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In-vivo, in-situ, light-tunable manipulation of cells' biomechanics on a photoactive azobenzene bio-substrate

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ABSTRACT

In-vivo, real-time study of the local and collective cellular biomechanical responses requires the fine and selective control of the cellular environment. Optical manipulation provides a suitable pathway to achieve non-contact, selective, local, temporal and spatial stimuli. The spectacular photomechanical properties of photoactive bio-substrates such as azobenzene-containing thin polymer films are a new promising strategy to achieve optically triggered local mechanical stimulation of cells. Excited cells exhibit spectacular morphological modifications and area shrinkage, which are dependent on the illumination. In this work we demonstrate that the capabilities of photomechanically active azo-containing substrates to optically stimulate cells' mechanical response can be strongly influenced by the adhesion binding agent used to deposit the living cells on the photoactive layer. This provides a further tool for the photomechanical control of the cellular environment and of the cellular response.

Keywords: Azobenzene, biomechanics, thin films, photoactive materials, tunable materials, smart materials, adhesion

1. INTRODUCTION

The interaction of cells with their chemical and mechanical environment influences their adaptive response and conditions the physiology and pathophysiology of the tissues. Several attempts to build simple systems to study the reciprocal influences of tissues and their environment [1-2], as well as to measure the consequences of environmental disturbances on cells, use technologies such as magnetic micropillars [3], PDMS stretching [4], optical and magnetic clamps, microstructured polymers culture substrates with controlled rigidity [2,5] and rigidity gradients [6]. These technologies do not allow in-situ, in-vivo and dynamic modifications of the substrate for a cellular or sub-cellular scale response to stress gradient change, which is relevant in many cell types [7].

The use of light as the control stimulus is a promising approach for cell stimulation, since it presents several advantages over the usual methods: non-contact operation (avoiding system perturbation or damage), remote actuation and sensing (compatible with restrictive environments and suitable for parallel addressing), high selectivity (wavelength, polarization, diffraction, etc.), local control down to submicronic scales. These features are particularly suitable for biological entities manipulation and analysis.

In this context, the photomechanical properties of photochromic materials such as azobenzene-containing systems offer a new way to optically achieve and control the mechanical stimulation of biological objects [8-13, 18]. Azobenzene-type molecules undergo photoisomerization between TRANS and CIS isomers. These interconversion processes induce changes in the molecule physical properties, such as the dipole moment, which, for example in a thin film, results in the variation of surface properties such as wettability [14]. In addition, in the case of the so-called push-pull systems, such as the Disperse-Red One (DR1) derivative, the TRANS isomer is stable while the CIS isomer is metastable. Therefore, a cyclic conformation change is produced under illumination with a single wavelength. When incorporated in a polymer-like matrix, the repeated photoisomerization of the chromophores induces a spectacular mechanical response of the host material. First, the photoinduced modification of the viscoelastic behavior of the matrix is observed. The mobility of

molecules due to photoisomerization induces a reduction in the hardness and viscosity of the material, that is restored when the illumination ceases [15]. Second, a deformation of the material occurs under illumination due to efficient polarization-directed photo-induced mass migration processes. This was exploited for optical nanopatterning of polymer thin films and for photomechanical actuation of micro-nano structures [8-13]. These phenomena offer a new and unique way of creating substrates whose mechanical properties can be dynamically and locally controlled by light.

Recently, by real-time, in-situ, fluorescence imaging of light-stimulated cells on PMMA-DR1 photoactive substrates, we have shown that optical stimulation of these photomechanical mechanisms in azo-polymers can be used to trigger optically stimulated area shrinking in living mouse *C2C12* cells deposited on the photoactive substrate [19].

In this work, we show that the adhesion protocol used for the deposition of living cells on the PMMA-DR1 substrate plays an important role on the control of the cell dynamics. In particular, the use of fibronectin as adhesion agent can slow down the cell response time and improve the stability and repeatability of the photo-mechanical actuation.

