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Binding of the cell adhesive protein tropoelastin to PTFE through plasma immersion ion implantation treatment

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ABSTRACT

The interaction of proteins and cells with polymers is critical to their use in scientific and medical applications. In this study, plasma immersion ion implantation (PIII) was used to modify the surface of polytetrafluorethylene (PTFE), enabling the covalent binding of a cell adhesive protein, tropoelastin, without employing chemical linking molecules. Tropoelastin coating of untreated or PIII treated PFTE simultaneously promoted and blocked cell interactions respectively, i.e. PIII treatment of the PTFE surface completely inverses the cell interactive properties of bound tropoelastin. This activity persisted over long term storage of the PIII treated surfaces. The integrin binding C-terminus of tropoelastin was markedly less solvent exposed when bound to PIII treated PTFE than untreated PTFE, accounting for the modulation of cell adhesive activity. This presents a new methodology to specifically modulate cell behavior on a polymer surface using a simple one step treatment process, by adjusting the adhesive activity of a single extracellular matrix protein.

1. Introduction

Synthetic polymers have many uses in scientific and medical applications. For example virtually all assays of cell adhesion and biological activity occur on adhesive polymer surfaces. Furthermore the polymeric material, PTFE is one of the most commonly used polymers for the fabrication of medical devices, with current applications in vascular prostheses, as tubular scaffolds for nerve regeneration, and for maxillofacial surgery [1]. Additionally PTFE is often employed in dermal applications such as reconstructive surgery and tissue augmentation [2,3].

Cells *in vivo* are surrounded by an extracellular matrix (ECM), which provides cellular cues vital for many biological functions such as cell adhesion, migration and proliferation, tissue organization, wound repair, development, and host immune responses [4]. To endow polymers with the biological activities of ECM proteins, simple physisorption is often used to attach ECM proteins onto polymer surfaces [5]. This physisorption is dependent upon the polymer chemistry, wettability [6], energy and topography [5] resulting in variable extents of attachment, persistence and conformational stability [7,8]. In contrast, covalent protein–polymer interactions offer the opportunity to increase the persistence of attached proteins, thereby overcoming some of the difficulties associated with the use of ECM proteins on synthetic surfaces. Previous covalent protein linkage methodologies required chemical linker molecules such as disulfides, silanization, epoxides or glutaraldehyde [9–11], which often involve multiple, relatively complex surface attachment processes. Aside from adding complexity to the protein attachment process, the incorporation of chemical linkers could result in toxicity. In contrast, plasma immersion ion implantation (PIII) enables the covalent linkage of proteins to polymer surfaces without the need for chemical linkers. In addition to forming covalent linkages this methodology retains the activity of bound enzymes and ECM proteins, presumably by providing a local environment that helps stabilize the protein structure [12–19].

PTFE surfaces are too hydrophobic to adhere cells directly [20]. To overcome this, physical methods of surface modification, including electrostatic treatment [21], carbon deposition [22], UV/gamma irradiation [1,23], plasma discharge [24], and ion implantation [25–28] are used as they are appealing for manufacture. Although physical treatments can improve cell adhesion to the

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polymer surface, this often occurs through passive, physiochemical interactions including hydrophobic, Coulombic and van der Waals forces between the surface and the cell. This passive adhesion occurs in a manner that is different to the active adhesion between a cell and a cell adhesive ligand [29]. Therefore the ability to attach an ECM protein to a substratum and tightly control its adhesive activity would facilitate biologically controlled cellular activity for scientific and medical applications [8].

Tropoelastin is a cell adhesive protein that is the major elastic constituent of elastic tissues such as skin, vocal fold, lung and elastic cartilage. Tropoelastin binds cells through multiple surface receptors and so has been studied as a biomimetic coating. The C-terminus motif of tropoelastin is recognized by fibroblasts and vascular cells through integrin $\alpha_V\beta_3$ and chondrocytes through heparan sulfate mediated interactions [30–33]. Additionally tropoelastin can bind cells through the elastin binding protein [34]. Tropoelastin is an asymmetric protein with an appended C-terminal cell binding foot [35–37]. Tropoelastin, elastin and elastin-derived peptides affect cell behavior to elicit morphological changes [38,39], chemotaxis [40], proliferation [41] and angiogenesis [42].

2. Materials and methods

2.1. Materials

0.1 mm thick PTFE sheets were obtained from Goodfellow. Recombinant tropoelastin was produced in-house as described in [43]. Mouse anti-human elastin antibody BA-4, goat anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP conjugated secondary antibodies were from Sigma. A polyclonal rabbit antibody raised to the C-terminal exon 36-encoded region of human tropoelastin was a kind gift from Dr. Robert Mecham (Washington University, USA).

2.2. Plasma immersion ion implantation (PIII) treatment

PTFE sheets were cut into 0.8 × 8 cm strips, mounted onto a substrate holder, with an electrically connected mesh placed 6 cm in front of the PTFE surface and immersed in an inductively coupled rF plasma with an rF power of 100 W. The working pressure was 2 mTorr of high purity nitrogen with a flow rate of 72 standard cubic centimeters (sccm). The samples were PIII treated by applying 20 kV pulses lasting 20 μs with a repetition rate of 50 Hz to the sample holder. Unless stated otherwise all treatments were for 800 s. The sample holder was earthed between pulses. Further details of the treatment process and its application to the surface attachment of bioactive enzymes and ECM proteins can be found in [12,13,15,18,19].

