European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 1) ISSN 1473-2262

The Role of In Vitro Techniques in Tissue Engineering Development

C.J. Kirkpatrick

[1] *Institute of Pathology, Johannes Gutenberg University, Mainz, Germany*

INTRODUCTION: As the field of biomaterial research becomes more sophisticated, including the approach of tissue engineering, there has been a necessary expansion of the concept of biocompatibility to address not only the biosafety issue, that is, the exclusion of cytotoxic and other deleterious effects of biomaterials, but also the biofunctionality component, which concerns the fulfilment of the intended function of the applied biomaterial. Careful scrutiny of this concept leads to the conclusion that relevant test systems for biofunctionality must centre on human cells, studied under conditions relevant to the situation in the living organism for which the medical device has been constructed. Thus, progress in bio-compatibility and tissue engineering (TE) would be inconceivable without the aid of *in vitro* techniques.

CURRENT DEVELOPMENTS:

Of paramount importance is proving the maintenance of the cell phenotype *in vitro*. Loss of essential characteristic functions of cultivated cells makes extrapolatory interpretations meaningless for the clinical situation. Until now much experimentation in vitro has concentrated on non-human cell systems in two-dimensional culture, very often with a view to excluding cytotoxic effects. For TE it is necessary to develop three-dimensional culture systems, in which, for example, confocal laser scanning microscopy with relevant immunocytological methods can greatly assist monitoring functional parameters [1,2], as well as co-culture systems [3] and dynamic cultures, as in bioreactors. This increased level of complexity is regarded as essential for a deepening of our understanding of biological mechanisms, without which a rational approach to TE design will not be possible.

Due to our interest in vascularization, much of our experimentation involves endothelial cells (EC) from microvascular sources as well as endothelial progenitor cells (EPC) from human peripheral blood. Among the biopolymers of interest are the silk protein, fibroin, in combination with collagen type I and chitosan-based scaffolds for bone TE. Examples will be given of experimental set-ups which enable cell functionality on the scaffold to be studied both at the gene transcript and protein level, thus illustrating the possibility to make *in vitro* methods more *in vivo*-like. A further example is the use of co-cultures of osteoblasts and EC to understand how these two essential cell types for bone regeneration influence each other, especially in the interaction with a biomaterial scaffold. Our initial studies indicate that EPC and adult EC behave differently in their interaction with cells of osteoblastic phenotype.

In the field of drug- and gene-delivery we have established a co-culture model of the air-blood (alveolo-capillary) barrier of the human lung [3] with a view to using it to study the mechanisms of nanoparticle uptake and transport. This basic knowledge is required to enable targeting to the lung to treat pulmonary disease as well as to use the lung as a portal of entry to the systemic circulation to target, for example, cancer in other organs.

FUTURE DIRECTIONS & CONCLUSIONS:

There is increasing interest in understanding how the regenerative potential in various tissues and organs can be specifically targeted. Of particular significance is an understanding of how the so-called stem cell niche is controlled, as this forms the basis for rational targeting strategies, using, for example, drug- or gene-delivery systems. In addition, novel bioreactor technologies will continue to be vital to the field of tissue engineering, as will the application of nanotechnologies. In all of these areas of interest *in vitro* methodology is an important component in development and testing approaches. Nevertheless, it must be stressed that even if human cells are employed under optimal culture conditions, the problem of extrapolation to the *in vivo* state should not be underestimated.

REFERENCES: [1] R.E. Unger, K. Peters, Q. Huang, et al (2005) *Biomaterials* **26**:3461-9. [2] K. Peters, H. Schmidt, R.E. Unger et al (2005) *Molec Cell Biochem* **270**:157-66. [3.] M.I. Hermanns, R.E. Unger, K. Kehe et al (2004) *Lab Invest* **84**:736-52.

ACKNOWLEDGEMENTS: The author wishes to express his thanks to the European Commission for various grants within the scope of its biomaterial programmes (esp. the NoE EXPERTISSUES), and the German Research Foundation (DFG; Priority Programme Biosystem 322 1100).

Ion channel agonist release scaffolds for bone tissue engineering.

Steven Hughes, Mairead Wood, Nadia Zghoul, Ying Yang and Alicia El Haj.
Centre for Science and Technology in Medicine, Keele University, Stoke-on-Trent, UK.

INTRODUCTION: Calcium influx through L-type VOCC ion channels is a key event in the transduction of mechanical stimuli by bone cells (1). The calcium channel agonist Bay K8644 has been shown to increase levels of mechanically induced bone matrix production (2), and thus manipulation of VOCC channels represents a potentially potent tool for tissue engineering. We have previously developed a Bay-encapsulated scaffold, which results in the up-regulation of bone matrix proteins in bone cell seeded constructs (3). In this study we use whole-cell electrophysiology to characterise the effects of 'scaffold released Bay' on L-type VOCC currents recorded from osteoblasts. Furthermore we also report the development of scaffolds incorporating a different class of L-type VOCC agonist, namely FPL.

METHODS: Collagen coated Bay-encapsulated PLLA scaffolds were produced as described previously (3). Scaffolds (n=4) were cultured in 1ml ddH_2O under cell culture conditions for 28 days. ddH_2O was collected and replaced weekly and the Bay concentration of individual samples determined via UV spectroscopy. Whole cell patch clamp electrophysiology of L-type VOCC currents recorded from ROS 17/2.8 cells was used as a means to assess the functionality of the released agonist. For isolation of calcium currents, barium was used as the charge carrier. Cells were bathed in either BaCl saline (108 mM $BaCl_2$ and 10 mM HEPES, corrected to pH 7.6 with NaOH) or BaCl saline containing 500nM, 1μM or 10μM Bay. Alternatively, cells were bathed in saline containing Bay released from scaffolds. Similar methods were also used to develop and evaluate FPL-encapsulated scaffolds.

RESULTS: For all scaffold salines, Bay concentrations remained within the physiological range required for agonist activity (1-10 μM) during 28 days in culture. L-type currents recorded in scaffold release salines showed characteristic differences when compared to recordings performed in the absence of Bay. These shifts are characteristic of the effects of Bay on L-type currents and were indistinguishable from currents recorded in the presence of known concentrations of Bay.

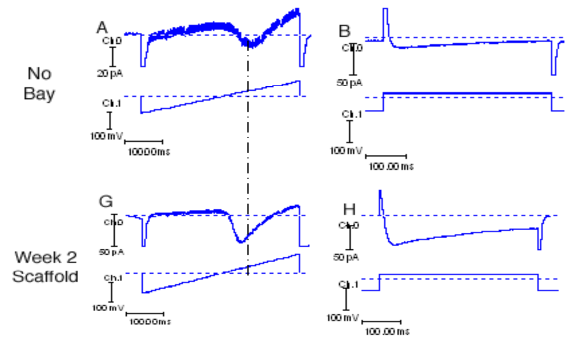


Fig 1 – L-type VOCC currents recorded in normal saline (top) and week 2 'Bay scaffold release saline' (bottom).

Whole cell recordings were also used to demonstrate the effects of FPL on osteoblast L-type currents and confirm the stability of this compound under cell culture conditions and following release form PLLA scaffolds. The effect of FPL on osteoblast proliferation, LDH production and apoptosis demonstrates that this compound is well tolerated by osteoblasts and represents another potential tool for augmenting bone matrix production in tissue engineered constructs.

DISCUSSION & CONCLUSIONS: In summary this work demonstrates that calcium channel agonists can be successfully incorporated and subsequently released from 3-D scaffolds without loss of functional activity. These findings confirm conclusions of our previous work suggesting that the enhancement of bone matrix production following mechanical conditioning of cells in Bay-encapsulated PLLA scaffolds is due to augmentation of L-type channel activity (3).

REFERENCES: El Haj et al, Med Biol Eng Comp, 37, 403-409, 1999; 2) Walker et al, J Cell Biochem, 79(4), 648-661, 2000; 3) Wood et al, J Control Release, 112(1), 96-102 2005.

ACKNOWLEDGEMENTS: This work was supported by the EPSRC (grant no.)

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 3) ISSN 1473-2262

Ultra-Rapid Pre-Fabrication of Biomimetic Collagen 'Tissues': Generating Meso-Scale Structure with Hyaluronan.

N.Anandagoda, U.Cheema and R.A. Brown

[1] University College London, UCL Tissue Repair and Engineering Centre (TREC), Institute of Orthopaedics, Stanmore Campus, London. UK.

INTRODUCTION:

Plastic compaction of hyper-hydrated native collagen gels (Brown et al 2005) represents a radical and new approach to the fabrication of biomimetic tissues & templates (as opposed to biomaterials-based tissue-equivalents). It allows the *rapid engineering* of meso-structure by directional water removal. The deformation is plastic (ie constructs retain their new structure) since the collagen has little inherent swelling potential. However, a further level of biomimesis could be achieved by local incorporation of osmotically active (swelling) macromolecules – a common example being hyaluronan. In this study, the GAG, hyaluronan, was locally incorporated into high density, ***cell-free,*** compressed collagen constructs with the aim both of identifying new techniques for rapid tissue engineering and as a model to test how connective tissue cells might utilise such mechano-osmotic fluid flow, in vivo.

METHODS:

Freeze-dried hyaluronan was applied to localised areas of freshly prepared Plastic Compacted (PC) collagen sheets (~50um thick), particularly along the short edges. Constructs were immediately formed into spirals by routine rolling from the short edge, ie., leaving a strip of hyaluronan at the core of the spiral. Spiral constructs were then incubated in water for periods from 1 min. to 3hrs at which point they were fixed for scanning electron microscopy.

RESULTS

welling of the core region was rapid, within the first 10 -30 min and largely complete in 1-2h. Over this time the thickness of outer collagen layers appeared to reduce dramatically, forming a clear and continuous channel along the full-length of the construct – essentially producing a tube structure (Fig. 1).

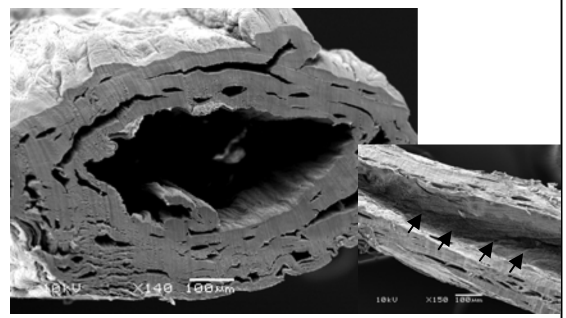


Fig. 1: SEM (main picture) of the core channel in TS. formed within a spiral collagen PC construct by hyaluronan, post compaction re-swelling. INSERT: LS view showing the full length channel (arrows).

DISCUSSION & CONCLUSIONS:

The prediction here was that hyaluronan would re-swell those areas of the PC collagen construct where it was localised, leading to cavity formation. This was indeed the experimental result, representing a rapid and localisable technique for inducing new structures and interfaces within the constructs, in this case as a tube. However, it was also found that the collagen layers themselves were further compressed, presumably by additional loss of fluid to the swelling polysaccharide gel. This presents the intriguing possibility that cells might *naturally* use a timed secretion of such GAG molecules to manipulate local tissue structure, and to form interfaces between fibril bundles. Local structural re-swelling represents important new biomimetic approach for engineering tissues and a model for investigation of natural cell systems.

REFERENCES:

RA. Brown, M. Wiseman, C-B. Chuo, U. Cheema, SN.Nazhat. (2005), *Adv.Funct. Material* **15**:1762-1770

ACKNOWLEDGEMENTS: This study is funded by BBSRC/EPSRC as part of TIBS Programme.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 4) ISSN 1473-2262

Development of fibrous porous silk scaffolds

B.J. Ainsworth and J.B. Chaudhuri

Centre for Regenerative Medicine, Department of Chemical Engineering, University of Bath, UK.

INTRODUCTION: Pore structure is very important when considering scaffolds for tissue engineering. High porosity and well connected pores create good mass transfer properties increasing cell viability. Pore size helps to determine cell differentiation[1]. Previous work with silk scaffolds with defined pore sizes has made scaffolds with small pores with limited connections[2], or large (>500µm), well connected pores[3]. This work has developed a technique for making silk scaffolds with small, well connected pores. These scaffolds have an unusual fibrous structure.

METHODS: Silk scaffolds were fabricated from freeze dried silk fibroin (Hobbycraft) *(prepared as in the literature[2]).* The silk fibroin was dissolved at either 10% (w/v) or 7.5% (w/v) in 20% (v/v) formic acid. It was then added to a circular mould (15mm diameter) either before or after NaCl was added as a porogen. Scaffolds were then left covered in the mould for 24 hours, when the cover was removed. After another 24 hours the bottom plate of the mould was removed. After a final 24 hours the scaffolds were removed from the mould and either placed in methanol or propanol (Fisher) for 30minutes or transferred immediately for salt leaching. Scaffolds for cell work were cut to 2mm height after 30 minutes in methanol. The scaffolds were then salt leached by placing into water, which was changed six times over 24 hours. Scaffolds for cell proliferation measurement were autoclaved, and then soaked in media (DMEM + 10% FCS, 100mM ascorbate-2-phosphate) overnight. Scaffolds were then seeded with 800,000 P3 ligament fibroblasts and placed on an orbital shaker at 100rpm. Media was changed every 2 days and proliferation was measured by determined the DNA content after 7 days with the picogreen assay (Invitrogen).

For SEM scaffolds were freeze dried, and then freeze fractured and gold coated. Images were taken with a JEOL JSM6310. For FTIR discs were pressed using roughly 2mg of powdered scaffold and 30mg of KBr (Sigma). FTIR spectra were an average of 100 scans at a resolution of 4cm^{-1} recorded on a Bruker Equinox 55 spectrophotometer.

RESULTS: SEM images comparing adding salt to the mould before and after silk showed that adding silk before salt resulted in a more replicable structure. The images also confirmed that it was possible to make scaffolds with small well connected pores. The scaffolds have an unusual fibrous structure; reducing the concentration of silk in the solvent results in sparser fibres (Figure 1).

FTIR spectra show that silk in the scaffolds is in silk II form (β-sheets), adding methanol does not change the spectra.

There was no significant difference between the DNA produced after 7 days: 10% w/v silk 330 +/- 94ng and 7.5% w/v silk 454+/-293ng.

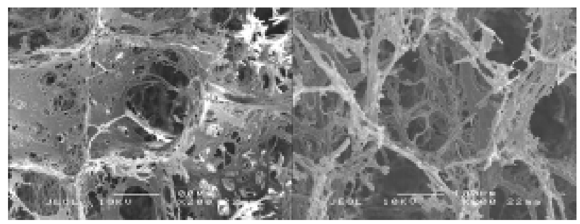


Fig. 1: SEM images of fibrous silk scaffold: 10% w/v silk (left) and 7.5%w/v silk (right)

DISCUSSION & CONCLUSIONS: A method to fabricate novel fibrous porous silk scaffolds has been developed. By changing the salt particle size it is possible to fabricate scaffolds with a large range of pore sizes. These pores are smaller than those of previous silk scaffolds with well connected, defined size pores. In addition the pore structure can be changed by varying the concentration of silk. Cell proliferation data suggests that these scaffolds are suitable for tissue engineering. Future work is to use these scaffolds to examine the effects of different pore sizes and pore structures on ligament fibroblast proliferation and matrix synthesis.

REFERENCES: [1] Karageorgiou, V. and D. Kaplan: *Biomaterials* 26:5474-91, 2005. [2] Nazarov, R., H.J. Jin, and D.L. Kaplan: *Biomacromolecules* 5:718-26, 2004. [3] Kim, U.J., Park, J., Kim, H. J., et al: *Biomaterials* 26:2775-85, 2005.

Zonal release of proteins within tissue engineered scaffolds

T. Suciati, D. Howard, J.J.A. Barry, N.M. Everitt[1], F.R.A.J. Rose, K.M. Shakesheff

Tissue Engineering Group, School of Pharmacy, and [1]Bioengineering Group, School of Mechanical, Materials and Manufacturing Engineering The University of Nottingham, NG7 2RD, UK

INTRODUCTION: The concept of releasing growth factors (GFs) over extended and controlled periods of time using microparticles is widely pursued within the field of tissue engineering[1]. Our approach uses growth factor loaded microparticles that may be built layer-by-layer into a 3D structure. The loose aggregate of microparticles is then sintered to form an interconnected porous matrix. These scaffolds could provide GF gradients to direct cell movement or to provide zonation of cell response to engineer multiple tissues in a single device that may be applied in multiphase tissue repairs (i.e. osteochondral defects). The aim of this study was to establish the methodology of producing such scaffolds with zonal protein release for tissue engineering.

METHODS: Horseradish peroxidase (HRP) or recombinant human bone morphogenetic protein-2 (rhBMP-2) was loaded into $P_{DL}LA$ microparticles using a Solid-in-Oil-in-Water (S/O/W) emulsion method[2]. The microparticles were then heat sintered to form scaffolds. The release profiles of HRP from both microparticles and scaffolds were performed by incubation in PBS and analysis of the protein concentration and activity using the micro-bicinchoninic acid (BCA) assay and 3,5,3',5'-tetramethylbenzidine (TMB) substrate, respectively. C2C12 mouse myoblasts were cultured on scaffolds consisting of rhBMP-2 loaded and rhBMP-2 free microparticles and the level of osteoblast induced differentiation measured using alkaline phosphatase (ALP). Zonal release of a tri-layered scaffold consisting of a layer of protein-free microparticles sandwiched between two layers of HRP loaded microparticles and a bi-layered scaffold consisting of a rhBMP-2 loaded zone and a rhBMP-2 free zone was analyzed by recording the sequential colour changes of TMB substrate and observing the staining of ALP-induced expression on C2C12 cells, respectively. The C2C12 cells cultured scaffolds were also stained for cell distribution using Toluidine Blue.

RESULTS: HRP was released in a controlled manner from microparticles and scaffolds over a 30-day period and the activity of released protein was maintained throughout this period. The analysis of C2C12 cell response on scaffolds consisting of various portions of rhBMP-2 loaded microparticles showed a linear relationship between increasing ALP induced expression and the ratio of these particles. An intense TMB yellow product was formed almost exclusively in the HRP loaded zones of the triple layer HRP scaffold which confirmed the zonation of protein release within this scaffold. Fig 1 shows the zonal cell response within the bi-layer rhBMP-2 scaffold; a significant increase of ALP activity was observed in the rhBMP-2 loaded zone.

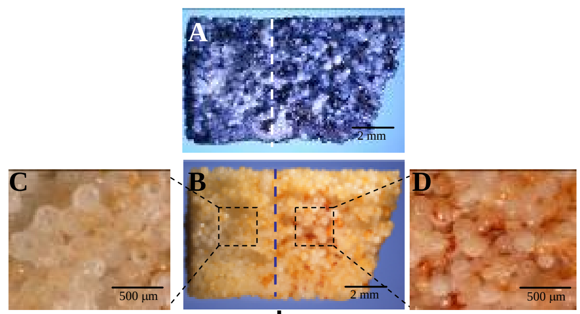


Fig. 1: *Homogenous cell distribution (A) and location of ALP staining (B) demonstrating the lack of osteoblast induced differentiation of C2C12 cells in the non-rhBMP2 loaded zone (C) and positive ALP staining (red) in the rhBMP-2 loaded zone (D).*

DISCUSSION & CONCLUSIONS: This study has demonstrated that zonal release of proteins to induce a location specific cell response can be achieved by organizing protein loaded microparticles into layered scaffolds. The doses of proteins within these scaffolds can be tuned by varying the ratio of protein-loaded and protein-free microparticles.

REFERENCES: [1]P.Q. Ruhe, et al, (2005) *J. Contr. Rel*. **106:**162. [2]T. Morita, et al. (2000) *J. Cont. Rel* **69**: 435.

ACKNOWLEDGEMENTS: EPSRC and the Islamic Development Bank for funding.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 6) ISSN 1473-2262

Biocompatibility of Normal Human Urothelial (NHU) and Urinary tract-derived Smooth Muscle (USM) cells grown on two and three dimensional (Poly (lactic-co-glycolic) acid (PLGA) scaffolds

J.Pettit[1], G.Rohman[2] , F.Isaure[2], N.Cameron[2] & J.Southgate[1]

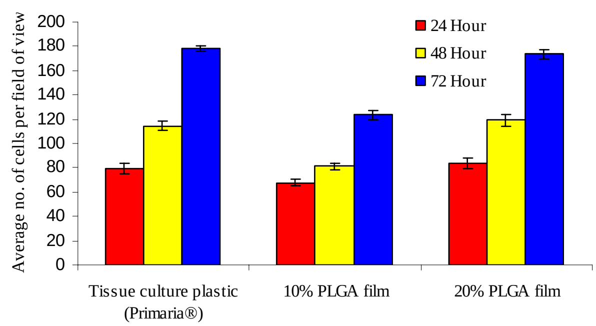
[1] *Jack Birch Unit of Molecular Carcinogenesis, University of York, England.*
[2] *Dept of Chemistry, University of Durham, England*

INTRODUCTION: The main function of the bladder as a low pressure storage reservoir for urine can be severely compromised as a result of severe dysfunction, trauma, cancer and congenital abnormalities. Tissue engineering strategies are consequently being developed in an attempt to rebuild and surgically repair the bladder. This investigation concentrates on the development of synthetic biomaterials as scaffolds for bladder repair/reconstruction. We report the preparation of non-porous two dimensional films and porous three dimensional foams and assess their biocompatibility in terms of supporting the adhesion and growth of NHU and USM cells.

METHODS: PLGA materials were prepared using two different methods: spin-coating to prepare non-porous 2D flat films of 10% and 20% (w/v) PLGA and emulsion freeze drying to prepare porous 3D foams. Initially, NHU and USM cells were seeded onto the 2D films and cell numbers were analysed at 24, 48 and 72 hour time points to construct growth curves. Film topography was assessed by Atomic Force Microscopy (AFM) and film degradation studies were undertaken using size exclusion chromatography. USM cells were seeded on to the 3D PLGA foams and maintained in submerged culture for up to 7 days to achieve cell infiltration and proliferation. Scanning Electron Microscopy (SEM) analysis was employed to visualise the USM cells within the 3D scaffold and USM-seeded 3D foams were embedded in polyester wax and sectioned by adapting a protocol by Steedman [1], and analysed by immunofluorescence for the expression of cell and extracellular matrix (ECM) proteins.

RESULTS: The 2D film study demonstrated that NHU and USM cells were able to adhere and proliferate on the PLGA non-porous 2D membranes, with both cell types growing significantly better on the 20% (w/v) PLGA films compared to the 10% (w/v) films at each time point ($p<0.05$; figure 1). AFM showed that there were no differences in surface topography between the 10% and 20% (w/v) films. Degradation studies showed that 10% (w/v) PLGA films degraded faster due to their greater surface area to volume ratio.

Fig. 1: NHU cell growth on 10% and 20% (w/v) PLGA 2D flat films.



Following 7 days of incubation, SEM analysis showed that USM cells were found evenly distributed within the 3D PLGA scaffolds and immunofluorescence studies demonstrated that ECM proteins had been deposited within the scaffold in association with the cells.

DISCUSSION: This study has shown that PLGA is a suitable synthetic material for supporting the growth of USM and NHU cells. The 2D film study suggests that although the degradability properties can be tuned by altering the PLGA concentration, this may affect how well the materials support cell growth. We suggest that PLGA foams provide a suitable scaffold environment to investigate whether cell interactions in 3D will lead to the development of more differentiated, functional tissue facsimiles. However, methods to monitor and assess the growth and phenotype of cells seeded in 3D scaffolds will require further development.

REFERENCES: [1] HF.Steedman (1957) *Nature*

ACKNOWLEDGEMENTS: This research was funded by the BBSRC.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 7) ISSN 1473-2262

Nanoscale Biomimetic Modification of the Cell-Substrate Interface for Bone Tissue Engineering

M.J.P Biggs[1,2,*], **R.G Richards**[2], **N Gadegaard**[1], **C.D.W Wilkinson**[1], **M.J Dalby**[1]

[1]*Centre for Cell Engineering, Institute of Biomedical and Life Sciences, University of Glasgow, UK.*

[2]*AO Research Institute, AO Foundation, Davos, CH.*

INTRODUCTION: Topographical modification may be particularly exploited in fracture fixation to improve osseointegration and implant stability. Cell-substrate interactions are mediated through transmembrane integrin receptors coupled to the extracellular matrix (ECM). Experimentally, nanofeatures have been shown to affect contact guidance *in vitro* and directly influence cellular adhesion[1]. Here experimental nanotopographies of varying order have been fabricated by electron-beam lithography (EBL), photolithography (PL) and polymer demixing in polymethylmethacrylate (PMMA). This study is concerned with the effects these nanotopographies have on adhesion formation in S-phase osteoblasts.

METHODS: 100 nm deep nanopit topographies were fabricated by EBL in square (Sq), hexagonal (HEX) and near-square (N-Sq) symmetries. Grooved substrates produced by PL were 327 nm deep, 100 µm, 10 µm and 25 µm wide. Two Poly(styrene) and poly(bromostyrene) polymer demixed topographies were fabricated. Primary human osteoblasts (HOBs) were cultured on all substrates, and S-phase cells identified by bromodeoxyuridine (BrdU) labelling. Immunocytochemistry and SEM were used to observed vinculin, S-phase nuclei and F-actin. Adhesion size and number were quantified by Image analysis[#]. Adhesions were designated as focal contacts (FX), focal adhesions (FA) of fibrillar adhesions (FB) according to size[2].

RESULTS: Highly ordered arrays of nanopits disrupted cytoskeletal organisation and cellular adhesion relative to controls. Grooved substrates induced contact guidance and adhesion formation dependant on groove width. Both polymer demixed topographies induced cell flattening, but not increased adhesion formation, (Table 1.).

DISCUSSION & CONCLUSIONS: Polymer demixed substrates induced increased HOB spreading relative to controls; adhesion size and number however were not seen to increase. Adhesion formation on nanogrooves was increased with intergroove distance and cell density, possibly as a result of increased protein adsorption. Narrow grooves increased contact guidance and influenced adhesion orientation (Fig 1.). Nanotopographical conformation can regulate adhesion formation and contact guidance.

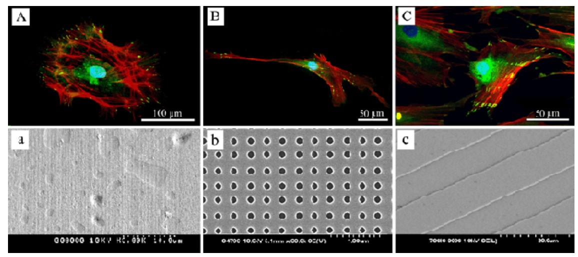


Fig. 1. (A) HOBs were spread on polymer demixed topographies (a). (B) Adhesion formation was reduced on Sq arrays of nanopits (b). (C) Grooved substrates influenced adhesion orientation (c).

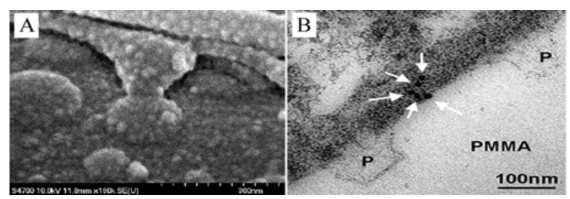


Fig. 2. (A) SEM of filopodial interaction with nanoislands. (B) Immuno-TEM interpit adhesion (P).

Table 1. *Adhesion Subgroup Size and Number*

Substrate Topography	Adhesion No			HOB Morphology
	FX	FA	FB	
Control	35	38	9	Spread
Square	25	15	2	Elongated
Hex	18	19	3	Rounded
N-Square	26	29	19	Spread
3% 1000 rpm	15	32	5	Spread
1% 3000 rpm	21	24	2	Spread
100 µm grooves	30	34	3	Spread
25 µm grooves	23	27	4	Elongated
10 µm grooves	14	25	3	Elongated

REFERENCES: [1] Zhu (2004) *Biomaterials* **25**, 4215-23. [2] Diener (2005) *Biomaterials* **26,** 383-392 [#]http://rsb.info.nih.gov/nih-image

*e-mail: m.biggs.1@research.gla.ac.uk

ACKNOWLEDGEMENTS: MJPB is supported by a grant from the AO Research Fund, Switzerland. MJD is a supported BBSRC David Phillips Fellowship.

Cellular Mechanotransduction in Bone: Moving Towards a Molecular Mechanism

C Jacobs[1,2], J You[3], G Reilly[4], N Batra[12], Y Li[12], A Malone[12], S Themiyasathit[12], J Tang[12], T Stearns[5], & C Anderson[5]

[1] *Dept of Mechanical Engineering, Stanford University, Stanford, USA*
[2] *Bone and Joint R&D Center, Department of Veterans Affairs, Palo Alto USA*
[3] *Dept of Orthopaedics, Pennsylvania State University, USA*
[4] *Dept of Engineering Materials, University of Sheffield, England*
[5] *Dept of Genetics, Stanford University, Stanford, USA*

INTRODUCTION: Bone cells occupy fluid filled voids (lacunae) in the mineralized matrix, interconnected by small tubes (canaliculi). As the bone matrix is cyclically loaded, fluid flows in the lacunar-canalicular network from regions of high matrix strain to low matrix strain and back in an oscillatory fashion. We have shown that this dynamic fluid flow is a potent signal for bone cell[1] regulations and that there appears to be important differences between the response to oscillatory flow and flows that do not incorporate a reversal of flow direction. We have characterized the biochemical signaling pathway activated by oscillatory fluid flow as involving IP3 mediated calcium signaling and MAP kinase signaling leading to osteogenic gene regulation.[2] Finally, we found that PGE2 release (critical to bone adaptation to mechanical loading) occurs in response to oscillatory fluid flow independent of intracellular calcium signaling, but does involve membrane associated extracellular proteoglycans[3] suggesting that at least two cellular signaling pathways are activated by oscillatory fluid flow. Currently our work is focused on uncovering the molecular mechanism by which these signaling pathways are activated.

DYNAMIC VS STEADY FLOW AND THE CYTOSKELETON: Interestingly, we have found that in contrast to unidirectional flow, the signaling pathway activated by oscillatory fluid flow does not involve the stretch activated membrane calcium channel. We speculate that one reason for this might be the viscoelastic mechanical nature of cells. Chronic unidirectional flow is likely to result in much larger cellular deformations than short-term reversing flow, thereby activating different cellular signaling pathways, perhaps associated with fracture healing. We have also recently found that while an hour of unidirectional flow leads to the formation of actin stress fibers in the cytoskeletons of bone cells, this does not occur with reversing oscillatory flow supporting the view that cellular viscoelasticity may be an important consideration for mechanotransduction.

STEM CELLS: Mechanical disuse is known to result in decreased numbers of bone forming cells. Thus, loading induced fluid flow may be an important regulator of osteoprogenitors as well as mature bone cells. In our recent work, we have found that oscillatory fluid flow does indeed increase the proliferation rate of bone marrow stromal cultures as well as the expression of markers of osteogenic differentiation.[4]

PRIMARY CILIA AS MECHANOSENSORS: Primary cilia are non-motile flagella-like structures found in most mammalian cell types. Although their existence has been known for over a century, they have had no clear function suggesting that they may be vestigial. However, recently they have been found to be involved in polycystic kidney disease and may act as sensors of fluid flow. This has prompted us to examine the potential role of the primary cilium as a mechanotransducer in bone. In our preliminary studies we have verified the existence of primary cilia in bone cells *in vivo*. Furthermore, we have found primary cilia in MC3T3-E1 osteoblastic and MLO-Y4 osteocytic cell lines and found that removal of the cilia results in reduced flow sensitivity in the cells. We have recently created a bone-specific conditional knock-out of the cilia protein Kif3A and are examining the osteogenic response of these mice to loading.

REFERENCES: [1]C Jacobs et al (1998) *J Biomech* **31:**969-976. [2]J You et al (2001) *J Biol Chem* **276:**13365-13371. [3]G Reilly et al (2003) *Biorheology*, **40:**591-603. [4]Y Li et al (2004) *J Orthop Res* **22:**1283-1289.

ACKNOWLEDGEMENTS: This work was supported by NIH AR45989, NASA NAG2-1601, and US Army Medical Research Award DAMD 17-98-1-8509.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 9) ISSN 1473-2262

Effect of tissue thickness and mechanical stimulation on porcine ligament tissue homeostasis

J Ingram, J Fisher & E Ingham

Institute of Medical and Biological Engineering, University Of Leeds, UK

INTRODUCTION: Acellular natural tissue scaffolds offer a promising solution to tissue engineering of the ACL. Previous work has shown that porcine patella tendons can be decellularised using low concentration SDS[1]. Following ultrasound treatment, the acellular tendons retained biomechanical properties and tenocytes were shown to migrate into the scaffold[1]. However the matrix became disorganised and cell viability was reduced to 50%[1].

In order to determine the conditions for culture of tissue engineered ACL, the aim of this study was to investigate the effects of dynamic culture and tissue thickness on the maintenance of cell viability and tissue histioarchitecture of porcine tendons.

METHODS: Patella tendons were dissected from hind legs of large white female pigs (35kg) within 1h of humane killing. Firstly, whole tendons (n=3) were cultured under four different conditions: a) clamped static culture b) clamped dynamic culture (1Hz, 6% strain for 4/24h) c) clamped rolled culture and d) unclamped rolled culture(12 rpm) for 6, 48 or 96 hours and 2 weeks. Secondly, tendons were split into individual fascicles or grouped fascicles.(~500µm) and cultured under a) clamped static culture b) clamped dynamic culture (1Hz, 6% strain for 4 /24h) or c) unclamped static culture (n=3; all groups) for 96 h and 2 weeks. Prior to harvest (2h) all tissues were injected with hypoxyprobe^{tm}-1(Chemicon). Tissues were harvested, split and samples were cryoembedded, processed for histology, or stained with Live/Dead stain (Molecular Probes) for examination by confocal microscopy. Immunoperoxidase labelling for MMP-1, -2 and -13 and TIMP-1.was carried out on cryostat sections. Histology sections were stained with H&E and labelled with a monoclonal antibody against hypoxyprobe^{tm}-1(Chemicon).

RESULTS: No change in histioarchitecture of any of the whole tendon groups was seen up to 96h. Only those tissues that had been cultured dynamically maintained normal histioarchitecture over the two week period, when clamped static tissues had a looser crimp than clamped dynamic tissues. Unclamped rolled tissues exhibited total loss of histioarchitecture at two weeks. Tissues which had not been rolled had significantly lower percentages of viable cells (clamped static 70.3±6.91, clamped dynamic 70.0±7.1) compared to rolled tissues (unclamped 99.33±0.65, clamped 99.16±0.8 $p<0.05$) following two weeks of culture. Hypoxia was observed in non-rolled samples at 48 and 96 hours at depths of >600µm although cell viability was unaffected at 48 hours.

The overall histioarchitecture of dynamically cultured fascicles (individual and grouped) was not affected. The clamped static fascicles showed some relaxation of collagen crimp. The non clamped statically cultured fascicles showed complete breakdown of the extracellular matrix Hypoxyprobe labelling of fascicles (individual and grouped) showed no evidence of hypoxia and cell viability was maintained in all fascicles for 2 weeks. Labelling for MMP-1, -2 and -13 and TIMP-1 at 2 weeks demonstrated strong positive labelling for MMP-1 and -2 in the non clamped samples. No other labelling was positive.

DISCUSSION & CONCLUSIONS: This study has shown that whilst static tension was able to maintain tissue histioarchitecture in the short term, in order to maintain normal tendon tissue histioarchitecture over two weeks dynamic mechanical stimulation was required. Dynamic culture was able to maintain tendon tissue histioarchitecture even with reduced cell viability. Tissue thickness was a limiting factor for maintenance of cell viability in whole tendon tissue and cell viability could be increased by rolling the tissues, presumably due to an increase in mass transport. Culture of individual tendon fascicles (individual or grouped) prevented hypoxia and cell viability was maintained. The lack of hypoxia in grouped tendon fascicles and the ability to maintain tissue histioarchitecture by dynamic culture *in vitro* is an important step forward in tissue engineering of the ACL.

REFERENCES:[1]Ingram *et al* (2004) WBC, Sydney .

‘External loading determines specific ECM genes regulation’

D.Karamichos, J.Skinner R.Brown and V.Mudera

TREC (Tissue Repair and Engineering Centre), Institute of Orthopaedics,University College London,UK

INTRODUCTION: Bio artificial matrices embedded with cells are simulated in bioreactors to facilitate ECM production. As cells attach, they develop forces, which are dependent on cell type and matrix stiffness. External forces (i.e strain), however, are critical for tissue homeostasis and elicit specific cellular responses, such as gene expression[3] and protein production. Collagen Type I is a widely used scaffold in Tissue engineering. The aim of this study was to study the mechanical and molecular responses, of different cell types to increasing collagen substrate stiffness[2].

METHODS: Cultured Human Bone Marrow Stem Cells (hBMSC) and Human Dermal Fibroblasts (HDF) were embedded in collagen constructs in 10% and 20% Fetal Calf Serum(FCS). Constructs were then pre-strained (0%, 5% and 10%) to increase matrix stiffness. Contraction forces generated by cells were quantified for 24 hours on the tensional Culture Force Monitor[1] after allowing for visco-elastic relaxation of collagen. ECM regulatory molecular genes were quantified using real time RT-PCR.