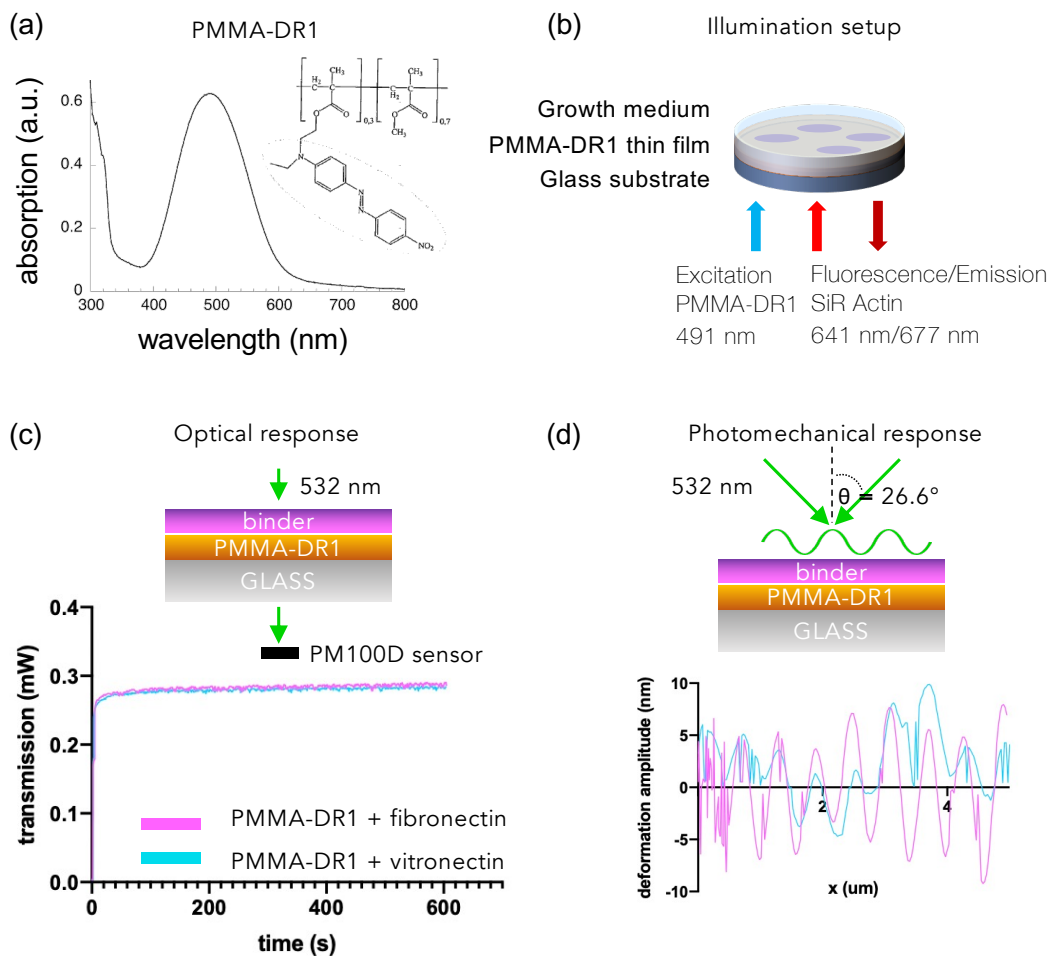


Figure 1. (a) PMMA-DR1 molecule UV-VIS absorption spectrum. (b) Schematic of the experimental setup. (c) Measured transmission optical response of PMMA-DR1 thin polymer film coated with fibronectin (purple) and vitronectin (blue) binding agents. (d) Measured AFM topography of surface gratings patterned by holographic projection of an optical interference pattern (each beam's wavelength: 532 nm, incidence angle: 26.6 °, linear polarization: $\pm 45^\circ$, power density: 0.9 mW/mm²; illumination time: 10 min) on PMMA-DR1 thin polymer film coated with fibronectin (purple) and vitronectin (blue) binding agents.

