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Crosstalk between focal adhesions and material mechanical properties governs cell mechanics and functions

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ABSTRACT

Mechanical properties of materials strongly influence cell fate and functions. Focal adhesions are involved in the extremely important processes of mechanosensing and mechanotransduction. To address the relationship between the mechanical properties of cell substrates, focal adhesion/cytoskeleton assembly and cell functions, we investigated the behavior of NIH/3T3 cells over a wide range of stiffness (3–1000 kPa) using two of the most common synthetic polymers for cell cultures: polyacrylamide and polydimethylsiloxane. An overlapping stiffness region was created between them to compare focal adhesion characteristics and cell functions, taking into account their different time-dependent behavior. Indeed, from a rheological point of view, polyacrylamide behaves like a strong gel (elastically), whereas polydimethylsiloxane like a viscoelastic solid. First, focal adhesion characteristics and dynamics were addressed in terms of material stiffness, then cell spreading area, migration rate and cell mechanical properties were correlated with focal adhesion size and assembly. Focal adhesion size was found to increase in the whole range of stiffness and to be in agreement in the overlapping rigidity region for the investigated materials. Cell mechanics directly correlated with focal adhesion lengths, whereas migration rate followed an inverse correlation. Cell spreading correlated with the substrate stiffness on polyacrylamide hydrogel, while no specific trend was found on polydimethylsiloxane. Substrate mechanics can be considered as a key physical cue that regulates focal adhesion assembly, which in turn governs important cellular properties and functions.

1. Introduction

Cells adhering to the extracellular matrix (ECM) probe the elasticity of the substrate, anchoring and pulling on their surroundings through trans-membrane adhesion receptors (integrins), which provide a structural connection between external cellular contacts and internal cytoskeleton (CSK) [1–14]. Focal adhesions (FAs) are adhesion plaques formed by an assembling complex of integrins and proteins. They act as a dynamic interface between CSK and ECM transmitting mechanical forces across the cell membrane. Several studies indicate that FAs sense substrate rigidity [15–19]. Pelhalm and Wang were among the first to demonstrate that the different responses of NIH/3T3 and NKR cells to the elasticity of the surrounding matrix originate at FA sites. Forces generated by acto-myosin stress fibers (SFs) and transmitted to the ECM through FAs are involved in important cell functions, such as migration and ECM remodeling [1,4,11–13]. Cells, in turn, sense the substrate rigidity and respond re-assembling FAs and SFs, closing the very intricate series of feedback loops between substrate stiffness and FA/SF assembly and growth [3,20]. It is well established that the interplay of ECM rigidity, FAs and CSK assembly regulates these series of mechanosensing and mechanotransduction feedbacks [21,22] that are responsible for regulating many cellular functions such as migration [23], spreading area [24], and differentiation [25,26].

There has been great progress in understanding how and which mechanosensitive molecules are involved in the transduction of mechanical forces into biochemical signals [20,27], but it remains partially unknown if and how the extracellular matrix mechanics, in terms of viscoelastic behavior, can regulate cytoskeletal organization, force generation, intracellular mechanics and, then, cell functions. In many cases, substrates and scaffolds presenting similar elastic modulus, but made of different materials, return to cells diverse mechanical feedback leading to not uniform range of cell responses and behaviors [25,26].

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Herein we investigated how NIH/3T3 fibroblasts modulate their shape, motility and cytoskeletal organization in response to different stiffness degrees of two of the most widely used types of material in the literature (polydimethylsiloxane, PDMS and polyacrylamide, PA). In particular, starting from the different mechanical behavior, elastic for PA and viscoelastic for PDMS, we focused our attention on (1) the existence of a direct relationship between substrate stiffness and characteristics of FAs; (2) the correlation between cell mechanics, motility, spreading and FAs; To this aim, a stiffness range spanning three orders of magnitude was created (1–1000 kPa) tailoring the elastic moduli of the two materials to obtain an overlapping region, between 25 and 200 kPa. A comparative analysis of some structural parameters and functions in the cells on PA and PDMS was performed.

2. Materials and methods

2.1. Substrate preparation and mechanical characterization

PA substrates were prepared by mixing PBS and acrylamide at a final concentration of 15% w/v and bis-acrylamide at a final concentration of 1.2% w/v starting from 40% w/v acrylamide and 2% w/v bis-acrylamide stock solutions. 1-[4-(2-hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propanone, commercially known as Irgacure 2959, was used to activate photopolymerization reaction at a concentration of 0.5% w/v. To control the mechanical properties of PA substrates we used three photomasks with different grayscale levels of opacity to filter the UV light exposure.

SYLGARD 184, purchased from Dow Corning (Midland, MI), consists of a base and a curing agent. To prepare PDMS substrates with different elastic moduli, the silicone elastomer base and the cross-linker were mixed at various ratios (1:10, 1:30, and 1:50), degassed under vacuum for 1 h and cured at 60 °C overnight.

