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Substrate topography: A valuable *in vitro* tool, but a clinical red herring for *in vivo* tenogenesis

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ABSTRACT

Controlling the cell–substrate interactions at the bio-interface is becoming an inherent element in the design of implantable devices. Modulation of cellular adhesion *in vitro*, through topographical cues, is a well-documented process that offers control over subsequent cellular functions. However, it is still unclear whether surface topography can be translated into a clinically functional response *in vivo* at the tissue/device interface. Herein, we demonstrated that anisotropic substrates with a groove depth of ~317 nm and ~1988 nm promoted human tenocyte alignment parallel to the underlying topography *in vitro*. However, the rigid poly(lactic-co-glycolic acid) substrates used in this study upregulated the expression of chondrogenic and osteogenic genes, indicating possible tenocyte trans-differentiation. Of significant importance is that none of the topographies assessed (~37 nm, ~317 nm and ~1988 nm groove depth) induced extracellular matrix orientation parallel to the substrate orientation in a rat patellar tendon model. These data indicate that two-dimensional imprinting technologies are useful tools for *in vitro* cell phenotype maintenance, rather than for organised neotissue formation *in vivo*, should multifactorial approaches that consider both surface topography and substrate rigidity be established.

Statement of Significance

Herein, we ventured to assess the influence of parallel groves, ranging from nano- to micro-level, on tenocytes response *in vitro* and on host response using a tendon and a subcutaneous model. *In vitro* analysis indicates that anisotropically ordered micro-scale grooves, as opposed to nano-scale grooves, maintain physiological cell morphology. The rather rigid PLGA substrates appeared to induce trans-differentiation towards chondrogenic and/or steogenic lineage, as evidence by TILDA gene analysis. *In vivo* data in both tendon and subcutaneous models indicate that none of the substrates induced bidirectional host cell and tissue growth. Collective, these observations indicate that two-dimensional imprinting technologies are useful tools for *in vitro* cell phenotype maintenance, rather than for directional neotissue formation, should multifactorial approaches that consider both surface topography and substrate rigidity be established.

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1. Introduction

Given the poor inherent regeneration capability of tendons, largely attributed to low vascularity and low activity cellular content, intervention strategies should be developed to promote functional tendon repair and regeneration. Given that tissue graft based therapies have failed to restore native tendon function, it is anticipated that the tissue-engineering arpeggio (scaffolds, cells, biologics alone or in combination) would provide a functional therapy in the years to come [1–13].

Biomaterials' design and development is coming ever closer to mimicking native extracellular matrix (ECM) assemblies, as advancements in engineering have allowed development of two- and three-dimensional substrates with precise mechano-architectural and chemical properties. Indeed, current biomaterial fabrication technologies not only achieve structural support, but also maintain permanently differentiated cell phenotype and/or direct lineage commitment of stem cells. For example, topographical features have been shown to maintain Oct4 expression in human embryonic stem cell culture, even in the absence of basic fibroblast growth factor supplementation [14]; topographical cues alone [15,16] or in combination with neurotrophic signals [17] have also been shown to enhance contact guidance and neuronal differentiation of human neural stem cells *in vitro*. Topographical cues have been shown to enhance myogenic differentiation and maturation of myoblasts [18] and to induce myogenic commitment of human mesenchymal stem cells *in vitro* [19]. Surface topography alone [20] or in combination with substrate rigidity [21] have been shown to control mesenchymal stem cell lineage commitment. Recently, multi-scale patterned substrates have been shown to control adhesion and differentiation of human mesenchymal stem cells [22] and topographical features combined with hyaluronic acid have been shown to enhance chondrogenic differentiation of dental pulp stem cells [23]. Furthermore, two-dimensional and three-dimensional patterning technologies have been shown to enhance osteo-induction of stem cells [24], whilst proliferation and osteogenic differentiation of human mesenchymal stem cells have been shown to be dependent on the size of the underlying structures [25]. However, optimal feature geometries and conformations (e.g. grooves, pillars) and dimensionality (e.g. nano, micro) of such topographical features remain elusive, despite significant scientific achievements and technological innovations in fabrication processes and *in vitro* analysis.

