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Regulating the mechanical properties of cells using a non-UV light-addressable hydrogel patterning process

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***Abstract*- The determination of the mechanical properties of cells plays an important role in biological studies and has gained acceptance recently as a possible label-free biomarker for cell status determination or diseases detection. Investigations on how external cellular properties affect cell mechanics are helpful in understanding cell disease processes and cell morphogenesis, which are of large significance in medical science. Although most researchers have focused on individual cell mechanics, or the effect of substrate stiffness on cells, cell mechanical response due to interactions among cells is yet to be examined. A reason for this is that the study of cell mechanical response to cell shape requires one to use a cell patterning process. However, existing cell patterning methods are very complex and time-consuming. In this paper, we describe a practical and rapid technique that can easily pattern cells into desired shapes, which allows investigations of the effect of external environment on cell stiffness. In the new technique, Poly-(ethylene) glycol diacrylate (PEGDA) hydrogel film with thickness 70–100 nm is controllably patterned on a hydrogenated amorphous silicon (a-Si:H) substrate by polymerizing PEGDA molecules in-situ using programmable visual light patterns. The idea is to enable the confinement of cells cultured on the hydrogels into special areas. The elastic modulus of the patterned cells is measured using an atomic force microscope. Experimental results have demonstrated the versatility of the technique as a tool for cell pattering and exploration of cell mechanics under external mechanical stimuli.**

I. Introduction

A large number of cells sense not only biochemical stimuli but also the mechanical cues arising from the external cellular matrix. The cues prompt the cells to grow through a process known as mechanotransdution. In particular, the rigidity of the substrate to which the cells get attached can induce the cell to regulate their shape, proliferation, internal cytoskeletal tension and stiffness. The stiffness of the substrate on which cells prefer to grow is different for cells from different tissues. For example, brain cells grow and spread better on a softer substrate in comparison to with cells from other tissues such as skin. The same cells growing on a substrate with a different stiffness often exhibit different behaviors. Stem cells can sense the elasticity of the matrix and transduce this signal into a morphological change and specific differentiation [1]. Additionally, mechanical properties of cells reflect their unique physiological state and can also indicate some diseases [2], [3]. All this suggests that studies on how the external cellular matrix elasticity regulates cell mechanics is important in understanding disease processes, morphogenesis, and tissue-repair strategies [4]. At the cellular level, normal tissue cells probe elasticity as they anchor and pull on their surroundings. Focal adhesions are formed and provide a physical connection between a cell and its external substrate, which provide the physical support needed for cytoskeletal deformation as cells migrate or contract. Vice versa, cell deformation always accompanies cytoskeletal tension, which senses their external environment. Cytoskeleton also plays a pivotal role in defining cell mechanical properties [5]. Although the tight relationship existing between external elastic and cell stiffness has been demonstrated, it is still unclear whether the external elastic modulus actually modulates cell shape to regulate cell stiffness, or whether substrate rigidity and cell shape regulate cell stiffness independently. Focusing on this issue, many methods have been invented for studying how external cellular mechanics affect cell mechanics. For example, [6] and [7] report methods based on micro-post arrays and micro-contact printing. These have demonstrated that both substrate stiffness and spreading area can affect cell stiffness. Techniques based on micro-fludic channels have also been used to study relationships connecting substrate stiffness, cell migration speed and cell spreading area [8], [9]. However, these often render the study of cell mechanics more complex, because processes such as adsorption of specific proteins and photolithography are usually required in these methods for confining cell adhesion and fabricate micro-scale environment.

