Novel cell patterning using microheater-controlled thermoresponsive plasma films

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Abstract: A novel approach is reported for cell patterning based on addressable micro heaters and a poly(N-isopropyl acrylamide) (pNIPAM) thermoresponsive coating. This thermoresponsive coating is created by a radio frequency NIPAM plasma and is denoted as plasma polymerized NIPAM (ppNIPAM). Films of ppNIPAM with a good retention of monomer side-chain functionality are produced using low-power continuous plasma deposition. Cell adhesion and cell detachment tests indicate that the surface switches between adhesive and nonadhesive behaviors as a function of temperature. The use of a photolithographically fabricated microheater array allows the ppNIPAM transition to occur spatially under the control of individual heaters. This localized change in the surface adhesive behavior is used to direct site-specific cell attachment. Patterned adhesion of two types of cells has been visualized on the array through fluorescent markers. Applications for diagnostic devices, cell-based sensors, tissue engineering, and cell transfection are envisioned. © 2004 Wiley Periodicals, Inc. J Biomed Mater Res 70A: 159–168, 2004

Key words: thermoresponsive polymer; poly(N-isopropyl acrylamide); cell adhesion; microheater array; cell patterning; biochip

INTRODUCTION

Micropatterning of different types of cells on a surface is a valuable technique for controlling cellular growth, developing high throughput cell-based assays, tissue engineering, and designing bioelectronic devices. Various strategies and devices such as micropatterned protein1 and DNA2 surfaces, micro wells,3 elastomeric stencils,4 microchannels, and micronetworks5,6 have been applied for this purpose. Each method has its advantages and inherent limita-

tions. Micropatterned protein and DNA surfaces are inexpensive to develop and simple to use. However, the different types of cells patterned on protein and DNA microarrays need to have intrinsic differences in their adhesion properties to the substrate. This limits the available combinations of cell types.7 Patterning two cell types with stencils requires no surface modifications. However, it is not applicable in some microsystems, as it requires the physical removal of the elastomeric membrane.8 Microchannels and micronetworks offer great flexibility in simultaneously working with multiple types of biomolecules and cells. However, the geometry of the patterns and the spacing between different cell types are limited by the constraints of the microfluidic devices.8 Therefore, there is still a need for new technologies that allow inexpensive, noninvasive, and automated approaches to pattern multiple types of cells on a surface with little restriction on the cell type combinations.

Several groups have recently explored the use of dynamic stimulus-responsive surface chemistries for
cell patterning to overcome those obstacles. Approaches include thermo-active, electrical-active, and photo-active chemistries. In general, all of these chemistries operate under the same principle: they can be switched from a state that prevents cell attachment to a state that promotes it. The general strategy to pattern two cell populations on dynamic surfaces is to prepare patterned stimulus-responsive chemistries where the unstimulated state is cell nonadhesive and the stimulated state is adhesive. The first cell type is seeded prior to activation of the stimulus-responsive regions that mask the direct attachment of cells. Stimulation directs the first cell type to adhere only to switched-on adhesive areas. Then, an environmental stimulation switches another stimulus-responsive area cell adhesive, directing the second cell type to the newly exposed adhesive regions. As the surface property change is the key driving force in these processes, instead of physical barriers or cell adhesion based on specific receptor chemistries, patterning cells with dynamic surface chemistries requires no particular cell type combination or invasive manipulation. Furthermore, this method places no restrictions on the shape of the patterns. However, most of the work to date has been performed by applying the environmental stimulus to the whole substrate. All the stimulus-responsive regions are activated at the same time and the advantages of the dynamic chemistry is not fully realized.

Several researchers have proposed combining microcontroller arrays with stimulus-responsive chemistries for protein and cell patterning, which allows individual areas on the dynamic surface to be activated independently. Compared with stimulating the whole substrate, the microcontrollers greatly enhance the information content on a surface. Recently, Bunker et al. reported a study utilizing this strategy for patterning proteins. However, very little work has been done using microcontroller arrays to specifically site-activate dynamic surface chemistries for cell patterning.

