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# Novel dynamic rheological behavior of individual focal adhesions measured within single cells using electromagnetic pulling cytometry

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**Abstract**

The rheology of cells and sub-cellular structures, such as focal adhesions, are important for cell form and function. Here we describe electromagnetic pulling cytometry (EPC), a technique to analyze cell rheology by applying dynamic tensional forces to ligand-coated magnetic microbeads bound to cell surface integrin receptors. EPC utilizes an electromagnetic microneedle that is integrated with a computerized control and image acquisition system and an inverted microscope and CCD camera to monitor bead displacement. Arbitrary force regimens may be defined over a wide range of frequency (DC to 10 Hz) and force (100 pN to 10 nN). With EPC, the viscoelastic creep response of individual focal adhesions was measured over three decades in time using RGD-coated magnetic microbeads bound to integrins that induce local focal adhesion assembly and coupling to the internal cytoskeleton. These data were compared to the power-law-like predictions from the soft glassy model of cell rheology proposed by Fabry et al. [14]. Although power-law-like behavior was observed in some focal adhesions, 52% of these structures did not exhibit power-law-like behavior, but instead exhibited either a multi-phase response characterized by abrupt changes in slope or experienced a retraction in the opposite direction to the applied force, especially in response to prolonged force application. These data suggest that while the soft glassy model may provide reasonable estimates for aggregate mechanical behavior of living cells, the rheological behavior of individual focal adhesions may be more heterogeneous and complex than suggested by the soft glassy model. These results are considered in context with the hierarchical nature of cytoskeletal architecture.


*Keywords:* Cell mechanics; Cytoskeleton; Focal adhesion; Creep; Magnetic

## 1. Introduction

The mechanical properties of living cells govern how they sense and respond to mechanical stress because they determine how cells deform in response to force, as well as how stresses are transmitted to molecular support elements inside the cell. Cells sense mechanical stress through transmembrane integrin receptors that link extracellular matrix (ECM) proteins to intracellular focal adhesion components which, in turn, transfer stress to and from the internal cytoskeleton [1]. The

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structural backbone of the focal adhesion also orients much of the cell's signal transduction machinery [2,3]. This architectural arrangement facilitates mechanochemical conversion when mechanical signals are applied to integrins; these signals influence virtually all cell behaviors, including growth, differentiation, contraction, motility and apoptosis [4–8].

Early studies of cell mechanics typically relied on measurements of cell deformation in response to static loads applied to the whole cell membrane, and early investigators routinely proposed simple viscoelastic models to describe the cell mechanical response [9,10]. Later studies using more sensitive techniques, such as optical tweezers and magnetic cytometry, permitted mechanical force application to specific cell surface receptors, and revealed that the mechanical properties of the cell differ greatly depending on the receptor probed for analysis. For example, cells were found to be relatively stiff when probed through transmembrane adhesion receptors, such as integrins, that link ECM to the internal cytoskeletal lattice composed of linked microfilaments, microtubules and intermediate filaments [1,11]. In contrast, when the same stresses were applied to transmembrane metabolic receptors, growth factor receptors, or histocompatibility antigens that only link to the submembranous cortical cytoskeleton (actin–spectrin–ankryin lattice) the same cells were found to be highly flexible. Thus, cells appear to exhibit complex mechanical behavior due to the load-bearing functions of discrete molecular networks inside the cell and their hierarchical structure [12].

On the other hand, recent measurements with oscillatory magnetic twisting cytometry have led to a more complex, viscoelastic structural dampening model of the cell that describes the generalized mechanical behavior of cells as a soft glassy material [13,14]. Specifically, the viscoelastic storage and loss moduli ($G'$ and $G''$, respectively) of cells were found to exhibit power-law-like behavior when analyzed in the frequency domain, and the slopes of these relationships are well-described by structural damping theory. However, it is not clear how this generalized model relates to the structural complexity of living cells at the sub-cellular scale. For example, it is not clear whether the structural damping theory applies to the behavior of the internal cytoskeleton, the cortical shell, or both. This is a particularly pertinent question because the analysis of data from the magnetic twisting cytometry studies used to develop the soft glassy model of cell mechanics excluded results from magnetic beads that were most tightly bound to the cytoskeleton and moved little in response to magnetic stress [14,15]. In these experiments, the viscoelastic response also was averaged over many beads, potentially masking, or altogether excluding, those beads that exhibited markedly smaller displacements. Additional support for the structural dampening law has been provided by atomic force microscopy, however, the probes used in this study were not directly coupled to the internal cytoskeleton via integrin linkages and therefore likely probed the cortical membrane rather than the internal cytoskeleton [16]. The question of whether structural damping theory applies to the whole cell or to particular sub-cellular domains remains unanswered.

