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# Cell-collagen networks breakdown by cell remodelling

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## Abstract

Collagen model tissues are analyzed, which consist of cells embedded in a collagen matrix at different concentrations (of cells and collagen). Rheological properties are measured and complementary confocal microscopy analyses are carried out. An important feature is observed, corresponding to the breakdown of the collagen network (i.e. decrease in network elasticity) for high collagen concentrations, due to the presence of cells. Thanks to confocal microscopy, we show that cells elongate within the gel and can remodel it, this being a concentration-dependent feature: the anchoring of cells is enhanced when collagen concentration increases, but cells have less space to deform themselves. These two mechanisms compete and can explain the observed rheological features.

**Key words:** tissue; collagen; rheology; confocal; reflectance; remodelling

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## 1. Introduction

The rheology of biological systems is of major importance for bioengineering and materials research; it has been a source of interest for many years [1], for it concerns a wide variety of human tissues, such as muscles, arteries, connective tissues, but also new biomaterials like scaffolds to be used for restoration medicine, wound healing, and various therapies. The rheological properties of such tissues cover a wide range, going from simple elastic materials, to fluid-like ones, but in the majority of tissues, linear and nonlinear viscoelastic effects [2] as well as viscoplastic effects (gel-like) seem to be ubiquitous [3]. The essence of these properties rely on the fact that cells live in a fluid material filled with fibres (i.e. collagen, and other matrix proteins), which give rise to both viscous and elastic properties. It is the complex interplay between cell deformation, fibre reorganization, and fluid flow [4] which leads to the understanding of such viscoelastic properties and to possible modelling [5-7].

In order to understand the complexity of such materials, model systems are commonly studied including basic solutions with components of the extra cellular matrix (ECM) which are collagen, laminin, elastin, fibronectin, etc. or other biopolymer gels present within cells, such as actin [8-9], tubulin, fibrin [10] which are basic cell elements. The rheological properties of such systems depend on mechanical and chemical properties of the polymer network, on the ability of components to assemble and persist, and can therefore exhibit either uncrosslinked polymer features or rheological properties as in crosslinked gels, in particular different crosslinks (e.g.  $\alpha$ -actinin, myosin II) holding the network together are shown to lead to a rich variety of such properties.

Commonly used techniques for studying biological gels range from microrheology devices (Optical tweezers [11], magnetocytometry [12], AFM [13], etc.) to conventional rheometry, compression tests [14], uniaxial tension or sometimes home-made devices [15]. Although relaxation tests [16-17] or cycle tests [18] are commonly used, dynamic tests – in a range of angular frequencies  $\omega$  - are the most versatile. Frequency-dependent viscoelastic properties ( $G'$  and  $G''$ ) have been obtained previously for F-actin solutions [19] and scaling laws for the elastic modulus  $G'$  vs. concentration were found for various filamentous biopolymer networks [20], using oscillatory shearing. For example, several researchers studied the rheology of Type I collagen [14,21-22] or modified collagen [23] and found similarities with other semi-flexible polymer networks. This motivated attempts to account for such rheology using models based on polymer networks theories [2,5,24-25]; indeed the arrangement of long protein filaments into networks of different geometries can be applied to such materials.

Going towards a more sophisticated model tissue implies to embed cells into the protein network and requires particular techniques (control of temperature, percentage  $\text{CO}_2$ , pH, etc.). Under such proper conditions, it is found that unlike classical fillers (or particles) in composite materials, cells play an active role by interacting with the extra cellular matrix (or fibre network), leading to growth/shrinking, reorganization or proteolytic activity [26]. For example, chicken embryo fibroblasts were found to give rise to an additional contribution to the stress in fibroblast-populated collagen matrices [27]. Therefore, the stiffness of the extracellular matrix can profoundly influence cell and tissue behaviors, and conversely. Another example is the microrheometric (magnetic bead-twisting) assay used to measure changes of a cell-collagen network [28]. The authors found that

cells elongate within the matrix and change the local micromechanical properties of type I collagen gel, in a cytokin-dependent manner. In this respect, the ECM is a rich signalling medium, where both biochemical and mechanical properties can rule physiological and pathological processes [29]. Therefore the ECM mechanical properties can be tuned for proper functions, thus strongly influencing the behavior of cells [30-31]. As a particular example, cellular sub-structures in myoblasts can be strongly affected by the rigidity of the underlying tissue [30].

To characterize a cellular network, one needs to measure the rheological properties, and relate these mechanical behaviors with respect to microstructure changes, using relevant techniques. The most classical ones used are SEM (Scanning electron microscopy) [32] or conventional biology tools such as histology [33-34] which require special preparations, meaning possible artefacts. Therefore such techniques are nowadays replaced by confocal microscopy (including laser reflectance for visualizing collagen fibres in the matrix) [35-38] leading to amazing observations of the scaffolds microstructure [39]. These observations have recently been coupled with micromechanical tools to analyze the stress effects on collagen microstructure [40].

Previous studies concern different cell types [41-42], but most of them usually focus on fibroblasts - which are major repair cells - embedded within 3D scaffolds containing matrix proteins [16,18,26,38,43-45]. Such model networks have been used to understand the migration/deformation of fibroblasts and the remodelling/compacting effect they can have on the ECM [38,46]. Collagen gels were used extensively with fibroblasts for studying the cell-matrix mechanical interactions. However, no results about the overall viscoelastic properties of such model networks using both conventional rheology and confocal microscopy have been carried out on systems with various concentrations.

Therefore, in this study, we propose to develop a 3D model network that mimics a normal tissue (e.g. connective tissue) and study its viscoelastic properties using both dynamic rheometry and confocal reflectance microscopy. To undertake such investigations, we used a collagen network at various concentrations, filled with different amounts of CHO cells. It was then possible to investigate the behavior of cells in different micro-environments. Materials and methods are described in §2, whereas the model networks viscoelastic properties are investigated in §3. At the same time, we used complementary confocal scanning microscopy (combined reflectance and fluorescence) to investigate cell-matrix interactions. In §4, some interesting properties are analyzed within the framework of a simple model, explaining how possible interactions can compete and give rise to complex behaviors. Finally, conclusions are presented in §5.

## **2. Materials and methods**

### **2.1 Cells, collagen, and gels**

We used a CHO cell line (Chinese Hamster Ovary) - an established line of fibroblasts – which was previously investigated by the authors, as a suspension at high concentrations [36]. CHO cells were grown in culture medium (DMEM containing 10% Fetal Calf Serum) using standard T75 flasks under proper conditions (37°C, 5% CO<sub>2</sub>) in a cell incubator, until they reached 70% confluence. Cells were then detached with trypsin, and suspensions at different concentrations were prepared using CHO cells and culture medium. Cell concentration was determined using a Neubauer's cell chamber, then the required concentration was adjusted.

Collagen (type I) was prepared from a rat tail collagen solution (BD Biosciences) and was mixed with culture medium. Then neutralization was carried out using NaOH

(0.1M) to reach a pH of 7.4 while maintaining the temperature at 4°C. After adding cells, the system was transferred onto the rheometer at 37°C for collagen polymerization during 30 mn.

Two kinds of tissues/networks were prepared in order to investigate collagen networks alone as well as cell-populated collagen matrices :

a) Collagen gels at four different concentrations (0.42-0.95-1.38-1.8 mg/mL)

b) Collagen gels including cells at different cell concentration. Three different cell concentrations were used:  $0.7 \cdot 10^7$  cells/mL,  $1.17 \cdot 10^7$  cells/mL and  $1.8 \cdot 10^7$  cells/mL. These concentrations correspond to cell volume concentrations of 4.7%, 7.8%, 12% respectively, and were adjusted so that collagen concentrations remained the same as in a).

