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Biomembrane-mimicking lipid bilayer system as a mechanically tunable cell substrate

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ABSTRACT

Cell behavior such as cell adhesion, spreading, and contraction critically depends on the elastic properties of the extracellular matrix. It is not known, however, how cells respond to viscoelastic or plastic material properties that more closely resemble the mechanical environment cells encounter in the body. In this report, we employ viscoelastic and plastic biomembrane-mimicking cell substrates. The compliance of the substrates can be tuned by increasing the number of polymer-tethered bilayers. This leaves the density and conformation of adhesive ligands on the top bilayer unaltered. We then observe the response of fibroblasts to these property changes. For comparison, we also study the cells on soft polyacrylamide and hard glass surfaces. Cell morphology, motility, cell stiffness, contractile forces and adhesive contact size all decrease on more compliant matrices but are less sensitive to changes in matrix dissipative properties. These data suggest that cells are able to feel and respond predominantly to the effective matrix compliance, which arises as a combination of substrate and adhesive ligand mechanical properties.

1. Introduction

Adherent cells actively probe the mechanical properties of the extracellular matrix (ECM) by imposing traction forces and detecting the resulting mechanical responses. These mechanical signals are converted into intracellular biochemical signals by a process termed mechano-transduction [1,2]. By this process, cells are able to mechanically adapt to the substrate they adhere to. The influences of matrix mechanical properties on cells have been extensively studied using polyacrylamide (PAA) or structured polydimethylsiloxane (PDMS) substrates. These studies showed that the matrix elasticity has implications on cell morphology [3–5], cell mechanical properties [6,7], migration [8,9], adhesion [5,10], contractile force generation [8,11,12], and differentiation [13].

In addition to substrate rigidity, the anchorage and binding details of the adhesion ligands may also play an important role in cellular mechano-sensing. For instance, softer PAA gels are more porous and, therefore, provide sparser anchoring points to adhesive ligands compared to stiffer PAA gels [14]. It has been argued that sparsely anchored adhesive ligands on soft PAA gels stretch differently and behave more compliant when exposed to lateral force than ligands anchored firmly to stiffer PAA gels. Thus, the mechanical cue to which cells respond may not be the stiffness of the underlying matrix but instead the amount of ligand extension or possibly the opening of cryptic binding sites as the ligands unfold under force [14,15].

The purely elastic PAA and PDMS substrates with immobilized, static adhesive ligands used in previous studies fall short of replicating the viscoelastic and dynamic nature of tissues and cells [16–20]. In contrast to elastic substrates where deformations come to a halt when cell tractions reach a steady state, cell adhesion ligands anchored to viscoelastic or plastic substrates remain mobile and thus provide a different mechanical stimulus. It has been shown that cellular traction forces decrease with increasing mobility of adhesion ligands anchored non-covalently to different polymeric substrates [21], although the bulk mechanical properties of the polymeric substrates were not characterized in that report.

We use a biomembrane-mimicking cell substrate based on a polymer-tethered multi-lipid bilayer system to study cell behavior in response to viscoelastic matrix properties [22]. As a cell adhesion ligand, laminin is coupled to the top lipid layer via amine-to-sulfhydryl crosslinkers. The material properties of the multi-bilayer cell substrate can be tuned by increasing the number of bilayers in the stack, which decreases the frictional coupling between the top

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layer and the supporting glass substrate and, therefore, increases substrate fluidity. Importantly, stacking does not alter the density or binding properties of adhesive ligands. Therefore, any responses of cells grown on bilayers with different stacking numbers can be attributed solely to changes in substrate bulk mechanical properties. Preliminary experiments revealed that the number of stacked bilayers affect fibroblast spreading, morphology, and migration [23], but the viscoelastic properties of the multi-bilayer systems has not been studied thus far.

