All-in-one rheology of multicellular aggregates

Gaëtan Mary

Matière et Systèmes Complexes (MSC), UMR 7057 CNRS, Université de Paris

François Mazuel

Matière et Systèmes Complexes (MSC), UMR 7057 CNRS, Université de Paris

Vincent Nier

Laboratoire Physico Chimie Curie, UMR 168, CNRS, Institut Curie, PSL University, Sorbonne Université

Florian Fage

Matière et Systèmes Complexes (MSC), UMR 7057 CNRS, Université de Paris

Irène Nagle

Matière et Systèmes Complexes (MSC), UMR 7057 CNRS, Université de Paris

Louisiane Devaud

Matière et Systèmes Complexes (MSC), UMR 7057 CNRS, Université de Paris

Jean-Claude Bacri

Matière et Systèmes Complexes (MSC), UMR 7057 CNRS, Université de Paris

Sophie Asnacios

Matière et Systèmes Complexes (MSC), UMR 7057 CNRS, Université de Paris

Atef Asnacios

MSC-Université Paris-Diderot <https://orcid.org/0000-0002-3146-476X>

Cyprien Gay

University Paris 7 & CNRS

Philippe Marcq

Sorbonne Université <https://orcid.org/0000-0002-3232-9156>

Claire Wilhelm

University of Paris and CNRS <https://orcid.org/0000-0001-7024-9627>

Myriam Reffay (✉ myriam.reffay@u-paris.fr)

Matière et Systèmes Complexes (MSC), UMR 7057 CNRS, Université de Paris <https://orcid.org/0000-0002-3695-2789>

Article

Keywords: all-in-one rheology, multicellular aggregates, magnetic rheometer

Posted Date: January 7th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-130138/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
[Read Full License](#)

All-in-one rheology of multicellular aggregates

Gaëtan Mary^{1‡}, François Mazuel^{1‡}, Vincent Nier², Florian Fage¹, Irène Nagle¹,
Louisiane Devaud¹, Jean-Claude Bacri¹, Sophie Asnacios¹, Atef Asnacios¹, Cyprien Gay¹,
Philippe Marcq^{2,3}, Claire Wilhelm^{1*} and Myriam Reffay^{1*}

1 Laboratoire Matière et Systèmes Complexes, UMR 7057, CNRS and Université de Paris, 75205 Paris cedex 13, France

2 Laboratoire Physico Chimie Curie, UMR 168, CNRS, Institut Curie, PSL University, Sorbonne Université, 75005 Paris, France

3 Laboratoire Physique et Mécanique des Matériaux Hétérogènes, CNRS, ESPCI Paris, PSL University, Sorbonne Université, Université de Paris, 75005 Paris, France

‡ These authors contributed equally to this work

* Authors for correspondence: claire.wilhelm@univ-paris-diderot.fr,
myriam.reffay@univ-paris-diderot.fr.

Abstract

Tissues are generally subjected to external stresses, a potential stimulus for their differentiation or remodelling. While single-cell rheology has been extensively studied, mechanical tissue behavior under external stress is still poorly known because of a lack of adapted set-ups. Herein we introduce magnetic techniques designed both to form aggregates of controlled size, shape and content (magnetic molding) and to deform them under controlled applied stresses over a wide range of timescales and amplitudes (magnetic rheometer). We explore the rheology of multicellular aggregates (F9 cells) using both standard assays (creep and oscillatory response) and an innovative broad spectrum sollicitation coupled with inference analysis. We find that multicellular aggregates exhibit a power-law response with non-linearities leading to tissue stiffening at high stress. Comparing magnetic measurements on aggregates to isolated F9 cells characterization by parallel-plates rheometry, we reveal the role of cell-cell adhesions in tissue mechanics. Thanks to its versatility, the magnetic rheometer thus stands as an essential tool to investigate model tissue rheology.

Introduction

Mechanical behavior of biological tissues governs various processes ranging from developmental biology to cell migration or tumor invasion [1, 2, 3, 4, 5]. Biological tissues are complex 3D multicellular, heterogeneous and active materials. Their intrinsic complexity makes them challenging to characterize as this necessarily entails to perfectly control and reproduce the tissue microscopic and macroscopic architecture. In that sense, multicellular aggregates are highly relevant models of biological tissues for quantitative exploration [6]. Organized in three dimensions and usually composed of a single cell type, their cell number, cell-cell interactions, and overall size are well-controlled. They allow to decouple mechanics from genetic regulation. All these features make them simple, fully-controlled systems of choice, as testified by their widespread current use in developmental biology [7], organogenesis, tumorigenesis [8, 9], drug screening and biophysical research [9, 10, 11, 12].

Tissue mechanics is generally found to be time-dependent: elastic on short times scales (seconds to minutes) [13, 14, 15, 16, 17] and viscous on longer time scales (minutes to hours) [18]. For longer time scales (days) cell division and death may also contribute to tissue fluidization [19]. Aggregate rheology should integrate these multiscale contributions as both intracellular and intercellular parameters are involved [20].

Many different techniques were dedicated to determine living cells mechanical features [21], evidencing a power law rheological behavior over a wide range of time scales [22, 23, 24, 25, 26,

27]. Such a power-law ousts the spring-dashpot models to impose the image of a cell with no dominating timescale [22] but rather a continuum of relaxation times and processes. Single cells are thus characterized by two parameters only: the prefactor and the exponent of the power law. The prefactor quantifies the overall stiffness of the cell (its typical modulus), while the power-law exponent indicates the relative contributions of elastic (energy storage) and viscous-like (dissipation) behaviors. For single cells, the typical moduli are hundreds of Pascals while the exponent α is found in the range $0.15 - 0.3$, thus closer to a solid ($\alpha = 0$) than to a viscous liquid behavior ($\alpha = 1$). Both depend on the level of applied stress, with evidence of stress-stiffening [28, 29] as well as of stress-softening [26], and attempts to reconcile the two opposite observations [30].

The rheology of multicellular aggregates certainly depends on cell-scale mechanical properties and should be influenced as well by the mechanical properties of intercellular junctions [31] which exhibit viscoelastic properties [32]. However the link between cellular and junctional contributions is not straightforward. The response of suspended epithelial cell monolayers has been characterized by combining conventional viscoelastic and fractional elements with an exponent close to 0.3 [33, 34]. Plus power-law rheology has been observed at the organ scale in a shear rheometer [35, 36] with an exponent close to 0.2. Understanding how cell-scale mechanical properties are translated at the tissue scale remains an open question. On the experimental side, one major hurdle stems from the difficulty to apply controlled stress solicitations to controlled tissues and to accurately measure their resulting deformations.

Herein we investigate multicellular aggregates rheology. We developed an innovative magnetic compression rheometer providing an unprecedented range and accuracy of the possible stimuli, as well as a two-fold versatility. First, the rheometer provides a wide range of accessible stimulations both in terms of frequency and amplitude. Second, magnetic molding guarantees the formation of controlled cellular spheroids. Combining both features, we subjected magnetized aggregates to standard rheological tests, as well as to broad-spectrum stimulations and tested the interplay between cell-scale mechanics and tissue-scale rheology. These protocols were cross-validated using a micro-plate experiment implemented for multicellular aggregates. Overall, the results demonstrate that multicellular aggregates exhibit a power-law rheological behaviour presenting a stress-stiffening. Interestingly, the stiffness of this cohesive cell assembly is lower than that of the corresponding individual cell. While cell-cell adhesion and individual cell stiffness do not affect the exponent governing the power law response, both contribute to the aggregate stiffness.

A magnetic compression rheometer at the tissue scale

In order to measure the rheological parameters of a whole multicellular aggregate, we stimulate this material mechanically with an arbitrary signal. We remotely apply magnetic forces on all cells through internalized superparamagnetic nanoparticles [37]. Previous works have evidenced that incorporating magnetic nanoparticles does not modify the cell biological activity [38]. The tissue can be stimulated at will as the magnetic cellular force is created by an electromagnet whose magnetic field is varied in a time-dependent manner.

The magnetic field develops a tissue magnetization $M_V(B)$ and exerts a roughly constant pulling force $f_V = M_V(B) \frac{dB}{dz}$ per unit volume of aggregate (Figure 1 and Methods). The maximal exerted force is 2.2 kN.m^{-3} . For z -independent sections (flat surface), no simple shear is induced and deformations are limited to uniaxial squeezing. We use a magnetically inspired molding strategy [16] to design cylindrical (with a typical diameter of 2 mm and a typical height $h_0 = 1 \text{ mm}$) as well as cuboid (1 mm long square base and 0.6 mm height) multicellular aggregates, corresponding to 10^6 and $2.5 \cdot 10^5$ cells respectively. Cells in these aggregates display well developed cell-cell adhesions (Figure 1e). The stress is always compressive, and we take a positive notation for simplicity. Its local value at any altitude z above the glass plate is simply given by the sum of all magnetic forces exerted on cells located higher within the aggregate:

Figure 1: The magnetic rheometer. **a-d** Side and top views of magnetic multicellular aggregates. The magnetic molding technique provides the desired aggregate shape, here a cylindrical aggregate of $900\ \mu\text{m}$ radius (**a-b**) or a cuboidal aggregate of lateral extension 1 mm and height $680\ \mu\text{m}$ (**c-d**) (scale bar: $500\ \mu\text{m}$). **e** Quarter sections of a cylindrical aggregate stained with SiR-actin (red), E-cadherin antibody (green) and DAPI (blue), merged channels (Scale bars: $100\ \mu\text{m}$). **f** Left panel: Schematics of the magnetic rheometer. An aggregate is seeded in a 37°C thermostated tank filled with culture medium. Its sides are sealed with glass to allow fast camera imaging. An electromagnet composed of a central soft iron polar piece surrounded by a coil is positioned underneath to generate the magnetic field. The current supplied to the coil is delivered by an amplified signal generator. Imaging and magnetic signals are synchronized. Right panel: The force is almost constant over the aggregate height (less than 20% variation around the mean value f_V) while the stress increases approximately linearly with the distance from the top, with a mean stress value $f_V h_0/2$. **g-h** Magnetic forces per unit volume imposed to the whole aggregate (gray area) and measured deformation of the aggregate (black markers) are superimposed. The versatility of the rheometer allows to impose various signals, here a step (**g**) and a combination of a step with a superimposed 1 Hz sinusoid (**h**).