## 2.2 Rheology

Rheometrical measurements of the networks were conducted with a Bohlin Rheometer (Malvern, Gemini 150, controlled stress). The temperature was kept at 37°C (peltier system, +/- 0.1°C) which allows for collagen polymerization as stated above, and throughout the experiments. A closed chamber saturated with water was placed around the sample in order to avoid evaporation. Dynamic oscillations were performed, i.e. the sinusoidal strain  $\gamma = \gamma_0 \sin(\omega t)$  was measured corresponding to the applied shear stress  $\tau = \tau_0 \sin(\omega t + \phi)$ , where  $\omega$  is the angular frequency, and  $t$  is time.  $\gamma_0$  and  $\tau_0$  are respectively the amplitude of the deformation and the amplitude of the stress. Therefore, dynamic moduli ( $G'$ ,  $G''$ ) can be obtained at different frequencies  $f$  (where  $\omega = 2\pi f$ ) in the range [0.01-2Hz] by selecting the in-phase elastic deformation ( $G'$ ) and its out of phase viscous counterpart ( $G''$ ):  $G' = \tau_0 \cos\phi / \gamma_0$  and  $G'' = \tau_0 \sin\phi / \gamma_0$ . The viscoelastic properties ( $G'$ ,  $G''$ ) of our materials were measured using parallel

plate geometry (20mm diameter) and a gap ranging between 500 $\mu$ m-800 $\mu$ m, since samples are of limited volumes. This is similar to the geometry previously used with CHO cell suspensions [36]. Experiments were started immediately after polymerization. The gels have a linear domain at moderate deformations  $\gamma_0$ , therefore collagen samples were tested under low strains ( $\gamma_0=0.01$ ) within the linear domain, in agreement with previous work [44], and similarly for cell-filled networks. Gels typically exhibit a flat plateau for  $G'(f)$  – or slowly increasing  $G'(f)$  - and a smaller  $G''(f)$  modulus (see supplementary data S1), with  $G''(f)$  also slowly increasing. This is the signature of a viscoelastic behavior. In what follows, we chose to show only the dependence of  $G'$  vs. frequency  $f$ , for this is the most significant modulus, and also in order to have a limited number of curves in each graph for clarity.

### **2.3 Confocal Microscopy**

To characterize structural changes in our model tissues, confocal microscopy was used in addition to rheometry. The idea was to analyze cell shapes as well as their behavior within the collagen network, and compare with the rheological data. The collagen matrix organization could also be analyzed. Birefringence is indeed an intrinsic optical property of many natural ordered biopolymers, including collagen fibers [47-48]. The collagen-induced depolarisation of the linearly polarized incident light allows the imaging of nascent 3D fibres in amorphous medium with high signal-to-noise ratio in confocal reflection mode. Confocal reflection microscopy and fluorescence were proposed earlier as powerful coupled tools to study 3D cell migration [35], and were used here to examine the organization of cells within the matrix. When combined with confocal microscopy, reflection images taken at sequential focal planes along the  $z$  axis can be reconstructed in 3D for visualization



and for image analysis. For an easy visualization of cells inside collagen gels, we have developed a stable CHO transfected cell line expressing GFP. Therefore reflected light from the collagen matrix was collected simultaneously with fluorescence from cells in a time-lapse mode to monitor the dynamic process of cell behavior through the 3D collagen matrix. In order to do so, collagen gels with and without cells were polymerized in a Lab-Tek chamber with coverglass and imaged using a confocal microscope (inverted Zeiss Axiovert 135M, LSM 510 model). The method was performed to visualize matrix fibres and cells using a 40X objective lens (Zeiss, C-Apochromat, water immersion). During the experiments, samples were placed in a temperature and CO<sub>2</sub> controlled on-stage incubator (37°C, 5% CO<sub>2</sub>). Two lasers were coupled to the microscope: an Argon laser at 488 nm was used to visualize the GFP-marked cells and a HeNe laser at 633 nm to visualize collagen structures.

## **2.4 Image processing**

In order to get more precise data on cell morphology, an image processing method was used. Cells from confocal images (z-stack) were first extracted and subdivided into many building blocks using Metamorph<sup>TM</sup>. To process the data, we used the Edit3D\_quant software (kindly provided by Yves Usson, TIMC-IMAG, Grenoble). The analysis and 3D visualisation software were written in standard C language using the GL library (Silicon Graphics library) and the FORMS user interface library (courtesy of Marck Overmars, Department of Computer Science, Utrecht University, The Netherlands) [49-51]. The software gives the 3D shape of the cell, so we obtained cell volumes including the nucleus, as well as other relevant

parameters (surface, major and minor axes, etc.) from which the cell elongation was calculated (see for example Fig.7a-7b).

### 3. Results

#### 3.1. Rheology

The collagen solutions were tested first. The frequency dependence of  $G'$  and  $G''$  at 1% deformation was measured for frequencies  $f$  ranging from 0.01 to 2 Hz at 37°C. The elastic modulus was always larger than the loss modulus. Both moduli increase very slowly with  $f$  and are almost parallel (see supplementary data S1), a result quite similar to a previous one [14,23] obtained for collagen gels (2mg/mL) in oscillatory shear at a constant strain of 1%. The order of magnitude of the plateau modulus  $G_0$  (i.e. the value for which  $G'$  is constant or has an inflexion point, here the value at 0.1 Hz was used) at a collagen concentration of 1.8mg/mL is of the order of 75 Pa, in good agreement with previously reported values [5,21,23,28]. The four collagen gels tested here (0.42-0.95-1.38-1.8 mg/mL) all exhibit increasing viscoelastic moduli with collagen concentration. This can be seen as part of the data in Fig. 1 (and also in supplementary data S1) where the evolution of the elastic modulus  $G'$  is presented vs. frequency at four different concentrations (and three cell concentrations).

The cells-filled collagen networks were characterized next. Measurements of storage and loss modulus  $G'(f)$  and  $G''(f)$  were respectively carried out in the frequency range  $0.01 \text{ Hz} < f < 2 \text{ Hz}$  at three cell concentrations ( $0.7 \cdot 10^7$ ,  $1.17 \cdot 10^7$ ,  $1.8 \cdot 10^7$  cells/mL) for each collagen network. The results are also shown in Fig 1. We observe that for both situations, with cells and in the absence of cells, the viscoelastic moduli always increase with collagen concentration, when matrices with the same cell concentration are tested.

An interesting feature is found when solutions with different cell concentrations are analyzed, as can be seen in Fig. 1.

- At small collagen concentration ( $c \leq 0.95$  mg/mL), the collagen elastic modulus  $G'$  is lower than the cell-collagen networks modulus. As more cells are included (from  $0.7 \cdot 10^7$  cells/mL to  $1.8 \cdot 10^7$  cells/mL), the elasticity of the network increases further.

- On the other hand, at higher collagen concentrations ( $c \geq 1.38$  mg/mL), the collagen elastic modulus  $G'$  is reduced when cells are included into the network. This is shown by the dramatic decrease of the elastic modulus  $G'$  when  $0.7 \cdot 10^7$  cells/mL are included within the matrix. When more cells are added (i.e.  $1.38 \cdot 10^7$  cells/mL and  $1.8 \cdot 10^7$  cells/mL), the network elasticity increases again and can reach its initial level ( $c=1.38$  mg/mL) or higher ( $c=1.8$  mg/mL). This feature therefore seems to be a concentration-dependent phenomenon and will be discussed in the next part.

In Fig. 2, we present the evolution of the elastic modulus  $G'$  at fixed cell densities, when varying the collagen concentration.  $G'$  increases with frequency  $f$  as usual, and always increases with collagen density.

Since the frequency dependence (slow increase of  $G'$ ) is in agreement with previous works, we clarify this analysis further by representing  $G'$  (more precisely its value  $G_0$  at  $f=0.1$  Hz) as a function of concentration, in the 16 different cases studied. This is shown in Fig. 3 using a log-log scale. This representation is interesting because it can allow one to determine the exponent of the power law model. Experimental data from other works have indeed shown the concentration dependence of the plateau shear modulus to vary like  $c^{2.2}$  in the case of actin gels,  $c^{2.1}$  for fibrin networks [20], and  $c^3$  for collagen networks [40]. Our data gives a dependence  $G' \approx c^{2.6}$ , in excellent agreement with previously reported data.

