Duration of microbead seeding on endothelial cells significantly affects their response to magnetic excitation

Yaniv Reichenberg[*] and Yoram Lanir[†]

Faculty of Biomedical Engineering, Technion-Israel Institute of Technology, Haifa 32000, Israel

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Our investigation of endothelial cell rheology using optical magnetic twisting cytometry revealed that with time following incubation of ferromagnetic beads on the cells, beads were sinking into the cells and an increasing number of beads demonstrated apparent absurd negative rheological properties. In parallel, the beads' average rheological response changed considerably over time, both in magnitude and in distribution. It was hypothesized that the apparent negative rheological response was related to the above sinking process of seeded beads into the cells, resulting in an elevation of the beads' rotation axis, thus causing a reversal of the beads' lateral movement direction in response to twisting external magnetic excitation. The results suggest that microbead-based rheological characterization of cells should be interpreted with caution, while considering the time of data acquisition.

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I. INTRODUCTION

Mechanical properties of cells determine the cell deformation under applied external and internal forces. They thus play a key role in cellular processes such as cell shape changes [1], cell locomotion [2], adhesion [3], and cell division [4]. Internally, these cell properties are a key factor in controlling cytoskeleton architecture via reorganization of the actin network [5], formation of stress fibers [6], activation of cellular motor units, and the growth of cellular protrusions such as philipodia [7] or axons [8]. Biochemically, cell properties are involved in mechanical signal transduction to the nucleus, thereby affecting cell growth, differentiation, proliferation and apoptosis, wound healing, protein synthesis, and gene expression [9–16].

When cells are deformed by forces they store a part of the mechanical energy in their cytoskeleton, and dissipate the rest. The energy dissipation is believed to be associated with cytoskeleton remodeling, cytoplasmic viscous mechanisms, and motor protein activity [17,18]. The combined response of the cell is represented by its viscoelastic properties. These properties determine the time evolution of mechanically controlled cellular processes and provide insight into the rate of cytoskeleton remodeling under cell stimulation and applied forces [6,19,20].

Several experimental methodologies have been developed for probing the mechanical properties of cells. One class of methods applies controlled forces on cell surfaces (deformable culture substrate [21], fluid shear stress [22–24]). To deal with the significant variability in properties among individual cells, single cell probing methodologies have been developed (micropipette aspiration [25,26] and microrheometric techniques such as cell poking [27,28], laser tweezers [20,29], atomic force microscopy, and magnetic tweezers [30,31]). The latter allow for viscoelastic characterization of cells under either linear [6,32] or twisting [33] excitation.

Optical magnetic twisting cytometry (OMTC) is an oscillating magnetic twisting methodology developed to measure the viscoelastic properties of the cytoskeleton (CSK) of living cells, on a scale ranging from whole cell populations to local sites in the CSK [34,35]. Application of the OMTC technique entails the study of the nanomovement of ferromagnetic microbeads, adhered to CSK elements, under controlled magnetic torque. OMTC enables the measurement of both elastic and viscous rheological properties over a wide range of frequencies and loadings [36], and over a wide range of time scales [37].

On a local scale, OMTC has been used to demonstrate structural anisotropy in the CSK and the buildup of displacements and stresses at discrete sites in the cell [38]. OMTC was also used to show that local stress induces cytoskeletal remodeling and stiffening across the cell [39]. On a global scale, OMTC revealed a power-law viscoelastic behavior of various cell types [including endothelial cells (ECs)], both under oscillatory and step loadings [40,41].

In addition, observing the spontaneous nanoscale movements of the microbeads (without the application of magnetic fields) was used in the characterization of molecular events and remodeling dynamics of the living cytoskeleton [37,42].

OMTC measurements are typically characterized by a significant scattering of results, which is set off by a large sample size of hundreds of beads [40,42]. This scattering has been reported to be log-normally distributed [34,39,43,44]. This distribution must be considered in data analysis (e.g., in estimating standard error) and in statistical hypotheses testing.

