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3D cancer cell culture in high-yield multi-scale scaffolds by shear spinning

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Abstract

Polymeric scaffolds comprising two size scales of microfibres and submicron fibres can better support 3D cell growth in tissue engineering, making them an important class of healthcare material. However, a major manufacturing barrier hampers their translation into wider practical use: scalability. Traditional production of two-scale scaffolds by electrospinning is slow and costly. For day-to-day cell cultures, the scaffolds need to be affordable, made in high yield to drive down cost. Combining expertise from academia and industry from the UK and USA, this study employs a new series of high-yield, low-cost scaffolds made by shear-spinning for tissue engineering. The scaffolds comprise interwoven sub-micron fibres and microfibres throughout as observed under scanning electron microscopy and demonstrate good capability to support cell culturing for tumour modelling. Three model human cancer cell lines (HEK293, A549 and MCF-7) with stable expression of GFP were cultured in the scaffolds and found to exhibit efficient cell attachment and sustained 3D growth and proliferation for 30 days. Cryosection and multiphoton fluorescence microscopy confirmed the formation of compact 3D cell clusters throughout the scaffolds. In addition, comparative growth curves of 2D and 3D cell cultures show significant cell-type-dependent differences. This work applies high-yield shear-spun scaffolds in mammalian tissue engineering and brings practical, affordable applications of multi-scale scaffolds closer to reality.

Introduction

Three-dimensional (3D) instead of 2D cell culturing of mammalian cells has been advocated for decades as a better model for studying *in vivo* 3D cell morphology, physiology, cell-cell interactions and cell-matrix interactions.[1] It is well recognised that 2D cell culture can give misleading and non-predictive results[2] and culturing cells in 3D has demonstrated a dramatic improvement in mimicking *in vivo* cell behaviour. For instance, breast epithelial cells cultured in 3D form acini in the presence of laminin which are able to produce milk *in vitro*,[3] whereas those cultured in 2D do not. To-date, many 3D cell culture platforms have been developed, which can be divided into three categories: multicellular spheroid-based platforms (e.g. hanging drop technique), gel-based 3D culture platforms (e.g. Matrigel and other hydrogels) and polymeric fibre-based platforms (e.g. electrospun nanofibres).[1,4,5]

In particular, polymeric fibres in the micro- and sub-micron scale can mimic the extracellular matrix and support cell proliferation into 3D tissues, making them an important class of healthcare material. However, a major manufacturing barrier hampers their translation into wider practical use in daily healthcare: scalability. Traditional bench-top production methods such as electrospinning are slow (mg/hour) and costly (>£100/kg).[6] To use sub-micron scaffolds for day-to-day cell culture tests, they need to be made affordable, in high yield to drive down cost. Furthermore, despite extensive research into polymer fibrous scaffolds, few high-yield (kg/hour productivity) scaffold production methods can generate both submicron fibre diameters and scaffold thickness above 0.3 mm. The nanofibre-only scaffolds are more akin to a film-like structure and often have pore-sizes smaller than the cell diameter,[7] leaving cells unable to move through the nano-sized pores, preventing 3D cell migration and infiltration beyond the scaffold surface.

In contrast, microfibre at 10 μm length scales can provide cell-size relevant pores for cell infiltration and migration inside the 3D scaffold[8] and their stiff mechanical properties can mitigate cell-adhesion-induced clumping and tearing of the 3D structure. However, scaffolds comprising microfibres at 10 μm length scales no longer mimic the extracellular matrix and do not facilitate cell proliferation as effectively as submicron fibres. Therefore, a strategy to generate scaffolds comprising microfibres uniformly mixed with sub-micron fibres could better initiate cell attachment and cell-scaffold interactions while allowing 3D cell infiltration. An additional advantage of scaffolds made of multiple fibre diameters is that different cell types can have different preference for the scaffold fibre diameter range. For example, oligodendrocytes have been reported to prefer fibres with diameters above 400 nm and more preferentially 2 – 4 μm,[9] whereas fibroblasts were found to prefer submicron fibres.[10]

Various strategies have been tried which result in bi-layered or multi-layered scaffolds with alternating layers of nanofibres and microfibres.[8,11,12] The sub-micron fibres, however, are not truly integrated with the microstructures, as two different technologies are required respectively to produce the nanofibrous and microfibrous features layer by layer, also making the process multi-step, complicated and time-consuming.

Combining expertise from academia and industry from the UK and USA, this work employs a continuous rather than a batch process called shear spinning for tissue engineering,[13] with a remarkable high yield exceeding 10 kg/hour, when other composite scaffold production methods such as electrospinning are struggling with a productivity in gram or milligram per

hour. The technology uses a simple one-step wet-laying procedure to integrate microfibres and sub-micron fibres to generate multi-scale composite scaffolds with readily adjusted scale ratios as required. Fibres of selected size scales generated in the coagulating liquid medium are mixed directly and subsequently condensed by wet-laying and drying to form the composite scaffolds.

Three model human cancer cell lines (HEK293, A549 and MCF-7) were cultured in the scaffolds and found to exhibit efficient cell attachment and sustained 3D growth and proliferation for 30 days. The scaffolds were found easy to handle and infiltrate with cells for incubation. Continuous monitoring of same colonies of different cell lines over 30 days were carried out to investigate cell viability and 3D infiltration inside the scaffolds. Cryosection and multiphoton fluorescence microscopy were carried out to further confirm the formation of compact 3D cell clusters throughout. In addition, comparative growth curves of 2D and 3D cell cultures were developed to understand cell-type-dependent behaviour in 2D versus in 3D. This work applies the high-yield shear-spun scaffolds in mammalian cancer tissue engineering and brings practical, affordable applications of multi-scale scaffolds closer to reality.

Materials and Methods

Fabrication of the shear-spun scaffolds

Shear-spun fibrous scaffolds are received as dry, thick (> 500 μm thick) A4 fibre mat sheets, of interwoven homogeneously entangled PET microfibres with PLA sub-micron fibres (Xanofi Ltd., North Carolina, USA). The 3D scaffold comprises of homogeneously

distributed polyethylene terephthalate (PET) microfibres and polylactic acid (PLA) sub-micron fibres. Shear-spun PLA and PET materials are chosen for their biocompatibility and commercial availability from Xanofi Ltd. The scaffold types are (by weight) 10% PLA sub-micron fibres, 90% PET microfibres; 30% PLA sub-micron fibres, 70% PET microfibres; 50% PLA sub-micron fibres, 50% PET microfibres, and 70% PLA fibres, 30% PET microfibres (Figure 1a-d). The production principle of shear spinning is described in an earlier work and illustrated in Figure 1e.[14] Briefly the polymer solution is extruded into a coagulating liquid medium containing non-solvent(s). The coagulating medium is sheared inside a cylinder that generates an elongational shear force to elongate the polymer solution into fibres of micro, sub-micron or nano-scale diameters. The length and diameter of the produced fibres are controlled depending on the material and process parameters.[15,16]

Preparation and sterilisation of PLA-PET scaffold discs

Shear-spun sub-micron fibre-microfibre scaffolds are provided as A4 size fibre mat sheets, which required puncturing and sterilisation. The fibre mats are punctured into circular discs using hollow stainless steel punches of appropriate diameters, (1.4 cm and 0.7 cm for 48-well and 96-well plates respectively). To sterilise the scaffolds, they are incubated with 10 mg/ml streptomycin and 10000 U/ml penicillin overnight, washed thoroughly with PBS and dried. The sterile scaffold plates are sealed and stored in a clean plastic box in a 4 °C cold room for future use.

Cell culture

A549 human lung cancer, MCF-7 human breast cancer and HEK 293 human embryonic kidney cells are grown in Dulbecco's Modified Eagle's Medium (DMEM) with 2 mM L-

glutamine (Lonza). All media are supplemented with 10% foetal bovine serum (FBS) (Hyclone) and 0.1 mg/ml streptomycin and 100 U/ml penicillin (Lonza). Media used to grow cells in the scaffolds are also supplemented with 0.25 μg/ml amphotericin B to prevent fungal contamination. Cells are grown in 37 °C humidified incubators at 5% CO_2.

Generation of stable cell lines

To generate stable cell lines with nuclear localised GFP expression, a lentiviral expression system for mammalian cells is used. The recombinant lentiviruses directing the expression of nuclear localised GFP are produced based on a method described previously.[17] To transduce cells, recombinant lentiviruses are added to cells together with 2 μg/mL polybrene to enhance viral infectivity. 24 hrs post-transduction, the medium containing lentiviruses is replaced with selective medium containing 1-2 μg/mL puromycin, which is changed every 3-4 days for a period of two weeks. After confirming recombinant protein expression using fluorescence imaging, the selected cells are used as a stable cell line. Then cells are amplified and cryopreserved in DMEM medium containing 40% FBS and 10% dimethyl sulfoxide and stored in liquid nitrogen.