The presence of cells embedded into the matrix shows a different dependence vs. collagen concentration (Fig. 3), leading to a smaller slope of order 1 (more precisely the slopes of the fits are respectively 1.11, 1.08 and 0.99 for cell concentrations respectively  $0.7-1.17-1.8 \cdot 10^7$  cells/mL) at the three cell concentrations studied. Again the dramatic effect of cells embedded in the network is outlined by the crossover of the collagen curve with the cell-filled network data at  $c > 1.38$  mg/mL.

To understand this cell effect, it seemed important to study the microstructure of the matrices, including the way cells organize within the gels, in particular through interactions with the filaments. This was possible thanks to complementary confocal microscopy data which is presented next.

### **3.2 Confocal Microscopy**

To understand first how the matrices behave, collagen gels with the same concentrations as above (0.42-0.95-1.38-1.8 mg/mL) were prepared and analyzed using confocal reflectance microscopy. The images were taken 120  $\mu$ m deep into the sample and revealed the presence of the collagen fibres. A simple visual inspection suggested that the matrices present an increase in fibre density (or decrease in mesh size) with increasing collagen concentration, as expected [47]. Figures 4 display this clear effect and the analysis of global pixel intensity versus concentration revealed a monotonous increase (data not shown), confirming the validity of our data.

The mechanical impact of the cells on their microenvironment (i.e. collagen fibres) was investigated next. Cells ( $1.8 \cdot 10^7$  cells/mL or 12% volume concentration) were embedded within gels (see Materials and Methods) with collagen concentrations ranging from 0.42 mg/ml to 1.8 mg/mL. This is shown in Figs 5a-d at a low magnification first.

- It seems obvious that the elongation of the cells (around 3 and 3.5) in Fig. 5a at low concentration is important. Cells adhere easily and show long protrusions. Such cells seem to deform the collagen fibres in their close neighbourhood and probably also pull collagen towards them, i.e. they remodel it.
- On the other hand, cells seem to be less elongated (aspect ratio or elongation slightly higher than 1) in the next two Figures 5b-c. Stiffer matrices seem to lead to round cells because they might be affected by a lack of space. Still cells are able to make short protrusions.
- Finally, at large collagen concentration (Fig.5d) cells seem to be stuck within the concentrated collagen. They keep a round shape (aspect ratio or elongation close to 1) similar to the ones observed previously in a dense collagen matrix 5mg/mL [34]. Interesting new regions filled with fluid (i.e. culture medium) appear and maybe due to the interplay between cell tractions/remodelling and fluid pressure.

To quantify these observations we analyzed image stacks by selecting single cells (zoom X3) embedded into collagen gels at the different concentrations (0.42-0.95-1.38-1.8 mg/mL). Fig.6a confirms that, in a less concentrated collagen gel, cells present a large deformation/elongation, in contrast with higher collagen concentrations where cells keep their almost round shape with very small protrusions (Fig.6b-d) corresponding to an amoeboid motion. In addition, Fig.6d shows a high density of fibres around the cell in agreement with the fact that cells play an attractive remodelling role of the collagen. To quantify these effects more precisely, an image processing method was used. Cells were isolated from the images (see Materials and Methods) and their global parameters (positions, surface, volume, etc.) were calculated. We focused on their elongation which seems a relevant parameter here. Fig. 7a shows typical projected shapes of the most elongated cells found for the

small collagen density (0.42mg/mL). Collecting results for a sufficient number of cells in different collagen matrices (18-16-19-16 for collagen concentration of 0.42-0.95-1.38-1.8 mg/mL respectively) leads to the chart in Fig. 7b where the number of cells vs. elongation is shown for the four collagen concentrations. Finally the data is collected in Fig. 8 where cell elongation is represented against collagen concentration. Different parameters from the statistical analysis can be used (maximum, minimum, mean, etc.). It appears that the cell elongation has a mean value around 2 at 0.42 mg/mL, then decreases down to 1.2 at 0.95 mg/mL then it increases slightly again to reach a plateau of 1.5 at the higher concentrations (1.38 or 1.8 mg/mL). Probably the explanation of such a complex shape for this curve needs to be understood based on the competition between different effects. This will be discussed in the next part.

#### **4. Discussion**

The first part of this work deals with the intrinsic properties of collagen gels. Such properties result from the interactions between two components: a network of collagen fibrils and an interstitial fluid, i.e. the culture medium. When mechanical stress is applied, the network and the medium deform together and the resistance to the interstitial flow of the solution through the network can lead to high pressure, which allows the gel to withstand compressive loads [14]. In shear, the resistance is quite important and the elastic moduli  $G_0$  can reach rather large values, in agreement with previous works [14,23]. The ability of the network to resist such stresses is due to its particular dense crosslinked microstructure as exhibited in Figs. 4. Our data for collagen gels is also comparable with the results obtained in laser trap microrheology [21] where the authors obtained an increasing shear modulus  $G$  with collagen

concentration  $c$  (as in our Figures 1 and 4), although they found a variation in shear moduli using different samples at the same concentration. The data obtained for  $G'(0.1 \text{ Hz})$  vs.  $c$  with a typical slope of 2.6 (see Fig. 3) is in agreement with previous works on actin and collagen networks [20,40].

In the second part of this work, cells are embedded into the gel. It is shown, for the first time, using rheological measurements, that the matrix stiffness is globally reduced by a rather large amount at high values of  $c$  (the collagen concentration), this effect being due to the presence of cells (see Figures 1-2-3). This effect, although surprising, might be due to the ability of cells to remodel the matrix as already discussed in the literature, where fibroblasts were earlier shown to be able to condense a tissue into a fibrillar structure [43], i.e. to compact it [38] or colonize it [33-34]. Note that other living cells (like bacteria) can also modify the viscoelasticity of certain gels [52]. This effect has been observed by Leung and co-workers using a different technique [28] : in their study, they used bead twisting microrheology to investigate the effect of cells (fibroblasts) included in a collagen matrix. The results are rather similar : the local properties of the collagen matrix are more important before including cells, and decrease when cells are included into the matrix (25K, 50K and 100K cells/mL, less than what was used in the present study). The authors also carried out this analysis as a function of incubation time (1-7 days) and they found the same effect except for very long durations. We restrict here the analysis to short term behavior (<24h) of cell-filled collagen matrices because it is known that over longer periods other mechanisms may take place, for example the expression of collagenase (MMP1) and gelatinase A (MMP2) leading to matrix degradation/hydrolysis [33]. Finally it can be concluded that the modulus reduction

observed for short times [28] is in agreement with our experiments (carried out within 24h).

The effect found in Fig. 3 seems to be similar to results obtained experimentally by Evans and Barocas [38], although their values are slightly higher than ours: their data indeed shows a small decrease of the Young Modulus  $E$  (difficult to detect) with cell concentration, then an increase at the higher cell concentrations, in the case  $c=3$  mg/mL. At lower values of  $c$  (1-2 mg/mL),  $E$  increases as in our case. Note that their model only predicts an increase with cell concentration, because it relies on theories with elastic inclusions, which are non living particles. Therefore, this paper confirms previous works [28,38] showing that the local collagen elastic modulus can be affected (i.e. decreased) by the presence of cells due to their interactions with the matrix.

Another interesting effect was the slope change observed in Fig. 3, corresponding to the elastic shear modulus  $G_0$  vs. collagen concentration  $c$ . This slope changes drastically from a value of 2.6 (case without cells) to a value of 1 when cells are embedded into the gel. This effect further enhances the fact that cells have a definitive role on the organization of collagen fibers. This effect may be due to the expression of integrins bound to the collagen fibres, and to the active behavior of CHO cell cytoskeleton through Myosin II-B enhanced contraction, as suggested earlier [45].

Let us now discuss these effects in view of the confocal microscopy analysis. It was found (see Figs 5-6) that cells behave differently depending on the collagen matrix concentration. Indeed, low concentrations lead to more elongated cells (Fig. 5a) whereas larger concentrations lead to smaller round cells (Fig. 5b-c); finally for even larger concentrations, cells might elongate again (Fig. 7-8). It has also been



emphasized that new regions appear in the cell-filled matrix (Fig. 5) as compared to the collagen networks (Fig. 4) which are more regular with a periodic well-defined mesh size.