$\sigma_{\text{loc}}(z) = \int_z^{h(t)} f_V(z') dz'$. For a uniform volume force f_V , the stress increases linearly with the distance from the upper surface. We define σ as the spatially averaged value of the stress

$\sigma \equiv \langle \sigma_{\text{loc}}(z, t) \rangle_z$ in the magnetic rheometer which reduces to $\sigma = \frac{1}{2} h(t) f_V \approx \frac{1}{2} h_0 f_V$. When $h_0 = 1 \text{ mm}$, it reaches about 1 Pa for the maximal value of the applied magnetic field.

With this set-up (Figure 1f), the aggregate deformation varies in response to the compressive force stimulation imposed through the electromagnet. The displacement $\delta h(t)$ of the upper aggregate surface is recorded by fast camera imaging and quantified through image analysis. The threshold of detection in displacement is of the order of $0.5 \mu\text{m}$ while the overall aggregate strain $\varepsilon \equiv \delta h/h_0$ (positive with the conventions used) does not exceed 5% (*i.e.*, $\delta h \leq 50 \mu\text{m}$).

Our aim is to measure the rheological response $\mathcal{R}$ relating the local strain ε_{loc} along the compression axis to the present and earlier values of the stress σ_{loc} along the same axis at the same location: $\varepsilon_{\text{loc}}(z, t) = \mathcal{R}(\{\sigma_{\text{loc}}(z, t')\}_{t' \leq t})$. The magnetic rheometer provides the applied stress signal $\sigma(t)$ and the measured strain $\varepsilon(t) = \delta h(t)/h_0$ at the scale of the aggregate. The latter is equal to the spatial average of the local deformation, $\varepsilon(t) = \langle \varepsilon_{\text{loc}}(z, t) \rangle_z$. Hence, assuming a linear local rheological response $\mathcal{R}$, $\mathcal{R}$ relates directly the macroscopic strain to the macroscopic applied stress: $\varepsilon(t) = \mathcal{R}(\{\sigma(t')\}_{t' \leq t})$. (For the nonlinear regime, see Methods).

Thanks to the electromagnet, this set-up is very versatile and aggregates were subjected to various force signals: steps, ramps, sinusoidal and broad spectrum signals with frequencies ranging from 0.1 to 10 Hz (Figure 1g-h). In this work, measurements were carried out on F9 murine cells [39], but the magnetic rheometer can be used with other cell types, provided that the cells are able to form stable multicellular structures and have a sufficient magnetic potency based on endocytosis.

Power-law rheology of multicellular aggregates

We first use two classic signals: stress steps and single-frequency stress sinusoids.

The response to a force step signal of 1.1 kN.m^{-3} , corresponding to a σ stepping up to $\sigma_0 = \frac{1}{2} h_0 f_V = 0.55 \text{ Pa}$ is recorded, (Supplemental Movie 1). Here, for $t \geq 0$ the applied stress is a constant, $\sigma = \sigma_0$. Hence, $\mathcal{R}$ is simply a function of time, and we define the function $J_{\sigma_0}(t)$ through: $\varepsilon_{\sigma_0}(t) \equiv \sigma_0 J_{\sigma_0}(t)$. We observe (Figure 2a) that it coincides with a power-law function of time over four orders of magnitude (0.01 to 100 s):

$$J(t) = J_0 (t/\tau)^\alpha, \quad (1)$$

Here we introduce the classical creep function $J(t)$, and drop the σ_0 subscript for J , assuming that the response is linear. The dimensional parameter τ is set to 1 s by convention.

We repeated the same assay over 40 aggregates to sample the distribution of both the exponent α and the prefactor J_0 . Figures 2b and c show their histograms and cumulative histograms (with J_0 in logarithmic scale). The latter are fitted by an error function, yielding the average values as well as the standard deviation. We find $\alpha = 0.21 \pm 0.03$ (mean $\pm$ sd) and $J_0 = (4.8 + 2.3, -0.9) 10^{-2} \text{ Pa}^{-1}$ (mean $\pm$ sd). Our sampling is consistent with normal and log-normal probability laws for α and J_0 respectively, in agreement with [24, 25]. As the dispersion between several measurements on a given aggregate is smaller than that observed between different aggregates, the magnetic rheometer is suitable to estimate the inherent distribution of rheological parameters. Further, by comparing the responses of cylindrical and cuboid aggregates, we demonstrate that the rheological parameters are independent of shape (Supplementary Figure S4).

As long as the aggregate is stimulated in its linear regime, its deformation $\varepsilon(t)$ in response to an arbitrary imposed stress history $\sigma(t)$ can be expressed in terms of the above creep function. Indeed, the stress can be viewed as a series of stress steps $d\sigma(t') = \frac{d\sigma}{dt}(t') dt'$ at earlier times $t' \leq t$, and the deformation is expected to coincide with the sum of the corresponding creep functions:

$$\varepsilon(t) = \int_0^t J(t-t') \frac{d\sigma}{dt}(t') dt' \quad (2)$$

Figure 2: **Creep function and power-law rheology.** **a** Creep function of a multicellular cylindrical aggregate submitted to a step of a 1.1 kN.m^{-3} force per unit volume, plotted in logarithmic scale. Experimental data (black crosses) are fitted with a power-law of time $J(t) = J_0 (t/\tau)^\alpha$ with the measured exponent $\alpha = 0.21 \pm 0.03$ and compliance $J_0 = 0.048 \pm 0.015 \text{ Pa}^{-1}$. **b-c** Cumulative distribution function (orange diamonds) and histogram (in black) of the exponent α (**b**) and of the compliance J_0 presented in logarithmic scale (**c**). The cumulative distribution functions of the exponent and of the logarithm of the compliance are fitted by an error function while their histograms are described by a normal distribution.

To test the power-law response obtained with one step-like stress signal, we superimposed to it a sinusoidal force with amplitude σ_{osc} at angular frequency ω (corresponding to 3 Hz), starting a few seconds after the force step (Figure 3b). Eq. (2) predicts that in the linear regime, an oscillatory stress yields a sinusoidal strain response with complex amplitude $\varepsilon_{\text{osc}}^* = J^*(\omega) \sigma_{\text{osc}}$, where:

$$J^*(\omega) = \frac{1}{E^*(\omega)} = \frac{J_0 \Gamma(1 + \alpha)}{(i\omega\tau)^\alpha} \quad (3)$$

with $E^*(\omega)$ the complex Young modulus and Γ the Euler gamma function. The measurements (Figure 3a,b,d) are consistent with the superposition of the earlier power-law response to a force step given by Eq. (1) and the oscillatory response at 3 Hz. It is thus possible to extract a complex modulus value $E^*(\omega)$ for each tested angular frequency ω .

Figure 3d displays the amplitude $|E^*|$ and phase shift φ of the complex modulus for several frequencies $\omega/(2\pi)$ ranging from 0.1 to 10 Hz. The amplitude exhibits a power-law dependence on frequency $|E^*| \propto \omega^\alpha$ over two orders of magnitude, while the phase shift φ appears independent of

frequency, consistently with Eq. (3) which predicts $\varphi = \alpha\pi/2$. E_0 is defined as the amplitude at 1 Hz, *i.e.*, $E_0 = |E^*(\omega = 2\pi/\tau)|$. It is related to J_0 through Eq. (3): $E_0 J_0 = (2\pi)^\alpha/\Gamma(1+\alpha)$. The oscillatory experiments yield $\alpha = 0.22 \pm 0.02$, hence $(2\pi)^\alpha/\Gamma(1+\alpha) \approx 1.64$, and $E_0 = 40 \pm 6$ Pa, which corresponds to $J_0 = 0.042 \pm 0.010 \text{ Pa}^{-1}$. The result from the oscillatory regime is thus in reasonable agreement (15% less compliant) with the one from the creep experiment. While agreement between these two rheometry protocols has been observed at the single cell level [25], we extend this result to the multicellular scale.