The past studies that generated data consistent with the structural dampening theory were analyzed in the frequency domain [13,14,17]. However, a direct consequence of this theory is that the viscoelastic creep in the time domain also will follow a power-law relationship [13,18]. We therefore set out to evaluate whether the viscoelastic creep response of individual integrin-dependent focal adhesions exhibits a temporal power-law-like relationship in living endothelial cells, as predicted by the structural dampening model. To accomplish this goal, we developed a new computerized system for electromagnetic pulling cytometry (EPC) that is capable of applying dynamic tensional forces to ligand-coated magnetic beads bound to receptors on the cell membrane, while measuring lateral nanometer scale bead displacements with high temporal resolution.

## 2. Materials and methods

### 2.1. Experimental procedure

Bovine capillary endothelial cells were cultured in 10% $CO_2$ at 37 °C on gelatin-coated tissue culture dishes in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with 10% calf serum (CS; Hyclone), 10 mM Hepes (JRH-Biosciences), 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (GPS, Gibco-BRL), as previously described [19]. Cells were serum-deprived for 12–24 h in defined medium containing DMEM containing 1% BSA, GPS, 10 μg/mL human high density lipoprotein (Intracell), and 5 μg/mL holo-transferrin (Collaborative Research) prior to experiments. The cells were then trypsinized (trypsin-EDTA, Gibco-BRL) and plated ($5 \times 10^4$ cells per dish) in defined medium on 35 mm glass-bottomed dishes (MatTek) that were pre-coated with 500 ng/cm$^2$ fibronectin [19]. In some experiments, cells were plated on uncoated dishes and 10% CS was included in the medium.

Tosyl-activated super-paramagnetic beads (4.5 μm; Dynal) were coated with synthetic RGD-peptide (Peptite-2000, Integra Life-Sciences) overnight at 4 °C in bicarbonate buffer (pH 9.4) to mediate binding to cell surface integrin receptors, as described [1,20]. The magnetic beads (10–20 beads/cell) were added to the adherent cells for 10–15 min at 37 °C, and then the unbound beads were removed by multiple washings with PBS. Experimental measurements were carried out on the

microscope stage platform within one hour of bead addition in bicarbonate-free medium consisting of Hank's balanced salt solution (Sigma), 1% BSA, 10 mL/L MEM non-essential amino acids, 20 mL/L MEM essential amino acids, 2 mM L-glutamine (Sigma), and 10 mM Hepes, pH 7.3.

### 2.2. Electromagnetic pulling cytometry

EPC was used to apply tensional forces to ligand-coated magnetic beads bound to receptors on the surface of cultured cells, and to measure resulting stress-induced bead displacements using a modified version of previously published techniques [10,21]. Tensional magnetic forces require high gradient magnetic fields, and for EPC, the magnetic field gradient was generated using a temperature-controlled electromagnetic microneedle with a pole tip (~10 μm radius of curvature) that was microfabricated using a controlled electrochemical polishing technique, as previously described [22]. The experimental setup is described in Fig. 1. The tip of the electromagnetic microneedle was positioned relative to a 4.5 μm diameter, RGD-coated magnetic bead bound to cell surface integrins within the working distance of an objective (60×, NA 1.4, Nikon) on an inverted microscope (Eclipse TE2000E, Nikon) using an electronic micromanipulator (Eppendorf). Images of the bead position were recorded using IPLab (Scanalytics, Fairfax, VA) and an externally-triggered CCD camera (CoolSnap HQ, Roper Scientific). A computer-controlled amplifier (Model SRL40-6, Sorensen, San Diego, CA) was used to provide electric current to the electromagnetic microneedle, and LabVIEW software (ver. 5.0, National Instruments, Austin, TX) was used to program the desired voltage waveform that was digitized and sent to the input terminals of the amplifier. MATLAB software (ver. 6, The MathWorks, Natick, MA) was used to analyze the image sequence, quantitate bead displacements and temporally align the bead displacements and force waveforms. To calibrate the applied magnetic force, 4.5 μm beads were magnetically pulled through a glycerol solution of known viscosity, as previously described [23,24].