In this study, we demonstrate the feasibility of patterning one or more types of cells using a microheater array controlled thermoresponsive chemistry. This thermoresponsive chip concept for cell patterning was first introduced earlier. This report presents a complete description of this concept. The thermoresponsive polymer we use is pNIPAM. The polymer shows a lower critical solution temperature (LCST) of 31°C in an aqueous environment. Below the transition temperature, pNIPAM-grafted surfaces are hydrophilic, swollen, and nonfouling. As the temperature increases above the transition temperature, the polymer collapses and the grafted surfaces increase in hydrophobic character. In this manner, pNIPAM can be switched from a state that prevents cell attachment to a state that promotes cell attachment, merely by changing the temperature of the surface. The use of a microheater array underneath the polymer allows the localization of the phase transition and cell adhesion to the microheater-heated regions. The unheated regions at room temperature serve as a cell attachment mask. By sequentially seeding different cell types and turning on different heaters, multiple cell types can be patterned. The combination of microheater arrays with pNIPAM increases the types of cells that can be patterned on the thermoresponsive surface in addition to improved flexibility in the feature geometry.

pNIPAM coatings are synthesized here by vapor-phase plasma deposition. This is a one-step coating process that produces conformal, sterile and pinhole-free pNIPAM coatings. Plasma deposition is also relatively independent of the nature of the substrates. In a previous study, we demonstrated plasma-polymerized NIPAM (ppNIPAM) has an exceptional retention of the monomer side chain structures and its surface wettability changes with temperature. In this report, we compare cell behavior on ppNIPAM films at different temperatures first. Based on the drastic cell adhesion difference at 23° and 37°C on the polymer, a strategy to create cell patterns is presented on a microheater array controlled ppNIPAM coating.

MATERIALS AND METHODS

Reagents and materials

Ninety-seven percent N-isopropyl acrylamide (NIPAM) was purchased from Aldrich (Milwaukee, WI) and used as received. The cell culture supplies were purchased from Gibco Invitrogen Corporation (Carlsbad, CA) and filtered through 0.2 μm filters before use. The cytotoxicity detection kit [lactate dehydrogenase (LDH) Assay Kit] was obtained from Roche Diagnostics Corporation (Indianapolis, IN). The 1,1’-dioctadecyl-3,3,3’-tetramethylindocarbocyanine (Dil)-conjugated acetylated low-density lipoprotein (Dil-Ac-LDL) was purchased from Biomedical Technologies (Stoughton, MA) and the FITC-conjugated mouse monoclonal antibody to α-smooth muscle actin from Sigma (St. Louis, MO). Poly(ethylene terephthalate) (PET) was purchased from Fisher Scientific Company (Houston, TX). Glass coverslips were obtained from VWR scientific (West Chester, PA).

Surface Preparation

ppNIPAM grafted surfaces were prepared by exposing 0.8- by 0.8-mm poly(ethylene terephthalate) (PET) substrates, tissue culture polystyrene dishes, or microheater chips to the vapor phase continuous NIPAM plasma as described previously. In brief, the powered electrode is connected to a 13.56-MHz radio frequency power source
and a manual impedance matching network. The deposition process included an 80 W methane plasma deposition, followed by NIPAM plasma deposition with stepwise decreasing powers from 80 to 1 W with a processing pressure of 140 mTorr. The ppNIPAM-grafted surfaces were rinsed three times with cold deionized water to remove uncrosslinked molecules before use.

Cell culture

Bovine aortic endothelial cells (BAECs) and bovine smooth muscle cells (BASMCs) were received for this research from Dr. Cecilia Giachelli, University of Washington, Seattle, WA. BAECs were cultured in DMEM supplemented with 4.5 g/L glucose, 10% fetal bovine serum (FBS), 0.1 mM MEM nonessential amino acids, 1 mM MEM sodium pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin. Primary BASMCs were maintained in DMEM supplemented with 4.5 g/L glucose, 15% fetal bovine serum, 10 mM sodium pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin. HEK-293 cells were maintained in DMEM supplemented with 4.5 g/L glucose, 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. BASMCs and HEK-293 cells used in the experiments were between passages 3 and 10. BAECs used in the study were between passage 7 and 15. Cell incubation was performed at 37°C in a humidified atmosphere with 5% CO₂. The cells were dissociated from the culture flasks with trypsin/EDTA, washed with DPBS, and resuspended in the respective culture media prior to the adhesion or patterning experiments.