Cell seeding in PLA-PET scaffolds

Prior to cell seeding, sterile PLA sub-micron fibre and PET microfibre scaffolds are incubated for 24 hrs with culture medium to fully soak the scaffolds and remove trapped air. When cell suspensions are prepared, they are passed through 40 μm cell strainer (BD Biosciences) to remove large cell clusters. Figure 1f summarises the main steps of cell seeding. 10% PLA – 90% PET scaffolds are used for cell growth analysis by seeding 1.0×10^5/well in a 48-well plate and cultured for 20 days. For monitoring the cell growth of 3D cell

clusters/colonies over a long period of time, low cell number (1000-3000 cells/well in 96-well plate) is seeded in PLA-PET scaffolds respectively with 10% PLA and 30% PLA and cultured for up to 30 days. Once the cell culture in scaffolds is no longer maintainable by the highest medium volume the well can hold (e.g. 250 µl/well for 96-well pale), they are transferred to a larger well size (24 well-plates) and supplied with more medium (> 2 ml per sample per well, refreshed every 3 days). Cells in scaffolds are grown at 37 °C and 5% CO_2 in a cell culture incubator.

Cryosectioning analysis of 3D cultures

Scaffolds are removed from the medium and washed with PBS. Cells in scaffolds are fixed with 4% paraformaldehyde (PFA) at 4 °C overnight and then washed with PBS. Prior to sectioning the fixed scaffolds are incubated with 20% sucrose at room temperature. Scaffolds are embedded in Tissue-Tek® optimum cutting temperature O.C.T ™ Compound (Sakura Finetek). The samples are frozen at -20 °C for 30 min and equilibrated to -15 °C inside the cryostat (Leica CM1850) for 30 min prior to cutting. Sections are cut at 30 µm thicknesses, mounted and dried on Superfrost glass slides (VWR) for 30 min at room temperature. Phase contrast and fluorescent images are taken at 5× and 20× magnifications.

Analysis of cell attachment efficiency to the scaffold

For the initial testing of cell attachment, high cell number of GFP-HEK293 cells (1×10^5 cells/well in 96-well plate) is seeded in a panel of PLA-PET scaffolds with different sub-micron fibre : microfibre ratios: 10% : 90%, 30% : 70%, 50% : 50% and 70% : 30%. After 2 days in culture, the scaffolds are removed and fluorescent images are taken of the plate wells' bottom surfaces. To determine the optimal sub-micron fibre : microfibre ratio for efficient

attachment, an indirect method is used to measure the cell number of the attached cells. This is because counting the cell number directly in the scaffold is not possible or accurate due to the opaque, 3D nature of the culture. PLA-PET scaffolds cut into the shape of discs, with a disc diameter of approximately 1.4 cm, are placed in a 48-well plate and incubated with medium for 24 hrs. High cell number (2.5×10^5 cells/well in 48-well plate) of three cell lines HEK293, MCF-7 and A549 are seeded in the panel of PLA-PET scaffolds and incubated for 18 hrs. The cells which infiltrated and attached to the scaffolds are estimated from the initial number seeded after subtracting those that left the scaffold and attached to plate surface. The optimal PLA sub-micron fibre : PET microfibre ratio was determined based on three criteria: 1) high attachment efficiency, 2) thorough infiltration, and 3) adequate internal volume for cell growth.

Immunofluorescence staining analysis

The 3D cell culture in shear-spun scaffold is investigated with immunofluorescence staining of E-cadherin, a marker of cell-cell interaction and F-actin filaments. After fixing 3D cell cultures with 4% PFA, the cultures are permeabilised and blocked at the same time for 60 min with 0.3% Triton X-100 and 5% (w/v) BSA at room temperature. Then samples are incubated with primary anti-E-cadherin antibody (1 : 200 dilution, Cell Signaling, USA) at 4 °C overnight, followed by applying the secondary anti-rabbit Alexa Fluor® 555 conjugated IgG antibody (1 : 500 dilution, Cell Signaling, USA) for 1-2 hrs. All incubations are done on a rocker in the dark and samples are washed three times with PBS for 10 min after each step. The nuclei are already labelled by stable expression of nuclear localised GFP. After the final washing step, the samples are mounted on a glass slide and observed using multiphoton SP8 confocal microscope (Leica Microsystems, UK). The F-actin filaments are stained by

phalloidin-TRITC (1 : 200 dilution, Sigma, USA) for 1-2 hrs at room temperature and the other steps are as mentioned above except the secondary antibody step.

Multiphoton fluorescence microscopy

Samples are imaged on a Leica SP8 confocal microscope (Leica Microsystems, UK) with Coherent Chameleon Vision II Multiphoton Laser (Coherent UK Ltd). Image acquisition is performed with 25× 0.95NA water immersion objective at 870 nm with two photon excitation. The 3 fluorescent signals are simultaneously acquired using the internal confocal PMT and HyD detectors (autofluorescence of the PET microfibres: 415 – 470 nm, GFP nuclei: 500 – 550 nm, red signal for F-actin filaments or E-cadherin: 590 – 690 nm). Z-series are performed at 1 μm intervals to depths of approximately 100 μm with an imaging average of 3 frames. Z-projections are performed using the Leica software and images are saved in tif format. Additional x,z-, y,z-, orthogonal-projection images and 3D movies are performed using Imaris 3D software (BitPlane AG, EU).

Scanning Electron Microscopy (SEM)

Scanning electron microscope (SEM, model: FEI Nova NanoSEMTM) is used to image scaffold samples. Each SEM sample is coated with gold using a sputtering machine (Emitech K550, Emitech Ltd, UK) for 120 s prior to observation. The coating is approximately 15 nm in thickness. Gun emission is set to 5 – 10 kV. The micrographs are analysed using ImageJ, a public domain Java image-processing program.

Cell viability assay

Cell viability in shear-spun scaffolds is examined using LIVE/DEAD® Viability/Cytotoxicity Kit (Invitrogen). 10^4 cells are cultured in shear-spun scaffold composed of 30% PLA sub-micron fibres and 70% PET microfibres for 10 days. Viable and dead cells are live stained with 1 μM calcein-AM and 2 μM ethidium homodimer-1 (EthD-1), respectively and incubated with the stains for 30 min. As a positive control for EthD-1 and negative control for calcein-AM staining, control cells are treated with 70% methanol for 30 min to achieve 100% death prior to live cell staining. The cultures are observed with fluorescence microscopy.

Comparative growth curves analysis

The number of viable cells is assessed by CellTiter 96® AQueous One Solution Cell Proliferation assay. The metabolically active cells bio-reduce a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, (MTS)]. The product is a dark formazan compound and its quantity is directly proportional to cell number when its absorbance is measured at 490 nm. Cells bioreduce MTS solution at different rates in 2D cultures and 3D scaffolds and as a result growth curves cannot be compared using the absorbance values. Therefore, the fold change relative to day 2 is calculated to compare growth curves in 2D and 3D cultures.

4.0×10^3 cells of each of HEK293, MCF-7 and A549 cell lines are seeded in 2D cell culture plates and in 30% PLA and 70% PET scaffolds. All culturing is carried out in 96-well plates. One day post-seeding, the scaffolds are transferred to new wells to ensure that only the cells growing in the scaffold are being detected. Cells are cultured for 2, 4, 6, 8 and 10 days and

medium is changed for all plates every 2-3 days. The experiment is carried out in quadruplicate and repeated four times. At each time point, the medium is replaced with 40 µl of MTS solution diluted in 200 µl of fresh medium per well. After the addition of MTS solution, cultures are incubated for 1 hr (A549), 2 hrs (MCF-7) and 4 hrs (HEK293) at 37 °C humidified incubators with 5% CO_2. After incubation, 120 µl of the incubated medium is transferred to a new 96-well plate and the formazan absorbance is measured at 490 nm in a microplate reader (Magellan™, Tecan).

Statistical analysis

The porosity of scaffolds with various percentage ratios of sub-micron fibres and microfibres are characterised. Specifically, the mass of a dry scaffold, m, is measured using a digital balance. The width, w, and length, L, of the scaffold are measured using digital calipers. The thickness of the scaffold (h) is imaged by SEM. Three measurements are taken per sample and the average value is recorded. The porosity is calculated using the following equation 1:

$$\text{Porosity (\%)} = (1 - ad/bd) \times 100 \qquad (1)$$

where, ad is the polymer mat's apparent density (gcm^{-3}), calculated as $ad = m / (h \times w \times L)$; bd is the bulk density of pure amorphous polymer(s) prior to fibre formation. The amorphous densities of the polymers are provided by the supplier Xanofi Ltd, respectively 1.24 g cm^{-3} and 1.38 g cm^{-3} for PLA and PET polymers.

The doubling time of cell number between two time points is calculated using the following equation 2:

$$\text{Doubling time (hrs)} = (t_2 - t_1) \times [\log 2 / \log(n_2/n_1)] \qquad (2)$$

Where n_1 is the cell number at time t_1 (hrs) and n_2 is the cell number at time t_2 (hrs), assuming a constant growth rate. The absorbance measurements obtained for the growth curve analysis are used to determine n_1 and n_2 because the absorbance of formazan product is directly proportional to cell number.