To measure the viscoelastic creep response of living cells and individual focal adhesions, cells were placed on a 37 °C heated stage in $CO_2$-independent medium and imaged on an inverted microscope. A cell was first located that had a single magnetic bead bound to the apical cell surface nearest the microneedle tip and separated by at least 3 bead diameters from all other beads (this criterion eliminated dipole–dipole interactions that would otherwise affect the local magnetic field and force

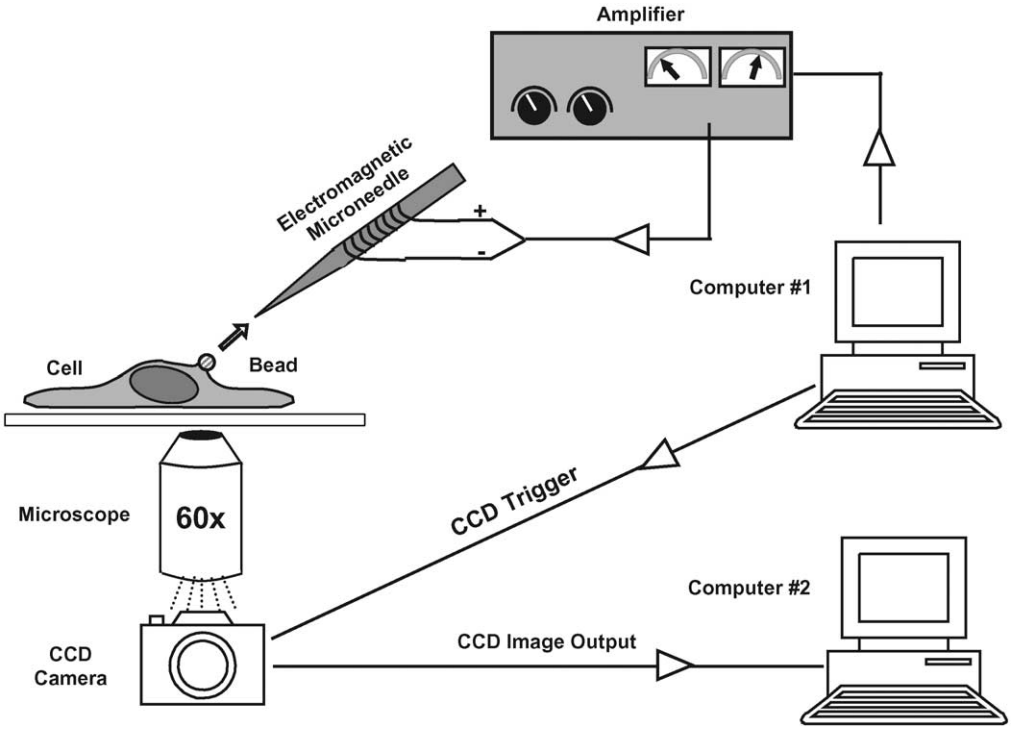


Fig. 1. Experimental setup for electromagnetic pulling cytometry. A 4.5 μm RGD-coated super-paramagnetic bead is bound to integrin receptors on the surface of a living endothelial cell, while a magnetic force (arrow) is applied to the bead using the electromagnetic microneedle. An amplifier supplies current to the electromagnetic microneedle and is controlled using LabVIEW on computer #1 to generate any arbitrary user-defined force regimen. The bead displacement is optically recorded using a microscope and an externally triggered CCD camera, and the images are stored on a computer #2 using IPLab. Connections and arrowheads indicate the direction of information flow. See text for further details.

exerted on the particle). The tip of the electromagnet microneedle was then positioned to a pre-determined height (30–100 μm) and horizontal distance (~60 μm) from the magnetic bead. A small region of interest (approximately $100 \times 100$ pixels) was selected around the bead, and IPLab was programmed to capture a defined number of frames (typically 4000) with a 1 ms exposure time. The LabVIEW program was then executed, which simultaneously initiated the waveform to the electromagnetic microneedle, image acquisition by the CCD camera, and digital data acquisition. Typical force regimens included one or more prolonged periods (ranging from 100 ms to 60 s) of constant force (ranging from 100 pN to 1 nN in the direction to the needle tip), and the resulting bead displacement was later analyzed to determine the horizontal component of the viscoelastic creep response.

