Surface Patterning of Self-healing P(MMA/nBA) Copolymer for Dynamic Control Cell Behaviors

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Abstract Cell behaviors are regulated by a dynamic and complex environment characterized by biophysical, mechanical and biochemical properties. However, most works regulate cell behaviors under static conditions or by external factors. To control cell adhesion and proliferation with a dynamic and mechanical environment, we pattern the surface on self-healing copolymer P(MMA/nBA). The copolymer P(MMA/nBA) with the composition of 48/52 (MMA/nBA) recovers nearly 100% of its original tensile strains after 86 h of recovery from deformation. The physical patterns on P(MMA/nBA) film are obtained over large areas and the size of the hole and the width of connecting bar are in line with the copper grid specifications. The patterned surface tends to be flat after 12 h with almost 75%–80% recovery. Compared with cell incubation on polystyrene flat and patterned surface of P(MMA/nBA), the number and morphology of cells are well manipulated on the patterned surface of self-healing P(MMA/nBA) film. This approach provides a convenient method for dynamically regulating the cell behaviors on the surface of self-healing materials without chemical or biological modifications.

Keywords Surface pattern; Self-healing; Cell behavior; Dynamic

INTRODUCTION

Cell behavior is regulated by a dynamic and complex environment characterized by biophysical, mechanical and biochemical properties.\textsuperscript{[1–6]} Many approaches to regulate cell-matrix interactions have been developed based on surface architecture.\textsuperscript{[2,3,5,6]} The surface architecture provides spatially and temporally resolved stimuli to cells. Spatial control is commonly obtained by surface pattern and temporal manipulation is accessed through the application of responsive switchable surface chemistry.\textsuperscript{[7]} Generally, responsive switchable surface regulates cell behavior in a short period. However, in the living system, cells sense and respond to the dynamic surface usually for a long time. In addition, the mechanical properties of substrate can itself modulate cell behavior.\textsuperscript{[5,6]} For example, it is at least several weeks for endothelial cell to form pulse blood vessel through cell migration, proliferation, polarity and differentiation.\textsuperscript{[7,8]} Recently, the progress on dynamic control of cell behavior with patterned surface has been made and the strategy for tissue regeneration and vascularization has proven to be efficient.\textsuperscript{[9–12]} Therefore, construction of surface architecture to provide dynamic and mechanic environment for cell behavior modulation is highly desired.\textsuperscript{[13–16]}

The surface of self-healing polymer is an ideal matrix for dynamic regulation of cell function because self-healing polymers possess notable ability to recover from damage or deform with external forces over a long period.\textsuperscript{[17–22]} Self-healing polymers can be prepared by embedding reactive encapsulated fluids,\textsuperscript{[23,24]} incorporating covalent or supramolecular dynamic bonds,\textsuperscript{[19,25–29]} dispersing nanomaterials,\textsuperscript{[30]} introducing phase-separated morphologies and incorporating living organisms.\textsuperscript{[24,31,32]} Recently, the commodity copolymers, poly(methyl methacrylate)/n-butyl acrylate [P(MMA/nBA)] and their derivatives, are reported to self-heal after physical or chemical damage.\textsuperscript{[33]} Self-repair occurs in a narrow 45/55 to 50/50 MMA/nBA compositional range for polymers produced using atom transfer radical polymerization (ATRP). P(MMA/nBA) synthesized with free radical polymerization exhibits similar behavior. And within this range, the copolymer topologies are preferentially alternating with a random component, which is due to favorable van der Waals forces to form key-and-lock interchain junctions. Compared with the strategy of supramolecular or covalent rebonding or encapsulation.
sulated reactants, the use of van der Waals forces eliminates chemical and physical alterations and is capable of multiple recovery on mechanical damage. In addition, the recovery induces the architecture reconstruction and mechanical variation on the copolymer surface dynamically, which offers defined control over the cell behavior at the interface.\textsuperscript{36-37} Thus, self-healing copolymer P(MMA/nBA) is a good candidate to provide dynamic and mechanical environment for cell metabolism.