Data from the fibre diameter distribution graphs and porosity percentages are expressed as the mean ± standard deviation (SD) of three independent experiments. Data from cell attachment efficiency and cell proliferation experiments are expressed as the mean ± standard error of the mean (S.E.M) of three or four independent experiments. Statistical significance is determined by a two-tailed student's t-test, (* $p < 0.05$).

Results and discussion

Characteristics of shear-spun integrated sub-micron fibre-microfibre scaffolds

Figure 1a-d presents an overview of the shear-spun scaffolds with varying compositional ratios of the PLA submicron fibres and PET microfibres, showing the general distribution of PLA versus PET fibres. The sub-micron fibre diameters used in the shear-spun scaffolds are close to those of ECM fibrils. Figure 2a-d presents SEM images showing front and cross-sectional views of the four types of shear-spun multi-scale scaffolds employed. The fibre scaffolds are thick and made of interwoven microfibres and sub-micron fibres, approximately 0.5 mm in thickness and with an average fibre of 12±1 μm for PET and 820±410 nm for PLA (Figure 2e). Figure 2e1 and e2 also respectively show the chemical structures of PLA and PET polymers. The sub-micron fibres and microfibres are randomly oriented and tangled together, especially at microfibre cross-junctions. Scaffolds comprising only submicron fibres have pore sizes smaller than cell diameter and do not facilitate 3D cell migration and

infiltration.[7] Therefore, microfibres are incorporated in the scaffold to form a two-scale architecture to provide structural support and allow infiltration and migration of cells.[8,11,12]

Cell growth pattern in 2D and 3D cell cultures

Three human cell types with different growth patterns are used in this study: lung cancer A549 cells which are highly invasive and exhibit migratory behaviour;[18] weakly invasive breast cancer MCF-7 cells and immortalised human embryonic kidney HEK293 cells.[19] To aid visualisation of cells in the scaffold, a panel of stable cell lines expressing nuclear localised GFP (NLS-GFP) is generated. Figure 3a-i show fluorescent, phase contrast and merged images of A549, MCF-7 and HEK293 cells with stable expression of NLS-GFP (Figures 3a-c, d-f and g-i, respectively).

To explore the cell growth pattern in the 3D scaffold, the three cell lines are cultured for 20 days in a shear-spun 10% PLA sub-micron fibre and 90% PET microfibre scaffold. Cells in tested 3D scaffolds exhibited similar cell growth patterns as in 2D cell culture; HEK293 and MCF-7 cells formed compact colonies/clusters whereas, the highly invasive A549 cells scattered along the microfibres (Figure 3k-m). This indicates that the 3D scaffold allows cells to exhibit their physiological behaviour without imposing geometrical confinement. Additionally, the compact cell clusters are denser at microfibre cross-junctions where networks of PLA fibres are located, resembling “bird nests”. One such PLA submicron fibre cluster is illustrated in Figure 3j (black arrow). This observation suggests preference for the PLA sub-micron fibres where cells attach, grow and then migrate outwards along the microfibres. This observation is consistent with the literature; cells attach better, spread and form more focal points on nanofibrous structures than microfibres.[8] Note that the “bird nest”

fibre clusters can only be observed in scaffolds with lower composition (10%) of the submicron fibres; we have used the 10% submicron fibre composition to better distinguish the cell-fibre interaction between the two fibre size scales. Scaffolds with 30-70% submicron fibres show uniform interwoven distribution between the multi-scale fibres with no clustering (Figure 2).

Growing large cell colonies in shear-spun 3D scaffold

To monitor growth of HEK293 cells over a long period of time, a low cell number is seeded in a 10% PLA sub-micron fibre and 90% PET microfibre scaffold to achieve single and well separated cells. It is observed that individual HEK293 cells or a cluster of several cells formed small colonies within the scaffold during 14 days in culture, as shown in Figure 4m-p. The expansion of a HEK293 cell colony is monitored for additional 14 days (Figure 4q-t). This experiment is repeated but with the three cell lines cultured in PLA-PET scaffold with 30% sub-micron fibre : 70% microfibre ratio instead of 10% sub-micron fibres : 90% microfibres ratio. All three cell lines form cellular colonies which keep growing during the 30 days of culturing (Figure 4a-i). In contrast to HEK293 and MCF-7 cells, A549 cells form less compact colonies which do not increase significantly in size after 20 days in culture.

The 3D cell culture inside the scaffold

To confirm the 3 dimensionality of the cell culture inside shear-spun scaffold, a protocol is developed for preparing cryosections of the scaffold with cultured mammalian cells. The 3D cell culture of HEK293 grown in a PLA-PET scaffold for 27 days (Figure 4t) is used to cut 30 μm cryosections. The cryosectioning analysis confirmed the three-dimensional growth of cells, as illustrated by the phase and fluorescent images at 5× and 20× in Figure 4u-v and w-

x, respectively. Immunofluorescence staining of E-cadherin is also performed to show the cell-cell interactions in the 3D culture. MCF-7 cells are grown in PLA-PET scaffold for 15 days and stained for E-cadherin (Figure 5). The presence of E-cadherin protein between the plasma membranes of neighbouring cells affirms their direct cell-cell interactions. Few cells did not show E-cadherin at their periphery (indicated by arrowheads in Figure 5). This is because cells which are in the process of cell division lose their contact with neighbouring cells.

If low cell number is used, the cell cultures in the 3D scaffold are patchy, with empty spaces between cell clusters. Thick continuous 3D cell culture throughout the scaffold can also be accomplished by saturating the scaffold with a high cell number ($>10^6$ in 96-well plate). To visualise cultured cells in a 3D PLA-PET scaffold, multiphoton fluorescence microscopy is utilised which has the ability to image samples up to 1 mm in thickness. Figure 6 shows that the cells are able to infiltrate and grow inside the scaffold. Supplementary Video 1 shows the distribution of cells at different depths, as the xy focal plane moves down through the scaffold, as well as spatial distribution by moving crossections in other orthogonal planes. In addition, depending on the scaffold topology, interconnected clusters were found resembling 3D cell culture across the scaffold, reaching up to approximately 80 μm in thickness. This is mostly found in areas with relatively large pores where cells can infiltrate and accumulate to form 3D cell clusters, as shown by 3D side view and orthogonal images in Figure 6e-i. It was possible to stain F-actin filaments across the thickness of the 3D culture (Figure 6b and f), indicating that the immunofluorescence staining can be applied directly to a 3D culture in a scaffold. This is a less invasive and more appropriate method for 3D cell culture analysis than using immunocytochemistry which involves sectioning and then staining; affecting the true structure of 3D culture in the scaffold.

All the above cell growth analyses confirm the suitability of shear-spun integrated sub-micron-microfibre scaffold for 3D cell culturing. The scaffold provides the third dimension for cells to form 3D cell clusters/colonies, resembling *in vivo* conditions. The vast majority of fibrous scaffolds which are developed and used for 3D cell culturing are comprised of nanofibres only or have film-like structure and, as a result, cells grow only on the top surface of the scaffold. Such scaffolds lack a true, third dimension and the 3D cell growth is limited. As discussed before, few groups have demonstrated the usefulness of combining nanofibres with microfibres for cell growth and functionality and we have shown that uniformly interwoven sub-micron-microfibre scaffolds can promote 3D cell growth.

The optimal sub-micron fibre to microfibre ratio for well-balanced 3D cell culture is determined

In general, the ideal 3D scaffold with the optimal sub-micron fibre : microfibre ratio should fulfil three criteria: 1) high attachment efficiency, 2) minimum pore size to allow cell infiltration and 3) adequate internal volume for well-balanced 3D cell growth. Achieving high cell attachment efficiency by a sub-micron fibre-microfibre scaffold is an important feature for cell-based assays e.g. cell proliferation, especially when seeded cell number needs to be tightly controlled. To investigate this, a panel of shear-spun PLA-PET scaffolds with different sub-micron fibre to microfibre ratios are used (Figure 1a-d, Figure 2). To vary the sub-micron fibre to microfibre ratios in the scaffolds, the mass of PET microfibres is kept the same and the mass of PLA sub-micron fibres is increased to achieve 10%, 30%, 50% and 70% sub-micron fibre content (by weight).

The outcome clearly demonstrates that the increase in the sub-micron fibre to microfibre ratio in the PLA-PET scaffolds results in better attachment or trapping of GFP-HEK293 and GFP-A549 cells to the scaffold (Figure 7a). This effect is less obvious for GFP-MCF-7 cells that show very efficient attachment to the scaffold even with the lowest sub-micron fibre : microfibre ratio (1:9). This may be because MCF-7 cells express higher levels of integrin receptors than A549 cells,[20] allowing MCF-7 cells to bind promptly even to microfibres alone.

