Let us further present the construction of the correction of the Hertz formula for a double layer system in Eq. (2) starting from the corrected Hertz formula for a single layered substrate proposed in (37). For the double layer system, one can first consider the apparent contact modulus \bar{E}_{12} of the double layer (made of 1 and 2), which can be decomposed as in (37):

$$\bar{E}_{12(\delta)} = \bar{E}_2 + \frac{\bar{E}_1 - \bar{E}_2}{1 + \left(\frac{1}{\beta_0} \frac{\delta}{h_1}\right)^{\eta_0}} \quad (7)$$

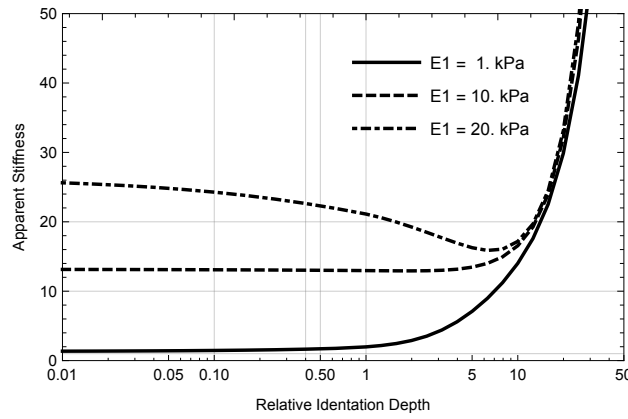


Figure 7: LogNormal plot of the apparent contact modulus \bar{E} vs. reduced indentation depth δ/h_{12} for different moduli of the first layer $E_1 = 1, 10, 20$ kPa (cell) and a second layer of $E_2 = 8$ kPa (gel) on a very rigid substrate $E_3 = 70$ MPa (glass). $h_1 = 10 \mu\text{m}$ and $h_2 = 70 \mu\text{m}$. Note the minimum of the curve when $E_1 > E_2$.

where $\bar{E}_1 = \frac{E_1}{1-\nu_1^2}$, $\bar{E}_2 = \frac{E_2}{1-\nu_2^2}$ and β_0 and η_0 are adjusting constants as described earlier in (37). Constructing a similar formula, by adding the substrate, leads to the final representation of the apparent modulus $\bar{E}(\delta)$:

$$\bar{E}(\delta) = \bar{E}_3 + \frac{\bar{E}_{12} - \bar{E}_3}{1 + \left(\frac{1}{\beta_1} \frac{\delta}{h_{12}}\right)^{\eta_1}} \quad (8)$$

where $\bar{E}_3 = \frac{E_3}{1-\nu_3^2}$ is the apparent modulus of the substrate, $h_{12} = h_1 + h_2$ is the combined height of the two layers and β_1 and η_1 are adjusting parameters. Combining equations (7) and (8), one obtains using simple algebra:

$$\bar{E}(\delta) = \bar{E}_3 + \frac{\bar{E}_2 + \frac{\bar{E}_1 - \bar{E}_2}{1 + \left(\frac{1}{\beta_0} \frac{\delta}{h_1}\right)^{\eta_0}} - \bar{E}_3}{1 + \left(\frac{1}{\beta_1} \frac{\delta}{h_{12}}\right)^{\eta_1}} \quad (9)$$

The adjusting parameters, i.e. β_0 , η_0 , β_1 and η_1 , are obtained by fitting the apparent contact modulus in Eq. (9) with the numerical results of the double layered model using a least square method. Two typical adjustments are displayed in Fig. 8 A and B.

The four fitting parameters β_0 , η_0 , β_1 and η_1 are obtained and plotted against E_1 . Fits are made, to be used in the iteration procedure, as explained in the Appendix 2 below.

