**1. Title:**

**Materials-driven Fibronectin Assembly on Nanoscale Topography Enhances Mesenchymal Stem Cell Adhesion, Prevents Biofilm Formation and Protects Cells from Quorum Sensing Virulence Factors**

**2. Authors:**

*Laila A. Damiati1,2, Monica P. Tsimbouri1, Mark Ginty3, Virginia Llopis-Hernandez1, Peter Childs4,5, Vineetha Jayawarna4, Yinbo Xiao1, Karl Burgess6, Julia Wells7, Mark R. Sprott1, R.M. Dominic Meek8, Peifeng Li4, Richard O.C. Oreffo7, Angela Nobbs3, Gordon Ramage9, Bo Su3, Manuel Salmeron-Sanchez4 and Matthew J. Dalby1\**

Dr. L.A. Damiati, Dr. M.P. Tsimbouri, Dr. V. Llopis-Hernandez, Dr. P. Childs, Dr. V. Jayawarna, Y. Xiao, Dr. M.R. Sprott, Prof. M.J. Dalby

Centre for the Cellular Microenvironment, Institute of Molecular, Cell and Systems Biology, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow G12 8QQ, UK.

Dr. L.A. Damiati

Department of Biology, Collage of Science, University of Jeddah, Jeddah 23890, SA. <https://orcid.org/0000-0002-4746-0915>

Dr. P. Childs

Department of Biomedical Engineering, University of Strathclyde, Glasgow G1 1QE, UK.

M. Ginty, Dr. A. Nobbs, Prof. B. Su

School of Oral and Dental Sciences, University of Bristol, Bristol, BS1 2LY, UK.

Dr. P. Li, Prof. M. Salmeron-Sanchez

Centre for the Cellular Microenvironment, Division of Biomedical Engineering, School of Engineering, University of Glasgow, Glasgow G12 8LT, UK

Prof. K. Burgess

Glasgow Polyomics Facility, College of Medical, Veterinary and Life Sciences, University of Glasgow, Switchback Rd, Bearsden, Glasgow G61 1BD, UK.

J. Wells, Prof. R. O.C. Oreffo

Bone and Joint Research Group, Centre for Human Development, Stem Cells and Regeneration, Institute of Developmental Sciences, University of Southampton, Southampton SO16 6YD, UK.

R.M. D. Meek

Department of Orthopedics, Queen Elizabeth II University Hospital, Glasgow G51 4TF, UK.

Prof. G. Ramage

Oral Sciences Research Group, Glasgow Dental School, School of Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, G12 8TA, UK.

*\*Corresponding author* [*matthew.dalby@glasgow.ac.uk*](mailto:matthew.dalby@glasgow.ac.uk)

**Abstract**

Post-operative infection is a major complication in patients recovering from orthopaedic surgery. As such, there is a clinical need to develop biomaterials for use in regenerative surgery that can promote mesenchymal stem cell (MSC) osteospecific differentiation and that can prevent infection caused by biofilm-forming pathogens. Nanotopographical approaches to pathogen control are being identified, including in orthopaedic materials such as titanium and its alloys. These topographies use high aspect ratio nanospikes or nanowires to prevent bacterial adhesion but these features puncture adhering cells, thus also reducing MSC adhesion. Here, we use a poly(ethyl acrylate) (PEA) polymer coating on titanium nanowires to spontaneously organise fibronectin (FN) and to deliver bone morphogenetic protein 2 (BMP2) to enhance MSC adhesion and osteospecific signalling. This nanotopography when combined with the PEA coating enhanced osteogenesis and reduced adhesion of *Pseudomonas aeruginosa* in culture. Using a novel MSC–*Pseudomonas aeruginosa* co-culture, we also show that the coated nanotopographies protect MSCs from cytotoxic quorum sensing and signalling molecules. We conclude that the PEA polymer-coated nanotopography can both support MSCs and prevent pathogens from adhering to a biomaterial surface, thus protecting from biofilm formation and bacterial infection and supporting osteogenic repair.

**3. Funder information:**

L.A.D. was supported by a scholarship from Jeddah University and the Saudi Arabian Government. The work was also supported by grants from EPSRC (EP/K034898/1) and MRC (MR/S010343/1).