Porosity analysis of the scaffolds is performed to ensure that the increased mass of PLA sub-micron fibres does not reduce the scaffold porosity significantly and hence does not reduce the scaffold internal volume. PLA sub-micron fibres are puffy material compared with the stiff PET microfibres, so addition of PLA also increases the thickness of the scaffolds, which compensates for the increase in mass, keeping porosity almost constant. Porosity (%) = $(1-ad/bd) \times 100$, where *ad* is the polymer mat's apparent density (g cm^{-3}), *bd* is the constant, amorphous density of bulk polymers. Since apparent density, *ad* = *mass* / *volume,* and upon addition of puffy sub-micron fibres mass is increasing and volume is also increasing, the resulting *ad/bd* remains relatively constant, and a similar porosity is observed (69±2%, see Figure 7b and Methods) for scaffolds with 0-50% PLA (up to 50% PLA sub-micron fibre content). At 70% PLA, porosity decreases to 58% as a result of the increase in mass overtaking the compensating increase in volume thickness.

It has been reported that the optimal pore size can vary from 5-500 µm for cell attachment and migration, depending on the cell type.[21] Cells can only pass through pores with sizes similar or greater than the diameter of the cell. However, when the pores in a scaffold are very big, cells can fall through the scaffolds and attach to the plate surface (Figure 7f). As the

percentage of PLA sub-micron fibres increases, the effective pore sizes are smaller and fewer cells fall through the scaffold (Figure 7c). An indirect strategy is followed to quantify the cell attachment percentage to the scaffold because cell retrieval from sub-micron fibre-microfibre scaffold (e.g. trypsinisation) is not efficient. Accordingly, after seeding a known high cell number, the scaffolds are removed and the cells attached to the plate surface are counted. This number is subtracted from the initial seeding number in order to obtain the number of cells attached to the scaffold.

Although the highest PLA sub-micron fibre : PET microfibre ratio (7:3) accomplished the highest attachment efficiency for all tested cell lines, cells grow mainly on the surface and fail to infiltrate the scaffold (data not shown); the material also has the lowest percentage porosity (58%) among all scaffold types tested. Similarly, the second highest PLA sub-micron fibre : PET microfibre ratio (1:1) achieved slightly better in terms of cell attachment efficiency than the 30% PLA sub-micron fibre : 70% PET microfibre ratio. However, the presence of high amount of PLA sub-micron fibre (50%) in the scaffold makes the scaffold dense, which reduces cell migration and infiltration. Therefore, the optimal and well-balanced PLA-PET scaffold is determined to comprise 30% PLA sub-micron fibres and 70% PET microfibres.

Cell viability in shear-spun scaffolds

For a sub-micron fibre-microfibre scaffold to be suitable for cell culture, it is important that it does not have cytotoxic effect on cell viability. The effect of PLA-PET scaffolds on the cell viability of three cell lines is assessed using a LIVE/DEAD viability/cytotoxicity kit (Invitrogen). Cells are cultured in the scaffold for 10 days prior to Live/Dead staining. Green

fluorescence reveals viable cells due to hydrolysis of calcein-AM by ubiquitous intracellular esterases, whereas dead cells associated with disintegrated membranes fluoresces red due to binding of ethidium homodimer-1 (EthD-1) to DNA. Methanol treatment kills all cells in the culture and, therefore, it is used as a negative and positive control for calcein-AM and EthD-1 staining, respectively. 2D cell cultures typically become confluent in 3-4 days, whereas Figure 8 demonstrates that the 3D PLA-PET scaffold shows cells with an estimated 95% viability after culturing for 10 days. This is likely to the cells being able to adhere to the scaffold, and the extra space the scaffold provides for growth, indicating the suitability of these 3D scaffolds for culturing mammalian cells.

Growth curve analysis in 2D and 3D cell models

The growth curves of HEK293, A549 and MCF-7 cells are compared between 2D tissue culture plates (2D culture) and 3D scaffolds with 30% PLA sub-micron fibre : 70% PET microfibre. Cell proliferation is measured over the course of 10 days using the MTS CellTiter 96® Aqueous One Solution assay (Promega). Figure 9 indicates that the pattern of growth in 3D and 2D cell cultures depends on the cell type. Furthermore, recent results using cellulosic scaffolds instead of PLA-PET scaffolds (unpublished observations) indicate that the growth pattern of different cell lines is also dependent on the material composition of the scaffold. After 6 days in culture, the growth of HEK293 cells in 3D scaffolds overtakes those growing in 2D; the difference is statistically significant at day 10 ($*p < 0.05$) (Figure 9a). Similarly, for the first 6 days, the growth of MCF-7 cells in 3D scaffold is the same as those in 2D; from day 8-10, cell growth significantly increased in the 3D scaffold compared to the 2D culture ($*p < 0.05$); the difference in cell number is nearly double at day 10 (Figure 9c). On the other hand, the growth curves for highly invasive A549 cells are comparable in both models

(Figure 9b). It is interesting to note that when cultured in cellulosic 3D scaffolds in another study (unpublished observations), A549 cell growth was slightly slower in 3D than 2D. This indicates that, based on promoting cell growth, the PLA-PET material is more suitable for A549 cell line than the cellulosic materials.

Based on the growth curves, the doubling time was calculated between different time intervals during the exponential growth, for up to day 6 (Table 1). Between 2 – 4 days, the doubling times of HEK293 and MCF-7 cell lines are longer in the 3D scaffolds (26.6 hrs and 48.8 hrs, respectively) than those in 2D cultures (23.6 hrs and 39.3 hrs, respectively). This may indicate that cells required a longer time to acclimatise to the scaffold before commencing cell proliferation. Between 4 – 6 days, HEK293 doubling time has become shorter in 3D scaffold (19.3 hrs) than in 2D culture (25.4 hrs). Cells naturally slow their doubling times when they reach high densities. This first occurs in 2D where there is less space for cells to divide. The doubling time of MCF-7 cells in 3D remained the same about 49 hrs until day 6, whereas in the 2D model it slowed to 73.7 hrs. In addition, the doubling times of A549 cells have remained the same (33.2 hrs) in both 2D and 3D models between 2 – 4 days, and slowed down between 4 – 6 days to 81.6 hrs in 3D and 87.9 hrs in 2D culture.

Other groups have reported the doubling time of HEK293, A549 and MCF-7 cells in 2D tissue culture plates to be 24 hrs, 27 hrs and between 32-48 hrs, respectively.[18,22,23] The calculated values for 2D culture are in agreement with these reports. It is known that the doubling time becomes slower gradually as cells increase in number and less space remains available for growth. This is observed for all three of the cell lines in 2D culture. On the other hand, for 3D cultures, the doubling time remained steady, signalling they provide additional space for cells to grow. This has led to a significant increase in cell number in 3D culture that exceeded the 2D culture for HEK293 and MCF-7 cells. The highly invasive A549 cells are an

exception here, perhaps since they limit their growth rate once they have a couple of neighbours to form a network, thus effectively reducing the effect of extra dimensionality for increased growth rate.

Most cancers are heterogeneous, containing sub-populations which are thought to cooperate together to promote tumour growth and progression via paracrine or juxtacrine crosstalks.[24] Chen *et al*. has reported that MCF-7 cells display continued proliferation in 3D collagen scaffolds resulting in much larger number of cells in comparison to 2D cultures. This is in agreement with the findings in the 3D shear-spun scaffolds. Chen *et al*. have also found that in breast cancer MCF-7 cell populations, the stem cell fraction is enriched when grown in 3D collagen scaffolds.[25] The same finding is also seen when MCF-7 cells are cultured on a thin layer of electrospun polycaprolactone-chitosan nanofibre scaffolds.[26] Growing MCF-7 cells in shear-spun integrated multi-scale scaffolds provides enough space for growth and mobility and could perhaps resulted in similar enrichment of certain cell sub-population (e.g. stem-like cells) that promote cell growth, a point which requires further investigation.

Conclusions

This study demosntrates the potential of shear-spun multi-scale scaffolds as a promising, high-yield, and affordable platform for 3D cell cultures. The scaffolds are generated with high productivity above 10 kg/hour to manufacture and integrate multi-scale submicron fibres and microfibres in one-step to form a truly 3D structure with > 300 μm thickness. They are found to provide uniform sub-micron fibres throughout to promote cell attachment, and an overall microfibrous architecture to effectively support 3D growth and migration. This work paves the way towards breaking one of the major translational barrier in the practical

application of 3D tissue engineering: the lack of affordable, high-yield, commercially available scaffolds with demonstrated effectiveness in 3D cell culture. Future work will further characterise and develop high yield scaffolds with innovative functions from a materials science perspective and explore their application in cell biology and high throughput screening assays for drug discovery programmes.

Acknowledgments

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6. Conflict of interest

SS declares he is one of the inventors of the shear spinning method and is on the board of directors of Xanofi Inc, which is commercialising it.