APPENDIX 2: LINEARIZATION OF THE MODIFIED HERTZ MODEL OF INDENTATION

Once analytical relations for $\bar{E}(\delta)$ have been found, a linearization can be done. The indentation force F_0 applied to our sample, as a function of the indentation δ_0 , is:

$$F_0 = \frac{3}{4} \bar{E}(\delta_0) \tan \theta \delta_0^2 \quad (10)$$

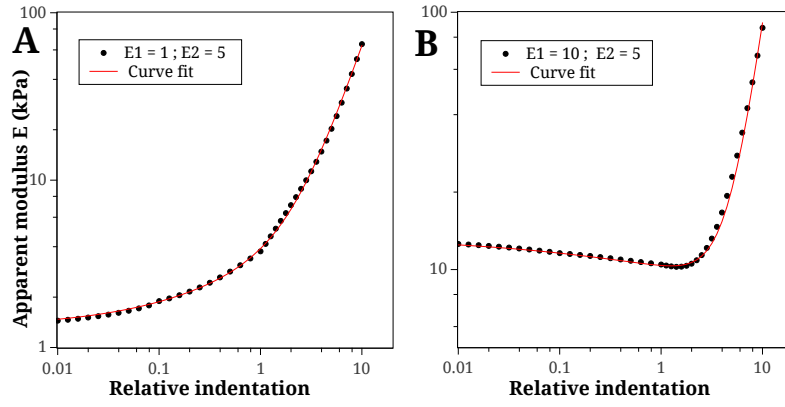


Figure 8: Comparison of the apparent contact modulus $\bar{E}(\delta)$ obtained from the double layer model (*points*) and Eq. (9) (*line*). (A) $E_1 = 1$ kPa, $E_2 = 5$ kPa (and $E_3 = 70$ MPa). The fitted parameters for this curve are $\beta_0 = 3.7$, $\eta_0 = 0.6$, $\beta_1 = 5.9$ and $\eta_1 = 1.8$. (B) $E_1 = 5$ kPa, $E_2 = 10$ kPa (and $E_3 = 70$ MPa). The fitted parameters for this curve are $\beta_0 = 1.55$, $\eta_0 = 0.38$, $\beta_1 = 109$ and $\eta_1 = 2.88$.

where θ is the pyramid half-angle. A small oscillatory amplitude δ is superposed to the indentation δ_0 . Eq. (10) becomes:

$$F_0 + F = \frac{3}{4} \bar{E}_{(\delta_0+\delta)} \tan \theta (\delta_0 + \delta)^2 \quad (11)$$

Only terms of the first order are kept, therefore:

$$\frac{F}{\delta} = \frac{3}{2} \tan \theta \delta_0 [\bar{E}_{(\delta_0)} + \frac{\delta_0}{2} \bar{E}'_{(\delta_0)}] \quad (12)$$

Let us now differentiate the apparent modulus $\bar{E}(\delta)$ with respect to the indentation δ , from Eq. (9):

$$\bar{E}'_{(\delta)} = \frac{d\bar{E}}{d\delta} = \frac{1}{[1 + (\frac{\delta}{\beta_1 h_{12}})^{\eta_1}]^2} \times \frac{1}{\delta} \left[(\bar{E}_2 - \bar{E}_1) \eta_0 \left(\frac{\delta}{\beta_0 h_1}\right)^{\eta_0} \frac{1 + (\frac{\delta}{\beta_1 h_{12}})^{\eta_1}}{(1 + (\frac{\delta}{\beta_0 h_1})^{\eta_0})^2} + \eta_1 \left(\frac{\delta}{\beta_1 h_{12}}\right)^{\eta_1} (\bar{E}_3 - \bar{E}_2 - \frac{\bar{E}_1 - \bar{E}_2}{1 + (\frac{\delta}{\beta_0 h_1})^{\eta_0}}) \right] \quad (13)$$