**4. Dataset and readme information**

**Data folders: SEM images for Ti Fig1a, Fig 2 and sup1**

**Nanowires production** The Ti disk samples were prepared from a 0.9 mm thick, and 10 mm dimeter; ASTM grade one Ti sheet (Baoji HeQiang Titanium Industry Co., China). The discs were polished to a mirror image and ultrasonically cleaned in water and ethanol. Nanowires were created by immersing the Ti disks in 1 M NaOH in a PTFE lined steel vessel, at a temperature of 240 °C for 1 hr or 2 hr. The samples were rinsed in water and ethanol, sequentially and they were subsequently heat treated at 300 °C for 1 hr prior to ion exchange in HCl. To convert the sodium Ti nanowires to TiO2 the samples were immersed in 0.6 M HCl for 1 hr, rinsed in water and ethanol, and finally heat treated at 600 °C for 2 hr

**Figure 1-a:** SEM images of flat control, 1hr nanospikes and 2 hr nanospikes showing the formation of high aspect ratio nanofeatures with increasing size.

**Figure 2:** Schematic depiction of fabrication of plasma PEA coated nanowires.

**Supplementary Figure 1:** SEM imaging of Ti nanowire surfaces with and without PEA coating. The images show that height of the nanofeatures increase proportionally with anodisation time and that the thin PEA coatings to not effect nanowire morphology.

**Data folders: AFM images and calculations, fig1-b&c**

**AFM characterisation:** AFM images optioned on flat and nanowires samples pre- and post- coating with PEA. AFM experiments were performed using a Multimode AFM equipped with NanoScope IIIa controller from Veeco (Manchester, UK) operating in tapping mode in air; the Nano- scope 5.30r2 software version was used. Si-cantilevers from Veeco were used with force constant of 2.8 N/m and resonance frequency of 70 kHz. The phase signal was set to zero at a frequency 5% to 10% lower than the resonance one. Drive amplitude was 600 mV and the amplitude set point Asp was 1.8 V. The ratio between the amplitude set point and the free amplitude Asp/A0 was between 0.4 - 0.8.

**Figure 1-b&c: b:** AFM images of uncoated, 90 sec and 3 min PEA coated flat controls, 1 hr and 2 hr topographies showing that the topography survives the PEA coating process. **c:** AFM Ra measurements.

**Data folders: WCA measurements, fig1-d**

**WCA:** Ti surface wettability was characterized by measuring WCA statically using the sessile drop method with 3 μL drops for 30 seconds (Optical Tensiometer Theta, Biolin Scientific).

**Figure 1-d:** Sessile drop contact angle measurements showing increased hydrophobicity with addition of the PEA coatings.

**Data folders: XPS, fig1-e&f**

**XPS:** XPS used here to analyse the chemical components of the metal alloys pre- and post- plasma PEA coating. The measurement spectra were obtained at the National EPSRC XPS Users’ Service (NEXUS) at Newcastle University, an EPSRC Mid-Range Facility. XPS was performed using a K-Alpha apparatus (Thermo Scientific), with a micro-focused monochromatic Al Kα source (X-ray energy = 1486.6 eV) at a voltage of 12 kV, current of 3 mA, power of 36 W, and spot size of 400 μm × 800 μm. Spectra analysis and curve fitting were performed using CasaXPS software version 2.3.16.

**Figure 1-e&f:** **e:** XPS spectra generated from PEA reference. **f:** XPS measurements of uncoated Ti controls with and without topography showing the presence of Ti and O. XPS spectras for 90 sec and 3 min PEA coatings showing that with the shorter, 90 sec, coating, while peaks related to PEA were clearly seen, Ti could still be detected, while at 3 mins, only peaks related to PEA were observed. The data shows that high aspect ratio topographies were successfully fabricated and coated with pPEA.