To date, two- and three-dimensional scaffold fabrication technologies (e.g. electro-spinning [26–31], fibre extrusion [32–35], isoelectric focusing [36,37] and imprinting [38–40]) have been at the forefront of scientific and technological research and innovation to recapitulate native tendon extracellular matrix (ECM) supramolecular assemblies. Although fibrous constructs (e.g. electro-spun polymeric fibres, extruded collagen fibres and isoelectrically focused collagen fibres) have been shown to maintain tenocyte phenotype and to differentiate stem cells towards tenogenic lineage *in vitro* and to induce acceptable regeneration in preclinical models, none of these technologies offers precise control over the spatial distribution of the fibres. Imprinting technologies, on the other hand, have demonstrated a diverse effect on a range of permanently differentiated and stem cell functions, including adhesion, orientation, secretome expression and lineage commitment [41–48] and offer significantly greater control over feature dimension and spacing. Specifically to tendon repair, such technologies have been shown to maintain tenocyte phenotype [38]; to promote aligned tendon-specific ECM deposition [39]; and to differentiate stem cells towards tenogenic lineage [40]. Despite these advancements, a comprehensive study on the influence of surface features with respect to the modulation of tenocyte phenotype *in vitro* through anisotropic nano- to micron-scale topographies and on tissue response *in vivo* has yet to be elucidated. Thus, in the present study, we employed imprint lithography to create anisotropically grooved substrates with constant width and spacing and varying depth, as opposed to isotropic topography, to study tenocyte function *in vitro* and the host tissue response *in vivo*.

2. Materials and methods

2.1. Anisotropic substrate fabrication

The process of substrate fabrication has been described previously [49]. Briefly, Si master moulds with anisotropic topographies were fabricated via a photolithography process, followed by reactive ion etching (RIE). $1.5 \times 1.5\ cm^2$ regions were patterned with lines/gratings of 2101.78 ± 35.21 nm and 1911.42 ± 37.50 nm widths respectively, and variable groove depths (37.48 ± 3.4 nm, 317.29 ± 7.05 nm and 1988.2 ± 195.3 nm). Silicon wafers ($3.0 \times 3.0\ cm^2$) were spin-coated with a positive photoresist (S1813 PR, Shipley) and then exposed using OAI Mask Aligner (Model MBA800). Following photoresist development, the master mould was etched by RIE (Oxford ICP etcher) using CHF3 + SF6 ionised gas. The moulds were silanised with 5 mM octadecyltrichlorosilane (OTS, Sigma Aldrich, Ireland) solution to facilitate imprint release. A thermal imprinting process was used to transfer the master pattern into a $2.0 \times 2.0\ cm^2$ PLGA substrate (85:15, Sigma Aldrich, Ireland) using a Specac Hydraulic Press (15 T and 25 T) at 120 °C and a pressure of 5 MPa, for 5 min. The imprinted gratings on polymer were subsequently analysed by SEM and AFM. Non-imprinted PLGA substrates were used as isotropic control substrates.

2.2. Surface chemical analysis

X-ray photoelectron spectroscopy (XPS) analysis was performed in a Kratos AXIS 165 X-ray photoelectron spectrometer using monochromatic Al Kα radiation of energy 1486.6 eV and operated at beam voltage of 15 kV and beam current of 10 mA. High-resolution spectra of O 1s, C 1s and Si 2p were taken at fixed pass energy of 20 eV. Construction and peak fitting of synthetic peaks in narrow region spectra used a Shirely type background and the synthetic peaks were of a mixed Gaussian-Lorenzian type. Relative sensitivity factors used are from CasaXPS library containing Scofield cross-sections. Binding energies were determined using C 1s peak at 284.8 eV as charge reference.

2.3. Human tenocyte culture

Human primary tenocytes (positive for tenomodulin, scleraxis and tenascin C; Cambridge Biosciences, UK) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented 10% foetal bovine serum and 1% penicillin/streptomycin (all Sigma Aldrich, Ireland). Cells were maintained at 37 °C and 5% carbon dioxide, with the media being changed every 3 days. Tenocytes were sub-cultured when 80% confluency was reached. Tenocytes were detached from the culture flask with trypsin–EDTA solution (Sigma Aldrich, Ireland) and then seeded on the imprinted and isotropic substrates in 12-well (Ibidi®, Germany) and 8-well (Lab-Tek™, Thermo Scientific, UK) chamber slides at a cell density of 20,000 cells per $1\ cm^2$ for morphometric, viability, proliferation and metabolic activity analyses and 25,000 cells per $1\ cm^2$ for gene expression analysis (sufficient quality and quantity of RNA was obtained at this density), respectively. All *in vitro* experiments were conducted for 1, 5, and 10 days and cells at passage 3 were used.