Introducing the following variables $\chi_0 = \frac{\delta_0}{\beta_0 h_1}$ and $\chi_1 = \frac{\delta_0}{\beta_1 h_{12}}$, the apparent modulus in Eq. (9), is evaluated at δ_0 :

$$\bar{E}_{(\delta_0)} = \frac{\bar{E}_1 + \bar{E}_2 \chi_0^{\eta_0} + \bar{E}_3 (1 + \chi_0^{\eta_0}) \chi_1^{\eta_1}}{(1 + \chi_0^{\eta_0})(1 + \chi_1^{\eta_1})} \quad (14)$$

and $\bar{E}'_{(\delta)}$ from Eq. (13) is evaluated at $\delta = \delta_0$:

$$\begin{aligned} \bar{E}'_{(\delta_0)} = \frac{d\bar{E}}{d\delta_{(\delta_0)}} &= \frac{1}{[1 + \chi_1^{\eta_1}]^2} \frac{1}{\delta_0} \times \\ & \left(-\bar{E}_1 \left[\eta_0 \chi_0^{\eta_0} \frac{1 + \chi_1^{\eta_1}}{(1 + \chi_0^{\eta_0})^2} + \eta_1 \chi_1^{\eta_1} \frac{1}{1 + \chi_0^{\eta_0}} \right] + \bar{E}_2 \left[\eta_0 \chi_0^{\eta_0} \frac{1 + \chi_1^{\eta_1}}{(1 + \chi_0^{\eta_0})^2} - \eta_1 \chi_1^{\eta_1} \frac{\chi_0^{\eta_0}}{1 + \chi_0^{\eta_0}} \right] + \bar{E}_3 [\eta_1 \chi_1^{\eta_1}] \right) \end{aligned} \quad (15)$$

Now let us replace the apparent modulus $\bar{E}(\delta_0)$ and $\bar{E}'(\delta_0)$ from Eq. (14)–(15) into Eq. (12). We obtain:

$$\frac{F}{\delta} \frac{2}{3 \tan \theta \delta_0} = \bar{E}_1 k_1(\chi_0; \chi_1) + \bar{E}_2 k_2(\chi_0; \chi_1) + \bar{E}_3 k_3(\chi_1) \quad (16)$$

where we have defined:

$$\begin{cases} k_1(\chi_0; \chi_1) = \frac{2(1 + \chi_0^{\eta_0})(1 + \chi_1^{\eta_1}) - \eta_0 \chi_0^{\eta_0}(1 + \chi_1^{\eta_1}) + \eta_1 \chi_1^{\eta_1}(1 + \chi_0^{\eta_0})}{2(1 + \chi_0^{\eta_0})^2(1 + \chi_1^{\eta_1})^2} \\ k_2(\chi_0; \chi_1) = \frac{2\chi_0^{\eta_0}(1 + \chi_0^{\eta_0})(1 + \chi_1^{\eta_1}) + \eta_0 \chi_0^{\eta_0}(1 + \chi_1^{\eta_1}) - \eta_1 \chi_1^{\eta_1} \chi_0^{\eta_0}(1 + \chi_0^{\eta_0})}{2(1 + \chi_0^{\eta_0})^2(1 + \chi_1^{\eta_1})^2} \\ k_3(\chi_1) = \frac{\chi_1^{\eta_1}(2 + \eta_1 + 2\chi_1^{\eta_1})}{2(1 + \chi_1^{\eta_1})^2} \end{cases} \quad (17)$$

It is now possible to obtain the apparent moduli \bar{E}_1 from Eq. (16):

$$\bar{E}_1 = \frac{F}{\delta} \frac{2}{3 k_1 \tan \theta \delta_0} - \bar{E}_2 \frac{k_2}{k_1} - \bar{E}_3 \frac{k_3}{k_1} \quad (18)$$

Using $E_1 = 2 G_1 (1 + \nu_1)$, and the expressions of \bar{E}_1 , \bar{E}_2 and \bar{E}_3 , Eq. (18) can be rewritten:

$$G_1 = \frac{F}{\delta} \frac{1 - \nu_1}{3 \tan \theta \delta_0} \frac{1}{k_1} - E_2 \frac{k_2}{k_1} \frac{1 - \nu_1}{2(1 - \nu_2^2)} - E_3 \frac{k_3}{k_1} \frac{1 - \nu_1}{2(1 - \nu_3^2)} \quad (19)$$

