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Surface plasma modification and tropoelastin coating of a polyurethane co-polymer for enhanced cell attachment and reduced thrombogenicity

Daniel V. Bax [a,b,*], Alexey Kondyurin [a], Anna Waterhouse [c], David R. McKenzie [a], Anthony S. Weiss [b,d,e], Marcela M.M. Bilek [a]

[a] Applied and Plasma Physics, School of Physics, University of Sydney, NSW 2006, Australia
[b] School of Molecular Bioscience, University of Sydney, NSW 2006, Australia
[c] Wyss Institute for Biologically Inspired Engineering, Harvard, Boston, MA 02115, USA
[d] Bosch Institute, University of Sydney, NSW 2006, Australia
[e] Charles Perkins Centre, University of Sydney, NSW 2006, Australia

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ABSTRACT

Polymers currently utilized for dermal and vascular applications possess sub-optimal biocompatibility which reduces their efficacy. Improving the cell-binding and blood-contacting properties of these polymers would substantially improve their clinical utility. Tropoelastin is a highly extensible extracellular matrix protein with beneficial cell interactive and low thrombogenic properties. We transferred these benefits to the polyurethane block copolymer Elast-Eon E2A through a specific combination of surface plasma modifications and coating with human tropoelastin. The cell-binding activity of bound tropoelastin was modulated by ion implantation of the underlying polymer, and correlated with surface hydrophobicity, carbon and oxygen content. This combined treatment enhanced human dermal fibroblast (HDF) and human umbilical vein endothelial cell (HUVEC) attachment, cytoskeletal assembly and viability, combined with elevated PECAM-1 staining of HUVEC cell junctions. The thrombogenicity of the polymer was ameliorated by tropoelastin coating. We propose that a combination of metered plasma treatment and tropoelastin coating of Elast-Eon can serve to improve the biological performance of implantable devices such as vascular conduits.

1. Introduction

Polyurethane elastomers are widely used for a range of clinical applications. For example polyurethanes and polyurethane blends are used extensively in cardiovascular devices such as heart valves and vein replacements, as an encapsulating material, for orthopedic and dermal application, for transdermal drug delivery patches, and for wound dressings [1–3]. They possess good biocompatibility, are easy to prepare and process and have a wide range of tunable physical and mechanical properties [1,4]. PDMS (polydimethylsiloxane/silicone) has been incorporated into polyurethane to produce a non-cytotoxic block copolymer with enhanced degradation resistance and *in vivo* biostability [4–7]. Additionally when coated onto stents these polyurethane containing siloxanes have been shown to possess low thrombogenicity [8]. Incorporation of silicon based chain extenders has led to the development of materials such as Elast-Eon which comprise a group of very flexible, low modulus polyurethanes. Despite these efforts to enhance the biocompatibility in their pristine forms, polyurethane and PDMS are inherently resistant to cell adhesion and support poor cell growth [1,5,9].

Thrombosis is the major cause of short-term failure of heart valves, coronary stents and small diameter synthetic grafts [10]. Currently this is managed by anti-platelet therapy, however this presents a risk of bleeding [11]. Therefore, there remains a pressing need for non-thrombogenic materials that can reduce this reliance on pharmacological intervention. *In vivo* the entire vascular system is protected by a non-thrombogenic lining of endothelial cells. In its untreated form polyurethane possess blood contacting properties that make it suitable for short term applications, however their

* Corresponding author. Current affiliation: Cambridge Centre for Medical Materials,
Materials Science and Metallurgy, University of Cambridge, UK.
E-mail address: dbax@physics.usyd.edu.au (D.V. Bax).

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long-term thrombotic nature can cause emboli and vessel occlusion [1,5,9,12]. As such, its use in cardiovascular replacements is limited to applications where fast endothelialization is not required. Plasma treatment of polyurethane has been shown to improve cellular interactions of human coronary artery endothelial cells [9], HUVEC [13], and bovine aortic endothelial cells [10] and has been used to pattern endothelial cells on treated polymer regions [14]. Ion beam implanted polyurethane shows improvements when investigated *in vivo* as an endoprosthesis [15].