RESULTS: hBMSCs showed FCS dependent contraction forces, in contrast to HDFs which showed matrix stiffness dependent. 10% FCS concentration significantly (p=0.05) reduced peak contraction as pre strain was increased in both hBMSC’s (Figure 1) and HDF’s (0%>5%>10%). HDF’s had significantly increased force generation (twice as much) to increasing serum concentration (i.e 10% to 20%) however contraction was still significantly reduced with increasing substrate stiffness in 20% FCS. In contrast, hBMSC’s at 20% FCS generated similar peak force contraction at 24h irrespective of pre strain (0%=5%=10%, Figure 2). ECM regulatory genes MMP2 and MMP9 showed up regulation at 5% pre strain, but a 50% down regulation when pre strain was increased to 10%. COL3 was down regulated at 5% and up regulated at 10% pre-strain.

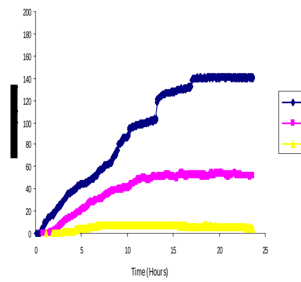


Figure 1. hBMSC’s contraction force response to 0%-10% pre-strain (10% FCS)

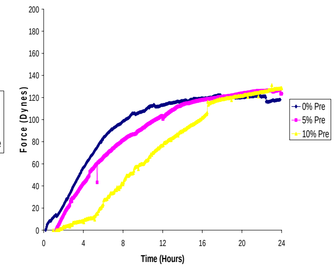


Figure 2. hBMSC’s contraction force response to 0%-10% pre-strain (20% FCS)

DISCUSSION & CONCLUSIONS: We have shown significantly differential mechanical and molecular response of hBMSC’s to increased substrate stiffness and increased FCS (20%) stimulation compared to HDF’s. For predictable cellular responses, mechanical stimulation of cells will have to be tailored to take into account the increasing stiffness of the matrix as ECM is deposited, as well as cell phenotype and serum concentration.

REFERENCES:

[1]M.Eastwood,V.C.Mudera,D.A.McGrouther,R.A.Brown (1998) Effect of precise mechanical loading on fibroblast populated collagen lattices: Morphological changes. *Cell motility and the cytoskeleton* **40**:13-21

[2]Karamichos D, Mudera VC, Brown RA. 2006. Complex dependence of substrate stiffness and serum concentration on cell-force generation. *J Biomedical Materials Research: Part A* Accepted for publication

[3]V.C.Mudera,R.Pleass,M.Eastwood,R.Tarnuzzer,G.Schultz,P.Khaw,D.A.McGrouther,R.A.Brown (2000) Molecular responces of human dermal fibroblasts to dual cues:Contact guidance and mechanical load.*Cell motility and the cytoskeleton* **45**:1-9

ACKNOWLEDGEMENTS: This study is funded by a Engineering and Physical Sciences Research Council Grant.

 ISSN 1473-2262

Measurement of collagen synthesis by cells grown under different mechanical stimuli

F. Foroughi, V. Mudera & R. Brown

University College London, Tissue Repair and Engineering Centre, Institute of Orthopaedics, Stanmore Campus, London HA7 4LP

INTRODUCTION: The use of scaffolds in tissue engineering is essential to provide cells with a matrix for cell proliferation and differentiation resulting in tissue regeneration. Normally this process involves seeding cells onto an artificial biodegradable scaffold providing mechanical support for cells until there is sufficient extracellular matrix deposition (ECM) to replace the artificial scaffold. Collagen is the bulk protein found in the ECM and measurement of its synthesis is the most direct, absolute indicator of ECM production.

METHODS: An HPLC method has been used for assay of hydroxyproline as a measure of collagen synthesis. At this stage 2D cell cultures were used as an initial screen. Four cell types (two CHO cell lines, human adult dermal fibroblasts and human neo natal dermal fibroblasts) were grown for 11 days in well plates. Culture media were sampled every 2-3 days for cumulative production of soluble collagen. At the end point of culture cell pellets were hydrolysed to give total collagen levels. Production rates, total collagen deposition and % deposition efficiencies were derived from these data for the four cell types.

These were compared with fibroblasts grown on polyglycolic acid (PGA) and polyethylene terephthalate (PET) meshes under static conditions, with and without mechanical stimulation in a pulsatile mechano-bioreactor.

RESULTS: The HPLC assay system was effective in quantifying collagen synthesis by cells in 2D culture. Comparison of the four cell types indicated that human adult dermal fibroblasts (HDFa) produced the largest quantity of total collagen (1331 ng) at day 11 but at low deposition efficiency (10.7%). The deposition efficiency of human neo natal dermal fibroblasts (HDFn) was highest (18.5%) from 957 ng of total collagen produced at day 11.

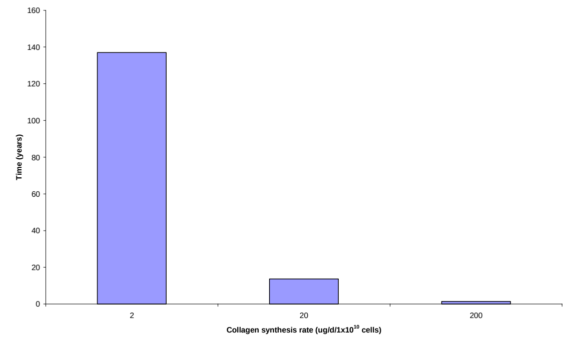


Fig. 1 The time required to produce 100 mg of collagen by cells at synthesis rates of 2, 20 and 200 µg per day.

Based on these results we have calculated the time required to produce 100 mg of collagen if cells were depositing 2, 20 or 200 µg of collagen per day by $1x10^{10}$ cells (Figure 1). On this basis the HDFn cells would take 54 years and the adult fibroblasts 67 years to produce a 100 mg construct.

Results for collagen synthesis by fibroblasts seeded onto static PGA and PET meshes are ongoing.

DISCUSSION & CONCLUSIONS: The results indicate that the collagen synthesis rates obtained using existing culture systems are insufficient to produce adequate collagen for a tissue engineered construct.

An alternative strategy may be to seed cells onto a collagen scaffold and culture in a bioreactor for expression of other ECM proteins (e.g. elastin, growth factors) in order to speed up the process of fabricating a tissue engineered construct.

ACKNOWLEDGEMENTS: This work was supported by an EU Framework 6 Programme grant - 3G-SCAFF: Third generation scaffolds for tissue engineering and regenerative medicine.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 12) ISSN 1473-2262

Non-Destructive Quantitative 3D Analysis of Structural, Flow and Mechanical properties of Porous Scaffolds

G. Poologasundarampillai, P. D. Lee, J. R. Jones
Department of Materials, Imperial College London, South Kensington Campus, London SW7 2AZ, UK

INTRODUCTION: Bioactive glass scaffolds have been produced with open macropores with a high degree of interconnectivity and large apertures for tissue engineering (TE) applications. The size of the porosity, and more importantly the interconnecting apertures, is critical to the success of 3D cell seeding and the survivability and growth of the new tissue. Non-destructive X-ray micro-tomography (XMT) and novel 3D image analysis have been used to quantify the pore networks. Fig 1 compares 3D XMT images of a scaffold and human trabecular bone. XMT data was input into a control volume model to predict the flow rate of culture medium through the scaffolds, which can be used for the design of optimised bioreactors for *in vitro* growth.

MATERIAL & METHODS: Glass Foaming: Bioactive glass scaffolds of composition 70 mol% SiO_2, 30 mol% CaO were made with 92% porosity using the sol-gel method with different final sintering temperatures (T_s); 600, 800, 1000°C [1].

XMT Scanning: One scaffold was scanned and sintered repeatedly so that the change in a specific pore network with temperature could be observed. Foams were cut into cuboids and scanned using a commercial XMT unit (Phoenix systems). Two different resolution scans were performed (high: 9.5-12.5 µm; low: 17.5-22.5 µm).

Analysing 3D Data: Quantification of the pore network, i.e., pore and aperture sizes, was achieved by thresholding and applying a dilatation algorithm to create a distance map of the image, which was then fed into a watershed algorithm [2]. Pore and aperture distributions for samples were plotted. Simulations were done on the recon-structed data to calculate flow and mechanical properties. Accuracy of the results was checked using manual measurements of individual pores and mercury porosimetry (MIP).

RESULTS & DISCUSSION: Pore size and aperture size distributions were obtained using the novel 3D image processing techniques. The modal pore diameter (D_{mode}) and modal aperture (A_{mode}) sizes decreased as T_s increased (Table 1). The % porosity also decreased. A_{mode} was greater than 100 µm for all the samples, as required for TE applications. The A_{mode} found from mercury porosimetry (MIP) was 100 µm or higher, but lower than the values obtained from 3D image analysis - MIP calculates the equivalent aperture diameter from flow analysis of mercury, rather than aperture length. Scaffold compressive strength increased, as T_s increased to 800°C, but aperture sizes were still suitable for TE.

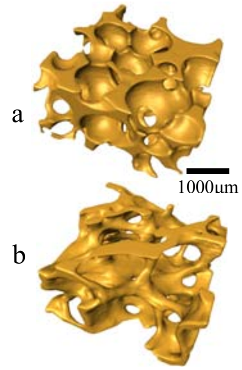


Fig 1. 3D image of (a) a scaffold and (b) human trabecular bone

Fig. 2 shows predicted fluid flow through a scaffold. Apertures were found to dictate flow properties. Table 1 shows mean value of the perme-ability tensor ($\overline{K}_{comp}$), which correlate to the MIP results.

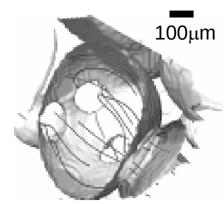


Fig. 2. Modeled flow shown in a scaffold pore using streak lines

Table 1. Characterisation of one scaffold heated to different T_s.

T_s [°C]	% Porosity	D_{mode} (3D) [µm]	A_{mode} (3D) [µm]	A_{mode} (Hg) [µm]	$\overline{K}_{comp}$
600	92.0	689	287	153	918
800	85.0	567	227	97	638
1000	84.5	537	228	119	721

Finite element modeling successfully predicted compressive strength of the scaffolds (data not shown).

CONCLUSIONS: XMT and novel image analysis allowed non-destructive 3D characterization of bioactive glass scaffolds and prediction of flow as a function of the pore network. These techniques are applicable to many types of scaffold material.

REFERENCES:

1. Jones JR, Ehrenfried LM, Hench LL, Biomaterials, 2006;7:964-973,
2. Jones JR, Lee PD, Hench LL. Phi T Roy A 2006: 364:263-281.

ACKNOWLEDGEMENTS: Royal Academy of Engineering, EPSRC (funding) and Dr Dominique Bernard (permeability model).

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 13) ISSN 1473-2262

Pulmonary Artery Smooth Muscle Cells Response to Vaso-active Stimulations In a Real-time 3-Dimensional Model

A. Soueid[1], T. Smith[1], S. Hall[2], V. Mudera[1]

[1]Tissue Regeneration and Engineering Centre, Institute of Orthopaedics, UCL, Stanmore.

[2]Institute of Child Health, Great Ormond Street Hospital, London.

INTRODUCTION: The tone of the pulmonary arteries is the summation of the activity of each smooth muscle cell (PASMC) within a vessel wall and its interaction with the endothelial cells and extracellular matrix (including collagen). There are reported phenotypic differences between PASMC in the inner & outer layers of pulmonary artery walls[1]. The response of a tissue engineered blood vessel to contractile and relaxing stimulants in-vitro is essential to predicting the response of the physiological and pathological vessels in-vivo. Previous work showed that pulmonary artery relaxation to nitric oxide is inhibited after exposure to chronic hypoxia [2]. We hypothesised that PASMC will differ in their ability to contract or relax a 3D collagen gel. Using a Culture Force Monitor (CFM) we sought to quantify the cellular response of PASMC derived from inner and outer normal and hypoxic arteries, harvested from piglet models, over 24 hours in response to contractile agonists and relaxing antagonists.

METHODS: Piglets were exposed to hypoxia (50KPa) for 3-14 days then sacrificed at day 14. Large intrapulmonary arteries were dissected and SMC derived from inner & outer layers were then cultured in DMEM/F12 medium with 10% FCS. 5 ml rectangular Collagen gels2 (rat tail collagen type I, 10x minimal essential medium, sodium hydroxide) were prepared in a sterilised silicone polymer mould and seeded with 5 million cells (passage 3-6). The gel was allowed to set with 2 A-frames (layered polyethylene mesh with a stainless steel frame) on either side and then suspended in DMEM with 10% FCS. One A-frame was connected to a fixed point in the CFM while the other is connected to a transducer. Real time contractile force generated (1 per second) and cellular response were recorded over 24 hours (at 37 ^{o}C, 5% CO^2). Once the cells reached tensional haemostasis an agonist (U46619) or antagonist (Sodium Nitropruside, SNP) drug was added to the system and the response was measured in real time.

RESULTS: Normal Outer PASMCs generated an immediate and tri-phasic contractile response to agonists with a mean peak force generated of 74 dynes ± SEM 24. Normal Inner PASMCs showed a similar response to the agonist (mean peak force 172 dynes ± SEM 61). In response to the antagonists, Normal Outer PASMCs relaxed to a mean of 236 dynes ± SEM 123 and Normal Inner to 153 dynes ± SEM 20. Hypoxic Outer PASMCs generated an increase in contractile force of 31.2 dynes ± 10.7 SEM and Hypoxic Inners a contractile force of 37.4 dynes ± SEM 11. Neither the Hypoxic Outer nor Inner PASMCs showed a response to SNP.

DISCUSSION AND CONCLUSIONS: We demonstrated that PASMCs derived from the normal pulmonary vessels respond to contractile and relaxing stimulants. On exposure to chronic hypoxia, the cells retained their ability to contract in response to agonists however they were unresponsive to antagonist stimulation. These findings suggest relaxing mechanisms of PASMC has been permanently altered as a response to chronic hypoxia, which will have implications in tissue engineering of blood vessels or treatment of pulmonary hypertension.

REFERENCES: [1] S.M. Hall, A. Hislop, C. Pierce et al (2000) prenatal origins of human intrapulmonary arteries. *Am J Respir Cell Mol Bio* **23**:194-203. [2] Tulloh, R.M., Hislop, A.A., Boels, P.J. et al (1997) Chronic hypoxia inhibits postnatal maturation of porcine intrapulmonary artery relaxation. *Am J Physiology* **272**, H2436-H24

TRANSLATING TISSUE ENGINEERING TO THE CLINIC

Professor Sheila MacNeil

Department of Engineering Materials and Division of Clinical Sciences (North), University of Sheffield and Research & Development Director, CellTran Ltd

Tissue engineering of skin is almost 25 years old. Based on a 1975 methodology for the production of thin sheets of integrated keratinocytes (cultured epithelial autografts, CEA) cells have been expanded in the laboratory and used successfully in the treatment of patients with extensive full thickness burns since the early 80s. However, attempts at developing commercial tissue engineered products have achieved only modest success. Unfortunately this has impacted negatively on perceptions of what is possible in this field. Thus the field has gone from a basic pragmatic approach into over inflated commercial expectations. Despite this, cultured cells and tissue engineered skin have significant benefits to offer patients with extensive burns and patients with chronic non-healing ulcers.

The approach that we have been working on within the University of Sheffield since the early 90s is to take existing methodologies for culturing keratinocytes and delivering them to patients and for making reconstructed human skin and seek to develop approaches which are significant improvements on these technologies with a view to making them more patient and clinician friendly. Thus for cultured epithelial autografts, through the spin-out company CellTran Ltd, we have sought to improve on this early methodology for culturing cells and transferring them from the laboratory to the patient. Using a chemically defined plasma polymerised surface containing 20% carboxylic acid groups, cells can be delivered on this surface to patients following initial expansion in the laboratory using conventional methodologies (1). This approach has been tried for repeated applications of autologous keratinocytes for chronic non-healing diabetic foot ulcers with considerable success (2) and more recently has been launched (Myskin) for the treatment of patients with extensive burns injuries. Supplying the cells on an easy to handle polymer disc obviates the necessity of the surgeon or district nurse handling spray-on cells or fragile sheets of cultured cells. The application of Myskin to the wound bed has also been made compatible with other ongoing treatment regimes for burns therapy and chronic wounds.

With respect to reconstructed skin, beginning initially with skin based on sterilised de-epidermalised donor dermis, we have reconstructed skin with either epidermal keratinocytes and fibroblasts or oral mucosa keratinocytes and fibroblasts and have preliminary data with the use of both in reconstructive surgery. Based on this our current plans are to replace the human dermis with a synthetic biodegradable scaffold produced by electrospinning and our research on this shows that its possible to culture keratinocytes, fibroblasts and endothelial cells together in electrospun 3D scaffolds under serum free conditions with a promising degree of cell organisation.

Fig. 1: This shows the appearance of a diabetic foot ulcer which had been non-healing for 3 years and then after 7 applications of Myskin. Myskin was delivered once per week to fit in with the patient's visit to a diabetic foot clinic.

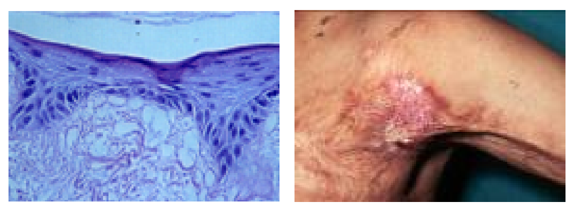


Fig 2: This shows the appearance of reconstructed human skin which was grafted into the axilla of a patient who had suffered skin contraction due to earlier burns injuries. As can be seen the reconstructed skin has survived and become vascularised but is beginning to contract.

REFERENCES: [1]France, R.M., Short, R.D., Dawson, R.A. and Mac Neil, S. (1998) Attachment of human keratinocytes to plasma co-polymers of acrylic acid/octa-1,7-diene and allyl amine/octa-1,7-diene. J. Materials Chemistry 8, 37-42. [2]Higham, MC, Dawson, R, Szabo, M, Short, R, Haddow, DB and MacNeil, S,(2003) "Development of a stable chemically defined surface for the culture of human keratinocytes under serum free conditions for clinical use". Tissue Engineering 9, 919-930 [3]Moustafa M, Simpson C, Glover M, Dawson RA, Tesfaye S, Creagh FM, Haddow D, Short R, Heller S and Mac Neil S. (2004)A new autologous keratinocyte dressing treatment for non-healing diabetic neuropathic foot ulcers. Diabetic Medicine 21,786-789.

 ISSN 1473-2262

DETAILED ANALYSIS OF THE GLYCOSAMINOGLYCAN CONTENT OF HUMAN AUTOLOGOUS CHONDROCYTE IMPLANTATION REPAIR TISSUE.

A Sharma[1] J B Richardson[2] S Roberts[3] N J Goodstone[1]
[1] *Institute of Science and Tecnology in Medicine (ISTM), University of Keele, England.*
[2]*Institute of Orthopaedics &* [3]*Centre for Spinal Studies, Robert Jones Agnes Hunt (RJAH) Orthopaedic & District Hospital NHS Trust, ISTM, University of Keele, England.*

INTRODUCTION: Cartilage repair strategies such as autologous chondrocyte implantation (ACI) are routinely used to treat damaged cartilage. To date, techniques to assess the glycosaminoglycans (GAGs) in the repair cartilage are limited because they often require large amounts of starting material and are time consuming. Fluorophore-Assisted Carbohydrate Electrophoresis (FACE) is a rapid and sensitive technique [1], which is useful for profiling GAGs from repair cartilage biopsies. Since the GAG component of any repair tissue is likely to have a major influence on its physiological functioning[2], we used FACE to quantitate and profile the amount and nature of GAGs in ACI repair cartilage removed 1-yr post-operatively. **METHODS:** Using a bone marrow biopsy needle (Manatech), biopsies (1.8 mm in diameter) were taken perpendicularly from the articulating surface through the full depth of cartilage and subchondral bone, from 8 patients who had undergone ACI 12 months previously. In all of the patients biopsies were taken from femoral condyle (5 medial, 3 lateral). Biopsies were selected from patients between 22 and 52 years in age. They were assessed comparatively with age and anatomical site matched cadaver tissue.

RESULTS: The repair tissue in all ages was composed predominantly of chondroitin sulphate (CS) (Fig 1A). Hyaluronan (HA) accounted for significantly more of the total GAG ($p<0.01\%$) when compared with the age matched cadaver samples (Fig 1). Disaccharides and non-reducing termini of all GAG populations; HA, CS and keratan sulphate (KS), were significantly reduced when compared with the age and anatomical site matched cadaver tissue ($p<0.01\%$) (Fig 2). Within the ACI repair tissue decreases, although not significant, were detected in concentrations of both unsulphated and sulphated HA, CS and KS disaccharides with increasing age (Fig 2).

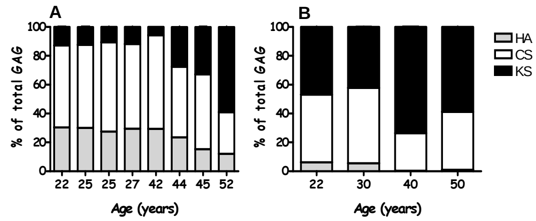


Fig 1:The percentage of HA, CS, and KS in cartilage biopsies taken from (A) patients 12 months post-ACI and (B) age and anatomical site matched cadver tissue.

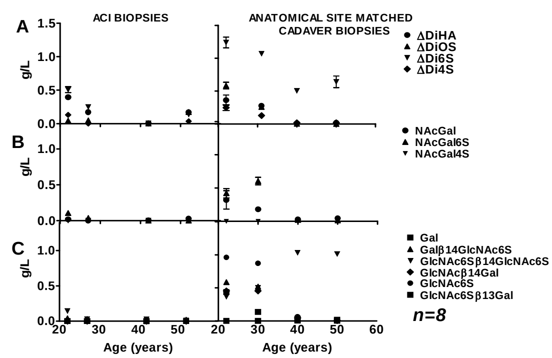


Fig 2: Levels of (A) HA and CS disaccharides, (B) CS non-reducing termini and (C) KS disaccharides in both ACI repair and cadaver cartilage.

DISCUSSION & CONCLUSIONS: FACE has provided new information about GAGs within repair tissue. The increase in the proportion of HA observed in the repair tissue may be important because the early stages of wound healing involve the generation of a HA rich matrix which aids cell migration and proliferation into the site of injury[3].

REFERENCES:.[1]Calabro A, Hascall VC, Midura RJ. *et al., Glycobiology* 2000; 10(3):283-293. [2]Bader DL, Kempson GE, Egan J *et al., Biochim Biophys Acta* 1992, 1116:147-152. [3]Toole BP. *Semin Cell Dev Biol* 2001; 12(2):79-87.

ACKNOWLEDGEMENTS: This work was funded by the Arthritis Research Campaign (16181).

 ISSN 1473-2262

Permacol™ Paste – a New Dermal Substitute in Full-Thickness Wounds?

R V Shevchenko[1], J R Sharpe[1], P D Sibbons[2], & S E James[1]

[1] *Blond McIndoe Centre, Queen Victoria Hospital, East Grinstead, RH19 3DZ, Sussex, UK.*
[2] *Northwick Park Institute for Medical Research, Harrow, HA1 3UJ, Middlesex, UK.*
Tel: +44 (0) 1342 414295 Fax: +44 (0) 1342 414550
liz.james@qvh.nhs.uk ross.schevchenko@qvh.nhs.uk www.blondmcindoe.com

INTRODUCTION: Insufficient skin for autologous grafts often makes treatment of full-thickness extensive burns difficult. Although cultured epithelial sheet grafts or sprayed keratinocyte suspensions have been applied to such wounds, poor long-term results have been achieved in most cases. This is thought to be due to the absence of a dermal layer [1]. In this study a new formulation of the porcine collagen-based biomaterial, Permacol™, was investigated for its potential role as a dermal substitute in full-thickness wounds. Permacol™, in the form of a sheet, has been useful as a stable implant in reconstructive surgery [2]. However, its use as a dermal substitute has been hindered by slow cell penetration and vascularisation [3-4]. A paste formulation was tested in our experiments in order to encourage cellular infiltration.

METHODS: Permacol™ was applied as a paste to the wound bed and covered by a split-thickness skin graft in the biopsy model and a silicone dressing in the large wound-chamber model in the Large White pig. The biopsy wound model allowed 24 samples of 8mm diameter to be tested per pig and the wound chamber model allowed 6 samples of 4cm diameter per pig. Suitable control wounds, which included Integra®, were set up in parallel. Excision biopsies were taken from punch-biopsy model wounds, and intrachamber punch biopsies with final excision biopsies were taken at specific time points up to day 28. To assess biointegration and neovascularisation, histological and immuno-fluorescent analysis of frozen and paraffin-fixed tissues was undertaken.

RESULTS: Permacol™ paste was well penetrated by cells by day 2, unlike Permacol™ sheet, and was integrated into the host tissue without causing excessive inflammation. Cellular infiltration of Permacol™ paste was superior to Permacol™ sheet and similar to Integra®.

The structure of Permacol™ collagen appeared to be similar to native dermis. Permacol™ paste was visible and intact within the tissue samples up to day 28, indicating the presence of a stable biomaterial.

Early neovascularisation in Permacol™ paste was noted at day 4, and functional newly-formed microvessels with circulating blood cells were discovered in some samples at day 7. Immunostaining for vascular endothelium confirmed early vascularisation of Permacol™ paste. This was similar when compared to Integra®.

DISCUSSION & CONCLUSIONS: This study indicates that a paste formulation of Permacol™ successfully bio-integrated into full-thickness wounds.

It was noted neovascularisation of Permacol™ paste at day 7 seemed to be superior to vascularisation of Integra®, which may be advantageous in the clinic for an earlier application of a thin split-thickness skin graft or sprayed keratinocyte suspension in heavily burned patients.

Paste formulation may have advantages in treating so-called “difficult” areas, where spreading a paste can allow for the variations in body contours as well as wound surface roughness, where sheet materials may fail to adhere and “take”.

Our findings suggest that Permacol™ in the form of a paste may act as a suitable alternative to current dermal substitutes for full-thickness wounds.

REFERENCES: [1] C.C. Compton, et al. (1993) *J.Burn Care Rehabil*. 14 (6):653-662. [2] S.H. Liyanage, et al. (2005) *Br.J.Plast.Surg.* Nov. 22. [3] T.M. Macleod, et al. (2004) *Burns* 30 (7):704-712. [4] M.L. Jarman-Smith et al. (2004) *J.Mater.Sci.Mater.Med.* 15 (8):925-932.

 ISSN 1473-2262

Improved technologies for translating culture of human melanocytes to the clinic

P. Eves, A.J. Beck, A.G. Shard and S. MacNeil.

Department of Engineering Materials, University of Sheffield, Sheffield, S3 7HQ, UK.

INTRODUCTION: Obtaining pigmentary function in autologous skin grafts is a current challenge in the healthcare industry as is developing reliable robust grafting strategies for patients with vitiligo. Vitiligo is an extremely common skin condition in which melanocytes stop producing pigment and are eventually destroyed by the immune system. Whilst not life threatening, it has a very negative psychological impact on patients.

In this paper we present ongoing work aimed at developing a simple methodology for delivering cultured cells to the patient that is clinically effective with respect to pigmentation and wound healing, is low risk for the patient but also user friendly for the surgeon or dermatologist when applying cells to the patient.

METHODS: Chemically defined substrates were produced by plasma polymerisation of either allylamine, acrylic acid or a plasma co-polymerisation of two of these monomers. Four culture media were employed, two designed to support keratinocytes, Green's (10% serum) and keratinocyte defined media (KDM – serum free), and two designed to support melanocyte expansion – MCDB153 (which cannot be used clinically) and melanocyte media (M2 – serum free). Cells were seeded either as monocultures, or as a 1:1 or 2:1 ratio of keratinocytes to melanocytes. Cell attachment and melanocyte function were assessed by MTT-ESTA assays and S100 staining.

RESULTS: Melanocytes and keratinocytes both in mono- and co-cultures attached and grew well on both acid and amine plasma polymers. There was no significant preference between these surfaces but co-cultures of the two cell types on these plasma polymers fared better than individual cell cultures. Explicitly, total cell numbers (keratinocytes plus melanocytes) in Greens, KDM and M2 media were 14%, 34% and 103% respectively greater than individual cultures of either melanocytes or keratinocytes. Also, with 2:1 co-cultures of keratinocytes:melanocytes on either acid or amine surfaces the survival rate of melanocytes in the presence of keratinocytes was much higher in M2 compared to Greens media. On an acid surface, melanocyte numbers ranged from 1700 cells/cm^2 in Greens to 5700 cells/cm^2 in M2. Melanocyte numbers on an amine surface ranged from 2500 cells/cm^2 in Greens to 4300 cells/cm^2 in M2.

Assessment of transfer of co-cultures of melanocytes and keratinocytes to an *in vitro* model of human dermis showed that melanocytes and keratinocytes can be successfully transferred from chemically defined carriers onto human dermis. This *in vitro* model of skin was then cultured in Greens media for 10 days at an air liquid interface. At this point the skin model was sacrificed for histological analysis. Figure 1 illustrates the presence of melanocytes (stained for S100 – brown endpoint – see red arrows) within the human skin model after a period of 10 days.

Fig 1: in vitro model of human skin

DISCUSSION: So far, we have demonstrated that chemically defined substrates can be used to culture both melanocytes and keratinocytes in medium (Greens) currently used in the clinic (which contains fetal calf serum sourced from New Zealand), but also in a serum free alternative – M2. We have also achieved successful transfer of melanocyte:keratinocyte co-cultures from flexible plasma polymerized silicone carriers onto an *in vitro* human wound bed model. However, in order to progress this work into a clinical environment we need to establish culture protocols within a GMP clean room and develop a cell transport media based on agar/hyaluronic acid for the transport of cells over a wide geographical area. A similar gelled media has recently been established by the group and used in a clinical environment for the treatment of chronic wounds with autologous keratinocytes.

CONCLUSION: We have developed a pre-clinical methodology for the delivery of a co-culture of autologous melanocytes and keratinocytes using a flexible and user-friendly chemically defined carrier surface. We now hope to translate this work from the laboratory into the clinic.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 18) ISSN 1473-2262

Biocompatibility of decellularised human amniotic membrane

S. Wilshaw[1], J. Fisher[1], J. Kearney[2] & E. Ingham[1]
[1] *Institute of Medical and Biological Engineering, University of Leeds, Leeds, England*
[2] *NHS Blood and Transplant, Liverpool, England*

INTRODUCTION: There is a clinical need for an immunologically compatible surgical patch with a wide range of uses including, use as a wound dressing and as a substrate for cell delivery. A novel detergent based protocol was modified to remove all cellular components from human amniotic membrane (HAM) in order to render it non immunogenic. The study aim was to investigate the biocompatibility of acellular human amniotic membrane *in vitro* and *in vivo*.

METHODS: HAM was decellularised according to the method developed by Wilshaw *et al.*, (2006).
Sodium dodecyl sulphate quantification: Radiolabelled C^{14} SDS was used to spike the wash buffer during decellularisation. The amount of radiolabelled SDS in each wash buffer and in the decellularised tissue was determined using a micro-plate scintillation counter and a standard curve of known C^{14} SDS concentrations.
In vivo biocompatibility study: Samples of fresh, decellularised and fresh glutaraldehyde-fixed HAM were implanted subcutaneously into groups of three mf-1 mice (two samples per mouse). The mice were sacrificed after three months. Explants were fixed, paraffin embedded and serially sectioned. Sections were stained using haematoxylin and eosin, Von Kossa's and labelled using monoclonal antibodies against CD 3, CD 4, CD 34 and F4/80.
Cell seeding: Primary human keratinocytes were seeded onto the basement membrane side of decellularised HAM. Following 24 hours the tissue was processed for SEM analysis. Primary human fibroblasts were also allowed to proliferate on the stromal side for up to four weeks, the rate of proliferation was monitored using the commercially available ATPLite-M® kit (PerkinElmer). The cell viability of the seeded cells was determined by means of the commercially available live/dead kit (Molecular Probes) and viewed using a confocal microscope and a conventional fluorescein longpass filter. Replicate samples (n=3) were fixed, paraffin embedded and serially sectioned. Sections were stained using haematoxylin and eosin.

RESULTS: The SDS assays indicated there to be 60 pg.g^{-1} SDS present in decellularised HAM and 3.19×10^{-4} % (w/v) was found to be present in the final wash buffer. Two of the decellularised samples, three of the fresh samples, whereas all six of the glutaraldehyde fixed samples were recovered from the mice after three months. The histology demonstrated a thick fibrous capsule and poor tissue integration of fresh HAM along with evidence of calcification. Decellularised HAM demonstrated good integration and a thin fibrous capsule. Immunohistochemical labeling of explanted decellularised HAM showed a low T-cell infiltrate with higher numbers of macrophages and endothelial cells, suggesting that the tissue might be being remodelled. Primary human keratinocytes demonstrated good attachment to decellularised HAM following 24 hours (Figure 1). Proliferation studies indicated that fibroblast numbers increased from 1.9×10^3 cells to 3.5×10^4 cells over a period of 14 days (Figure 2). Live/dead staining showed that decellularised HAM was able to maintain the viability of seeded human fibroblasts and keratinocytes over a period of 21 days.

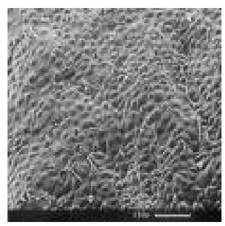


Figure 1 *SEM micrograph of primary human keratinocytes seeded onto basement membrane of decellularised human amniotic membrane after 24 hours.*

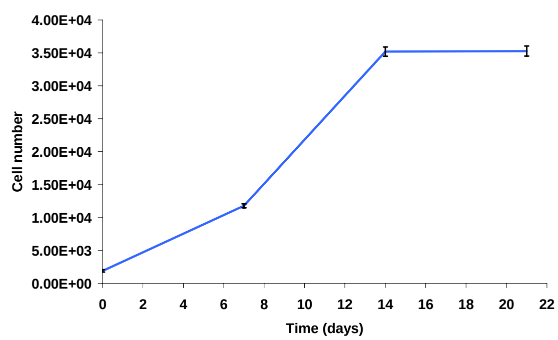


Figure 2 *Proliferation rate of primary human fibroblasts cultured on the stroma of decellularised HAM.*

DISCUSSION & CONCLUSIONS: The acellular matrix appeared to be biocompatible *in vivo*. The acellular matrix has shown potential for the attachment and proliferation of primary human fibroblasts and keratinocytes, as well as maintaining the viability of seeded cells.

REFERENCES: [1]S. Wilshaw, J. N. Kearney, J, Fisher & E. Ingham. (2006) *Tissue Engineering*.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 19) ISSN 1473-2262

BioDynamic Test Instrument for the Characterization of Tissues and Biomaterials

S. Williams, D. Burke, W. Conrads, T. Nickel, and L. Mejia

Bose Corporation, ElectroForce Systems Group, Eden Prairie, Minnesota, USA.

OBJECTIVE: The objective of this work was to employ the novel design of the BioDynamic testing platform to evaluate the mechanical properties of hydrogels and other composite biomaterials. The testing platform allows for continuous test and stimulation in a fully integrated and instrumented configuration by providing material characterization (viscoelastic properties, strength, creep and stress relaxation) within a physiological environment (nutrient flow, pressure loading, pH, dissolved oxygen, and temperature).

METHODS: An advanced BioDynamic testing platform has been designed that can be used to evaluate the mechanical properties of tissue-engineered constructs for both cardiovascular and musculoskeletal applications. The BioDynamic instrument was used to test a variety of specimens to demonstrate its versatility and advanced features. The dynamic mechanical properties of polyvinyl alcohol hydrogels (Cambridge Polymer Group, Boston, MA) were evaluated with our unique computer-controlled moving magnet linear motor that provides load, displacement, strain or pressure profiles. The hydrogel samples were 3-4 mm in diameter and 3-4 mm in height, and testing was performed in compression with a 5 mm displacement transducer and a 250 gram force transducer. Vascular graft distension with increasing pressure was also evaluated in a BioDynamic instrument using a laser micrometer. The graft material used (Gel-Del Technologies, Inc., St. Paul, MN) is composed of proteins and polymers fabricated to mimic the viscoelastic properties of native blood vessels.

RESULTS / DISCUSSION: The ability to perform very low force applications is illustrated in Figure 1. The peak-to-peak loading on the hydrogel was approximately 2 mN with a corresponding peak-to-peak displacement of 28 μm. A distinct linear region was not observed with a displacement ramp as the specimen made a very gradual change in stiffness as a function of % strain. Figure 2 shows the dynamic mechanical analysis software analysis as a 0.001 N compressive contact force was applied to the specimen. Upon completion of data acquisition, the software calculated the modulus and tan delta for the specimen, which appeared to exhibit resonance between 20 and 100 Hz. Figure 3 shows two cycles of a sinusoidal pressure waveform from 0 to 25 mmHg followed by a cycle of pressure increase to 250 mmHg. The diameter response followed the pressure changes very closely throughout the test. After each cycle, OD did not return to its initial value within the test's time frame, indicating potential creep behavior. A cycle of pressure increase from 0 to 295 mmHg is also shown. The specimen is again exhibiting creep by not returning to its initial diameter over the time frame studied.