Figure 3: Harmonic regime and comparison with parallel plate rheology and single cell rheology. **a** Kymograph of the upper interface of the aggregate submitted first to a force step of 834 N.m^{-3} and then to a force oscillation at a frequency of 3Hz and with an amplitude 280 N.m^{-3} around the step force. **b** Extracted oscillatory contribution of the deformation (in black) superimposed with the harmonic part of the applied force. Phase shift φ and amplitude A are measured at each frequency. **c** Respective geometries of the magnetic rheometer (left) and the parallel-plates rheometer (right). Cuboid aggregates are obtained by magnetic molding and form along z -axis. In the magnetic rheometer, the aggregates are compressed along their formation axis z while in the parallel-plates rheometer, aggregates are compressed along an orthogonal direction. **d** Frequency dependence of the modulus (purple symbols) and phase (green symbols) of the complex viscoelastic modulus E^* of the aggregates measured in two different geometries : cylinder (diamonds) and cuboid (squares) and with two distinct set-ups (magnetic and parallel-plates rheometers). Experiments were repeated for 45 cylindrical aggregates and 7 cuboid aggregates. Experimental data are fitted by a power-law for the modulus (purple lines) and by a constant for the phase shift (green lines) giving an exponent $\alpha = 0.22 \pm 0.01$ and a stiffness $E_0 = 40 \pm 6$ Pa for the magnetic rheometer. Results obtained with a single-cell microplates rheometer for F9 single cells loaded with nanoparticles (open circles) are included for comparison.

Back-to-back comparison with a parallel-plates rheometer

As a cross-validation, we used a set-up initially designed for single-cell rheology [40], the parallel-plates rheometer [41], and extended it to the scale of multicellular aggregates. In their principle, measurements on cellular aggregates are similar to measurements on single cells. A cuboid aggregate is compressed between two glass microplates, a rigid one and a flexible one whose stiffness has been previously calibrated. Knowing the force applied to the cellular aggregate and its deformation, its compression viscoelastic modulus is estimated. The design of the flexible glass microplate (the force probe) had to be adapted specifically: while aggregates are far larger (around $500 \mu\text{m}$ radius) than single cells, they are softer (Figure 3d). Thus, original spatula-like plates with wide tips (1 mm in width) and a thin neck around 5mm long and $100 \mu\text{m}$ width) were designed to reach a typical stiffness of tens of $\text{nN}/\mu\text{m}$ (see Methods for details).

The sinusoidal stimulations exerted with the parallel-plates rheometer closely paralleled those exerted with the magnetic rheometer: aggregates were first compressed, then an oscillatory deformation in the 1 – 2 % range was applied. Studying the response of 7 cuboid aggregates, we find the same power-law rheology, characterized by an exponent $\alpha = 0.27 \pm 0.04$ and an elastic modulus $E_0 = 45 \pm 12$ Pa. Quantitatively, cellular aggregates thus exhibit a slightly more prominent viscous-like component (higher α) than when tested with the magnetic rheometer, while estimates of the linear elastic modulus E_0 are consistent between the two instruments. This comparison corroborates the results obtained with the magnetic rheometer and confirms the power-law rheology of multicellular aggregates.

Contribution of single cell mechanics

Figure 4: **Contribution of cell-cell contacts and cell cytoskeleton on aggregates rheology.** **a-d** Immunofluorescence images of aggregates both in the center and on the periphery. Scale bars indicate 20 μ m. Nuclei (DAPI labelling), cadherins (GFP E-cadherin antibody) and actin filaments (SiR-Actin) are labelled. **b-c** Impact of maturation time (from 16 to 48 hours), and disruptive agents (EGTA and latrunculin A) on the exponent α (**e**) and compliance (**f**), expressed in fold increase as compared to the value at 24 hours maturation time (light red), chosen as standard.

Since multicellular aggregates are essentially made of cells adhering to each other, their mechanical behavior is expected to depend on the rheological properties of the individual cells as well as on cell-cell adhesion. To test these dependencies, we first investigate isolated F9 cells mechanical behavior.

We probe individual F9 cells in a single-cell microplate rheometer [40] (Figure 3d). Briefly, the applied deformation on individual F9 cells is in the 10 – 50 % range, and the elastic modulus is

measured at different frequencies up to 0.5 Hz. The exponent found for F9 single cells ($N = 11$), $\alpha^{\text{SC}} = 0.27 \pm 0.03$, is consistent with the one measured in the microplate rheometer for F9 cell aggregates. However, F9 single cells are significantly stiffer than F9 cell aggregates, with an elastic modulus $E_0^{\text{SC}} = 240 \pm 80 \text{ Pa}$, leading to $J_0^{\text{SC}} = (8 \pm 2) 10^{-3} \text{ Pa}^{-1}$.

At the single-cell level, α is robust to most inhibitors of cytoskeletal activity [26]. We test it at the scale of multicellular aggregates by investigating the role of the actin cytoskeleton. Aggregates are submitted to latrunculin A, an inhibitor of actin polymerization. On single cells, latrunculin A softens the cells by 2 to 5-fold, depending on cell type and inhibitor concentration [26, 22, 42]. On aggregates, latrunculin A disrupts the dense cortical actin meshwork visible on normal conditions (small patches appear, Figure 4d). Quantitatively, the exponent α does not change significantly (Figure 4e). By contrast, latrunculin A-treated aggregates exhibit a twice higher compliance (Figure 4f), as also found in single cells. Changes of individual cell mechanics thus have a direct impact on aggregate stiffness.

Contribution of cell-cell adhesion

We next examine the role of cell-cell adhesion in the mechanical properties of cell aggregates, first by varying the maturation time from 16 h to 48 h, second adding EGTA to inhibit the formation of intercellular adhesions. Indeed, increasing the maturation time leads to more cohesive aggregates : cell-cell adhesions develop with time from the periphery to the center of the aggregate (Figure 4a-b). Conversely, under the action of EGTA, a calcium ion chelator decreasing intercellular adhesion forces without affecting single cell mechanical features [26], the aggregates appear looser, with a lower expression level of cadherins at the membrane (Figure 4c). In these conditions, while the exponent α is not significantly modified by the degree of intercellular adhesion (Figure 4e), the compliance factor J_0 depends on the cell-cell contacts (Figure 4f): when the aggregate cohesiveness is reduced, either by shortening the maturation time or under the action of EGTA, the aggregate becomes softer (larger value of J_0). Conversely, the maturation of cell-cell adhesions stiffens the aggregate and at 48 h of maturation, the compliance of the aggregate becomes remarkably close to the one of isolated F9 cells.

Non-linear and broad spectrum rheology

After determining the linear rheological features of the multicellular aggregates, we next probe the non-linearity of the power-law response.

A stress ramp $\sigma(t) = \dot{\sigma}_0 t$ (where $\dot{\sigma}_0$ is a constant stress rate) is expected to generate a power-law response in the linear regime. Indeed, Eq. (2) predicts:

$$\varepsilon(t) = \frac{J_0 \dot{\sigma}_0 \tau}{(1 + \alpha)} \left(\frac{t}{\tau} \right)^{1+\alpha} \quad (4)$$

The actual deformation (Figure 5a) shows an apparent deviation from linearity for volume forces above 1.2 kN.m^{-3} (corresponding to 0.6 Pa stress when $h_0 = 1 \text{ mm}$): the observed deformation is smaller than expected from Eq. (4). This stress stiffening is confirmed by recording the creep responses to different force steps corresponding to stress amplitudes ranging from 0.3 to 1.2 Pa . For each value of the stress, the creep deformation still follows a power-law with almost unchanged exponent (Figure 5c), while the compliance prefactor J_0 is reduced for higher stress levels (Figure 5d).

To systematically quantify the nonlinearity of the aggregate rheology in a single experiment, we introduce an original protocol in which we impose a large amplitude, broad spectrum stress signal (example on Figure 6a, see Methods for analytical expression and details). The rheological differential equation between strain and stress is inferred by identifying the relationship between

Figure 5: Non linearity of the rheological parameters. **a** Displacement (black crosses) of the upper surface of the aggregate submitted to a stress ramp from 0 to 1.1 Pa in 100 s (grey signal). The expected linear answer is superimposed in green. The insert is a linear zoom of the signal corresponding to maximal stress. **b** Creep functions obtained for four different stress steps ($\sigma = 0.21, 0.42, 0.63, 0.84$ Pa). **c-d** Variation of the exponent α and the compliance J_0 with the stress. Mean data obtained from the independent creep experiments performed at 3 different stress levels with the applied stress ($\sigma = 0.27, 0.54$ and 1.1 Pa) are superimposed on individual data obtained from aggregates which have experimented four stress steps (pink curves). One has to pay attention to the fact that for J_0 variations, the discrepancies related to the individual variation of the critical stress σ_c are almost wiped out by fitting individually this parameter in the range $\sigma_c = 0.45 \pm 0.10 \text{ Pa}^{-1}$. These results are compared with the nonlinear fractional model prediction delimited by the light blue area and corresponding to the possible range of stiffness extracted from broad range spectrum response fits.

their relevant times derivatives or integrals (Figure 6c,d). These signals and their time derivatives form a trajectory in the system phase space. Using a broad spectrum signal allows to explore the phase space efficiently and to thus sketch the manifold that corresponds to the constitutive equation.

Various, possibly non-linear, rheological models can be tested and compared quantitatively (Methods). Inspired by the power-law creep linear response and by the literature [43, 44], we tested a linear fractional model, and obtained a differentiation exponent $\alpha = 0.25 \pm 0.02$ and a compliance prefactor $J_0 = 0.045 \pm 0.010 \text{ Pa}^{-1}$ ($N = 8$). It provides a fit with reasonable, yet far from perfect, quality (Figure 6b, blue line). Therefore, more general, nonlinear equations with one fractional element were investigated. Using the Akaike Information Criterion (Table 1), we identify as the best model the following nonlinear fractional stress-strain relationship (Eq. (8) in Methods):

$$\varepsilon_{\text{loc}}(t) = J_1 I_\alpha^\tau \left(\frac{\sigma_{\text{loc}}(t)}{1 + \sigma_{\text{loc}}(t)/\sigma_c} \right) \quad (5)$$

where $I_\alpha^\tau(f(t))$ is the α -order fractional integral of the signal $f(t)$ [44]. Maximal likelihood optimization yields the following values of model parameters: the dynamical exponent $\alpha = 0.22 \pm 0.01$, the compliance $J_1 = 0.13 \pm 0.02 \text{ Pa}^{-1}$ and the critical stress for non-linear effects $\sigma_c = 0.5 \pm 0.1 \text{ Pa}$ ($N = 8$). Note the good agreement between the value of the order of differentiation α and previous estimates obtained with classical rheological stimulations. Including either strain

or stress thresholds in the model did not improve agreement with experimental data, which ruled out the relevance of plastic behaviour [6] in the F9 multicellular aggregates in that range of deformations.