Coating polymers with ECM proteins has been used in a biomimicary approach to endow the polymers with biological activities such as the ability to stimulate cell adhesion, migration and proliferation, tissue organization, wound repair, development, and host immune response [16,17]. There is a paucity of ways to utilize intact ECM molecules to confer biologically relevant cell interactions to polymer surfaces. We have recently demonstrated plasma ion immersion implantation (PIII) as a method to covalently link ECM proteins to polymeric surfaces without the need for conventional linker chemistry [18,19]. As such PIII mediated covalent linkage mitigates the need for multiple, relatively complex surface attachment processes and removes the potential for chemical modification of vital side chains and toxicity associated with chemical linkage.

Tropoelastin, the precursor of mature elastin fibers, can comprise ~50% of the dry weight of the artery wall where it has a role in signaling and regulating luminal endothelial cells. The C-terminus of human tropoelastin binds to fibroblasts and vascular cells through integrin $\alpha_V\beta_3$ [20]. The N-terminus of tropoelastin can also support HUVEC adhesion [21]. Additionally, tropoelastin can bind to cells and modulate cell activity through VGVAPG motifs which bind to the cellular elastin binding protein [22]. Although other ECM proteins such a fibronectin and collagen support cell adhesion, these ECM proteins lead to clot formation. By contrast, elastin is a low thrombogenic material and as such is being assessed for its potential as a vascular conduit component [23]. Here we explore the potential of tropoelastin as a polymer coating to promote endothelial cell attachment and reduce thrombogenicity.

2. Materials and methods

2.1. Materials

Elast-Eon E2A was provided by AorTech Biomaterials (Scoresby, Australia). It incorporates hard segments of 4,4′-methylenediphenyl diisocyanate and 1,4-butanediol, and a mixed soft segment of PDMS and poly(hexamethylene oxide) as a compatiblizing macrodiol in the ratio of 80:20 [4].

Recombinant human tropoelastin comprising amino acid residues 27-724 of GenBank AAC98394 (gi182020) was produced in-house as described in Ref. [24]. The mouse anti-human elastin antibody (BA-4) and the goat anti-mouse IgG-HRP conjugated secondary antibody were from Sigma. Human dermal fibroblasts (HDFs) GM3348 were 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 every 3–4 days up to passage 14. Human umbilical vein endothelial cells (HUVECs) were harvested enzymatically from umbilical cords [25]. HUVECs were cultured in a humidified 5% CO_2 atmosphere and passaged 1 in 3 every 3–4 days, up to passage 5.

Whole blood was obtained from healthy non-smoker male volunteers who had not taken aspirin for 2 weeks prior to donation with informed consent in accordance with the Declaration of Helsinki. Approval for obtaining blood samples was granted by the University of Sydney, Human Research Ethics Committee (protocol 05-2009/11668).

2.2. Plasma and plasma immersion ion implantation (PIII) treatment

Elast-Eon E2A sheets were cut into 0.8 × 8 cm strips, mounted onto a substrate holder, covered by a mesh and immersed in an inductively coupled RF plasma powered at 100 W with reflected power 12 W when matched. The mesh was placed 5.5 cm in front of the polymer surface and electrically connected to the substrate holder. The working gas was high purity (99.999%) nitrogen at a pressure of 2 mTorr and with a flow rate of 72 standard cubic cm. Where applicable the samples were PIII treated by applying 20 kV pulses each lasting for 20 μs with a repetition rate of 50 Hz to the sample holder during plasma treatment. The sample holder was earthed between the pulses. Untreated controls did not undergo treatment in the plasma chamber.

The water contact angles for untreated and PIII treated samples were measured as described in Ref. [26].

2.3. Electron paramagnetic resonance (ESR) measurement

20 mm × 40 mm Elast-Eon E2A samples were rolled along their widths and the electron spin resonance spectra were recorded using a Bruker Elexsys E500 electron paramagnetic resonance (EPR) spectrometer which had been precalibrated using a weak pitch KCl sample. Measurements were taken in X band with a microwave frequency of 9.35 GHz and a central magnetic field of 3330 G at 20 °C. Ten scans were taken per sample. The sweep time per scan was 41.94 s. The signal intensity was numerically integrated to plot the number of spins vs. field, and integrated twice to calculate the total number of spins. For estimation of unpaired spin concentrations in the Elast-Eon E2A samples, a strong pitch α,α′-diphenyl-β-picrylhydrazyl sample was used.