Fig. 8 shows that elongation vs. collagen concentration  $c$  implies complex mechanisms due to the presence of a minimum of elongation (for the collagen concentration 0.95 mg/mL). To explain this feature, a simple analysis is proposed based on the existence of two competing effects leading to a minimum, a phenomenon also observed for 2D cell migration [53-54].

- Effect 1: binding to collagen

(a) when collagen density is low, cells have few anchoring contacts available and have difficulties to adhere, so they do not elongate.

(b) when the collagen density is high, cells can adhere easily because they have more anchoring contacts, and spread more easily.

- Effect 2: size effects

(a) when the collagen density is low, cells have more space and spread/elongate.

(b) when the collagen density is high, cells do not have any space to move so they do not elongate.

These two competing effects result in the minimum of the curve representing the elongation vs. collagen concentration.

Another important consequence affecting the microstructure of the network is the attachment of cells to the fibres and the tensions they generate (Figures 5c-d and 6c-d), which leads to remodelling of the collagen network, and possible breakdown in elasticity by creation of larger aggregates made of cells/collagen (Fig. 9) in between which the interstitial fluid can circulate (Fig 5d). This effect is shown to lower the global elasticity of the tissue.

Let us summarize all this information:

- **Low collagen concentration:** cells have space but can make less contacts. It is the space effect which dominates. Cells elongate and adhere strongly. They do not move fast and do not remodel the network very much. The network rigidity remains low.

- **Intermediate collagen concentration:** cells have less space but more contacts. They do not have enough space to elongate. They remain round (small elongation) and are quite motile. They remodel the network.

- **high collagen concentration:** cells have hardly any space, but a lot of contacts to adhere. Cells are slightly elongated, pull on the collagen fibers, and eventually they remodel the network strongly (or could undergo apoptosis [34]). The rigidity of the network is strongly reduced (Fig. 1).

Finally, the drastic reduction of the elastic moduli obtained previously can be explained by the ability of cells to attract collagen fibers and bring them closer. Cells reorganize the initial network (Fig.9, left) where the collagen exhibited strong interacting crosslinks (Figs 4) corresponding to a rigid gel. This remodelling by cells leads to a loose network including weakly connected islands drained by the interstitial fluid (Fig. 9, right) as observed in confocal microscopy (Fig 5d). This idea seems to be true for two of the collagen densities tested (1.38 mg/mL and 1.8 mg/mL) and applies to all three cell concentrations studied ( $0.7 \times 10^7$ - $1.17 \times 10^7$ - $1.8 \times 10^7$  cells/mL).

## 5. Conclusions

In this work, we focused on the mechanical impact that CHO cells (a fibroblast type) can have on their microenvironment, i.e. a collagen network, and conversely. Bulk

rheology, as well as confocal reflectance/fluorescence microscopy were used to explore the complex interplay between the different network elements.

Collagen matrices were found to exhibit viscoelastic properties with an elastic modulus following a nonlinear relation with collagen concentration in agreement with previous works.

Regarding cell-filled matrices (tissue-like), we found that cells can affect the collagen rheology dramatically. This was shown by a strong decrease of the elastic shear modulus  $G'$  of cell-collagen networks at the highest collagen concentrations used ( $c > 1$  mg/mL). Confocal microscopy enabled to explain this dramatic change by revealing the cell-collagen microstructure. In particular, the elongation of cells within the gel was found to be a possible mechanism for gel remodelling: interactions between cells and their micro-environment seemed to be based on the competition between “matrix anchoring” (i.e. adhesion) and “spacing”, giving rise to non trivial deformation of cells. In the high concentration collagen matrices, remodelling of the network was enhanced by the ability of cells to attract the fibers, thus leading to new microstructures and to the breakdown of the elastic modulus.

Perspectives of this work could focus on the ability of the confocal method to capture matrix fiber heterogeneities or orientations in cell-populated collagen gels [40,55]. But they could also find interest in the combined effects of cell remodelling, proteolytic degradation [56] and growth [26,48] on longer time scales [33-34]. Finally they would be ideally suited for studying cell migration in 3D collagen matrices.

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## Figure captions

**Fig. 1:** Elastic modulus  $G'(f)$  as a function of frequency  $f$  at four fixed collagen concentrations (0.42-0.95-1.38-1.8 mg/mL) using different cell concentrations:  $0-0.7 \times 10^7-1.17 \times 10^7-1.8 \times 10^7$  cells/mL.

**Fig. 2:** Elastic modulus  $G'(f)$  as a function of frequency  $f$  at four fixed cell concentrations ( $0-0.7 \times 10^7-1.17 \times 10^7-1.8 \times 10^7$  cells/mL) using different collagen concentrations : 0.42-0.95-1.38-1.8 mg/mL (same as Fig. 1 but shown in a different way).

**Fig. 3:** Concentration dependence of the shear elastic modulus  $G_0$  (0.1 Hz) vs collagen concentration (log-log scale) with and without cells. The fits indicate power law with respective exponents 2.6 for collagen gels alone and 1.11-1.08-0.99 for cell concentrations  $0.7 \times 10^7-1.17 \times 10^7-1.8 \times 10^7$  cells/mL.

**Fig. 4:** Confocal reflectance images at four collagen concentrations demonstrate the architectural differences between matrices prepared from type I collagen. Bar = 40  $\mu\text{m}$  for all images. (a) 0.42 mg/mL (b) 0.95 mg/mL (c) 1.38 mg/mL (d) 1.8 mg/mL.

**Fig. 5:** Confocal reflectance and fluorescence microscopy for collagen gels with cells; (magnification 40x, 1  $\mu\text{m}$  z-slice, xy: 358 x 358  $\mu\text{m}$ , Zoom 0.7). (a) 0.42 mg/mL (b) 0.95 mg/mL (c) 1.38 mg/mL (d) 1.8 mg/mL.

**Fig. 6:** Confocal reflectance and fluorescence microscopy for collagen gels with cells; (magnification 40x, 1  $\mu\text{m}$  z-slice, xy: 76.8 x 76.8  $\mu\text{m}$ , Zoom 3). (a) 0.42 mg/mL (b) 0.95 mg/mL (c) 1.38 mg/mL (d) 1.8 mg/mL.

**Fig. 7:** (a) Examples of increasingly elongated cells in a low density collagen gel (0.42 mg/mL) obtained by surface projection using Edit3D. (b) Histograms showing cell elongation for different collagen concentration, N number of cells.

**Fig. 8:** Cells elongation vs. collagen concentration. Boxes outline the 25–75% data range, squares in the boxes correspond to the mean elongation value, crosses (x) delimit the 99% data range, and hyphens (-) represent minimum and maximum elongation values.

**Fig. 9:** Sketch of the possible structure formed by cells surrounded by collagen network: Cells reorganize the initial collagen matrix (left) by attracting collagen thus depriving other regions from collagen, and creating less dense regions where fluid can flow. The rigidity of the network on the right is smaller.

**Supplementary data S1:** Rheometrical data ( $G', G''$ ) for the four collagen gels considered (0.42-0.95-1.38-1.8 mg/mL).