This paper reports on a rapid, automatic technique for investigating how cell shape affects cell mechanics. This technique is capable of regulating signal cell shapes without changing substrate stiffness and substrate biochemical properties, thus enabling the study of the independent influence of cell shape on cell mechanics. In this technique, an addressable polymerizing PEGDA hydrogel film with thickness 70–100 nm is deposited in situ on a-Si:H substrate by using a series of programmable visual light patterns. Since PEGDA hydrogel is protein passive, adherent cells can't spread on it owing to its electronic neutrality. By contrast, the a-Si:H substrate is stiff and has a net charge on the interface with liquid which makes the cell adhesive. Employing this technique, hydrogel film with an arbitrary geometry and pattern can be modified on the substrate

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within 1–5 seconds to control cells to grow into defined shapes. In this report, MCF-7 cells from human breast cancer cell lines are cultured by utilizing the new technique and the effects on their stiffness values are investigated using an atomic force microscope.

II. Methods and Material

A. Experimental assembly

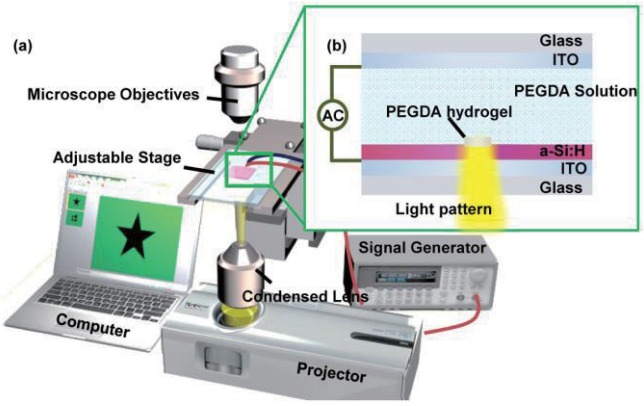

Fig. 1 The experimental system: (a) The components of the system. (b) The two dimensional structure of the assembly for light-addressable PEGDA hydrogel polymerization.

Fig. 1 shows the assembly for realizing digitally light-addressable modification of PEGDA hydrogel film. The a-Si:H substrate is used as a photo-switchable electrode —see Fig. 1(b). In the figure, (1) is the top glass (3 cm × 3 cm) coated with a 120 nm indium tin oxide (ITO) film connected to an AC signal, (2) is the microfluidic chamber with a height of 60μm into which the polymer is injected, and (3) is the bottom substrate (3 cm × 3 cm) with a 1 μm photoconductive film of a-Si:H deposited onto an ITO glass substrate by using a plasma the enhanced chemical vapor deposition (PECVD) process [10]. The a-Si:H substrate contacts the solution and obtains power from an ITO electrode placed beneath it. The assembled chip is placed on a translational platform which can be programmed to move in three dimensions—see Fig. 1 (a). The AC electrical signal generated from a signal generator (Agilent 33522A, U.S.A.) is applied between the top and bottom ITO electrodes. Next, a series of digital light patterns, generated by a commercial computer software (Microsoft PowerPoint or Adobe Flash) are projected from a LCD projector (VPL-F400X, Sony, Japan) onto the a-Si:H substrate through a condenser lens (Nikon 50X/0.55). Being a photoconductive material, the conductivity of the illuminated a-Si:H can increase by several orders (from 10^{-11} S/m to 10^{-5} S/m) under irradiation. Thus, the programmable light patterns projected onto a-Si:H substrate can indeed work as photo-switchable and photo-confined electrodes [11].

B. Material

Poly-(ethylene) glycol diacrylate (PEGDA) is a commonly used biomaterial in view of its good biocompatibility and high tunability. PEGDA can be photo-crosslinked to form a three-dimensional network with good oxygen permeability. Further, the mechanical properties of the cross-linked hydrogel can be adjusted via its molecular weight and the concentration of the polymer. The elastic modulus increases with increasing polymer concentration or decreasing polymer molecular weight. The PEG chains in PEGDA molecules are neutral, highly mobile, and heavily hydrated by an aqueous solution. They also have a large exclusion volume, which makes the crosslinked hydrogel passive in terms of protein adsorption [12], [13]. In our technique, PEGDA hydrogel with arbitrarily defined patterns are polymerized on a-Si:H substrate under both light irradiation and electric field. The resistance of PEGDA to protein adsorption enables it to act as blank platform so as to inhibit cells adhesion and alter cells to grow in a pattern [14].