To measure bead displacement, image sequences were converted into TIFF format and analyzed using MATLAB Image Processing Toolbox (ver. 6, The MathWorks) to track the displacement of the bead centroid as a function of time. For each frame of the image sequence, MATLAB was programmed to identify the perimeter of the bead using either an intensity threshold technique or an edge detection routine. The two-dimensional, non-weighted centroid was then calculated for each frame based upon the position of all pixels contained within the defined perimeter. To compute bead displacement during a single creep period, MATLAB was programmed to identify the precise starting and ending time of each force pulse based upon the recorded voltage waveform from the amplifier. The position of the bead centroid in the frame immediately preceding the onset of the force pulse was defined as the origin, and the displacement for all subsequent frames during the force pulse was calculated with respect to this position. To examine the power-law behavior of the viscoelastic creep response, the displacement during the creep period was graphed on logarithmic axes and fit to a power-law model of the form: $at^b$, where $a$ and $b$ are fitted parameters and $t$ is time in seconds. Fits were calculated using a non-linear optimization routine executed on MATLAB.

## 3. Results

We have previously developed and used magnetic twisting cytometry [1], a magnetic tweezer [23] and a permanent magnetic needle [24] to measure the static mechanical properties of living cultured cells. In all of these methods, controlled mechanical stresses were applied to specific cell surface receptors by exposing cells bound to ligand-coated magnetic beads to oriented magnetic fields. To more effectively probe the dynamic viscoelastic behavior of living cells, we developed a new form of magnetic cytometry—EPC. This method utilizes an electromagnetic microneedle with a pole tip that can be shaped using an electrochemical polishing technique to apply a broad range of magnetic forces and magnetic field gradient configurations [22]. The electromagnetic microneedle was integrated into a computerized control system that allows the user to specify the electromagnetic force waveform while rapidly (~45 Hz) imaging bead motion (Fig. 1). Post-processing analysis yielded the bead displacement waveform from the image sequence and aligned the force and displacement waveforms (error <1 ms) to provide accurate temporal resolution of the dynamic force–displacement relationship. Calibration studies revealed that magnetic beads experience a pulling force in the direction of the microneedle tip that increases as a function of electric current and as the bead approaches the tip of the electromagnetic microneedle (Fig. 2). Normalizing the magnetic force by the electric current collapsed all data onto a single curve (not shown), indicating that the magnetic force increased linearly as a function of current in the range investigated (250–750 mA). Forces ranging up to 10 nN or more can be reproducibly applied to magnetic beads using this approach [22].

In the present study, we used this system to apply controlled mechanical forces to RGD peptide-coated magnetic beads (4.5 μm diameter) that were bound to integrin receptors on the surface of cultured capillary endothelial cells. RGD peptide is the cell binding sequence from the extracellular matrix protein, fibronectin; it binds and activates transmembrane integrin receptors on the cell surface to promote focal adhesion formation and thereby link integrins to the internal cytoskeleton [25–27]. To accurately measure the stress-induced displacement of the surface-bound magnetic beads, image sequences recorded on an optical micro-

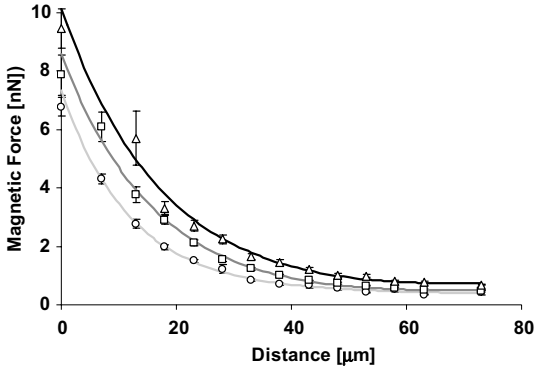


Fig. 2. Calibration of electromagnetic force acting on magnetic microbeads. The force acting on a 4.5 μm diameter magnetic bead (Dynal) decreases as a function of distance from the microneedle tip and increases as a function of the electromagnetic current: 250 mA (circles, light gray curve); 500 mA (squares, dark gray curve) and 750 mA (triangles, black curves). At least 15 beads were analyzed for each value of the electric current (error bars indicate SEM).