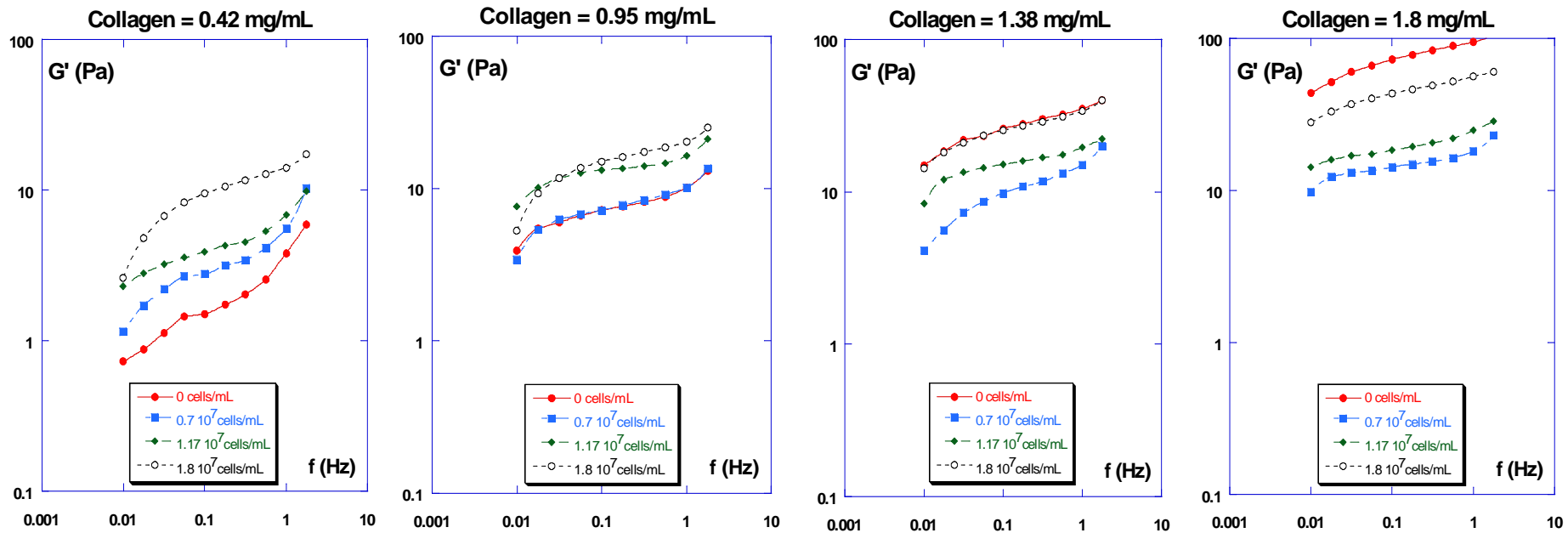
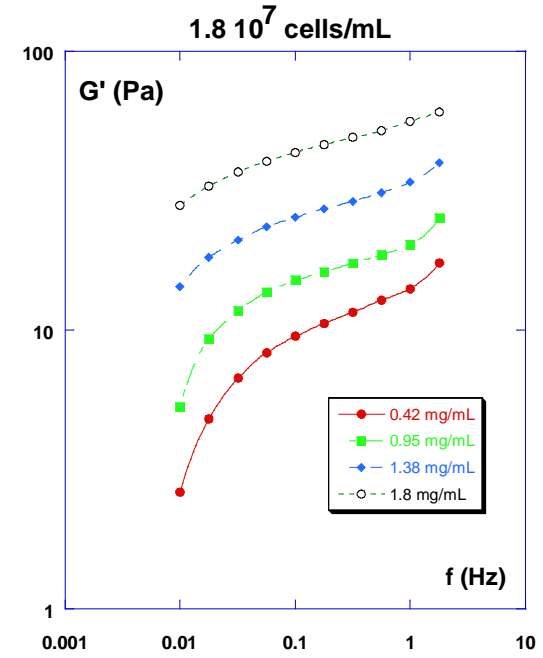
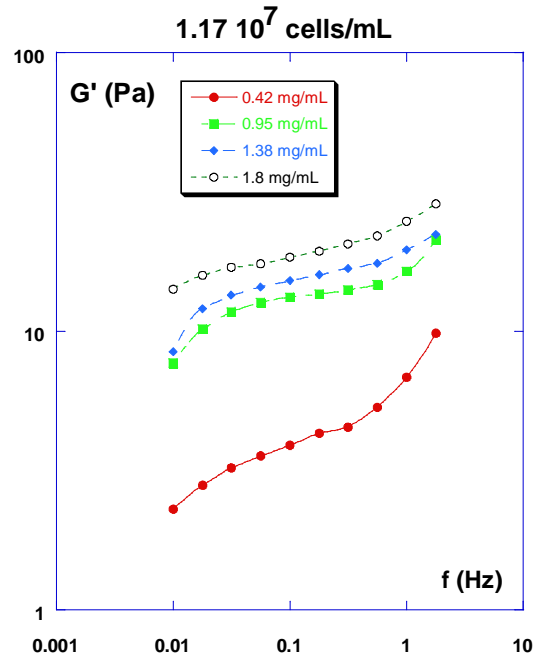
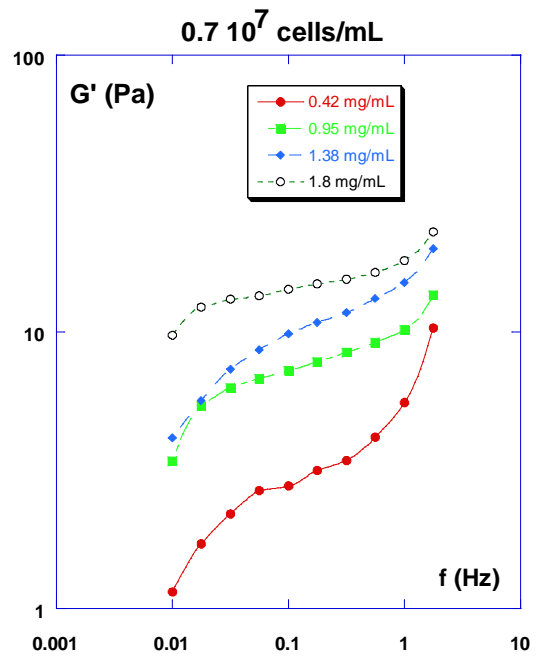