Let us introduce the complex force $F^*(\omega)$ and indentation $\delta^*(\omega)$; the complex shear modulus $G^*(\omega)$ of the first layer is:

$$G^*(\omega) = G_1^*(\omega) = \frac{F^*(\omega)}{\delta^*(\omega)} \frac{1 - \nu_1}{3 \tan \theta \delta_0} \frac{1}{k_1} - E_2^* \frac{k_2}{k_1} \frac{1 - \nu_1}{2(1 - \nu_2^2)} - E_3^* \frac{k_3}{k_1} \frac{1 - \nu_1}{2(1 - \nu_3^2)} \quad (20)$$

where E_2^* and E_3^* can also be complex. When using elastic gels, we use $E_2^* = E_2$ which is real. When using endothelial cells, E_2^* is complex and was measured separately. Finally $E_3^* = E_3$ is real for the glass substrate. Eq. (20) is used to determine $G^*(\omega)$ based on an iterative process. This requires to initiate with a given value of G_1^* (close to previous ones), determine k_1 , and plug it into the r.h.s. of Eq. (20) to find the new $G^* = G_1^*$. A few iterations (around twenty usually) are necessary to converge to the desired value $G^*(\omega)$.

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SUPPLEMENTAL INFORMATION**Mechanosensitivity of cancer cells in contact with soft substrates using AFM**

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SUPPORTING INFORMATION: INFLUENCE OF SUBSTRATE RHEOLOGY AND THICKNESS

Simulations were carried out to characterize the influence of the substrate rheology and height in the three-layer model described in our study. Here we considered an apparent shear complex modulus $G^*(\omega)$ for the first layer ($G^* = G_{cell}^*$), and calculated the corrected modulus $G_1^*(\omega)$ depending on different parameters of the substrate.

As an example, $G^*(\omega)$ is generated using the simplified fitting model described in Eq. (6-7), with the following values of the parameters: $G_N^0 = 2000$ Pa, $n_f = 0.1$, $k_0 = 200$ Pa, $g_1 = 5$, $a = 1.2$, $b = 1.5$, consistent with classical cell data. Thus, the value of the shear modulus $|G^*|^1$ at low frequencies is $|G^*(1Hz)| \simeq 2$ kPa and $E \simeq 3 G_N^0 = 6$ kPa.

The three different gels used in our study i.e. $E_2 = 5, 8, 28$ kPa were considered. Then an elastic substrate as well as a viscoelastic substrate were simulated. Finally, the sensibility to the height of the substrate was studied using three different values $h_2 = 2, 8, 70 \mu m$. In all these cases, the height $h_1 = 1000$ nm of the first layer (the cell) and the indentation $\delta = 400$ nm of the tip into the first layer were kept constant.