Eq. (5) also predicts strain histories when the aggregate is subjected to simpler stress signals. The creep response to a step stress solicitation is a single power-law function, as in the experimental observations, with a constant exponent α (Figure 5c) while the stress dependence of the estimated effective compliance reproduces the one established by step forcing (Figure 5d). Interestingly, an effective exponent that decreases with the applied force is obtained by complementing the model with a second fractional integral acting on the strain (Figure S5, see Eq. (14) in Methods for additional details).

For each sample, a single broad spectrum experiment allowed us to recover the rheological features identified from stimulations based on both creep functions and harmonic signals and to generalize them in the form of a differential equation (Eq.5). Taken together, the analysis of all collected data confirms that the rheology of multicellular aggregates is best described by a fractional rheological law with a force-dependent prefactor (stress stiffening).

Discussion

Our work shows that magnetic cells may act as actuators to apply controlled internal forces on the aggregate they form. This set-up is thus able to test the rheology of model tissue. Given that estimates of single cell rheological parameters often differ by orders of magnitude when measured by different techniques [21], the agreement between magnetic and microplate rheometry is remarkable and validates this internal magnetic rheometry method. The magnetic rheometer allows to exert arbitrary compressive stress signals in the range from 0.1 to 10 Hz. It offers a unique possibility to implement broad spectrum rheology, an approach which aims at providing the constitutive equation of the material using a single experiment per sample. This is reminiscent of the optimally windowed chirp protocol introduced in [45, 46], a protocol carried out in Fourier space. In contrast, the present broad spectrum protocol is performed in real space, thereby allowing to fully characterize nonlinearities with only one signal.

The direct measurements of model tissue rheology reported here show that multicellular aggregates behave as viscoelastic materials well described by a nonlinear fractional model with stress stiffening. The nonlinear form given in (5) is reminiscent of the dependence found experimentally between stiffness and stress for single cells [27, 47]. The creep function of the aggregate is a power law function. This behavior is the hallmark of the presence of a continuous distribution of relaxation times, already identified at the individual cell level [25]. The value of α close to 0.2 puts multicellular aggregates in the same range as individual cells in terms of dynamics. This dynamic exponent also proves robust to most perturbations. By contrast, stiffness is remarkably sensitive not only to intracellular cytoskeleton (reflecting the single cell rheology change in the case of latrunculin-A actin disruption) but also to cell-cell adhesions. The compliance J_0 decreases (stiffening) as the aggregate matures (Figure 4). Indeed as cellular adhesions develop, individual cells become more sharply faceted and expulse interstitial fluid from the aggregate [48] and reduce their sliding possibilities in the interstitial regions. This change in geometry, as in liquid foams, corresponds to a stiffening of the entire structure since it increases the level of local deformations required to match a given macroscopic deformation. Alongside, we observed a softening (Figure 4) in the presence of EGTA, which is known to weaken cell-cell adhesion and results in less faceting cells.

Single cells appear stiffer than the multicellular aggregates (Figure 3), even after maturation up to 48 hours, (Figure 4f). That may result from boundary conditions being very demanding in the single-cell experiment, where a significant fraction of the cell surface is connected to rigid plates and the cell has to largely deform. By contrast, in measurements on the entire aggregate, the overall deformation imposed can be reached through a combination of cell deformations

Figure 6: **Broad spectrum rheology.** **a** Stress signal and **b** corresponding strain response of the aggregate (black curve) fitted by different fractional models : linear power law model (blue curve), nonlinear fractional model defined by Eq. (10) (red curve). **c-d** Phase space representation where the colored curves correspond to the functional form given by Eq. (19) in the linear (blue) and nonlinear (red) fractional model. In respect with the use conventions, α -order derivatives of strain have the same dimension as the strain. Black points in all subfigures correspond to the same data. Differentiation exponents are different in **c** ($\alpha = 0.271$) and **d** ($\alpha = 0.217$) since their values were obtained by fitting different models to the same data set. The absolute fit quality is estimated by R^2 -determination leading to an $R^2 = 0.94 \pm 0.02$ for linear fractional model and a $R^2 = 0.992 \pm 0.006$ for the non-linear fractional model (Eq. (19))

modes since the surroundings of each cell is then softer. Note that this argument also applies to 2D situations, making 2D epithelia softer than single epithelial cells, as measured in a suspended geometry [49].

Finally, both intercellular and intracellular components contribute to the multicellular aggregates. Stress stiffening, observed here with cell aggregates, has previously been reported for complex polymeric materials as well as living cells [50, 51, 52]. For polymers, alignment along the direction of the applied force leads to an increased resistance, which contributes to the stiffening of the network. This contribution, present at the scale of the cytoskeleton, may extend to the cellular, as well as to the multicellular scale. In tissues, cell-cell arrangements may also play a role as stress can disrupt adhesion and facilitate in turn cell-to-cell movements leading to a stress softening. However, since maximal deformations in our experiments are in the 5% range, the disruption of cell junctions seems unlikely to be relevant but cell deformations and sliding

may contribute. Observed non-linearities may therefore be mainly associated with the cellular contribution. One *caveat* concerns the threshold stress values above which stress stiffening is observed, of the order of 1Pa here, *vs.* 10 – 1000 Pa in single cells [47, 53] and several hundreds Pa in monolayers [33, 34]. However, the magnetic rheometer allows to exert comparatively small stresses, while most other techniques involve larger stresses. Thus a multi-step stress stiffening at different force levels may be implied.

Conclusion

The rheology of multicellular aggregates presents many similarities with single cell and cell monolayer rheology in spite of marked differences in terms of scales and structures. We find that their responses to a step-compression both follow a power-law in the range from tenths of seconds to minutes, with a comparable dynamical exponent. The similarity in behavior extends in the non-linear regime as multicellular aggregates exhibit stress stiffening above stresses of the order of 1Pa. However, the behaviour of multicellular aggregates differs from that of single cells. Their effective compliance is higher than that of single cells, and depends on the maturation of cell junctions.

The magnetic rheometer gives the possibility to exert small forces and study precisely the small deformations of millimeter scale multicellular aggregates. Together with model inference and model selection, broad spectrum analysis allows to decipher in a single acquisition the rheological model that best describes a sample. Thanks to its versatility both with respect to the nature of applied signals (from step to multi frequency force signal) and to the nature of biological samples (cell types, drugs and inhibitors), this all-in-one magnetic rheometer – either on its own, or coupled with inference analysis – could become a key player for the mechanical exploration of biological model tissues.

References

- [1] TA Engstrom, T Zhang, AK Lawton, A.L. Joyner, and JM Schwarz. Buckling without bending: a new paradigm in morphogenesis. *Phys. Rev. X*, 8:041053, 2017.
- [2] Valerie Marie Weaver. Cell and tissue mechanics: the new cell biology frontier. *Molecular Biology of the Cell*, 28(14):1815–1818, jul 2017.
- [3] Kaoru Sugimura, Pierre-François Lenne, and François Graner. Measuring forces and stresses in situ in living tissues. *Development*, 143(2):186–196, Jan 2016.
- [4] Otger Campàs. A toolbox to explore the mechanics of living embryonic tissues. *Seminars in Cell & Developmental Biology*, 55:119–130, Jul 2016.
- [5] A Saraswathibhatla and J Notbohm. Traction and stress fibers control cell shape and rearrangements in collective cell migration. *Phys. Rev. X*, 10:011016, 2020.
- [6] Tomita Vasilica Stirbat, Sham Tlili, Thibault Houver, Jean-Paul Rieu, Catherine Barentin, and Hélène Delanoë-Ayari. Multicellular aggregates: a model system for tissue rheology. *Eur Phys J E Soft Matter*, 36(8):9898, Aug 2013.
- [7] Vicard Du, Nathalie Luciani, Sophie Richard, Gaëtan Mary, Cyprien Gay, François Mazuel, Myriam Reffay, Philippe Menasché, Onnik Agbulut, and Claire Wilhelm. A 3d magnetic tissue stretcher for remote mechanical control of embryonic stem cell differentiation. *Nature Communications*, 8(1), sep 2017.