**Supplementary Figure 2:** *Enlargements of XPS images from Figure 1f*

**Data folders: qPCR, fig1-a, b &f**

**Cell culture**: Adult human bone marrow Stro-1+ MSCs were isolated with consent from patients at the Southampton General Hospital, UK. Cells were cultured in Dulbecco’s modified essential medium (DMEM) supplemented with 10% (v/v) FBS, 1% (v/v) MEM NEAA (non-essential amino acid), 1% (v/v) 200 mM L-glutamine, 2% (v/v) antibiotics (6.74 U/ml penicillin- streptomycin, 0.2 μg/mL Fungizone) and 1% sodium pyruvate (11 mg/ml) at 37 oC in 5 % CO2. Seeding on coated Ti was done in serum-free medium in 24-well plates at 104 cells/disc. After 2 h, the media were changed to low serum growth medium (1% FBS).

**qPCR:** Total RNA was extracted at day 21 and 28-time point culture using a Qiagen RNeasy Micro kit and protocol. To standardise for quantitative analysis, equal amounts of RNA from each sample were used for cDNA synthesis using the Qiagen QuantiTect RT-PCR kit and protocol. qRT-PCR was carried out using the Qiagen Quantifast SYBR Green kit and the reactions run in the 7500 Real Time PCR cycler from Applied Biosystems. Three biological and two technical replicates from each replicate were tested. Expression of test osteopontin (OPN), osteonectin (OSN) was normalised against housekeeping gene glyceraldehyde 3-phosphate dehydrogenase(GAPDH) (list of primers is shown in Table 1). QRT-PCR products were quantified using the 2-ΔΔCt method.

**Table 1: Primers used for qRT-PCR**

|  |  |
| --- | --- |
| Gene Name | Primer sequence |
| GAPDH | Forward: TCAAGGCTGAGAACGGGAA  Reverse: TGGGTGGCAGTGATGGCA |
| Osteonectin  (OSN) | Forward: AGAATGAGAAGCGCCTGGAG  Reverse: CTGCCAGTGTACAGGGAAGA |
| Osteopontin  (OPN) | Forward: ACAGCCGTGGGAAGGACAGT  Reverse: GACTGCTTGTGGCTGTGGGT |

**Figure 3-a,b,f:** Rapid qPCR screen at 21 and 28-days of culture for osteopontin (OPN) and osteonectin (OSN) transcripts allowing selection of the 2 hr nanowires with 90 sec pPEA coating as optimal for osteogenesis on high aspect ratio nanofeatures.

**Data folders: Alizarin Red, fig3-c**

**Alizarin Red:** Mineral production was studied using AR and CB staining. After 35 days in culture, cells cultured on flat and nanowires surfaces were washed with 1x PBS and fixed with 4% v/v formaldehyde 1x PBS for 15 min at 37 °C. 1 ml/well Alizarin Red Stain S solution (40 mM) was added and incubated at RT for 30 minutes. Excess dye was removed, and cells were carefully washed 5x with deionized water. Calcium deposits were visualised using a light microscope (Leica, Germany).

**Figure 3-c:** Alizarin red histology for mineralisation after 35 day MSC culture; arrows indicate nodule formation.

**Data folders: Calcein blue (CB), fig3-d&e**

For Calcein blue staining, 10 mg of CB were dissolved in 0.25 ml of KOH (1 M) and 9.75 ml of distilled water were added to make up a 3.1 X 10-3 M CB solution. Five microliters of the CB solution were added to the culture medium 1 h before cell fixation. The cells were then washed with PBS 3x and fixed with 4% formaldehyde in PBS for 10 min. After fixation, the specimens were washed with PBS three times and dried. Stained cells were imaged using a Zeiss immunofluorescence microscope. Images were processed using imageJ imaging software, and images that were to be compared against one another were taken with the same exposure.

**Figure 3-d&e: d:** Quantification of calcein blue stain histology for number of bone nodules after 35 day MSC culture. **e:** Typical images for calcein blue fluorescent histology showing less background that Alizarin red stain.

