

Red blood cells rheology measured using AFM A comparison with other studies

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1. Introduction

The rheology of red blood cells (RBC) has been a subject of interest for many years and was investigated early by Evans & Hochmuth (1976) using micropipettes coupled with microscopy to determine an intrinsic 2D elastic constant, the shear modulus ($\sim 10^{-5}$ N/m). An additional viscosity was also found on the order of $\sim 10^{-6}$ N.s/m, since the membrane was considered to be viscoelastic. Later, Hategan *et al.* (2004) used Atomic Force Microscopy (AFM) to analyze RBCs plated on poly-L-lysine-coated substrates to investigate shape, membrane fluctuations at various adhesion levels, and found complex dynamic patterns, this showing differences between suspended and adhered RBCs. More recently, Turlier *et al.* (2016) demonstrated the presence of metabolic active processes for frequencies below 10 Hz playing a role in red blood cells membrane fluctuations.

Viscoelastic data has not been obtained until the work of Puig-de-Morales-Marinkovic *et al.* (2007) using functionalized ferrimagnetic microbeads bound to RBCs and subjected to a torque. Their study provided dynamic moduli ($\sim 10^6$ Pa/m) as a stress per unit length. Their elastic modulus (G') was found rather constant whereas the loss modulus (G'') was shown to increase with frequency as a power law on the order of 1. Other studies using AFM have shown that it is possible to achieve viscoelastic measurements for cells as a way to monitor their mechanosensitivity (Abidine *et al.*, 2018).

Based on this latter work, we propose to carry out a systematic analysis of the viscoelastic properties of RBCs under controlled conditions, such as the adhesion to the substrate. A functionalization protocol is used to obtain such results. The data will be compared to previous works and the different methods used.

2. Methods

2.1 Red Blood Cells

RBCs were obtained from the EFS (Etablissement Français du Sang) on a regular basis. RBCs were washed thrice in PBS and were resuspended in Petri dishes at small concentrations for 5 minutes, then rinsed in order to have a few adhering RBCs.

2.2 Substrate preparation

After 10s of plasma treatment of the glass coverslips, 500 μ L of Poly-L-lysine prepared at different concentrations $C_{PLL} = [0.001-0.01-0.1-1]$ mg/mL with molecular weight 70-150 kDa was left 10 min to coat the dish, before being rinsed and air-dried for 2 hours. This enabled to obtain various adhesion effects, that can affect RBC spreading (Hategan *et al.*, 2004). Coverslips were glued into TPP Petri dishes, adapted for the AFM measurements.

2.3 AFM

Measurements were made on top of about 20 RBCs (from the same donor) adhering at the bottom of Petri

dishes, using a Nanowizard 4XP AFM (Bruker, Berlin) in contact and oscillary modes, using tips (QUEST20, UK) with a $3\mu\text{m}$ -diameter bead glued at the edge. Cantilever stiffness calibration gave $k=0.02\text{ N/m}$. A typical approach curve was made with a small set-point $F_0\sim 50\text{pN}$, followed by cantilever retraction at velocities of $1\mu\text{m/s}$. The analysis was made using Hertz model for a sphere i.e. where E is the Young modulus (Pa), ν is the Poisson ratio (~ 0.5), R the bead radius and δ_0 is the indentation. For microrheology, a similar approach was made, followed by application of small sinusoidal indentations $d=50\text{nm}$ at angular frequency $\omega=2\pi f$ (frequency f in Hz) superposed to the initial δ_0 . Linearization of Hertz equation leads to $G^*(\omega)=G'+iG''=[(1-\nu)(F^*/\delta^*-i\omega b(0))]/4(R\delta_0)^{1/2}$, the dynamic modulus, with $b(0)$ determined as previously (Abidine *et al.*, 2018).

3. Results and discussion

3.1 Young modulus

The Young's modulus was measured using Hertz equation up to 20pN , corresponding to $d_0 < 1\mu\text{m}$, to reduce substrate effects. Measurements ($N\sim 20$) were made for 5 donors and shown in Table 1.

Table 1. Young's moduli (Pa) for five donors at different poly-L-lysine concentration C_{PLL} (mg/mL).

C_{PLL}	0.001	0.01	0.1	1
E (Pa)	$14\pm 8.7^\dagger$	$17\pm 7.8^\dagger$	$20\pm 10^\dagger$	$40\pm 21^\dagger$

† medians with $p < 0.05$.

