Design and Characterization of Synthetic Biodegradable Films for Soft Tissue Engineering

Sofia Ribeiro[1,2][a], Emanuel M. Fernandes[4,5][b], Manuela E. Gomes[4,5][c], Rui L. Reis[4,5][d], Yves Bayon[1][e] and Dimitrios I. Zeugolis[2,3][f]

[1]*Institute Medtronic, Sofradim Production, Trevoux, France*
[2]*Regenerative, Modular & Developmental Engineering Laboratory (REMODEL), National University of Ireland Galway (NUI Galway), Galway, Ireland*
[3]*Science Foundation Ireland (SFI) Centre for Research in Medical Devices (CÚRAM), National University of Ireland Galway (NUI Galway), Ireland*
[4]*3B's Research Group – Biomaterials, Biodegradables and Biomimetics, University of Minho Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, Guimarães, Portugal*
[5]*ICVS/3B's – PT Government Associate Laboratory, Braga/Guimarães, Portugal*

1 RESEARCH PROBLEM

To repair soft tissue, it is vital to ensure that the biomaterial is able to mimic the complex elasticity and topography of the native tissue. Huge efforts have been invested into the development and design of appropriate elastomeric biomaterials to match the tissue of choice. However most of past studies have used non-degradable polymers as substrates. In order to obtain an optimal tissue engineered approach it is vital to study the effect of biodegradable polymers.

The goal of the present study is to characterize extensively a set of biodegradable polymeric micro-grooved films and to assess its effect on cell adhesion, morphology and phenotype through optimal substrate rigidities.

2 OUTLINE OF OBJECTIVES

Production of biodegradable polymeric films with a range of different stiffness.

Characterization of the physico-chemical and mechanical properties of the polymeric films.

Assessment of the impact of substrate rigidity on cell adhesion, proliferation and differentiation potential.

Optimization of imprinting of micro-sized grooves in the polymeric films.

3 STATE OF THE ART

Current surgical interventions are based on tissue grafts; synthetic/natural biomaterials; direct cell injections; and combinations of cells and a carrier system. However, preclinical and clinical trials revealed that tissue grafts are characterised by delayed remodelling and substandard mechanical function (Zeugolis et al., 2011); natural/synthetic biomaterial-based substitution yields thinner and weaker neotissue (Zeugolis, Chan and Pandit, 2011); direct cell injections offer little control over localised retention and distribution of the injected cell suspensions, leading to scattered therapeutic efficiency; and the presence of the carrier in the cell/carrier system hinders normal tissue remodelling and function (Abbah et al., 2014). All in all, current surgical repairs do not restore soft tissue function, imposing the need for new functional and clinically relevant/viable regeneration strategies.

Research efforts have been directed towards reconstruction of more functional in vitro microenvironments using biopolymers (Gomes et al., 2017) with optimized surface topography (Vermeulen et al., 2019) and substrate stiffness (Li et

[a] https://orcid.org/0000-0002-0265-0416
[b] https://orcid.org/0000-0002-4296-2529
[c] https://orcid.org/0000-0002-2036-6291
[d] https://orcid.org/0000-0002-4295-6129
[e] https://orcid.org/0000-0002-0232-3079
[f] https://orcid.org/0000-0002-7599-5191

Ribeiro, S., Fernandes, E., Gomes, M., Reis, R., Bayon, Y. and Zeugolis, D.
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al, 2013) as means to control cellular growth, motility and phenotype maintenance (Figure 1). The rationale of using topographical features as means to control cellular functions are based on the fact that fundamental cellular substrata, the basement membranes, are not smooth structures, but are covered with the intertwined fibrillar meshwork of the ECM (Jin et al., 2018). Similarly, rigidity plays a crucial role in cell motility in vivo, as cells move from regions of high ECM compliance to more stiff regions (durotaxis) and in vitro studies have demonstrated that stem cells commit to phenotype with extreme sensitivity to elasticity; soft substrates that mimic brain are neurogenic, whilst rigid substrates that mimic bone are osteogenic (Engler et al., 2006; Lv et al., 2015). Thus, it has been postulated that in vitro recapitulation of physiological tissue topography and rigidity will enable clinical translation of cell-based therapies.

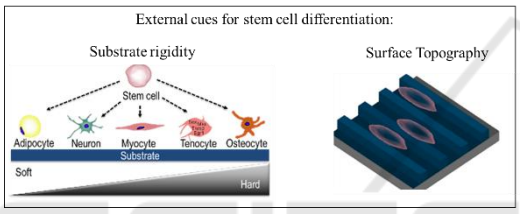

Figure 1: Substrate rigidity and surface topography as external cues for stem cell differentiation.