The mechanical properties of PDMS and PA substrates were evaluated by small-amplitude oscillatory shear experiments that allowed measurement of the response of the samples and hence of their linear viscoelastic properties [28]. The tests were performed by using a stress-controlled rheometer (Gemini, Bohlin Instruments) equipped with a parallel plate geometry (20 mm of diameter). The instrument was preheated to 37 ± 0.01 °C and maintained at a constant temperature throughout the test. Dynamic strain sweeps were performed at a frequency of 1 Hz with strain amplitude ranging from $1.5 \cdot 10^{-4}$ to $1.5 \cdot 10^{-1}$. The tests were repeated at least four times for each typology of sample. To measure the shear modulus G, a stress–strain test was performed on the samples applying a shear deformation in the range from 10^{-1}% to 80% at 1 Hz frequency. No hysteresis or non-linear phenomena were observed during the characterization of the samples.

The local elasticity of PA gels was also probed with a commercial AFM (JPK Instruments, Germany) mounted on an epifluorescence microscope (Olympus IX70). Gel stiffness was quantified by indenting each sample at sixty distinct points; the substrate stiffness was defined as the average of six measurements. We used glass sphere cantilevers with a force constant of 0.05 N/m (Novascan, USA). Cantilevers were calibrated by measuring the free fluctuations when unloaded. To quantify the stiffness, the Hertz model gives the following relation between the indentation δ and the loading force F in the case of an infinitely hard sphere of radius R (AFM tip) touching a soft planar surface

$$F_{\text{sphere}} = \frac{4}{3}\frac{E}{(1-\nu)}\sqrt{R}\delta^{3/2} \quad (1)$$

where E is Young's modulus and ν is the Poisson ratio (ν_{PA} = 0.457; ν_{PDMS} = 0.5 [29]) of the soft material.

2.2. Creation of a substrate step

To create a step in rigidity, we covered half a coverglass with PDMS (1:10 ratio), that was cured at 60 °C overnight. After PDMS curing, a droplet containing 10 μl of acrylamide/bis-acrylamide mixture was placed adjacent to the PDMS and confined to the other half of the coverglass. PA was cured using the photomask having the highest level of transparency. We thus obtained step substrates characterized by a difference in Young's modulus at the interface of about one order of magnitude (120–1000 kPa). 10 μl of acrylamide/bis-acrylamide mixture was assessed to be the right amount to obtain the same height of PDMS region once reaching the swelling equilibrium.

2.3. Conjugation of RGD peptides to PDMS and PA substrates

The conjugation of RGD peptides was performed through a two-step method by using a bifunctional photolinker, N-sulfosuccinimidyl-6-(40-azido-20-nitrophenylamino)hexanoate (sulfo-SANPAH, Thermo Fischer Scientific) as a cross-linking agent to immobilize RGD peptides. In the first step, a sulfo-SANPAH solution in deionized water was prepared in the following way: sulfo-SANPAH was first dissolved in dimethylsulfoxide (Sigma, St. Louis, MO) at a concentration of 0.25 mg/μl and then diluted with deionized water to 0.5 mg/ml concentration. The freshly prepared sulfo-SANPAH solution was placed onto PDMS and PA sheets and exposed to UV light for 20 min. A coupling solution was prepared by dissolving RGD peptides (Sigma–Aldrich) in a 50 mM (pH 8.5) bicarbonate buffer at a 1 mM final concentration. After washing, the PDMS and PA sheets were completely covered with the RGD peptide solution and incubated at 4 °C for 24 h. The RGD solution was removed and the membranes were washed with PBS three more times. Unreacted NHS groups were blocked by treating the polymer surfaces with a 0.2 mM ethanolamine (Sigma, St. Louis, MO) in the same condition as for the peptide at 4 °C for 30 min. Then, samples were stored dry until further use. Afterward, the substrates were sterilized for cell attachment incubating them with antibiotic solution for 24 h at 37 °C and thoroughly washed with ultrapure water and dried under vacuum for determination of conjugated peptides.

2.4. Determination of surface RGD density

The density of the RGD peptide on the PDMS surfaces was determined directly on solid support by using MicroBCA assay (Sigma, St. Louis, MO) as described by Tyllianakis et al. [30]. The absorbance of the peptide group present on surfaces was measured at 562 nm using a microplate reader (Victor, PE) and the evaluation of surface RGD bonds was carried out through the standard calibration curve, obtained using RGD solutions of known concentrations. The amount of immobilized short peptides containing the enhancing tyrosine residue (RGD) was measured by adding the MicroBCA working solution directly onto the samples in a reduced volumetric form of the assay. A nominal density was calculated by taking into account the area of each treated sample and referred to as RGD nmol/cm^2.

2.5. Surface roughness and hydrophilicity characterization

Surface roughness was measured using atomic force microscopy (AFM). Measurements were performed using a Nanowizard II AFM (JPK Instruments AG, Berlin, Germany), operating in contact mode and employing triangular tip with a nominal spring constant of 0.03 N/m (MLCT, Brukerprobes). To determine different roughness of surfaces 1.953 μm × 1.953 μm area were scanned at different sizes. The RMS (root-mean-squared) roughness was calculated