The PEGDA prepolymer solution used in this technique is easily obtained by dissolving pure PEGDA (Mw=10 KD，Mn=575; Sigma Aldrich) in deionized (DI) water at a ratio of 1:4 v/v, and stirring for about 10 minutes until the PEGDA is dissolved fully. The prepared solution has a conductivity of 1.5×10^{-3} S/m as measured by a Cond 3110.

C. Cell culture

Breast cancer cells from the MCF-7 cell line (obtained from Shenyang Pharmaceutical University, China) [15] are cultured in a CO2 constant-temperature incubator (Model 371, Thermo Scientific). A Roswell Park Memorial Institute RPMI-1640 culture medium containing 10% fetal bovine serum and 1% penicillin-streptomycin is used. The MCF-7 cells are detached from the 25 cm^2 flask when cells cover 80% space, using trypsin-EDTA solution. Next, the cells are dispersed using a cell culture medium to form a cell suspension solution. The cells are seeded onto the a-Si:H substrate and a moderate amount of culture medium is added to enable the substrate to be submerged. All cell manipulations are performed in a biological safety cabinet (Model 1384, Thermo Scientific). Finally, the cells are cultured in a constant-temperature CO2 incubator.

D. Measurement of mechanics

Although Atomic Force Microscopy (AFM) is a powerful and versatile tool capable of characterizing surface topography, there is also a strong interest in applying AFM to probe the mechanical properties of biological samples [16], [17]. AFM can work in a liquid environment to measure in situ the mechanical properties of living cells.

In our technique, AFM imaging of the hydrogel pattern is performed on a Nanoscope VI Dimension 3100 AFM (Veeco) using a tapping mode involving TESP tips (Veeco) with a triangular cantilever design.

The AFM system used for mechanical measurement is a Bioscope Catalyst Atomic Force Microscope (Bruker AXS, USA) equipped with a scanner with the maximum XY scan range equal to 150 μm × 150 μm and the maximum Z scan range equal to 21 μm. The AFM probe is a pyramid-shaped MLCT tip (Bruker AXS, USA) made of non-conductive silicon nitride, backside gold-coated, with spring constant equal to 10 pN/nm. The probe is localized onto the cell surface with the assistance of a charge-coupled device (CCD) camera, which avoids positioning probe on cell nuclei because they are much stiffer than the cytoskeleton [6]. The measurement of mechanical properties is performed at the fluidic contact mode. The procedure involves setting the trig

threshold of the cantilever as 50 nm so as to ensure that the force exerted on the cell is about 500 pN and obtain 16 indention force curves for each cell. Next the elastic modulus of the cell is calculated using the Hertz model:

$$F = \frac{3}{4} \times \frac{E \tan(\alpha)\delta^2}{(1-\nu^2)} \quad (1)$$

In the model, v is the Poisson ratio of the sample, which is frequently assumed to be 0.5 for a cell, δ is the indentation depth, α is the tip half angle in radians (estimated by the probe manufacturer), E is the Young's modulus, and F is the applied force.

III. Images and Data Analysis

The patterning process of the PEGDA hydrogel was monitored in real-time by a charged coupled device (DH-SV1411FC, DaHeng Image, China), a microscope (Zoom 160, OPTEM, USA), and a computer equipped with an image acquisition card. The optical cell images and the fluorescent images were obtained using a microscope (Ti-U Nikon, Japan). Optical pictures of the PEGDA hydrogel patterns were obtained using a digital microscope (KH-7700 Hirox, Japan). The structures were characterized using an atomic force microscope (AFM Dimension 3100 Veeco, USA). The sizes and spreading areas of cells were measured by a NIS-Element software (Nikon, Japan). Finally, the data collected were statistically analyzed using MATLAB programs.