Here we report on a study of a time-dependent phenomena occurring during several hours following microbead seeding and incubation on endothelial cells. The study will focus on the distribution of apparent beads rheological response and on the beads sinking into the cells. Although these phenomena were studied in relation to the OMTC technique, their implications are likely to be relevant to other research techniques using microbeads seeded on cells.

II. MATERIALS AND METHODS

The experimental system has been previously described in detail [45], and is briefly presented in the following.

[*]Yanivr@gmail.com
[†]Yoram@bm.technion.ac.il

A. Cell culture

Bovine aortic endothelial cells (BAEC, BME Technion, Israel) were grown until confluence in minimum essential medium (MEM, 01-050-1A, Biological Industries, Beit Ha'emek, Israel) supplemented with 1% L-glutamine (030201, Biological Industries), 1% penicillin-streptomycin (030311, Biological Industries), and 10% fetal bovine serum (040011, Biological Industries). The beads' medium contained bovine serum albumin to prevent nonspecific binding. The BAEC were seeded within transparent tubes (inner diameter of 5 mm, wall thickness of 0.5 mm, and length of 11 cm) made of silicone (RTV615, GE Silicones, Albany, NY). The tubes were prepared by silicone injection into a highly polished custom-made stainless steel mold and polymerized at an oven temperature of 70°C for one hour. The tubes inner surfaces were then precoated with 1.7 μg/cm^2 fibronectin (03-090-1-01, Biological Industries). BAEC (concentration above 30×10^3 cells/cm^2) in culture media were then seeded on the inner surface while rotating the tubes at 2 rpm, aimed to obtain uniform surface cell coating.

B. Beads seeding on cells

Ferromagnetic microbeads (4.5 μm diameter, Harvard University, School of Public Health) were coated with peptides containing Arg–Gly–Asp sequence (RGD, Peptide International, Louisville, KY) to allow adherence to the cells' integrins, and incubated on the silicone substrate for 30 min, while rotating at 2 rpm to obtain a uniform bead distribution. Following incubation the tubes were gently washed with a serum-free medium to remove unbound beads.

C. Imaging of bead sinking

The process of beads sinking into the cells was monitored in a confluent EC culture using an inverted microscope (Nikon Eclipse TE2000, Nikon Instruments, Melville, NY) with a 20×/0.45 objective (Plan Fluor ELWD, Nikon Instruments) at several focal depths. Images were taken for 7 h at a rate of one image every 8 min starting at 30 min after beads seeding (due to the incubation time required in the bead seeding protocol).

D. OMTC rheological investigation

Beads were magnetized by a strong vertical magnetic field (2 T) for 0.1 ms, followed by the application of a twisting moment imposed by a horizontal sinusoidal magnetic field (0.2 T). In this study we applied a twisting frequency of 2 Hz [34].

The beads position was imaged using a CCD camera (ORCA-ER, Hamamatsu Photonics, Hamamatsu City, Japan) mounted on the inverted microscope and phase locked to the twisting cycle. A bead tracking software (Jack Fairbank Software, Ontario, Canada) determined the beads' location with an accuracy of 5 nm [36] (root mean square).

Raw data was filtered using a high pass filter with a cutoff frequency equal to that of the magnetic field. The beads' lateral movement data was then analyzed in the frequency domain. Roughly half the beads did not show significant response to the applied magnetic excitation, presumably due to lack of attachment to the cell's cytoskeleton, or they presented significant higher order harmonic response which biased data analysis as the high harmonics represent nonlinear response to the applied torque. Beads were excluded unless their response at the magnetic field frequency was at least 200 times stronger than the median of their response at all other frequencies, and at least four times stronger than the strongest harmonic response [34,44,46]. On average, 30%–50% of the beads passed the acceptance criteria and were analyzed.