Figure 1



**Figure 2**

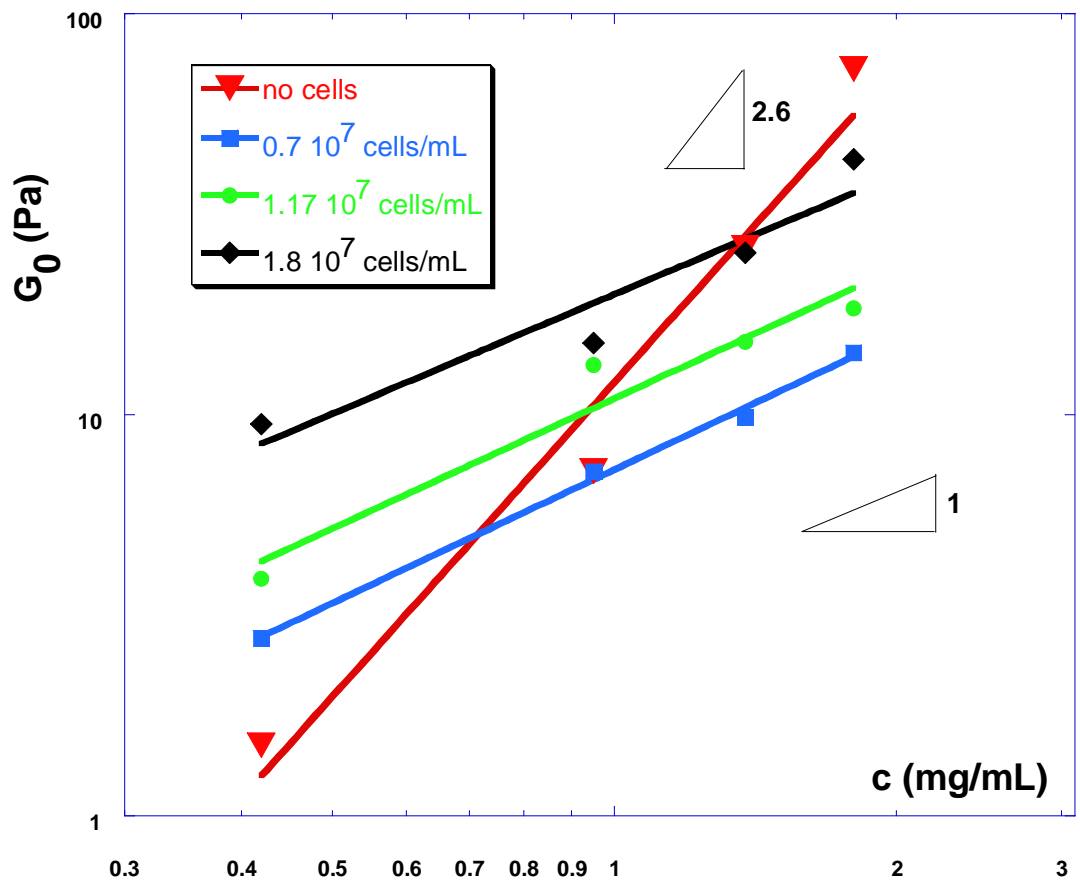


Figure 3

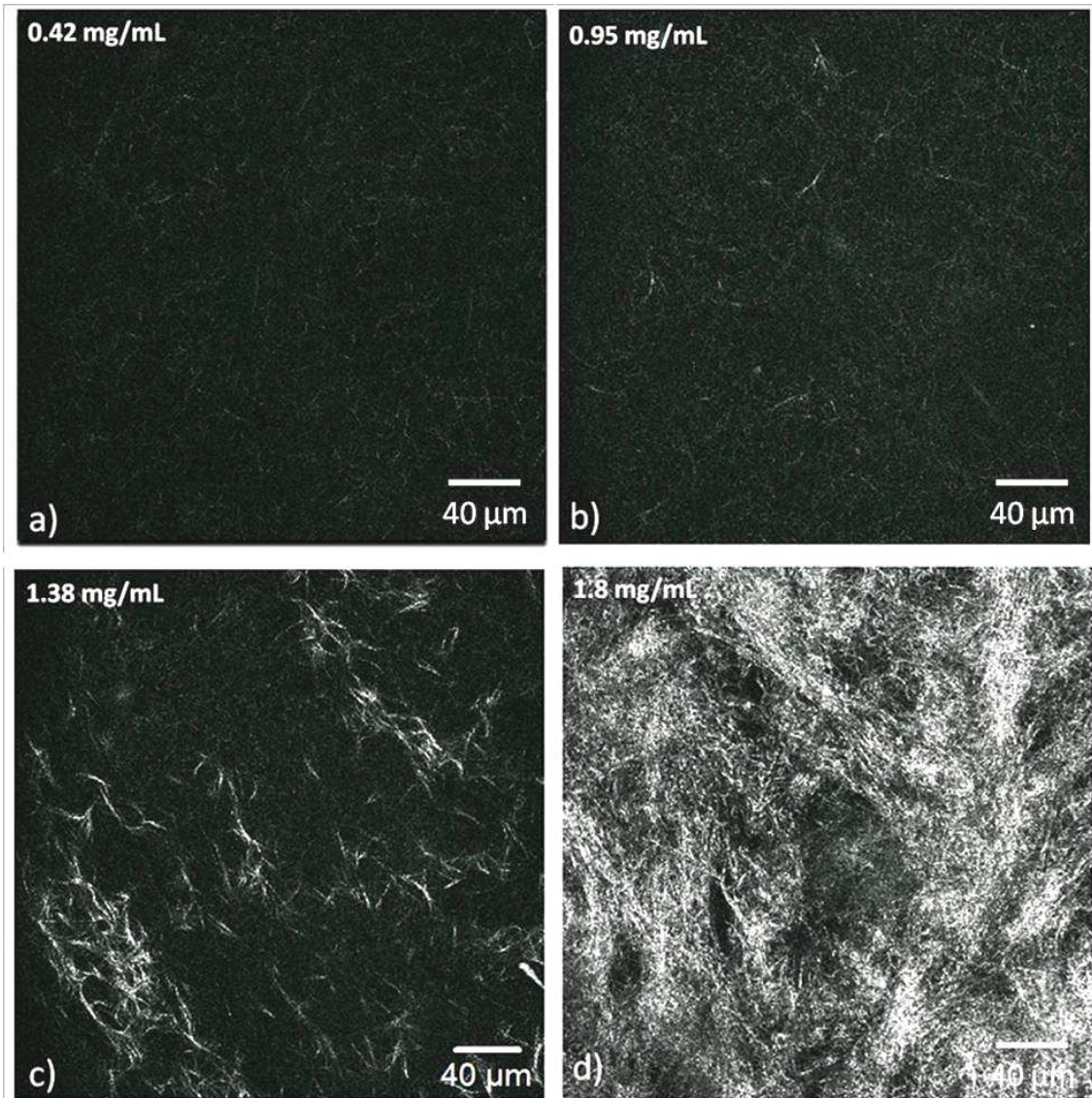


Figure 4