Cell adhesion, proliferation, and detachment

ppNIPAM-coated PET and bare PET samples were fitted into 48-well tissue culture polystyrene (TCPs) plates. One milliliter BAECs, BASMCs, or HEK-293 cells in either serum-free or serum-containing DMEM were seeded on ppNIPAM-coated PET, bare PET, or TCPs at a cell density of 5 x 10⁴/mL. The plates were cultured at either 37°C or room temperature for 3 h (BAECs and BASMCs) or 6 h (HEK-293 cells). Half of the 37°C incubated samples were transferred to room temperature and incubated for another 2 h to study the cell response upon temperature drop. The cell morphology was observed under a phase contrast microscope (Nikon TE200 Inverted Microscope). The number of attached cells was determined using an LDH assay, which is based on the releasing of the cytoplasmic enzyme LDH and measuring its activity in the supernatant. Briefly, the samples with adhered cells were washed twice with DPBS and cells were permeabilized with 500 μL 1% Triton X-100 for 30 min. One hundred microliters of the supernatant were transferred to a 96-well TCPs plate and mixed with the reaction solution for 30 min at room temperature. The absorbance of the solution was measured at 490 nm using a SpectraCount™ plate reader (Packard) and fit to a standard curve to determine the number of cells on each surface. The number of attached cells at each test condition was plotted with 6 replicates.

To test whether cells proliferate normally on the thermoresponsive polymer, ppNIPAM was directly coated on a 48-well TCPs plate. One milliliter of cells was seeded at a cell density of 5 x 10⁴ cells/mL in complete media. Cells cultured on untreated TCPs served as a control. The cells were cultured at 37°C in a humidified atmosphere with 5% CO₂ and observed every day under a phase contrast microscope (Nikon TE200 Inverted Microscope). To test the response of the confluent cell sheet to temperature drop, the wells with confluent cells sheets were rinsed twice with DPBS and replenished with serum-free DMEM at room temperature. The plates were left at room temperature for 2 h and observed.

Cell patterning

Microheater array chips with three independent resistive heaters were fabricated using standard microfabrication techniques. A 30-nm chromium layer was first evaporated onto 170-μm-thick glass coverslips followed by a gold layer of 100 nm thickness. The metal layers were patterned using photolithography. Four hundred nanometers of silicon nitride was sputtered over the surface of the patterned metals. ppNIPAM was deposited on the side opposite to the heaters as the functional layer. A Teflon chamber was built to hold the reaction solution only in contact with the ppNIPAM-coated side so that the heating wires were isolated from the electrolyte [see Fig. 5(B)]. To test the relationship between power input and surface temperature, a layer of thermochromic liquid crystal paint was applied on top of the ppNIPAM on a test chip.

Cell patterning was performed using BAECs and BASMCs from serum-free media. Four hundred microliters BASMCs suspended in serum-free DMEM were added to the chamber at a density of 2 x 10⁵/mL to promote confluent cell adhesion. One heater was turned on with a predetermined power input for 3 h. The chip was rinsed 3 times with DPBS and 400 μL BAECs suspended in serum-free DEME was seeded at the same density with a second heater on for another 3 h. The first heater was kept “on” during the whole process to prevent cell detachment.