- [8] Ruei-Zhen Lin and Hwan-You Chang. Recent advances in three-dimensional multicellular spheroid culture for biomedical research. *Biotechnology Journal*, 3(9-10):1172–1184, oct 2008.
- [9] Sritama Nath and Gayathri R. Devi. Three-dimensional culture systems in cancer research: Focus on tumor spheroid model. *Pharmacology and Therapeutics*, 163:94–108, jul 2016.
- [10] X. Cui, Y. Hartanto, and H. Zhang. Advances in multicellular spheroids formation. *Journal of The Royal Society Interface*, 14(127):20160877, feb 2017.
- [11] Adam A. Lucio, Alessandro Mongera, Elijah Shelton, Renwei Chen, Adele M. Doyle, and Otger Campàs. Spatiotemporal variation of endogenous cell-generated stresses within 3d multicellular spheroids. *Scientific Reports*, 7(1), sep 2017.
- [12] Francesco Pampaloni, Emmanuel G Reynaud, and Ernst H K Stelzer. The third dimension bridges the gap between cell culture and live tissue. *Nat Rev Mol Cell Biol*, 8(10):839–845, Oct 2007.
- [13] Ali Kalantarian, Hiromasa Ninomiya, Sameh M I Saad, Robert David, Rudolf Winklbauer, and A. Wilhelm Neumann. Axisymmetric drop shape analysis for estimating the surface tension of cell aggregates by centrifugation. *Biophys J*, 96(4):1606–1616, Feb 2009.
- [14] Karine Guevorkian, Marie-Josée Colbert, Mélanie Durth, Sylvie Dufour, and Françoise Brochard-Wyart. Aspiration of biological viscoelastic drops. *Phys Rev Lett*, 104(21):218101, May 2010.
- [15] Tomita Vasilica Stirbat, Abbas Mgharbel, Selena Bodennec, Karine Ferri, Hichem C Mertani, Jean-Paul Rieu, and Hélène Delanoë-Ayari. Fine tuning of tissues viscosity and surface tension through contractility suggests a new role for α -catenin. *PLoS One*, 8(2):e52554, 2013.
- [16] Francois Mazuel, Myriam Reffay, Vicard Du, Jean-Claude Bacri, Jean-Paul Rieu, and Claire Wilhelm. Magnetic flattening of stem-cell spheroids indicates a size-dependent elastocapillary transition. *Physical Review Letters*, 114(9), Mar 2015.
- [17] Miao Yu, Aria Mahtabfar, Paul Beelen, Yasir Demiryurek, David I. Shreiber, Jeffrey D. Zahn, Ramsey Foty, Liping Liu, and Hao Lin. Coherent timescales and mechanical structure of multi-cellular aggregates. *Biophysical Journal*, 114(3):652a, 2018.
- [18] Friedhelm Serwane, Alessandro Mongera, Payam Rowghanian, David A Kealhofer, Adam A Lucio, Zachary M Hockenbery, and Otger Campàs. in vivo quantification of spatially varying mechanical properties in developing tissue. *Nature Methods*, 14:181–186, Jan 2017.
- [19] Jonas Ranft, Markus Basan, Jens Elgeti, Jean-François Joanny, Jacques Prost, and Frank Jülicher. Fluidization of tissues by cell division and apoptosis. *Proc Natl Acad Sci U S A*, 107:20863–20868, Nov 2010.
- [20] Sham Tlili, Cyprien Gay, François Graner, Philippe Marcq, François Molino, and Pierre Saramito. Mechanical formalisms for tissue dynamics. *Eur Phys J E Soft Matter*, 38:121, 2015.
- [21] P-H Wu, D Raz-Ben Aroush, A Asnacios, W-C Chen, ME Dokukin, BL Doss, P Durand-Smet, A Ekpenyong, J Guck, NV Guz, PA Janmey, JSH Lee, NM Moore, A Ott, Y-C Poh, R Ros, M Sander, I Sokolov, JR Staunton, N Wang, G Whyte, and Wirtz. G. A comparison of methods to assess cell mechanical properties. *Nat Meth*, 15:491–498, 2018.

- [22] Ben Fabry, Geoffrey N. Maksym, James P. Butler, Michael Glogauer, Daniel Navajas, Nathan A. Taback, Emil J. Millet, and Jeffrey J. Fredberg. Time scale and other invariants of integrative mechanical behavior in living cells. *Phys. Rev. E*, 68:041914, 2003.
- [23] J. Alcaraz, L. Buscemi, M. Grabulosa, X. Trepas, B. Fabry, R. Farré, and D. Navajas. Microrheology of human lung epithelial cells measured by atomic force microscopy. *Biophysical Journal*, 84(3):2071–9, 2003.
- [24] Nicolas Desprat, Alain Richert, Jacqueline Simeon, and Atef Asnacios. Creep function of a single living cell. *Biophysical journal*, 88(3):2224–2233, 2005.
- [25] Martial Balland, Nicolas Desprat, Delphine Icard, Sophie Féréol, Atef Asnacios, Julien Browaeys, Sylvie Hénon, and François Gallet. Power laws in microrheology experiments on living cells: Comparative analysis and modeling. *Phys. Rev. E*, 74:021911, 2006.
- [26] Xavier Trepas, Linhong Deng, Steven S An, Daniel Navajas, Daniel J Tschumperlin, William T Gerthoffer, James P Butler, and Jeffrey J Fredberg. Universal physical responses to stretch in the living cell. *Nature*, 447(7144):592–595, May 2007.
- [27] Philip Kollmannsberger and Ben Fabry. Linear and nonlinear rheology of living cells. *Annu Rev Mat Res*, 41:75–97, 2011.
- [28] Viola Vogel and Michael Sheetz. Local force and geometry sensing regulate cell functions. *Nat Rev Mol Cell Biol*, 7(4):265–275, Apr 2006.
- [29] Benjamin D Matthews, Darryl R Overby, Robert Mannix, and Donald E Ingber. Cellular adaptation to mechanical stress: role of integrins, Rho, cytoskeletal tension and mechanosensitive ion channels. *J Cell Sci*, 119(Pt 3):508–518, Feb 2006.
- [30] Lars Wolff, Pablo Fernández, and Klaus Kroy. Resolving the stiffening-softening paradox in cell mechanics. *PLoS One*, 7(7):e40063, 2012.
- [31] G Charras and AS Yap. Tensile forces and mechanotransduction at cell-cell junctions. *Curr Biol*, 28:445–457, 2018.
- [32] K Bambardekar, R Clément, O Blanc, C Chardes, and Lenne PF. Direct laser manipulation reveals the mechanics of cell contacts in vivo. *Proc Natl Acad Sci*, 112:1416–1421, 2015.
- [33] N Khalilgharibi, J Fouchard, N Asadipour, R Barrientos, M Duda, A Bonfanti, A Yonis, A Harris, P Mosaffa, Y Fujita, A Kabla, Y Mao, B Baum, JJ Munoz, M Miodownik, and G Charras. Stress relaxation in epithelial monolayers is controlled by the actomyosin cortex. *Nat Phys*, 15:839–847, 2019.
- [34] A. Bonfanti, J. Fouchard, N. Khalilgharibi, G. Charras, and A. Kabla. A unified rheological model for cells and cellularised materials. *Royal Society Open Science*, 7:190920, 2020.
- [35] Marion Geerligs, Gerrit W M Peters, Paul A J Ackermans, Cees W J Oomens, and Frank P T Baaijens. Linear viscoelastic behavior of subcutaneous adipose tissue. *Biorheology*, 45(6):677–688, 2008.
- [36] S. Nicolle, P. Vezin, and J.-F. Palierne. A strain-hardening bi-power law for the nonlinear behaviour of biological soft tissues. *Journal of Biomechanics*, 43(5):927–932, mar 2010.
- [37] C Wilhelm and F Gazeau. Universal cell labelling with anionic magnetic nanoparticles. *Biomaterials*, 29:3161–3174, 2008.

- [38] D. Fayol, G Frasca, C LeVisage, F Gazeau, N Luciani, and C Wilhelm. Use of magnetic forces to promote stem cell aggregation during differentiation, and cartilage tissue modeling. *Adv Mater*, 25:2611–2616, 2013.
- [39] A Nagafuchi, Y Shirayoshi, K Okazaki, K Yasuda, and M Takeichi. Transformation of cell adhesion properties by exogenously introduced E-cadherin cDNA. *Nature*, 329:341–343, 1987.
- [40] O. Thoumine and A. Ott. Time scale dependent viscoelastic and contractile regimes in fibroblasts probed by microplate manipulation. *J Cell Sci*, 110:2109–2116, Sep 1997.
- [41] N Desprat, A. Guirouy, and A Asnacios. Microplates-based rheometer for a single living cell. *Rev. Sci. Instrum.*, 77:055111, 2006.
- [42] A. Berquand, A. Holloschi, M. Trendelenburg, and P. Kioschis. Analysis of cytoskeleton-destabilizing agents by optimized optical navigation and AFM force measurements. *Microscopy Today*, 18(02):34, mar 2010.
- [43] H Schiessel, R Metzler, A Blumen, and TF Nonnenmacher. Generalized viscoelastic models: their fractional equations with solutions. *Journal of physics A: Mathematical and General*, 28(23):6567, 1995.
- [44] Francesco Mainardi. *Fractional calculus and waves in linear viscoelasticity: An introduction to mathematical models*. Imperial College Press, 2010.
- [45] Mehdi Bouzid, Bavand Keshavarz, Michela Geri, Thibaut Divoux, Emanuela Del Gado, and Gareth H. McKinley. Computing the linear viscoelastic properties of soft gels using an optimally windowed chirp protocol. *Journal of Rheology*, 62:1037–1050, 2018.
- [46] Michela Geri, Bavand Keshavarz, Thibaut Divoux, Christian Clasen, Daniel J. Curtis, and Gareth H. McKinley. Time-resolved mechanical spectroscopy of soft materials via optimally windowed chirps. *Physical Review X*, 8:041042, 2018.
- [47] Philip Kollmannsberger and Ben Fabry. Active soft glassy rheology of adherent cells. *Soft Matter*, 5:1771–1774, 2009.
- [48] G. Frasca, V. Du, J-C. Bacri, F. Gazeau, C. Gay, and C. Wilhelm. Magnetically shaped cell aggregates: from granular to contractile materials. *Soft Matter*, 10:5045–5054, 2014.
- [49] Andrew R Harris, Loic Peter, Julien Bellis, Buzz Baum, Alexandre J Kabla, and Guillaume T Charras. Characterizing the mechanics of cultured cell monolayers. *Proc Natl Acad Sci U S A*, 109(41):16449–16454, 2012.
- [50] Pablo Fernández and Albrecht Ott. Single cell mechanics: stress stiffening and kinematic hardening. *Phys Rev Lett*, 100(23):238102, Jun 2008.
- [51] Karen E Kasza, Amy C Rowat, Jiayu Liu, Thomas E Angelini, Clifford P Brangwynne, Gijsje H Koenderink, and David A Weitz. The cell as a material. *Curr. Opinion Cell Biol.*, 19:101–107, 2007.
- [52] Daniel A. Fletcher and R. Dyche Mullins. Cell mechanics and the cytoskeleton. *Nature*, 463:485–492, 2010.
- [53] Pablo Fernandez, Pramod A. Pullarkat, and Albrecht Ott. A master relation defines the nonlinear viscoelasticity of single fibroblasts. *Biophysical Journal*, 10:3796–3805, 2006.