IV. Results and Discussion

A. Hydrogel film modified digitally on a-Si:H substrate

As described earlier, when an a-Si:H substrate is irradiated, the electrical resistance of the illuminated areas will decrease by several orders due to the generation of electro-holes pairs. This process enables the illuminated areas to function as virtual electrodes defined by the programmable light patterns. In the solution atop these virtual electrodes, a non-uniform electric field exists when an external signal is applied. The electric power and the electro-holes pairs thus activated can induce the polymerization of PEGDA molecules in situ. The polymerized hydrogel films are deposited onto the virtual electrodes. Additionally, the pattern of the polymerized hydrogel is defined by the light patterns. Based on this mechanism, cell-passive hydrogel films with arbitrary geometric shapes can be modified at any position on a-Si:H substrate by automatically controlling the generation of light patterns and displacing the three-dimensional adjustable platform.

The polymerized hydrogel patterns on a-Si:H substrate are shown in Fig. 2, where (a) shows the three-dimensional axes of the AFM imaging results shown in (c), (d), and (e). Axis z represents the height of the polymerized hydrogel. From the topography of the hydrogel imaging results, it can be seen that the hydrogel height is smaller than 80 nm. Fig. 2 (b) shows the polymerized hydrogel with different channel gaps. Note that the width of the naked a-Si:H ranges from 2.7–10.8 μm.

Owing to their resistance to protein adsorption, MCF-7 cells cannot spread on a PEGDA hydrogel film. In consequence, the technique of automatically fabricating PEGDA hydrogel has potential of becoming a versatile tool for controlling cells to grow along confined channels. This is useful in studying tumor migration processes and in investigating the relationship between cell shape and cell mechanics. Fig. 3 shows MCF-7 cells growing along a confined a-Si:H channel (width equals 10 μm) and spreading along it. Fig. 3 (f) shows a cell staining result obtained after the cell has been seeded for 72 hours by using calcein-AM/propidium iodide double fluorescence. Results indicate that the cells were able to grow healthily on a-Si:H substrate with PEGDA hydrogel film.

The technique based on light-addressable hydrogel modification on a-Si:H substrate can also be used to study migration processes in tumor cells. As shown in Fig. 4, MCF-7 cells are seeded on two rectangular spots separated by several vertical channels of width 7 μm. After spreading over the spots, the cells migrate to another spot across the channel. Figures 7 (a)–(e) display the migration process over a period of 43 hours. It is clear that, during migration, the cells protrude their lamellipodiums to detect the channel direction first and then regulate cytoskeleton deformation so as to adapt to the channel size. This process changes the cell's mechanics.

It is well known that cell shape has a significant impact on cell mechanics. Clearly, the technique reported in this paper is powerful in regulating the shape of a single cell. As mentioned before, the width of the channel can be customized conveniently by customizing the patterns of the projected light patterns. The ability to regulate cell shape without changing the substrate stiffness is important for studying the independent effect of cell shape on cell mechanics.

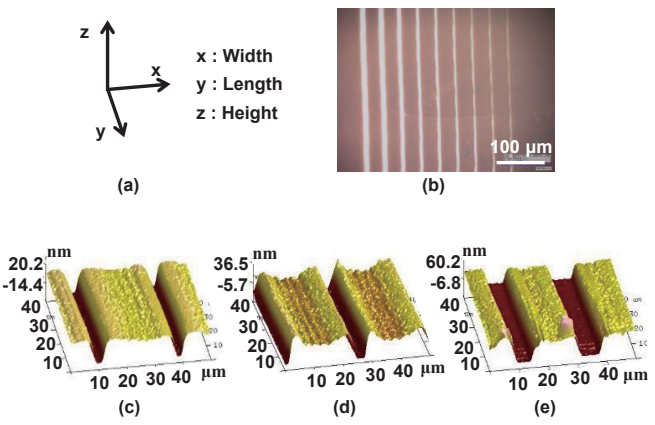

Fig. 2. Sample imaging results associated with a polymerized PEGDA hydrogel film. (a) Three-dimensional axes of the AFM imaging results shown in (c), (d), (e). (b) The optical figure of the hydrogel film with different gaps ranging from 2.7–10.8 μm. In this latter figure, the dark parts represent the polymerized hydrogel and the white channels are the naked a-Si:H substrate. (c), (d), and (e) respectively display the topography of the polymerized hydrogel film with different gaps.