The cells' cytoskeletal stiffness was determined based on the displacement dynamics of the cytoskeleton-attached microbeads in response to a given magnetic torque. The cells' complex apparent rheological response (not the cells' intrinsic modulus—see Discussion) was defined as $\tilde{g}(f) = \tilde{T}(f)/\tilde{d}(f)$, where $\tilde{T}$ is the Fourier transform of the applied twisting magnetic field, and $\tilde{d}$ is the Fourier transform of the resulting bead displacement. $\tilde{g}(f)$) is measured in T/nm and consists of an elastic (storage) component g', which is the real part of $\tilde{g}$ (the in-phase movement of the bead), and the loss component g'', which is the imaginary part of $\tilde{g}$ (the out-of-phase movement of the bead).

E. Time evolution of the cells' apparent rheological response

OMTC rheological measurements were carried out on between 150 and 250 beads seeded on a confluent EC culture at 45, 130, 190, and 490 minutes following the beads seeding on the cells. The distribution over the beads population of the rheological parameters (g' and g'') were tested for adherence to a normal distribution using a Lilliefors statistic [47].

III. RESULTS

A. Beads sinking into the cells

Images taken at several focal depths show that beads which appear at first "in focus" in a focal plane placed above cell height, gradually lost focus in that plane, while entering focus in a focal plane placed below cell height (Fig. 1). The beads' height changed over the course of three hours, after which no further change was discernible.

B. The cells' rheological response

At all time lapses, data of some cells yielded apparent anomalous results: a significant portion of the beads manifested negative elastic and loss moduli. These beads moved in an opposite direction to the expected one (phase shift of 180°–270°) under the applied magnetic field (Fig. 2). These negatively moving beads were not eliminated from the data analysis by the acceptance criteria as they showed a clear response in the magnetic field frequency, without significant higher harmonics.

The occurrence of negatively responding beads increased with time. The percentage of cells with apparent negative rheological response grew from 20% at one hour after seeding, to 63% at three hours (Fig. 3). After three hours there was no further apparent change in the percentage of cells with negative rheological response.

C. Time evolution of the apparent moduli distributions

Previous studies reported that the rheological moduli as evaluated from the beads' tracking were log-normally distributed over the bead population [36,37,39,48].

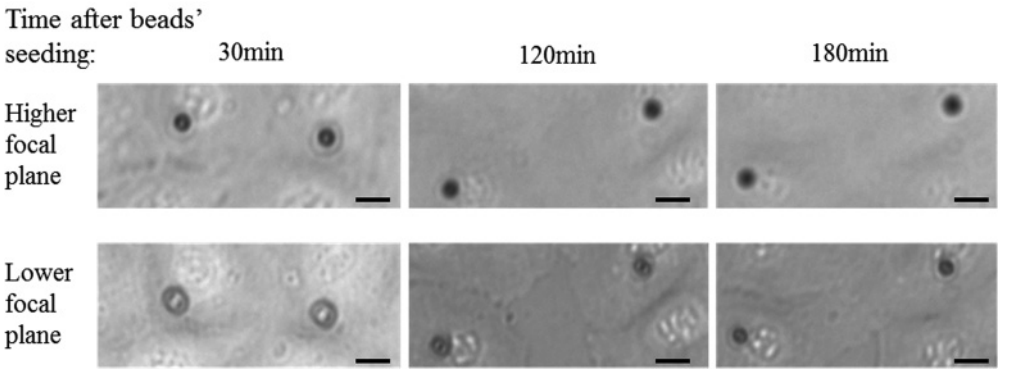

FIG. 1. Images of two beads adhered to ECs, taken 30–180 m after bead seeding (the beads' locations have shifted slightly over the course of the experiment as the cells moved). The images were taken at two focal planes, one above the cells and one below. The images show how the beads, which at first were in focus in the upper plane, gradually became out of focus, while gaining focus in the lower plane. Scale bar = 10 μm.

In the present study, while this distribution was observed in the first hour after beads seeding, data measured several hours later shifted to closer-to-normal distributions. This was the case for both positively (Fig. 4) and negatively responding beads (data not shown).

Concurrently, the average measured rheological response of the positively responding beads changed considerably. The median elastic response g' increased by 35%, from 7.7 ± 2.1 T/nm at 45 min after seeding to 10.5 ± 1.8 T/nm at 490 min after seeding, while the median of the loss response g'' increased by more than 500% at that period from 2.55 ± 0.76 to 14.8 ± 2.1 T/nm.