4 METHODOLOGY

4.1 Film Preparation

Absorbable polyesters made from different combination of monomers, such as lactic acid, glycolic acid, trimethylene carbonate, dioxanone & β-caprolactone, were selected for their physico-chemical intrinsic properties. Even though the selected polymers have similar chemistry they show different mechanical and degradation properties (Figure 2).

Polymeric films were produced by compression moulding using a thermal presser Carver 3856 CE. The presser was heated close to the polymer melting temperature. The polymer was placed between Teflon sheets and metal sheets inside the presser for 5 minutes. Then the system was placed under pressure. The sample was removed after gradually cooling down the system.

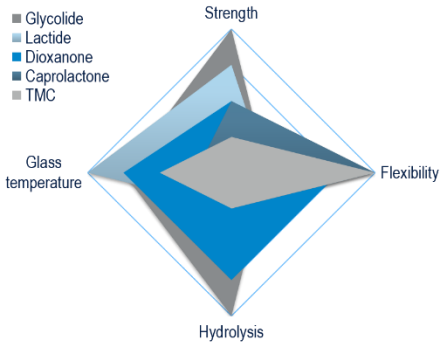

Figure 2: Monomers selected (Glycolide, Lactide; Dioxanone, Ɛ-Caprolactone and Trimethylene Carbonate (TMC)) and the representation of their intrinsic properties.

4.2 Chemical Characterization

NMR technique monitors the residual solvent concentration in films and the monomer content. The samples were prepared by dissolving the polymers in CDCL3 or TFA deuterated. 1H NMR spectra were obtained at 400 MHz. Measurements were performed on a Fourier 300 Bruker spectrometer.

Differential Scanning Calorimetry (DSC) technique was used to assess the glass transition (Tg) and melting temperature (Tm), variation of enthalpy of the polymeric films, as well as the crystallinity content.

The DSC equipment used, a DSC 1 Star System, Mettler Toledo, was programmed to perform two heating curves, with a cooling intermediated step. The temperatures used for each condition were optimized. The mass of the analysed sample was between 5 and 6 mg. The second heating curve was the reference for determining the Tg temperature, Tm and percentage of crystallinity.

FTIR measurements were obtained using ATR technique with a Spectrum 100 FT-IR Spectrometer, Perkin Elmer by averaging 32 scans over the range of 4000 cm-1 to 800 cm-1.

The wettability of the samples was characterized by static water contact angle measurements using a sessile drop method with an OCA15+ goniometer (DataPhysics, Germany) under ambient conditions at room temperature. A 3 µL drop of distilled water and diiodomethane (CH2I2) were dropped via a motor-driven syringe. The data presented was calculated using the final averaged values. The values for polarity of the surface and the surface tension were obtained by the Owens-Wendt method.

4.3 Mechanical Properties

4.3.1 Dynamic Mechanical Analysis (DMA)

The viscoelastic measurements were performed using a DMA Q800 from TA Instruments. The measurements were carried out at 37ºC in wet conditions. Samples were cut in rectangular shapes with about 14.5 x 5.3 mm (l,w) and 0.2 mm thickness and clamped in the DMA apparatus. The sample was deformed at constant stress-amplitude (25 µm) over 3 different frequencies (0.1, 1 and 10 Hz).

4.3.2 Tensile Tests

Mechanical properties were assessed under uniaxial tension, using a Zwick/Roell (Leominster, Herefordshire, UK) Z005 testing machine, loaded with a 10 N load cell, as has been described previously.

The samples were pre-cut into a dog-bone shape, as per ASTM D882−2010 guidelines. Prior to testing, all samples were incubated overnight at room temperature in PBS and tissue paper was used to remove excess PBS. The samples thickness was measured using digital callipers (Scienceware, Digi-Max, Sigma-Aldrich, Ireland). The samples were hand-tightened between the vertical grips, which were set at 50 mm gauge length. Scaffolds that broke at contact points with the grips were rejected from the analysis. The extension rate was set at 5 mm/min. The following definitions were used to calculate mechanical data: stress at break was defined as the load at failure divided by the original cross-sectional area (engineering stress), strain at break was defined as the increase in scaffold length required to cause failure divided by the original length, and modulus was defined as the linear region of the stress-strain curve using a stress at 0.02 strain divided by 0.02.

