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Directed cell attachment by tropoelastin on masked plasma immersion ion implantation treated PTFE

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ABSTRACT

The ability to generate cell patterns on polymer surfaces is critical for the detailed study of cellular biology, the fabrication of cell-based biosensors, cell separation techniques and for tissue engineering. In this study contact tape masking and steel shadow masks were used to exclude plasma immersion ion implantation (PIII) treatment from defined areas of polytetrafluoroethylene (PTFE) surfaces. This process enabled patterned covalent binding of the cell adhesive protein, tropoelastin, without employing chemical linking molecules. Tropoelastin coating rendered the untreated regions cell adhesive and the PIII-treated area non-adhesive, allowing very fine patterning of cell adhesion to PTFE surfaces. A blocking step, such as with BSA or PEG, was not required to prevent cell binding to the underlying PIII-treated regions as tropoelastin coating alone performed this blocking function. Although tropoelastin coated the entire PTFE surface, the cell binding C-terminus of tropoelastin was markedly less solvent exposed on the PIII-treated, hydrophilic regions. The differential exposure of the C-terminus correlated with the patterned distribution of tropoelastin-mediated cell adhesion. This new methodology specifically enables directed cell behavior on a polymer surface using a simple one-step treatment process, by modulating the adhesive activity of a single extracellular matrix protein.

1. Introduction

Synthetic polymers are used extensively for the fabrication of scientific and diagnostic platforms. For example virtually all assays of cell adhesion and biological activity occur on adhesive polymer surfaces. The polymeric material PTFE is used routinely for the fabrication of medical devices such as vascular prosthesis, maxiofacial surgery and dermal applications [1–3]. Often such polymer surfaces are inherently non-cell adhesive, so ECM protein coating is routinely utilized to endow polymers with their biological activity. Such coating is frequently accomplished through simple protein physisorption [4]. Physisorption is dependent upon the polymer chemistry, wettability, energy and topography resulting in variable degrees of protein adsorption, persistence and stability [4–7]. For example PTFE is very hydrophobic and so binds strongly to many proteins [8,9]. However upon adsorption to PTFE, proteins can undergo structural changes [10–14] with implications for *in vivo* foreign body response [15] and hemocompatibility [16]. We have previously shown that nitrogen PIII treatment of PTFE modifies the PTFE surface chemistry. Upon PIII treatment the surface is defluorinated and oxidized causing increased surface wettability with water contact angles decreasing from 114.4° to 87.8° [17,18]. This in turn modulates the cell binding activity of bound ECM proteins and of bound enzymes [17–24].

In contrast to simple protein physisorption covalent protein–polymer interactions offer the advantage of increased persistence of attached proteins, thereby overcoming a major difficulty arising from use of transiently resident ECM proteins on synthetic surfaces. We have previously shown that in addition to modifying the surface chemistry of PTFE, PIII treatment offers a methodology to covalently link ECM proteins to PTFE without the need for chemical crosslinkers [17].

Tropoelastin is the major constituent of many elastic tissues such as arteries, lung and skin dermis. Elastin is stable in adult humans with a very low turnover. Tropoelastin can bind to fibroblasts via integrin $\alpha_V\beta_3$ and heparan sulfate interactions at the C-terminus [25–27]. Such cell binding activity is sensitive to the surface properties of the underlying polymer substrate. Indeed we

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have shown that tropoelastin switches between states: from a cell adhesive protein on untreated PTFE to a non-adhesive cell adhesion blocker on PIII-treated PTFE [17]. This switching correlates with increased cell binding activity on hydrophobic surfaces and decreased cell binding activity on hydrophilic surfaces. This switching activity is attributed to exposure of the cell binding C-terminus on untreated PTFE and limited exposure on PIII-treated PTFE.