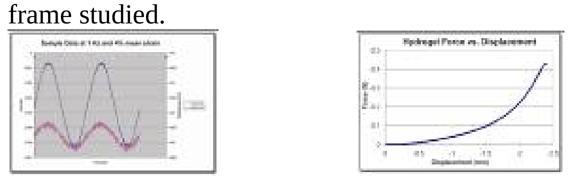


Figure 1: Dynamic material properties of a soft polyvinyl alcohol hydrogel. (Left) low force application, (right) displacement ramp from contact to 430 mN at a rate of 0.02 mm/s.

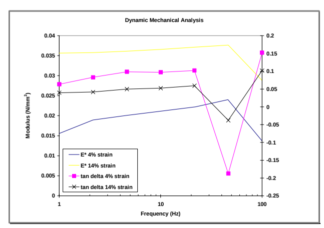


Figure 2: Hydrogel modulus and tan delta at various strain and frequency levels.

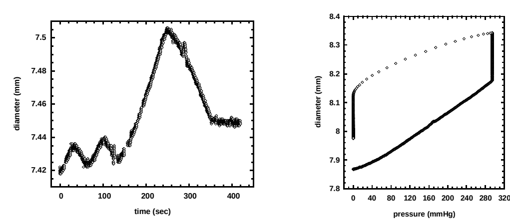


Figure 3: Vascular graft distension versus time in response to pressure changes and diameter change with pressure. (Left) pressure was increased to 25 mmHg for two cycles and to 295 mmHg during the third cycle, (right) linear pressure ramp from 0 to 295 mmHg.

CONCLUSIONS: Preliminary results with hydrogel disks for orthopaedic applications and vascular grafts show that the BioDynamic test instrument is a powerful tool for the integration of biochemical and mechanical stimulation and properties characterization in one system.

Alginate Scaffolds and Perfusion Bioreactors: A Promising System For Cartilage Engineering With Stro-1+ Progenitors From Human Bone Marrow

R.W. Forsey[1], R.Tare[2], R.O.C Oreffo[2] & J B. Chaudhuri[1]

[1] *Centre for Regenerative Medicine, Department of Chemical Engineering, University of Bath.*
[2] *Bone and Joint Research Group, University of Southampton.*

INTRODUCTION: The use of robust three dimensional (3D) constructs to produce viable hyaline cartilage models is vital in developing clinical approaches to cartilage regeneration. Previous work which examined the use of polyglycolic acid (PGA) fleece seeded with the Stro-1+ progenitor population of human bone marrow, showed production of fibrocartilage[1]. This study, therefore, examines the use of alginate/chitosan scaffolds, a widely used system in tissue engineering[2], using both perfused and static conditions to engineer 3D hyaline cartilage with Stro-1+ progenitors and ATDC-5 as a positive control.

METHODS: Stro-1+ cells were expanded in monolayer cultures, to confluence, in 10% FCS, α-MEM, while ATDC-5 cells were expanded as monolayer cultures in DMEM, 5% FCS, 1X ITS, before being harvested for encapsulation. Approximately 4 x 10^6 Stro-1+/ ATDC-5 cells were resuspended in 1ml of 2% alginate containing 10ng/ml TGF-β3. Alginate/chitosan cell beads were formed by carefully placing droplets into a 1.5% chitosan solution using a 1ml syringe and 25G needle. Beads were washed in media, placed either into a perfusion bioreactor (4 reactors/n=5) or a 6 well plate (n=10) and cultured for 21 days (ATDC5) and 28 days (Stro-1+). Bioreactors were set with a perfusion flow rate of 1ml/day. Samples of media were taken at regular time points for both bioreactor and static systems and frozen (-20°C) for later analysis. Following culture, the beads were analysed for cell viability (Cell tracker green/ Ethidium homodimer staining and media analysis) and chondrogenesis (histology; Alcian blue/Sirius red [A/S] staining).

RESULTS: Cell survival was evident in alginate/chitosan beads in both static and perfused cultures for both cell types. This was observed by confocal imaging of cells stained with Cell tracker green throughout the constructs. Confocal imaging showed that cell distribution was even in both static and perfused cultures of both cell types. In addition, cell metabolism was monitored by measuring changes in metabolites over time, as determined through analysis of stored media samples using a culture media analyzer (Bioprofile 400).

Histological analysis of A/S-stained sections of ATDC5 capsules cultured in the perfusion bioreactors revealed a distinctly hyaline cartilage-like morphology composed of chondrocytic cells lodged in lacunae. In comparison, in A/S-stained sections of ATDC5 capsules cultured under static conditions, the chondrocytic cells appeared to be in the initial stages of organising themselves in a 'cell within lacuna' morphology. Alginate capsules with Stro-1+ cells cultured under perfused and static conditions exhibited a cartilaginous morphology in which cells were lodged in lacunae.

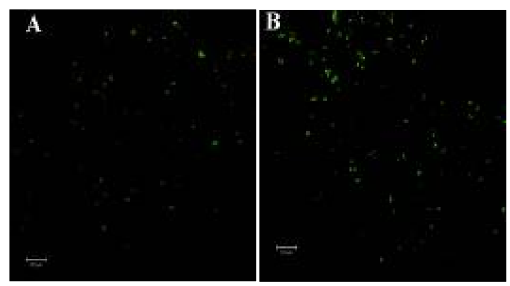


Fig. 1: Cell tracker green staining of Stro-1+ cells in alginate/chitosan beads A) Static B) Perfused cultures. Scale bar = 100 µm

DISCUSSION & CONCLUSIONS: This study has shown the suitability of the alginate/chitosan system as a potential 3D environment for chondrogenic differentiation of Stro-1+ progenitors from human bone marrow. The formation of hyaline cartilage-like structures with both cell types and the good dispersal of viable cells throughout the beads reinforces the use of alginate as a scaffold for tissue engineering. The observed hyaline cartilage-like morphology highlights the benefits of using a perfused bioreactor and underscores the potential of such systems within cartilage engineering.

REFERENCES: [1] R. Tare *et al.* (2005), Annual TCES Conference, London p38 [2]D W Green *et al.* Adv Func Mater 15, 917, 2005

ACKNOWLEDGEMENTS: We would like to thank the BBSRC all for research support.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 21) ISSN 1473-2262

Perfusion of Oxygen in 3D Plastic Compressed Collagen Constructs

U.Cheema [1], B. ALP [1], R.A. Brown [1], A. J. MacRobert[2]

[1]University College London, Tissue Repair and Engineering Centre, Stanmore Campus, London HA7 4LP, [2] National Medical Laser Centre,, UCL, London W1W 7EJ

Introduction

The development of 3D connective tissues *in vitro* is heavily dependent upon remodelling of the matrix, in particular collagen, by resident cells. We have developed a novel plastic compression (PC) technique, for the fabrication of dense cell-collagen based bio-mimetic tissues (Brown *et al*. 2005).

Cell survival in these PC collagen constructs is critical for successful tissue modelling and so the aim here is to understand, quantitatively their dynamic perfusion. This is important for the development of tissue bioreactors for the culture of PC constructs. We have used a fibre-optic oxygen sensor to measure changing oxygen levels in the core of such constructs. This effectively measures O_2 consumption by cells, and by extrapolation, gradients and diffusion properties in the model tissues, which can be correlated with cell death.

Materials and Methods

Acellular and cell-seeded type I collagen gels were made, as previously described, and routinely compacted by a combination of compression and blotting. The rate of compaction was controlled by the force applied and the extent of fluid removal to a porous 'sink'. Cellular collagen gels were made, using Human Dermal Fibroblasts (HDF), at densities from 0.5. million-2 million cells per construct. These constructs were compressed using standardised protocol and rolled to form tight spiral rods (final construct = 21mm length, 2.31mm diameter). pO_2 oxygen probes were inserted into the centre of gels, and oxygen monitored over a period of up to 5 days in static culture.

Results and Discussion

The consumption of oxygen by cells was statistically significantly different between 0.5 million, 1 million and 2 million cells per construct (figure 1, *= P<0.001). However even after 5 days of this culture, the level of cell death was modest, using analysis with live/dead staining and confocal microscopy.

Pulmonary Arterial Smooth Muscle cells were also studied to establish cell specific rates of oxygen consumption. These cells consume higher amounts of oxygen in comparison to Dermal fibroblasts. Oxygen diffusion gradients have been established to test the role of increasing collagen density in single and double compressed constructs. Marked differences in the oxygen diffusion gradient were seen when collagen was approximately twice as dense.

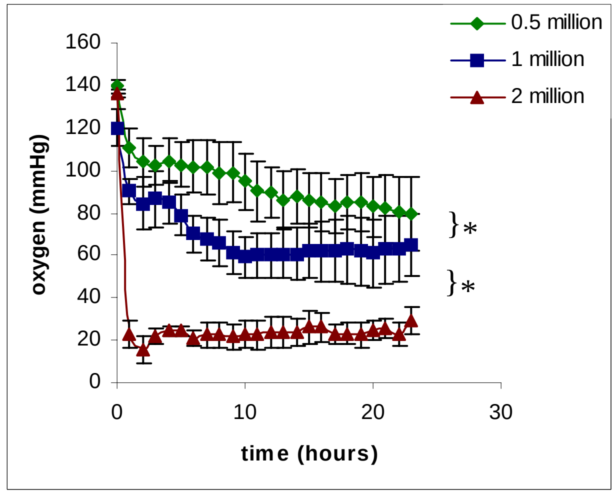


Figure 1. Oxygen partial pressure measured in the centre of HDF-seeded constructs.

Conclusions

This is a highly effective model of perfusion in 3D connective tissues. Gradients were cell type, cell density, collagen density and time dependent. PC collagen material and its laminated 3D structure, allows relatively rapid O_2 equilibrium across extended gradients (~1mm) even at high cell densities. Fibroblasts are able to tolerate and grow within these constructs at relatively low oxygen tensions for extended periods.

We have established a novel method for the monitoring of oxygen in the centre of model PC collagen tissue cultures, and shown that the high density PC collagen material allows good perfusion of oxygen. This model tissue offers unique insights to cell physiology in 3D through its extreme simplicity and controllability. Rapid cell death in this matrix-rich system does not seem to be such a major problem.

References

Brown, R.A. Wiseman, M. Chuo, C.B. **Cheema, U.** Nazhat, S.N. 'Ultrarapid Engineering of Biomimetic Materials and Tissues: Fabrication of Nano- and Microstructures by Plastic Compression.' 2005. *Adv. Funct. Mater*. 15 (11): 1762-1770

Acknowledgements

We are grateful to the BBSRC and EPSRC for funding of this project.

 ISSN 1473-2262

Femtosecond High Resolution Near Infrared Non-linear Optical imaging (NIR-NLOI): Applications in Cell and Tissue Engineering

Uday K. Tirlapur, Xia Xu and Zhanfeng Cui
Oxford Centre for Tissue Engineering and Bioprocessing, Department of Engineering Science
University of Oxford, Parks Rd., Oxford, OX1 3PJ, UK

INTRODUCTION: Current understanding and future advances in cell and tissue engineering require intricate knowledge of vital cellular, sub-cellular, extracellular (structural) and physiological (functional) changes in two-, three or even four-dimensions (in space and time). Precise high resolution monitoring of engineered cells and 3D tissue constructs can be accomplished by way of vital non-invasive near infrared (NIR) laser based optical techniques. Femtosecond NIR non-linear optical imaging (NIR-NLOI) is now emerging as a safe modality for imaging various living cells and tissue *in vivo* and *ex vivo* with minimal phototoxicity and photodamage. *In situ* NLOI enables simultaneous imaging of various endogenious and exogenious fluorophores by two- or multiphoton excitation[1] using a single NIR (700 – 1100 nm) wavelength. Importantly NLOI facilitates concomitant imaging of extracellular structural components such as collagen/elastin by utilizing their intrinsic optical signals such as second harmonic generation (SHG) as well[2].

METHODS: We have used the recently established (Mulholland, et al)[3] state-of-art femtosecond multiphoton laser scanning optical system modified and configured for the non-invasive 3D imaging of thick biological specimens with microspectral imaging capability. Sub-femtolitre excitation was realised using a high-numerical-aperture (N. A. 1.3) 60x water or 100 x Oil immersion objectives, with a working distance of c.a 1 mm and 200 micrometers respectively. In the Bio-Rad multiphoton dedicated system, a single 670 nm ultraviolet optimised long-pass dichroic mirror, placed in the excitation path within the infinity focus of the microscope head directed fluorescence emission signal in the UV to visible wavelength range towards non-descanned bi-alkaline and multi-alkaline Photomultiplier Tubes (PMTs).
Images were loaded either into Imaris 4.2 or the LaserSharpe software for processing and analysis. The Imaris software suite offers a range of highly advanced 3D image processing capabilities including volume rendering, orthogonal plane projections, and statistical analysis of three-dimensional surface-rendered objects. Each of these image analysis modalities was used to evaluate the *ex vivo* tissue samples and 3D cultures of stem cells and chondrocytes as well.

RESULTS:

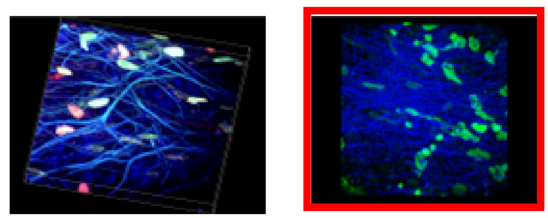


Fig. 1: Cell morphology and organisation of collagen in ex-vivo heart valve of pig (left) and mouse spleen (right).

DISCUSSION & CONCLUSIONS: During the meeting we will briefly describe the NIR-NLOI system-setup and present a few of the recent applications of this state-of-art imaging technology for non-invasive monitoring of (1) 3D orientation of cells and collagen in various living and *ex vivo* tissues by way of second harmonic generation imaging, (2) *in situ* changes in cell morphology and viability of chondrocytes cultured in 3D scaffolds; (3) 3D imaging of human bone marrow stem cells expressing eGFP, and (4) the unique possibility of optical gene transfer into target cells.

REFERENCES: [1]W. R. Zipfel., R. M. Williams, & W. W. Web (2003) Nature Biotec. **21**: 1369-1377. [2] P. J. Campagnola & Loew, L. M, (2003) Nature Biotec **21**: 1356-1360. [3]W. J. Mulholland, et al (2006 Apr 27). J. Invest. Dermatol. (Epub ahead of print)

ACKNOWLEDGEMENTS: This project is funded by the Biotechnology and Biological Science Research Council of the UK (LINK Grant 43/LKE 17445 and EBS 43/E18069).

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 23) ISSN 1473-2262

Application of Optical Coherence Tomography in Tissue engineering

Ying Yang, Pierre O Bagnaninchi, Alicia J El Haj

Institute in Science and Technology in Medicine, Keele University, England

INTRODUCTION: Monitoring cell profiles in 3D porous scaffolds presents a major challenge in tissue engineering. The fabrication of 3D thick tissue constructs has been limited by our ability to visualize the complex cellular dynamics and morphological organizational events occurring deep within these constructs by conventional imaging techniques, such as light microscopy. Optical coherence tomography (OCT) has recently emerged as a promising imaging technique, mainly for medical applications. The original development of OCT was for transparent tissues, such as cornea and retinal tissues. Current OCT technology enables non-transparent, soft and hard tissues to be examined. Several features in OCT are unique and highly attractive for tissue engineering. Measurements by OCT can be realized on-line and non-destructive; the resolution is up to the cellular dimension (0.9-10 μm); the penetration depth for a non-transparent object can be up to 2 mm. A number of different forms, but fundamentally identical OCT, have evolved over the past decade that are developed to image/quantify the different parameters of biological tissue, these including microstructures (time-domain OCT), flow (Doppler OCT), birefringence (polarization sensitive OCT), metabolic states (spectroscopic OCT), biomechanical properties etc. OCT operating in a single function or a combined function may tackle different monitoring tasks in tissue engineering. In this paper, we outline how OCT can be applied to monitor the parameters of tissue engineered constructs non-destructively and dynamically.

METHODS: Two types of OCT system were used for this investigation. One was a time-domain Michelson interferometer based and fiber-optic integrated OCT which employed a 1300 nm superluminescent diode with a bandwidth of 52 nm. The light source yielded 14 μm axial resolution in free space, or 10 μm in the tissue. Another system was a whole field optical coherence microscopy (WFOCM) based on a Linnik interferometer. The X×Y×Z imaging resolutions for the current system were experimentally determined at 0.9×0.9×0.7 μm when the objective lenses were immersed in water. Porous PLA scaffolds and chitosan scaffolds with micro-channel were produced in the lab. MG63 bone cells and tenocytes were seeded onto the scaffolds with different seeding density and culture conditions. The blank scaffolds and the cultured constructs were scanned by the OCT systems. The quantitative evaluation of the structure changes in the constructs has been undertaken.

RESULTS: OCT was capable of revealing the microstructure of blank scaffolds and the cultured constructs clearly (Fig 1). Quantitative calculation of the change in the porosity, pore size and microchannel-filling ratio reflected the profiles of cell proliferation and matrix production within the constructs (Fig2).

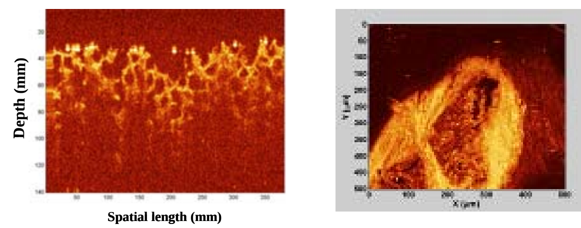


Fig. 1: Time-domain OCT image of a blank PLA scaffold (left), and WFOCM image of a PLA scaffold seeded with $4x10^6$ bone cells for 5 weeks (right)

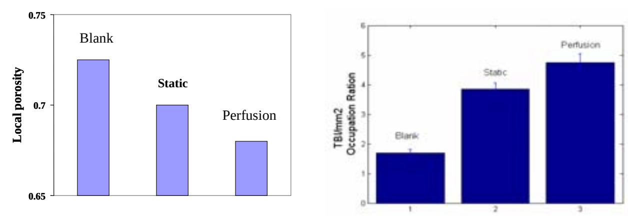


Fig 2: Quantitative evaluation of the changes of the porosity and microchannels in the constructs under different culture conditions

DISCUSSION & CONCLUSIONS: It is confirmed that OCT can monitoring cell growth profile based on its ability to reveal the change of pore architecture in the constructs. The mechanical properties and the components within the constructs can been characterized by OCT well. OCT demonstrate a great potential in tissue engineering. **ACKNOWLEDGEMENTS:** This work was supported by BBSRC (BBS/B/04277, BBS/B/04242) and EPSRC (GR/S11510/01).

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 24) ISSN 1473-2262

A Novel Confocal FRAP technique for the Measurement of Long-term Actin Dynamics in Stress Fibres

J.J. Campbell & M.M. Knight

[1] *Medical Engineering Division, Department of Engineering, Queen Mary University of London. E1 4NS*

INTRODUCTION: The actin cytoskeleton has been implicated in many aspects of cell function. Actin is biphasic, with globular (G) actin contributing subunits for fibrous (F) actin, which forms microfilaments and organises into stress fibres. This dynamic relationship can be examined using fluorescence recovery after photobleaching (FRAP). However, previous studies failed to target FRAP to defined structures and account for inherent cytoskeletal movement[1]. This study utilises advances in confocal microscopy to develop a novel FRAP technique for quantifying F and G actin dynamics.

METHODS: Passage 5 cells derived from a mature chondrocyte cell line[2] (H5) were transfected using Amaxa Nucleofector technology (Amaxa, Germany) with a p-eGFP-actin vector (Clontech, BD Biosciences). GFP-Transfected cells were visualized at 37°C by confocal microscopy (TCS SP2, Leica). Photo-bleaching was confined to 3 x 1µm areas overlying regions of stress fibre and interfibre space (fig 1.). FRAP analysis comprised 1 pre-bleach, 1 bleach and 50 post-bleach image frames. The frame-rate was 11.64 seconds. Fluorescence intensity was recorded though multiple linear profiles bisecting the long axis of each bleached area. The lowest mean intensity value over data points of 3µm length was used to indicate the level of fluorescence recovery. FRAP curves were described by fitting a two-phase exponential.

$$y = YMAX_1\left(1 - e^{k_1 x}\right) + YMAX_2\left(1 - e^{k_2 x}\right) \qquad (1)$$

RESULTS: The success of GFP transfection was confirmed by its conformity to rhodamine phalloidine stained actin filaments (data not shown). A comparison of FRAP in stress fibre (n=56) and interfibre space (n-13) (fig 2.), reveals two modes of recovery. Actin recovery was slower in the stress fibres compared to that in the interfibre space. Based on the curve fit, the mobile fractions within stress fibres and interfibre space comprised 45.9% and 69.4% respectively ($YMAX_1$+ $YMAX_2$).

DISCUSSION: The present study describes a powerful new FRAP technique for quantifying protein dynamics in cytoskeletal networks. This technique overcomes many limitations of previous studies and has been used to illustrate differences in long-term actin dynamics in and around stress fibres. This technique may now be used to exam the effect of factors such as mechanical loading and surface topography.

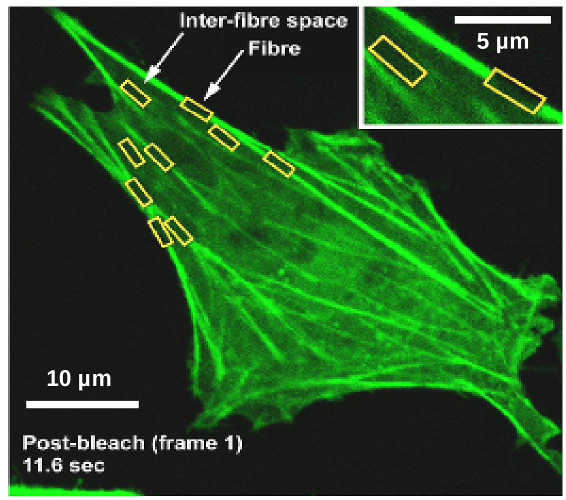


Fig. 1: Post-bleaching image indicating regions of FRAP.

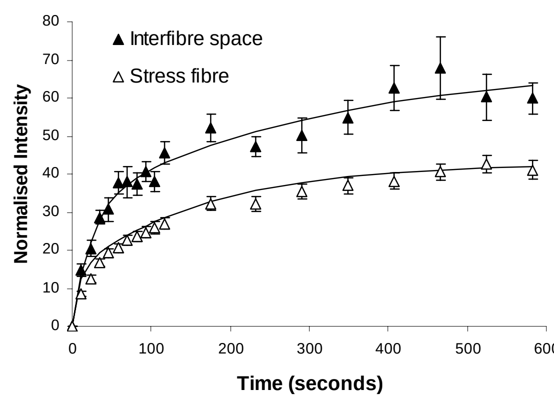


Fig. 2: Normalised FRAP curves, with two-phase exponential curve fit, showing temporal change in fluorescence intensity within stress fibres (n=56, R^2 0.999) and interfibre space (n=13, R^2 0.967). Error bars represent standard deviation.

REFERENCES: [1] T.E. Kreis et al. (1982) *Cell* **29**:835-45. [2]H.M. van Beuningen, et al (2002) *Osteoathritis & Cartilage*.**10**:977-86.

ACKNOWLEDGEMENTS: This work was funded from a BBSRC grant.

Enzyme Responsive Peptide Gels for Regenerative Medicine

V. Jayawarna, R.J. Williams, J.E. Gough, R.V. Ulijn*

[1] *School of Materials, University of Manchester, UK*

[2] *Manchester Interdisciplinary Biocentre (MIB)*

INTRODUCTION:

Spontaneous formation of macroscopic hydrogels from small molecule building blocks via self-assembly provides a route toward designed functional biomaterials. We will show that a number of small peptide amphiphiles, consisting of (mixtures of) dipeptides linked to fluorenylmethoxycarbonyl (Fmoc) spontaneously form fibrous hydrogels in physiological conditions. The self-assembly process is driven by π-stacking of the conjugated fluorenyl moieties and formation of helical conformations as is demonstrated by circular dichroism and fluorescence spectroscopy. The amino acid sequence within the Fmoc-dipeptide building blocks controls the architecture and the physical properties of the assembled structures. Combinations of Fmoc-dipeptides were identified that formed fibrous hydrogels that were i) stable under cell culture conditions, ii) of similar dimensions to the fibrous components of the extracellular matrix and iii) capable of supporting cell culture of chondrocytes in 3D.[1] We demonstrate that peptide gel formation can be triggered selectively by using proteases.[2]

METHODS: Characterisation of hydrogels: circular dichroism, fluorescence spectroscopy, cryo-scanning electron microscopy, rheology. Chondrocyte cell culture in 2D and 3D, collagen antibody staining, fluorescence microscopy, environmental scanning electron microscopy (ESEM).

RESULTS & DISCUSSION: We found that a number of peptide gels could be formed that were transparent and stable at pH7 in tissue culture medium. The physical properties and fibrous morphologies of these gels were strongly dependant on the nature of the amino acid side chains (see Figure 1). These peptide gels supported metabolically active chondrocyte cells for periods of up to two weeks (Figure 2). Peptide gels could undergo sol to gel transitions in response to changes in temperature and ionic strength, which is useful for minimal invasive surgery strategies.

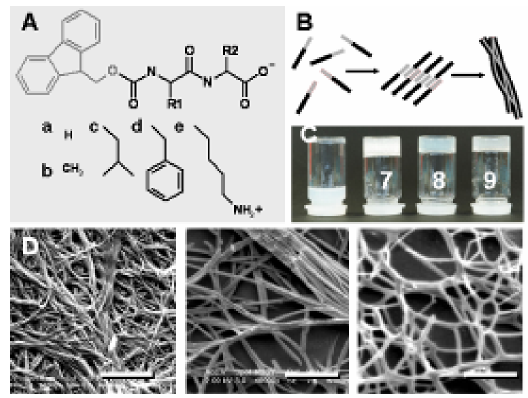


Figure 1: A: Molecular structure of Fmoc-dipeptides. The R groups are for amino acids Gly (**a**), Ala (**b**), Leu (**c**), Phe (**d**) or Lys (**e**). B: Proposed self-assembly mechanism. D: Cryo-SEM micrographs of nanofibrous materials.

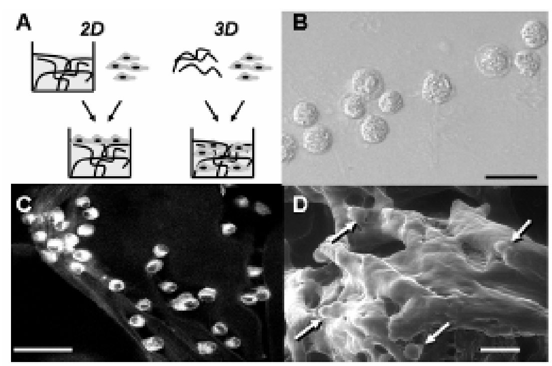


Figure 2: Chondrocyte cell culture on peptide hydrogel scaffolds. A: 2D and 3D cell culture. B: chondrocytes on hydrogel surface. C: DAPI stained chondrocytes in 3D culture. D: ESEM study of hydrated gel with chondrocytes (arrows).

CONCLUSIONS: We demonstrate that short peptide amphiphiles can form extracellular matrix mimics that support chondrocyte cell culture in 2D and 3D.

REFERENCES: (1) Jayawarna V, Ali M, Jowitt TA, Miller AF, Saiani A, Gough JE, Ulijn RV. *Adv. Mater.* **2006**, 5, *in press.* (2) Toledano, S.; Williams, R. J.; Jayawarna, V.; Ulijn, R. V. *J. Am. Chem. Soc.* **2006**; 128(4); 1070-1071.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 26) ISSN 1473-2262

Evaluation of Anti-Inflammatory Calixarene-Peptides for Biomaterial Modification

M. Charnley[1], K. Fairfull-Smith[2], N.H. Williams[2], J.W. Haycock[1].
[1]*Department of Engineering Materials, Sheffield University, S1 3JD, UK*
[2]*Department of Chemistry, University of Sheffield, S3 7HF, UK.*

INTRODUCTION: There is an increase in the use of implantable medical devices for the repair of soft and hard tissue. Many such devices can initiate acute inflammation, resulting in device failure. The co-delivery of anti-inflammatories together with the device is proposed as a therapeutic strategy to reduce excessive inflammation. α-Melanocyte-stimulating hormone (MSH) is a natural and potent anti-inflammatory hormone produced in the body with very short peptide sequences that make it amenable for easy laboratory synthesis. The aim of this work is to immobilise short MSH peptides onto medical device surfaces using molecules called calixarenes, which are known to attach to a wide variety of material surfaces. This is being approached by synthesizing MSH-calixarene molecules with the aim of being able to 'dip and dry' treat medical devices with an anti-inflammatory 'coating'.

METHODS: Initial studies indicated the anti-inflammatory properties of the analogue of MSH, GKP(D)V, were retained when joined to a PEG-350 tether. The PEG-GKP(D)V moiety was then attached to the calixarene molecules and coated onto glass coverslips. Surfaces were coated with two compounds, calixarene-PEG-OMe, without the peptide moiety, and calixarene-PEG-GKP(D) in varying molar ratios (0% to 100%).

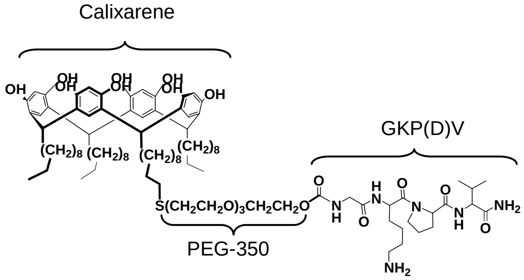


Figure 1: Schematic diagram of the GKP(D)V peptide attached to the calixarene compound via a PEG-350 tether, which is then coated onto glass.

The surface was characterised using XPS, MALDI-ToF-MS and contact angle goniometry. Human dermal fibroblast cells were then cultured onto the coated glass coverslips for 48 hours and stimulated with TNF-α ($1000 Uml^{-1}$) for 120 minutes. Cells were then immunolabeled for the p65 subunit of NF-κB to monitor acute inflammatory signalling. Whole cell counts were performed, cytoplasmic labelling indicated inactive NF-κB and nucleic labelling indicated active NF-κB.

RESULTS:
Surface Characterisation. XPS and MALDI-ToF-MS indicated that the GKP(D)V peptide was immobilized onto the glass surface.
NF-κB Activation. Unstimulated cells exhibited predominately cytoplasmic labelling regardless of the surface upon which they were cultured. Stimulation of the cells with TNF-α caused rapid translocation, and therefore activation, of NF-κB to the nucleus. Culturing cells on calixarene-PEG-OMe coated surfaces had no effect on NF-κB activity. In contrast culturing cells on calixarene-PEG-GKP(D)V coated surfaces inhibited TNF-α stimulated NF-κB activation by up to 14.5±3.0% ($n=3$, $p=0.001$). Levels of inhibition were comparable to those observed when cells were cultured on to glass and then incubated with both GKP(D)V at 10^{-9} M and TNF-α (9.4%±2.6; $n=3$, $p=0.003$).

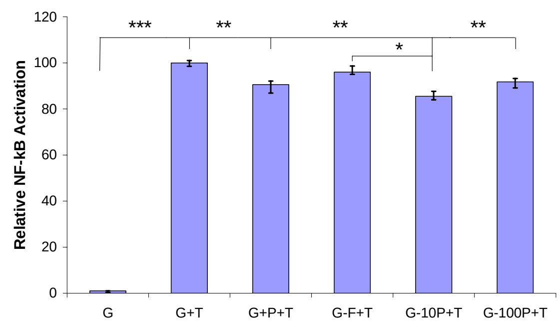


Figure 2: Immobilised GKP(D)V inhibits NF-κB activity. TNF-α ($1000 Uml^{-1}$); P, GKP(D)V peptide; G, glass; F, calixarene-PEG-OMe; G-P, calixarene-PEG-GKP(D)V; $n=3$, *$p\leq0.05$, **$p\leq0.001$, ***$p\leq0.005$.

DISCUSSION & CONCLUSIONS: Initial results indicate the GKP(D)V peptide has been immobilised onto a glass surface using calixarene chemistry and retains anti-inflammatory properties. This strategy supports ongoing research into its application as an anti-inflammatory coating for biomaterials.

ACKNOWLEDGEMENTS: We thank the BBSRC and EPSRC for funding this work and Dr. S. L. McArthur and Dr. P. Kingshott for surface characterisation.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 27) ISSN 1473-2262

The Effect of Sodium Trisilicate on Bone Resorption and Healing

G Ponirakis & J E Gough

School of Materials, University of Manchester, England

INTRODUCTION: Bone is capable of remodeling itself. Silicate ions (Si $[OH^-]_4$) might be one of the main factors which make bioactive glass very effective in enhancing bone healing. Bioactive glass is used in dental and orthopedic applications as bone fillers. In this study, we set out to examine the biocompatibility of silicate ions using sodium trisilicate ($Na_2Si_3O_7$) to macrophages and osteoblasts. Silicate ions from bioactive glass are released into the body or cell media which maybe linked to the osteoproductivity of bioactive glass. Our study showed that silicate ions, 20µg/ml, do not activate macrophages in secreting peroxide, or cytokine IL-1β. This suggests that silicate ions do not stimulate the inflammatory response. We found that silicate ions stimulate osteoblastic cells to secrete cytokines TGF-β2 and OPG. This suggests that silicate enhances collagen production by osteoblasts and bone augmentation, respectively. In addition, this study demonstrates that 20µg/ml silicate increases alkaline phosphatase activity and collagen production by osteoblasts. However, the rate of silicate ions released from an implant in the body must be regulated as silicate can be cytotoxic at 100µg/ml but osteogenic at 20µg/ml.

METHODS: 1. Cell proliferation was measured using a Hoechst based DNA assay. 2. Collagen production was quantified using the sircol assay. 3. Cytokine assay was studied using ELISA kits.

RESULTS: Bone Proliferation: Silicate at 100µg/ml down-regulated osteoblastic growth. 20µg/ml silicate increased bone growth but was not as rapid as the control without silicate. Figure 1 indicates osteoblast morphology in 20µg/ml silicate.

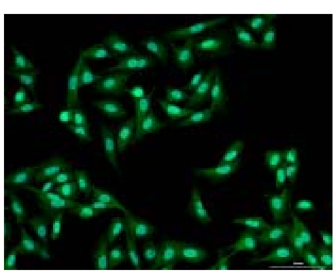


Fig. 1:Osteoblast morphology at 20µg/ml silicate Cells were stained with phalloidin for F-actin.

Bone Differentiation and Maturation Assay:

1. Alkaline Phosphatase: Throughout 3 weeks, silicate at 20µg/ml showed higher alkaline phosphatase production by osteoblasts compared to the control (without silicate). 2. Collagen production: Silicate at 20µg/ml stimulated collagen production by osteoblast more than the control throughout 24 days. Figure 2 demonstrates collagen type I production by osteoblasts after 12 days with 20µg/ml silicate.

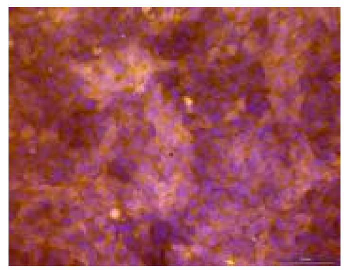


Fig. 2: Collagen type I production by osteoblasts with 20µg/ml silicate after 12 days.

Bone Differentiation and Maturation Assay:

1. IL-1β: 20µg/ml silicate showed no IL-1β production over 21 hours. 2. Peroxide: 20µg/ml silicate did not stimulate peroxide production by macrophages over 6 hours. 3. TGF-β1: 20µg/ml silicate induced TGF-β1 secretion by osteoblast within 21 hours. 4. OPG: 20µg/ml silicate up-regulated OPG production by osteoblast in 21 hours.

DISCUSSION & CONCLUSIONS: Our study confirms that silicate might be one of the main factors that is involved in the osteoproductivity of bioactive glass. Silicate down-regulates bone growth and up-regulates bone differentiation by increasing alkaline phosphatase and collagen secretion by osteoblasts. Silicate at 20µg/ml does not activate macrophages to secrete peroxide and IL-1β suggesting that silicate does not stimulate the inflammatory response. It is crucial to control the amount of silicate released at the surface of bioactive glass.

Development and validation of an agent-based computational model of normal human keratinocytes organisation *in vitro*

Tao Sun[1], Phil McMinn[2], Simon Coakley[2], Mike Holcombe[2], Rod Smallwood[2], Sheila MacNeil[1]

1: Department of Engineering Materials, Sheffield University, Kroto Research Institute, Broad Lane, Sheffield, S3 7HQ, UK.