Tables

Table 1: Cell doubling time in 2D and 3D cultures during growth

Cell line	Time Interval (days)	2D Culture (hrs)	3D Culture (hrs)
HEK293	2 – 4	23.6±4.9	26.6±6.7
	4 – 6	25.4±2.8	19.3±1.8
A549	2 – 4	33.2±4.1	33.2±8
	4 – 6	87.9±12.9	81.6±10.5
MCF-7	2 – 4	39.3±4.7	48.8±5.1
	4 – 6	73.7±3.3	49.5±9

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Figures

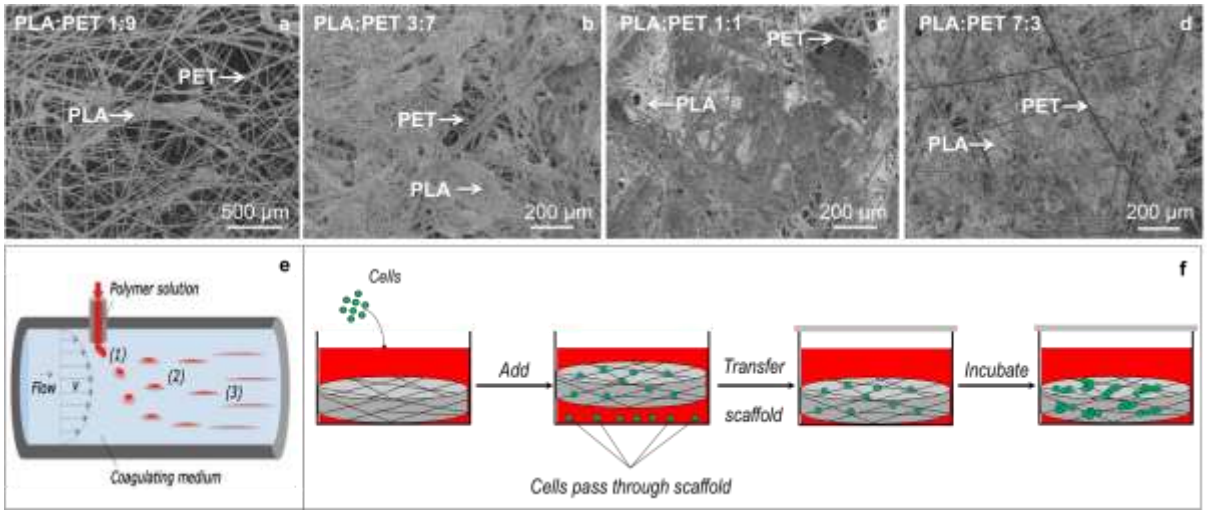

Figure 1. (a-d) SEM images showing overview of scaffolds with different compositions of PLA submicron fibres and PET microfibres. (e) Schematic diagram illustrating shear-spun fibre production principle: (1) The polymer solution is infused into coagulating medium that undergoes rapid flow to provide shear. (2) The polymer solution breaks into droplets, which become exposed to shearing force and break up into smaller elongating droplets. (3) As the solvent of the droplets exchanges with the nonsolvent in the coagulating medium, the elongating droplets solidify to form micrometre, sub-micron or nano- fibres depending on the materials and process parameters. (f) Schematic diagram illustrating cell seeding in shear-spun sub-micron fibre-microfibre scaffold.

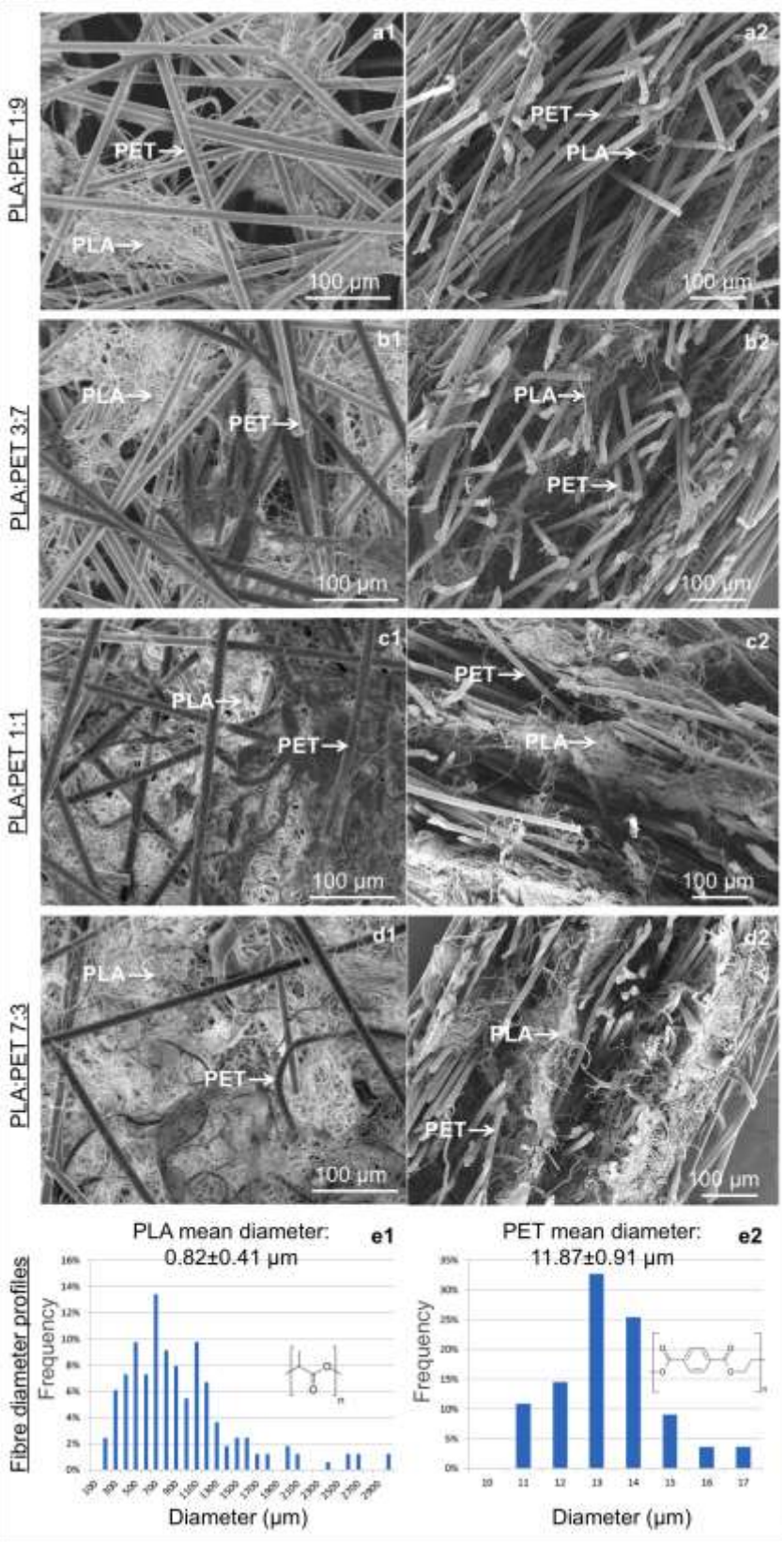

Figure 2. (a-d) Scanning electron microscope (SEM) images showing (1) front view, (2) cross-sectional view of scaffolds of varying PLA and PET compositions: (a1-2) 10 wt% PLA

and 90 wt% PET (PLA:PET 1:9); (b1-2) 30 wt% PLA and 70 wt% PET (PLA:PET 3:7); (c1-2) 50 wt% PLA and 50 wt% PET (PLA:PET 1:1); and (d1-2) 70 wt% PLA and 30 wt% PET (PLA:PET 7:3). (e1-2) Fibre diameter distribution profiles of (e1) PLA sub-micron fibres and chemical structure, and (e2) PET microfibres and chemical structure. Scale bars: 100 μm.