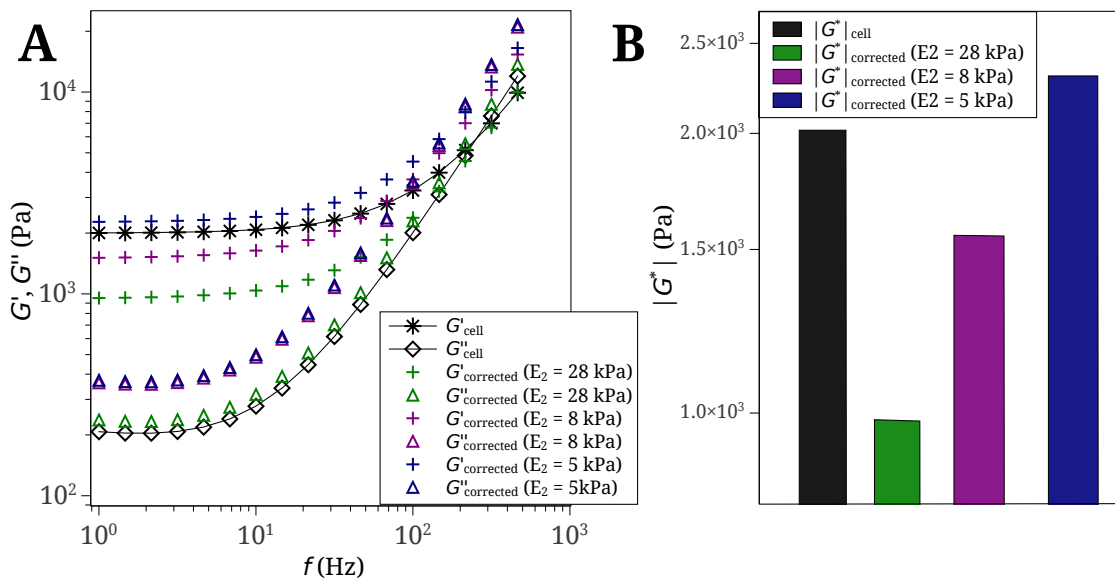


Figure S1: Substrate correction when the substrate has an elastic modulus E_2 higher, close to or smaller than the elastic modulus $E = 6$ kPa of the first layer (cell). (A) Generated viscoelastic moduli of the cell $G_{cell}^* = G^*$ (black), and corrected viscoelastic moduli considering three values of $E_2 = 5, 8, 28$ kPa. (B) The corresponding values of $|G^*|$ calculated at 1 Hz. In this simulation, $h_2 = 70 \mu m$, $h_1 = 1 \mu m$, and $\delta = 400$ nm.

Influence of the substrate elasticity

Fig. S1 shows the influence of the second layer elastic modulus E_2 on the viscoelastic properties of the first layer ($E_1 = E_{cell}$).

The results can be summarized as follows:

¹The modulus of the complex shear modulus G^* is $|G^*| = \sqrt{G'^2 + G''^2}$

- If $E_{cell} > E_2$, the cell rigidity is underestimated because of the presence of a softer substrate below. Thus the correction increases the values of G' and G'' (Fig. S1 A, blue symbols).
- If $E_{cell} < E_2$, the cell rigidity is overestimated because of the presence of a more rigid substrate below, and the correction will lower the values of G' (Fig. S1 A, purple and green symbols). The higher E_2 , the higher the correction (see larger correction of $E_2 = 28$ kPa as compared to $E_2 = 8$ kPa).

These results are also summarized in Fig. S1 B where the shear modulus $|G^*|$ at 1 Hz is represented in the three cases.

Comparison between an elastic substrate and a viscoelastic substrate

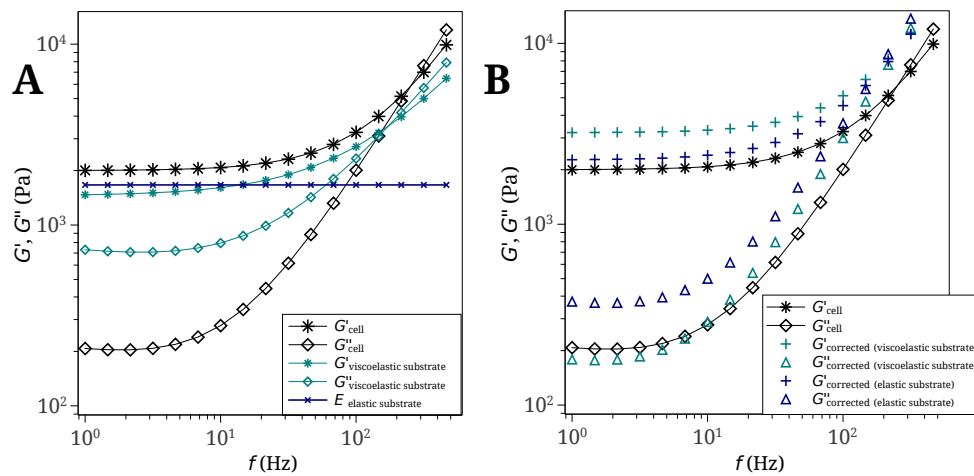


Figure S2: Correction of the apparent modulus G_{cell}^* considering an elastic substrate and a viscoelastic one. (A) Apparent viscoelastic moduli of the cell G_{cell}^* (black), and substrate with $|G_2^*| = 1.7$ kPa (turquoise), and modulus of the elastic modulus $E_2 = 5$ kPa (blue). The viscoelastic moduli of the substrate were generated using the fitting model and values were chosen so that $3|G_2^*| \simeq E_2 = 5$ kPa. (B) Corrected viscoelastic moduli considering the viscoelastic substrate (turquoise) and the elastic substrate (blue).