**Data folders: AFM and proteins adsorption, fig 4 a-f**

Evaluation of FN and BMP2 interactions with pPEA. FN nanonetworks were assessed by AFM. (a) FN nanonetwork on a glass coverslip with spin-coated PEA, showing a diffuse network. (b) left: FN nanonetwork on a glass coverslip with pPEA at 90 kJ showing dense FN networks. Right: The availability of the RGD (HFN7.1) and heparin binding sites (P5F3) on FN, as assessed by ELISA. These sites’ availability increased with a concomitant increase in BMP2 absorption when FN was present as a network on 90 kJ pPEA. (c) Coating thickness measurements showing thin films are achieved at 9 kJ and 18 kJ (90 sec and 3 min coatings respectively). (d) AFMs of uncoated and pPEA (90 sec and 3 min coatings) flat Ti samples showing very low surface roughness with nanoscale undulating topographies. AFMs of FN coated flat Ti samples with and without pPEA coatings (90 sec and 3 min). Addition of FN provides a change in surface appearance, potentially with more network morphology observable on pPEA coated samples, especially the 3 min coating. (f) ELISA was used to observe FN and BMP2 interactions with flat and 2 hr nanowire Ti surfaces, with and without 90 sec pPEA FN/BMP2 coating. From flat to 2 hrs (with or without coating), an increase in FN absorption was seen, perhaps reflecting increased surface area. Importantly, BMP2 absorption was seen to increase when samples (flat or nanowires) when pPEA coatings were used prior to FN coating.

**A. FN adsorption**

The protein adsorption properties of the FN coating were analysed by performing a bicinchoninic acid (BCA) protein assay by following the manufacture instruction (Thermofisher, UK).

**B. BMP2 adsorption**

The amounts of BMP2 bound to Ti/PEA, and Ti/PEA/FN surfaces were quantified by indirect enzyme-linked immunosorbent assay (ELISA) using DuoSet human BMP2 kit (R&D Systems, UK). After the samples were coated with BMP2, the coating solutions and the first wash solutions were collected for analysis. The amount of BMP2 bound to the samples was calculated as a difference between the total BMP2 used for coating and the amount of BMP2 in the collected solution.

**C. Integrin- and heparin-domain viability**

P5F3 and HFN7.1 antibodies were used to assess the availability of heparin binding domain and cell binding domain respectively. Primary antibody incubation P5F3 (Santa Cruze, Germany) and FN7.1 (Dr. David R. Soll, c/o DSHB, The University of Iowa) were incubated for 1 hr at RT. Washing with 0.5% Tween 20/PBS and subsequent incubation with secondary antibody for 1 hr. Followed by adding equal amount of substrate reagent: colour reagent A (H2O2) and colour reagent B (Tetramethylbenzidine) for 20 min in the dark at RT. Finally, the surfaces were incubated with stop solution (H2SO4) and read with fluorescence at 450 nm.

**Data folders: *P. aeruginosa* response to nanowire topography and pPEA coating**

**Bacteria culture:** *P. aeruginosa* ATCC 27853 was grown aerobically overnight in 10 mL DMEM in a 37 °C shaker incubator set at 220 rpm. The bacterial suspension was then diluted in DMEM to OD600= 0.1 and further incubated until mid-exponential phase was reached. At this time, bacterial cells were harvested by centrifugation (7 min, 5000 xg), washed twice in PBS buffer, and suspended in DMEM OD600= 0.3 (approx. 108 CFU/mL). 104 CFU/ml were used for culture.

**Subfolder: Bacteria Imaging and Quantification (SEM, LIVE/DEAD images, and ATP).**

**SEM:** After 24 hr of co-culture, the samples were fixed 2.5 % glutaraldehyde for bacteria buffer for overnight at 4 °C and then rinsed in 0.1 M sodium cacodylate. Samples were post fixed in 1% osmium tetroxide for 1 hr at RT then washed 3x with distilled H2O for 10 min. The samples were then stained by 0.5% uranyl acetate for 1 hr in the dark. For dehydration a 30-100% ethanol series were applied. A hexamethyldisiloxane step was conducted prior to sputter coating (20 nm gold/palladium). Samples viewed on a JEOL IT100 SEM running at 10-20 Kv, on Secondary Electron Detector (SED) mode, tiff Images captured using JEOL Intouch Scope software version 1.03.

**Figure 5-a:** SEM shows that the bacterium stays on top of the nanowires and lose flagellum (arrows) after 4-hour culture.