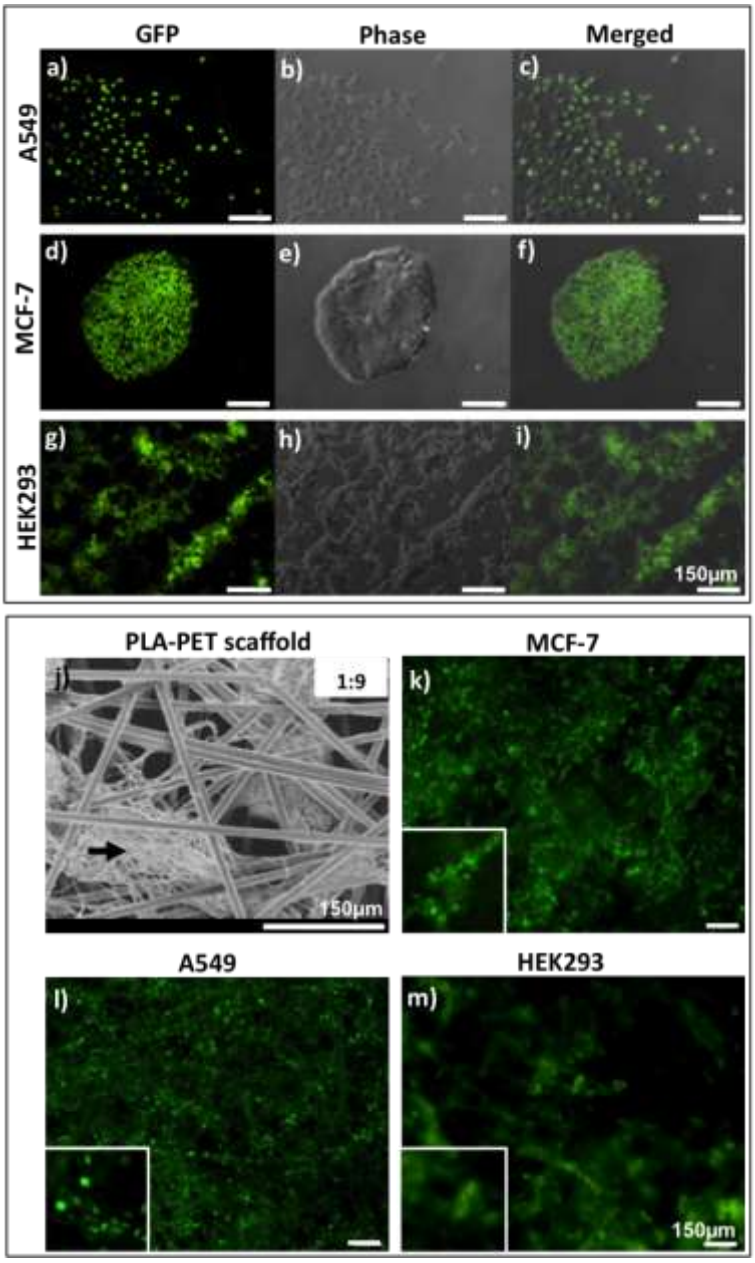

Figure 3. Cell growth of different human cancer cell lines in 2D culture plates and 3D PLA-PET scaffolds. (a-i) Fluorescent, phase and merged images of (a-c) A549 human lung cancer, (d-f) MCF-7 human breast cancer and (g-i) HEK293 human embryonic kidney stable cell

lines expressing nuclear localised GFP (NLS-GFP) generated using lentiviral expression system. (j) SEM image of 1:9 PLA-PET scaffold; black arrow indicates a cluster of PLA sub-micron fibres (bird's nest). Such clusters can only be observed in scaffolds with 10% PLA submicron fibres; scaffolds with 30-70% submicron fibres show uniform interwoven distribution between the multi-scale fibres with no clustering. (k-m) Fluorescent images showing cell growth pattern at 20 days in 1:9 PLA-PET scaffolds using (k) GFP-MCF-7, (l) GFP-A549 and (m) GFP-HEK293 cell lines. Cells were seeded at 1.0×10^5 cells/well in 48-well plates. Scale bars: 150 μm.

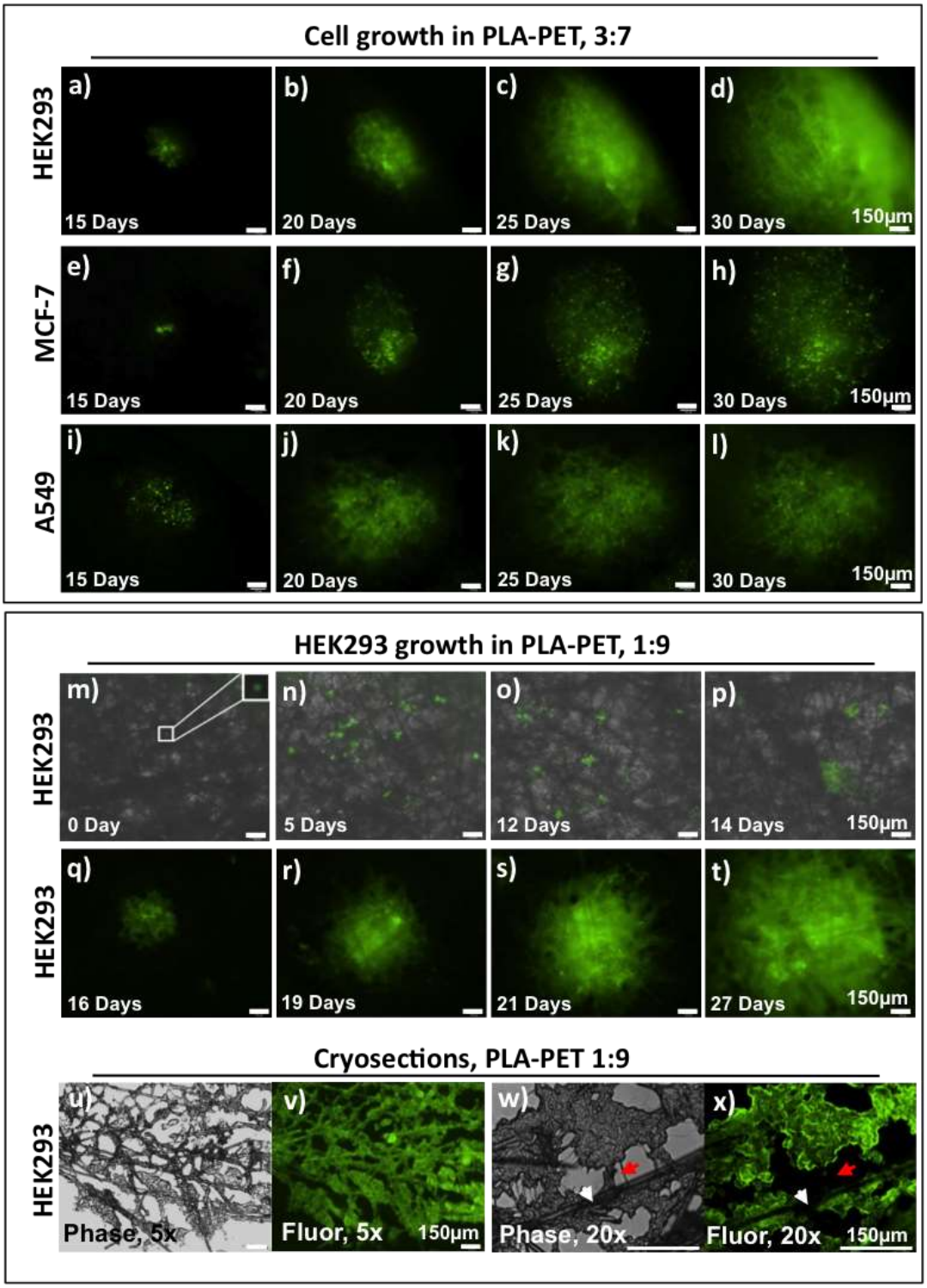

Figure 4. (a-l) Fluorescent images showing 30-day continuous monitoring of time-dependent growth of the same colonies in 3:7 PLA-PET scaffolds: (a-d) HEK293, (e-h) MCF-7 and (i-l) A549. A low cell density per cell type was seeded ($1x10^3$ cells/well in 96-well plate) to observe long-term colony development. (m-t) GFP-HEK293 cells seeded at low density

($2.5x10^3$ cells/well in 96-well plate) in 1:9 PLA-PET scaffolds. (m-p) Merged fluorescent and phase images showing formation of defined colonies from single or cluster of cells after 14 days in culture. (q-t) The fluorescent images show the time-dependent growth of an individual colony of GFP-HEK293 cells, monitored from day 16 – day 27 of culture. **(u-x)** Cryosection analysis of HEK293 3D cell culture in PLA-PET scaffold. Fluorescent and phase images showing cryostat sections of 3D cell clusters/colonies in 10% PLA sub-micron fibres and 90% PET microfibres scaffold at (u-v) 5× magnification and (w-x) 20× magnification. The 3D cell cultures from (q-t) at day 27 are used to cut 30 µm cryosections. Red arrows indicate locations of sub-micron fibres and white arrows indicate microfibres. All scale bars: 150 µm.