2.3. ELISA

Strips of untreated and PIII treated PTFE were cut into 1.2 × 0.8 cm samples and placed in a 24 well plate (Greiner). Tropoelastin was diluted in PBS and 0.75 ml added per well then incubated at 4 °C for 16 h. 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 mouse anti-elastin antibody (BA-4) 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 × 12 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 samples washed with 4 × 1 ml PBS. The samples were transferred to a new 24 well plate with 0.75 ml ABTS solution (40 mM ABTS [2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)] in 0.1 M NaOAc, 0.05 M NaH_2PO_4, pH5, containing 0.01% (v/v) H_2O_2). After 30–40 min the plates were agitated and absorbances were read at 405 nm.

2.4. ATR FTIR

Untreated and PIII treated PTFE were cut into 6 × 0.8 cm strips and placed into 15 ml Falcon tubes. Tropoelastin was diluted in PBS and 8 ml added and incubated at 4 °C for 16 h. Unbound tropoelastin was removed by aspiration, and samples were washed with 3 × 10 ml volumes of PBS. Samples were SDS-treated by transferring to 8 ml 5% SDS (w/v) in PBS and incubated at 90 °C for 10 min. Following SDS treatment, samples were placed into a new 15 ml tube and washed with 3 × 10 ml PBS then 3 × 100 ml QH_2O. Non-SDS-treated samples were washed with 3 × 10 ml PBS then 3 × 100 ml QH_2O. Samples were dried for 2 days prior to accumulation of spectra using a Digilab FTS7000 FTIR spectrometer fitted with an attenuated total reflection (ATR) accessory with a trapezium germanium crystal and incidence angle of 45°. To obtain sufficient signal/noise ratio and resolution of spectral bands, 500 scans with a resolution of 1 cm^{-1} were taken. Difference spectra, used to detect changes associated with the presence of tropoelastin, were obtained by subtracting spectra of treated samples not incubated in tropoelastin from the spectra of those incubated in tropoelastin solution. Spectral analysis was performed using GRAMS software.

2.5. Cell culture

Human dermal fibroblasts (HDFs – line GM3348) were obtained from the Coriell Research Institute (Camden, NJ, USA). 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.

2.6. Cell attachment analysis

Cell attachment methodology was adapted from [44]. 1.2 × 0.8 cm samples were tropoelastin coated as for ELISA analysis. After PBS washing of unbound tropoelastin, non-specific polymer binding was blocked with 10 mg/ml heat denatured BSA (80 °C for 10 min, then cooled on ice) in PBS for 1 h at room temperature. Near confluent 75 cm^2 flasks of HDFs were harvested by trypsinization, and the cell density adjusted to 5×10^5 cells/ml. The BSA blocking solution was aspirated, the wells washed 3 × 1 ml with PBS and 1 ml aliquots of cells added for 60 min at 37 °C in a 5% CO_2 incubator. To estimate percent cell attachment a minimum of three known cell number controls were plated by adding 0, 0.5 or 1 ml cells to unblocked PIII treated PTFE that possesses high cell binding activity. After incubation, the cell number controls were fixed by the addition of 100 μl 50% glutaraldehyde (w/v) directly to the media. Cells were removed from the experimental wells and non-adherent cells were removed with 2 × 1 ml PBS washes. Adherent cells were fixed with the addition of 1 ml 5% glutaraldehyde (w/v) in PBS for 20 min, then washed with 3 × 1 ml PBS. Cells were stained with 500 μl 0.1% (w/v) crystal violet in 0.2 M MES pH5.0 for 1 h at room temperature then washed 4 × 1 ml QH_2O. The samples were transferred to a new 24 well plate and the crystal violet was solubilized in 500 μl 10% (v/v) acetic acid and the absorbance measured at 570 nm using a plate reader. The data from known standards were fitted to a linear regression and the gradient was used to convert experimental absorbances into percentage attachment. In all experiments triplicate measurements were taken.

2.7. Cell spreading analysis

1.2 × 0.8 cm samples were coated with tropoelastin then BSA blocked as described for cell attachment analysis. Near confluent 75 cm^2 flasks of HDFs were harvested as for cell attachment analysis, and the cell density adjusted to $1–5 \times 10^5$ cells/ml. 1 ml aliquots of cells were added and incubated at 37 °C in 5% CO_2 for 90 min. Cells were then fixed with the addition of 81 μl 37% (w/v) formaldehyde directly to each well for 20 min. Formaldehyde was aspirated and the wells washed 3 × 1 ml PBS before staining with 500 μl 0.1% (w/v) crystal violet in 0.2 M MES pH5.0 for 1 h at room temperature. The crystal violet was aspirated and excess stain was removed with extensive washes of QH_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 2×10^5 cells/ml were added to the samples following BSA blocking.

2.8. Confocal microscopy

1.2 × 0.8 cm samples were tropoelastin coated then BSA blocked as described for cell attachment 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 permabilized in 500 μl 0.5% (w/v) Triton X-100 in PBS for 4 min. After aspiration of the Triton X-100, wells were washed 3 × 1 ml with PBS and 1 ml 1 μg/ml rhodamine conjugated phalloidin was added for 1 h at room temperature. 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 aspiratation, 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.

2.9. Water contact angle measurement

Water contact angles were measured as described in [17].

2.10. In vivo implantation and histology

Biopsy punched 0.8 mm diameter PIII (treated both sides) or untreated PTFE discs were sterilized in 70% ethanol and washed 4 × 1ml with PBS. Samples were incubated