Cell staining and fluorescence microscopy

The cell-patterned chip was incubated with DMEM supplemented with 10% FBS and 4 μg/mL Dil-Ac-LDL at 37°C for 4 h to label BAECs. BAECs specifically take up the Dil-Ac-LDL and store it in the endosomal granula. The chip was washed with DPBS and the cells were fixed with 4% paraformaldehyde/0.1% Triton X-100 for 10 min. Then the chip was blocked with 1% BSA in DPBS for 30 min and reacted with a FITC-labeled-rabbit-polyclonal anti-bovine smooth muscle actin antibody at 1:100 dilution for 30 min at room temperature. The chip was observed under a fluorescence microscope (Nikon TE200 Inverted Microscope). The double-stained images were superimposed with software (Meta-morph Image).
RESULTS

Cell adhesion

Cell adhesion on ppNIPAM-coated PET, bare PET, and TCPS were tested at either room temperature (below the LCST) or 37°C (above the LCST). At 37°C, BAECs and BASMCs behaved similarly on the thermoresponsive and control surfaces after 3-h incubation. Large numbers of cells were found to attach, adhere, and spread on ppNIPAM, PET, and TCPS from either serum-free [Fig. 1(A,B)] or low serum-containing media (1% FBS for BAECs and 1.5% for BASMCs). However at room temperature, the effect of the thermoresponsive surfaces on cell behavior was immediately apparent. After 3-h incubation at room temperature, adhesion of a large number of BAECs and BASMCs was again observed on TCPS and PET [Fig. 1(D)], while only a few cells adhered to ppNIPAM at room temperature [Fig. 1(C)]. Furthermore, when BAECs and BASMCs were first incubated at 37°C for 3 h and then cooled to room temperature for another 2 h, spread cells on TCPS and PET had only minor morphological changes [Fig. 2(B,D)]. In contrast, on ppNIPAM surfaces, most of the spread cells rounded up and detached from the surfaces after the temperature drop [Fig. 2(A,C)].

To quantify the number of adhered cells, the surfaces were rinsed with DPBS and an LDH assay was carried out. In serum-free or low-serum-containing media, the numbers of adhered BAECs and BASMCs on TCPS and PET were fairly constant, regardless of the incubation temperature and temperature drop. On ppNIPAM, however, the number of cells was an order of magnitude lower when the incubation was carried out at room temperature than at 37°C. This was true of cells cultured in both serum-free and low-serum-containing media. When 37°C incubated samples were moved to room temperature to incubate for another 2 h, 70% or more of the cells came off from ppNIPAM [Fig. 3 (A–D)]. When cell adhesion was tested with DMEM containing 10% FBS, the number of cells that adhered at room temperature and 37°C was not significantly different on ppNIPAM (data not shown).

In serum-free or low-serum-containing media, very few HEK-293 cells were found to adhere on any of the test surfaces. For this reason, subsequent cell adhesion experiments were carried out in DMEM supplemented with 10% FBS at 37°C and room temperature for 6 h. The number of HEK-293 cells adhered on TCPS and PET was 3 times lower at room temperature than at 37°C from 10% serum media. On ppNIPAM, the difference increased to a factor of 10. When 37°C incubated plates were brought to room temperature

Figure 1. BASMCs adhesion on ppNIPAM and TCPS from serum-free DMEM at both room temperature (bottom) and 37°C (top) observed with phase contrast microscopy. (A) ppNIPAM, 37°C; (B) TCPS, 37°C; (C) ppNIPAM, 23°C; (D) TCPS, 23°C. Bars = 100 μm.
for 2 h, the number of adhered cells on TCPS did not change significantly. In comparison, the number of adhered cells decreased by 68 and 50%, respectively, when ppNIPAM and PET were cooled from 37°C to room temperature [Fig. 3(E)].

Cell proliferation and cell sheet detachment

BAECs, BASMCs, and HEK-293 cells proliferated similarly on TCPS, PET, and ppNIPAM-coated TCPS from complete media at 37°C. All three cell types reached confluence after 72-h incubation on the three surfaces. The morphology of the cells was identical on ppNIPAM and control surfaces at confluence [Fig. 4 (A, B)]. When confluent HEK-293 and BAEC cells were transferred to room temperature, cell sheets were observed to come off immediately from ppNIPAM-coated TCPS [Fig. 4(C)], but not from TCPS or bare PET, even after 12 h at room temperature.