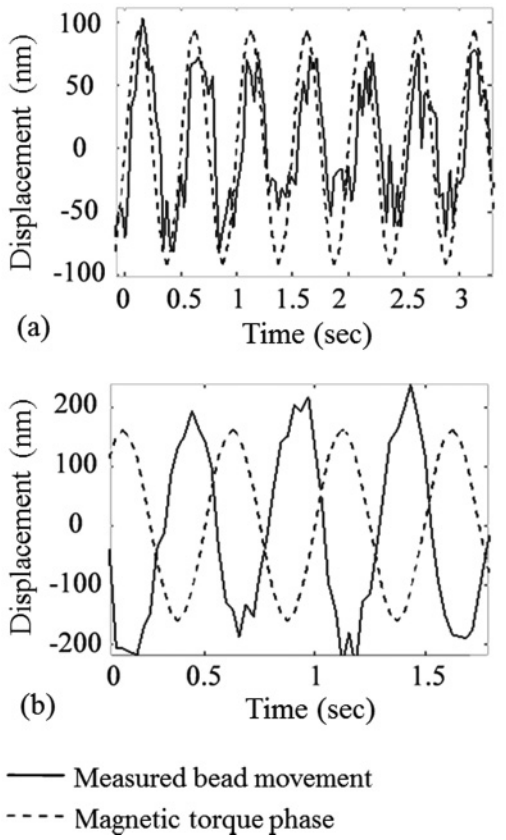

FIG. 2. Data of a bead movement (smooth line) under the applied magnetic twist (broken line): (a) an in-phase moving bead yielding positive moduli, and (b) an out-of-phase moving bead (seemingly moving opposite the magnetic torque) yielding apparent negative moduli.

IV. DISCUSSION

OMTC and direct microscopic inspection were applied to monitor the effect of time following beads seeding and incubation on the apparent rheological response.

In previous beads-based microrheometric investigations it was tacitly assumed that beads sinking into the cells (and their associated rheological response) stabilized by their incubation (commonly of 20–30 min duration) on the cells. The most important observation in the present study is that in cultured endothelial cells, beads continue to sink into the cells even after being incubated, and that simultaneously the rheological response evolves with time. The physical link between the two observations could be related to the fact that the cells' contact with the RGD coated beads induces CSK remodeling

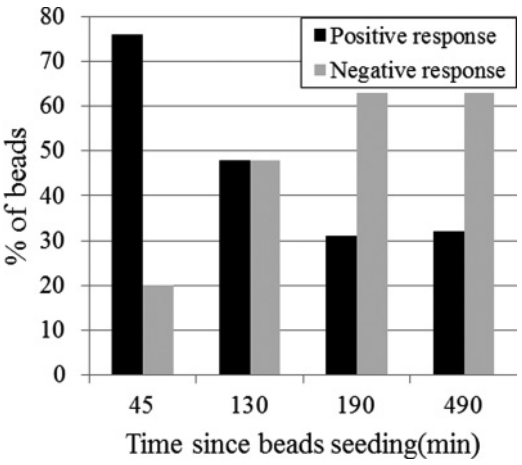

FIG. 3. Percentages of cells showing apparent positive and negative rheological responses, as a function of time after beads seeding. Results taken from 96 beads which passed acceptance criteria.