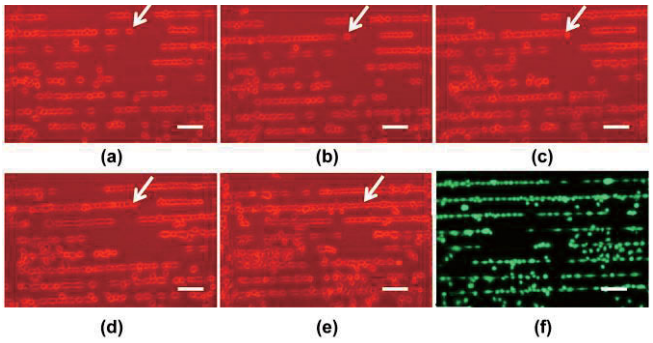

Fig. 3. Cell spreading processes observed in predefined channels. (a)–(e) show the growth status of the cells after they have been seeded on a-Si:H substrate for 28, 34, 46, 54 and 72 hours, respectively. The figures show cells spreading on an a-Si:H substrate along the directions of channels. (e) is a cell staining result obtained by using calcein AM/propidium iodide. The last figure shows that cells have high viability even after having grown for 72 h on a-Si:H substrate. Scale bar: 100 μm.

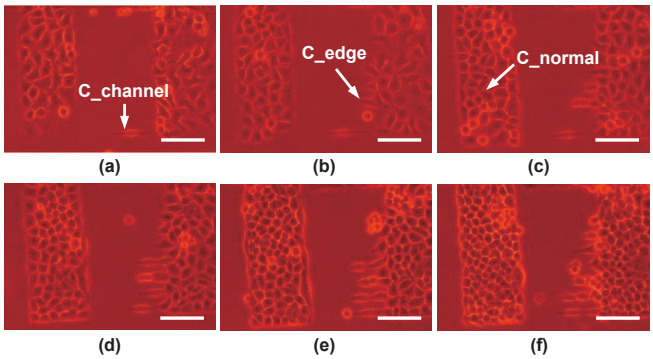

Fig. 4. Cell migration from one spot to another along predefined channels. (a)–(f) show the status of cells after they have migrated for 0, 7, 19, 25, 31 , and 43 hours respectively. Scale bar: 100 μm.

B. Mechanical properties

Cells growing on channels with different widths regulate their shapes to adapt the limited adhesion areas. This process is regulated by the deformation of the cytoskeleton. Further, cytoskeleton plays an essential role in determining cell mechanical properties. Cytoskeletal deformation is also important in tumor cell migration. To investigate the interplay among these physiological issues, we measured the stiffness values of MCF-7 cells growing under different conditions by means of an AFM. As shown in Fig. 4, there are three kind of cells: C_normal cells that spread normally which seems quadrate, C_edge cells that grow along the edge of a naked a-Si:H area, and C_channel cells that migrate along the channels defined by the PEGDA hydrogel (these cells have a spindly shape). The typical force curves of these three kinds of cells are shown in Fig. 5 with the x axis representing the indention depth of a cell under the exerted force along the y axis. We used Gaussian fitting to calculate the elastic modulus. The final results are shown in Fig. 6. Note that the average modulus of cell growth normally is 0.3±0.2 kPa, cells growing on the interface of hydrogel have stiffness values of 0.4±0.3 kPa, while cells spreading on a confined channel have stiffness values of 2.0±1.0 kPa. The results indicate that cells growing in a channel are stiffer than those growing at the edge as well as cells spreading normally. Cells growing on the edge are a little stiffer than normally growing cells, but the difference is not immediately obvious. It is also worth noting that the measured cells all have similar spreading areas (420–440 μm^2). This means that we can ignore the effect of spread area on cell mechanics. This result also suggests that cells exhibiting a spindly shape are stiffer. However, cells generating cytoskeleton deformation adapt to the external environment and this deformation can influences their shapes and mechanical properties. Comparing these three kinds of cells, it seems that both cell shape and their surrounding environment have observable effects on their stiffness values.