Cell patterning

Based on the drastically different number of adhered cells on ppNIPAM at below and above the LCST, we designed a microheater array chip underneath the polymer coating to spatially control the temperature, phase transition, and cell adhesion on the polymer surface. The microheater array used in this work had three independent heaters. The resistance of each heating element was measured to be around 100 Ω. The chip was connected to a power source and contacted solution only on the side opposite to the metal layers [Fig. 5(B)]. By monitoring the surface with thermochromic liquid crystal paint, we observed that a 50 mW input power was required to heat the ppNIPAM surface to 35°–40°C with 400 μL buffer in the chamber [Fig. 5(A)]. The surface heated to above the transition temperature almost instantaneously when the power was supplied, and the warm region was localized to around the heater with little expansion over time. As the warm area was restricted to directly on top of the heating element, the ppNIPAM transition to the nonfouling state was expected to occur in the same localized region.

The cell patterning was first tested with a single type of cell. After seeding BAECs on the ppNIPAM-coated microheater array chip for 3 h with two heaters "on," the cells were stained with Dil-Ac-LDL and observed under a fluorescence microscope. Ac-LDL is specifically taken up by viable endothelial cells, so it gives information about both the viability and local-
Figure 3. Quantification of BASMC, BAEC, and HEK-293 cell adhesion on ppNIPAM, PET, and TCPS using LDH assay. Adhesion on ppNIPAM, the control PET and TCPS surfaces were quantified using (A) BASMCs from serum-free DMEM, (B) BASMCs from DMEM with 1.5% FBS, (C) BAECs from serum-free DMEM, (D) BAECs from DMEM containing 1% FBS, and (E) HEK-293 cells from DMEM containing 10% FBS. In (A)–(D), the blank, gray and black bars were obtained by culturing cells at 23°C for 3 h, 37°C for 3 h, and 37°C for 3 h followed by 23°C for another 2 h. In (E), the blank, gray, and black bars were obtained by culturing cells at 23°C for 6 h, 37°C for 6 h, and 37°C for 6 h followed by 23°C for another 2 h; 1 mL cells at a density of $5 \times 10^4$ cells/mL were seeded in each case.

Figure 4. Morphology of BAECs on (A) ppNIPAM and (B) TCPS after 72-h incubation from complete media. (C) Confluent cells on ppNIPAM detach from the surface as an intact sheet when the culture plate was brought to room temperature. Bars = 100 μm (A,B), 500 μm (C).
Localized cell adhesion was observed [Fig. 6(A)], associated with the heaters that were turned on. The pattern shape resembled the heating area observed using the thermochromic liquid crystal paint [Fig. 6(B)]. This suggested that localized cell adhesion was driven by the temperature-induced polymer property change.

To pattern two types of cells, 400 μL BAECs and 400 μL BASMCs were sequentially added into the Teflon chamber with different heaters turned on in sequence. To prevent detachment of the first type of adhered cells, the first heater was left on during the whole process. BAECs and BASMCs were then stained with molecules specific to cell types. Localized adhesion of the two types of cells was observed after staining [Fig. 6(C)]. When a control experiment was performed with a microheater chip lacking the ppNIPAM coating, nonspecific adhesion was observed all over the chip regardless of the heated or cold area (data not shown).

**DISCUSSION**

Previously, we reported that plasma polymerized NIPAM (ppNIPAM) coatings maintained monomer side chain functionality and showed a surface wetta-
bility change with changes in temperature. The present study was undertaken to characterize cell behavior on ppNIPAM at different temperatures and explore the applications of ppNIPAM for cell patterning. We show that cell adhesion on ppNIPAM increases by an order of magnitude when cells are incubated at 37°C compared to 23°C. This is expected as the surface hydrogel characteristic has an important effect on protein adsorption and cell adhesion. It has been observed that generally hydrogel surfaces are nonfouling and repel nonspecific protein adsorption. On the other hand, moderately hydrophobic surfaces (contact angle slightly above 65°) promote protein adsorption, possibly due to hydrophobic interactions. Adhesion of many cell types obeys similar rules, possibly because the adhesion is mediated by ECM proteins adsorbed to a surface. Since ppNIPAM is a hydrophilic hydrogel below the LCST and hydrophobic above the LCST, ECM protein deposition and cell adhesion are poor at room temperature, but promoted at 37°C. On the nonthermosensitive surfaces, surface wettability and protein adsorption (paper in preparation) is fairly constant over this temperature range, which results in a relatively constant number of adhered cells at the two test temperatures.