2. Department of Computer Science, Sheffield University, Kroto Research Institute,

Abstract: An agent-based computational model was developed to describe the dynamic multicellular morphogenesis of normal human keratinocytes (NHK) in monolayer culture at low (0.09mM) and high (2mM) exogenous Ca++ environments. *In silico* hypothesis testing indicated that cell-cell contact, cell-substrate contact, and differentiation of transit amplifying (TA) cells were major mechanisms, whereas cell cycle times of both stem and TA cells had no obvious effects on the pattern of NHK distribution. The model suggested that the limited divisional capability of TA cells wasn't an internal cell property but a statistical phenomenon. By deliberately omitting the rules for the stem cell colony autoregulation and for cell differentiation mechanism, the population growth of a transformed keratinocyte cell line (HaCat cells) was also successfully described. The model of NHKs was then used in a predictive sense. Simulation of scratch wounds made to NHK cells indicated that wound healing in low [Ca2+] media would be achieved mainly by migration and subsequent proliferation. In physiological [Ca2+] media, a stem cell position dependent wound closure pattern was predicted. Both were then validated by *in vitro* experiments designed to check the model's predictions. This work demonstrates that an agent-based model, incorporating rules relating to relatively few mechanisms, is sufficient to describe, explain and predict some biological phenomena seen when keratinocytes and transformed keratinocytes are cultured *in vitro*.

Key words: Computational modelling, keratinocyte, HaCat cells, calcium, wound healing

Patterns of Genomic Regulation In Mesenchymal Stem Cells Cultured on Osteogenic Nanotopographies.

A. Andar[1], S. Affrossman[2], C.D.W. Wilkinson[1], R. Tare[3], R.O.C. Oreffo[3], M.J. Dalby[1]

[1]*Centre for Cell Engineering, Infection and Immunity, Institute of Biomedical and Life Sciences, Joseph Black Building, University of Glasgow, Glasgow, G12 8QQ, UK.* [2]*Department of Pure and Applied Chemistry, University of Strathclyde, Thomas Graham Building, Cathedral Street, Glasgow, G1 1XL, UK.* [3]*Bone and Joint Research Group, Developmental Origins of Health and Disease, University of Southampton, Southampton S016 6YD, UK.*

INTRODUCTION: A key tenet in orthopaedic implant design is differentiation of the native mesenchymal stem cells in to mature, bone producing, osteoblasts.

Previously, we have published immunohistological evidence of primary human mesenchymal stem cells producing increased levels of osteocalcin (OCN) and osteopontin (OPN) in response to a variety of nanotopographies when cultured in basal media alone[1, 2]. OCN and OPN are osteoblast specific matrix proteins and thus, our results allude to the nanotopographies having osteogenic properties.

In order to expand on our understanding of the genomic process of osteogenic differentiation on the nanotopographies, two further selection processes have been used. Firstly, stem cell response to a selection of nanomaterials was analysed with 1.7k gene arrays. The most interesting of these were subsequently analysed with both 19k gene arrays and also 101 gene osteospecific arrays and compared to stem cells treated with dexamethasone (dex).

METHODS: Stro-1 selected mesenchymal stem cells were cultured for 21 days on materials fabricated by photolithography and polymer demixing for 1.7k arrays and then both 14 and 28 days for 19k arrays. Cells treated with dex were also cultured for 14 and 28 days. Both the 1.7k and 19k arrays were from the Ontario Microarray Centre and contained general, well-characterised expressed sequence tags. Also, 101 gene osteospecific oligo arrays from Superarray Bioscience were used to detect early osteoblast differentiation at 14 days.

The topographies were all produced by embossing in polymethylmethacrylate (PMMA) via Ni intermediaries fabricated by sputter coating and electroplating of the master topographies. Flat PMMA was used as a control.

Cluster analysis and iterative group analysis were performed with the Ontario arrays. For the osteospecific arrays, numbers of gene hits for each gene were counted.

RESULTS & DISCUSSION: Cluster analysis revealed large distinct areas of similar differential expression of genes on the nanotopographies compared to planar control. These results infer that as the cells differentiate large numbers of similar genes are, for the most part, turned on (it is noted that smaller clusters of down-regulations were observed). Critically, up-regulations tended to include clusters involved in matrix regulation and cell signalling. Down-regulations were seen in areas such as proliferation. We demonstrate for the first time, using these osteo-specific arrays that the nanotopographies exhibit a similar effect to dex with significant implications for materials/cell science.

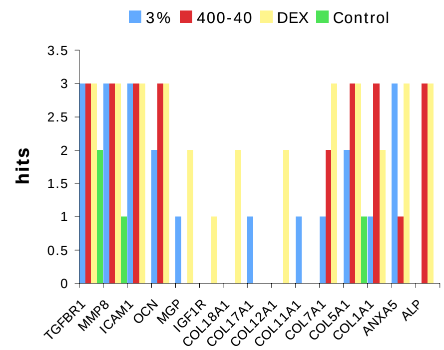


Fig. 2: Number of hits for osteospecific genes (just a small selection).

ACKNOWLEDGEMENTS: This work was funded by MJD's BBSRC David Phillips Fellowship. ROCO & RT are supported by funding from the BBSRC and EPSRC.

REFERENCES:

1. Dalby, M. J. et al. *Biomaterials* **2006,** 27, 1306-1315; 2. Dalby, M. J et al. *Biomaterials* **2006,** 27, 2980-7.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 30) ISSN 1473-2262

Multiscale Transport Modelling of Nutrient Transport in Bioreactor for Growing 3D Bone Tissues: Sub-Cellular to Laboratory scale

DB Das & DR Jones
Department of Engineering Science, University of Oxford, Oxford OX1 3PJ
(Email: Diganta.Das@eng.ox.ac.uk)

Recent experimental studies suggest that hollow fibre membrane bioreactors (HFMBs) may be used to grow bone tissues, which may then be implanted into patients to repair various skeletal defects. The HFMBs mimic the capillary network that exists in bones and are very effective in supplying nutrients to cells (to maintain cell metabolism) and removing waste products (e.g., excreta from micro-organisms, dead cells, etc). In order to guide the design of effective bone tissue engineering protocols, we need to elucidate the quantitative relationships between the cell environment and tissue behaviour in HFMBs and their relationship with nutrients supply. This necessitates that the appropriate bioreactor conditions for generating neotissues, and the mass transfer and chemical reaction during cell growth and extracellular matrix formation, are studied thoroughly. One should also be aware that the mass transfer processes in growing bone tissues in bioreactors take place at several scales – from the scale of the individual cell to the scale of the laboratory device (Figure 1). However, the significance of the mass transfer processes may be very different from scale to scale. For example at the sub-cellular scale, the transport processes are dominated by diffusive-reaction mechanisms. At the extracellular matrix, these processes are primarily diffusion dominated. At the scale of the laboratory device, the transport processes are governed by convection-diffusion and reaction. Therefore, to characterize the 'overall' mass transfer processes, one also needs an understanding of the processes at smaller scale and their manifestation at larger scale, such as the laboratory device.

This paper will present our attempt at modelling and simulation of nutrients transport in HFMB for growing 3D bone tissues using a finite element model. In specific, a computational framework will be presented which has been used to upscale the mass transfer processes at sub-cellular scale to larger scale. The framework is then to carry out a systematic analysis of the influence of various process parameters of HFMB used in bone tissue engineering, e.g., cell density, cellular size, hydrodynamics behaviour, etc.

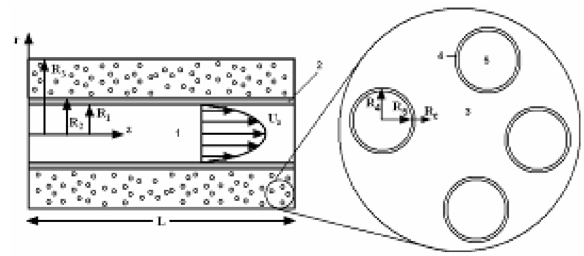


Fig 1: Cross Section of an individual hollow fibre

See, for example, Figure 2 our results for the effects of cell size on mass transfer behaviour. These simulations have been run with various radii of cells, i.e., 5×10^{-7} m, 10×10^{-7} m and 25×10^{-7} m, while the cell membrane thickness and cell density are kept the same. As expected, the figure shows that increasing cell size lead to decrease in nutrient concentration, glucose in this case. Figure 3 shows the distribution of glucose in the cells of HFMB.

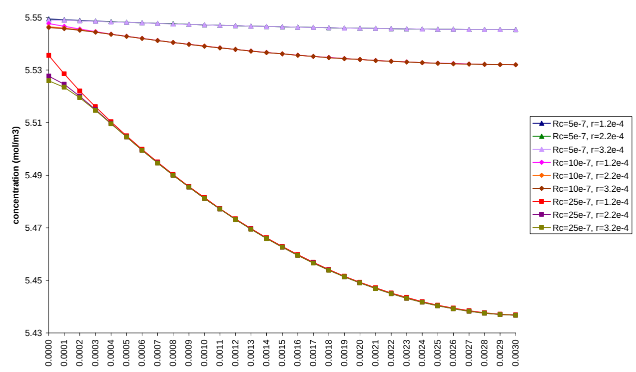


Fig 2: Axial glucose concentration profiles in cells of HFMB for various cells sizes

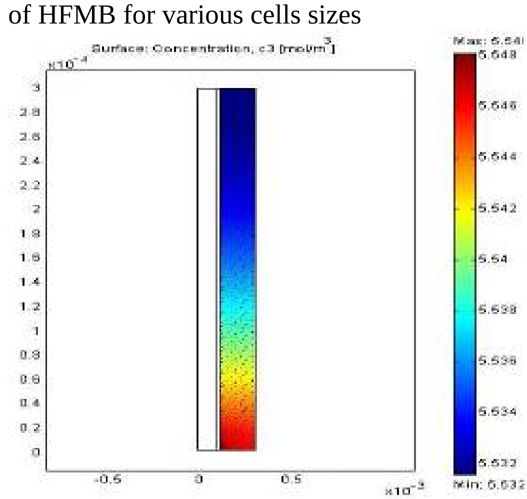


Fig 3: Distribution of glucose in the cells of HFMB

Individual Cell-Based Simulation of 3D Multicellular Spheroid Self-Assembly

J J Zhu [1], S Coakley[1], M Holcombe[1], S MacNeil[2], R H Smallwood [1]
[1]Department of Computer Science, University of Sheffield, UK.
[2]Department of Engineering Materials, University of Sheffield, UK

INTRODUCTION:

We present a three-dimensional agent-based, biophysical model to study the in vitro self-assembly of multicellular spheroids. We investigate how the collective cell migration and pattern formation originate in the behavior of a collection of individuals, each of which responds to a number of physical forces, such as specific and nonspecific adhesion forces between cells and cell-substrate; repulsive force between cells, resistance force between cell-ECM and cell-substrate.

METHODS:

Computer simulation is implemented by solving over-damping type equations for individual cell. We study, by variation of cell-specific parameters, which of them affect the spatial-temporal organization and self-aggregation.

RESULTS:

Self-aggregation of DU 145 human prostate carcinoma cells in liquid overlay culture are shown in Figure 1 (*t=0)* and figure 2 (*t=13.89* days). Spheroids formed in a short time interval ($t\approx 3$ hours), then reorganized and increased in size. Case study also was carried out for an in vitro LNCaP human prostate cancer cell aggregate. Numerical simulations are compared with experiment results in literature. Main parameters (unit: SI): cell radius $R_0=5\times10^{-6}$, ECM resistance $c_m=1.2$, Young modulus E=1000, adhesion coefficient $\varepsilon_s=6\times10^{-4}$, $\varepsilon_c=3\times10^{-4}$, resistance coefficient $\mu_s = 3\times10^{11}$, $\mu_c= 3\times10^{11}$, activation force $|\mathbf{F}_{A0}|=3\times10^{-9}$. Subscript s/c stands for substrate/cell.

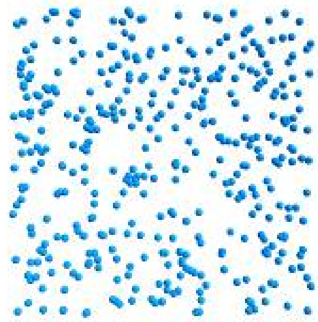


Figure 1. Randomly distributed 400 cells in a square 400μm×400μm

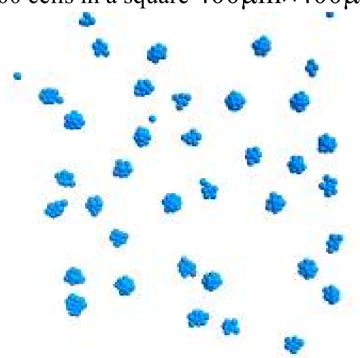


Figure 2. Spheroid formation at *t=13.89* days

DISCUSSION & CONCLUSIONS:

Adhesion forces and cell motility play a predominant role in self-assembly of multicellular spheroids. Self-aggregation could not occur if cell motility is too large or adhesion force between cells is too small. 3D spheroid will not form, if adhesion coefficient between cells is much smaller than adhesion coefficient between cell and substrate.

REFERENCES:

J Galle, M Loeffler, and D Drasdo (2005) Biophysical Journal. **88**: 62-75.

H Song, KC O'Connor, DJ Lacks (2003) Biotechnology Progress **19**: 1742-1749. RM Enmon, KC O'Connor, Lacks DJ, Schwartz, DK, and Dotson, RS (2001). Biotechnology and Bioengineering **72**: 579-591.

Mechanical loading determines collagen fibril diameter independent of cell activity

U.Cheema [1], CB Chuo[1], Padmini Sarathchandra[1], Showan N. Nazhat[2] R.A. Brown [1,]
[1]University College London, **UCL**, Tissue Repair and Engineering Centre, Stanmore Campus, London HA7 4LP, [2] Division of Biomaterials & Tissue Engineering, Eastman Dental Institute, London, WC1X 8LD, United Kingdom

Introduction

The mechanical properties of most vertebrate tissues are dominated by the fibrous protein collagen. It is important that the material properties of connective tissues fulfil a variety of tensile, compressive, shear and torsional load bearing functions. These are achieved by standard adaptations of the fibrous, anisotropic form of their collagen component. A key determinant of that is the distribution of fibril diameters. Collagen fibril diameter in native tissues varies considerably from tissue to tissue, and between age, repair and growth stages

We have tested the idea that fibril diameter can be regulated directly, using mechanical loading to promote fibril fusion in plastically compressed collagen materials (Brown *et al.* 2005).

Materials and Methods

Acellular collagen gels were made, as previously described, and routinely compacted by a combination of compression and blotting. The rate of compaction was controlled by the force applied and the extent of fluid removal to a porous ‘sink’. The compacted gel was cut into three strips of 7 mm x 33mm. Each of these strips were then loaded, and treated as N=1.

Load was applied parallel to the tethered axis of the collagen gels. A single pattern of cyclical load was applied, with each cycle lasting 20 minutes. The control regimen was application of one cycle and test regimens applied between 12 and 144 cycles. Collagen gels were analysed for fibril diameter (electron microscopy) or quasi-static tensile mechanical properties directly after the treatment

Results and Discussion

Collagen fibril diameter clearly increased with increasing load cycle number as frequency analysis and as median diameter. The median baseline fibril diameter (1 cycle) was 29 ± 4.6 nm and this increased > 2 fold to 70 ± 10 nm after 144 cycles ($P<0.001$) (figure 1).

The break stress, break strain and elastic modulus of the collagen material increased with increasing cycle number, particularly between 48 and 144 cycles. Break stress and modulus increased by 4.5 and 2 fold respectively.

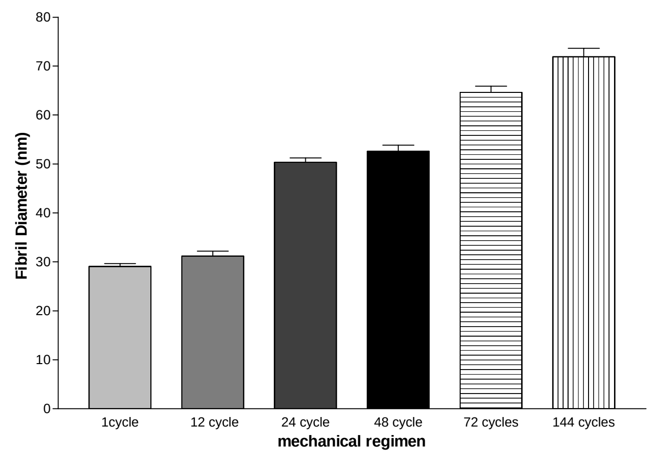


Figure 1. A histogram showing increasing fibril diameter as collagen is cyclically loaded.

Conclusions

This study represents the first demonstration, to our knowledge, that both fibril diameter and overall material properties can be directly controlled, without cells, through mechanical loading. This would suggest that material properties of the natural collagen polymers *in vivo* may also be controlled by a combination of local, cell generated strains and external loading. It also redirects how we can engineer biomimetic collagen materials for implants by providing previously impossible cell-independent control of mechanical properties.

References

Brown, R.A. Wiseman, M. Chuo, C.B. **Cheema, U.** Nazhat, S.N. ‘Ultrarapid Engineering of Biomimetic Materials and Tissues: Fabrication of Nano- and Microstructures by Plastic Compression.’ 2005. *Adv. Funct. Mater*. 15 (11): 1762-1770

Acknowledgements
We are grateful to the BBSRC and EPSRC for funding of this project.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 33) ISSN 1473-2262

Mechanical Properties of Transversalis Fascia and Hernia formation

A. Kureshi[1], P. Vaiude[1], S. Nazhat[2], S. Barker[3] & R. Brown[1]

[1]*University College London, Tissue Repair & Engineering Centre, Stanmore Campus, HA7 4LP London*
[2]*Dept of Biomaterials & Tissue Engineering, Eastman Dental Institute, UCL, London.*
[3]*St Luke's Hospital, 14 Fitzroy Square, London, W1T 6AH.*

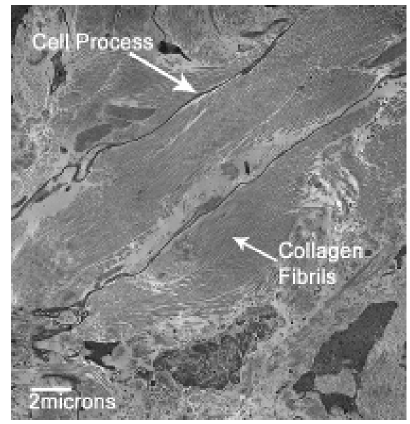
INTRODUCTION: The transversalis fascia (TF) has long been the focus of hernia patho-physiology and management. Of particular interest there appears to be, in many hernias, a dramatic 'stretching' or 'growth' of the TF. This has significant bio-mechanical implications and applications being a natural model of adult tissue expansion. Mechanical properties of connective tissues, including the TF matrix are primarily dependent on collagen architecture particularly orientation and fibril diameter. The mechanical properties of TF were studied using a Dynamic Mechanical Analyser (DMA, Perkin Elmer) and correlated with Transmission Electron Microscopy (TEM) imaging.

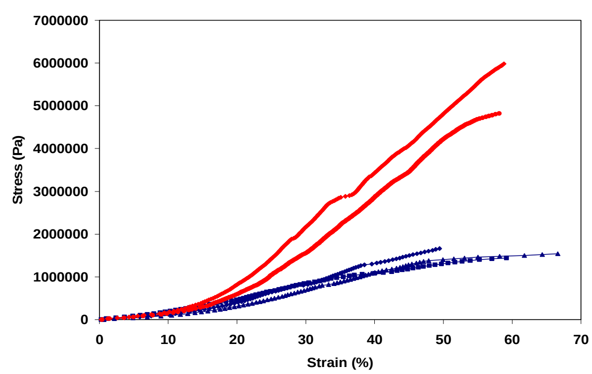
METHODS: TF specimens were harvested from 20 patients undergoing inguinal hernia repair surgery and 4 control specimens were obtained from organ transplant donors. The specimens were orientated in transverse and longitudinal anatomical planes. The tissue was then cut into 2mm wide strips and using a metal mesh clamped into a Perkin Elmer Dynamic Mechanical Analysis Instrument (DMA). Detailed mechanical parameters were analysed by putting the tissue under a uniaxial tensile load of 200mN/min to failure or until 6000mN was reached. Stress, strain, modulus and break stress/strain values were calculated for each specimen. These were statistically analysed comparing the two tissue strips in perpendicular planes. The results were correlated to Transmission Electron Micrographs of the same tissue.

RESULTS: The results of the mechanical testing revealed distinct differences between the properties of the TF cut in transverse and longitudinal anatomical planes. Tissue cut and tested in the transverse plane produced higher break stress values than tissue tested in the longitudinal plane for all patient samples. Similar strain values were observed in both planes.

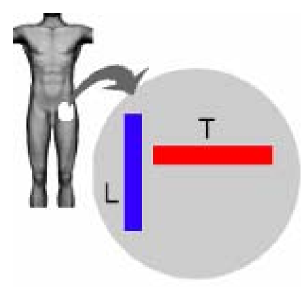
DISCUSSION & CONCLUSIONS: The TF displays anisotropy when comparing longitudinal to transverse orientations in the body.

Fig. 1: Micrograph of TF from a direct inguinal hernia showing predominant unilateral fibril direction. Mag: 4400x.





Graph 1: Representative stress-strain curves obtained from Mechanical Analysis of TF showing transverse (red) vs. longitudinal (blue) planes.



This suggests that the collagen fibrils are predominantly orientated in the transverse plane, making the tissue weaker and less stiff in the longitudinal plane. This explanation sheds some light on the pattern of tissue expansion in TF 'stretching' in the hernia.

ACKNOWLEDGEMENTS: Ethicon GmbH.

 ISSN 1473-2262

Micro-mechanical Analysis of PLLA scaffolds for Bone Tissue Engineering

E.Baas, J.H. Kuiper, M.A. Wood, Y. Yang & A.J. El Haj
Institute of Science and Technology in Medicine, School of Medicine, Keele University, England

INTRODUCTION: Many tissue engineering strategies rely on using combined cells and scaffold approaches. Bone is thought to use local mechanical strain as a cue for bone production, thus ensuring bone is laid down where it is needed most. It is reported that an agonist to strain-operated membrane channels, Bay K8466, enhances the sensitivity of bone cells to strain and increases matrix production [1]. Encapsulating this agonist into the scaffold may make bone cells more responsive to local mechanical strains in the scaffold. If so, mechanically loading the scaffold will lead to increased bone production at locations of apposite strain.

To test this strategy of enhancing bone regeneration in vitro, the relation between strain at the cellular level and micro-level bone matrix formation must be investigated. Because the scaffolds consist of random pores, we propose to derive the inhomogeneous surface strain distribution numerically by combining micro-compression experiments with micro-Finite Element (FE) models, both based on micro-Computed Tomography (µCT) reconstructions. A linear FE model is applied to estimate the local micro-level surface strains in wet poly (L-lactic acid) scaffolds. Bone regeneration is measured by the local degree of mineralization using µCT and related to the local surface strain environment at different locations throughout the scaffold.

METHODS: In an initial experiment six PLLA scaffolds, of which three enhanced with Bay, are scanned with µCT before seeding and culture of MG63 cells in a perfusion-compression bioreactor. The 15x15x15 µm voxels of the reconstruction of each scaffold are directly converted to 8-node brick element meshes. The linear FE model is applied using Scanco Medical FE software on a Pentium 4 - 3.2 GHz platform with 4GB RAM.

After fixation the samples are dried chemically prior to the second µCT scan. The mineralised bone is segmented from the scaffold and soft tissue. The reconstructions are realigned with the first scan, to locate the corresponding positions in pores in both reconstructions where mineralised bone is found. This is performed manually on 2D cross-sections, based on the calculated centre of mass of each mineralised bone segment.

RESULTS: Each scaffold retained its morphology during the whole experiment *(fig.1)*, allowing correlation of the positions of all formed mineralised bone segments (35-85 per sample) to the pore in the original scaffold *(fig.2)*.

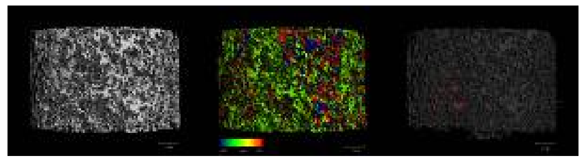


Fig. 1: Side views of a 3D reconstruction of a sample: the blank scaffold (left); FE-derived principal strain values in scaffold (middle); the tissue-scaffold construct (mineralised tissue is highlighted red)(right).

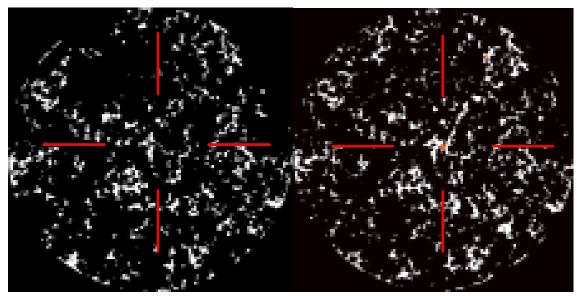


Fig. 2: Two corresponding cross-sections of a sample scanned before (left) and after (right) cell culture (mineralized bone is orange).

DISCUSSION & CONCLUSIONS: The model successfully generates local and sample specific strain values at locations of mineralised tissue formation. This allows investigation of the relevance of a number of parameters, like local strain distribution characteristics, how local the effect of apposite strain is and the effects of Bay enhancement. Also the effect of compressive strain well within the scaffold can be compared to fluid flow shear effects at the scaffold periphery with respect to bone mineralization.

REFERENCES: [1] L.M. Walker, S.J. Publicover, M.R. Preston, M.A. Said Ahmed & A.J. El Haj (2000) *J Cell Biochem*, **79**, pp 648-661.

ACKNOWLEDGEMENTS: This work is funded by EPSRC (GR/S11510/01).

A New Method for Tailoring the Degradation Rate of Chitosan Fibre-mesh Scaffolds

C. Cunha-Reis[1*], K. TuzlaKoglu[2], Y. Yang[1], A. El Haj[1] and R. L. Reis[2]

[1] Institute for Science and Technology in Medicine, School of Medicine, Keele University, Thornburrow Drive, Hartshill, Stoke-on-Trent ST4 7QB UK E-mail: c.m.reis@pmed.keele.ac.uk
[2] 3B's Research Group – Biomaterials, Biodegradables and Biomimetics, Department of Polymer Engineering, University of Minho, Campus de Gualtar 4710-057, Braga, Portugal
[*] Visiting researcher in 3B's Research Group

INTRODUCTION: The degradation of scaffolds depends on several parameters, namely the biomaterials intrinsic properties and the scaffolds morphology. It is possible to tailor the degradation rate by changing the polymers chemical structure. Nevertheless, that may be time consuming and difficult to control. In addition, that approach may lead to undesired changes in other properties of the polymer [1]. The aim of this study was to investigate the influence of porosity and fibre diameter on the degradation rate of chitosan fibre-mesh scaffolds, as a possible way to tailor the degradation rate of such type of scaffolds.

METHODS: *Scaffolds production*: The scaffolds with different porosities were produced by wet spinning 4.2 and 3.2 mL of a 3% chitosan solution in 2% acetic acid, placing the correspondent fibres in equal moulds and drying at 60 ºC. For each porosity, fibers with 200 µm and 90 µm of diameter were produced.
Scaffolds degradation: The scaffolds were weighted and immersed for 4 weeks in a 1mg/mL lysozyme solution – in Phosphate Buffer Saline (PBS), pH 7.4 - at 37 ºC. The degradation solution was changed weekly. The scaffolds were weighted before and after drying and the water uptake and the weight loss, respectively, were determined.
Morphological analysis: The morphological analysis of the scaffolds before and after degradation was performed by Scanning Electronic Microscopy (SEM) and micro CT.

RESULTS: Figure 1 evidences the differences in the scaffolds porosity that can be achieved by this method.

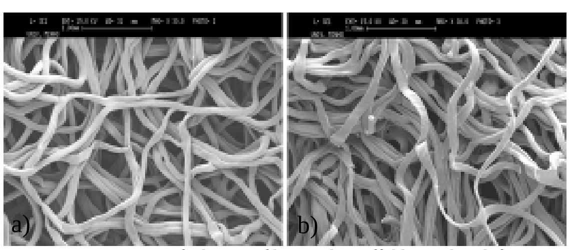


Fig.1. SEM picture of chitosan fibre-mesh scaffolds produced from different volumes of chitosan solution (a – from 3.2 mL;b – from 4.2 mL).

Scaffolds composed of fibres with different diameters were also achieved consistently.

The fibres with smaller diameter have shown higher values for the water uptake and the weight loss, independently of the scaffolds porosity (Figure 2).

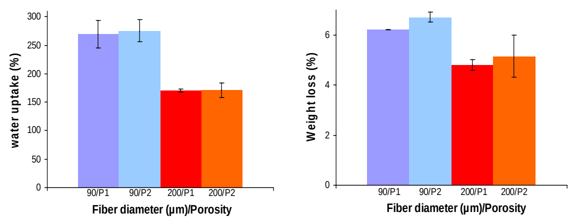


Fig.2. Water uptake (left) and weight loss (right) of the chitosan fibre-mesh scaffolds after 4 weeks of degradation in a 1 mg/mL lysozyme solution. P1 – low porosity;P2 – high porosity

DISCUSSION & CONCLUSIONS: It is of general acceptance that the higher the water uptake, the higher the degradation rate. The results obtained with this work are in accordance to that. The scaffolds produced with the thinner fibres have a higher surface area and consequently a higher water uptake comparing to the thicker ones. That leads to more contact points between chitosan and the lysozyme solution, giving rise to higher weight loss. Regarding the scaffolds porosity, no differences in the degradation rate were observed between the two obtained structures. It is possible that the difference in the porosity is not big enough to produce a structural difference capable of influencing the degradation rate.
In summary, it is confirmed that the fibre diameter influences the degradation rate of chitosan fiber-mesh scaffolds. Tailoring the fibre diameter will be a very useful tool to adjust the scaffolds degradation to a specific application without the need to change the polymers chemistry.

ACKNOWLEDGEMENTS : Marie Curie Actions: Alea Jacta EST – MEST-CT-2004-008104.

REFERENCES: [1] T.Freier, et al *Biomaterials* (2005) 26:5872–5878.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 36) ISSN 1473-2262

Characterization of Neuronal Repair within Injectable Fibronectin and Collagen Based Gels Implanted into the Injured Adult Rat Spinal Cord.

A. Alovskaya[1], D. Wei[2], V. King[2], J. Priestley[2], R.Brown[1]

[1] *Institute of Orthopaedics and Musculoskeletal Science, Tissue Repair and Engineering Centre, University College London*

[2] *Neuroscience Centre, Institute of Cell and Molecular Science, Queen Mary University of London*

INTRODUCTION: Injury of the adult mammalian spinal cord elicits a cascade of pathophysiological events that results in loss of neural tissue, and, consequently, partial or complete loss of neurological functions. Subsequent to injury of the spinal cord, fluid filled cavities often develop an inhibitory environment in the form of glial scar. These factors, in part, prevent regenerative repair in the spinal cord. In the current study we have begun to develop support materials that form gels when they are injected into lesion cavities in the spinal cord. The aim is to provide a substrate for axonal growth, infiltration of other beneficial elements (e.g. blood vessels) and reduces neural death.

METHODS: We examined three types of materials: (1) viscous fibronectin solution (2) collagen gel and (3) fibronectin/fibrin gels (Fn/Fb). Each of these materials was injected into a lesion cavity made in the dorsal aspect of the thoracic spinal cord. At one and four weeks post-injury we used immunohistochemistry to examine a variety of neuronal and non-neuronal elements at the implant site.

RESULTS: At one week post-injury both fibronectin containing materials had supported strong regeneration into the lesion site, with little cavitations within the spinal cord. In contrast, the collagen material resulted in very dense and cavity filled areas within the implant site itself. Immunostaining showed substantial axonal growth within both fibronectin groups as well as a infiltration of Schwann cells and blood vessels and the presence of the extracellular matrix molecule laminin. Within the dense areas of the collagen implant, little infiltration of these elements was seen. In addition, extensive macrophage infiltration was present within all implants. The amount of macrophage infiltration in the surrounding intact tissue was no more than that seen following injury alone, indicating that none of the materials increased the inflammatory response of the spinal cord. By four weeks post-injury, some large cavities had developed at the interface between the intact tissue and implant in the fibronectin group only, but this was minimal in animals with Fn/Fb injection. Infiltration of the various cellular and non-cellular elements at this time point was grossly similar between the two fibronectin materials.

DISCUSSION & CONCLUSIONS: The results indicate that injectable fibronectin gels are effective in filling in lesion cavities in the damaged spinal cord. However, the Fn/Fb material was superior both in terms of the completeness with which the cavities were filled and in the degree of axonal regeneration supported.

ACKNOWLEDGEMENTS: This work is supported by BBSRC project grant.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 37) ISSN 1473-2262

Matrix Proteins as Predictive Markers of the Mechanical Strength of Engineered Cartilage.

A.. Crawford[1], D. J. Kelly[2], S. C. Dickinson[3], T. J. Sims[3], J. Mundy , A. P. Hollander[3], P. J. Prendergast[2] and P.V. Hatton[1].

[1]*Centre for Biomaterials and Tissue Engineering, University of Sheffield, Sheffield. UK.*
[2]*Bioengineering Trinity, College, Dublin, Ireland.* [3]*Academic Rheumatology, University of Bristol, Avon Orthopaedic Centre, Southmead Hospital, Bristol. UK.*

INTRODUCTION: The mechanical properties of articular cartilage are dependent on the extracellular matrix composition. However, it is not clearly defined which matrix components are the most appropriate markers for predicting mechanical ability of tissue-engineered cartilage. The aim of this study was to investigate which matrix proteins correlate with mechanical quality of engineered cartilage. Engineered cartilage constructs were formed from scaffolds seeded with varying numbers of hyaline chondrocytes or; by seeding with a single cell concentration/scaffold and varying the length of time constructs were cultured. to yield varying matrix compositions. Changes in proteoglycan content [determined as glycosaminoglycan content (GAG)], total collagen (determined as hydroxyproline), collagen I and II concentrations and collagen cross-links were correlated with the mechanical properties of the constructs.

METHODS: Non-woven HYAFF 11® scaffolds (2mm depth, 5mm diameter, Fidia Advanced Polymers, Italy) were seeded dynamically with 2, 4, 8, or 16 x 10^6 bovine hyaline chondrocytes and cultured for 42 days as described previously [1]. 3mm cores were taken and tested under confined compression and analyzed [2] to determine proteoglycan content [by calorimetric assay with dimethylmethylene blue) total collagen (by amino acid analysis), collagens I and II (measured by inhibition ELISA) and collagen cross-links (measured by amino acid analysis). For varying the time in culture, scaffolds (2mm depth, 8mm diameter) were seeded with 16 x 10^6 chondrocytes and cultured for 20, 30, 40 or 80 days [1]. The constructs were tested under non-confined compression and analyzed as described.

RESULTS: In experiments changing the number of cells seeded, the aggregate modulus correlated with the of both GAG *(P*<0.0001) and collagen II (*P*<0.0001) but not collagen I expressed as a percentage of the matrix composition. Varying the length of culture showed that Young's modulus increased over the culture period and correlated with GAG (*P*<0.0001*)*, collagen II (*P*<0.0001), and ratio of mature to immature collagen cross-links (*P*=0.0001). No correlation was found between Young's modulus and matrix hydroxyproline.

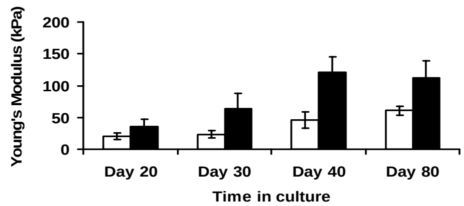


Fig. 1: The Effect of Time in Culture on Young's Modulus of Engineered Hyaline Cartilage.

DISCUSSION & CONCLUSIONS: The results indicated that measurement of collagen II and GAG are good predictive markers of the mechanical quality of tissue-engineered hyaline cartilage. However, there was no significant correlation between total collagen content and mechanical quality. Therefore, collagens other than type II probably compose a significant percentage of the matrix of immature engineered cartilage constructs

REFERENCES: [1] Crawford A. and Dickinson S.C. (2004) *Chondrocyte isolation, expansion and culture on polymer scaffolds.* Methods Mol. Biol. 238:147-157,

2. Kaffienah W. and Sims T.J. (2004). *Biochemical methods for the analysis of tissue-engineered cartilage.* Methods Mol. Biol. 238:217-230.

ACKNOWLEDGEMENTS: This project was supported by the European Commission under the 5th Framework Programme (SCAFCART project GRDR1-1999-00050.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 38) ISSN 1473-2262

Development and validation of a 3D lymph node-adipocyte co-culture system

S.Daya , A. Loughlin & H. MacQueen

Dept of Biological Sciences, The Open University, Milton Keynes, England

INTRODUCTION: White adipose tissue is involved in localised paracrine interactions with lymph node cells. Localised immune challenges with lipopolysaccharide stimulate the release of Tumour Necrosis Factor-α (TNF-α) and interleukins 4, 6, 8 and 10 from lymph node cells. This in turn directs adipocytes to respond by releasing fatty acids from their triacylglycerol stores. This mechanism is believed to be of great importance as it provides energy and metabolic precursors to the lymph node cells during the immune response.