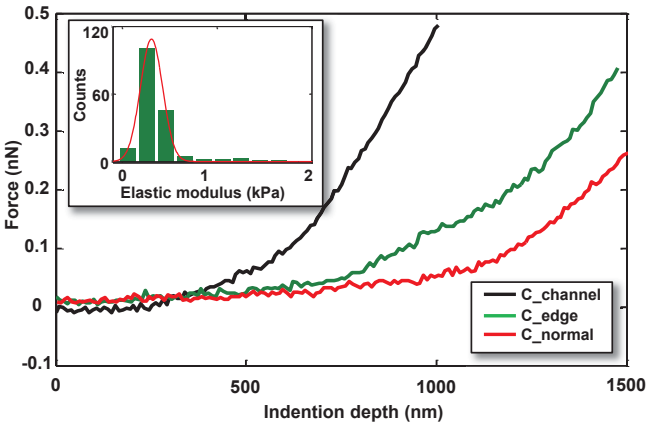

Fig. 5. Typical force curves of cells. As marked in Fig. 4, "C_channel" represents cells growing on channels of confined size, "C_edge" represents cells growing on the edge of a naked a-Si:H area, while "C_normal" means cells growing normally on a-Si:H substrate. The inserted figure shows the Gaussian fitting results used to calculate elastic modulus of cells.

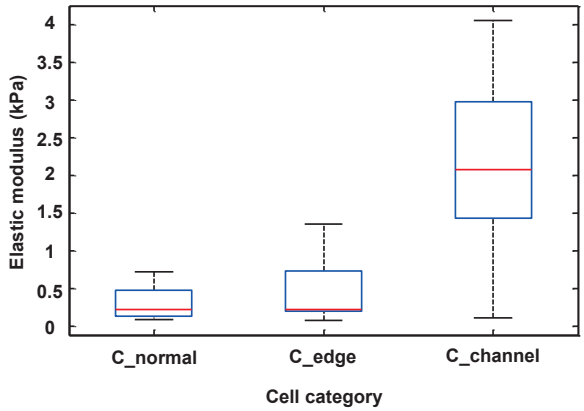

Fig. 6. Measured elastic modulus values for the three kinds of cells.

V. Conclusion

This paper has reported on an automatic hydrogel modification method capable of patterning cells into desired shapes. In comparison to existing methods, the proposed technique is easier, faster and cheaper. Furthermore, it has the ability to change the cell patterns dynamically by projecting a light, without the need for a physical mask. The effects on cell mechanical property due to cell interaction or shapes of cell can be easily investigated by the proposed method. Utilizing this technique, a cell-passive hydrogel can be modified on a-Si:H substrate and used to control the cell shape and status. In our work, the method was tested in the context of tumor cell migration. AFM was used to

characterize the elastic modulus of the tumor cells in different shape and different migration process. The results showed that the cells underwent cytoskeletal deformation so as to adapt to the external environment and that this deformation had affected their mechanical properties. This conclusion is consistent with previous findings that both cell external environment and spreading area are capable of regulating cell stiffness [6]. The technique proposed in this paper can be used to control a single cell's shape by confining their growth in predefined patterns. This is useful in studying the independent effect of cell shape on cell stiffness. For example, cells growing on narrow channels with small adhesion area stretch their bodies as much as possible along the channel direction. As a result, the cell body gets attached to the area and generates large traction forces to balance itself, while a larger traction force can increase cell stiffness [5, 7]. In short, the proposed method provides a versatile tool for cell patterning, which, in turn, makes it applied in a straightforward manner for cell mechanical study.

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