**Live/Dead:** Adhesion of bacteria on different types of material was observed using an inverted fluorescent microscope Zeiss Axiovert fluorescence microscope using live/dead staining (LIVE/DEAD BacLight bacterial viability kit, Molecular Probes), the green and red intensities were used to determine the threshold between live and dead bacteria, respectively.

Live/dead BacLight bacterial viability kit and fluorescence microscopy were used for the quantitative assays of bacteria on coated or uncoated Ti discs. The live/dead stain was prepared using by diluting 1 µl of staining component A (STYO 9) and 1 µl of staining component B (propidium iodide) in 1 ml of dH2O. 500 µl of reagent mixture was added to each disc before incubating them at RT for 15 min before taking images. The total attachment (of live and dead cells) on Ti surfaces was measured to quantify the percentage of dead bacteria using the following formula:

% of live or dead bacteria= (Number of live or dead bacteria)/ (Total number of cells)

**Figure 5-b:** Nanowires reduce *P. aeruginosa* viability after 8-hour culture

**ATP analysis:** To evaluate the viable bacterial cell numbers based on the ATP present, the nanowires surfaces, and controls were placed into a 24 well plate and submerged with the bacterial suspension. Plates were incubated for 18 hr at 37 °C under static conditions. After incubation, supernatants were removed into new 96 well and equal amount of BacTiter-Glo Reagent (Promage, UK). The plates were read within 5 minutes of adding the reagent using a luminescence plate reader (CLARIOstar, BMG LABTECH, Germany).

**Figure 5-c:** **ATP assay for planktonic bacteria showing more unattached bacteria with 24 hour nanowire culture**

**Metabolomics.** *P. aeruginosa* cultured on different Ti surfaces for 24 hr. After incubation, each Ti disc was transferred to a bijou and kept on ice. A 1 ml: 1g mixture of a chloroform, ethanol and ddH20 (CEW, ratio 1:3:1) and acid washed glass beads 0.1 mm was produced, and 1 ml was added to each bijou. Samples were placed on a cell disrupter (Disrupter Genie bead beater, Scientific industries Inc., New York, USA) operating at a speed of 3000 rpm, 3x for 30 sec. On removal all the liquid/bead mixture was transferred to microcentrifuge and centrifuged at 10,000 rpm for 3 minutes to remove the beads. The supernatant was subsequently transferred to a fresh microcentrifuge.

**Figure 5-d&e:** Untargeted metabolomic analysis for amino acids after 24-hour culture

**Supplementary Figure 3&4:** Untargeted metabolomics data for *P. aeruginosa* amino acid metabolism

**Data folders: MSC and PA co-culture**

**SEM:** After 24 hr of co-culture, the samples were fixed in 1.5% glutaraldehyde for MSCs for 1 hr at RT and then rinsed in 0.1 M sodium cacodylate. Samples were post fixed in 1% osmium tetroxide for 1 hr at RT then washed 3x with distilled H2O for 10 min. The samples were then stained by 0.5% uranyl acetate for 1 hr in the dark. For dehydration a 30-100% ethanol series were applied. A hexamethyldisiloxane step was conducted prior to sputter coating (20 nm gold/palladium). Samples viewed on a JEOL IT100 SEM running at 10-20 Kv, on Secondary Electron Detector (SED) mode, tiff Images captured using JEOL Intouch Scope software version 1.03.

**Figure 6-a&c: SEM images for MSC and Pa co-culture**

**Immunofluorescence (IF):** Two independent experiments with 3 technical replicate each, the discs of each substrate were analysed by immunofluorescence. Briefly, cells were fixed in 4% formaldehyde fixative at 37 °C for 15 min and permeabilized at 4 °C for 5 min. The samples were blocked with 1% BSA/PBS at 37 °C for 5 min and stained with the appropriate primary antibodies for pRUNX-2 or OPN (1:50 in 1% BSA/PBS, Autogen Bioclear). Actin filaments were stained with phalloidin (1:100, Invitrogen). Two washes in 1 × PBS/0.5% Tween-20 (3 × 5 min at RT) followed and a secondary, biotin-conjugated antibody (1:50, horse monoclonal anti-mouse (IgG), Vector Laboratories) was added for 1 hr at 37 °C. The samples were washed, and streptavidin was added (1:50, Vector Laboratories) at 4 °C for 30 min, before the samples were given a final wash and mounted in Vectashield mounting medium (Vector Laboratories) containing DAPI to stain the nucleus. Visualisation was via a fluorescence microscope (Zeiss Axiovert 200 M, 10x magnification, NA 0.5). Comparisons of staining intensity between substrates was analysed by Image J software version 1.42q.