Here, we present a novel strategy to control cell adhesion and proliferation with a dynamic and mechanic environment. Firstly, we synthesized copolymer P(MMA/nBA) with free radical polymerization. The copolymer P(MMA/nBA) with the composition of 48/52 (MMA/nBA) can recover nearly 100% of its original tensile strains after 86 h of recovery from deformation. Then, the physical patterns on P(MMA/nBA) film are obtained over large areas and the size of the hole and the width of connecting bar are in line with the copper grid specifications. The patterned surface tends to be flat after 12 h with almost 75%–80% recovery. Finally, cell incubation is performed on the patterned surface. Compared with cell incubation on polystyrene flat and patterned surface of P(MMA/nBA) film without self-healing capability, the number and morphology of cells are well manipulated on the patterned surface of self-healing P(MMA/nBA) film. Therefore, this work demonstrates a facile method for controlling cell behaviors spatially and temporally with patterned surface of self-healing polymer.

EXPERIMENTAL

Materials

Methyl methacrylate (MMA) was purchased from Tianjin Yongsheng Fine Chemical Co., Ltd. n-Butyl acrylate (nBA) was purchased from Xiqiao Chemical Co., Ltd. 2,2-Azobisis(2-methylpropionitrile) (AIBN) (Sigma-Aldrich) was recrystallized before use. Toluene, hexane, tetrahydrofuran (THF), and chloroform were purchased from Beijing Chemical Factory. Fetal bovine serum (FBS), 0.25% trypsin-EDTA, penicillin, streptomycin, and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from GibCO. 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) was purchased from Beyotime. Phosphate-buffered saline (PBS, 0.01 mol/L phosphate buffer, pH 7.4) was prepared freshly. The other solvents and reagents were of analytical grade and utilized without further purification. Milli-Q water (18.25 MΩ·cm) was used in all experiments.

Synthesis of P(MMA/nBA) Copolymer

The synthetic route to the copolymer refers to the literature.\textsuperscript{33} Specifically, a total of 0.168 mol of MMA/nBA monomers with 45/55 ratios was dissolved in 20 mL of toluene. After 5 min, 10 mg of AIBN initiator was added to the reaction vessel. The reaction was carried out at 75 °C for 8 h to obtain a P(MMA/nBA) copolymer with 48/52 ratios. The 60/40 and 47/53 compositions of MMA/nBA were employed to copolymerize P(MMA/nBA) copolymers with 63/37 and 49/51 actual ratios, respectively. The obtained copolymer was dissolved in 40 mL of toluene and precipitated in hexane, and the precipitated copolymer was placed in a vacuum oven to be dried until the solvent was evaporated. The actual monomer ratios (\( F \)) in these copolymers were determined using \(^1\)H-NMR.

Self-healing of P(MMA/nBA) Films

Dried P(MMA/nBA) copolymer (11 g) was dissolved in 70 mL of tetrahydrofuran to prepare a solution with a concentration of 15%. Then, the solution was dropped into a clean and flat glass plate in the ventilated kitchen for 48 h. After the solvent was volatilized, the glass plate was immersed in deionized water for 2 h, and a copolymer film having a thickness of about 250 μm was obtained. The P(MMA/nBA) membrane was cut to a size of 2 cm × 2 cm, soaked in deionized water with ultrasonic treatment for 15 min at room temperature, and the cleaned membrane was placed in a vacuum oven at 37 °C for 24 h. In order to determine the self-healing properties of P(MMA/nBA) copolymers, films were cut into 1.0 cm × 3.0 cm × 0.03 cm splines, and the stainless steel single-sided blade was drawn from the middle along the length of the spline to create a crack about 0.8 cm wide. The cuts were spliced together and the films were allowed to heal for 12 or 86 h at 37 °C. Finally, tensile stress-strain measurements were performed using an Instron 1121 testing machine. The same stress-strain measurements were also performed before damage. All measurements were conducted at a strain rate of 1 cm/min using a 1 kN load cell.

Physical Pattern and Surface Architecture Characterization

200 Mesh and 400 mesh round-hole copper grinds and 200 mesh square-hole copper grinds were applied to cover on the surface of film, and a small amount of deionized water was added to make them together with the film. Then, they were sandwiched between the two layers of polyester film, and placed in the mold of Flat vulcanizing machine repeatedly by punching 3 times with an impact pressure of 16 MPa. After 5 min, the copper grind was removed, and the patterned copolymer film was obtained. The probe profiler captures the contours of the physical patterned surface and the surface morphologies were observed with field emission scanning electron microscopy (FESEM) and polarized optical microscopy (Zeiss Axio Imager A2m, Carl Zeiss, Germany) equipped with a video CCD camera.