The ability to generate cell patterns on polymer surfaces is critical for the fabrication of biosensors based on living cells, such as fibroblasts, where it is necessary to monitor the status of these cells in closely packed, defined locations [28]. Accurate positioning of cells is also a prerequisite for cell-based screening [29], cell separation techniques and for the detailed study of cellular biology [30]. Efforts to pattern human cells on polymer surfaces have typically used aligned microcontact printing, plasma mechanical pattern generation [31], micro-lithography, PDMS micro-patterning and micro-fluidic patterning [32,33] but these methods are often associated with high cost, involve complex surface chemistry and may not retain proteins in preferred orientations [30]. There is a paucity of ways to utilize intact ECM molecules to confer biologically relevant cell interactions on the polymer surface. Those methods that do rely on patterned distribution of ECM proteins or protein-derived motifs on a non-adhesive, often PEG-coated, background material; this requires multiple complex chemical steps. Instead here we examine masked PIII modification of polytetrafluoroethylene (PTFE) in conjunction with tropoelastin coating with the aim of patterning the distribution of human cells without the need for dedicated blocking molecules.

2. Materials and methods

2.1. Materials

0.1 mm thick PTFE sheets were obtained from Goodfellows. Recombinant tropoelastin was produced in-house as described in Ref. [34]. The mouse anti-human elastin antibody BA-4, the goat anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP conjugated secondary antibodies were purchased from Sigma. A polyclonal rabbit antibody raised to the C-terminus of elastin was a kind gift from Robert Mecham (Washington University, USA). Unless stated otherwise all other reagents were purchased from Sigma. Human dermal fibroblasts (HDFs) were sourced as line number GM3348 from the Coriell Research Institute (Camden, NJ). HDFs were cultured in a humidified 5% CO_2 atmosphere in DMEM supplemented with 10%(v/v) fetal calf serum (Gibco). Cells were passaged 1 in 3, up to passage 14, every 3–4 days.

2.2. Plasma immersion ion implantation (PIII) treatment

PTFE sheets were cut into 0.8 × 8 cm strips for tape masking and into 6 × 8 cm rectangles for shadow masking and wiped with 100% ethanol. A contact mask in the form of 3 mm wide ADH Kapton blocking adhesive tape (Associated Gaskets, Australia), or shadow mask constructed from 130 μm thick laser cut steel (Mastercut Technologies, Australia) was used to limit PIII treatment to specific regions of the PTFE. These were mounted onto a substrate holder, covered by an electrically connected mesh 5.5 cm from the sample and immersed in an inductively coupled 100 W RF plasma with a working gas pressure of 2 mTorr of high purity nitrogen and a flow rate of 72 standard cubic centimeters (sccm). 20 kV pulses lasting for 20 μs with a repetition rate of 50 Hz were applied to the substrate holder. The sample holder was earthed between the pulses. Samples were PIII-treated for 800 s. Further details of the PIII treatment process and surface characteristics are in Refs. [17,18]. After PIII treatment the tape or steel mask was removed.

2.3. Cell spreading analysis

Samples of masked PIII-treated PTFE were coated in 10 μg/ml tropoelastin in PBS for 16 h at 4 °C. The tropoelastin solution was aspirated and the samples were washed with 3× PBS. Where stated samples were BSA blocked in 10 mg/ml heat denatured BSA (85 °C for 10 min then cooled on ice) for 1 h at room temperature. Near confluent 75 cm^2 flasks of HDFs were harvested by trypsinization and the cell density adjusted to 2 × 10^5 cells/ml. The BSA block was removed, the samples were washed with 3× PBS, then 1 ml aliquots of cells were added to the wells, and placed at 37 °C in a 5% CO_2 incubator for 90 min. Following incubation, the cells were immediately fixed with the addition of 81 μl 37% (w/v) formaldehyde directly to the well for 20 min. The formaldehyde was aspirated and the wells were washed with 3× 1 ml PBS before staining with 500 μl 0.1% (w/v) crystal violet in 0.2 M MES pH 5.0 for 1 h at room temperature. The crystal violet was aspirated and excess stain was removed with extensive washes of dH_2O. The samples were layered between two glass slides and visualized by phase contrast microscopy.