- [54] Ana Espinosa, Jelena Kolosnjaj-Tabi, Ali Abou-Hassan, Anouchka Plan Sangnier, Alberto Curcio, Amanda K. A. Silva, Riccardo Di Corato, Sophie Neveu, Teresa Pellegrino, Luis M. Liz-Marzán, and Claire Wilhelm. Magnetic (hyper)thermia or photothermia? progressive comparison of iron oxide and gold nanoparticles heating in water, in cells, and in vivo. *Advanced Functional Materials*, 28(37):1803660, jul 2018.
- [55] N. Bui, P. Durand-Smet, and A. Asnacios. Single-cell mechanics: the parallel plates technique. *Methods in cell biology*, 125:187–209, 2015.
- [56] Hirotugu Akaike. A new look at the statistical model identification. *IEEE Transactions on Automatic Control*, 19(6):716–723, 1974.
- [57] Kenneth P Burnham and David R Anderson. *Model selection and multimodel inference: a practical information-theoretic approach*. Springer, 2003.

Methods

Nanoparticles and reagents

The nanoparticles used to magnetically label the F9 cells are citrated iron oxide maghemite (Fe_2O_3) superparamagnetic nanoparticles with a characteristic diameter $\delta_0 \simeq 7 - 8$ nm (polydispersity 28 %, standard deviation $s \simeq 2$ nm). The magnetization curve (Figure S1) is described by a Langevin function weighted by the nanoparticles size δ :

$$\frac{M(|B|)}{M_{\text{sat}}} = \phi(|B|) = \int M^{\text{np}}(\delta) \mathcal{L}(\xi(\delta)) P(\delta) d\delta \quad (6)$$

where M^{np} is the magnetization at saturation of a maghemite nanoparticle, $\xi(\delta) = \mu_0 H M^{\text{np}}(\delta) / (k_B T)$ and $\mathcal{L}(x) = \coth(x) - 1/x$. The size distribution P is approximated by a log-normal law with the characteristic diameter δ_0 and the standard deviation s . As particles are superparamagnetic, M is independent of the direction of the magnetic field and applied forces are pulling ones.

To inhibit actin filament formation, Latrunculin A ($1 \mu\text{M}$) purchased from Sigma is used in some experiments during aggregate formation and stimulation. EGTA (0.4 mM) may be added at the same stage to act as a calcium chelator and decrease cell-cell cadherin mediated interactions as well as individual calcium influx.

Immunofluorescence was performed using SiR-actin ($2 \mu\text{M}$ for 1 hour) to stain actin filaments and DAPI to label nuclei. E-cadherin antibody (ECCD-2 from Thermofisher used at 1 : 50 for 2 hours) coupled with a secondary anti-rat GFP antibody is used to enhance this cell-cell adhesion molecule.

Figure S1: **Magnetization curve of the iron oxide nanoparticles.** The magnetization normalized by the saturation magnetization is plotted as a function of the external magnetic field.

Magnetic cell labeling and magnetic formation of multicellular aggregates

F9 murine cells are grown in DMEM supplemented with 5 % fetal bovine serum (FBS) and 1 % antibiotics (penicillin/streptomycin) at 37°C and 5 % CO_2 . When cells are at confluence, nanoparticles solution ($[\text{Fe}] = 2$ mM in RPMI) is added for 2 h. Nanoparticles are internalized in cells through endocytosis pathways and are finally localized within intracellular vesicles identified as endosomes [54]. After incubation, cells are washed with fresh RPMI and detached with trypsin to be seeded in 2 % agarose gel cylindrical molds (diameter 2 mm, height 1.5 mm) or cubic molds (1 mm square) obtained by forming a network of small magnetic cylinders (or cubes) within an agarose mold (Figure S2). Indeed by removing the magnets below, and taking off the magnetic cylinders or cubes within the gel, we achieve to have wells to seed cells within. The seeding of cells (1 million) in the wells is then magnetically driven by the magnets below the dish (Figure S1A-B). Cell-cell contacts develop overnight to produce a cohesive cylindrical tissue that can be removed from the mold (Figure 1a-d). The resulting aggregates are removed from the well after

Figure S2: **Magnetic molding of cylindrical multicellular spheroids.** **a** Schematic representation and **b** pictures of the different steps of the molding protocol. (1) A layer of 2% agarose solution in PBS is poured into a culture dish. (2) A cylindrical permanent magnet is placed under the dish while the agarose is still liquid to attract a calibrated steel cylinder (2 mm in diameter). (3) The agarose solidifies as it cools down and the cylinder is removed to form a cylindrical mold. (4) The dish is filled with DMEM medium without FBS and magnetically labeled cells are seeded in the wells. The magnet attracts and compacts the cells for 30 seconds before being removed. (5) The dish is incubated at 37°C and 5% CO₂ overnight to allow tissue formation.

16 h (overnight maturation), showing important cell-cell adhesions. Their size is reproducible: cylindrical aggregates of 2 mm diameter and 0.8 mm height for 10⁶ cells and cuboidal aggregates of 1 mm square base and *circa* 0.6 mm height. In addition, magnetic molding allows us to form multicellular aggregates in presence of chosen inhibitors. The resulting aggregates (Figure 1a-e) have a homogeneous bulk saturation magnetization ($M_V^{\text{sat}} = 103 \pm 16 \text{ A.m}^{-1}$) measured with a vibrating sample magnetometer (Quantum Design, Versalab).

Magnetic field applicator

The magnetic field used to deform the magnetized aggregate was generated with an electromagnet composed of a custom-made water-cooled coil (6 Ω, 14 mH) wrapped around a soft iron polar piece (diameter 55 mm, height 85 mm) allowing the passage of a current up to 10A. In its upper part, the diameter of the polar piece goes down from 55 mm to 5 mm. The top of the polar piece was 3 mm below the center of the aggregate. The electromagnet tension was controlled with a TGA 1242 signal generator (Aim&TTi) and amplifier (Kebco BOP).

The shape of the polar piece was optimized to maximize the magnitude of the magnetic field gradient dB/dz along the z coordinate (30 T.m^{-1} when the magnetic field reaches its maximum value 120 mT) as well as its homogeneity in the aggregate volume. Nanoparticles magnetize in the presence of the magnetic field B . As a result, the aggregate displays a magnetization $M_V = M_V^{\text{sat}} \phi(B/B^{\text{sat}})$ per unit volume (for ϕ , M_V^{sat} and B^{sat} , see above). The magnetic field generated at the center of the aggregate linearly depends on the applied tension (see Figure S3a) and the magnetic field gradient was almost homogeneous (less than 20% variation over a size exceeding one millimeter), whatever the applied tension (Figure S3b) ensuring a homogenous force on cells.

The set up was calibrated and characterized in order to determine the bulk flattening magnetic force generated as a function of the applied tension $V(t)$ using the set-up as a tensiometer [16]. Ferrofluid drops (aqueous solution of the nanoparticles used for cell labelling) of various size deposited on a superhydrophobic surface in a silicon bath, were flattened. Fitting each experimental profile with the Laplace equation for capillarity in non-wetting conditions provided the surface tension. This measurement was validated by comparison with classic sessile drop experiment (Figure S3c). The maximum value of the magnetic force density was measured to be close to 2.5 kN.m^{-3} .

Figure S3: **Calibration of the magnetic field generator.** **a** Magnetic field created by the electromagnet as a function of the distance to the top of the polar piece for different tensions applied to the amplifier. The center of the aggregate is 3 mm above the electromagnet so the aggregate position is emphasized in violet. **b** Force per unit volume extracted from the measured magnetic field and aggregate magnetization curve. **c** Ferrofluid drops ($[\text{Fe}] = 1.25 \text{ M}$) of three different volumes ($0.6, 1.5, 4.5 \mu\text{L}$) deposited on a superhydrophobic coated glass surface, in a silicon oil bath, are submitted to increasing magnetic flattening forces (under normal gravity, $f_{V1} = 6.9 \text{ kN.m}^{-3}$, $f_{V2} = 56 \text{ kN.m}^{-3}$, $f_{V3} = 107 \text{ kN.m}^{-3}$) corresponding to increasing tensions applied to the amplifier (respectively: 0, 1.2, 4.8, 8 V). Droplets experimental equilibrium profiles are fitted with theoretical capillary profile (yellow), obtained by integrating numerically the Laplace equation for capillarity in non-wetting conditions (using Matlab's function ode45).