The surface topography and recovery were measured by Surface S stylus Profilometer (KLA Tencor P-7). The scanning length was about 600 μm, the scan speed was 100 μm/s, the sampling rate was 200 Hz, and the applied mass was 2 mg. The vertical measurement range of profilometer was smaller than 1 mm. The depth of groove on patterned surface was in situ measured based on the profile data of patterned surface for 12 h.

Cytotoxicity of P(MMA/nBA) Films

The in vitro cytotoxicity of P(MMA/nBA) films to L929 Murine Fibroblast cells was evaluated by using a resazurin microtiter assay (REMA).\textsuperscript{38} All P(MMA/nBA) films were cut into 1 cm × 1.5 cm pieces, and immersed in pH 7.4 phosphate buffer solution (PBS) for 10 h to reach swelling equilibrium.\textsuperscript{39} L929 cells were cultured in high glucose Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS) and 1% antibiotics (100 mg/mL penicillin and 100 mg/mL streptomycin) for 24–48 h. The cells were removed from culture dish by addition of 0.25% trypsin-EDTA and centrifuged at 1500 r/min for 5 min with 5 mL of culture medium solution. The supernatant was removed and the cell suspensions were diluted to

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1 × 10^2 cells/mL with culture fluid (containing 10% FBS and 1% antibiotics). Then, 1 mL of cell suspensions were added to each well of a 24-well plate containing the P(MMA/nBA) films and the plate was incubated at 37 °C, 5% CO₂. The control experiment without the samples was conducted. After 24 or 48 h of culture, 100 μL of 5 mg/mL resazurin solution was added into the wells and another 4 h of incubation, then the resazurin-containing medium (200 μL) was transferred into a 96-well plate and the fluorescence intensity value at 530/590 nm excitation/emission wavelength was measured on a microplate reader (TECAN GENIOS, Austria). The results were represented as means compared with the control experiment.

**Cell Culture and Counting**

L929 murine fibroblast cells were used for investigation cell behavior on self-healing copolymer. Prior to cell seeding, patterned P(MMA/nBA) films were placed into 24-well plates with a small amount of autoclaved deionized water and then sterilized under ultraviolet 30 min. L929 cells were digested with 0.25% trypsin-EDTA, then centrifuged for 5 min at 1500 r/min in 5 mL of medium, and the bottom cells were collected and re-suspended in cell culture medium, and seeded on the membrane at a cell density of 5 × 10^4 cells/mL. 1 mL of the cell suspension was added to each well and cultured in a standard cell culture environment. For comparison, cell culture in the polystyrene plate was performed at the same conditions.

At 3, 6, and 12 h of cell culture, the cells were photographed with an inverted microscope, and then cell counts were performed by DAPI staining.[40] The cells were washed twice with sterile PBS and fixed in 4% paraformaldehyde for 30 min at 4 °C, followed by DAPI staining at room temperature for 10 min in the dark, and then washed with PBS three times. Finally, each sample was randomly captured with 5 fluorescence micrographs by using a confocal laser scanning microscope (CLSM) (LSM700-Zeiss, Germany). All samples were visualized using the same acquisition settings with a 10 × objective and the number of cells on five photographs was counted by Image J software, and the average value was taken as the cell number value of the sample of this group.

**Actin Cytoskeleton Staining**

After culturing for 12 h, cells were fixed with 4% paraformaldehyde for 20 min at 4 °C and gently rinsed with PBS twice. Later, the cells were permeated with 0.1% Triton X-100 for 5 min. The cells were again washed twice with PBS and stained with FITC-phalloidin, incubated for 30 min at room temperature in the dark and washed three times with PBS. Finally, DAPI staining solution was added at room temperature for 10 min in the dark, and the cells were washed with PBS twice to stain cell nuclei. The fluorescence micrographs were obtained with a confocal laser scanning microscope (CLSM) (LSM700-Zeiss, Germany).

**RESULTS AND DISCUSSION**

Cell behavior is modulated by a dynamic and complex environment characterized by biophysical, mechanical and biochemical properties.[1−3] To control cell adhesion and proliferation with a dynamic and mechanic environment, we physically pattern the surface on self-healing P(MMA/nBA) copolymer (Fig. 1). The patterned surface becomes flat due to the chain recovery of P(MMA/nBA) copolymer (Figs. 1A and 1B). The surface reconstruction on the patterned surface provides dynamic and mechanic environment for regulation of cell number and morphologies (Fig. 1C).