To characterize the mechanical properties of the bilayer substrates, we measure the creep response by applying lateral forces onto magnetic microbeads coupled to the laminin ligands. As a reference, we compare these properties to those of laminin-coated glass as well as polyacrylamide substrates. The responses of mouse embryonic fibroblasts (MEFs) to changes in matrix compliance of these substrates are studied in terms of spreading area, motility, cytoskeletal prestress, cell stiffness, and focal adhesion size. Finally, the relative influence of matrix elastic versus dissipative properties on cell behavior is analyzed.

2. Materials and methods

2.1. Cell culture

Mouse embryonic fibroblasts (obtained from Dr. W. Ziegler, University of Leipzig) [24] are maintained at 37 °C and 5% CO_2 in low glucose (1 g/L) Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin. Before plating, cells are rinsed with PBS and trypsinized with 0.05% trypsin/EDTA.

2.2. Bilayer assembly

Bilayers are fabricated as described in Ref. [22]. In brief, small unilamellar vesicles (SUVs) or giant unilamellar vesicles (GUVs) are used. SUVs are prepared by sonication [25,26] and GUVs by the sucrose hydration method [27]. Two complementary chloroform lipid stock solutions consisting of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC, Avanti Polar lipids) with either 5 mol% 1,2-dihexadecanoyl-*sn*-glycero-3-phosphothioethanol (sodium salt) (DPTE, Avanti Polar lipids) or 5 mol% 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-{maleimide(polyethyleneglycol)-2000} (ammonium salt) (DSPE PEG2000-Maleimide, Avanti Polar lipids) are prepared as SUVs and GUVs. To produce GUVs, the stock solution is dried, resuspended in 0.1 mM sucrose/1 mM $CaCl_2$ solution, heated for 2 h and then cooled down to room temperature. SUV vesicles are formed by resuspending the dried stock solution in Milli-Q water, followed by sonication for 10 min. For the first layer of every stack, SUVs are added to a solid glass support. They adsorb to the glass and break due to intrinsic instabilities to form a lipid bilayer [28,29]. Additional layers are formed through GUV fusion (Fig. 1). For these layers, GUVs are added in a 0.1 mM glucose/1 mM $CaCl_2$ solution and are allowed to bind for 2 h. Stacking is achieved by adding complementary GUV solutions, which are linked covalently by sulfhydryl-maleimide coupling chemistry, linking thiolated lipid DPTE with maleimide-functionalized lipopolymer PEG2000-Maleimide. Adding additional layers increases lateral linker mobility [22] and decreases frictional coupling of the top layer with the glass substrate [30] (Fig. 1). The top layer of the stacks is labeled with Texas-Red, allowing us to monitor the quality of the formed bilayer deposition through diffusion measurements (fluorescence recovery after photobleaching, FRAP). Each final substrate is tested for defects by laser bleaching of several 20 μm diameter spots at different locations and observing the subsequent fluorescence recovery. Substrates that did not fully recover within 2 min were discarded. The top layer always contains DPTE, allowing for the layers to be coated with cell-substrate linkers (here: laminin) that are linked via heterobifunctional quantum dots functionalized with PEG-Mal and PEG-NHS.

2.3. Polyacrylamide gels

Polyacrylamide gels are cast using a 40% acrylamide/bisacrylamide (ratio 37.5:1) solution (Sigma Aldrich). The solution is mixed with water at a final concentration of 4% or 6.1% acrylamide. To initiate polymerization, 0.2% tetramethylendiamin (TEMED) and 0.5% amoniumpersulfat (APS) is added. For traction measurements, gels are prepared by the same protocol at a concentration of 6.1% acrylamide/bisacrylamide, with fluorescent beads embedded in the gel (see below under traction microscopy).

2.4. Live cell imaging

Cells are imaged using an inverted microscope (DMI6000B, Leica) and kept at 37 °C and 5% CO_2 within an incubation chamber during measurements. To monitor the changes in cell spreading area, cells are plated for 24 h prior to measurements.