Signal generation and synchronization

The tension signal is transferred to the generator using the Waveform Manager Plus software. To synchronize the deformation signal with the force generation, a LED, powered by a pulse generator that is synchronized with the signal generator, was used to overexpose a single image of the movie at the beginning of the deformation sequence.

Image analysis

Deformations were analyzed using the image correlation based tracking algorithm on ImageJ: Tracker Install (developped by Olivier Cardoso <https://sites.google.com/site/oliviercardoso/Home/plugins>). It extracts the displacement of the aggregate upper interface as a function of time ($\delta h(t)$ in Figure 1f-h). Vibrations of the setup (mainly due to the water circulation use to thermalize the container) were compensated by tracking a fixed point on the container bottom (adsorbed dust for instance) and subtracting its motion from the interface displacement. The precision on the interface displacement is $\pm 0.5 \mu\text{m}$. Sampling frequencies are between 10 to 100 Hz depending on the experiments.

Shape-independance of cellular aggregates rheology

Figure S4: **Influence of the shape on the power law exponent.** Comparison of the compliance **a** and of the α exponent **b** in two different geometries : cuboids and cylinders. No differences are noticeable.

Microplate rheometer

A microplate rheometer was used both for single cell and for aggregate measurements [41]. In such a device, the sample is compressed between two parallel microplates. One is rigid. The other is flexible. Its deflection δ is related to the force $F = k\delta$ applied to the sample through the calibrated stiffness k .

However, since the set-up was initially designed to test single cells (size around $10\mu\text{m}$), the flexible microplate had to be adapted to both the size ($500\mu\text{m}$) and the stiffness of cell aggregates. Thus, new spatula-like plates have been created with wide tips (1 mm in width) and a thin neck (around 5 mm long and $100\mu\text{m}$ width) to be able to test large but very soft objects like cell aggregates. The stiffness of the spatula was $27.8\text{ nN}/\mu\text{m}$. For single cell measurements, as F9 cells are very soft, we used a microplate of $0.6\text{ nN}/\mu\text{m}$. The flexible microplates were obtained by heating and pulling borosilicate plates of $10\text{ cm} \times 1\text{ mm} \times 200\mu\text{m}$ [55]. The stiffness of the flexible microplates was calibrated using a standard microplate itself calibrated following the protocol detailed in [41]. The setup was mounted on a Leica DMIRB inverted microscope (Leica Microsystems, Rueil-Malmaison, France), and samples were visualized using a x10 objective for aggregates and a x63 objective for single cell measurements. The stress σ applied to the sample was defined as the force $F = k\delta$ divided by the contact area A between the sample and the plate: $\sigma = F/A$. For single cells, the contact areas between the plates and the cell were estimated by assuming a circular contact, i.e. $A = \pi D^2/4$. The apparent contact diameters D_F and D_R (respectively for the flexible and rigid plates) were measured on bright field images. Since D_F and D_R are usually different, we retained their mean square value, leading to $\sigma = \frac{8F}{\pi(D_F^2 + D_R^2)}$. As for the cuboid cell aggregates of dimensions $L \times L \times h$, they initially lie on their square face. They are seized laterally between both microplates (Figure 3c). Hence, the contact surface is rectangular and its area $A = Lh$ is determined through bright field imaging. L is measured directly during the experiment. It may slightly differ from the measurement obtained before the microplate handling. h is estimated by using images taken before the microplates grasped the aggregates: a correction is applied to take into account the possible deformation due to handling (it is computed from the corresponding L values). Finally, the uniaxial cell or aggregate strain perpendicular to plates was defined as $\varepsilon = \frac{L-L_0}{L_0}$, where L_0 (resp. L) is the distance between the parallel microplates before (resp. after) compression. The apparent modulus is then given by $E = \sigma/\varepsilon$. Dynamical measurements were carried out in the frequency range from 0.1 to 3.2 Hz for cell aggregates, and from 0.1 to 0.4 Hz for single cells. In the latter case, measurements at higher frequencies were difficult to analyze: the very low stiffness of the flexible plate (at the limit of the technique), required to measure the very low viscoelastic modulus of F9 cells, makes it very sensitive to all viscous flows induced by the plate motion in the complex geometry around the plates and between both plates around the aggregate.

Model	AIC ($\times 10^4$)	α	J (Pa $^{-1}$)	a (Pa $^{-1}$)	$\bar{a}$ (Pa $^{-2}$)	b (Pa $^{-1}$)	β
(9)	-5.69 ± 0.06	0.25 ± 0.02	0.045 ± 0.010	$\times$	$\times$	$\times$	$\times$
(10)	-7.20 ± 0.07	0.22 ± 0.01	0.13 ± 0.03	2.2 ± 0.5	$\times$	$\times$	$\times$
(11)	-6.73 ± 0.07	0.22 ± 0.02	0.07 ± 0.02	$\times$	0.7 ± 0.4	$\times$	$\times$
(12)	-6.78 ± 0.07	0.22 ± 0.01	0.08 ± 0.01	$\times$	$\times$	-0.47 ± 0.15	$\times$
(13)	-7.16 ± 0.08	0.22 ± 0.01	0.16 ± 0.08	3.4 ± 3.3	-0.6 ± 1.0	-0.1 ± 0.4	$\times$
(14)	-8.30 ± 0.05	0.29 ± 0.02	0.07 ± 0.01	0.5 ± 0.2	$\times$	$\times$	0.20 ± 0.06

Table 1: **Model selection.** The AIC and optimized parameter values are given for models (9-14) (mean $\pm$ standard deviation, $N = 8$).

Broad spectrum signal

The stress signal we applied for the broad spectrum method is a superposition of several sinusoidal signals:

$$\sigma(t) = \sigma_0 \varphi(t) \sum_{i=1}^n \rho_i \cos(2\pi f_i t + \phi_i), \quad (7)$$

where σ_0 is used to tune the amplitude and $\varphi(t) = \tanh(\lambda t)$ is an envelope function used to smooth the beginning of the signal over the first few seconds. The frequency range is chosen as 0.1-10 Hz. The $n = 32$ frequencies f_i are equally spaced logarithmically within this range. The phases ϕ_i are chosen randomly within $[0, 2\pi]$. In order to damp high frequencies, the magnitude of the real prefactor ρ_i is chosen to decrease with the frequency. More precisely, ρ_i^2 is drawn uniformly within $[0, f_{\min}^2/f_i^2]$, which ensures that the complex amplitude $\rho_i e^{i\phi_i}$ is distributed uniformly in the disk of radius $f_{\min}/f_i$, a scaling reminiscent of $1/f$ noise.

Rheological models

Generally speaking, a rheological model may be defined by a p -dimensional parameter vector $\vec{\theta}$ and by an equation of the form $\varepsilon_{\text{loc}}(t) = \mathcal{R}(\{\sigma_{\text{loc}}(t')\}_{t' \leq t}, \vec{\theta})$, where $\mathcal{R}$ is an integro-differential operator acting on the function $\sigma_{\text{loc}}(t)$.

The rheological models we considered, defined in the following paragraphs by order of increasing complexity, contain fractional integrals [44], defined as:

$$I_{\alpha}^{\tau}(f) = \frac{1}{\tau^{\alpha} \Gamma(\alpha)} \int_0^t (t - t')^{\alpha-1} f(t') dt'. \quad (8)$$

where an arbitrary time scale τ is set to $\tau = 1$ s and where $I_{\alpha}^{\tau}(f)$ has the same dimension as f .

Linear fractional rheology This model has two parameters (both positive):

$$\varepsilon_{\text{loc}} = J_1 I_{\alpha}^{\tau}(\sigma_{\text{loc}}) \quad \vec{\theta} = (\alpha, J_1) \quad (9)$$

where α is dimensionless and J_1 is in Pa $^{-1}$.

Nonlinear fractional rheology These are non-linear versions of the order $\alpha \geq 0$ fractional rheology:

$$\varepsilon_{\text{loc}} = J_1 I_\alpha^\tau \left(\frac{\sigma_{\text{loc}}}{1 + a \sigma_{\text{loc}}} \right) \quad \vec{\theta} = (\alpha, J_1, a) \quad (10)$$

$$\varepsilon_{\text{loc}} = J_1 I_\alpha^\tau \left(\frac{\sigma_{\text{loc}}}{1 + \bar{a} \sigma_{\text{loc}}^2} \right) \quad \vec{\theta} = (\alpha, J_1, \bar{a}) \quad (11)$$

$$\varepsilon_{\text{loc}} = J_1 I_\alpha^\tau (\sigma_{\text{loc}}(1 + b \sigma_{\text{loc}})) \quad \vec{\theta} = (\alpha, J_1, b) \quad (12)$$

$$\varepsilon_{\text{loc}} = J_1 I_\alpha^\tau \left(\frac{\sigma_{\text{loc}}(1 + b \sigma_{\text{loc}})}{1 + a \sigma_{\text{loc}} + \bar{a} \sigma_{\text{loc}}^2} \right) \quad \vec{\theta} = (\alpha, J_1, a, \bar{a}, b) \quad (13)$$

where a and b are in Pa^{-1} and $\bar{a}$ is in Pa^{-2} . These models give various functional forms to the nonlinear relationship between the fractional derivative $I_{-\alpha}^\tau(\varepsilon_{\text{loc}})$ and σ_{loc} . By construction, they all reduce to (9) when $a = \bar{a} = b = 0$.