To determine the effect of EDTA or heparan sulfate on cell spreading the same methodology was employed except that 0.5 ml aliquots of 10 mM EDTA or 20 μg/ml heparan sulfate followed with 0.5 ml HDFs, resuspended to a density of 4 × 10^5 cells/ml were added to the samples following BSA blocking.

2.4. Confocal microscopy

Samples were tropoelastin-coated then BSA blocked as described for cell spreading analysis. HDFs were prepared as for spreading analysis and 1 ml aliquots of cells were added to the samples and placed at 37 °C in a 5% CO_2 incubator for 180 min. Following incubation, the cells were immediately fixed as for spreading analysis then the cells were permeablized in 500 μl 0.5% (w/v) Triton X-100 in PBS for 4 min. After aspiration of the Triton X-100, the wells were washed with 3× 1 ml PBS then 1 ml 1 μg/ml rhodamine conjugated phalloidin was added at room temperature for 1 h. After incubation, the wells were washed with 3× 1 ml PBS and cell nuclei stained with 500 μl 3.5 μM DAPI for 30 s. After aspiration, the wells were washed with 3× 1 ml QH_2O. Samples were placed in a drop of Fluoromount between a glass slide and a coverslip, and then sealed with varnish before viewing on a Zeiss LSM 510 Meta confocal microscope.

2.5. Immunoblotting

Masked, PIII-treated samples of PTFE were tropoelastin coated as for cell spreading. Unbound tropoelastin was removed by aspiration, and the samples were washed with 3× 1 ml aliquots of PBS. Where specified samples were SDS washed by transferring to 1.5 ml 5% SDS (w/v) in PBS and incubated at 90 °C for 10 min. The SDS-treated samples were returned to the 24-well plate and washed with 3× 1 ml PBS. Untreated samples were washed in 3× 1 ml PBS at room temperature. Non-specific antibody binding to the PTFE was blocked with 3% (w/v) bovine serum albumin (BSA) in PBS for 1 h at room temperature. Following BSA blocking the samples were washed with 2× 1 ml PBS, then incubated in 0.75 ml 1:2000 diluted BA-4 antibody or 1:500 diluted rabbit anti-C-terminus antibody for 1 h at room temperature. Antibody solutions were removed and the samples were washed in 3× 1 ml PBS before incubation in 0.75 ml 1:10,000 diluted goat anti-mouse or anti-rabbit IgG-HRP conjugated secondary antibody for 1 h at room temperature. The secondary antibody solutions were removed and the samples were washed 4× 1 ml PBS for 10 min each wash. After washing the samples were incubated in Lumiglo (Cell Signaling Technologies) solution for 2 min then mounted between two transparent plastic sheets before exposing to CL-XPosure film (Pierce).

2.6. Surface wettability

A masked PIII-treated sample was dipped into water containing Coomassie Brilliant Blue. The water distribution was immediately photographed.

3. Results

3.1. Contact masking during PIII treatment

Layering low tack adhesive tape across PTFE samples during PIII treatment is a simple method for spatial patterning of PIII treatment. The distribution of cells on tape-masked PIII-treated samples was clearly altered by visual observation and phase contrast microscopy (Fig. 1). In the absence of tropoelastin or BSA blocker, the masked and unmasked regions each bound to cells, although the cells were larger and more spread on the unmasked areas than the tape-masked areas. BSA effectively blocked all cell adhesion and spreading on both regions of the PTFE surface in the absence of tropoelastin. This approach allowed detection of tropoelastin-mediated cell adhesion in the absence of cell adhesion to the underlying polymer. Consistent with a tropoelastin switch [17] tropoelastin coating of the tape-masked samples resulted in cell adhesion and spreading specifically to the masked (untreated) areas of the PTFE. The unmasked (PIII-treated) regions had few cells, which were small and rounded. This occurred in a manner that was not sensitive to BSA blocking, indicating that tropoelastin acted simultaneously as a promoter of cell spreading on the masked regions, and a blocker of cell adhesion on the unmasked areas. Indeed coating of masked PIII-treated PTFE with increasing