We also observe that at room temperature, the number of adhered BAECs and BASMCs on ppNIPAM increases with the serum content in the media. Hydrogel surfaces have been found to resist protein adsorption most effectively from a single protein solution. The amount of absorbed proteins on such surfaces increases in serum- or plasma-containing solution. This explains the observation that more cells adhere on ppNIPAM from serum-containing media than from serum-free media when the polymer is in the hydrophilic state. Furthermore, surface density of adsorbed adhesive proteins can be well below a monolayer and still support full-scale adhesion of some cell types. We believe that ECM proteins deposited on ppNIPAM from high-serum-containing DMEM at room temperature probably have reached such a level that the adhered BAECs and BASMCs number is comparable to that at 37°C from the same media.

Encouraged by the observation of temperature-dependent cell adhesion on ppNIPAM, we fabricated a microheater array-controlled ppNIPAM chip for cell patterning. Previously, researchers have patterned thermoresponsive polymers on a cell adhesive substrate for cell coculture purposes. In those strategies, the temperature change is applied to the whole substrate, which limits patterns to only two cell types that must be adjacent to each other. With the microheater array, the limitation on the number of cell types and pattern geometry is effectively removed. In our study, cell patterning was demonstrated with BAECs and BASMCs. However, the strategy is not limited to these two cell types. Cell culture on pNIPAM has been studied by various groups using different cells, such as granulocytes, chondrocytes, endothelial cells, neural cells, hepatocytes, fibroblasts, epithelial cells, monocytes and polymorphonuclear leukocytes, microglia, cardiac myocytes, and keratinocytes. The general observations have been similar to those reported in this study. Thus, this method combining microheater arrays with pNIPAM for cell patterning can most likely be applied to all these cells. It is straightforward to apply the technique for larger-scale cell chips with more heaters and other spacings.

Compared with commonly used cell patterning devices, such as micropatterned protein surfaces, stencils, and microfluidic devices, the strategy of using stimulus-responsive chemistry combined with microcontrollers has advantages. First, since all the addressing, masking, and adhesion information is included in the chip, the process eliminates complicated alignment procedures, solvent/elastomer contamination, and aggressive manipulation. This is especially desirable for certain microsystems where microfluidic devices are not applicable. Second, the entire process can be performed in a macroscopic media environment that can easily satisfy the cell’s nutrient and oxygen requirement. Third, the dimension of the patterned feature can be tailored by scaling the microcontroller size and choosing proper substrates. With routine submicrometer fabrication resolution in the semiconductor industry, it should be feasible to create feature sizes with the dimensions of a single cell. Fourth, by generating a functional polymer using the plasma deposition method, mass production of the chips should be straightforward. The chip used in our study can be integrated with microfluidic components, multiplexing circuitry, memory, and batteries for building an automated, compact, and portable “laboratory-on-a-chip” device.

In conclusion, we quantitatively characterized the adhesion behavior of three types of cells on a thermo-responsive polymer at different temperatures. An order of magnitude difference in cell adhesion was observed for all the tested cell types in serum-free and serum-containing media. Based on these observations, we developed a new cell patterning approach with which single or multiple types of cells can be directed to adhere at spatially localized sites on a surface. Our approach used a microheater array to control the polymer property transition and to direct site-specific cell adhesion. Patterning of single or multiple types of cells was demonstrated. We believe similar cell patterning strategies can be developed for other stimulus-responsive polymers by designing the corresponding microcontrollers.

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