**Synthesis of P(MMA/nBA) Copolymer and Self-healing Properties**

P(MMA/nBA) copolymer is synthesized through free radical polymerization with the molar feed ratios of 45/55 and 60/40, respectively. The 1H-NMR spectra of P(MMA/nBA) copolymer are shown in Fig. 2. The typical signals at 3.5−3.6 ppm (O―CH₂), 1.4−1.6 ppm (―CH₂―), 3.7−4.2 ppm (O―CH₂―CH₂―), and 0.7−1.0 ppm (―CH₂―CH₃) confirm the successful synthesis.[41] The monomer ratios (F) of copolymers are determined by the ratio of signal area at 3.5−3.6 ppm to that at 4.0−4.1 ppm in 1H-NMR spectra.[3] The monomer ratio of MMA to nBA is 48/52 at feeding ratio of 45/55, while 63/37 at feeding ratio of 60/40.

The self-healing properties of two copolymers are evaluated. The film of copolymers is cut into 1.0 cm × 3.0 cm ×
0.03 cm strips, and the ~0.8 cm wide crack in the middle of strips along the length direction is created with a stainless steel single-sided blade. Then the cracks are spliced together for 12 or 86 h at 37 °C. Finally, tensile stress-strain measurements are performed on a universal testing machine (Instron 1121, UK) with a crosshead speed of 1 cm/min. The stress-strain curves before damage and 12 or 86 h after repair for P(MMA/nBA) copolymer (48/52) are shown in Fig. 3. At the conditions of 37 °C and relative humidity of 50%, the P(MMA/nBA) copolymer (48/52) recovers 78% to nearly 100% of its original tensile strains, respectively (Figs. 3A and 3B). The insets of damaged and repaired P(MMA/nBA) copolymers confirm self-healing properties of the film. In contrast, self-repair does not take place for P(MMA/nBA) copolymer (63/37) even days after damage (Fig. S1, in the electronic supplementary information, ESI). It seems that irreversible chain dislocations and insufficient interchain van der Waals (vdW) forces suppress chain recovery and self-healing behavior. This results agree with the works by Urban et al., who reported that self-repair occurs without external intervention only within narrow MMA/nBA compositional ranges from 45/55 to 50/50.\textsuperscript{[33]}

**Surface Pattern and Reconstruction on Patterned P(MMA/nBA) Films**

Self-healing properties render the surface reconstruction available. Patterned P(MMA/nBA) film is generated with TEM copper grid as the mold. The copper grid is adhered to the P(MMA/nBA) film, and sandwiched between two layers of polyester film. Then, the sandwiched film is placed in the mold of flat vulcanizing machine and punched with an impact pressure of 16 MPa. After 5 min, the copper grid is removed, and the patterned surface with different sizes and shapes is obtained. The patterned surface is observed with field emission scanning electron microscopy (FESEM) and polarized optical microscopy (POM). Both the SEM and POM images reveal that the physical patterns are uniform over large areas (Fig. 4). The size of the hole and the width of connecting bar are in good agreement with the copper grid specifications, demonstrating excellent replication of the shape of the mold.\textsuperscript{[42]}

Surface reconstruction on patterned surface is measured by Surface Stylus Profilers (KLA Tencor P-7) with scanning length of 600 μm and speed of 100 μm/s. The vertical measure range of profilometer is smaller than 1 mm. Fig. 5(A) exhibits the optical image of patterned P(MMA/nBA) (48/52) film (Fig. 5Aa), and height-length curves before and after self-repair (Fig. 5Ab versus Fig. 5Ac). At ambient conditions, 9 μm-deep groove in the patterned P(MMA/nBA) (48/52) film can be recovered to flat after 12 h. In contrast, the height of peaks at the patterned P(MMA/nBA) (63/37) film changes slightly (~5%) after 12 h at the ambient conditions (Fig. 5B). Thus, surface reconstruction depends on self-healing properties of P(MMA/nBA) film resulted from chain configuration. It is reported the chain flexibility is the smallest for self-healing compositions when chains are in the equilibrium state. The helix-like conformations are formed for self-healing copolymer with the strong vdW inter-chain forces to create a viscoelastic response that energetically favors self-recovery upon chain separation due to key-and-lock associations of neighboring chains. When chains are separated as a result of an external force being removed, copolymer chains readily return to their initial conformations by restoring helix-like chain
conformations in a spring-like manner and reforming key-and lock junctions.\cite{33}

The recovery rate can be controlled by copolymer composition and patterning scale (Table S1, in ESI). The copolymer composition and patterning scale create the patterned surface with varied depth of groove. Thus, the spatial and temporal cell performance can be dynamically controlled on the patterned surface.