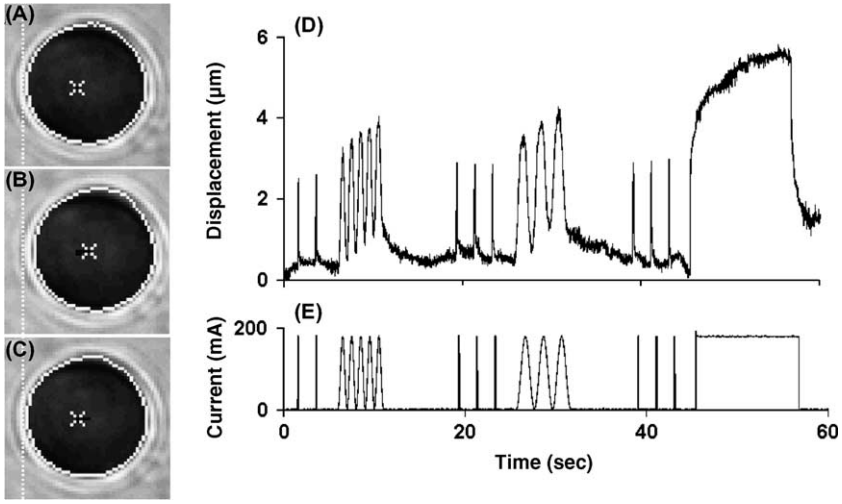


Fig. 3. Tracking of single magnetic microbeads during a dynamic force regimen. (A–C) Bright field microscopic image sequences are processed using MATLAB to outline the bead perimeter (○) and to compute the bead centroid (×), shown here before (A), during (B), and after (C) a 100 ms, ~300 pN force pulse applied to one RGD-bead bound to surface membrane integrin receptors on a living endothelial cell. The direction of magnetic force is to the right. (D) Bead displacements measured during a dynamic force regimen driven by an electric current waveform (E) consisting of multiple ($n = 8$) sub-second (100 ms) force pulses interspersed with two periods of sinusoidal oscillations (1.0 Hz, 0.5 Hz, from left to right) and ending with a single period of prolonged force (10 s). 200 mA corresponds to approximately 300 pN force.

scope were analyzed using MATLAB Image Processing Toolbox which was used to track the motion of the bead centroid and to compute the displacement of the bead as a function of time. An example of the tracking algorithm is shown in Fig. 3A–C, where the position of the bead centroid is identified for each frame during a brief force pulse (100 ms, ~300 pN). The tracking algorithm can be used to measure bead displacement (Fig. 3D) in response to various mechanical stimuli, including sinusoidal oscillations (DC to ~10 Hz), rapid (<100 ms) or prolonged (>10 s) magnetic pulses or almost any arbitrary user-defined force waveform by controlling the electric current to the electromagnetic microneedle (Fig. 3E).

Past studies have shown that when the mechanical properties of living cells are probed through integrins and their associated connections with focal adhesion structures, cells exhibit non-linear stiffening behavior in response to mechanical stress above a certain threshold [1]. To examine the range of linear force–displacement behavior, we measured bead displacement in response to 100 ms force pulses of varying magnitude (from ~100 pN to 1 nN; Fig. 4A and B). Immediately following the onset of each applied current wave and force pulse (Fig. 4A and B), the magnetic bead rapidly moved towards the needle tip; it also continued to slightly "creep" during the force period, and then quickly returned to its starting position after the current and force

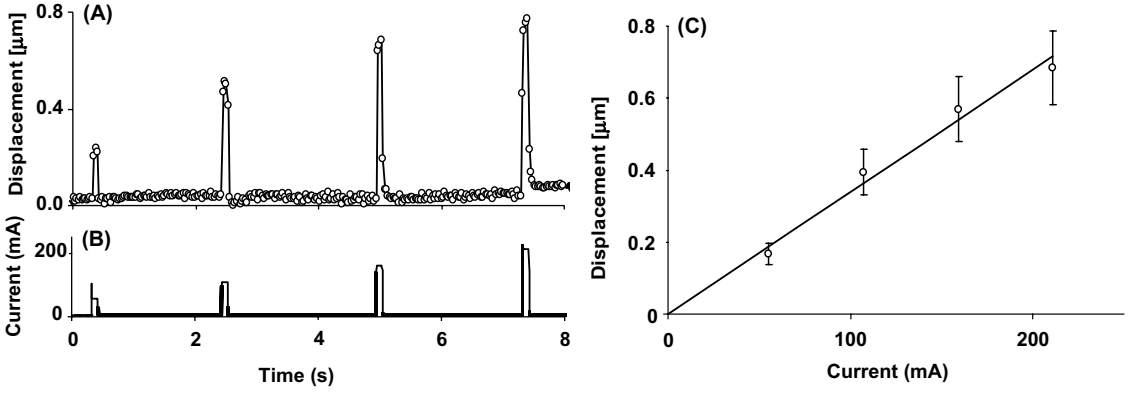


Fig. 4. Focal adhesions in living cells exhibit linear force–displacement behavior. (A) The measured bead displacement (○) during four 100 ms force pulses of increasing magnitude (~100 pN to 1 nN). (B) The electric current delivered to the electromagnetic microneedle corresponding to the displacement results shown in panel (A). (C) A plot of the maximum bead displacement as a function of electric current reveals a linear force–displacement relationship in the range of force investigated.