Nonlinear fractional rheology, two fractional integrals (orders $\alpha \geq \beta \geq 0$)

$$\varepsilon_{\text{loc}} = J_2 I_{\alpha-\beta}^\tau \left(\frac{\Sigma_{\text{loc}}}{1 + a \Sigma_{\text{loc}}} \right) \quad \vec{\theta} = (\alpha, \beta, J_2, a), \quad (14)$$

where we define $\Sigma_{\text{loc}} = I_\beta^\tau(\sigma_{\text{loc}})$, which reduces to $\Sigma_{\text{loc}} = \sigma_{\text{loc}}$ in the limit where $\beta = 0$. This model reduces to (10) when $\beta = 0$, and to (9) when $a = 0$.

Stress-strain relationship The volumic nature of the applied magnetic force implies that the stress is not uniform within the samples. Rather, it increases approximately linearly from the free surface of the aggregate up to its maximum value at the substrate surface: $\sigma_{\text{loc}}(z) = \sigma_{\text{max}}(1 - z/h_0) = 2\sigma u$, where σ is the spatially averaged applied stress and $u = 1 - z/h_0$ is the reduced distance from the upper surface of the aggregate. As a result, the local deformation ε_{loc} also depends on altitude *via* the (nonlinear) differential equation $\varepsilon_{\text{loc}}(z, t) = \mathcal{R} \left(\{\sigma(z, t')\}_{t' \leq t}, \vec{\theta} \right)$. Correspondingly, the overall aggregate deformation can be obtained by averaging the local deformation field. For a response $\mathcal{R}$ and a homogeneous magnetic force f_V , the macroscopic strain is given by:

$$\varepsilon(t) = \langle \varepsilon_{\text{loc}}(z, t) \rangle_z = \langle \mathcal{R} \left(\{2u \sigma(t')\}_{t' \leq t} \right) \rangle_{0 \leq u \leq 1}. \quad (15)$$

When the rheology $\mathcal{R}$ is linear, the spatial average commutes with $\mathcal{R}$, and global variables are also related through the same operator: $\varepsilon(t) = \mathcal{R} \left(\{\sigma(t')\}_{t' \leq t}, \vec{\theta} \right)$. For instance, for the linear fractional constitutive equation (9) one also has $\varepsilon = J_1 I_\alpha^\tau(\sigma)$ and for a step stress of magnitude σ_0 , using Eq. (8) we find a power law response:

$$\varepsilon(t) = \sigma_0 \frac{J_1}{\Gamma(1 + \alpha)} \left(\frac{t}{\tau} \right)^\alpha. \quad (16)$$

Comparing this result with definition (1), we obtain the creep compliance $J_0^L = J_1/\Gamma(1 + \alpha)$ for the linear case.

More care needs to be exercised when the rheology $\mathcal{R}$ is nonlinear. A useful example is the case of Model (10), for which the expected overall deformation reads:

$$\varepsilon(t) = \int_{u=0}^{u=1} J_1 I_\alpha^\tau \left(\frac{2u\sigma(t)}{1 + 2au\sigma(t)} \right) du \quad (17)$$

$$= J_1 I_\alpha^\tau \left(\int_{u=0}^{u=1} \frac{2u\sigma(t)}{1 + 2au\sigma(t)} du \right) \quad (18)$$

$$= J_1 I_\alpha^\tau \left(\frac{2a\sigma(t) - \log(1 + 2a\sigma(t))}{2a^2\sigma(t)} \right). \quad (19)$$

For a step stress, the compliance now depends on the step magnitude σ_0 in this non-linear case (see Figure 5e):

$$J_0^{\text{NL}} = \frac{J_1}{\Gamma(1+\alpha)} \frac{2a\sigma_0 - \log(1+2a\sigma_0)}{2a^2\sigma_0^2}. \quad (20)$$

Parametric inference

In order to assess to what degree the rheological model, defined by $\mathcal{R}$, is suitable to describe the experimental data $\{\varepsilon_{\text{exp}}(t)\}_t$, we assume that the experimental noise $\phi(t)$ is white and Gaussian (amplitude s) and contributes additively within the measured deformation value:

$$\varepsilon_{\text{exp}}(t) = \mathcal{R}(\{\sigma(t')\}_{t' \leq t}, \vec{\theta}) + \phi(t). \quad (21)$$

We then determine the values of the parameters $\vec{\theta}$ and s , *i.e.*, $p+1$ real numbers, that maximize the likelihood function

$$L\left(\{\varepsilon_{\text{exp}}(t)\}_t \mid \{\sigma(t)\}_t, \vec{\theta}\right) = \frac{1}{(2\pi s^2)^{N_t/2}} \exp\left(-\sum_{t=1}^{N_t} \frac{\|\varepsilon_{\text{exp}}(t) - \mathcal{R}(\{\sigma(t')\}_{t' \leq t}, \vec{\theta})\|^2}{2s^2}\right) \quad (22)$$

for N_t time points (typically $N_t \simeq 7 \cdot 10^3$ here). Indeed, the likelihood L indicates how likely it is to observe the actual experimental data $\{\varepsilon_{\text{exp}}(t)\}$, given the rheological model (encoded in $\mathcal{R}$), the parameter set $(\vec{\theta}, s)$ and the imposed stress history $\{\sigma(t)\}$. The larger the likelihood, the better the model and its parameters fit the experimental data.

Model selection

The rheological models defined by Eqs. (9-14) are tested against the experimental data by maximizing the likelihood function (22), yielding optimal parameters. Table 1 gathers the average values and standard deviations of these optimal parameters ($N = 8$).

Once the parameter values of each model have been optimized, we need to be able to select the best model. One difficulty is that the various models contain different numbers of parameters. In order to avoid both too simple models and too complex models (since overfitting may reduce reproducibility and/or predictability), we compute for each model the Akaike information criterion [56] defined as

$$\text{AIC} = -2\log L + 2(p+1). \quad (23)$$

The AIC provides an objective estimation of the quality of each model, penalizing the number of parameters p and favouring a high value of the likelihood function L [57]:

$$\text{AIC}(\mathcal{R}) = \sum_t \frac{\|\varepsilon_{\text{exp}}(t) - \mathcal{R}(\{\sigma(t')\}_{t' \leq t}, \vec{\theta})\|^2}{s^2} + N_t \log(2\pi s^2) + 2(p+1) \quad (24)$$

The absolute AIC values are not particularly meaningful, but their relative values provide a comparison between the different models, where the best model corresponds to the lowest AIC value.

On Figure 6b, the experimentally measured deformation $\varepsilon(t)$ (black points) is compared with the predictions provided by the linear fractional model (9) and the non-linear fractional models (10) given the applied stress $\sigma(t)$ displayed on Figure 6a. The linear fractional model (9) fails to reproduce the time evolution of the measured deformation, while the non-linear fractional model (10) yields satisfactory predictions. Inspection of Table 1 shows that estimated AIC values agree with these qualitative observations. Further, all nonlinear fractional models (10-12) have a lower AIC than that of the linear fractional model (9). Among (10-12) the lowest AIC is obtained by (10). The AIC obtained for (10) is consistent with that of the more complex model (13);

however most parameter values obtained for (13) are consistent with zero. For these reasons, we designate (10) as the best fractional model with one fractional integral.

Finally, introducing a second fractional order in model (14) allows to predict almost perfectly the deformation history (Figure S5a). The relevance of this model is confirmed by comparing phase space plots on Figures 6cd and S5b. However, numerical integration shows that the power-law behavior then predicted for step stress assays is characterized by an exponent that depends on the stress amplitude σ_0 , at variance with experimental observations (see Figure S5c). Further, the model fails to reproduce quantitatively the σ_0 dependence of the creep compliance J_0 (Figure S5d).

Acknowledgements

The authors thank O. Cardoso for the assistance he provided on edges tracking. Our thank to A. Fromain for help on VSM measurements. We thank Ali Abou-Hassan (PHENIX, UMR 8234, Paris) for providing us with the magnetic nanoparticles. The authors gratefully acknowledge funding from the city of Paris Research Program Emergence (Grant IDs ‘MAGIC’) and support from the European Union (ERC-2014-CoG project MaTissE 648779).

Supplementary

Figure S5: **Nonlinear behavior: model including two fractional exponents, Eq. (14).** **a** Strain response (black markers) of the aggregate to a broad spectrum strain fitted by a two exponents fractional model (pink line) (fit quality $R^2 = 0.9988 \pm 0.0004$) **b** Phase space representation of the same data (black) with the corresponding fit by the two exponents fractional model (pink). Variation of the exponent α (**c**) and of the compliance J_0 (**d**) fitted from the three creep functions with the applied force per unit volume. Note that the predicted value of α decreases with f_V .

Figure S6: **Supplemental movie of an aggregate submitted to a force step corresponding to a 2 Pa solicitation during 10 s** Large field movie of an aggregate while it is flattened by a maximal force step for 10 s.

Figures

Figure 1

[Please see the manuscript file to view the figure caption.]

Figure 2

[Please see the manuscript file to view the figure caption.]

Figure 3

[Please see the manuscript file to view the figure caption.]

Figure 4

[Please see the manuscript file to view the figure caption.]

Figure 5

[Please see the manuscript file to view the figure caption.]

Figure 6

[Please see the manuscript file to view the figure caption.]

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [creaneau250100fps.avi](#)