We have developed a long term 3-dimensional co-culture system with adipocytes and lymph node cells for the purpose of investigating interactions between these cells *in vitro*. (Patent application number 0606764.9).

METHODS: Preadipocytes were isolated from the popliteal adipose tissue depot. The cells were expanded then seeded into a type I collagen gel. The preadipocytes were induced to differentiate over a 14 day period. The lymph node cells were isolated by teasing apart the node and releasing the cells into a petri dish containing culture medium. Present experimental work with the 3D system is aimed at introducing lymph node cells, in proportions similar to those found in intact lymph nodes, among differentiated adipocytes.

RESULTS: Differentiating preadipocytes *in vitro* showed multiple lipid droplet accumulation and similar protein expression patterns to those of mature adipocytes *in vivo*.

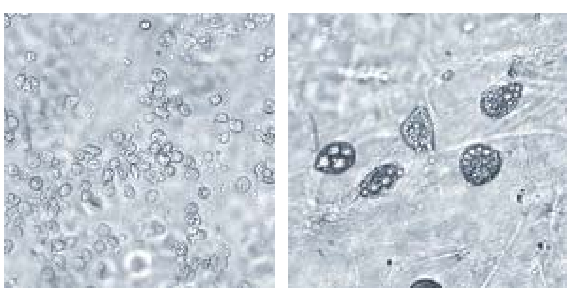
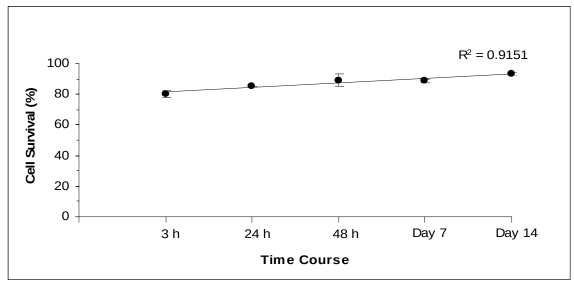


Fig. 1: Differentiation of preadipocytes in 3D culture (phase): Day 0 (left), Day 14 (right).

Differentiated adipocytes expressed and showed upregulation of the S100 protein, insulin receptor and caveolin-1 and TNF-α receptors (TNFRI and TNFRII) and the chemokine receptor CCR5 during differentiation, when assessed by immunofluorescence and western blot (1). Prior to the induction of differentiation, preadipocytes showed good proliferation, assessed by BrdU incorporation and maintained high cell viability during differentiation (Graph 1).

Graph 1. Cell survival of differentiating preadipocytes in a collagen gel.



Over 80% (n=10000) of lymph node cells were viable after isolation from the popliteal node and over 75% (n≥5000) of these cells remained viable in the system. T cells were the major cell type (52.1%) among the mixed cell population isolated from the popliteal lymph node (assessed by flow cytometry), followed by B cells, dendritic cells and macrophages.

DISCUSSION & CONCLUSIONS: Present experimental work with the culture system is aimed at introducing lymph node cells, in proportions similar to those found in intact lymph nodes, among differentiated adipocytes and observing interactions and the establishment of a spatial relationship between them. Co-cultures will be used to investigate the lymph node adipocyte interactions following immune stimulation (lipopolysaccharide treatment) measuring production of inflammatory mediators (cytokines) and lipolytic activity.

REFERENCES: [1] S.Daya, A.J. Loughlin, H.A. MacQueen (2006) Culture and differentiation of preadipocytes in 2-dimensional and 3-dimensional *in vitro* systems. Submitted to Differentiation.

ACKNOWLEDGEMENTS: This work is supported by a BBSRC studentship.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 39) ISSN 1473-2262

'Fusion of Primary Human Skeletal Muscle Cells within a 3D-Construct'

M.Brady[1,2], R.Brown[1], M. Lewis[2] and V.Mudera[1]

[1] *Tissue Repair and Engineering Centre (TREC), Institute of Orthopaedics, University College London, UK*

[2] Division of Biomaterials and Tissue Engineering, *Eastman Dental Institute, University College London, UK*

INTRODUCTION: To date there have been two approaches to tissue engineer lost or damaged muscle: the *in vitro* approach to create differentiated muscle tissue constructs for implantation by inducing the fusion of myoblasts to myotubes in 3D culture (Bach *et al.* 2004) and the *in vivo* approach injecting muscle-precursor cells into sites of dysfunction – the hope here is that the cells will reorganize spontaneously to form new muscle tissue. The aim of this study was to induce fusion of CD56+ primary human muscle derived cells (PHMDCs) by investigating the effect of increasing cell density and Plastic Compression (PC) (Brown *et al.* 2005) to create a 3D differentiated muscle tissue construct. Key to this was the need to demonstrate the appearance of myogenin as a marker of myoblast differentiation.

METHODS: PHMDCs were seeded at increasing densities in 3D-collagen gels. The optimal cell density was determined by monitoring the force contraction profile generated by the constructs on a culture force monitor (CFM). To further induce myoblast fusion PC was used to increase cell density and decrease total volume of the construct, to facilitate fusion. RT-PCR was used to detect myogenin, a marker of myoblast differentiation. Finally, TEM was used to identify multinucleated (fused) cells.

RESULTS:

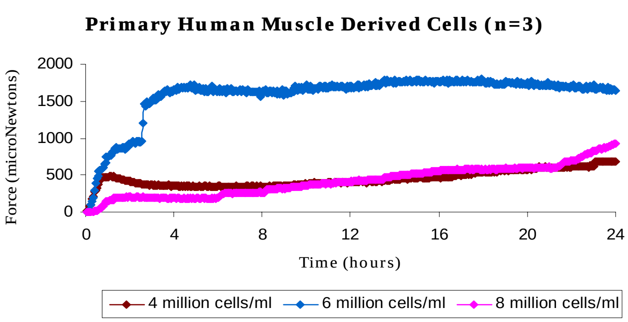


Fig. 1: Force Contraction profiles of Primary Human Skeletal Muscle Cells with changing cell density

The contraction profile of PHMDCs seeded at densities of 4, 6 and 8 million cells/ml (*Fig. 1*) generated peak forces of 675, 1700 and 930µN respectively over 24 hours. Myogenin expression was identified in constructs at a density of 6 million cells/ml and in the equivalent PC constructs. Multinucleated cells within 3D collagen and PC constructs using TEM were identified (*Fig. 2*).

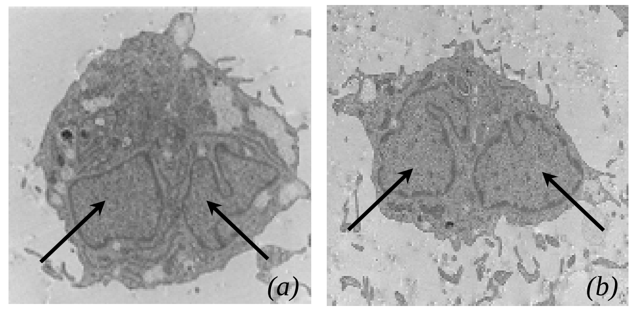


Fig. 2: TEM depicting multinucleated cell in a 3D (a) normal and (b) PC collagen construct at 24 hours. Arrows indicate the presence of two nuclei within a single cytoplasm.

DISCUSSION & CONCLUSIONS:

We have established that fusion of PHMDCs within a 3D construct is strongly dependent upon cell density and proximity. The optimal cell density within our defined 3D collagen construct was determined to be 6 million cells/ml. These constructs were then used for PC to further increase cell density and improve mechanical strength. The tissue engineering of a new 3D differentiated muscle tissue construct was verified by the presence of the gene myogenin. This construct will be used as a model of skeletal muscle to investigate and test the effect of mechanical stimulation on muscle cell differentiation, growth and mechanical strength.

REFERENCES:

[1]AD. Bach, JP. Beier, J. Stern-Staeter, RE. Horch (2004) *J.Cell Mol. Med.* **8(4)**:413-422

[2]RA. Brown, M. Wiseman, C-B. Chuo, U. Cheema (2005), *Advanced Functional Materials* **15(11)**:1762-1770

ACKNOWLEDGEMENTS: Funding from the Royal National Orthopaedic Hospital (PhD Studentship). Thanks to Dr. Suhel Miah for his TEM expertise

 ISSN 1473-2262

Influences of buffer systems on chondrocyte growth during long-term culture in alginate

Xia Xu[1], Jill, P.G.Urban[2], Uday Tirlapur[1], J.A. Browning[2]
Min-Hsein Wu[1], Robert Wilkins[2], Zhanfeng Cui[1]
[1]*Department of Engineering Science, University of Oxford, Parks Rd., Oxford, OX1 3PJ, UK*
[2]*Physiology Laboratory, University of Oxford, Parks Rd., Oxford, OX1 3PT, UK*

INTRODUCTION: Chondrocyte behaviour is very sensitive to culture environment such as physical and biochemical conditions. To determine the influence of buffer systems on chondrocyte fate during long-term culture, HEPES buffered media in the absence and the presence of bicarbonate were used, respectively.

METHODS: Bovine articular chondrocytes were cultured in 1.2 % alginate beads for up to 12 days, at the density of 4 million cells/ml. Culture medium A was DMEM buffered by with HEPES (25 mM). Culture medium B had the same compositions as Culture medium A, but buffered by a combination of $NaHCO_3$ (44 mM) and HEPES (25 mM). The pH was adjusted to pH 7.4 and the osmolarity of both culture media was adjusted to 380 mOsm. The alginate beads were cultured in 24-well microplate (3 beads/well) with 2 ml culture medium A in a humidified air incubator and 2 ml culture medium B in a 5% CO_2 humidified incubator at 37^{o}C up to 12 days, respectively. The culture medium was replaced every 2-3 days. Cell density was measeured by DNA content using Hoechst 33258. Intracellular pH, glysaminoglycan (GAG) and collagen production were measured at day 5 and day 12. Cell morphology, distribution and viability in alginate beads were monitored using multiphoton microscope over 12 days of culture.

RESULTS: The cell density in the presence of $NaHCO_3$ was dramatically greater than that in the absence of $NaHCO_3$ at the end of 12 days of culture from DNA assay and multiphoton microscope analysis (Fig. 1). In the presence of bicarbonate, the intracellular pH was more alkaline, about 0.2 pH unit (Fig. 2). Although there was no significant difference in collagen production with culture time in the presence of $NaHCO_3$, about 50 % more GAG was deposited in alginate beads when chondrocytes were cultured in the combination of HEPES and bicarbonate, compared to chondrocytes cultured in the absence of $NaHCO_3$ at the end of 12 days of culture.

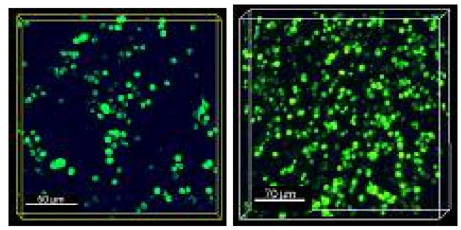


Fig. 1: Cell morphology and viability after 12 days of culture: HEPES only vs. Bicarbonate and HEPES (right).

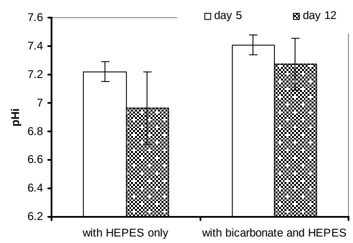


Fig. 2: Intracellular pH of bovine chondrocytes in alginate beads during 12 days of culture under HEPES only and HEPES and bicarbonate together

DISCUSSION & CONCLUSIONS: Culture medium buffered with a combination of HEPES and bicarbonate provides a relatively stable culture environment to chondrocyte seeded in the hydrogel scaffolds in the presence of CO_2. The presence of sodium bicarbonate results in more alkaline in the intracellular pH of bovine chondrocytes after long-term culture. The combination and HEPES in culture medium improves cell growth, matrix production in 3D alginate beads, and more chondrocytes are grown in pairs and clusters, similar to the state in native articular cartilage.

ACKNOWLEDGEMENTS: This project is funded by the Biotechnology and Biological Science Research Council of the UK (EBS 43/E18069).

Surface Chemical Gradients to Optimise Substrata for Self-Renewal of ES Cells

P. Murray,[1] D. Edgar,[1] R. McGreal,[1] R D Short,[2] & K.Parry[3]

[1] *Stem Cell Consortium, School of Biological Sciences, University of Liverpool, England*
[2] *Dept of Engineering Materials, University of Sheffield, England,*
[3] *Plasso Technology, Innovation Centre, Sheffield, England*

INTRODUCTION: Various reports detail how the culture conditions for mouse ES (mES) and human (hES) may be manipulated to maintain these cells in an undifferentiated state.[1,2] Significant differences between mES and hES cells have been commented upon, as well as common mechanisms in maintaining self-renewal. It has been recently shown[3] that the self-renewal of mES and hES cells can be promoted by restricting the degree to which these cells spread. This result implies that the self-renewal mES and hES cells can occur when their spreading is restricted by culture on weakly adhesive substrates. Herein, we show how using surface chemical gradients, of varying carboxylic acid density, an optimal chemistry is readily identified whereby cells can be maintained in compact small colonies, retaining cell-cell contact, without loss of the key ES cell markers alkaline phosphatase and Oct-4.

METHODS: Surface chemical gradients of carboxylic acid were fabricated by means of plasma deposition on 13 mm plastic coverslips.[4] High functional group retention was achieved by the use of low plasma power. Exact control was maintained over the start and endpoints of gradients, and a batch of identical gradients was produced for this study. X-ray photoelectron spectroscopy (Kratos Ultra) with a monochromated x-ray beam was used to obtain line-scan spectra along the length of the gradient, from which the carboxylic acid gradient was reconstructed, and optimal acid density and spacing of acid groups calculated. mES and hES were seeded on chemical gradients and after 7 days, were examined by optical microscopy and stained for AP activity and Oct-4.

RESULTS: Fig. 1 shows hES on a surface chemical gradient of carboxylic acid. The cells were maintained in serum free media (Advanced media, Invitrogen). At the top end of the coverslip, where the surface is purely hydrocarbon, cells have failed to attach. Attachment occurs at the mid point, where the surface comprises a mixture of carboxylic acid and hydrocarbon. These cells have formed compact colonies and retain tight cell-cell contact. At the bottom of the coverslip, cells are much more fully spread, and have reduced cell-cell contact. Cells at the mid-point have stained strongly for AP, whilst at the bottom the cells do not stain strongly. hES and mES cells have been cultured on homogeneous surfaces with the chemistries of the mid-point and various positions towards the bottom of the coverslip. Using stains AP and Oct-4, it is shown that on surfaces of increasing surface acid density, cells spread and there is loss of expression of AP and Oct-4.

Fig. 1: hES cells on a surface chemical gradient of varying acid density. At top, 0% carboxylic acids, at mid-point ca. 8%, towards the bottom ca. 12%. Cells stained for AP.

DISCUSSION & CONCLUSIONS: These preliminary results are strongly suggestive that the capacity of ES cells for self-renewal may be maintained by surface chemistry alone. If true, this has the important implication that geometric control (ie control over cells spreading) is an important factor in the maintenance of self-renewal. Surface chemical gradients are an ideal tool for rapid (high throughput) screening.

REFERENCES: [1] A.G. Smith, et al, *Nature* (1988) **336**, 688-690 [2] T Burdon et al. T*rends Cell Biol.*,(2002) **12**, 432-38 [3] Murray P et al, in preparation [4]J D Whittle et al. *Chem. Comm.*, (2003) **14;** 1766

Local Delivery of Anti-inflammatory Drugs for Epithelial Cells

S.Roberts[1], N. Gavalas[2], E.H. Kemp[2] & J.W. Haycock[1]

[1] *Dept of Engineering Materials and* [2]*Clinical Sciences (North), University of Sheffield, England.*

INTRODUCTION: Acute peripheral inflammation creates an extremely hostile environment in the wound bed, which in addition to its remedial functions can harm healthy cells. This can result in increased healing times, hypertrophic scarring and graft rejection. Anti-inflammatory drugs can modulate inflammation to reduce the above problems, associated with acute tissue damage. The control of inflammation via the melanocortin receptors (MCRs) has been a topic of recent interest following observations that α-MSH has anti-inflammatory activity in a number of cell types. This has led to further investigation of the properties of the α-MSH peptide, its analogues and novel compounds designed to specifically target the MCRs. It is known that α-MSH acts by binding to the MC1R, causing an intracellular increase in cyclic AMP and calcium that interrupt proinflammatory pathways. There are five MCRs cloned to date[1,2] and an increasing amount of literature suggests that MC3R and MC4R may have roles in inflammatory control (as well as other effects such as control over satiety and sexual behaviour). In order to delineate these signalling pathways and resultant functions we are investigating MC1R, MC3R and MC4R individually, using CHO cell lines stably transfected with each receptor. Elevation of cyclic AMP and Ca^{2+} was investigated in response to stimulation with α-MSH, Nle^{4}-$_{D}$-Phe^{7}-α-MSH (a potent analogue of α-MSH) and BMS-470539, a novel MC1R agonist developed by Bristol-Myers-Squibb[3].

METHODS: Relative NF-κB activity was assessed by measuring light output from HBL melanoma cells transfected with an NF-κB-luciferase reporter construct, stimulated with TNF-α. The inhibitory effect of BMS-470539 was investigated by pre-incubating cells with the compound prior to TNF-α addition. Stably transfected CHO clones were developed by co-incubating cells with transfection reagent and a DNA prep coding the receptor of interest. Clones were selected using G418 sulphate. For cyclic AMP assays, cells were labelled using ^{3}H-adenine. Cyclic AMP production was measured by stimulating cells with either α-MSH, Nle^{4}-$_{D}$-Phe^{7}-α-MSH or BMS-470539 (10^{-12}M to 10^{-6}M), to form labelled cAMP. This was separated from other nucleotides by neutral alumina chromatography and the radioactivity of resultant samples measured using a beta counter. For intracellular calcium measurement, cells were grown on glass cover slips and labelled with Fura-2AM. Ca^{2+} elevation in response to drug addition was measured in real time using cell-averaged fluorimetry.

RESULTS: BMS-470539 was found to significantly reduce NF-κB activation stimulated by TNF-α in a dose dependent manner. Results indicate a potential for elevating cAMP by stimulation of MC1R, MC3R and MC4R with α-MSH and Nle^{4}-$_{D}$-Phe^{7}-αMSH. Interestingly, no cyclic AMP elevation was observed in response to BMS-470539, contrary to findings by Bristol-Myers-Squibb[3]. All three agonists were found to elevate intracellular calcium levels through MC1R. Work is ongoing to investigate intracellular Ca^{2+} signalling via MC3R and MC4R.

DISCUSSION & CONCLUSIONS: In conclusion, our results show that novel MC1R agonists cause intracellular elevations that may inhibit cellular responses linked to inflammation, suggesting that these compounds may have therapeutic potential in controlling the inflammatory response. These novel compounds are ideal to deliver locally for preventing inflammation as they are straightforward to manufacture. Future work is underway to link intracellular events measured from each receptor with functional control over p65/NF-κB, and in turn in situ delivery to establish suitability for local delivery.

REFERENCES: [1]K. Mountjoy, L Robbins et al (1992) *Science* **257**:1248-51.[2]V. Chhajlani, J. Wikberg (1992) *FEBS Lett* 309:417-20. [3]T. Herpin, G. Yu, K. Carlson et al (2003) *J Med Chem* **46**:1123-26.

ACKNOWLEDGEMENTS: EPSRC for funding, Bristol-Myers-Squibb and Kenneth E. Carlson for BMS-470539.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 43) ISSN 1473-2262

Contractile Properties of fibroblasts derived from Primary frozen shoulder and effects of TGF beta 1 stimulation

M Bains[1], S Lambert[2], V Mudera[1]

[1] *Tissue Repair and Engineering Centre, IOMS, University College London, England*

[2] *Royal National Orthopaedic Hospital, Stanmore, England.*

INTRODUCTION: Primary Frozen Shoulder (PFS) is a debilitating disease of unknown aetiology. There is fibrosis and contracture of the coracohumeral ligament, tissues of the rotator interval and the glenohumeral ligaments, leading to restrictive shoulder movements requiring surgical intervention [1]. Frozen shoulder has been postulated to be dupuytren's disease of the shoulder with an association inferred since 1936. The purpose of the study was to test the hypothesis that cellular mechanisms of fibroblasts derived from primary frozen shoulder exhibited similar activity in terms of contraction and response to cytokine (transforming growth factor beta1) to fibroblasts derived from dupuytren's disease. Understanding of cellular responses is critical to developing non surgical treatment strategies.

METHODS: Primary explant cultures of fibroblasts from six patients with PFS and four control patients were obtained using standard tissue culture techniques. Fibroblasts were seeded in 3-D collagen constructs and contraction force generated over 24hours measured using a culture force monitor (CFM) in real time. Increasing concentrations of TGF-beta1 were added to cell seeded gels and force generated measured using the CFM over 24hours. These mechanical output data were statistically compared to data available from Dupuytren's disease.

RESULTS: Compared to Dupuytren's fibroblasts [2], PFS fibroblasts showed a statistically reduced ability to contract a 3-D collagen gel over 24hours ($p<0.01$). In Dupuytren's disease, fibroblasts derived from nodules and cords generate peak forces of 140dynes and 110dynes respectively, while PFS fibroblasts generated peak force of 8dynes. The response to TGF-beta1 stimulation, which has been shown to enhance peak force contraction in Dupuytren's fibroblasts had no effect on PFS fibroblasts and this was statistically significant ($p<0.01$).

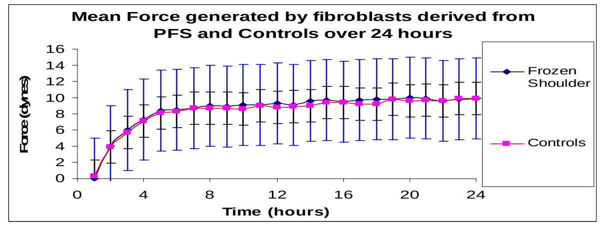


Fig. 1: Contraction Profiles of fibroblasts derived from primary frozen shoulder (n=6) as compared to control derived fibroblasts (n=4)

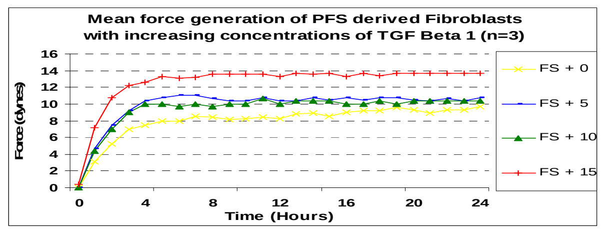


Fig. 1: Contraction Profiles of PFS derived fibroblasts (n=3) to increasing concentrations of TGF beta 1 (0, 5, 10 & 15ng/ml)

DISCUSSION & CONCLUSIONS: These data suggest intrinsic differences in cellular activity and mechanisms between Dupuytren's and Primary Frozen Shoulder even though clinically they both manifest with a contracted extracellular matrix affecting function and requiring surgical intervention. This may explain increasing post surgically recurrence in Dupuytren's as compared to Primary Frozen Shoulder release.

REFERENCES:

[1] Ozaki J *et al* (1989) *JBJS* **71A**:1511-1515

[2] Bisson M *et al* (2004) PRS **113(2)**:611-620

ACKNOWLEDGEMENTS: Royal National Orthopaedic Hospital, Stanmore for kindly funding the research.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 44) ISSN 1473-2262

Use of Simvastatin for Enhanced 2D and 3D Bone Tissue Engineering Constructs

S. L. Griffiths & S. H. Cartmell

ISTM, University of Keele, Thornburrow Drive, Hartshill, Stoke-on-Trent, ST4 7QB

INTRODUCTION: Bone tissue engineering is a potential emerging therapy for patients suffering from bone loss as a result of trauma or disease. Statins are a commonly prescribed cholesterol lowering drug, however it has recently been shown that they also have the beneficial side effect of enhancing bone matrix formation(1). The inhibition of the cholesterol biosynthesis pathway by statins also interacts with other pathways to produce an upregulation of BMP2. This study has looked at using this bone enhancing effect of statins for the novel use in tissue engineered scaffold constructs. A 5µM concentration of Simvastatin was added as a supplement to media of human mesenchymal stem cells (hMSC's and human osteoblasts (hOB's) which had been seeded in the 2D cultured onto 6 well plates and cultured for 7days *in vitro* and the gene expression of various bone related genes analysed. In the 3D culture the cells were seeded onto PLLA scaffolds and cultured *in vitro* with Simvastatin over a 7-week period, then the volume and location of mineralized matrix were analysed.

METHODS: 2D culture: MSCs and hOBs seeded at 100,000 cells/well. Media: DMEM for hMSCs, αMEM for hobs. 5µM simvastatin supplement to media, controls no simvastatin. Media supplements: 0.1mM human sera, 0.01mM antibiotic, 0.01mM ascorbic acid, 0.01mM β-Glycerophosphate. Real-time-RTPCR was used to analyse expression of Osteopontin (OP), Bone-morphogenic-protein-2 (BMP2), Osteocalcin (OCN), RUNX2, Collagen II (Coll II) and bone-sialoprotein (BSP) normalised to 18S. 3D culture: PLLA scaffolds (porosity of 88±2%, pore size of 250-350µm, manufactured using a salt leaching technique). hMSC's seeded at ~200,000 cells/scaffold, hOB's at ~850,000 cells/scaffold. Media as above, again with 5µM simvastatin added as a supplement to media. Scanco microCT40 was used to determine the volume and location of matrix mineralization. A picogreen DNA assay was used to determine DNA concentration.

RESULTS: 2D culture: hMSC and hOB gene expression of OP and BMP2 significantly increased in the statin group, whilst OCN, BSP, Coll II and RUNX2 showed significant or greatly suppressed expression. 3D culture: hMSC's, no significant difference between statin and control groups for volume of mineralised matrix (VMM) and rate of proliferation. Normalisation of VMM to DNA also showed no significant difference. hOB's, no significant difference between statin and control VMM, however the rate of proliferation was significantly lower in the statin group (p=6.26x10^{-9}). Normalisation of the VMM to DNA revealed a trend of increased VMM being produced per cell in the statin group (p=0.087).

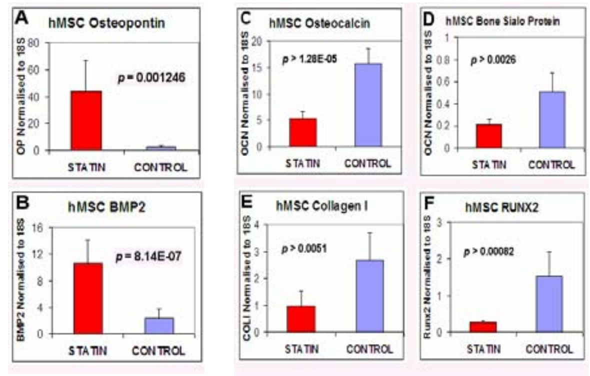
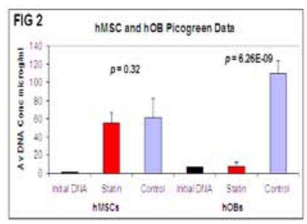


FIGURE 1A-F *7day real time bone related gene expression of human MSC's with or without exposure to simvastatin*



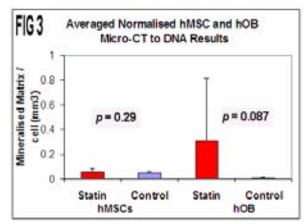


FIGURE 2 *hMSC and hOB DNA results 7week culture on 3D PLLA scaffolds with and without exposure to simvastatin*

FIGURE 3 *hMSC and hOB microCT mineralised matrix results normalised to DNA, 7week culture on 3D PLLA scaffolds with and without exposure to simvastatin*

DISCUSSION: Results suggest that in the long-term 3D cultures, 5µM simvastatin produces a trend of increased VMM/cell in hOBs whilst causing a significant reduction in cell proliferation, whilst this was not seen in the 3D hMSC cultures. Simvastatin when added to hMSC and hOB 2D cultures significantly upregulated expression in genes that are used as markers of osteoblastic differentiation and mineralization, whilst down regulating others. This suggests that statins play a role in altering the coupled regulation, of cell cycle progression and osteogenic differential gene expression. Further studies are underway to optimise the type, concentration and timing interval of statin addition to culture, in human cells so that more significant effects can be produced and the mechanisms of action better understood. We anticipate that optimising the statin administration timing to 2D/3D bone cell constructs to allow for cell proliferation prior to statin induced osteoblast maturation has great potential for bone tissue engineering applications.

REFERENCES: (1) Mundy, Garrett, Harris, Chan, Chen, Rossini, Boyce, Zhao, Gutierrez (1999) Science 286: 1946-1949.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 45) ISSN 1473-2262

Anti-inflammatory peptide approaches for preventing vascular inflammation

S. E. W. Grubb[1], C. M. Holt[2], N. Malik[2] & J. W. Haycock[1]

[1] *Department of Engineering Materials, Mappin Street, University of Sheffield, UK*

[2] *Cardiovascular Medicine, Stopford Building, University of Manchester, UK*

INTRODUCTION: Inflammation is implicated in the development of atherosclerotic lesions and restenosis [1]. It is proposed that by inhibiting the local inflammatory process e.g. using a drug-eluting stent platform, neointima formation will be reduced. Melanocyte stimulating hormone (MSH) peptides are potent inhibitors of inflammation, and act via the melanocortin-1 receptor (MC1R) [2]. Therefore, the aim of this work was to confirm whether MSH peptides can inhibit inflammatory signaling *in vitro* and upregulation of the E-selectin adhesion molecule.

METHODS: To confirm that α-MSH inhibits TNF-α stimulated NF-κB activation porcine vascular smooth muscle (VSM) cells were transiently transfected in vitro with an NF-κB dependent luciferase reporter construct. After transfection cells were stimulated with pTNF-α +/- α-MSH ($10^{-6}/10^{-9}$ M) for 4 hours and luciferase activity analysed. In addition, E-selectin expression was assessed by flow cytometry (Guava PCA) using porcine endothelial cells grown in culture for 3 days and pre-incubated with α-MSH (10^{-8} M/10^{-10} M/10^{-12} M) for 15 minutes prior to stimulation with pTNF-α (2 ng/ml) for 24 hours.

RESULTS:

Transient Transfection: Stimulation with pTNF-α increased the relative NF-κB dependant luciferase activation by 37 % (Figure 1). α-MSH (10^{-9} M) was found to significantly inhibit pTNF-α stimulated relative NF-κB dependant luciferase activation by 29 % (n=3, p>0.01). α-MSH alone (without TNF-α) did not alter the activation.

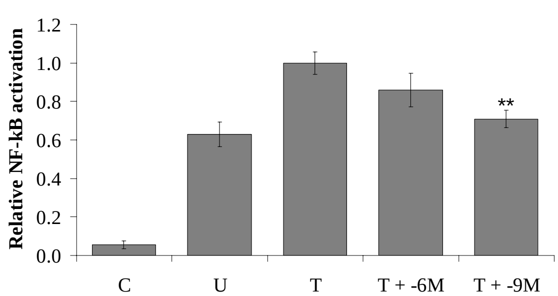


Fig. 1: α-MSH inhibits TNF-α stimulated relative NF-κB dependant luciferase activation. C, Control; U, Unstimulated; T, pTNF-α (2 ng/ml); M, α-MSH ($10^{-6}/10^{-9}$M). n=3. **p>0.01

E-selectin upregulation: α-MSH inhibited TNF-α stimulated E-selectin upregulation in a dose responsive manner from 10^{-8} M to 10^{-12} M (Figure 2). Complete inhibition was observed at 10^{-8} M.

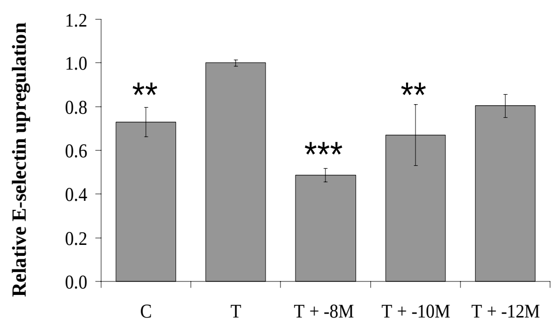


Fig. 2: α-MSH inhibits TNF-α stimulated E-selectin upregulation. C, Control (unstimulated); T, pTNF-α (2 ng/ml); M, α-MSH ($10^{-8}/10^{-10}/10^{-12}$M). n=3. **p>0.01, ***p>0.0001

DISCUSSION & CONCLUSIONS: α-MSH decreased inflammatory signaling and adhesion molecule upregulation in TNF-α stimulated VSM and endothelial cells. Work is currently underway to: (1) extend previous work on adhesion molecules with E-selectin to ICAM-1 and P-selectin on endothelial cells and ICAM-1 on VSM cells; (2) to look at the effect of α-MSH on cellular apoptosis; (3) to investigate the effect of MSH on inflammatory signaling *in vivo*. This work suggests that MSH may be of potential therapeutic value in the prevention of vascular inflammation, especially in restenosis from drug eluting stents.

REFERENCES: [1] T. Inoue et al (1996) *J Am Coll Cardiol* **28**:1127-33. [2] J. Wikberg et al (2000) *Pharmacol Res* **42**:393-420.

ACKNOWLEDGMENTS: We gratefully acknowledge the EPSRC for financial support.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 46) ISSN 1473-2262

Characterisation of a decellularised xenogeneic scaffold for tissue engineering of small diameter vessels.

C. Derham[1], J. Ingram[1], J. Fisher[1], S. Homer-Vanniasinkam[2] & E. Ingham[1]

[1] *Institute of Medical and Biological Engineering, University of Leeds, Leeds, England*

[2] *Vascular Surgical Unit, Leeds General Infirmary, Leeds*

INTRODUCTION: Autologous vascular tissue remains the gold standard for small diameter (<6mm) arterial bypass. The medium and long term results using prosthetic, biologic, and allograft alternatives are not satisfactory when compared to vein bypass. The aim of this study was to characterise a decellularised xenogeneic ureteric scaffold for use in the development of a tissue engineered small diameter living vascular graft.

METHODS: Porcine ureters were treated with hypotonic buffer, low concentration SDS (0.1% w/v) in the presence of protease inhibitors, and nuclease solution (RNase/DNase) to render the tissue acellular. Biomechanical properties of the porcine ureter were determined by uniaxial tensile testing to failure, compliance and suture retention strength of fresh and decellularised ureters. The ureter was assessed for the presence of the galactose α1,3galactose(α-gal) epitope using immunoperoxidase labelling (antibody to α-gal).
Porcine smooth muscle and endothelial cells were isolated from thoracic aortas, cultured and characterised using immunofluorescence (α smooth muscle actin, desmin and vimentin for smooth muscle cells, and vWF and CD34 for endothelial cells). The cytotoxicity of the decellularised porcine ureter was assessed by contact cytotoxicity using porcine smooth muscle and endothelial cells.
Porcine endothelial cells were seeded onto acellular ureters at a seeding density of 0.5 $x10^6$-1.0 $x10^6.cm^{-2}$ for 24h and analysed by scanning electron microscopy.
Data was analysed using Student's t-test and ANOVA.

RESULTS: Histological analysis of the decellularised porcine ureter revealed preservation of the histioarchitecture whilst showing no evidence of cellularity (Figure 1.). The absence of cells in the decellularised ureteric scaffold was confirmed using Hoechst stain and agarose gel electrophoresis of DNA. The ultimate tensile strength and compliance of the decellularised porcine ureter was not significantly different from fresh ureter (UTS fresh=7.32MPa, decell=5.48MPa, p= 0.095) (Figure 2.). However, there was a significant reduction in the elastic phase slope of decellularised ureter in the circumferential direction (Fresh=2.78x10^{-4}GPa, decell=3.71x10^{-4}GPa, p=0.018). The compliance of the decellularised ureter was significantly reduced at higher pressure (>160mmHg) compared to fresh ureter (strain of fresh=18.38, decell=7.00, p=0.020).Suture retention strength of decellularised ureter was significantly greater than fresh ureter (fresh= 0.89N, decell=1.67N, p=0.008). Decellularised ureter showed no evidence of α-gal staining when compared to control tissue (fresh porcine pericardium). There was no evidence of contact cytotoxicty exhibited by the decellularised porcine ureter to porcine smooth muscle and endothelial cells. Attachment of porcine endothelial cells was seen following 24h incubation of endothelial cells on the luminal side of decellularised ureter.

Figure 1: Fresh ureter x400 Decellularised ureter x400

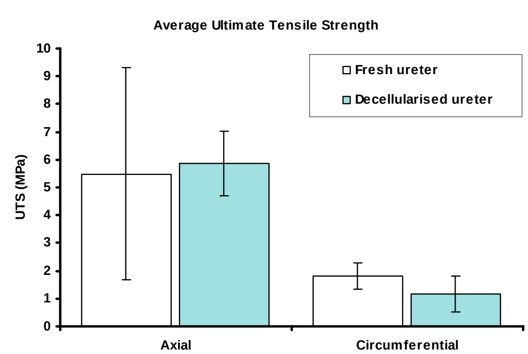


Figure 2: Ultimate tensile strength of fresh and decellularised ureter in axial and circumferential directions.