2. MATERIALS AND METHODS

2.1 Photoactive substrate

An organic polymer film is obtained by spin-coating a solution of PMMA-DR1 (Sigma Aldrich 570435) in dichloromethane (25 mg/ mL) at 5000 rpm for 40 s at room temperature on a glass coverslip. The obtained thin film has a thickness of about 250 nm [12]. The volumic concentration in DR1 molecules is of the order of 1 molecule.nm⁻³. The light absorption spectrum of the film, shown in Fig. 1(a), is peaked at 491 nm.

2.2 Cell culture

i) Fibronectin binding

PMMA-DR1 glass coverslips were coated by human fibronectin thanks to an overnight incubation at 4°C with 100 µl of 40µg/ml solution of human fibronectin (Sigma) in PBS in a wet atmosphere. Control glass coverslips were first activated by 30 sec plasma treatment (Plasma Cleaner, Harrick) and immediately coated by human fibronectin as previously described. Before the use, coverslips were rapidly rinsed in water and placed in a bath of PBS to avoid drying.

ii) FBS binding

C2C12 (ATCC) mouse myogenic cell line was cultured at 37°C in 5% CO₂ in DMEM supplemented with 10% fetal bovine serum (FBS) and vitronectin [16]. After trypsinization, cells were deposited on a glass coverslip coated with PMMA-DR1 at 3x10⁴ cells/ml and cultured overnight.

2.3 Actin staining

Before light stimulation of the substrate and observations, the actin cytoskeleton was stained by 45mn incubation with 6µM of SiR Actin (Cytoskeleton). Cells were washed 3 times with DMEM 10% FBS without phenol red and incubated in this medium. Coverslips were mounted in a ChamSlide holder (GATACA Systems) for microscopic observations.

2.4 Laser stimulation of azobenzene-containing polymer and wide-field microscopy

Samples are observed with an inverted iMIC *TILL Photonics* microscope provided by the Imag'IC facility (Institut Cochin, Paris). Laser stimulation was performed using multi-wavelength laser (including 491 nm - Cobolt Calypso 50 mW and 641 nm - Toptica iBREAM 100 mW). A schematic of the experimental setup is shown in Fig. 1(b). Two EMCCD cameras ANDOR Technology coupled with two lenses 1.5x (TuCam Andor Technology) are used to acquire images. Samples are observed with a x40 immersion objective throughout the experiment. The azobenzene substrate was excited with a 491 nm laser (3mW) for 4, 8 or 15 mn as described in the "Experimental results" section. The fluorescence image of the SiR Actin at 677 nm was performed with the same rate all along the illumination sequence and well after the 491 nm laser was switched off.

Experiments were performed with the help of the BioMecan'IC facility (Institut Cochin, Paris).

2.5 Image analysis

For each image, we extract the area of the cells, by using the routine area measurement algorithm of FIJI software suite. We then calculate the averages of the measured cell areas for each illumination time, and we compute the statistical significance by two-way ANOVA with Tukey's multiple comparisons test.

2.6 AFM setup

We used an AFM system (Bruker DI3100) in "Tapping mode" with a Si AFM tip (Nanosensor PPP-NCHR). We worked in air at room temperature with a low free amplitude (about 200 mV) to be soft enough not to damage or modify the measured surface. The tetrahedral tip with the sharper tip (tip radius less than 10 nm) was used for high lateral resolution. The nominal resonant frequency and the spring constant of the AFM cantilever were 330 kHz and 42 N/m.