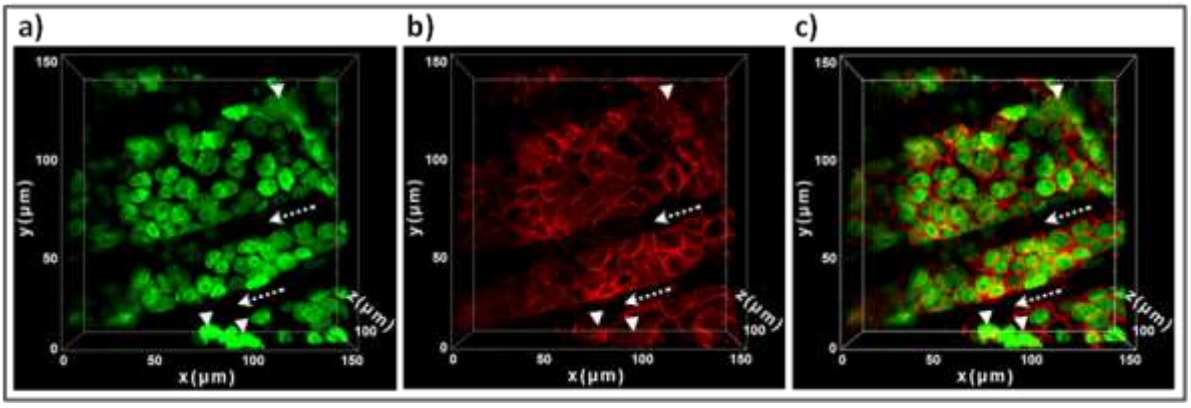

Figure 5. Cell to cell interaction and active division in shear-spun PLA-PET scaffold. (a-c) 3D view images of (a) nuclei (green), (b) E-cadherin (red) and (c) merged. Dotted arrows indicate the PET microfibres and arrowheads indicate actively dividing cells, which do not have E-cadherin at their periphery. GFP-MCF-7 cells ($4×10^3$ cells /well in 96-well plate) were seeded in 30% PLA sub-micron fibres and 70% PET microfibres scaffold and cultured for 15 days. 3D cultures were fixed and then immunofluorescently stained for E-cadherin. Images were taken and analysed using multiphoton confocal microscope and Imaris 8.0.1 software.

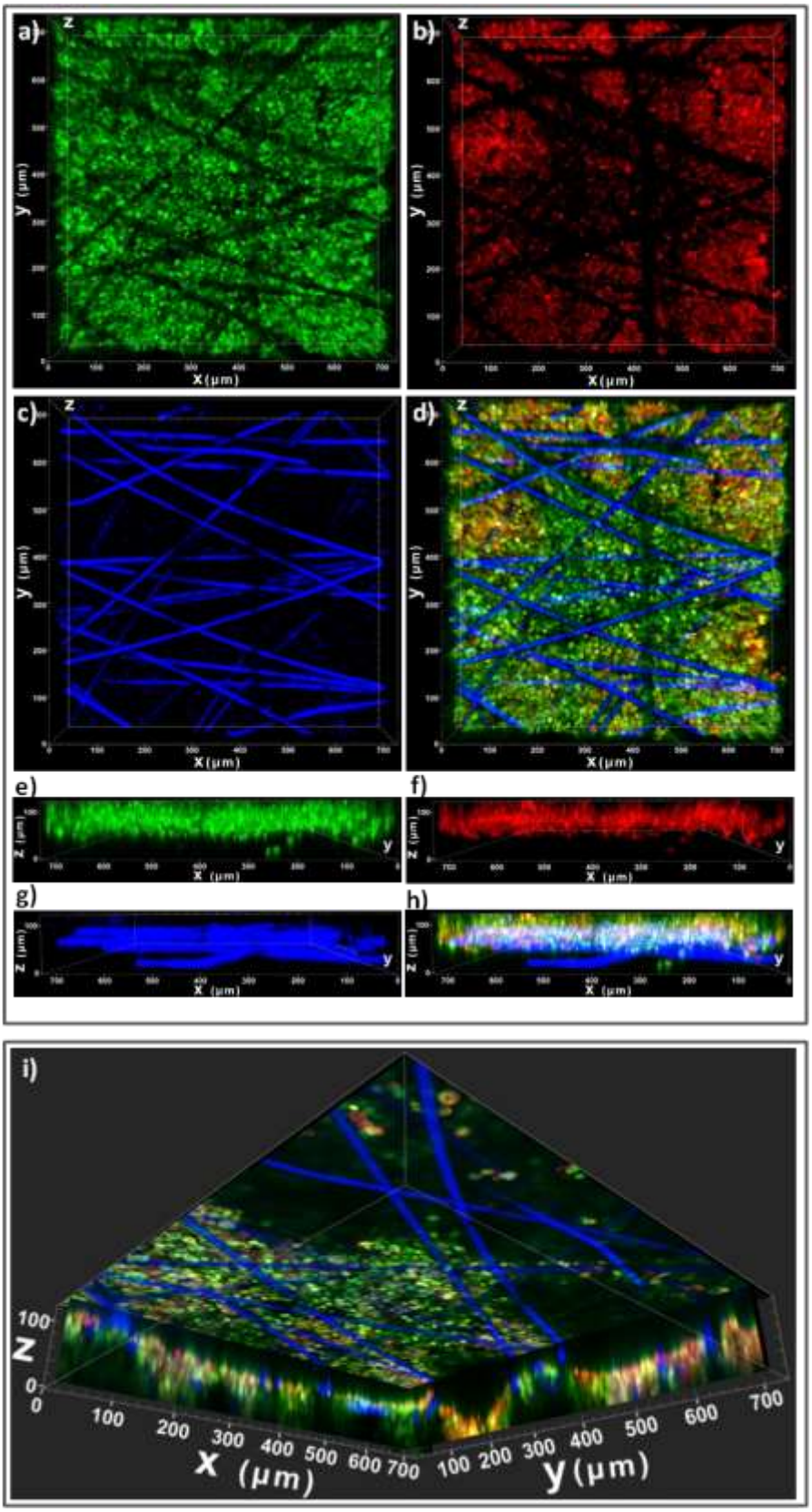

Figure 6. Multiphoton fluorescence microscope images of the 3D culture of HEK293 cells in PLA-PET scaffold. (a-i) GFP-HEK293 cells (2×10^6 cells /well in 96-well plate) are seeded in

3:7 PLA-PET scaffolds and cultured for 10 days. Nuclei were labelled green by stable expression of nuclear localised GFP, actin filaments were stained red with phalloidin-TRITC and PET microfibres auto-fluoresced blue. Images were taken and analysed using multiphoton confocal microscope and Imaris 8.0.1 software. (a-h) 3D projection images for top and side view of (a and e) nuclei, (b and f) actin filaments, (c and g) PET microfibres and (d and h) merged image. (i) Projection image showing the orthogonal xy, xz and yz planes.

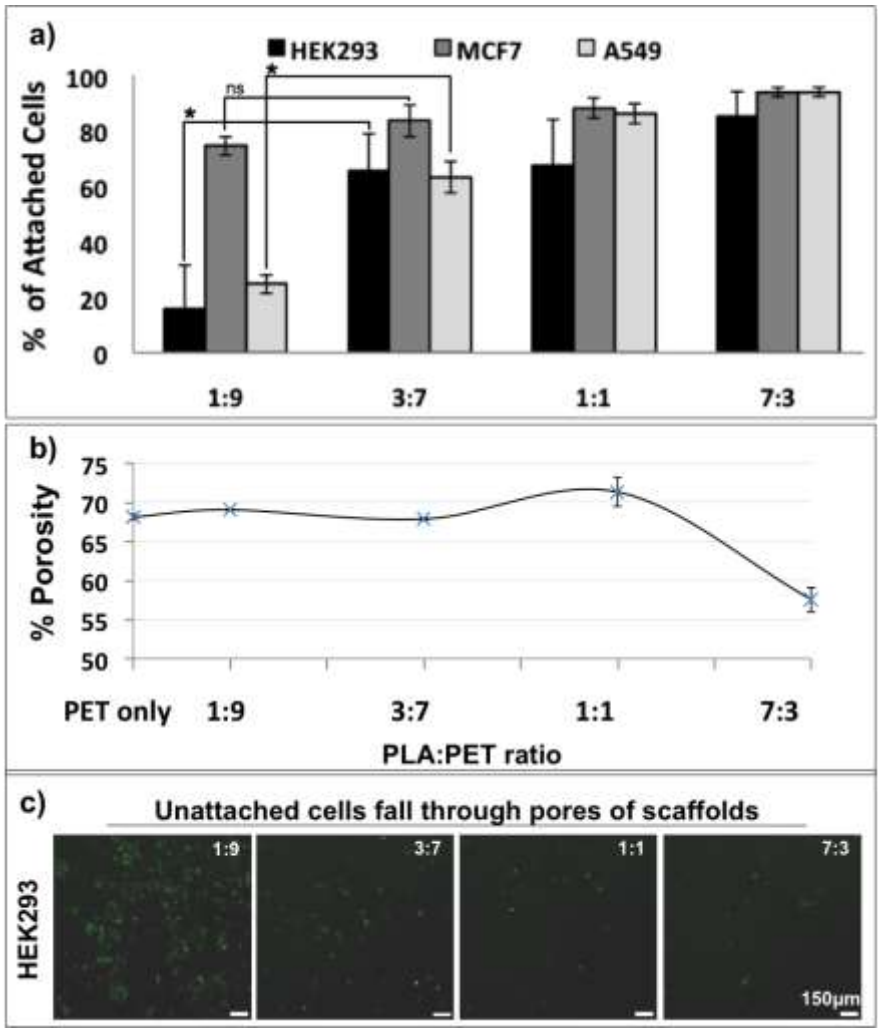

Figure 7. Determination of optimal PLA:PET ratio in scaffolds for efficient cell attachment. (a) Cell attachment efficiency of human cancer HEK293, MCF-7 and A549 cell lines in different PLA:PET ratio scaffolds. Cells were seeded at high densities of 2.5×10^5 cells/well in 48-well plates. Error bars represent the mean ± S.E.M of three independent experiments.