DISCUSSION & CONCLUSIONS: Decellularisation of the porcine ureter was complete without disruption of the histioarchitecture. There was no significant change in ultimate tensile strength or the average collagen phase slope of decellularised ureter compared to fresh ureter. The decellularised porcine ureter was biocompatible with porcine smooth muscle and endothelial cells, and endothelial attachment occurred when these cells were seeded on the lumen of decellularised ureter. This study has demonstrated the feasibility of using the decellularised porcine ureteric scaffold in tissue engineering a small diameter living vascular graft.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 47) ISSN 1473-2262

Short-term responses of stem cells and osteoblasts to a novel mechanical force application technique.

G. R. Kirkham[1], K. J. Elliot[2], A. Keramane[1] D. M. Salter[2], J. Dobson[1], A. J. El Haj[1], S. H. Cartmell[1]

[1]*ISTM, University of Keele, Thornburrow Drive, Stoke-on-Trent, ST4 7QB*

[2]*The Queen's Medical Research Institute, University of Edinburgh, Edinburgh, EH16 4TJ*

INTRODUCTION: This study describes short-term changes in the membrane potentials of both MG63 human osteoblast-like and human mesenchymal stem cells (hMSCs). Magnetic particles were attached to individual cells and an oscillating magnetic field was applied, allowing force application to specific regions of the cell membrane at controlled frequencies. The degree of force was theoretically [2,3] calculated to be in the order of in the order of a few piconewtons.

METHODS: Ferromagnetic microparticles (4.0-4.5µm diameter) were coated with Arg-Gly-Asp (RGD). Time-varying magnetic fields were applied using a computer controlled drive system (Fig1). Magnetic source used in all experiments was a rare earth NdFeB magnet. Commercially purchased (Cambrex) human mesenchymal stem cells (hMSCs) and the osteosarcoma cell line MG63 were tested. The stretch activated ion channel (SAC) antagonist Gadolinium (Gd) was used for both MSCs and MG63. Large conductance calcium-activated potassium channel (BK ion channel) antagonists Tetraethylammonium chloride (TEA) and 4-Aminopyridine (4-Ap) were only used with MG63 cells [1]. All electrophysiological recording were taken by impaling cells with a glass electrode, as previously described [4]

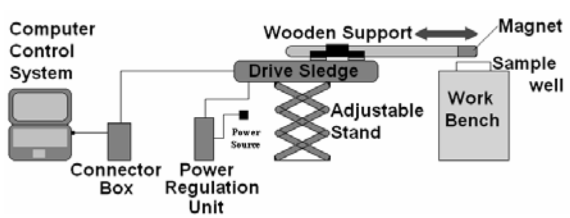
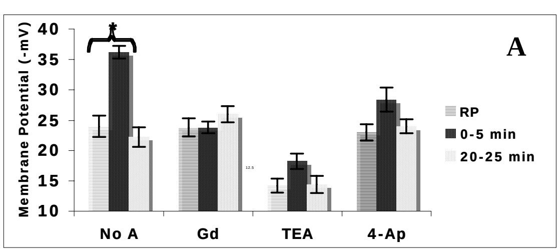


Fig. 1: A schematic representation of the computer controlled magnetic field delivery system.

RESULTS:



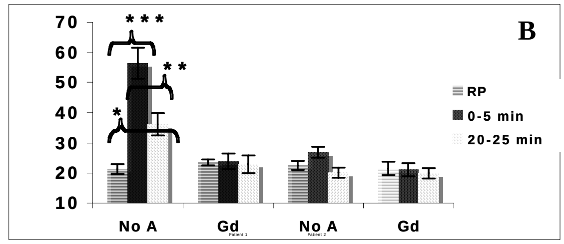


Fig 2: A summary of the effects of magnetic particle based force application with and without antagonists on: ***A*** *MG63 cells *p < 0.01,* ***B*** *hMSCs *p < 0.01 ** <0.01 *** 0.01. No A = No Antagonist, Gd = 10µm Gadolinium, TEA = 20mM TEA, 4-Ap = 3mM 4-Ap*

DISCUSSION & CONCLUSIONS: Both patient 1 hMSCs and MG63 cells showed a significant increase in membrane hyper-polarisation in the experimental group. The presence of gadolinium completely abrogated the hyper-polarisation response in both hMSCs and MG63 cells, indicating the change in membrane potential may be due to stretch activated ion channels (SAC), possibly resulting in calcium flux[5]. The presence of TEA and 4-Aminopyridin significantly reduced the response in MG63 cells, indicating that BK ion channels may play a role in their hyper-polarisation. The exact mechanisms regulating BK channel induction remains unclear but may be due to the activation of specific cell signaling cascades mediated by the opening of SAC.

REFERENCES: [1]M. Wright, R. Stockwell, G. Nuki. (1992). *Connect Tissue Res* **28**: 49-70. [2]J. Dobson, A. Keramane, A, A. El Haj. (2002) *European Cells and Materials* **4**: 42-44. [3]Q. Pankhurst, J. Connolly, S. Jones, J. Dobson. (2003) *Journal of Physics D: Applied Physics* **36**: R167-R181. [4]Salter.D.M. et al (2000) *Journal of Bone and Mineral Research* 15:9: 1746-1755. [5]Hughes. S et al (2003) *European Cells and Materials* 6: 2:43.

ACKNOWLEDGEMENTS: Dr Steven Hughes for his advice on the electrophysiology. Jon Dobson acknowledges the support of a Wolfson Foundation – Royal Society Merit Award.

 ISSN 1473-2262

'Novel scaffolds for tissue engineering of human skeletal muscles'

R. Shah, I. Ahmed, J.C. Knowles, N.P. Hunt, A.C.M. Sinanan and M.P. Lewis
Division of Biomaterials and Tissue Engineering, *Eastman Dental Institute, University College London, UK*

INTRODUCTION: Tissue engineering is a multidisciplinary approach aimed at producing new organs and tissues for implantation in order to circumvent the limitations imposed by current techniques such as surgical tissue transfer.

Structure begets function and highly ordered skeletal muscle (SkM) consists of elongated, multinucleate muscle cells (fibres) that are arranged in bundles surrounded by connective tissue sheaths. It is therefore of no surprise that tissue engineered SkM complexes are often designed around fibre containing scaffolds. This work is the natural continuation of strategies introduced at TCES 2002[1].

METHODS: Primary human jaw (masseter) muscle derived cells (hMDC) were obtained using our well-established protocols. 3D organoids were generated using a degradable artificial scaffold (soluble phosphate glass), Type I collagen, or a composite of glass fibres and Type I collagen. Using immunocytochemistry and microscopy, the organoids were tested to determine structure and expression of relevant proteins, and qPCR was used to determine transcription of functional genes.

RESULTS: By day 17, hMDC cultured on glass fibre scaffolds *in vitro* had attached, proliferated and differentiated to form organised prototypic muscle fibres attached to the glass fibres reminiscent of a "myotendinous" junction (Figure 1).

In comparison, hMDC cultured in 3D collagen scaffolds showed a random disorganised arrangement by day 17. A composite of both fibres and collagen did not enhance either situation; in fact the resulting cells population was highly disorganised and not associated with the glass fibres (Figure 2).

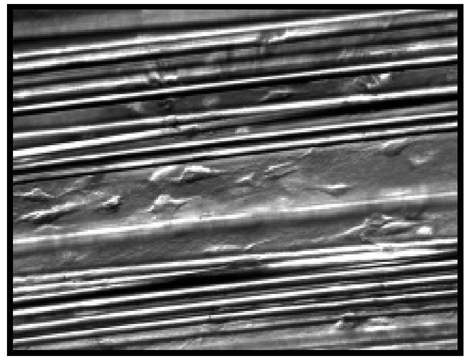


Fig. 2: Composite scaffolds with single cells within the collagen encasing the glass fibres – day 17, 20x modulation contrast

DISCUSSION & CONCLUSIONS: Artificial phosphate-based glass fibres have been produced to form a biomimetic scaffold. The parallel nature of the fibres has encouraged hMDC to form organised muscle fibre-like structures along the length. The size of the fibre in figure 1 is comparable to fibre lengths found in the eye muscles. This is another step closer to engineering human skeletal muscle. Work is in progress to refine the system further.

REFERENCES: [1] Shah, R., Knowles, J.C., Hunt, N.P. and Lewis, M.P. (2002) European Cells and Materials **4[S2]**: 3.

ACKNOWLEDGEMENTS: Patients at the Eastman Dental Hospital.

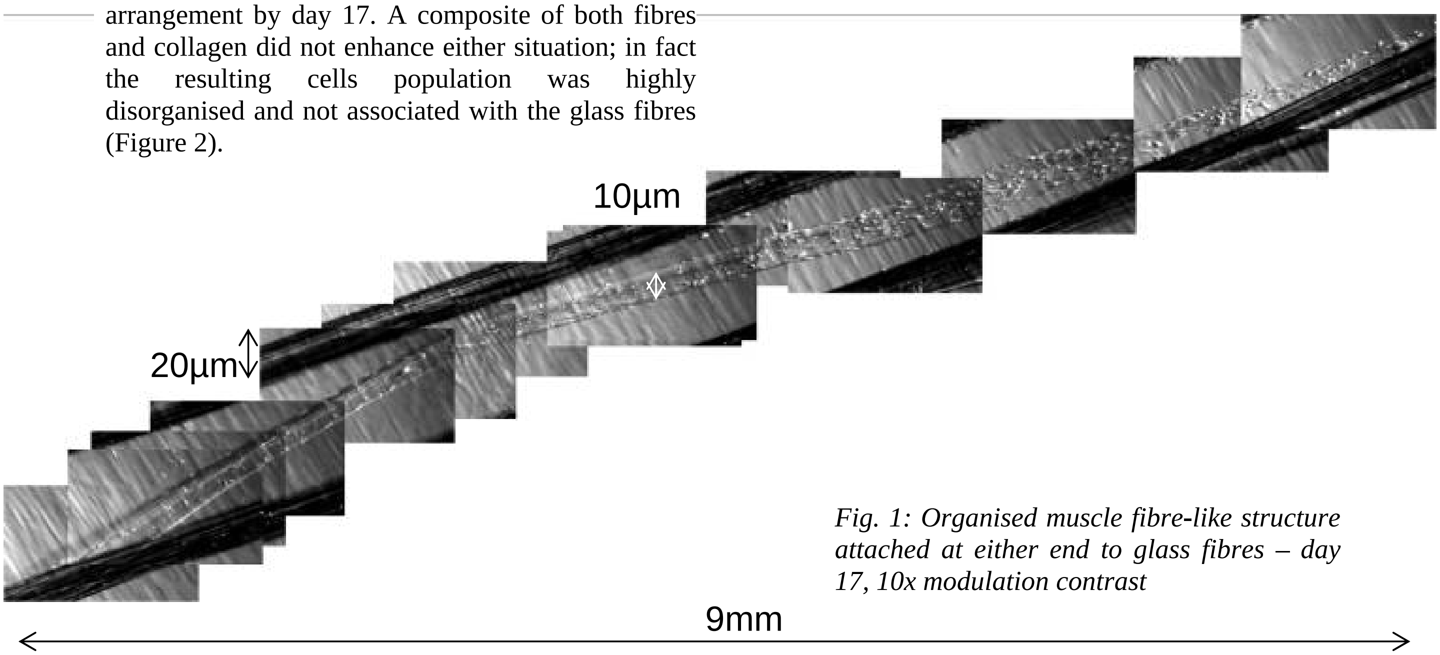


Fig. 1: Organised muscle fibre-like structure attached at either end to glass fibres – day 17, 10x modulation contrast

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 49) ISSN 1473-2262

CHARACTERIZING mRNA PROFILES IN BOVINE INTERVERTEBRAL DISC CELLS

S.Stephan[1,2], W.E.B Johnson[1], J.Menage[1], J.P.G. Urban[3] and S. Roberts [1,2]

[1]*Centre for Spinal Studies, Robert Jones & Agnes Hunt Orthopaedic Hospital, Shropshire.UK*
[2]*Institute for Science and Technology in Medicine, Keele University Medical School.UK*
[3]*Laboratory of Physiology, Oxford University, UK*

INTRODUCTION: To date there is no marker specific for intervertebral disc, as the matrix components are very similar to those of articular cartilage. Since this is an important deficiency in terms of tissue engineering, the aim of this study is to isolate mRNA from intervertebral disc cells in the nucleus pulposus in order to determine potential markers. These will be compared with expression of the same markers in low passage (<3) cultured primary intervertebral disc cells and other primary bovine cell lines including skin dermal fibroblasts, adipose cells, chondrocytes and bone marrow stromal cells. We have primers available to examine a panel of mRNA profiles of ECM proteins, proteases (MMPs and ADAM-TS) / protease inhibitors (TIMPs), cytokines / cytokine receptors and transcription factors that can be compared to a house keeping gene. Measurement of these markers in primary cell lines, together with observations on cell morphology, may enable identification of a candidate cell phenotype for introduction into damaged intervertebral disc tissue to facilitate repair; this could be considered to treat some patients with back pain or spinal deformities.

METHODS: Bovine nucleus pulposus tissue was dissected from the intervertebral disc, snap frozen for RNA extraction and ground to powder in $N_{(L)}$. Sample temperature was elevated to room temperature and mRNA extracted in tri reagent and chloroform, before running on Qiagen RNA mini-prep columns. RNA concentration from disc tissue is low due to low cell number in the nucleus pulposus. Therefore, use of global PCR amplification to create a library of 3` biased cDNAs, whose relative quantities are representative of the original mRNA prep, has been investigated. Bovine nucleus pulposus tissue was dissected, cells extracted via collagenase digestion and filtration, and cells were cultured in DMEM: F12-Hams with L-glutamine, 10% foetal calf serum, amphotericin B, gentamicin and ascorbic acid. Dermal tissue, articular cartilage and subcutaneous adipose tissue were exposed to an identical cell extraction regime and culture

conditions. Cell populations were assessed for morphology in addition to mRNA profile.

RESULTS: RNA has been extracted from disc tissue samples and the global PCR amplification methodology optimized to allow measurement of the aforementioned panel of markers. Distinct cell phenotypes have been photographed (Fig.1) for all established primary cells lines and RNA extractions are being performed to build a library of mRNA to compare with extracted disc tissue mRNA.

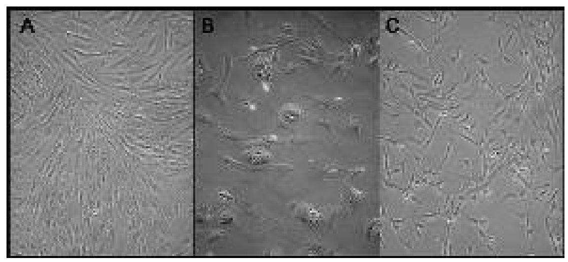


Fig.1. *Images of primary bovine cell cultures established from **A:** dermal layer **B:** subcutaneous adipose **C:** nucleus pulposus.*

DISCUSSION & CONCLUSIONS: Established primary bovine cell lines have shown distinct phenotypes when cultured *in vitro.* Both adherent dermal cells (Fig. 1A) and nucleus pulposus cells (Fig. 1C) have a fibroblastic-like phenotype, whereas subcutaneous fat cells (Fig 1B) have two distinct populations of cells *in vitro*: mesenchymal-like cells surrounding granular cells with a rounded phenotype, recapitulating a similar cell orientation to their *in vivo* environment. It is anticipated that our primary cells may possess similar RNA profiles to RNA extracted from disc tissue and that these cells may display plasticity in their phenotype and genotype. Primary cells may be induced to revert into disc-like cells when placed into a nucleus pulposus ECM environment and these may be used to regenerate damaged disc tissue.

ACKNOWLEDGEMENTS: This work is funded by the BBSRC.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 50) ISSN 1473-2262

Astrocyte responses to dorsal root ganglia in 3-dimensional co-culture models

K L Lazenby[1,2] & J B Phillips[1]

[1]Biological Sciences Department, The Open University, Milton Keynes, U.K.
[2]University College London, London, U.K.

INTRODUCTION: A key impediment to repair following central nervous system (CNS) injury is the formation of a glial scar which inhibits neuronal growth beyond implanted tissue engineered devices [1]. Astrocytes, which support neuronal function in healthy tissue, undergo characteristic changes to form this physical/chemical barrier at the boundary of regeneration, but the precise nature of this response is poorly understood. One of the principle limitations to research in this field is the lack of an effective cell culture model; astrocytes in conventional culture support the growth of neurones despite expressing features of the reactive phenotype. However, astrocytes in 3-dimensional (3D) culture can inhibit neuronal growth [2]. The aim here is to grow astrocytes in a 3D co-culture model in order to mimic the host CNS repair environment where they encounter Schwann cells and regenerating neurones at the interface of implanted conduits [1].

METHODS: Primary astrocytes were prepared from neonatal rats [3] and seeded within 1 ml cylindrical collagen gels formed from type I rat tail collagen (1.92 mg/ml). Dorsal root ganglia (DRGs) were harvested from adult rats and either (i) dissociated using collagenase and injected into the centre of the astrocyte gel, or (ii) embedded as intact ganglia within the astrocyte gel during setting. These co-culture systems were maintained for 11 days, then astrocyte, neurone and Schwann cell morphologies were examined using immunofluorescence (antibodies against GFAP, βIII-tubulin and S100 respectively) detected by fluorescence and confocal microscopy.

RESULTS: A halo of GFAP immunofluorescence was detected around the DRG positions. Astrocytes adjacent to DRGs were larger and displayed a more ramified phenotype than those in comparator regions of the same gels (fig 1). Classification of astrocyte morphology in the DRG-adjacent region confirmed that there were significantly more ramified cells here than in the control areas (fig 2).

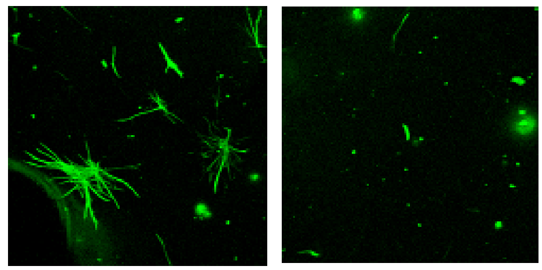


Fig. 1: Confocal micrographs showing morphology of astrocytes adjacent (left) and 2mm distal (right) to embedded DRG.

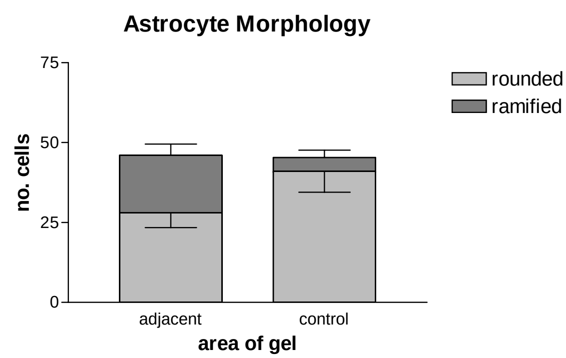


Fig. 2: Assessment of astrocyte morphology in adjacent and control regions.

DISCUSSION & CONCLUSIONS: Astrocytes in 3D culture exhibited a localised response to the presence of co-cultured DRGs. They became larger and more ramified than comparator cells in a manner reminiscent of the reactive gliosis observed at damage sites in vivo [1]. Recapitulation of the astrocytic response in this simple model will enable triggers and therapeutic interventions to be investigated in a highly controlled environment providing a useful tool for future studies.

REFERENCES: [1] J.B. Phillips, V.R. King, Z. Ward et al. (2004) *Biomaterials* **25**:2769-79. [2] J.W. Fawcett, E. Housden, L. Smith-Thomas et al. (1989) *Dev Biol* **135**:449-58. [3] J.B. Phillips & B. Pearce (2005) *Inflammopharmacol* **13**:431-9.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 51) ISSN 1473-2262

Investigating the effect of photodynamic therapy on nerves using tissue engineered culture models

K E Wright[1], E Liniker[2], A J MacRobert[2], R A Brown[2], M J Saffrey[1] & J B Phillips[1]

[1]Biological Sciences Department, The Open University, Milton Keynes, U.K.
[2] National Medical Laser Centre, UCL, London, U.K.
[3]Institute of Orthopaedics and Musculoskeletal Science, UCL, London, U.K.

INTRODUCTION: Photodynamic therapy (PDT) shows potential as an effective treatment for prostate cancer [1]. Clinical observations indicate that this approach causes fewer nerve damage related side effects than conventional treatments. The aim here is to investigate the effect of PDT on nerve tissue using engineered 3-dimensional cell culture models. Initial experiments focussed on establishing photosensitiser localisation in neurones and Schwann cells, then developing a model for simulating nerve PDT in culture.

METHODS: Neurones and Schwann cells were cultured from the dorsal root ganglia and sciatic nerves of 200g rats. Tissues were dissociated using collagenase then seeded onto glass coverslips for localisation experiments using 4 µg/ml meso tetra hydroxyl phenyl chlorine (mTHPC) for 4 hours. Intracellular localisation of mTHPC fluorescence was visualised using fluorescence and confocal microscopy and quantified using digital image analysis. Schwann cells and neurones were identified using immunoreactivity for S100 and βIII tubulin respectively. A 3-dimensional (3D) cell culture system was developed to enable PDT to be directed against distinct parts of neurones. DRG explants were embedded within tethered aligned Schwann cell-populated type I collagen gels [2].

RESULTS: Localisation experiments revealed photosensitiser fluorescence within Schwann cells and the cell bodies of neurones, but not in neuronal cell processes (fig 1 & 2).

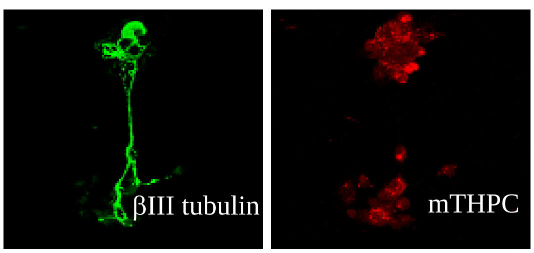


Fig. 1: Neurone in culture (left) with mTHPC localisation in cell body and underlying Schwann cells but not in neuronal cell process (right).

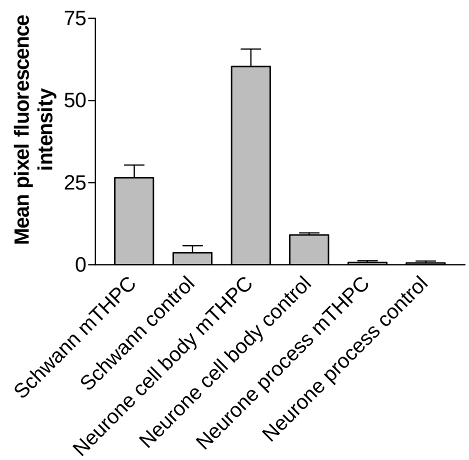


Fig 2: Quantifiaction of mTHPC localisation in neurons and Schwann cells

DRG explants embedded within aligned Schwann cell populated collagen gels extended linear neuronal processes (fig 3), forming an effective model with which to investigate PDT in distinct parts of neurones.

Fig 3: DRG explant extending processes in 3D culture model

DISCUSSION & CONCLUSIONS: This ongoing investigation into nerve sparing indicates that mTHPC may not be localised within neuronal processes. A 3D model has been created to investigate whether this protects the neuron from PDT damage when light is directed at the axon.

REFERENCES: [1] C.M. Moore, T.R. Nathan, W.R. Lees et al. (2006) *Lasers Surg Med* (in press). [2] J.B. Phillips, S.C.J. Bunting, S.M. Hall et al. (2005) *Tiss Eng* **11**:1611-17.

Design of Polyester-based Non-Porous Films and 3D Porous Scaffolds for Soft Tissue Engineering

G. Rohman[1] & J. Pettit[2] & F. Isaure[2] & N. R. Cameron[1] & J. Southgate[2]
[1] *Dept. of Chemistry, University of Durham, England*
[2] *Department of Biology, University of York, England*

INTRODUCTION: The present study reports the design of poly(lactide-*co*-glycolide) (PLGA) non-porous films and porous 3D scaffolds for soft tissue engineering using the solvent casting technique and the emulsion processing route. Different parameters such as the concentration of PLGA or the amount of water were studied to assess their influence on the properties of the films or the porous scaffolds, respectively.

METHODS: The films were prepared by casting a solution of commercial PLGA (75:25, M_w = 90,000-126,000 g.mol^{-1}, PDI = 2.8) and dichloromethane (DCM) on a microscope glass slide using the spin-coating method. The porous scaffolds were prepared by the emulsion freeze-drying technique. Briefly, the porous matrixes were prepared by mixing PLGA in DCM (17 wt/v %) and a surfactant (span 80) into a reactor equipped with a vertical steel stirrer and then adding water drop-wise. Thereafter, the emulsion was poured in a Teflon mould and placed in a freeze-dryer for 24 h.

RESULTS: The increase of PLGA concentration in the DCM solution leads to the increase of the thickness but the decrease of the film storage (E') and loss (E'') moduli (Table 1). After 7 days of incubation at 37 °C, thinner films exhibit a mass loss of 39 wt % while the mass loss of thicker films does not exceed 6 wt %. Environmental Scanning Electron Microscopy (ESEM) analysis revealed that no features were present on the film surface before or after degradation.

Table 1: Thickness and moduli of different PLGA films.

[PLGA] (wt/v %)	Thickness (µm)	E' (MPa)	E'' (MPa)
10	25	5,400	480
20	50	650	210

Due to the stability of the emulsion or the fragility of the foam, the content of water in the emulsion can not be increased above 56 %, which leads to porous structures with pore diameters smaller than 100 µm (Figure 1). After 14 days of incubation at 37 °C, the mass loss of foams does not exceed 13 wt %.

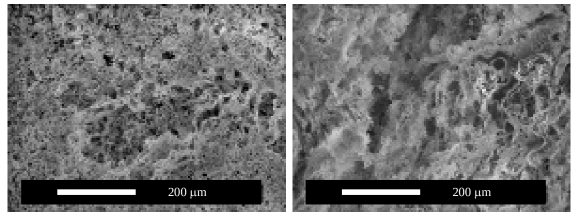


Fig. 1: ESEM micrographs of PLGA porous scaffolds obtained with a 40 % water-based emulsion: surface (left) and cross-section (right).

DISCUSSION & CONCLUSIONS: The increase in moduli with decreasing film thickness may be attributed to the increased importance of polymer microstructure [1]. The material's strength and stiffness are significant as they should ideally approach those of the tissue it is to replace [2]. At the beginning of the degradation process, the hydrolysis of thinner films is higher than the one of thicker films because thin films have a greater surface area to volume ratio and thus a greater extent of water uptake [3]. The absence of pores on the surface after degradation can be explained by the heterogeneous bulk degradation of PLGA films.

After freeze-drying, the molecular weight of the foams decreases and the polydispersity index increases up to 14.2 compared to raw PLGA, showing the degradation of the polyester during the foam process. The presence of pores at the surface of the 3D scaffold may allow the diffusion and the growth of the cells within the foams.

First results of bladder cell seeding onto the films or the 3D scaffolds show that the cells attach onto and survive on the non-porous films for at least one week. Moreover, cells were present on the surface and within the 3D scaffold structure after 7 days of incubation.

REFERENCES: [1] R.C. Thomson, G.G. Giordano, J.H. Collier, S.L. Ishaug, A.G. Mikos, D. Lahiri-Munir, C.A. Garcia (1996) *Biomaterials* **17**: 321-27. [2] D.E. Discher, P. Janmey, Y.-L. Wang (2005) *Science* **310**: 1139-43. [3] L. Lu, C.A. Garcia, A.G. Mikos (1999) *J. Biomed. Mater. Res.* **46**: 236-44.

ACKNOWLEDGEMENTS: The authors acknowledge the support of the Biotechnology and Biological Sciences Research Council (BBSRC).

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 53) ISSN 1473-2262

Bioactive Glass Fiber Reinforced Composite

H. Ylikauppila[1], L. Nikkola[1], M. Tukiainen[1], M. E. Gomes[2], R. L. Reis[2], M. Kellomäki[1], N. Ashammakhi[1]

[1]*Institute of Biomaterials, Tampere University of Technology, Tampere, Finland*
[2]*3B's Research Group, University of Minho, 4710 Braga, Portugal and Dept of Polymer Eng, Campus de Azurém, U. Minho, 4800 Guimarâes, Portugal*

INTRODUCTION: For bone regeneration and repair, combinations of different materials are often needed. In many applications it is useful to combine biodegradable polymers with osteoconductive materials. Bioactive glass (BaG) is one such material. A related issue is to improve the mechanical properties of polymer matrix by reinforcing it with BaG fiber. Thus, the aim of this work was to develop a BaG fiber reinforced starch-polycaprolactone (SPCL) composite.

METHODS: The composite was produced by extruding thick sheets from SPCL (30/70 wt%) blend. Sheets were cut and heat-pressed in layers with BaG fiber mats to form composite structures, with the following different combinations: 6xSPCL+5xBaG, 3xSPCL+6xBaG, 3xSPCL+4xBaG, 3xSPCL+2xBaG. 3xSPCL and 6xSPCL were used as non-reinforced controls. Thermal, mechanical, and degradation properties of the composite were studied. In addition, the actual amount of BaG in the composites was determined using simple burning tests.

RESULTS: A strong endothermic peak indicating melting at about 56^{o}C was observed by DSC analysis. TGA showed that thermal degradation of SPCL started at 300^{o}C with the degradation of starch and continued at 380^{o}C with the degradation of polycaprolactone (PCL). Mechanical properties of the reinforced composites were considerably better than the properties of the non-reinforced composites. Reinforcing increased shear strength by 50%, tensile strength by 52%, and bending strength by 67%. However, the mechanical properties of the composites after two weeks of hydrolysis were comparable to the properties of the non-reinforced samples. The degradation time of SPCL, as expected, was long; during the 6 weeks hydrolysis the mass only decreased by about 5%. The decrease of mass will of course occur faster at a later stage. The amount of glass in the composites remained the same for the 6 weeks period of hydrolysis.

DISCUSSION & CONCLUSION: It is possible to enhance initial mechanical properties of SPCL by reinforcing it with BaG fibers. However, mechanical properties need to be further improved for allowing long-lasting bone applications.

ACKNOWLEDGEMENTS: This work is in the framework of the European Comission Network of Excellence (EXPERTTISSUES Project).

Electrospun Starch-polycaprolactone Nano Fibers

H. Ylikauppila[1], L. Nikkola[1], M. Gomes[2], R. Reis[2], N. Ashammakhi[1]

[1] *Institute of Biomaterials, Tampere University of Technology, Tampere, Finland.*

[2] *3B´s Research Group, University of Minho, 4710 Braga, Portugal and Dept of Polymer Eng, Campus de Azurém, U. Minho, 4800 Guimarâes Portugal.*

INTRODUCTION: Starch based polymers have been widely studied for several different applications within the biomaterials field, including as scaffolds for tissue engineering. Recently electrospinning has been gaining interest as a promising method to manufacture highly porous 3D structures which provide high surface area for cell attachment and proliferation, being adequate for several uses in tissue engineering. The aim of this work is to develop nano-fiber based constructs from starch-polycaprolactone (SPCL 30/70 wt%) blends using electrospinning.

METHODS: SPCL was dissolved in acetic acid to form 14 w/v-% solution and stirred to produce an homogenous solution. About 0.1g of polymer in solution was electrospun onto substrate. The distance between needle tip and the substrate was 15 cm and the electric field was 13kV/cm. The microstructure of the obtained constructs was characterized by using scanning electron microscopy (SEM).

RESULTS: The electrospinning of SPCL produced a highly porous 3d scaffold with a typical nanofiber-mesh structure. SEM analysis revealed also the presence of starch particles (with an average size 6µm) which are interconnected by the polycaprolactone nanofibres of about 150nm. More details will be presented.

DISCUSSION & CONCLUSION: It is possible to produce highly porous nano-fibre based constructs from SPCL using electrospinning. Such constructs may have applications in tissue engineering of different tissues such as bone, skin and cartilage.

ACKNOWLEDGEMENTS: This work is in the framework of the European Comission Network of Excellence (EXPERTTISSUES Project).

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 55) ISSN 1473-2262

Drug Release from Multicomponent Implant

L. Nikkola[1], P.Viitanen[1] and N. Ashammakhi[1]

[1] *Institute of Biomaterials, Tampere University of Technology, Tampere, Finland*

INTRODUCTION: In our previous study (Viitanen et al. 2005) we have reported on developing DS releasing bioabsorbable rods. However, their drug release properties were unsatisfactory. We have thus assessed the use of sintering technique of enhancement of drug release in the current study.

METHDOS: Melt extruded PLGA 80/20 rods were compounded 8wt-%DS. Three different components were produced by self reinforcing (SR) some of compounded 8wt-%DS rods and sterilize some of the SR-rods. These three different rods were sintered with heat and pressure to form one multicomponent rod. Thermal properties were analyzed using differential scanning calorimetry (DSC) to determine glass transition temperature (Tg), melting temperature (Tm) and heat of fusion (ΔH). Drug release measurements were performed using UV-Vis spectrophotometer. There were three different specimen groups: A1 constructed from even parts of components, B1 and B2 from 47 volume-% of compounded and 32 volume-% of SR and 21%-volume of sterilised SR rods. B2 specimens were sterilized. Five parallel samples of three different specimen groups (A1, B1, and B2) were measured first at 6 hour intervals then on daily basis and later about three times a week. Mechanical strength was measured during two weeks after which the components disintegrated each other.

RESULTS: Release rate consisted of three different phases: 1) sharp start peak, 2) second smoother peak, and 3) the last smooth peak (Fig. 1). The form of the profile depended on the fractions of different components.

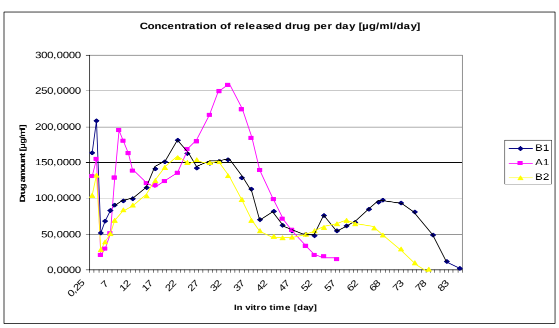


Fig. 1 Drug release profiles of the three specimen groups.

Released DS concentrations reached local therapeutic levels and maintained at that stage for 24-36 days depending on the fraction of different components. A11 DS was released from the rods during 50-70 days. Notable was also the accelerative effect of sterilization to the release.
The drug release profiles of initial components and sintered multicomponent differs from each other dramatically. It is easily seen that the drug release of multicomponent implant is more stable and begins earlier, which are the properties desired.

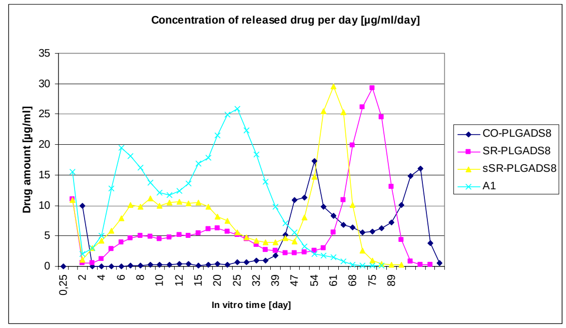


Fig 2. Comparison of drug release profiles of initial components to sintered multicomponent implant.

Initial shear strength was 82MPa and it decreased to 15MPa during two week in hydrolysis when after the components disintegrated. The mechanical attachment accomplished by sintering was sufficient although the components disintegrated too fast.

CONCLUSIONS: By sintering different PLGA/DS components, which have different release rates it is possible to construct a truly controlled release implant for bone fixation with anti-inflammatory properties.

REFERENCES: P. Viitanen, E. Suokas, P. Törmälä, N. Ashammakhi. Release of diclofenac sodium from polylactide-go-glycolide 80/20 rods. Journal of Materials Science: Materials in Medicine as permanent record of the 10th International Meeting on Polymers in Medicine & Surgery (submitted).

ACKNOWLEDGEMENTS: Research funds from the European Commission (EXPERTISSUES project NMP3-CT-2004-500283), Technology Development Center in Finland (TEKES) and the Academy of Finland (Center of Excellence 73948) are greatly appreciated.

Anti-Inflammatory drug releasing nano fibrous sheet

L. Nikkola[1], K. Vapalahti[1], J. Seppälä[2], N. Ashammakhi[1]
[1] *Institute of Biomaterials, Tampere University of Technology, Tampere, Finland*
[2] *Helsinki University of Technology, P.O. Box 6100, FIN-02015 HUT, Finland*

INTRODUCTION: The aim of this study was to develop a nanomat with the ability of diclofenac sodium releasing properties.

METHODS: A bioabsrbable polymer 1 was dissolved into a solvent. Test drug 1 was added. Nano-fibers were made by electrospinning onto substrate. Microstructure of the sheet was studied using SEM and drug release profiles with UV/VIS spectroscopy.