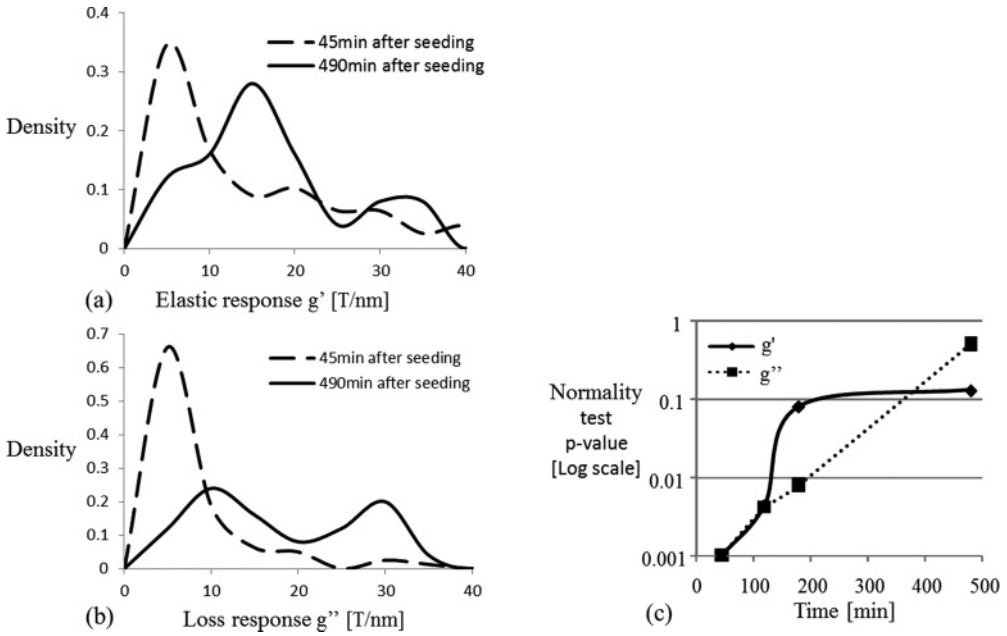

FIG. 4. Evolution of the density distributions of ECs' (a) elastic and (b) loss rheological responses over the positively responding bead population. The results are presented at different times after beads seeding on the cells, revealing a pattern change from close to log-normal distribution to a nearly normal one. (c) Lilliefors normality test [47] p values, for rejection of null hypothesis of normal distribution, changed from values smaller than 0.001 at 45 min (for both moduli) to 0.12(g') and over 0.5(g'') at 490 min.

which is likely to evolve with the beads sinking, thereby affecting the CSK measured response [40,49]. If applicable to other cell types, this observation calls for caution in data analysis. Specifically, since the relationship between the apparent (measured by the bead tracking) and intrinsic (true) viscoelastic moduli of the cells strongly depends on the degree of bead internalization (embedding) [32,36,39,50], and since this degree of embedding was found in the present study to change continuously with time, it is thus impossible to estimate unique and reliable mechanical moduli based on the beads tracking data alone. It is for this reason that the present study focused on the apparent moduli.

The observed simultaneous evolution of the beads sinking and rheological response raises the possibility that the observed dispersion of the beads rheological response (Fig. 4) is related to the observed dispersion of the level of beads sinking. Investigation of this possibility is left for future studies.

The correspondence between sinking duration and the time required for stabilization of the percentage of anomalous responding beads—roughly three hours—may not be accidental. As speculated above, the rheological anomaly may be associated with the beads sinking, which could vertically shift their rotational axis under the applied magnetic field. Anomaly in the beads response would result from their sinking since the rotation axis above the midheight of the bead could mean that under the same torque, the lateral motion of the bead would be in the opposite direction compared to a bead with a rotation axis below the midheight (Fig. 5). Since the microscope-coupled camera measures only lateral motion, it would seem that the bead moves opposite to the expected direction.

An objection to the above speculation could arise from the relatively low height of endothelial cells (~3–4.5 μm [51]), compared to bead diameter (~4.5 μm). However, a heightened rotation axis would cause opposite lateral motion if it is located at any point above the midheight of the bead (~2.25 μm). As ECs in a culture have been previously found [51] to have a maximum height >2.25 μm above culture base, and 85% of ECs have a maximum height >3 μm above culture base, ECs are probably high enough to accommodate bead sinking to an extent that would raise its rotation axis sufficiently to alter its lateral movement.