3. EXPERIMENTAL RESULTS

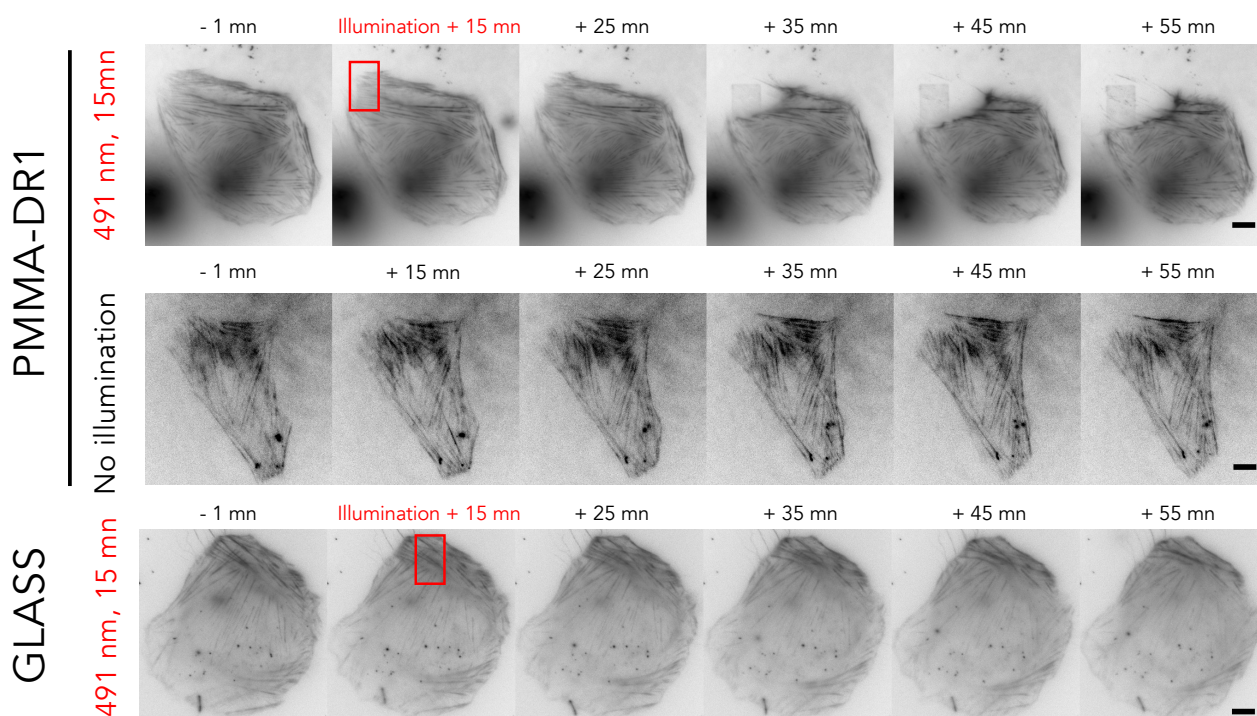


Figure 2. Temporal evolution of SiR Actin fluorescence images of C2C12 cells in three representative experimental conditions, at different times. The red rectangle ($15\mu\text{m} \times 20\mu\text{m}$) shows the light-stimulated area, with linearly polarized blue light (491 nm), $3\text{ mW}/\text{mm}^2$. The black scale bar is $10\mu\text{m}$. (Top) Cells deposited on a PMMA-DR1/FN substrate, before illumination (- 1mn); after 15min of illumination; at different times after illumination is switched OFF (+25, ..., +55 mn). (Middle) Cells deposited on a PMMA-DR1/FN substrate, without illumination. (Bottom) Cells deposited on a glass coverslip without PMMA-DR1/FN, coated with fibronectin, before illumination (- 1mn); after 15min of continuous illumination; at different times after illumination is switched OFF (+25 ...+55 min).

In our recent work, we have demonstrated the use of optically triggered photomechanical phenomena in DR1-containing polymers to achieve photoinduced area shrinking of cells deposited on a PMMA-DR1 photoactive substrate. In these systems, cell adhesion was achieved by physiological serum and vitronectin binding agent [19]. In this work we aim at studying the effect of fibronectin binding agent on the cellular response.