RESULTS: Thickness of the electrospun sheet was about 2 mm. SEM analysis showed that polymeric nano-fibers containing drug particles form very interconnected porous nano structure. The average diameter of nano-fibers was 130nm. In the beginning of drug release test a high start peak was observed. But, after this the rate was decreased. More details will be presented.

DISCUSSION & CONCLUSIONS: The nano-fibrous porous structure made of bioabsorbable polymer loaded with test drug is feasible to develop. This structure may have potential in analgesic drug release applications.

ACKNOWLEDGEMENTS: Research funds from the European Commission (EXPERTISSUES Project NMP3-CT-2004-500283), Technology Development Center in Finland (TEKES) and the Academy of Finland (Center of Excellence 73948) are greatly appreciated.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 57) ISSN 1473-2262

Nanofibres prepared using electrospinning

A. Ndreu[1], L. Nikkola [2], H. Ylikauppila[2], N. Ashammakhi[2], V. Hasirci[1]
[1] Department of Biological Sciences, Middle East Technical University, Biotechnology Research Unit, Ankara 06531, Turkey
[2] Institute of Biomaterials, Tampere University of Technology, P.O.BOX 589, 33101 Tampere, Finland

INTRODUCTION: There are a variety of manufacturing techniques used to produce fibers in nanoscale such as phase separation, drawing, template synthesis and electrospinning. Electrospinning has been shown to be more advantageous recently due to its simplicity, high porosity of the obtained structure, nanometer scale of fibers and versatility in that it can be applied to a variety of polymers. One of the most important application areas of nanofibers fabricated by electrospinning is tissue engineering[1], which deals with producing scaffolds that mimic both biological functions and structure of naturally existing extracellular matrix (ECM). However, this process has many parameters that have to be controlled in order to obtain a good structure that can enhance adhesion, proliferation and differentiation of cells. The aim of this study is to construct a nanofibrous scaffold that will play a significant role in new bone tissue regeneration.

METHODS: Biodegradable poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV8) polymer, containing 8% of hydroxyvalerate, was dissolved in different amounts of chloroform or a mixture of chloroform/ N,N-dimethyl formamide (DMF) (96:4) in order to observe the effect of solvent on fiber morphology. Moreover, the effect of presence of salt in the polymer solution was studied. Nano/micro fibers were collected onto a metal substrate in the form of an interconnected, non-woven mat. The morphology of electrospun fibers was observed by scanning electron microscopy (SEM) and their diameter was measured via an Image J analyzer program.

RESULTS: SEM analysis demonstrated that increase in polymer concentration results in an increase of fiber diameter and a change in bead formation. However, bead presence is not desirable in tissue engineering scaffolds, therefore, their formation should be prevented. It was observed that addition of salt led to the disappearance of the beads but in this case some fiber fusion occurred due to slow solvent evaporation. On the other hand, addition of a solvent with a higher dielectric constant (DMF) improved electrospinning conditions and more straight fibers without beads were obtained. This was chosen to be the best condition for the future tissue engineering studies.

DISCUSSION & CONCLUSIONS: It is possible to produce nano/micro fibers from PHBV8 polymer that can be used as a scaffold in tissue engineering applications. Conductivity of polymer solution was an important parameter in this case. The best condition for proper fiber formation was found to be 15% polymer concentration in a 96:4 (v/v) mixture of chloroform and DMF.

REFERENCES: [1] F. Yang, C. Y. Xu, M. Kotaki, S. Ramakrishna (2004) *J Biomater. Sci. Polymer Ed.* **15**:1483

ACKNOWLEDGEMENTS: Research funds from the European Commission Network of Excellence (EXPERTISSUES Project) are greatly appreciated.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 58) ISSN 1473-2262

ISOLATION AND PRELIMINARY CHARACTERISATION OF STEM CELLS FROM HUMAN DENTAL PULP

A. Bowen[1], A. English[2], E. Jones[2], S. Wood[1], J. Kirkham[1] and X.B. Yang[1]*

[1]*Depatment of Oral Biology, Leeds Dental Institute, University of Leeds, Leeds LS2 9LU.*
[2]*Academic Unit of Musculoskeletal Disease, St James Hospital, Leeds LS9 7TF.*
**E-mail: denxy@leeds.ac.uk*

INTRODUCTION: Therapies based upon cell replacement and tissue engineering, underpinned by stem cell biology, are emerging as potentially powerful strategies in modern regenerative medicine. Recently, existing concepts of cell lineage, commitment and differentiation have been challenged by the use of adult stem cells as a source of pluripotent cells. The aim of this study was to investigate the potential of using human dental pulp tissue as a source of pluripotent stem cells.

METHODS: Fifty dental pulps were extirpated from healthy permanent teeth (third molars and premolars) extracted at Leeds Dental Institute. After splitting the teeth, the pulps were removed and divided into six transverse segments in an apical-coronal direction to investigate any site-specific differences in stem cell potential. Primary cells were isolated using standard organ culture methods, establishing a total of 100 cultures. Cells were cultured in basal media or under osteogenic, chondrogenic and adipogenic conditions and assessed by enumeration of colony forming units fibroblastic (CFU-F) formation, histological staining (alkaline phosphatase - ALP, alcian blue/sirius red, von Kossa, Oil Red, Toluidine Blue), biochemical assays (ALP, DNA content, sGAG), flow cytometry and light microscopical analysis.

RESULTS: The majority of human dental pulp derived stem cells (hDPDSCs) demonstrated classic spindle shaped fibroblast-like morphology and growth characteristics. The proliferative potential of both the dental pulp derived CFU-F and primary cell cultures varied from tooth-to-tooth and from site-to-site within individual pulps. Cells derived from the middle segments of the pulp demonstrated stronger ALP positive activity compared with those derived from the more apical and coronal regions. Microscopical analyses of the hDPSDCs following culture in specialised media indicated osteogenic, chondrogenesis neural and epithelial morphologies whereas adipogenesis was low. Mineralized deposits were observed when the cells were cultures under osteogenic conditions. hDPSDCs had surface phenotype similar to human mesenchymal stem cells ($CD45^{-}CD34^{-}CD133^{-}$ $CD105^{+}CD73^{+}CD166^{+}$).

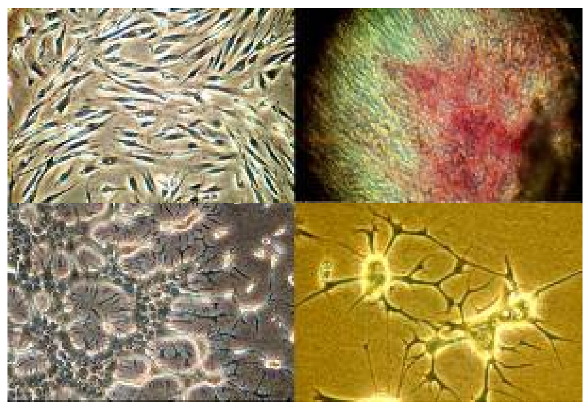


Fig. 1: Plasticity of human dental pulp stem cell.

DISCUSSION & CONCLUSIONS: We conclude that pluripotent stem cells are present within human dental pulp. The reasons for the observed site-specific differences in proliferative and osteogenic potential are unknown but they may reflect differences in the relative contributions of the different tissue compartments at these locations. This study, together with the work of others, indicates the potential for using dental pulp as a source of stem cells for future tissue replacement therapies and tissue engineering strategies.

ELECTROSPINNING OF FIBRINOGEN NANOFIBERS

Tatjana Sindelar[1], Lila Nikkola[2], Nurredin Ashammakhi[2], Martijn van Griensven[1], Heinz Redl[1]
[1] *Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, Vienna, Austria*
[2] *Institute of Biomaterials, Tampere University of Technology, Tampere, Finland*

INTRODUCTION: Electrospinning has been recognized as an efficient technique for the fabrication of polymer nanofibers. It uses an eletric field to control the deposition of polymer fibers onto a target substrate. This electrostatic processing strategy can be used to fabricate fibrous polymer mats composed of fiber diameter mostly between 100 nm and 3 µm. In this study we describe electrospinning of fibrinogen nanofibers in an attempt to create biomimicking tissue in vitro for use as a tissue scaffold.

METHODS: We have used lyophilized human fibrinogen of the product Tisseel® VH (Baxter AG, Austria) to demonstrate fibrinogen electrospinning. Fibrinogen dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol and sodium chloride solution was eletrospinned under various conditions. Electrospun fibers of fibrionogen were processed for scanning electron microscopy (SEM) evaluation and analyzed by native gelelectrophoresis.

RESULTS: The SEM evaluation showed that formed fibers were flat and had large diameter distribution from 120-1000µm resulting in approximate fiber diameter of 550µm. With some conditions bead formation occurred.

DISCUSSION AND CONCLUSION: The efficacy of this process, as well as the final fiber product, are affected by many factors, including, but not limited to solution polymer concentration, viscosity of solution, voltage between solution and ground electrode, the distance between the Taylor cone and the ground electrode, and environmental conditions such as humidity and temperature. Nano fiber similarity in size to native extracellular matrix components and the 3-dimensional structure allows cells to attach to several fibers in a more natural geometry. In summary, the electrospinning process is a simple and efficient technique for the fabrication of 3D structures composed of fibrinogen fibers.

ACKNOWLEDGEMENTS: This work is in the framework of the European Comission Network of Excellence (EXPERTTISSUES Project).

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 60) ISSN 1473-2262

The Use of Green Fluorescent Protein to Determine Chondrocyte Fate in a Sheep Model of ACI

J R Sharpe [1], AA Shetty [2] & S E James [1]

[1] *Blond McIndoe Centre, Queen Victoria Hospital, East Grinstead, W. Sussex*
[2] *Medway Maritime Hospital, Gillingham, Kent*

INTRODUCTION: The use of autologous chondrocyte implantation (ACI) is becoming increasingly widespread clinically, however a number of questions still exist as to its clinical efficacy and how implanted chondrocytes might contribute to the repair. Furthermore, little is understood about the fate of the implanted chondrocytes once the joint is closed and weight bearing motion is resumed. A recent study which looked at the fate of chondrocytes implanted within an alginate matrix into osteochondral defects in rabbits found that the number of implanted chondrocytes decreased in number with time[1].

METHODS: In our study a 5mm defect was created in the medial femoral condyle of a sheep knee. Chondrocytes isolated from cartilage harvested during this procedure were cultured *in vitro* and labelled by using a gene encoding for a green fluorescent protein (GFP) which is expressed by the transduced cells. As is the case clinically, these cultured autologous chondrocytes where implanted into the defect using a sutured periosteal graft, which was sealed with fibrin glue. At the time of re-implantation 1% of the $3.7\text{x}10^6$ cells expressed GFP. One week after ACI the animal was killed and the defect was retrieved, decalcified and freeze sectioned in entirety.

RESULTS: Histology of the retrieved tissue revealed the presence of numerous GFP positive chondrocytes at the site of the defect (Figure 1). Counting of cells showed that the total number of GFP+ cells in the MFC defect to be 695 which would equate to approximately $7\text{x}10^4$ cells in total. This indicates that approximately 2% of the cells initially injected below the periosteum were retained with 98% being lost into the synovial capsule. No GFP positive cells could be found in any of the samples of synovial fluid, synovium or meniscus.

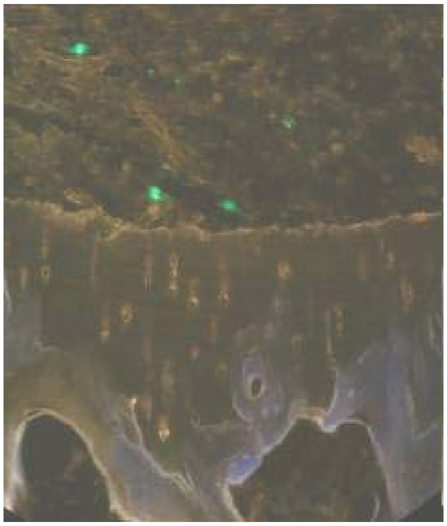


Fig. 1: Detection of GFP+ cells in defect.

DISCUSSION & CONCLUSIONS: This model was designed to determine what proportion of implanted chondrocytes remain at the defect site following ACI. Following wound closure and resumption of the stresses that occur between the femoral condyles and the tibial plateau it seems inevitable that when chondrocytes are implanted below a periosteal flap as in ACI some are lost into the surrounding synovial capsule. The fact that only 2% of the implanted chondrocytes injected could be detected does indeed indicate that the vast majority are lost into the synovial fluid. This experiment does show categorically however that implanted chondrocytes persist at the implant site for at least one week. Further work currently underway will examine the longer term fate and contribution of GFP labelled chondrocytes to cartilage repair.

REFERENCES: 1. Mierisch *et al.* JBJS [Am]2003;85-A:1757-67

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 61) ISSN 1473-2262

A Pilot Study of In-Vitro Gingival Fibroblast Differentiation in Spheroid Culture

Z. Berahim, A.K. Jowett & A. Rawlinson

School of Clinical Dentistry, University of Sheffield, England.

INTRODUCTION: Periodontal diseases are a group of related inflammatory conditions affecting the tooth supporting tissues. Currently available treatments aimed at the regeneration of lost tooth support have limited clinical success, and there is a need to develop further methods. However, the regeneration of periodontal tissue is a complex process involving multiple cells and extra cellular matrix. Traditional two dimensional models for cell culture have limited application in this area of research. Spheroid culture is a form of three dimensional cells culture that promotes cells matrix interaction which could recapitulate the aspect of cell homeostasis in vivo[1]. While this technique has been widely used in cancer research, it has not been used to date in order to evaluate the application to in-vitro research on periodontal tissue regeneration.

METHODS: Normal cell lines of gingival fibroblasts were seeded in tissue culture flasks until confluent. Then they were trypsinized and cultured in multi well plates by liquid overlay technique. Each plate was divided into three sections, each section containing a different concentration of cells; 25 000, 50 000, 100 000 cells. The spheroids were fixed and processed for histological examination at interval of 2, 14 and 30 days.

RESULTS: Gingival fibroblasts formed spheroids in a period between 12 and 48 hours. After 2 weeks an inner dead cell zone, an outer live cell zone and a mixed cell zone could be observed in spheroids of different ages and different cell number. No fibril formation can be detected from the histological sections. Alcian blue staining showed the present of glycosaminoglycan at the centre of spheroids at 14 and 30 days.

DISCUSSION & CONCLUSIONS: This study concluded that cell line gingival fibroblasts can be grown consistently in a spheroid form. However the cells appeared to differentiate along a chondrogenic rather than fibrous connective tissue lineage. This result may be used for further investigation of gingival fibroblast cells in this 3-dimensional model.

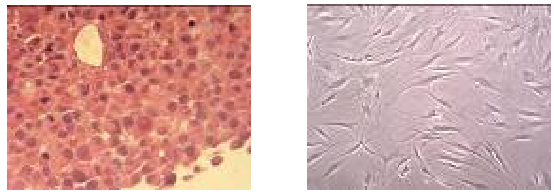


Figure 1: Section of gingival fibroblast in spheroid culture (left) versus gingival fibroblast in 2-dimensional culture (right).

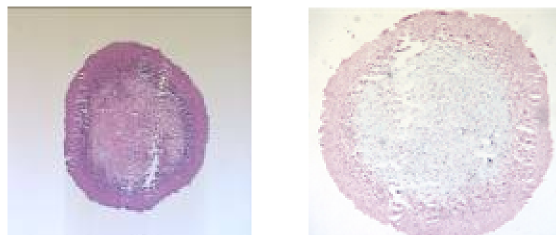


Figure 2: Gingival fibroblast stained with H&E showed three different zones (left) and Alcian Blue showed glycosaminoglycan (right).

REFERENCES: [1]Carlsson J and Yuhas JM (1984). *Liquid overlay culture of cellular spheroids.* In: Spheroid in Cancer Research, Springer-Verlag Berlin.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 62) ISSN 1473-2262

Engineered & Chemically Modified Porous Cellulose Fibrous Networks For Controlled Cell Adhesion

D. M. Kalaskar, J. E. Gough, R. V. Ulijn, W.W. Sampson, S. J. Eichhorn*
School of Materials, University of Manchester, Grosvenor Street, Manchester, M17HS, UK

INTRODUCTION: This study uses an interdisciplinary approach to investigate the potential of cellulose based fibrous scaffolds for controlled mammalian cell attachment and proliferation. Fibrous networks can be made with varying porosity and mass per unit area. Interconnectivity between pores can be tailored to manipulate the distance between pores to provide the opportunity for cells to bridge between them. Even though cellulose has been used as material for tissue engineering, a complete study of fibrous networks for this purpose in terms of geometry and surface chemistry has not been addressed to date. Thus an attempt has been made to enhance the inherent properties of cellulose fibre surfaces.
Furthermore, the potential this of this technology as a high throughput process for tissue engineering scaffolds is discussed as a standard papermaking processes can be used to generate these structures.

METHODS: By introducing charge on a scaffold material cell adhesion is improved. Glycine residues are coupled to the hydroxyl surface of cellulose to introduce charge, showing increased cell growth. Similarly glycine residues were coupled as Fmoc-Gly via carboxylate termini using a conventional ester coupling method followed by removal of Fmoc in piperidine to expose amino groups. In present study 3T3 Murine Fibroblast cell were used. Cell morphology was studied by using Scanning Electro Microscopy. Cell penetration studies were done by using confocal laser scanning Microscopy.

RESULTS: Chemical modification is achieved by using glycine and 9-fluorenylmethoxycarbonyl (Fmoc) protected glycine, using a conventional ester coupling method. Deprotection of Fmoc is carried out by using 20% piperidine to expose glycine. The use of ToF-SIMS (Time of flight Secondary ion mass spectrometry) has confirmed the presence of both species. Fibroblast cells are cultured onto the scaffolds, and cell attachment and proliferation are assessed by using scanning electron microscopy and confocal laser scanning microscopy. It is shown that glycine treatment favours cell attachment on the surface of the fibres, and Fmoc treatment the aggregation of cells in the porous structure of the networks.

DISCUSSION & CONCLUSION: In present study, amino acids are use to modify fibrous cellulose scaffolds, where capability of Fmoc (flurenylmethoxycarbonyl) functionality to render hydrophobic surface was achieved successfully. The ability to 'switch-off' the 'surface activity' of the cellulose fibres using Fmoc-proteced glycine, so that cells aggregate within the pores. Similarly, by simple chemical modification to deprotect Fmoc, surface can be activated with glycine for cellular proliferation. The modification is shown to be capable of binding protein to cellulose surfaces without resource to complex chemistry.

(a) **(b)** **(c)**

Fig. 1: Scanning Electron micrographs of cell attachment to a cellulose scaffold. (a) untreated cellulose, (b) cellulose sample modified with Fmoc protected glycine, (c) glycine modified cellulose sample

FUTURE WORK: Cell binding can also be improved by introducing different functionalities such as aliphatic, aromatic, polar, basic, acid groups. In addition the effect of conventional functional peptides such as RGD can also be tried in future with cellulose for improving cell adhesion .

REFERENCES: [1]Wan, J .L., Laurencin, C. T., Caterson,E.J., Tuan,R.S., Ko., F. K. 2002. J Biomed Mater Res, 60, 613-621. [2]Entcheva, E., Biena, H.,Yina, L., Chiung,Y. C., Farrella, M., Kostovc, Y., 2004. Biomaterials, 25, 5753–5762

ACKNOWLEDGMENT: This work is being funded by the ORS, EPSRC and The University of Manchester.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 63) ISSN 1473-2262

Modelling the Periodontal Defect for Drug Delivery & Regenerative Medicine Research

F. Mirvakily*, A. Rawlinson P.V. Hatton & K. Hurrell-Gilingham

Centre for Biomaterial and Tissue Engineering, School of Clinical Dentistry, University of Sheffield, S10 2TA, UK

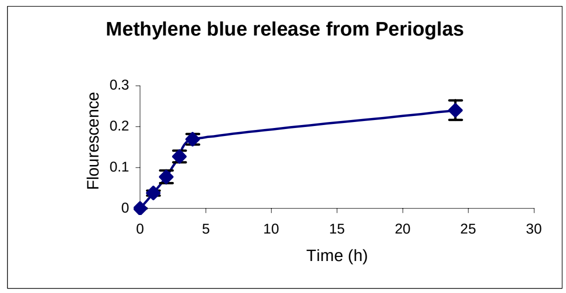
INTRODUCTION: Periodontitis is a common cause of tooth loss in adults. This is due to the destruction of the supporting tissues of teeth including alveolar bone and connective tissues. Currently available treatments are based on surgical approaches such as open flap techniques or guided tissue regeneration. These are effective in some cases, but evidence for substantial bone regeneration is limited. Bioactive materials including bioglasses, bioglass-ceramics, and calcium phosphate ceramics have been reported for the reconstruction of bones and tissues. *In vivo* data are promising, but this is not always reflected by improved clinical outcomes following treatments.

The aim of this research is to investigate new regenerative therapies that improve upon the currently available treatments for periodontitis. There are however, no sound models of periodontal defects for the evaluation of drug release or cell-based therapies. Therefore the objective of this study was to develop an *in vitro* model and perform pilot drug delivery experiment using potential bone substitutes and scaffold biomaterials.

METHODS: We designed a model of a bone defect 3 mm in depth and 4 mm diameter using cylindrical acrylic moulds based on data from published clinical studies[1, 2]. *In vitro* drug release from 45S5 bioglass® was investigated using methylene blue dye. 45S5 bioglass® was melt-derived, milled and sieved to obtain particle sizes of (150-425 μm) comparable to commercial Perioglas. The composition was confirmed using X-ray fluorescence spectroscopy (XRF), and the detailed morphology was characterized by scanning electron microscopy (SEM).

RESULTS: Dye release from the bioglass was greatest at early time points, indicating that a "surface burst" occurred before sustained release of much lower level but very slow levels of dye release.

DISCUSSION & CONCLUSIONS: We concluded that the model reported here has great promise for periodontal drug release studies, and it is therefore a significant advance on current approaches based on simple elution from a disc or tablet. Further work will explore cell and tissue engineering approaches.



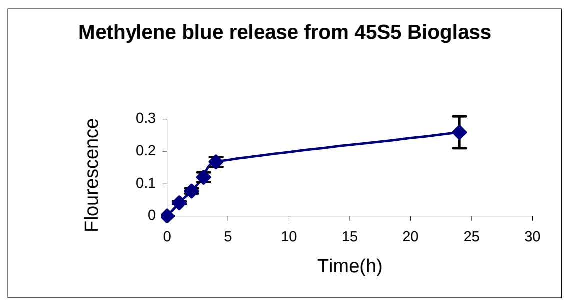


Fig. 1: Comparison of methylene blue release from perioglas (top) and 45S5 bioglass® (bottom)

REFERENCES: [1]Young SJ, Chaibi MS et al (1996) **67**(8): 763-9. [2] Becker.W, Becker.BE, Berg L, Samsam C (1986) **57**(5): 277-85

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 64) ISSN 1473-2262

A self-assembling peptide-based scaffold to support cell growth

Gribbon, C.,[1] Banwell, E.F.,[1] Ryadnov, M.G.,[1] & Woolfson, D.N.[1,2]
[1]*School of Chemistry, University of Bristol, UK.*
& [2]Department of Biochemistry, School of Medical Sciences, University of Bristol, UK.

INTRODUCTION: For tissue engineering (TE) purposes, self-assembling scaffolds are desirable. Many current strategies for such scaffolds concentrate on carbohydrate-based or peptidic materials; several groups are pursuing the construction and use of β-amyloid-like structures in the area[1]. By far the most abundant natural protein-assembly motif, however, is the α-helical coiled coil. Indeed, many of the proteins of the extracellular matrix (ECM) have coiled coils (laminins, matrillins, thrombospondins, fibrinogen/fibrin)[2]. Sequence-to-structure "rules" for the folding and assembly of coiled coils are now available, and this understanding permits the rational design of coiled-coil peptides and structures. One such design from our own laboratory is the SAF (**S**elf-**A**ssembling **F**ibrillar) system[3]. This comprises two complementary peptides that, when mixed, combine to form a "sticky ended" building block for fibres. The resulting structures are tens of nanometres thick and tens of microns long. One obstacle to using the SAFs in TE is stability. The work described here addresses the issue of assembling the SAFs in physiological conditions of pH, temperature and salt.

METHODS: Natural coiled-coil assemblies are "blunt-ended"; that is the helices associate side-to-side in register. To promote end-to-end association in the SAF system, the two SAF peptides were design to assemble offset, leaving complementary "sticky ends", Fig. 1. We make the SAF peptides by peptide synthesis, and characterise them using a combination of spectroscopy and microscopy.

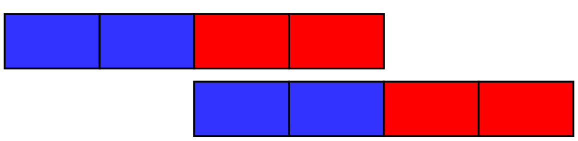


Fig. 1: Basic principle of "sticky ended" assembly, where red and blue blocks represent oppositely charged regions of peptide sequence.

RESULTS: A typical transmission electron micrograph for a SAF preparation is shown in Fig 2. We used this method to assay the assembly of several iterations of SAF designs engineered for increasing stability. The third-generation design both folds into α-helical structures and assembles into supramolecular fibres under physiological conditions and in cell-culture media. We are currently testing these fibres in culture with cells.

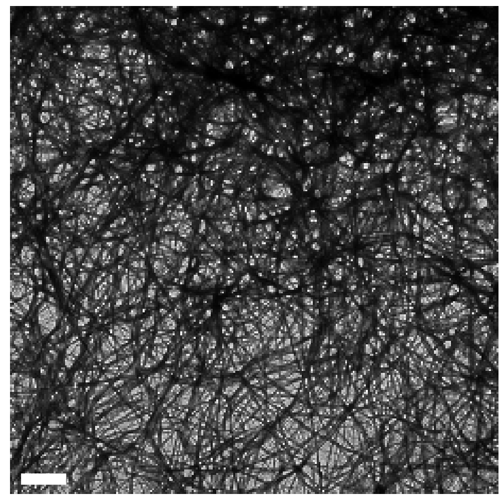


Fig. 2: Electron micrograph of SAFs in phosphate-buffered saline at 37°C. Scalebar 2μm.

DISCUSSION & CONCLUSIONS: The ECM is composed of a complex of proteins and carbohydrates. Many current scaffolds for 3D cell culture use polymers and carbohydrates as scaffold material. Peptide-based strategies for making scaffolds include β-sheet based peptides[1], which, whilst robust and straightforward to construct, have the disadvantage of being linked to amyloid disorders. The SAF system presents an alternative candidate for peptide-based scaffolds. In addition to straightforward fibres, it is possible to design additional features, such as branching and networks of fibres, and to decorate them with recognition motifs[4]. Thus, systems like this are becoming real alternative to synthetic polymers and ex vivo scaffolds.

REFERENCES: [1] Zhang, S., et al. (2002). *Curr. Op. Chem. Biol.* **6**: 865-871. [2] Engel, J. (2004) *Internat. J. Biochem. & Cell Biol*. **36**: 997-1004. [3] Pandya, M.J., et al. (2000). *Biochemistry* **39**: 8728-8734. [4] Ryadnov, M.G & Woolfson, D.N. (2004) *JACS* **126**: 7454-7455.

ACKNOWLEDGEMENTS: This work is supported by the BBSRC and the MRC.

Generation of Thymus Cell Aggregates Using an *In Vitro* Co-Culture System

M. J. Tomlinson[1], L. D. K. Buttery[1], D. I. Pritchard[2] & K. M. Shakesheff[1]

[1] *Tissue Engineering Group, School of Pharmacy, The University of Nottingham, Nottingham, NG7 2RD, U.K.*

[2] *Infection and Immunity Group, School of Pharmacy, The University of Nottingham, Nottingham, NG7 2RD, U.K.*

INTRODUCTION: The thymus is a multicellular organ which acts as the central site for T cell lymphopoiesis from haematopoietic stem cell precursors, transformed via a series of interactions and gene rearrangements into thymocytes [1]. These T cells have a large array of reactivity to foreign antigens and form the basis of immunity. Depletion of the circulating T cell population is the causative factor of Acquired Immunodeficiency Syndrome (AIDS) and is caused by the Human Immunodeficiency Virus (HIV) binding to and destroying T helper cells via the CD4 receptor [2]. Formation of a controllable *in vitro* thymic system for the generation of T cells could allow for the immune reconstitution of carriers of this virus.

Therefore the aim of the current study is to generate aggregates of thymus cells using co-culture systems.

METHODS: Thymuses were isolated from 8 week old CD1 mice before being digested with trypsin/EDTA for 30 minutes at 37°C. The resulting cell suspension was filtered through a 70µm cell strainer (BD Biosciences) before incubation with Calcein AM / Ethidium Homodimer-1 (Molecular Probes). Thymus cells were then incubated with either mitomycin C incubated SNL fibroblasts or on tissue culture plastic with no other cells present using RPMI-1640 media supplemented with 10% FCS on orbital shakers (60rpm, 2 cm radius) at 37°C. Cells were maintained for 3 weeks and images were taken at 2 day timepoints

RESULTS: Figure 1A shows that at day 0 thymus cells in SNL fibroblast co-culture are in a single cell suspension, but figure 1B shows that at day 6 they formed large, approximately 400µm aggregates. Figure 1C shows thymus cells grown in monoculture at day 0 again the cells are in a single cell suspension but figure 1D shows that at day 6 the cells have only formed small aggregates of approximately 100µm.

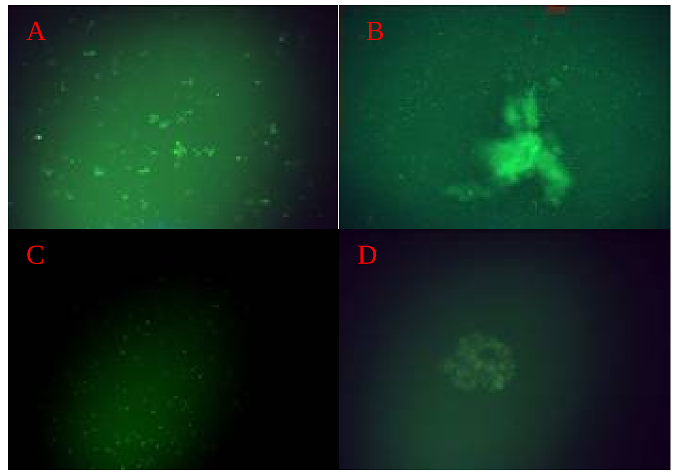


Fig. 1: Calcein AM stained thymus cell isolates cultured on SNL fibroblasts at 0 days (A) and 6 days (B; x10 magnification) and thymus cell isolates in monoculture at 0 days (C) and 6 days (D; x20 magnification).

DISCUSSION & CONCLUSIONS: From these observations it can be seen that thymus cells grown in a co-culture system with SNL fibroblasts more readily form large aggregates of cells. This is possibly due to signals received from the fibroblasts, mimicking the *in vivo* environment. Whether these aggregates are formed due to increased cell binding or proliferation is currently being investigated. Once the system for generating these aggregates is optimised they could then be used for further investigation.

REFERENCES: [1] Anderson, G., Moore, N.C., Owen, J.J.T. and Jenkinson, E.J., (1996). *Annu. Rev. Immunol.* **14**: 73–99. [2] Dalgleish, A.G., Beverley, P.C., Clapham, P.R., Crawford, D.H., Greaves, M.F. and Weiss, R.A., (1984). *Nature* **312**: 763-7.

ACKNOWLEDGEMENTS: This work was funded by the BBSRC.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 66) ISSN 1473-2262

Development of Poly(N-vinylpyrrolidinone) hydrogels for treatment of skin graft contracture

L.E. Smith[1], S. Rimmer[2] & S. Mac Neil[1]

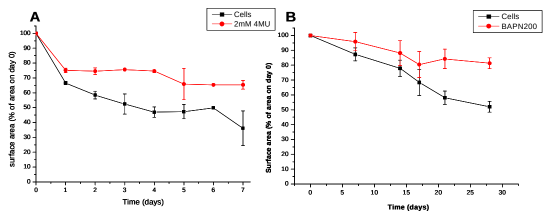
[1] *Kroto Research Institute, North Campus, Broad Lane, University of Sheffield, Sheffield, South Yorkshire, S3 7HQ, UK.*

[2] *Polymer and Biomaterial Laboratories, Chemistry Department, Brook Hill, University of Sheffield, Sheffield, South Yorkshire, S3 7HF, UK*

INTRODUCTION: Skin graft contracture is a major post healing complication associated with burns treatment. The aim of this project was to develop and characterize a hydrogel wound dressing that could be used to deliver potential anti-contraction agents. Poly(N-vinylpyrrolidinone) (PNVP) was chosen to form the hydrogel due to its long history in biomedical applications. Two different crosslinking agents, ethylene glycol dimethacrylate (EGDMA) and diethylene glycol bisallylcarbonate (DEGBAC) were used to produce two different hydrogels with very different material properties. The agents selected to reduce contraction were β-aminopropionitrile (βAPN) , a competitive lysyl oxidase inhibitor which has been shown to significantly reduce contraction of a human reconstructed skin model and 4-methyl umbelliferone, a hyaluronan synthase inhibitor.

METHODS: For details of hydrogel synthesis, material characterisation and cell culture see [1]. **Contraction models.** Collagen gels are formed from a stock solution of 5mg/ml rat tail collagen I in 0.1M Acetic acid to a final concentration of 1.5mg/ml in standard Greens medium and cast into a 24 well plate. Gels were cast containing 12,500 fibroblasts with 37,500 keratinocytes in 20µl medium added to the culture system after the gels had set. After 1 hour the gels were released from the sides of the well and 1ml of cell culture medium added. The reconstructed skin model was formed from seeding de-epithelialised acellular donor dermis with cultured fibroblasts and keratinocytes and was cultured submerged for two days. After two days the seeded area was excised and raised to an air liquid interface. At this point treatment with βAPN and 4MU commenced. For both models contraction was measured using image analysis at regular time points.

RESULTS AND DISCUSSION: The two different crosslinkers used enabled us to produce two classes of polymer network with different material properties. Culture of fibroblasts in indirect contact with the hydrogels showed them to be non-cytotoxic and even stimulatory to cell viability and this effect was not altered by the presence of fetal calf serum in the culture system. [1]. 4-MU decreased contraction in both the collagen gels (Figure 1A) and the reconstructed



skin model however βAPN was found to decrease contraction only in the reconstructed skin model (Figure 1B).

Fig. 1: Effect of 4-MU on contraction of collagen I gels (**A**) and effect of βAPN on contraction of reconstructed skin (**B**)

Preliminary data for the release of 4MU from P(NVP-co-DEGBAC) showed that after an initial burst the release rate dropped to negligible levels. Hydrogels could provide release of potential anti-contraction agents for up to 48 hours.

CONCLUSIONS: Results to date suggest that this hydrogel can successfully take up and release these two agents which have been shown to moderate contraction in both models. The challenge now will be to evaluate the drug releasing hydrogels against both contraction models to develop an approach for topical clinical use following skin grafting.

REFERENCES: [1] L.E. Smith, S. Rimmer, S. Mac Neil, (2006) *Biomaterials* **27**: 2806-2812.

ACKNOWLEDGEMENTS: We would like to thank the EPSRC for a doctoral training award for L. Smith.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 67) ISSN 1473-2262

Development of an *in vivo*-like *in vitro* Intestinal Epithelial Model

S.Buck[1], LD Buttery[1], C Hawkey[2] and FRAJ Rose[1]

[1]*Tissue Engineering Group, School of Pharmacy, The University of Nottingham, NG7 2RD, U.K*

[2]*Wolfson Digestive Diseases Centre, School of Medical and Surgical Sciences, QMC, Nottingham, NG7 2RD, U.K.*

INTRODUCTION: The gastrointestinal tract is one of the most important and convenient routes for drug administration. However current models for drug testing, mostly based around the use of established cell lines, are unrealistic of the multicellular structure of the intestine *in vivo.* According to some previous work with the intestinal epithelial cell line HCA-7, transepithelial resistance (TER) is increased in co-culture growth with intestinal myofibroblasts[1]. Here we investigated changes in TER over an extended period of time and assessed the affect of co-culture growth at the gene expression level. A further intestinal cell line, Caco-2, was also investigated for its potential in the system. Additional primary intestinal cells were isolated from mice and the growth of these in co-culture was assessed.

METHODS: Isolation of myofibroblasts: Colonies of human myofibroblasts were established from human lamina propria cultured for 4 weeks[2]. **Cell Lines:** HCA-7 and Caco-2 cells were maintained in culture in 10% FCS-DMEM. **Isolation of Primary Intestinal Cells:** Colons were excised from adult male mice. Luminal contents were flushed out using HBSS and the tissue was cut into 2mm^2 segments[3]. Collagenase-dispase digestion followed by a sedimentation procedure was used to produce a pure crypt preparation. **Co-culture:** Co-cultures were established on poly(ethylene terephthalate) filter supports. Myofibroblasts were trypsinised and resuspended in 10% FCS-DMEM. This cell suspension was seeded onto the inverted filter insert and allowed to adhere for 24 hours[3]. The filters were turned and grown in wells containing 10% FCS-DMEM. Epithelial cells or primary intestinal cells were seeded on the opposite side of the filter at a density of $3x10^5$ cells/ml. **TER Measurements:** An Epithelial Voltohmeter (World Precision Instruments) was used to measure TER across the membrane. **DNA Microarray:** Microarray analysis was carried out using epithelial RNA following co-culture.