**Figure 6-b,d-f:** IF images for MSC and *P. aeruginosa* co-culture and pRUNX measurements

**Supplementary Figure 4:** PA culture on agar plates to determine the best antibiotic concentration for co-culture experiments.

**Data folders: MSC and PA QSSMs**

**Apoptosis assay by flowcytometry:** MSCs were incubated with 200 µM C12-HSL for 24 hr and then stained with an antibody against Annexin V to detect apoptosis. After incubation, the cells were harvested by adding 200 µl of trypsin and washed with PBS and another wash was applied using 1x binding buffer (the Annexin V binding buffer is a 10x concentrate composed of a 0.2 µm sterile filtered 0.1 M Hepes (pH 7.4), 1.4 M NaCl, and 25 mM CaCl2 in PBS solution). The cells were incubated with 5 µl of Annexin V staining for 15 mins/ dark/ RT. Another 500 µl of binding buffer were added and the cells were resuspended at 419 xg for 4 mins. Fresh 200 µl of binding buffer were added and 1 µl of PI before flow cytometry running. The excitation/emission for Annexin V is 494/518, and 535/617 for PI. The cells were gated, and standard compensation was applied (Figure 6b and Supplementary figure 5a) (Attune NxT, life technologies).

**Figure 7 a-c:** Effects of C12-HSL QSSM on MSCs culture on uncoated and pPEA coated nanowires. (a) Structure of C12-HSL. (b) Gating strategy for flow analysis allowing for selection of MSC sized cells and viability status attribution. (c) Quantification of viable, early apoptotic, late apoptotic and necrotic MSCs after 24 hour culture on control and test surfaces with and without C12-HSL.

**MTT assay:** MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) assay is used to evaluate the metabolic activity of cells in the presence of C12-HSL at the 50, 200,400,800 µM concentration. After 24 hr incubation the 10 µl of MTT dye solution (5 mg/ml MTT in PBS, pH 7.4) was added to each well of a 96-well plate and incubated for 2 hr. After the incubation, formazan crystals were solubilised with 200 µl DMSO. The absorbance of each well at 550 nm was read on a Dynatech MR7000 microplate reader.

**Sub 7-a:** MTT metabolic assay at 2, 24 and 48 hours of culture with C12-HSL

**LIVE/DEAD staining:** The MSCs cultured with C12-HSL, the cells were washed twice in warm 1x PBS, which was then replaced with live/dead assay reagents (2 μM calcein AM and 4 μM ethidium homodimer-1 in DMEM) and incubated at RT for 15 min in the dark, then imaged using a Zeiss Axiovert fluorescence microscope.

**Sub 7-b&c:** live/dead viability quantification from use of calcein AM and ethidium homodimer fluorescence stain

**JC-1 analysis:** At the end of incubation period, the media contained the C12-HSL removed and a fresh media containing 200 μM of JC-1 (2 μM final concentration) were added to the cells, for the control tube, 1 μL of CCCP (50 μM final concentration) and incubated at 37 °C, 5% CO2, for 30 mins. Followed by single wash with PBS and pellet the cells by centrifugation at 419 xg for 4 mins. The cells resuspended in fresh PBS and analysed on a flow cytometer with 488 nm excitation using emission filters appropriate for Alexa Fluor 488 dye and R-phycoerythrin. The cells were gated, excluding debris using the CCCP-treated sample, and standard compensation was applied.