When we considered a viscoelastic substrate with high values of G'' (like the layer of HUVECs, Fig. S2 A), the cell viscous component was affected as follows:

- If $G_{cell}'' > G_{substrate}''$, the cell viscous component is underestimated, and the correction increases G_{cell}'' .
- If $G_{cell}'' < G_{substrate}''$, the cell viscous component is overestimated, and the correction decreases G_{cell}'' as shown in Fig. S2 B (turquoise).

Considering only an equivalent elastic substrate (same $|G^*| = 1.7$ kPa) gave rise to different G' and G'' (blue). Therefore the precise contribution of the viscoelasticity of the substrate is important since it affects both G' and G'' accurately.

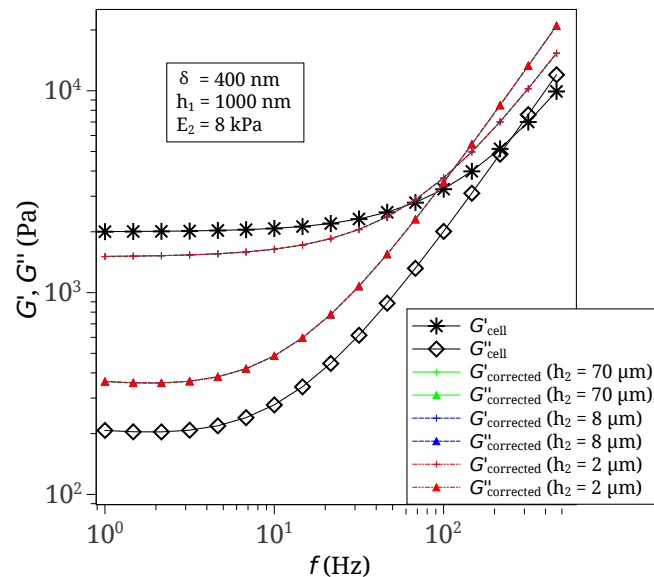


Figure S3: Correction of the apparent modulus G_{cell}^* considering three different substrate heights $h_2 = 2, 8, 70 \mu\text{m}$. The other parameters are: $E_2 = 8 \text{ kPa}$, $h_1 = 1 \mu\text{m}$ and $\delta = 0.4 \mu\text{m}$.

Influence of the substrate height – Justification of the HUVEC thin layer model

Fig. S3 shows the effect of substrate height h_2 when cells are in contact with a 8 kPa thin layer. The thickness has almost no influence on the correction of the apparent modulus, but the elasticity does. This is due to the fact that the relative indentation depth $\frac{\delta}{h_{12}}$ is always very small in this case. Indeed, as shown in Fig. 7 of the Appendix 1, the apparent stiffness remains almost constant for small relative indentation depths (lower than 0.5), which is the case in most experiments involving the HUVEC monolayer simulated here. The correction mainly comes here from the elasticity E_2 of the underlying substrate ($E_2 = 8 \text{ kPa}$), as already seen in Fig. S1 B, but the thickness is not so important. This is a justification of our approach of the endothelial layer, which can be mimicked by a thin layer, even though it has a waviness corresponding to hollows and bumps (thickness between 2 and 8 μm). So we can assume that the substrate height does not influence much the corrected values of the viscoelastic moduli.

CANCER CELL TRANSMIGRATION THROUGH THE ENDOTHELIUM

Movie S1 provides adequate evidence of the cancer cell passage through the endothelium.

Transmigration of cancer cell. A J82 cancer cell is tagged with GFP (green) and endothelial cells (purple) are stained using CellTrace Far Red DDAO-SE from Life technologies. Beads also appear in red and indicate the gel (8 kPa) upper surface. At the beginning of transmigration, the endothelial cells are seen from below showing no trace of tumor cell. Then a view from the side shows the dynamic process followed by the cancer cell, penetrating through the endothelial monolayer. At the end, a view from below shows the cancer cell (green) after eventually passing through the endothelium.