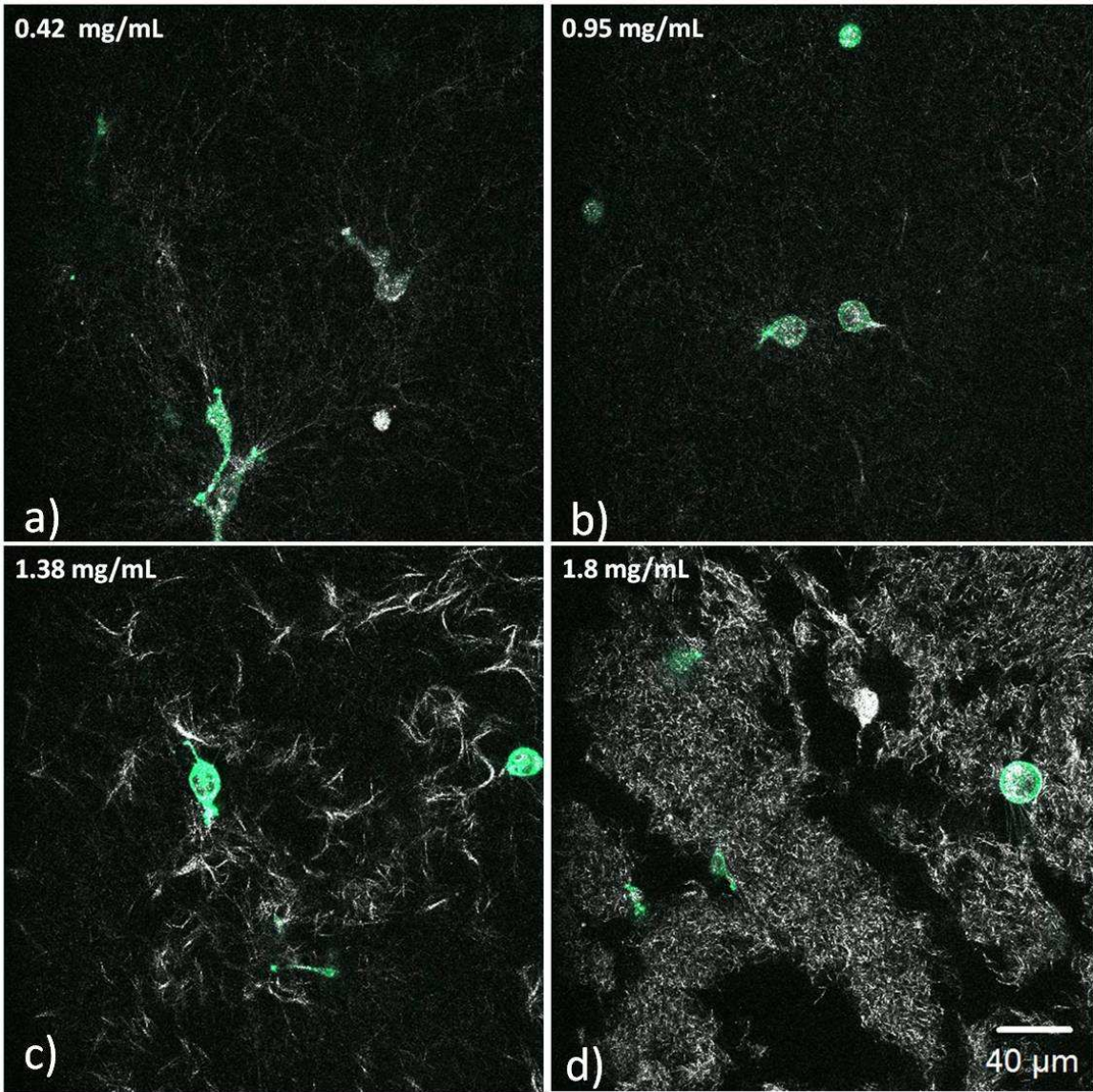


Figure 5

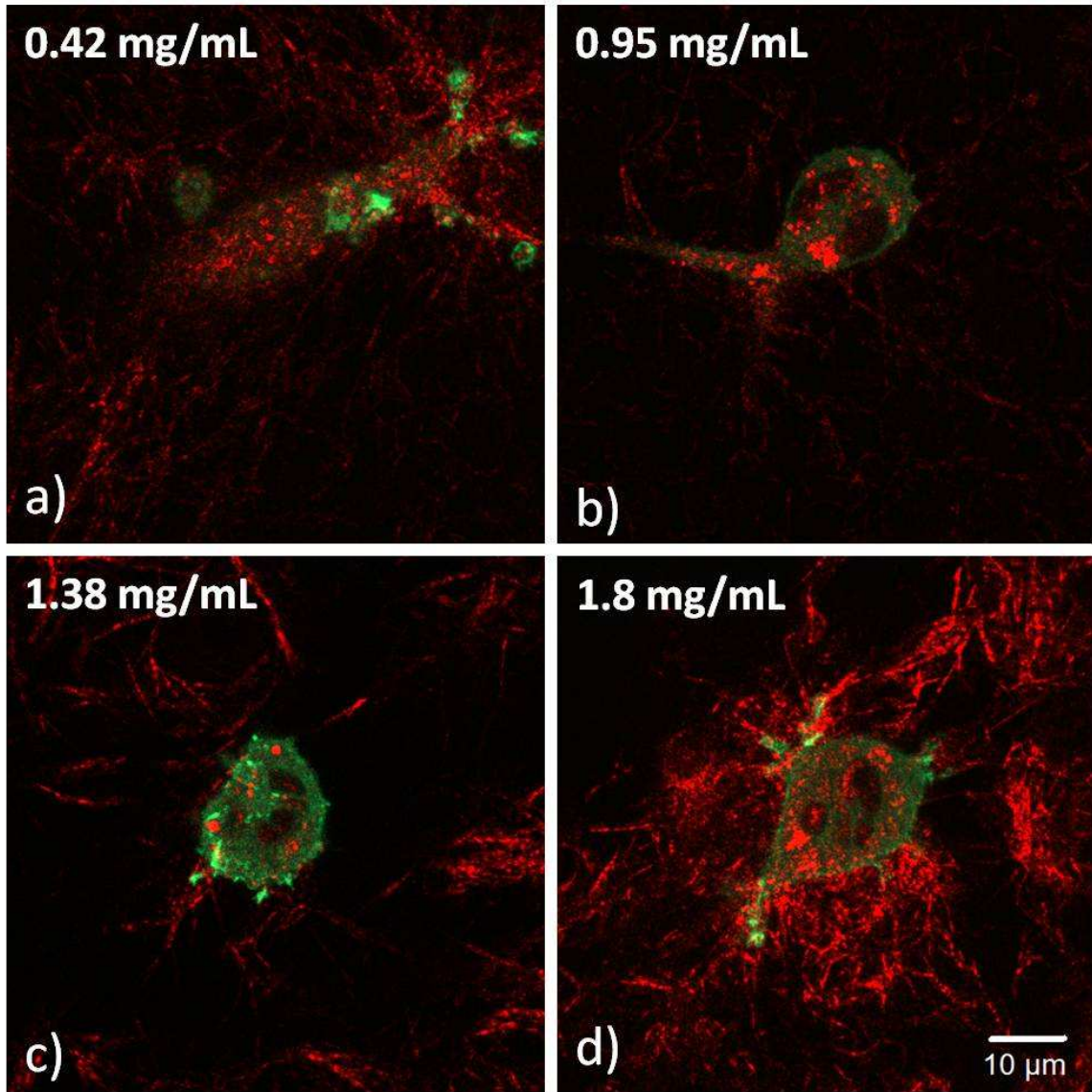
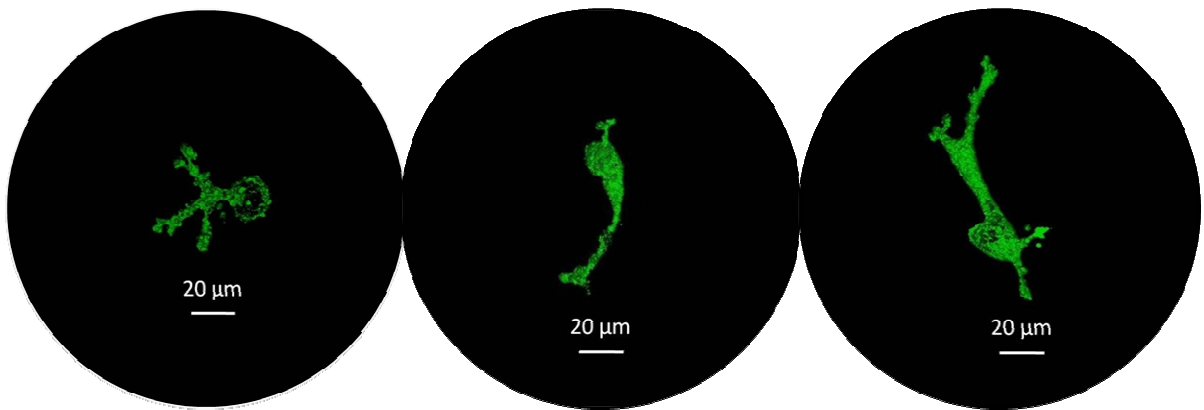


Figure 6



(a)



(b)