We first compare the optical response of the PMMA-DR1/vitronectin (PMMA-DR1/VN) and PMMA-DR1/fibronectin (PMMA-DR1/FN) systems. Measuring the optical transmission kinetics for both systems (Fig. 1c), shows no significant differences in the optical activity. We then compare the photomechanical response of both systems, by optical inscription of a 1D surface relief grating by holographic projection of an optical interference pattern (Fig. 1d). Each beam's wavelength is 532 nm, the incidence angle is 26.6° , beams are linearly polarized at $\pm 45^\circ$, optical power density is $0.9\text{ mW}/\text{mm}^2$ and the illumination time is 10 min. By Atomic Force Microscope measurements, we determine that the amplitude of the obtained gratings in both systems is of the same order of magnitude (average of about 8 nm). This shows that both systems have similar photomechanical responses.

In order to determine the effect of the fibronectin on the cellular response to the optical triggering of the substrate's photomechanical effects, we optically stimulate the photoactive substrate while monitoring the cellular response by optical and fluorescence microscopy, before, during and after blue laser illumination (Fig. 1b). SiR Actin fluorescence images are captured every minute, which allows to measure the evolution of the cell's area. Optical stimulation is obtained by projecting a rectangular illumination pattern (wavelength: 491 nm, power density: $3\text{ mW}/\text{mm}^2$, illumination time: 15 min) as in our previous work. This triggers locally both the photoactive material's photodeformation by mass migration, and the photoinduced change of the substrate's viscoelastic properties [19]. The typical positioning of the

photomechanical excitation pattern with respect to a living cell is shown in Fig. 2 (top). The red rectangle indicates the optically excited area on the cell's lamellipodia, where the motility is higher.