Cell Incubation on Patterned Surface of P(MMA/nBA)

The cytotoxicity of P(MMA/nBA) films to L929 murine fibroblast cells is evaluated by using a resazurin microtiter assay. P(MMA/nBA) films are put in 24-well plate for cell incubation, and the polystyrene plate without P(MMA/nBA) films is used for control experiment. As shown in Fig. 6(a), both P(MMA/nBA) films with self-healing capability [P(MMA/nBA) films (+)] and without [P(MMA/nBA) films (−)] have negligible cytotoxicity.

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after incubation with L929 cells for 48 h (> 90% viability), demonstrating high biocompatibility of P(MMA/nBA) films for cell manipulation.\textsuperscript{[43]}

L929 murine fibroblast cells are selected to examine the cell growth behavior on polystyrene plate, P(MMA/nBA) films with self-healing [(P(MMA/nBA) film (+)) and without self-healing [(P(MMA/nBA) film (−))], respectively. Due to the tendency of cells to adhere on the groove of the patterned surface, the number of L929 cells on polystyrene flat surface is larger than that on patterned surfaces of P(MMA/nBA) film. Moreover, cells on polystyrene flat surface proliferate continuously from 3 h to 12 h, while the number of cells remains steady on patterned [P(MMA/nBA) film (−)]. In contrast, cells on patterned [P(MMA/nBA) film (+)] spread and proliferate continuously from 3 h to 12 h with a slightly slower speed within a smaller space. This result indicates that patterned surface with self-healing properties can tune the cell spreading and proliferation. DAPI staining images were obtained by confocal laser scanning microscopy to record the number variety of cells on the three substrates over time (Fig. S3, in ESI). The cells after 12 h incubation are stained with FITC-phalloidin and DAPI successively, and then observed with a confocal laser scanning microscope (Fig. 7). Cells seeded on polystyrene plate adopt a well-spread, lamellar morphology with large cell area (Fig. 7a). For patterned [P(MMA/nBA) film (+)] with self-healing properties, the grooves become flat after 12 h. Thus, the surface nano-architecture provides spatially and temporally resolved response of the material, and offers defined control over the behavior of cells at solid-liquid interface. The groove-flat transition induces cell adhesion and proliferation and cells adopt spindly morphology with thin, elongated processes that are terminated in branched protrusions (Fig. 7b). It seems that vinculin-rich focal adhesions are sequestered to the tips of these protrusions. In contrast, because nano-architecture remains stable on the patterned [P(MMA/nBA) film (−)] without self-

![Graph showing cell viability and proliferation of P(MMA/nBA) copolymer films.](https://doi.org/10.1007/s10118-020-2382-1)
healing properties, cell adhesion is limited in the groove area (Fig. 7c). Thus, the groove structure induces focal adhesion of cells with low cell adhesion area.13

CONCLUSIONS

We synthesized copolymers P(MMA/nBA) with free radical polymerization. The copolymer P(MMA/nBA) with the composition of 48/52 (MMA/nBA) exhibited self-healing properties and recovered nearly 100% of its original tensile strains after 86 h of recovery from damage. Then, the P(MMA/nBA) film was used as a substrate for physical pattern with TEM copper grid as the mold. The physical patterns were uniform over large areas and the size of the hole and the width of connecting bar were in good agreement with the copper grid specifications. The patterned surface tended to be flat naturally after 12 h with almost 75%–80% recovery. Compared with cell incubation on polystyrene flat and patterned surface of P(MMA/nBA) film without self-healing capability, the number and morphology of cells could be well manipulated on the patterned surface of self-healing P(MMA/nBA) film. Therefore, this work paved a facile way to designing surface micro/nano-architecture of self-healing polymer with physical pattern for well-defined control over the cell behavior with spatial and temporal responses, which cannot be obtained with static systems or conventional stimuli ones.

Electronic Supplementary Information

Electronic supplementary information (ESI) is available free of charge in the online version of this article at https://doi.org/10.1007/s10118-020-2382-1.

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