4.4 Degradation Studies

Films were incubated at 37°C in PBS for up to three months. After predetermined periods of time samples were removed from the solution, rinsed with distilled water and dried at 37°C for 48 hours. Membrane mass was weighted, and the percentage of weight loss was calculated following equation 1:

$$\% \text{ weight loss} = (m_i - m_f)/m_i \text{ X } 100 \quad (1)$$

Where m_i and m_f are the initial and final mass of the sample, respectively.

4.5 *In Vitro* Assessment

4.5.1 Human Bone Marrow Stem Cells (hBMSCs) Isolation and Culture

BMSCs were isolated according to standard protocols. Briefly, bone marrow was flushed from femurs and the flush-out solution was thoroughly resuspended in complete basal medium and passed through a 70 µm cell strainer. Cells were washed in PBS- and were subsequently plated in complete basal medium. After 2 days in culture non-adherent cells were removed by several washes in PBS- and cultured to near confluence (approx. 80%). Cells were trypsinised, pooled, and re-plated (passage 0). Cells were subsequently passaged at approximately 70-80% confluency and were never allowed to reach full confluency. Cells at passage 2 to 4 were used for all experiments.

4.5.2 hBMSCs Seeding on Polymeric Films

Prior to cell culture studies, the films were sterilized by ethylene oxide. hBMSCs at passages 3–4 were harvested from culture flasks using trypLE Express (Thermo Fisher, USA). Cells were washed with PBS and centrifuged at 1200 rpm for 5 minutes. The cell pellet was resuspended in α-minimal essential medium (αMEM) supplemented with 10% fetal bovine serum (FBS) and 2mM GlutaMAX and the cells were seeded at different concentrations described below onto the polymeric films, previously placed into wells of a 24 well plate. The cells were then cultured at 37 °C in a humidified atmosphere of 5% CO_2. Subsequently, a drop of 100 µl of the cell solution was seeded on top of the films and the cells were allowed to attach at 37 °C, 5% CO2, 90% humidity for 2,5 h prior to adding 900 µl complete basal medium. The medium was changed every other day.

4.5.3 Cell Adhesion

To evaluate the influence of the substrate stiffness in cell morphology, 500 cell/cm^2 were seeded into the polymeric films and incubated for 6 and 24 hours. hBMSCs morphology was assessed by F-actin and DAPI staining. Cells were fixed using a 10% formalin solution for 30 minutes at 4°C. The samples were then washed with PBS. For cell permeabilization it was used a solution of 2% BSA in PBS. 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) and Rhodamine B isothiocyanate were used to stain the cell nuclei and F-actin filaments, respectively. The samples were incubated with 1 mL of PBS containing

5 µL of Rhodamine for 20 minutes at room temperature and protected from light. After extensive washing, samples were stained with 1µL or DAPI in 1 mL of PBS for 20 minutes at room temperature in the dark. After staining the samples these were washed again with PBS. The cells were visualized using a fluorescence microscope.

4.5.4 Cell Proliferation

After each time point, the metabolic activity was assessed by Alamarblue® assay. The samples were placed in a new well-plate with 0.1M phosphate buffer saline solution (PBS). 1 mL of 10% Alamarblue® solution in PBS was added and a negative control of Alamarblue® at 10% alone. The cell culture plates were incubated for 3 hours at 37°C at 5% CO2. A microplate read (Bio-Tek, USA) was used to read the Optical Density (OD). Three replicates of each condition were analysed.

To assess the hBMSCs proliferation seeded on polymeric films and coverslips as control samples, a fluorometric double-strand DNA quantification kit (PicoGreen, Molecular Probes, Invitrogen) was used. For this purpose, cell lysates were collected at 7, 14 and 21 days by transferring the samples into 1.5 mL microtubes containing 1 mL of ultrapure water. The samples were incubated for 1h at 37 °C in a water bath, and next stored at -80 °C. Cell lysates and standards (ranging from 0 to 2 mg mL-1) were prepared and mixed with a PicoGreen solution in a 200:1 ratio and placed in an opaque 96-well plate. The plate was incubated for 10 min in the dark, and fluorescence was measured on a microplate ELISA reader (Synergy HT, BioTek, USA) with excitation at 485/20 nm and emission at 528/20 nm. The DNA values were calculated using a calibration curve.

4.5.5 Differentiation

For assessing the in vitro differentiation capacity, cells were seeded onto polymeric films, as described above, and were kept in complete basal medium for 48 h. Subsequently, the samples were placed in appropriate differentiation media. The trilineage differentiation capacity was confirmed after 21 days in culture by and semi-quantitative qPCR.