were removed. Because of the extremely brief duration of applied force (100 ms), very little viscoelastic creep occurred and the bead returned to almost its exact starting position after the force was removed, indicating that this mechanical response was almost purely elastic. For the range of force investigated (100 pN to 1 nN), bead displacement increased linearly with increasing electromagnetic force (Fig. 4C). Furthermore, because several images (four or five) were acquired during the 100 ms force pulse, these data also demonstrate that EPC can be used to obtain accurate temporal resolution of bead displacements over a wide frequency range (0–10 Hz).

To measure the viscoelastic creep response of living cells, the electromagnetic pulling cytometry system was programmed to apply prolonged (5–60 s) force pulses to individual magnetic beads bound to cell surface integrins using a force magnitude (~300 pN) that was in the linearly elastic range (Fig. 4C). For each pulse period, the creep displacement response was computed as described in Methods and plotted on logarithmic axes (Fig. 5). For some focal adhesions (48% of 27), the creep response was consistent with a power-law model, with a power-law slope that was nearly constant over three decades (Fig. 5A). Fitting these creep responses to the form $at^b$ yielded an average power-law slope, represented by the power-law exponent $b$, for integrin-associated focal adhesions in endothelial cells of $0.21 \pm 0.03$ (mean ± SEM). These results are consistent with past studies analyzing large populations of beads bound to integrins on cells which demonstrate similar power-law-like behavior in the frequency domain using oscillatory magnetic twisting cytometry [13,14,17] and the viscoelastic analysis of individual beads [28].

While some focal adhesions exhibited a power-law-like creep response, 52% of the RGD-coated microbeads analyzed in the present study did not exhibit a power-law-like relationship and failed to show a consistent slope for the creep response when plotted on a logarithmic scale. For example, many focal adhesions (32% of the total) exhibited a "biphasic" creep response that was characterized by two, abruptly changing slopes during the creep period when plotted on logarithmic axes (Fig. 5B). For these focal adhesions, the power-law exponent ($b$) representing the slope of the creep response began at $0.18 \pm 0.03$ and then increased to $0.50 \pm 0.05$ ($p = 0.0002$) after $4.5 \pm 1.5$ s of applied force. These data indicate a time-dependent "loosening" of focal adhesion mechanical properties in response to prolonged force. Interestingly, the slope at the beginning of the creep period for these bead-associated adhesions was not significantly different ($p > 0.44$) from the slope of the other focal adhesions that exhibited a consistent power-law slope throughout the entire creep period, most of which lasted for 10 s or more (Fig. 5A). In addition, 36% of the bead-associated focal adhesions that did not display consistent power-law-like behavior actually exhibited retrograde motion away from the direction of applied force (Fig. 5C) that began on average $15.5 \pm 6.8$ s after the onset of the force pulse.