Significant differences were found, and show an increase of the Young's modulus (E) which scales with Poly-L-lysine concentration. The higher the concentration, the higher E , as expected, corresponding to firm adhesion on the glass leading to a higher membrane tension (Hategan *et al.*, 2004).

3.2 Viscoelastic data

Further microrheology measurements were carried out in the range $[0.5-100]\text{Hz}$. Fig. 1 shows the typical behavior corresponding to almost constant G' and linearly increasing G'' . The slope of G'' is smaller than 1, but overall this corresponds to a viscous effect with viscosity $\sim 0.3\text{ Pa}\cdot\text{s}$, probably due to spectrin effects. We observe that a Voigt model (spring and dashpost in parallel) is sufficient to describe well this behavior. The

dependence on the poly-L-lysine concentration (C_{PLL}) is clear as the viscous moduli are almost the same (except for 0.001 mg/mL) whereas G' increases with C_{PLL} . A soft glassy rheology model such as $G^*=G_0(i\omega\lambda)^n + \eta\omega$ provides a better fit of the data (Figure 1, solid lines). G_0 takes its values accordingly to those in Table 1, exponent n (~ 0.1) is close to 0 as expected from previous observations, and η decreases from $0.51\text{ Pa}\cdot\text{s}$ to $0.35\text{ Pa}\cdot\text{s}$ with increasing C_{PLL} .

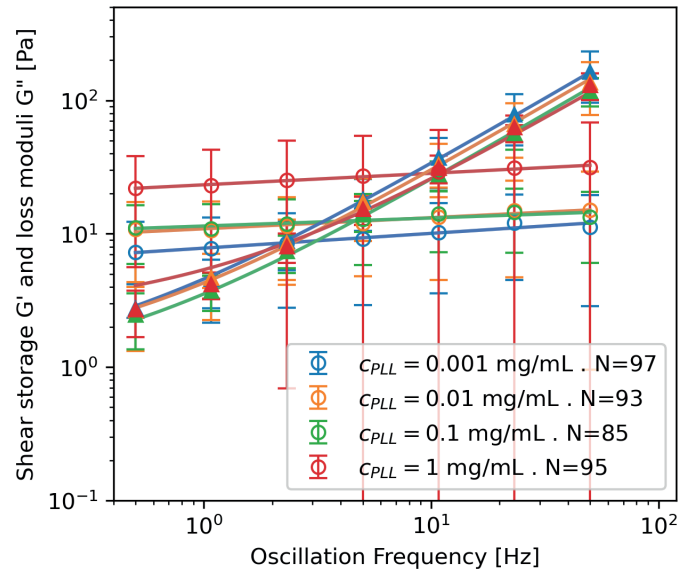


Figure 1. Elastic (G' , circle) and viscous (G'' , triangle) moduli (Pa) at different C_{PLL} .

To sum-up, these results are in very good agreement with Puig-de-Morales-Marinkovic *et al.*, 2007 but have a different dimension (Pa/nm). Since the torque applied to the bead produces shear, moduli are obtained by dividing their stress by shear deformation, i.e. displacement by RBC height ($\sim 2\mu\text{m}$). This gives both quantitative and qualitative agreement (within a factor ~ 5). A bottom correction to take into account substrate effects could further improve results and comparisons with authors.

4. Conclusions

In this work, it was shown that viscoelastic data using AFM can give similar results as compared to magnetic twisting cytometry. The elastic shear modulus G' was rather constant in the range considered, while the shear loss modulus G'' increased with a power slope roughly equal to one. Interestingly the value of the loss modulus did not depend so much on the poly-L-lysine coating that was used for surface coverage. But the elastic shear

modulus G' was correlated significantly with the poly-L-lysine content. This means that rheological properties depend strongly on the tension developed during adhesion to the surface governing membrane properties.

Further works are needed to model the shape of RBCs more precisely and account for this effect, and results should also consider possible substrate effects.

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Conflict of Interest Statement

The authors declare no conflict of interest.

Contributor Roles

AN: Conceptualization, Data curation, Investigation, Formal analysis, Methodology, Writing-review & editing; MF: Methodology, Investigation, Validation; TB: Funding acquisition, Project administration; CV: Conceptualization, Methodology, Formal analysis, Supervision, Writing- review & editing.

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