*p<0.05, ns = not significant. After 18 hrs of seeding, scaffolds were removed and cell attachment number was estimated indirectly by counting cells that did not attach to scaffold found at the bottom of the well plates. (c) Analysis of scaffold porosity at different PLA:PET ratios. Data were expressed as means ± S.D of three independent experiments. (c) Fluorescent images showing decreasing number of GFP-HEK293 cells fallen through the pores of scaffolds as PLA submicron fibre content increased; cells were seeded at high density of 1×10^5 cells/well in 96-well plates and images were taken of cells remaining on the plate surface at day 2 after removal of cell-seeded scaffolds. Scale bars: 150 µm.

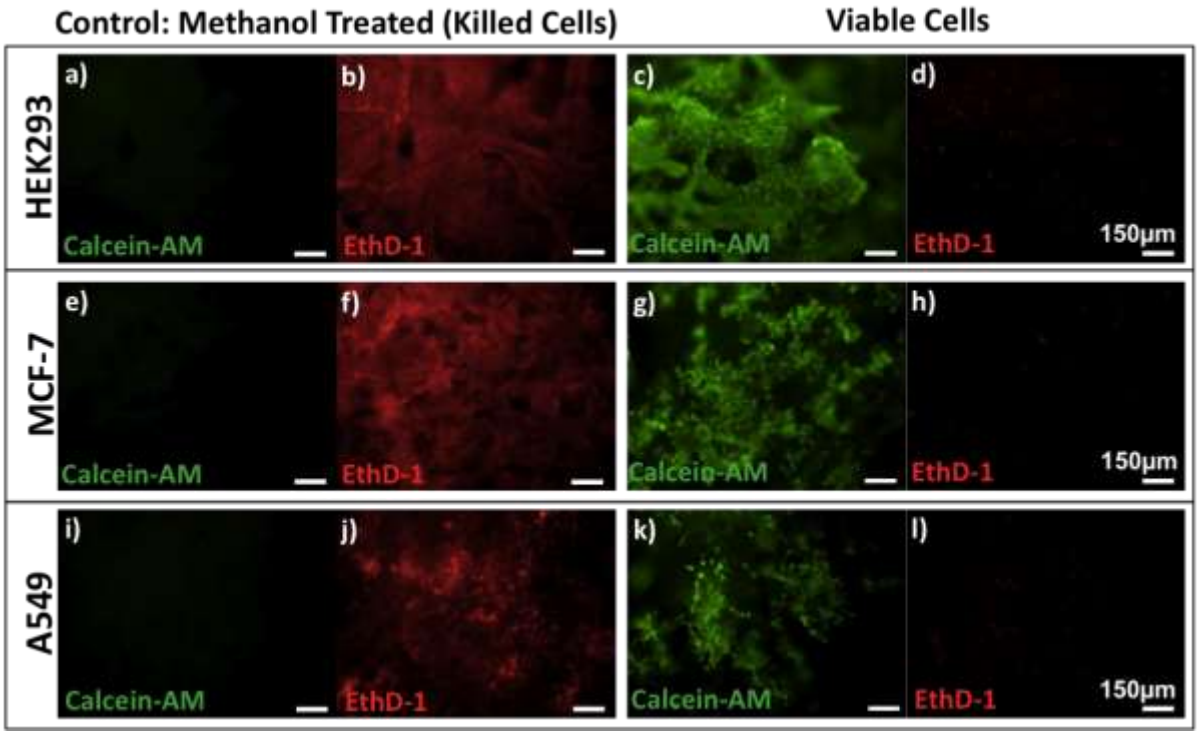

Figure 8. Cell viability staining of cell lines cultured in PLA-PET scaffold for 10 days. The cell viability of (a-d) HEK293, (e-h) MCF-7 and (i-l) A549 cells are tested using LIVE/DEAD viability/cytotoxicity kit (Invitrogen). Parental cells are seeded in shear-spun PLA-PET scaffold and are live stained with (a, c, e, g, i, k) calcein-AM (green) for viable cells and (b, d, f, h, j, l) ethidium homodimer-1 (EthD-1) (red) for dead cells. Methanol

treated cultures are negative and positive control for calcein-AM and EthD-1 staining respectively. The scale bars indicate 150 µm.

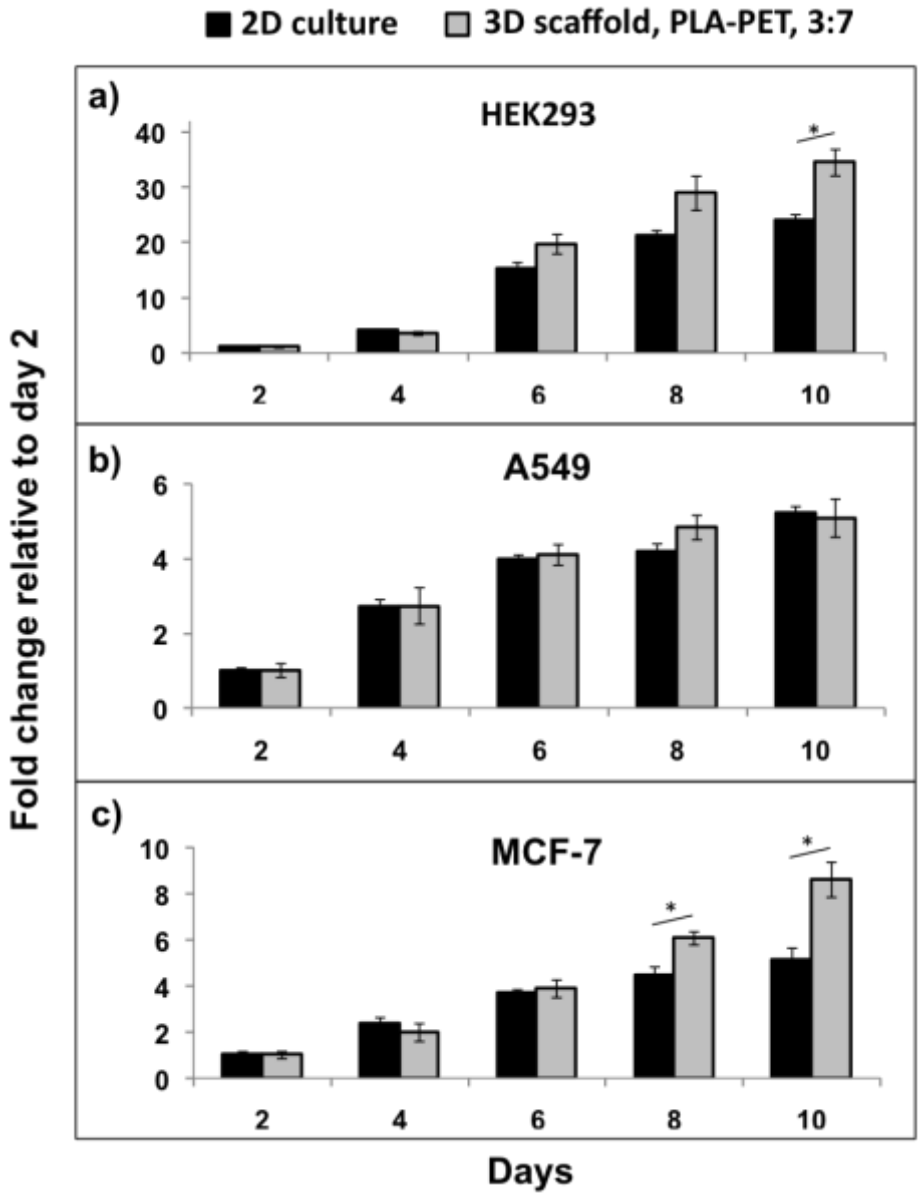

Figure 9. Comparative growth curves between 2D culture and 3D shear-spun PLA-PET scaffold. (a-c) 4000 cells of (a) HEK293, (b) A549 and (c) MCF-7 cell lines are seeded in 2D culture and in 3D scaffold with 30% PLA sub-micron fibres and 70% PET microfibres (3:7). Cell proliferation is measured at the indicated time points using the MTS CellTiter 96® Aqueous One Solution assay. The absorbance is measured at 490 nm. As absolute comparison of bioreduction of MTS solution is not possible between 2D and 3D cultures

directly, growth curves of each are plotted as the fold change relative to day 2 versus number of days. Data are expressed as mean number of cells ± S.E.M of (a-c) 4 independent experiments, each performed in quadruplicate. *$p < 0.05$.