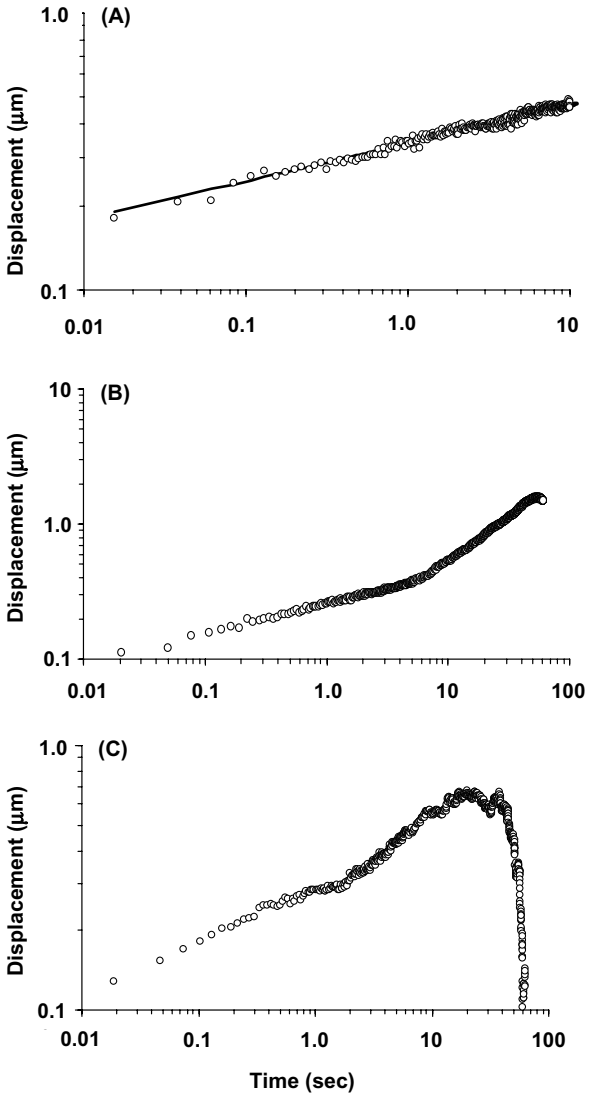


Fig. 5. The viscoelastic creep response of individual focal adhesions is more complex than predicted by the soft glass rheology model. (A) The viscoelastic creep response of a single RGD-coated magnetic bead (open circles) plotted on logarithmic axes exhibits a single power-law-like relationship during a prolonged force pulse (11 s, ~300 pN). The creep response was fitted to the form $at^b$ (black curve), where $a = 0.25$ μm and $b = 0.19$, $R^2 = 0.94$. (B,C) The creep response of two different RGD-coated magnetic beads that do not exhibit a power-law-like relationship during a prolonged force pulse (60 s; ~300 pN) but instead exhibit either multiple power-law-like periods having different slopes (B) or retrograde bead motion in the opposite direction to the applied force (C).

## 4. Discussion

To probe the rheological behavior of living cells and individual focal adhesions, we developed a form of mag-

netic cytometry—EPC—that incorporates a computer-controlled electromagnetic microneedle [22] to apply a wide range of static or dynamic tensional forces (up to 10 nN) via ligand-coated magnetic microbeads bound to membrane surface receptors. These studies show that when combined with high-speed image and data acquisition, EPC is capable of measuring the dynamic rheological behavior of individual focal adhesions exposed to sub-second (100 ms) or prolonged (>60 s) magnetic forces as well as any arbitrary, user-defined, dynamic force regimen (up to 10 Hz). By incorporating heterodyning imaging techniques and by using either a faster camera shutter or strobe to shorten the exposure time, it is possible to extend the frequency ceiling to 1 kHz or more [13,14].

In comparison to magnetic twisting cytometry that uses uniform, perpendicular magnetic fields to generate torque on ferromagnetic microparticles, EPC is distinct because it can apply strong tensional forces (up to 50 nN on 4.5 m dynabeads [22]) to either super-paramagnetic or ferromagnetic particles and it can generate large forces (~1 nN) on nanomagnetic particles as small as 250 nm [22]. Moreover, on account of the non-linear relationship between torque and bead orientation in a uniform magnetic field [29], the viscoelastic creep studies performed in the current study would be much more complicated using magnetic twisting cytometry. Other techniques for magnetic cytometry also generate tensional forces on magnetic microparticles, but generally these methods rely on fixed, non-interchangeable pole arrangements to generate the required magnetic field gradients [10,21,23,30]. In contrast, EPC uses a customizable and interchangeable electromagnetic microneedle where specific pole tip geometries can be electrochemically microfabricated to generate particular magnetic field configurations as would be needed to apply force on magnetic nanoparticles or to capture a single magnetic particle from a population of similar particles [22]. Like other techniques for magnetic pulling cytometry [10,21], EPC may apply only attractive forces to magnetic particles, and thus pure oscillatory forces cannot be applied. Finally, in comparison to other non-magnetic cytometry techniques, such as optical traps and micropipette pulling, EPC is able to generate stronger forces without light-induced heating effects [31] and it can more precisely define the site of force application using small micro- or nanomagnetic particles.

Using EPC, we focused on the viscoelastic creep response of individual focal adhesions and set out to evaluate the predictions of the soft glassy model of cell rheology proposed by Fabry et al. [13,14]. We found that the creep response follows a power-law-like relationship for up to three decades in time in almost half of the focal adhesions exposed to forces for up to 10 s in duration, consistent with the soft glassy model. A recent report similarly observed power-law behavior when the creep response of individual magnetic beads exposed to force for up to 3.2 s was analyzed using magnetic twisting cytometry [28]. In the present study, however, many focal adhesions exhibited a creep response that was characterized by either multiple, abruptly changing slopes when plotted on logarithmic axes or retrograde motion and retraction of the bead in the direction away from the applied magnetic force. These data demonstrate that the rheological behavior of individual focal adhesions is more heterogeneous and complex than suggested by the soft glassy model, especially when analyzed over longer time scales that are relevant to cell physiology.