**Sub 7-d:** JC-1 staining for mitochondrial activity showing gating strategy and % mitochondrial activity with increasing C12-HSL concentration

**Supplementary figure 8:** MSC viability by annexin V and propidium iodide flow cytometry with exposure to different concentrations of C12-HSL

**Data folders: 3D Ti lattices**

**Compression test.** The compression on the micro lattices was performed at the quasi-static rate 0.001 s−1 in an INSTRON machine, with the deformation history recorded by a JAI camera. Note that the bottom of the micro lattice was a plate when manufactured whilst the top was open.

**Figure 8-a&b:** Young’s modules measurements and SEM images for 3D lattices.

**Figure 8-c-e:** SEM and IF images of MSCs cultured on 3D Ti lattices.

**Alamar blue:** The alamar blue solution was mixed 1:10 in DMEM, 900 μl were added to each scaffold and incubated for 6h (37°C, 5% CO2). The supernatant was transferred to a 96 well plate in triplicate (3 x 200 μl) and analysed using a Thermo- Scientific, Multiskan FC. Absorbance was analysed at A1= 570 nm and A2 = 600 nm. % reduction was calculated using the following equation;

Where:

O1 = molar extinction coefficient (E) of oxidized alamarBlue® (Blue) at 570 nm

O2= E of oxidized alamarBlue® at 600 nm

R1 = absorbance of reduced alamarBlue® (Red) at 570 nm

R2= absorbance of reduced alamarBlue® at 600 nm

A1 = absorbance of test wells at 570 nm

A2 = absorbance of test wells at 600 nm

N1 = absorbance of negative control well (media plus alamarBlue® but no cells) at 570 nm

N2 = absorbance of negative control well (media plus alamarBlue® but no cells) at 600 nm

**Figure 8-f:** Alamar blue analysis at 14 days of culture indicating that the pPEA coating has no detrimental effect on viability in 3D.

**Figure 9:** *P. aeruginosa* culture on 3D Ti lattices.

**Statistical Analysis.** All experimental results were interpolated and analysed using GraphPad Prism (GraphPad Software Inc.). Means and standard deviations were calculated, and data were analysed by t test, one- or two-way analysis of variance (ANOVA) test with Tukey’s or Dunnett's multiple comparison post-test. All results are shown in mean ± standard deviation with 95%, 99%, and 99.9% of accuracy (\* P ≤ 0.5, \*\* P ≤ 0.01, \*\*\* P ≤ 0.001, \*\*\*\* P ≤ 0.0001). Replicate details for each experiment are shown in Table 2.

**Table 2: Details of sample replicates and statistical tests used in figures**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Figure | Sub figure | Name | Statistical analysis | Donors | Material replicates | Technical replicates |
| 1 | c | AFM (Ra) | - | - | 4 | 2 |
| 1 | d | WCA | - | - | 4 | 2 |
| 2 | A, b | qPCR | One-way ANOVA | 1 | 4 | 2 |
| 2 | d | Calcein Blue | T-test | 1 | 4 | 15 |
| 3 | b | ELISA | One-way ANOVA | - | 3 | 1 |
| 3 | f | Coating thickness | One-way ANOVA | - | 4 | 1 |
| 3 | g | ELISA | One-way ANOVA | - | 3 | 2,3 |
| 4 | b | Live/Dead bacteria | T-test | - | 3 | 12 |
| 4 | c | ATP release | T-test | - | 4 | 3 |
| 4 | d,e | Metabolomics | - | - | 4 | 1 |
| 5 | e,f | Co-culture | T-test | 2 | 3 | 17,23 |
| 6 | c | Apoptosis (on Ti) | One-way ANOVA | 2 | 3 | 1 |
| 7 | a | Compression test | - | - | 2,3 | 1 |
| 7 | f | AlamarBlue | T-test | 1 | 4 | 3 |
| S2 | - | Metabolomics | - | - | 4 | 1 |
| S4 | a | MTT | One-way ANOVA | 1 | 4 | 3 |
| S4 | c | Live/dead mammalian cells | One-way ANOVA | 1 | 4 | 12 |
| S4 | d | JC-1 | One-way ANOVA | 1 | 4 | 1 |
| S5 | b | Apoptosis (on glass) | One-way ANOVA | 3 | 4 | 1 |