Another explanation for anomalous responding beads could be related to the one suggested by Heinrich and co-workers [17]. They found that in dictyostelium discoideum cells force pulses triggered fast deflections of intracellular beads bound to the microtubules, in directions opposite or perpendicular to the external force. These events were attributed to the active viscoplastic behavior of the cells' cytoplasm. It is highly doubtful if these mechanisms are applicable to the present study. The beads in their study were much smaller (1.42 μm) and established contact and moved along the cell microtubules, unlike our study where the beads (4.5 μm), although in tight contact with the cell membrane via integrins, were still exterior to the cell and thus expected to be less or not directly affected by the microtubules. More importantly, Heinrich *et al*. applied force pulses of 17 sec duration which allowed for viscoplastic effects to take place, unlike our study, where the shorter oscillatory excitation (at a frequency of 2 Hz), allowed for much smaller viscoplastic effects, which most probably were counterbalanced by an opposite effect in the next half of the cycle period.

In light of the observed beads sinking, the time-dependent changes in the cells rheological response are to be expected, since sinking of the beads alters their attachment strength to the cytoskeleton. Therefore, the "real" rheological moduli of the cell's cytoskeleton would reflect differently in beads positioned

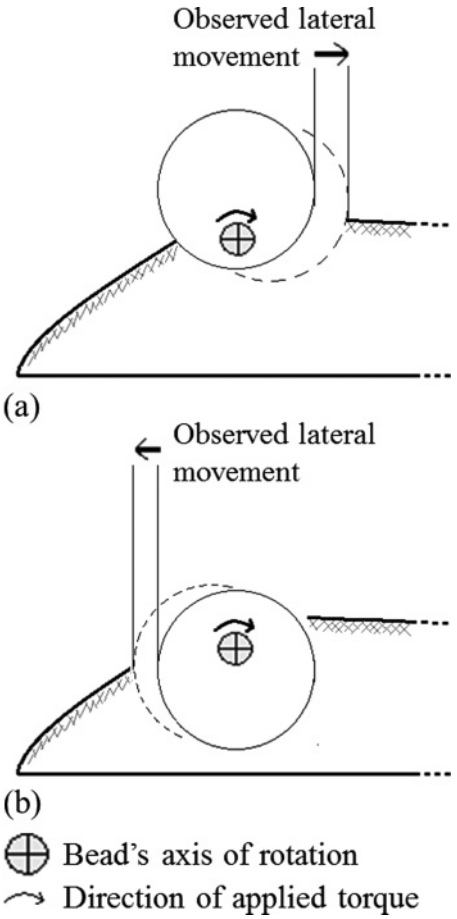

FIG. 5. Illustration of the effect on the bead lateral motion of a vertical change of the rotation axis that may occur due to sinking of the bead. The same torque is applied to both beads, but the beads revolve around different axes, thereby manifesting lateral movements in opposite directions.

at different heights. In that context, it should be recalled that OMTC results are indicative of the rheological properties and are related to the true moduli values through a geometric factor which is a function of bead embedment [36,50].

These changes in the cells rheological response and in the associated apparent moduli and their dispersion, even after exclusion of the negatively responding beads, indicate that measured data taken during bead sinking are transient and may introduce bias in data analysis. However, it should be noted that since the sinking process takes approximately three hours, these changes should not be significant when all data is acquired during a short duration.

The study has a number of limitations. One is that beads were coated with a protein containing the RGD sequence. RGD sequences are recognized by several members of the integrin families. But for the purposes of the present study this nonspecifity is not a disadvantage since the sole purpose of bead coating by RGD sequence in the present study was to tightly bind the bead via integrins to the cell cytoskeleton. All integrins which recognize the RGD sequence contribute to the strength of binding. Another limitation is that the methodology used to observe the bead sinking into the cell is not state of the art. It was chosen since the sole goal of observing beads was to show that they do sink into the cell. As no mathematical analysis was done, there was no need for more precise quantitative data.

In summary, our visual results suggest that sinking of microbeads into cultured endothelial cells takes place during a three hour period following the bead seeding. This process is accompanied by a parallel increase in the number of negatively responding beads in the OMTC setup, and by changes in the distributions of the rheological properties over the cell population. These findings shed light on an essential role of the sinking process of beads seeded on ECs, which may have a substantial effect on OMTC results if the data is taken prior to stabilization of the sinking process.

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