We have previously shown that the viscoelastic properties of individual focal adhesions vary depending on the degree of local coupling to the internal cytoskeleton as measured by focal adhesion assembly, despite integrin ligation to identical RGD-coated beads [24]. External forces applied bead-associated focal adhesions that couple to the contractile cytoskeleton induce an active strengthening response and cellular retraction in a direction opposite to applied force [7,32–34]. This intrinsic heterogeneity in RGD-induced focal adhesion assembly and mechanical linkage to the cytoskeleton may be the basis for the different power-law slopes observed in the present study. For example, the EPC system may measure the properties of the submembranous cortical cytoskeleton in some beads, whereas it might probe the deeper cytoskeleton in others, either due to active contractile strengthening responses, or to progressive bead internalization. This hypothesis is consistent with the finding that retrograde bead motion occurred at later times (~15 s), interrupting the slope of the power-law during prolonged exposure to force.

The bead-to-bead variations of dynamical rheological behaviors observed in the present study may not have been detected previously in studies using oscillatory magnetic twisting cytometry that were originally used to promote the soft glassy model of cellular rheology due to use of ensemble analysis of large populations of beads [14]. In addition, because that method only detects displacements due to bead ''tipping'', the beads that formed the stiffest focal adhesions and were most tightly linked to the internal cytoskeleton would move very little and hence would have been largely ignored or excluded during the analysis [11]. Thus, those past oscillatory magnetic twisting data may have been inadvertently biased towards those focal adhesions that exhibit weak connections to the internal cytoskeleton [11] and thereby would be most representative of the submembranous actin–spectrin–ankryin shell [35]. While additional support for the soft glassy model has been obtained using atomic force microscopy, this technique also primarily probes the generalized structural features of the surface membrane of living cells because it does

not specifically ligate integrin receptors, and thus most likely measures the dynamic mechanical behavior of the cortical cytoskeleton [16].

In summary, the EPC technique provides an inexpensive and effective means to analyze static and dynamic mechanical behaviors of single living cells, and of specific subcellular structures, such as focal adhesions. Careful analysis of the rheology of individual focal adhesions using this method revealed novel dynamic behaviors that were not observed in previous analysis of large populations of bead-associated focal adhesions which suggested that all cells exhibit power-law-like mechanical behavior over a wide range of frequencies consistent with soft glass theory [13,14]. Instead, we found that the predicted power-law-like creep behavior was only exhibited by a subset of beads, and in contrast to the soft glassy model, many beads exhibited multiple power-law-like slopes, indicating that time- and force-dependent changes occur in the local mechanical properties of focal adhesions. These subtleties were likely not observed in the past because of the use of ensemble analysis of data from large populations of surface-bound magnetic beads, and because the displacements of the most well anchored magnetic beads are not detectable using oscillatory magnetic twisting cytometry. The switch between distinct power-law like periods having different slopes, when observed, occurred approximately 4–5 s after the application of applied force and may be related to dynamic changes in the structural linkages between the focal adhesions and various cytoskeletal elements, or to biochemical remodeling events induced by force (e.g., protein phosphorylation). Retraction forces that lead to retrograde bead motion tended to occur after longer times ($15.5 \pm 6.8$ s) and are likely due to actomyosin-dependent cell strengthening responses that are known to occur in response to applied force [4,7,32,34]. Both the internal cytoskeleton and cortical actin shell are three-dimensional networks (or foams) composed of many different load-bearing molecules and multi-molecular assemblies that likely exhibit a wide range of different viscoelastic time constants and thus, it is not surprising that they exhibit power-law-like behavior when their rheology is analyzed. It is, however, possible that the viscoelastic behavior of a focal adhesion that is associated with the cortical actin–spectrin–ankryin shell will exhibit a different power-law-like slope compared to focal adhesions that are coupled to the internal actin-microtubule-intermediate cytoskeleton. Alternatively, the changes in slope we observed could be due to internal rearrangements within the focal adhesion itself which effectively alter the degree of order within this structural lattice. In accordance with the soft glass theory, this would in effect cause the mechanical properties of the cell measured through the bead to shift between solid-like and fluid-like behaviors. In any case, uncovering the molecular and architectural basis for this rheological behavior should be an important target in future studies of cell mechanics.

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