4.6 Imprinting

Si master moulds with grooved topography (2x2 µm) were fabricated via a photolithography process, followed by reactive ion etching (RIE). Silicon wafers (3.0 _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 enable imprint release. A thermal imprinting process was used to transfer the master pattern into the polymeric films using a nanoimprinter at optimized temperatures and pressures. The imprinted gratings on polymer were subsequently analysed by SEM and AFM. Non-imprinted substrates were used as isotropic control substrates.

5 EXPECTED OUTCOME

It is expected to obtain polyester films with a range of mechanical properties that will mimic the intrinsic mechanical properties of the native tissue, such as adipose, tenogenic and osteogenic tissue (Figure 3). Chemical characterization and degradation studies will provide information about the polyester prolife. In vitro studies will shed some light on the impact of the film's stiffness to the cell fate.

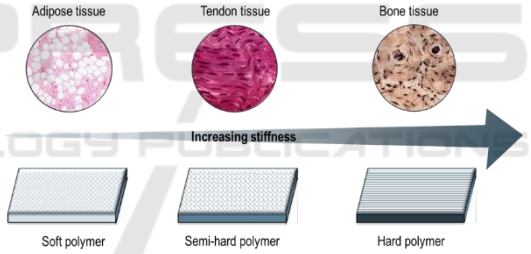

Figure 3: Polyester films with increasing stiffness in order to mimic adipose, tendon and bone tissue.

6 STAGE OF THE RESEARCH

It was possible to successfully produce polymeric films using a large range of polyesters using a thermal presser. Chemical analysis was used to trace the chemical profile for each polymer. The selection performed lead to materials with very distinct profiles, regarding their crystallinity content and degradation rate.

The mechanical properties of the materials were analysed regarding at their macro level by DMA at 37°C in a PBS bath. The results show that the developed films have a storage modulus ranging from 0.1 up to 2.6 GPa.

Biological assays showed good cell adhesion, cell proliferation and cell viability. Cell morphology and cluster formation were very different from one to another polymer. The focal adhesion pattern has been analysed as well, which means that the behaviour of cells was strongly influenced by the nature of the polymer and its associated stiffness, while other parameters remained equal.

In the future, the combined effect of stiffness and topography will be assessed on cell phenotype maintenance.

This project is entering its final stage and is set to finish in May 2019. As a disclosure, results cannot be presented in details since 2 papers are under preparation. In addition to this work, I co-authored two book chapters and an additional review paper is under preparation.

The results have been orally communicated at these conventions: European Orthopaedic Research Society, November 2018, Galway, Ireland; 4th International Conference on Biomedical Polymers & Polymeric Biomaterials, July 2018, Krakow, Poland; and Future Investigators of Regenerative Medicine (FIRM) Meeting, September 2017, Girona, Spain. This work received funding from H2020-MSCA-ITN-2015, Tendon Therapy Train Project (Grant Agreement Number: 676338).

REFERENCES

Abbah, S. A., et al. 2014. Assessment of stem cell carriers for tendon tissue engineering in pre-clinical models. *Stem Cell Research & Therapy*. 5. 38.

Engler, A. J., et al. 2006. Matrix Elasticity Directs Stem Cell Lineage Specification. *Cell*. 126. 677-689.

Gomes, M. E., et al. 2017. Tissue Engineering and Regenerative Medicine: New Trends and Directions-A Year in Review. *Tissue Engineering Part B: Reviews.* 23(3). 211-224.

Jin, G., et al. 2018. Electrospun three-dimensional aligned nanofibrous scaffolds for tissue engineering. *Materials Science & Engineering C*. 92. 995-1005.

Li, Z., et al. 2013. Differential regulation of stiffness, topography, and dimension of substrates in rat mesenchymal stem cells. *Biomaterials.* 34. 7616-7625.

Lv, H., et al. 2015. Mechanism of regulation of stem cell differentiation by matrix stiffness. *Stem Cell Research & Therapy*. 6:103.

Vermeulen, S., et al. 2019. Identification of topographical architectures supporting the phenotype of rat tenocytes. *Acta Biomaterialia*. 83. 277-290.

Zeugolis D.I., et al. 2011. Xenogenic tissues and biomaterials for the skeletal system. *In Comprehensive Biomaterials*. Volume 2. Oxford. Elsevier. 387–404.

Zeugolis D.I., Chan, J.C.Y., Pandit, A. 2011. Tendons: engineering of functional tissues. *In Tissue Engineering*. Berlin. Springer. 537–572.