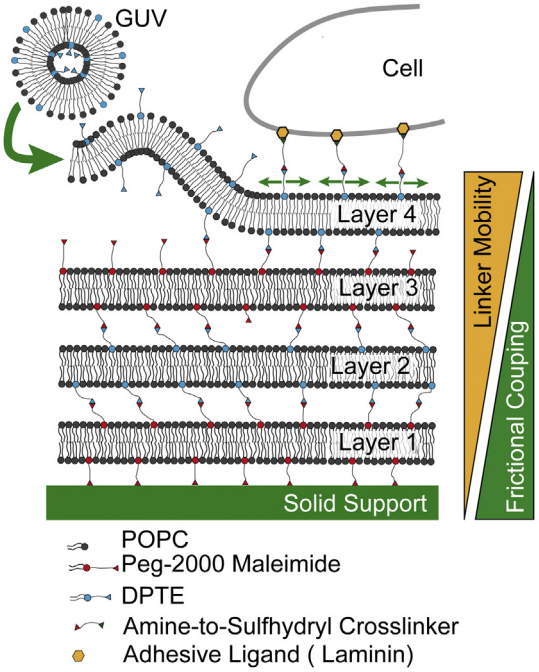

Fig. 1. Phospholipid bilayer stack assembly and properties. Bilayer substrates are built by stacking alternating GUV solutions on top of each other and linking them via sulfhydryl-maleimide coupling chemistry. Previous work suggests that with increasing number of layers, frictional coupling to the solid support decreases, and linker mobility increases [22]. The top layer is functionalized with laminin as a ligand for cell adhesion. Each bilayer has a thickness of 4 nm, and each polymer layer has a thickness of 3.4 nm [22].

Phase contrast images of the cells are obtained and analyzed using a customized Matlab program.

For measuring cell migration, cells are monitored in Hoffmann contrast mode using a 20× 0.4 NA objective with a 0.5× video coupler. Images are taken every 300 s over a time period of up to 24 h. Cell movements are tracked with custom image processing software written in Matlab. From the cell trajectories, the mean squared displacement (MSD) is calculated [31] and described with a power-law relationship of the form

$$\mathrm{MSD} = D^*(\Delta t/t_0)^{\beta} \quad (1)$$

with D denoting the apparent diffusivity (the MSD at the reference time $t_0 = 1$ min), and β denoting the power-law exponent. The time interval Δt ranges from 300 s to 300 min. D characterizes the speed of cell movements at short time intervals, and β characterizes the persistence of cell movement at long time intervals [32]. β typically ranges from a value of 1 for randomly migrating cells to a value of 2 for persistent, ballistically migrating cells [32]. As D is log-normal distributed [32], the geometric mean and geometric standard error of D is computed for each measurement condition. For each trajectory, we also compute the cosine of the turning angle of cell movements between subsequent time intervals Δt ranging from 300 s to 300 min [32]. A value near unity characterizes a persistent motion; a value near zero characterizes a random motion.

2.5. Magnetic tweezer microrheology

Magnetic tweezers are used to characterize the mechanical properties of substrates and cells [33]. In brief, the magnetic tweezer device is attached to an inverted microscope equipped with a 40×, 0.6 NA objective. The device consists of a solenoid with 250 turns of 0.4 mm diameter copper wire around a high-permeability μ-metal core (HyMu80 alloy, Carpenter, Reading, PA) with a sharp tip.

Magnetic pulling forces in the horizontal direction are exerted on superparamagnetic beads (epoxylated 4.5 μm Dynabeads, Invitrogen). For coupling the beads to the substrates or cells, beads are coated with laminin or fibronectin (Roche Diagnostics), respectively, at a concentration of 5 μg protein in 1 ml PBS for 1×10^7 beads, at 4 °C overnight in a shaker. Prior to measurements, beads are sonicated for 10 s and added to the substrate or the cells at a density of 3000 beads per mm^2. After 30 min of incubation, unbound beads are gently washed off.

Cells are seeded at a density of 4000 cells/cm^2. Measuring time is limited to 30 min per dish since the cells are kept at 37 °C but without CO_2 on the microscope stage. For measuring the mechanical properties of the bilayer substrates, a series of 3