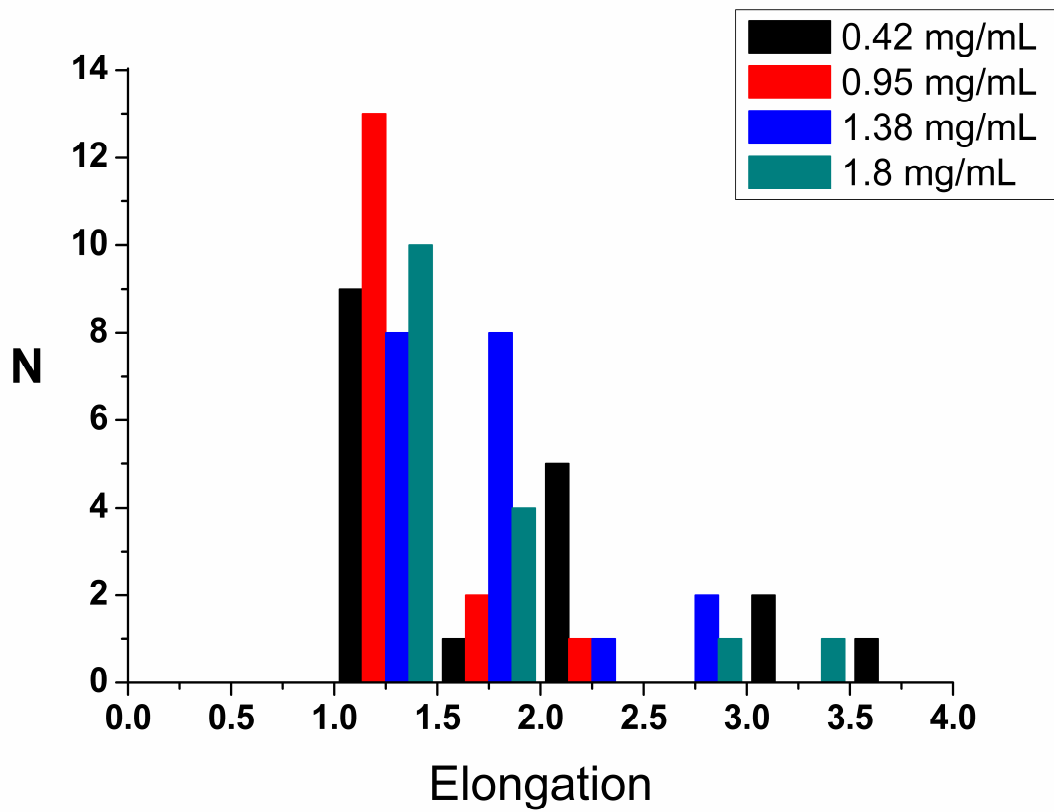


Figure 7

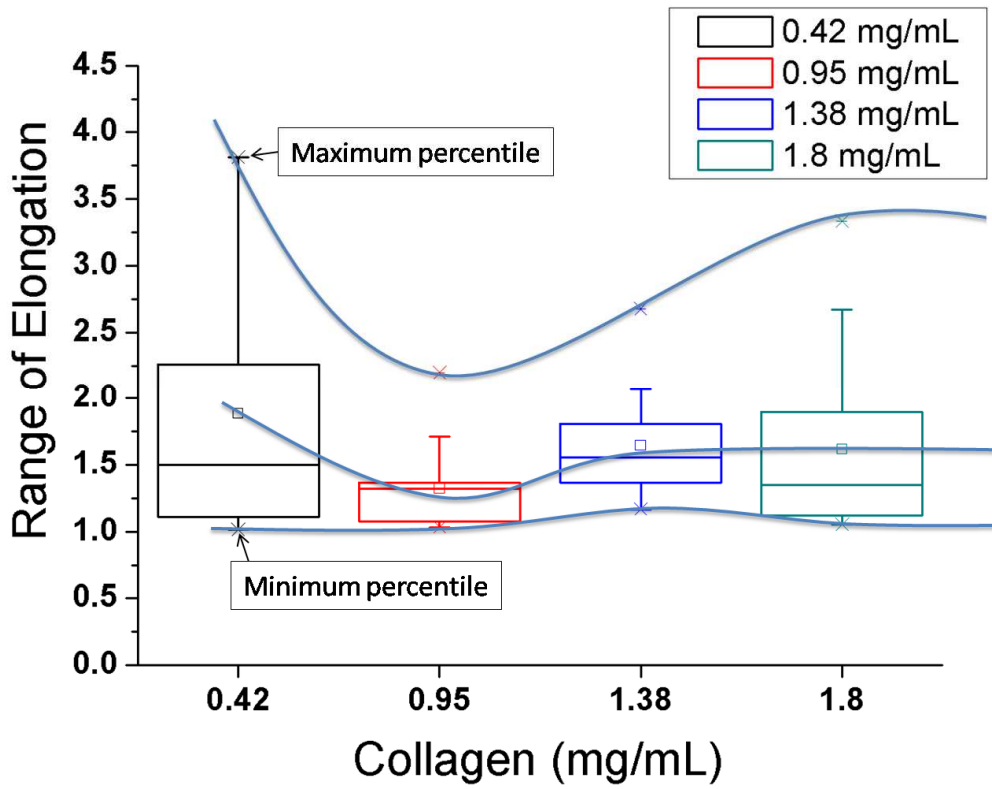
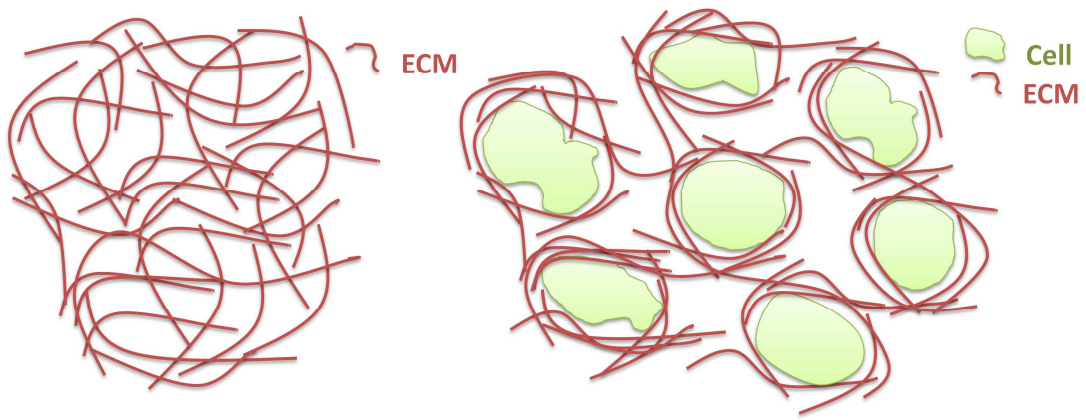
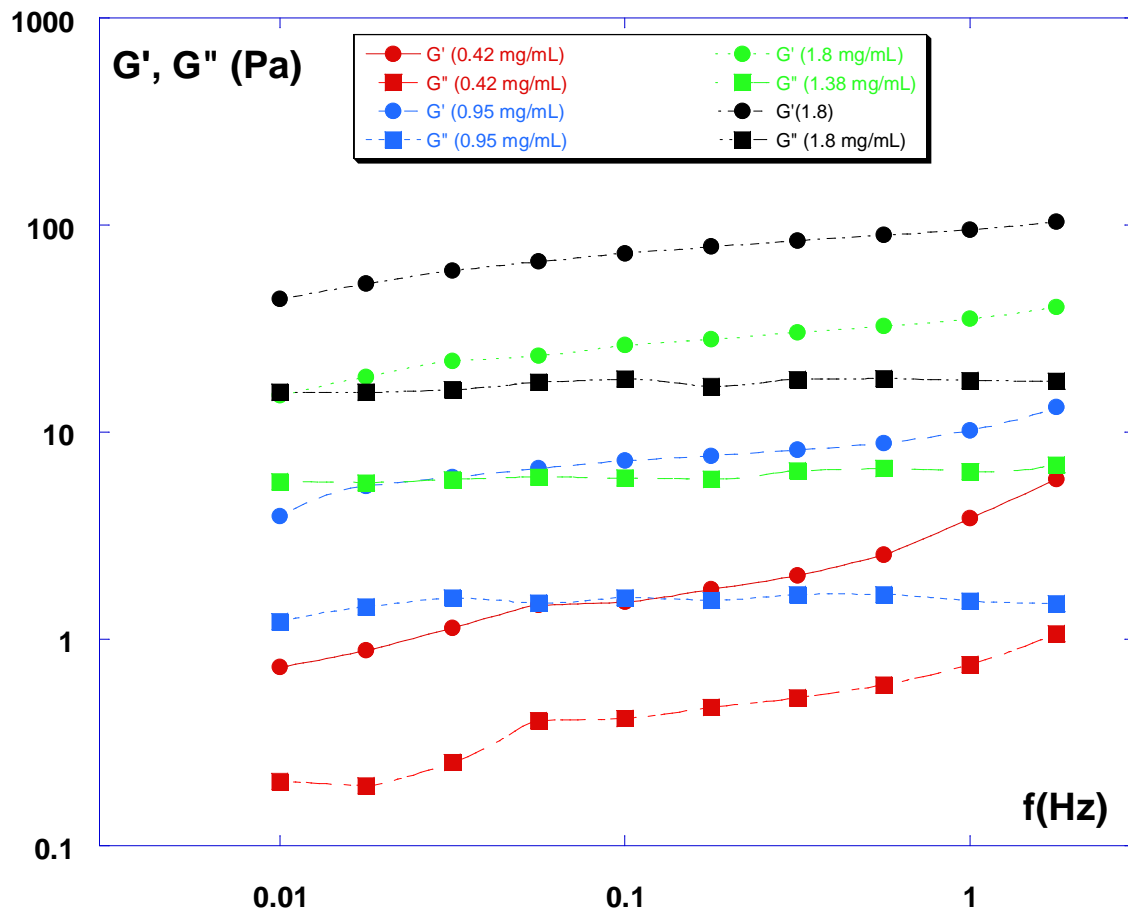


Figure 8



**Figure 9**



Supplementary data S1