2.4. XPS measurement

XPS measurements were performed using an XPS spectrometer (SPECS, Berlin, Germany), equipped with a FOCUS 500 (Al Kα, hν = 1486.6 eV) monochromatized X-ray source operating at 200 W, a PHOIBOS 150 MCD-9 hemispherical analyzer and a delay line detector. Survey spectra of each sample were acquired using a binding energy range of 0–1400 eV and pass energy of 30 eV. Higher resolution scans (0.01 eV steps) were taken for O1s, N1s, C1s, Si2s and Si2p regions at a pass energy of 23 eV. Data analysis and peak fitting was performed using Casa XPS software. Scans were calibrated using the C1s C–C/H peak at 285 eV as the reference peak for all spectra taken. Peaks were fitted using a G-L (Gaussian − Lorentzian) function and a Shirley baseline. Peaks were quantified using relative sensitivity factors (RSF) supplied by the spectrometer manufacturer.

2.5. FTIR ATR spectra

FTIR ATR spectra were recorded using a Digilab FTS7000 FTIR spectrometer fitted with an ATR accessory (Harrick, USA) and a trapezium Germanium crystal employing an incidence angle of 45°. 500 scans at a resolution of 1 cm^{-1} were taken to generate spectra with sufficiently high spectral band resolution and signal-to-noise ratio. The surface of the samples was dried using dry air flow before data collection. Differences, obtained by subtraction, between spectra of samples before and after PIII treatment, were used to characterize the effect of surface treatments. All spectral analyses used GRAMS software.

2.6. ELISA

0.8 × 1.2 cm samples were placed into the wells of a 24 well plate (Greiner). Tropoelastin was diluted to 10 μg/ml in PBS and 0.75 ml added to each well, then placed at 4 °C for 16 h. Unbound tropoelastin was aspirated and bound tropoelastin was detected by ELISA [27].

2.7. Cell attachment and spreading

0.6 × 0.8 cm samples were placed in a 48 well plate and coated with tropoelastin as described for ELISA. Where stated, non-specific cell binding to Elast-Eon E2A 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 then cell attachment and spreading were quantified [28].

2.8. Confocal microscopy

Elast-Eon E2A samples and cells were prepared as for attachment analysis. 0.5 ml aliquots of cells at a density of 1 × 10^5 cells/ml were added to the wells and placed at 37 °C in a 5% CO_2 incubator for 150 min. Cells were fixed then permeabilized in 500 μl 0.5% (w/v) Triton X-100 in PBS for 4 min, washed 3 × 1 ml with PBS then blocked with 3% (w/v) BSA for 1 h. The BSA was aspirated and 1 ml of 1:500 mouse-anti human PECAM-1 primary antibody or 1:500 mouse-anti human vWF primary antibody were added for 1 h. The primary antibody was aspirated and 1 ml of 1:400 FITC- conjugated goat-anti mouse secondary antibody containing 1 μg/ml rhodamine conjugated phalloidin was added for 1 h at room temperature. Samples were washed 3 × 1 ml with PBS, the cell nuclei were stained with 500 μl 3.5 μM DAPI for 30 s then washed 3 × 1 ml with water. They were then mounted on a drop of fluoromount between a glass slide and a coverslip, sealed with nail varnish and viewed using a Zeiss LSM 510 Meta confocal microscope.

2.9. Cell viability

0.5 ml aliquots of cells at a density of 1x10^5 cells/ml were added to wells and incubated at 37 °C for 4 h. Trypan blue was added at a final concentration of 0.1% (w/v) and the numbers of clear and blue cells were counted. Percent cell viability was derived after dividing the number of clear cells by the total number of cells.

2.10. Static blood incubation

0.8 × 1.2 cm samples were tropoelastin coated as for cell adhesion assays then incubated in 2 ml heparinized (0.5 U/ml) whole blood for 30 min [25]. Following incubation the samples were washed, fixed in 2.5% (w/v)