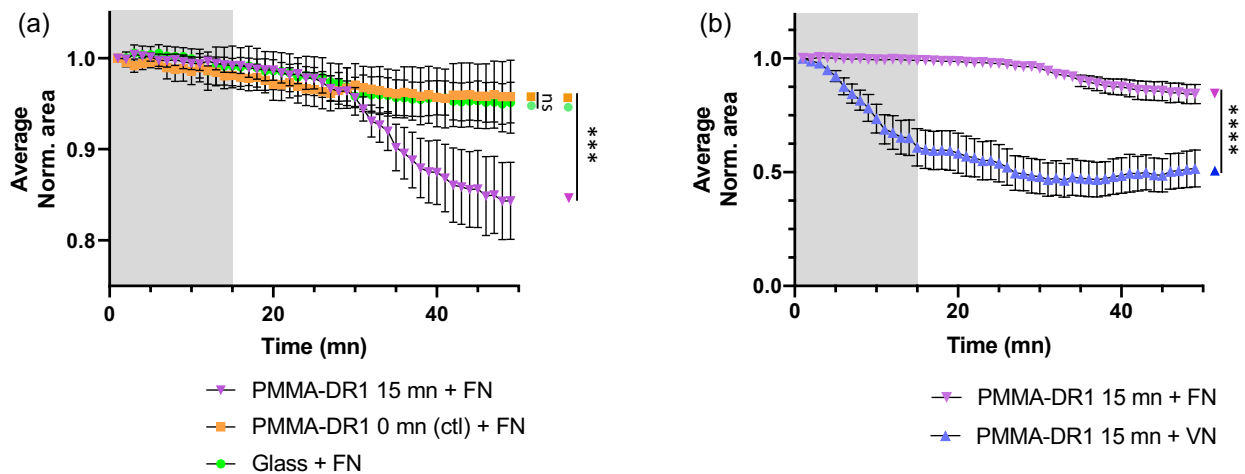


Figure 3. Statistical analysis of the measured C2C12 cells' area on SiR Actin fluorescence images. Average evolution of area parameter over time was normalized relatively to the mean area value measured 5 mn before illumination is switched on. (a) Average cells' area measurements in three experimental conditions: (i) 15 min optical stimulation of PMMA-DR1/FN substrate (purple); (ii) without optical stimulation on PMMA-DR1/FN (orange); (iii) 15 min optical stimulation on a glass coverslip without the photoactive layer. (b) Comparison of cells' area shrinkage obtained on PMMA-DR1/FN substrate (purple) and PMMA-DR1/VN substrate (violet) under the same illumination conditions. "ns" indicate non statistically significant, *** p-value<0.001 and **** p-value<0.0001 by two-way ANOVA with Tukey's multiple comparisons test. All error bars are SEM. Illumination area is 15 μ m X 20 μ m, light is linearly polarized, wavelength is 491 nm, optical power density is 3 mW/mm².

Figure 2 shows typical SiR Actin fluorescence images of C2C12 cells in three representative experimental conditions, at different times. (i) Cells deposited on a PMMA-DR1/FN system are illuminated for 15 min (Fig. 2 – top). The red rectangle indicates the illuminated area. 35 min after the illumination is switched OFF, a clear shrinkage of the cells' lamellipodium is observed at the top-left of the image near the illumination area. The visible rectangle on the substrate confirms the optical activation of the PMMA-DR1 photomechanical response [19].

We measure also two "control" experimental conditions: (ii) we perform the same experiment as in (i) but without optical stimuli (Fig. 2 – middle): no cellular response is observed; (iii) we perform the same experiment as in (i) but without the PMMA-DR1 layer, i.e., with cells deposited on the glass coverslip with the fibronectin binding agent (Fig.2 – bottom): no significant cellular response is obtained.

Note that, during these experiments, cells continue their natural biological functions, which causes random area variations, which may be independent from any optical stimuli.

Figure 3 shows the statistical analysis of the measured area, performed on several experimental conditions. Average evolution of area parameter over time was normalized relatively to the mean area value measured 5 mn before illumination is switched on. Fig. 3a compares results for experimental conditions (i-iii). In the case of 15 min optical stimulation of PMMA-DR1/FN, we observe area shrinkage starting at about 15 min after the illumination is switched OFF and reaching about 15% area shrinkage in the subsequent 20 min. On the other hand, no significant cellular response is observed without illumination or without the PMMA-DR1/FN layer. These observations clearly show the effect of the optical actuation of the photoactive PMMA-DR1 material on the cell mechanical response, with respect to the control experiments, where no significant effect on the cell behavior is observed (p-value<0.001).

Figure 3b compares the cellular response obtained in the same illumination conditions in the case of PMMA-DR1/FN (purple) and PMMA-DR1/VN (violet). PMMA-DR1/FN induces a slower and less efficient cellular response, starting at about 15 min after the illumination is switched OFF, and reaching 15% area shrinkage after 20 min, while PMMA-DR1/VN triggers a cellular area shrinkage starting at about 8min during illumination, and reaching about 50% area

shrinkage in about 22 min. On the other hand, PMMA-DR1/FN seems to give much more reproducible and stable cellular responses, which may allow a more reliable optical control of the cellular behavior.

4. DISCUSSIONS AND CONCLUSIONS

These experiments show that the use of fibronectin as a binding agent for the deposition of living cells on azo-polymer substrates does not inhibit the optical and photomechanical properties of the substrate. It provides a suitable way to control the dynamics and the reliability of the cellular mechanical response to the optical stimuli. Fibronectin provides a stronger mechanical interaction with the cells with respect to vitronectin, especially at the cellular focal points. The enhanced adhesion provided by fibronectin may be at the origin of the observed “slower” and more stable cellular mechanical response.

In conclusion, this work brings new evidence on the possibility of using binding agents as means of controlling the cellular mechanical response to the environment photoinduced modifications obtained by means of the light-stimulation of photoactive polymer bio-substrates. The quantitative determination of the role of fibronectin on the cellular area shrinkage and the mechanisms at play at the cell/substrate interface and their influence on the intracellular and intercellular dynamics will require further investigations.

5. ACKNOWLEDGMENTS

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