RESULTS: Co-cultures of epithelial cell lines and intestinal myofibroblasts showed an enhanced TER by comparison with those in monoculture. Intact crypts were successfully isolated from the intestinal tissue over a period of 1 – 2 hours of digestion. Spreading of the crypts in this system was observed. Cell ‘clusters’ rather than crypts were present after only 15 hours of culture. These were then maintained for periods of up to 3 weeks. Initial studies suggest that there is an interaction between the two cell types present in the co-culture.

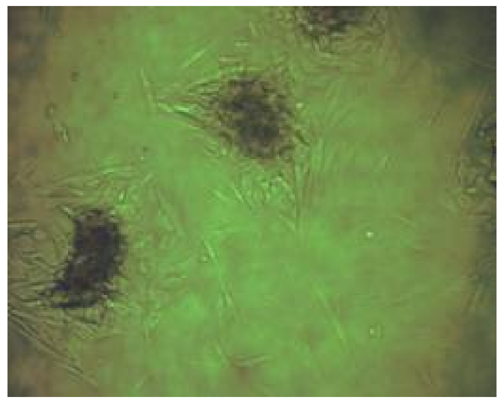


Fig. 1: Growth of intestinal crypts in co-culture with human myofibroblasts

DISCUSSION & CONCLUSION: The co-culture of primary intestinal crypts with intestinal myofibroblasts represents a novel way of recreating the intestinal environment *in vitro.* This system can be expanded upon in the future to create a 3D model of the intestine that can be used for pharmaceutical and potentially even medical benefits.

REFERENCES: [1] J. Beltinger, B.C. McKaig, S. Makh, W.A. Stack *et al* (1999) *Am. J Physiol.* **277**: 271 – 279 [2] Y.R. Mahida, J.Beltinger, S. Makh, M. Goke *et al* (1997) *Am. J. Physiol.* **273**: G1341 – G1348 [3] C. Booth, S.Patel, G.R. Bennion and C.S. Potten (1995) *Epith Cell Biol.* **4**: 76 – 86

ACKNOWLEDGEMENTS: Michelle Jackson, Dr Paddy Tighe, Dept. of Immunology, QMC, Histopathology QMC, Biomedical Services Unit, QMC.

TISSUE ENGINEERING OF HYPERTROPHIC CARTILAGE FOR BONE REPAIR

A. Kwarciak, A. Crawford, I. Brook, P.V Hatton

Centre for Biomaterials and Tissue Engineering, School of Clinical Dentistry University of Sheffield, Claremont Crescent - S10 2TA Sheffield

INTRODUCTION: An alternative approach to bone tissue engineering may be to find a suitable cell source to engineer a cartilage-based construct (e.g. hypertrophic cartilage) that can mineralise *in vitro* or *in vivo*. Cartilage has the advantage that it can survive in a relatively hypoxic environment, which may allow more time for vascularisation of the engineered graft to develop post-implantation. The aim of this research was to investigate the conditions that promote generation of a cartilage construct with characteristics of hypertrophic tissue

METHODS: Chondrocytes isolated from sternum, articular and nasal cartilage were expanded in monolayer and then cultured as pellets under chondrogenic (DMEM with 10% FCS, 1μg/ml insulin, 50 μg/ml L-ascorbic acid) and osteogenic (chondrogenic medium with 10mM B-glycerophosphate and 10^{-8}M dexamethasone) conditions for 28 days. The stage of their differentiation was evaluated by immunolocalisation of collagens, histochemical detection of alkaline phosphatase and biochemical analysis of glycosaminoglycans content. Development of collagen X assay is in progress.

RESULTS: Preliminary data shows that successful process of chondrocytes re-differentiation can occur using this culture system with cells of different passages (bovine nasal P_1 chondrocytes pellet, see Fig.1). Toluidine blue staining demonstrated the presence of sulfated glycosaminoglycans in the extracellular matrix. Immunohistochemical analysis of pellets revealed little staining for collagen type I and strong for collagen type II. Alkaline phosphatase activity detected in a limited proportion of the samples (mainly from cells at early passages, see Fig.1 D) was indicative of chondrocytes entering the hypertrophic stage. No calcium deposit was detected.

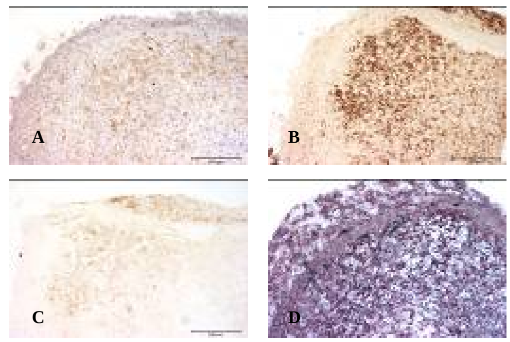


Fig. 1: Bovine nasal chondrocytes pellet, cultured under standard chondrogenic conditions; (A) collagen I; (B) collagen II; (C) alkaline phosphatase

DISCUSSION & CONCLUSIONS: In conclusion, chondrocytes can be induced to express alkaline phosphatase suggesting that they follow the process of hypertrophic differentiation and therefore show some potential for use in bone tissue repair. Ongoing work is directed at determining culture conditions to accelerate process of chondrogenic and osteogenic differentiation

REFERENCES: Kato Y, I.M., Koike T, Suzuki F, Takano Y (1988). “Terminal differentiation and calcification in rabbit chondrocyte cultures grown in centrifuge tubes: Regulation by transforming growth factor/8 and serum factors.” Cell Biology 85: 9552-9556.

ACKNOWLEDGEMENTS: The authors are grateful to the European commission (Marie Curie Alea Jacta EST, contract MEST-CT-2004-008104) for funding. The work was performed as part of the EXPERTISSUES Network of Excellence.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 69) ISSN 1473-2262

Optimizing Degradable Scaffolds for Cranium Tissue Repair

H M Aydin[1], Y Yang[2] E Piskin[1] & A El Haj[2]

[1] *Chemical Engineering Department and Bioengineering Division, Hacettepe University and TUBITAK, Centre of Excellence: Biyomuh, Turkey*

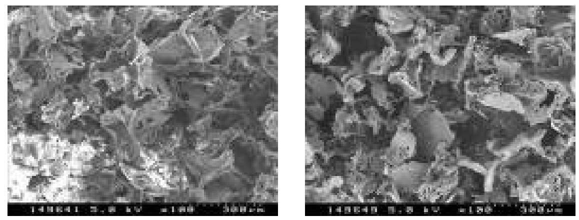
[2] *Institute in Science and Technology in Medicine, Keele University, England.*

INTRODUCTION: The issue of repairing deformities in the craniofacial area continues to be a challenging one for reconstructive surgeons. To date many methods and materials have been used to obtain a waterproof seal of bone defects in the cranium [1]. Various methods have been used for the preparation of porous polymeric structures for biomedical applications [2]. The aim of this study is to develop a new scaffold formation method for cranium tissue repair, so that the scaffolds with high mechanical properties and high pore interconnection can be obtained.

METHODS: Medical grade poly(lactic acid) (PURASORB™) (PLA) was used to generate scaffolds by using two porogens. NaCl was used to control macro-pore size as to 100 to 250 μm; whilst naphthalene was utilized as a porogen to increase pore interconnections. After the salt was leached out by water, naphthalene was removed by tetrahydrofuran (THF) and this step followed by the removal of THF by ethanol. The morphology of the scaffolds manufactured with and without naphthalene was observed by SEM. MG63 osteoblastic cells were seeded in the scaffolds with the density of $1x10^6$ cells per scaffold. Cell attachment and viability were investigated by live/dead fluorescent dye kit and observed in confocal microscopy.

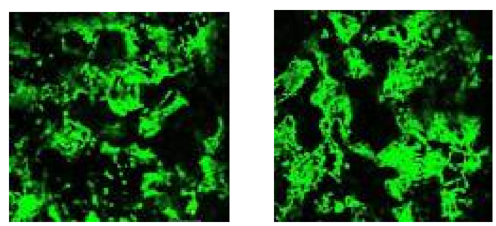
RESULTS: Introducing naphthalene as the second porogen in solvent-casting and salt-leaching process resulted in improvement of the pore interconnectivity. By using 20% naphthalene, highly interconnected pore structures achieved. SEM images of the scaffolds prepared with and without naphthalene are given in Figure 1. Extra small pores presented in the pore wall were visualized in the scaffolds produced with naphthalene (Fig1 b). Both cell-scaffold constructs made with and without naphthalene exhibited strong green fluorescence (Figure 2), indicating that the cells were alive and healthy, and the naphthalene in the scaffold process did not affect cell viability.

DISCUSSION & CONCLUSIONS: Application of two porogens may produce mechanically strong porous scaffolds for cranium repair by using small NaCl particulates. The nutrient diffusion efficiency can be compensated by the smaller pores generated by the second porogen, naphthalene, which enhance the pore interconnection.



(a) *(b)*

Fig. 1: SEM images of scaffolds prepared by solvent-casting and salt-leaching technique. (a) without naphthalene; (b) with naphthalene



(a) *(b)*

Fig. 2: Comfocal microscopic images staining with live/dead fluorescent kit. (a) without naphthalene; (b) with naphthalene

REFERENCES:[1] S. Velayudhan, T. V. Anilkumar, T. V. Kumary, P. V. Mohanan, A. C. Fernandez, H. K. Varma, P. Ramesh (2005) *Biological Evaluation of Pliable Hydroxyapatite-ethylene Vinyl Acetate Copolymer Composites Intended for Cranioplasty*. Acta Biomaterialia [2] Q. Hou, D. W. Grijpma, J. Feijen (2003) *Porous Polymeric Structures for Tissue Engineering by a Coagulation, Compression Moulding and Sal Leaching* Technique, Biomaterials.

ACKNOWLEDGEMENTS: EXPERTISSUES Network of Excellence (NoE)–NMP3-CT-2004-500283

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 70) ISSN 1473-2262

Repair of a Wounded Heart; An Investigation of Cardiomyocyte Differentiation of Embryonic Stem Cells.

M. R. R. Self, L.D. Buttery & K. M. Shakesheff

Tissue Engineering Group, School of Pharmacy, Nottingham University, England.

INTRODUCTION: Repair of heart tissue after myocardial infarction is complicated by the cells lack of regenerative capability and the need for constant activity. Insertion of a patch of ex-vivo cultured cardiomyocytes into the area of damage has shown promise, however the lack of suitable cardiomyocytes is problematic. It is foreseeable that differentiated embryonic stem cells (ESC) could be used to supply cardiomyocytes. Differentiation requires an aggregation stage, therefore a variety of methods of aggregation of stem cells for differentiation were investigated.

METHODS: Mouse ES14 cells were grown on a feeder layer of mouse SNL cells and maintained using DMEM supplemented with 10% FCS, 1% Penicillin/Streptomycin, 1mM L-Glutamine and 0.1mM Mercaptoethanol. Once 80% confluency was attained the cells were trypsin dissociated and differentiated by aggregation in one of the following ways; **Mass suspension:** cells were aggregated in 2 ml of media in a 6 well non-tissue culture treated plate. **Hanging droplet:** cells were aggregated in droplets of 25µl on the lid of a 24 well plate (each well contained 700 µl of PBS to prevent drying; using a method modified from ref [1]). **Microtube:** cells were aggregated by settling in 1.5 ml micro-centrifuge tubes [2]. Aggregation was performed at cell concentrations of 10,000 25,000, 100,000, 250,000, 1,000,000 and 2,500,000 cells per ml, with and without enhanced chemical binding of the cells using sodium periodate, biotin hydrazide and avidin [3].

After 2 days aggregation cells were transferred to non-tissue culture treated plates for a 5 days. They were then transferred to tissue culture treated plates and allowed to settle out and differentiate. The cells were grown for a further period of 19 days and viewed daily for areas of beating cells, using a microscope. Beating areas were measured using an estimate of the generation of beating areas, where zero indicates no beating areas, 1 is one small area of beating cells, 3 a few small areas or one medium sized area, and 5 either 1 very large area or many smaller areas of beating cells that would in total cover up to half of a well of a 6 well plate.

RESULTS: Beating cells were derived using all methods of aggregation; however the number and size of these differed considerably from method to method. Figure 1 shows the beating area for the different methods with and without biotin aggregation at 100,000 cells per ml. Overall, from 10,000 to 25,000 cells per ml typically generated small areas of beating cells, from 100,000 to 250,000 cells per ml large areas and/or high numbers of beating areas were seen. Above 1 million cells per ml few beating areas were seen.

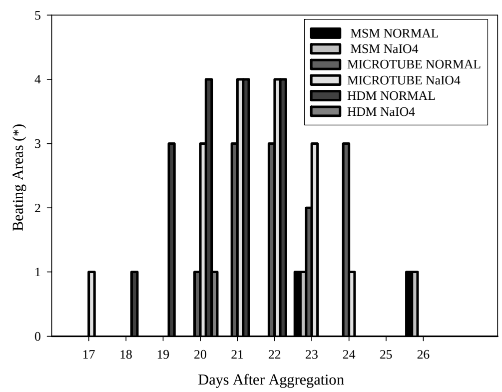


Figure 1: *ES14 cells aggregated at 100,000 cells per ml, using mass suspension (MSM), micro-centrifuge tubes (MICROTUBE) or hanging droplets (HDM) either treated with sodium periodate (NaIO4) or left untreated (NORMAL). The beating area scale is described in the Method.*

DISCUSSION & CONCLUSIONS: From this initial experiment it appears that aggregation by micro-centrifuge tube between 100,000 and 250,000 cells per ml will produce beating cells at about 17 days after aggregation.

REFERENCES: [1]V. Maltsev, J. Rohwedel, J. Heschler and A.M. Wobus (1993) *Mech Dev* **44:**41-50. [2] H. Kurasawa, T. Imamura, M. Koike et al. (2003) *J Biosci Bioeng* **96:**409-411 [3]P. De Bank, B. Kellam, D. Kendall (2002) *Biotechnol Bioeng* **30:**800-808

ACKNOWLEDGEMENTS: I would like to thank Dr. Dan Howard for helpful discussions and acknowledge the BBSRC for funding.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 71) ISSN 1473-2262

The Effect Of Cell Support Geometry On Osteogenic Differentiation Of H1 Human Embryonic Stem Cells.

D. Howard, K.M. Shakesheff, L.D. Buttery.

Tissue Engineering Group, School of Pharmacy, University of Nottingham, England.

INTRODUCTION: Differentiation of Human Embryonic Stem Cells (HESC) to the osteogenic lineage could allow repair of bone defects that could otherwise not be remedied. The associated cell delivery devices may have an influence upon the outcome of repair, not only by chemical composition but also the geometry and associated characteristics [1].

METHODS: To test osteogenic differentiation of HESC on a range of scaffold geometries a series of $P_{(D,L)}LA$ scaffolds were manufactured (Fig 1) and Human Embryonic Stem Cells (HESC) grown and differentiated upon them *in vitro*.

Scaffolds were prepared from the same batch of $P_{(D,L)}LA$ (60K Mw) and included; supercritical CO_2 scaffolds, salt leeched scaffolds (containing; square, round, square interlinked and round interlinked porogens), spherical and irregular, heat sintered (60ºC) scaffolds and PLA fleece (from Cellon).

To each of these scaffold types 100,000 H1 HESC were added using an orbital shaker (2 cm radius at 60 rpm; n=6; simultaneously to minimise culture variables). HESC were prepared by culture upon mitomycin-C inactivated MEF feeder layers as undifferentiated cells, “semi-differentiated” in pellet culture and culture expanded in monolayer, before being added to the scaffolds in the presence of ascorbate (10 µM) dexametahasone (10 nM) for 14 days (Differentiation protocol modified from ref [2]).

To estimate cell number, metabolism was measured via the Alamar Blue (AB) assay (as per manufacturers instructions; Serotech Ltd).

To observe the position of cells within the scaffolds Live/Dead (Calcein-AM and EthD1; Molecular probes) was used (as per manufacturers instructions).

To estimate the osteogenic response, activity was measured using conversion of pNPP in an alkaline buffer with comparison to known concentrations of pNP (as modified from ref [3]).

RESULTS: The results suggest that geometry alone can have an influence upon the osteogenic response, indicated by alkaline phosphatase activities in comparison to cell metabolism. Scaffolds in order of osteogenic permisivity (AP/AB) were - cube pore, irregular sintered particles, spherical pore, sintered spheres, spherical fused pores, CO_2 foamed, fleece and fused cube porogen. The range of scaffolds tested all supported cell growth and differentiation and none appeared to specifically inhibit osteogenesis.

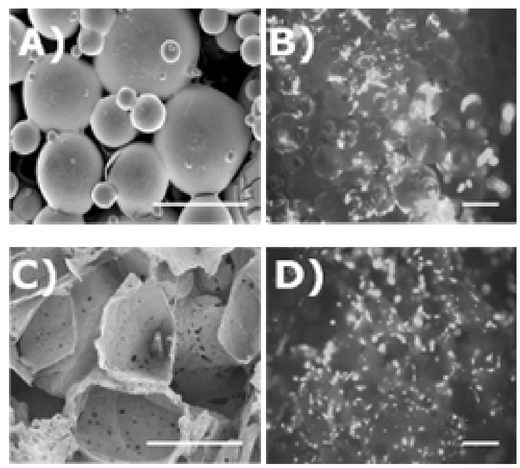


Fig. 1: Examples of two different scaffold geometries of convex-spherical and concave-square (A and C) with HESC location highlighted using live/ dead staining (B and D, Bar = 300 µm).

DISCUSSION & CONCLUSIONS: The scaffold range varied in other aspects, such as cell seeding, pore connectivity and sub-micron scaffold surface topography all of which could influence the outcome.

REFERENCES: [1] U. Ripamonti, (2004) J Cell Mol Med. 8:169-80. [2].C. Bielby, A.R. Boccaccini, J.M. Polak, L.D. Buttery. (2004) *Tissue Eng.* 10:1518-25. [3] ROC Oreffo A. Bennet, A.J. Carr, J.T. Triffit. (1998). Scand J Rheumatol. 27:415-24.

ACKNOWLEDGEMENTS: Many thanks to Lloyd Hamilton for helpful discussion of polymer characteristics. This work was supported by the B.B.S.R.C.

Mechanical Characterisation of UVA-Riboflavin Crosslinked Collagen Hydrogels

M.Ahearne[1], Y.Yang[1], A.J.El Haj[1], K.Then[2] & K.K.Liu[1]

[1] *Institute for Science & Technology in Medicine, Keele University, England.*
[2] *Birmingham and Midland Eye Centre, Birmingham City Hospital, England.*

INTRODUCTION: Collagen hydrogels have been investigated for growing numerous tissue equivalents in-vitro, including skin, cornea and vascular tissue. One of the major difficulties to be overcome in using collagen to engineer tissue equivalents is to replicate the native tissues mechanical strength. A UVA-riboflavin technique has been developed to crosslink collagen fibers in the cornea, which does not damage corneal cells [1]. We propose that this same technique can be modified to improve the mechanical strength of collagen hydrogels for use in tissue engineering applications. A non-destructive technique is also required to characterize the changes of mechanical properties in the hydrogels.

METHODS: Rat-tail collagen type 1 (BD Bioscience) was used to make collagen hydrogels of concentration 3.5 mg/ml. The hydrogels were submerged for 15 minutes in a 0.1% riboflavin photosensitizing solution. Hydrogels were then placed under a UVA light source with a maximum irradiance of 3 mW/cm^2 for 30 minutes.

The mechanical properties of the collagen hydrogels were obtained using a novel indentation system [2]. This consists of a sample holder and an image acquisition system (Fig. 1). The holder clamped the hydrogel around its outer edge while it was submerged in buffer solution at 37ºC. A ball of known weight and size was placed on top of the hydrogel causing it to deform. The image acquisition system, consisting of a long focal distance objective microscope linked to a CCD camera, recorded side-view images of the deformation profile from outside the incubator through a glass window. A theoretical model was derived to calculate the mechanical properties of the hydrogels from their deformation profiles.

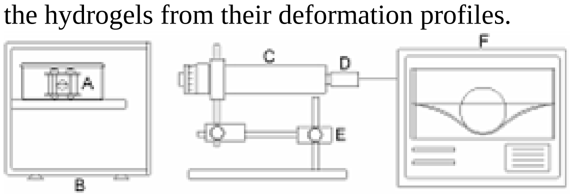


Fig. 1: Schematic of instrument system: (A) Sample holder and ball; (B) incubator at 37°C; (C) long focal microscope; (D) CCD camera; (E) X-Y translation stage; (F) image analysis system.

RESULTS: Our initial results demonstrate that the UVA-riboflavin crosslinking technique improved the mechanical strength of collagen hydrogels. The Young's modulus of the collagen hydrogels was measured before and after UVA-riboflavin crosslinking (Fig 2). A student T-test (95% confidence interval) was used to verify that there was a significant increase in the modulus of the hydrogels after crosslinking.

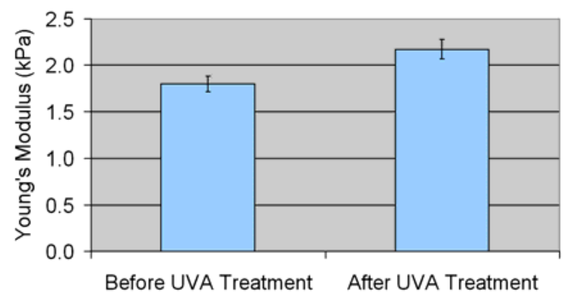


Fig. 2: Young's modulus of collagen hydrogels (n=4) before and after UVA-riboflavin crosslinking treatment.

DISCUSSION & CONCLUSIONS: This experimental technique potentially has many applications in tissue engineering, as collagen hydrogels are much weaker than the native tissues they are used to replicate. In addition to demonstrating a method of improving the mechanical strength of collagen hydrogels, this work also demonstrates the capabilities of the long focal indentation system for measuring the mechanical properties of hydrogels non-destructively, at evaluate temperatures and in biological solution. Work still needs to be carried out to optimise the crosslinking conditions and to determine the effect this technique has on cells when they are seeded to the collagen hydrogels.

REFERENCES: [1] G. Wollensak, E. Spoerl, T. Seiler (2003) *J Cataract Refract Surg* **29**:1780-85. [2]M. Ahearne, Y. Yang, A.J. El Haj, et al (2005) *J R Soc Interface* **2**:455-63.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 73) ISSN 1473-2262

Investigation of the Frictional and Mechanical Properties of Tissue Engineered Cartilage

M. Plainfosse[1,2], J. Katta[2], Z. Jin[2], J. Fisher[2], A. Crawford[1], P.V. Hatton[1].
1. Centre for Biomaterials and Tissue Engineering, University of Sheffield, England.
2. Institute of Medical and Biological Engineering, University of Leeds, England.

INTRODUCTION: The main function of articular cartilage is to provide low friction and wear between articular surfaces. Native cartilage is sometimes subject to degradation or trauma, and its natural capacity for regeneration is relatively poor. One aim of regenerative medicine is to develop implantable engineered cartilage which matches the properties of native articular tissue to repair defects. The purpose of this study was to investigate the frictional and mechanical properties of tissue engineered cartilage using bovine chondrocytes seeded on poly(glycolic acid) scaffolds (PGA). In addition, the nature and quantity of debris generated during the friction tests were examined.

METHODS:
Construct synthesis: Articular cartilage slices were harvested from bovine metacarpophalangeal joints. Chondrocytes were isolated via enzymatic digestion and expanded at 37°C in 5% CO_2 - 95% air, in expansion medium[1] until a sufficient number of chondrocytes available. Poly-(Glycolic-Acid) scaffolds (Ø 6mm × 2mm) were seeded at 112×10^6 cells per cm^3, enclosed in an agarose gel plug and cultured in differentiation medium[1] for 33 days. *Friction tests*: The engineered constructs were attached to metal backing pins and loaded on to a reciprocating stainless steel plate in a bath of lubricant (PBS). A piezoelectric force transducer was used to measure the frictional force between the pin and the counterface during sliding. A normal load of 13.2 N (contact stresses of 0.3 MPa) was applied on the pins and a sliding speed of 4 mm/s was adopted. Startup friction measurements were recorded after 5 s, 30 s, 2 min, 5 min, 10 min and 20 min of static loading. Between each measurement a load removal period, at least equal to the time of loading, was applied. During this interval, the constructs were immersed in lubricant to recover. Additionally, dynamic friction tests involving one hour of continuous reciprocating sliding between the constructs and the plate under load were carried out and friction measurements taken at 5 s, 30 s, 2 min, 5 min, 10 min and every ten minutes thereafter. Native cartilage and the unseeded material scaffold were used as controls. *Biochemical analysis*: Histology, scanning electron microscopy (SEM) observations and glycosaminoglycan (GAG) quantification were performed on the constructs to compare the structure, composition and properties before and after the mechanical tests. The lubricant and debris generated were harvested in order to examine their composition (histology and GAG assay).

RESULTS: All the constructs tested exhibited a time-dependent increase in the coefficient of friction and reached an equilibrium final value. While this phenomenon is similar to native cartilage, the values are different: $\mu_{\text{start-up eq}}=0.120$ and $\mu_{\text{dynamic eq}}=0.175$ for the engineered constructs, $\mu_{\text{start-up eq}}=0.240$ and $\mu_{\text{dynamic eq}}=0.038$ for the native cartilage.

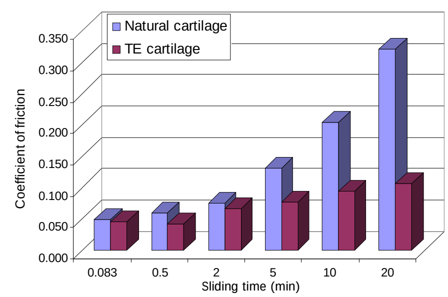


Fig. 1: Initial coefficient of friction vs. sliding time for tissue engineered and native cartilage.

DISCUSSION & CONCLUSIONS: Our conclusion was that engineered cartilage shared similar frictional behaviour to native tissue, although the precise mechanism may differ. More advanced constructs, with properties closer to those of native cartilage, should however be developed for further investigation and clinical use.

REFERENCES: [1] A. P. Hollander, P. V. Hatton *Biopolymer Methods in Tissue Engineering*. Humana Press (2004).

ACKNOWLEDGEMENTS: This work was funded by the White Rose University Consortium and was performed as a part of the EXPERTISSUE Network of Excellence [project reference 500283].

 ISSN 1473-2262

Chondrocytes in Monolayer Culture on a Thermoresponsive Polymer vs. Conventional TCPS

L.R.M. Elowsson[1], J. Collett, S. Rimmer[2], A. Crawford[1], & P.V. Hatton[1]

[1] *Dept of Adult Dental Care, University of Sheffield, England*

[2] *Dept of Chemistry, Polymer & Biomaterials Chemistry Lab, University of Sheffield, England*

INTRODUCTION: Expansion of cells is an essential step in tissue engineering to provide sufficient numbers from a tissue biopsy. In monolayer culture, trypsin is commonly used to detach cells. To remove the potential risk of cross infection[1], alternatives to animal-derived products are being investigated. Culture of cells on thermoresponsive polymer grafted surfaces offers one such alternative method of cell detachment[2]. When cooled below their lower critical solution temperature (LCST), approximately 32ºC, these hydrogels become swollen due to hydration and cause cell detachment. The aim of the study was to compare the detachment of bovine articular chondrocytes (BAC) from tissue culture polystyrene (TCPS) surfaces using trypsin/EDTA (0.5 g/L trypsin/0.2 g/L EDTA), with the detachment from a thermoresponsive polymer surface.

METHODS: A thermoresponsive hydrogel system (poly(NIPAM)-g-GMMA-co-EGDMA) was synthesised[3]. Chondrocytes were isolated from bovine metacarpophalangeal joints, expanded, and seeded onto the different surfaces (as described previously[4]). When confluent, the cells were stained with CellTracker™ Green CMFDA (Molecular Probes, C2925). Live imaging was acquired to follow cell detachment, using a fluorescence microscope (Zeiss Axiovert 200M). Cell viability by Alamar blue assay was also studied as a result of the different culturing conditions.

RESULTS: Cultures with similar morphologies were obtained on both surfaces looking at the micrographs.

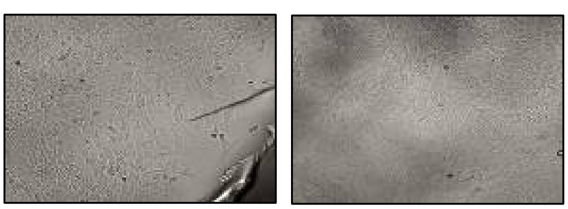


Fig. 1: Micrographs showing BAC (passage 2) after 7 days of culturing on (a) poly(NIPAM)-g-GMMA-co-EGDMA and (b)TCPS

Assessment of cell viability with Alamar blue indicated that cells were equally viable on both surfaces.

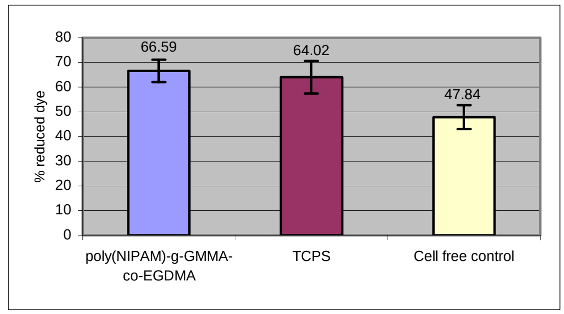


Fig. 2: Cellular activity of BAC on poly(NIPAM)-g-GMMA-co-EGDMA and TCPS respectively was determined by Alamar blue assay after five days of culture as percentage reduction of the stain.

DISCUSSION & CONCLUSIONS: The results indicated that poly(NIPAM)-g-GMMA-co-EGDMA grafted surfaces offered a promising alternative for the expansion of chondrocytes. Our interim conclusion was that the mechanism may be more complex than previously reported, encompassing changes in both hydrophilicity and dimension. Work is in progress to study the arrangement of the cytoskeleton by following the actin filaments on the different surfaces.

REFERENCES: [1]Jochems, CEA, et al, Altern Lab Anim, 2002. **30**(2): p. 219-227. [2]Okano, T, et al., Biomaterials, 1995. **16**(4): p. 297-303. [3]PhD Thesis of Jon Collett, 2005. [4]Hollander, A.P. and Hatton, P.V., (2004). Biopolymer Methods in Tissue Engineering. Totowa, New Jersey, Human Press Inc.

ACKNOWLEDGEMENTS: The authors are grateful to the European Commission (Marie Curie Alea Jacta EST, contract MEST-CT-2004-008104). The work was performed as part of the EXPERTISSUES Network of Excellence [project reference 500283].

*l.elowsson@sheffield.ac.uk

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 75) ISSN 1473-2262

Establishing An Osteoblast:Osteoclast Co-culture System For Use In Bone Tissue Engineering

G. L. Jones[1], M. J. Marshall[2], A. J. El Haj[1], S. H. Cartmell[1]

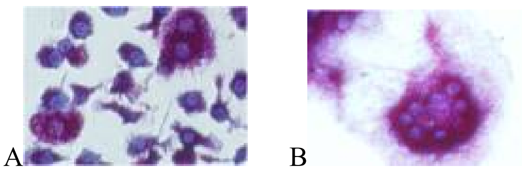
1. ISTM, University of Keele, Thornburrow Drive, Hartshill, Stoke-on-Trent, ST4 7QB

2. Charles Salt Centre, RJAH Orthopaedic Hospital, Oswestry, Shropshire, SY10 7AG

INTRODUCTION: Current bone tissue engineering strategies often focus on creating constructs consisting of a degradable scaffold and a single cell type, usually osteoblasts, the mineralised matrix producing cell of bone tissue. We hypothesise that by co-culturing osteoclasts on the same construct with osteoblasts, that the cell signalling pathways that are inherent in the physiological body, will, at a basic level, be replicated in the in-vitro 3D environment. This cell-cell communication has the potential to actively assist in the creation of a functional tissue engineered bone construct. The resorbing of the calcium based scaffold by the osteoclasts on which the co-culture is presiding may in turn promote the osteoblasts to produce an increased amount of mineralised matrix in comparison to a scaffold seeded with just osteoblasts alone. In this way we anticipate that this dynamic co-culture system will have synergistic effects on mineralised matrix deposition thus creating a functional tissue engineered construct in a shorter preparation time in-vitro whilst maintaining the potential of an increased acceptance rate into the body once implanted into a fracture site in-vivo. In this study, we describe our initial experiments in setting up this co-culture where we have analysed osteoclast activity on a 2D dentine slice using traditional analysis of SEM and histology as well microCT imaging.

METHODS: Monocytes were isolated from murine bone marrow, seeded onto dentine slices (5mm diameter, 3μm thick, IDS) at a density of $1x10^6$ cells per slice. The monocytes were then differentiated towards osteoclasts using macrophage colony stimulating factor (MCSF) and receptor activator of NFκB ligand (RANKL)[1]. Osteoclasts were visualised using Tartrate Resistant Acid Phosphatase (TRAP) positivity in multinucleate cells after 4 and 7 days culture in conjunction with microCT and SEM analysis for resorption pit determination.

RESULTS: *Figure 1 MicroCT image of dentine slice prior to culture*



A B

Figure 2 A. TRAP positive cells B. TRAP positive multinuclear osteoclast cell (x40)

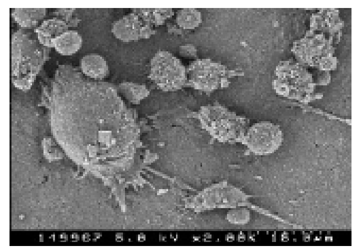


Figure 3 SEM image of cells adhered to a dentine slice after 4 days of culture. (x2000)

DISCUSSION & CONCLUSIONS: We have established a murine system of differentiating osteoclasts from bone marrow derived monocytes that is consistent with the published literature. MicroCT analysis of the calcium phosphate matrices used in this project is proving an effective high resolution tool to locate and quantify mineralised matrix turnover. Our future studies now will look into the cell seeding ratio of osteoblast (derived from both murine bone, mesenchymal progenitors in the bone marrow) versus osteoclasts (derived from haemopoetic progenitors in the murine bone marrow) on dentine slices using these same techniques. Once we have established this information we will begin to quantitate the efficacy of this technique on novel scaffolds for bone tissue engineering including collagen/hydroxyapatite scaffolds in collaboration with Dr Jan Czernaska, Oxford University and Dr Antonnella Motta, University of Trento.

REFERENCES: 1. Dotard et al. 2005. Clinica Chimica Acta. 356(1-2): 154-63
ACKNOWLEDGEMENTS: Karen Walker, Keele for her advice on SEM analysis. EXPERTISSUES EU Vth framework Integrated Network.

 ISSN 1473-2262

Biomechanical characterisation of decellularised and cross linked bovine pericardium.

D Oswal[1], S Korossis[1], S Mirsadraee[2], H Wilcox[2], K Watterson[3], J Fisher[1], E Ingham[2]
Institute of Medical and Biological Engineering, Schools of Biochemistry & Microbiology[2] and Mechanical Engineering[1], University of Leeds, Leeds, England and Leeds General Infirmary[3], Leeds, England.

INTRODUCTION: Bovine pericardium is used extensively to repair cardiac defects and to make artificial heart valves because of its ready availability and its strength. Commercially available pericardium is treated with 0.5% glutaraldehyde which cross-links the collagen fibres, kills the resident cells, reduces antigenicity and improves the shelf life. The major limitation to the continued clinical use of glutaraldehyde cross-linked pericardium is that it undergoes calcification and degeneration. The dead cells, cell fragments and glutaraldehyde treatment all contribute to this deterioration *in vivo.*

The aims of this study were to develop a protocol for decellularisation of bovine pericardium and determine the biomechanical properties of pericardium treated with a reduced concentration of glutaraldehyde (0.05% instead of 0.5%) with a view to producing biomaterials with improved biocompatibility for cardiovascular applications.

METHODS: Bovine pericardia were decellularised using an established protocol involving treatment with hypotonic buffer; 0.1% (w/v) SDS plus proteinase inhibitors and nuclease treatment. Histological analysis and Hoescht staining was performed to validate the adequacy of decellularisation. Matched samples of fresh and acellular pericardium were treated with 0, 0.05 or 0.5 % (w/v) glutaraldehyde for 24h (n=6 per treatment). Following treatment the biomechanical properties (Youngs elastic modulus; collagen phase slope, transition stress and strain, ultimate tensile strength and failure strain) were determined by uniaxial testing to failure. Data were analysed by ANOVA. Contact cytotoxicity was used to determined the *in vitro* biocompatibility of the variously treated pericardia.

RESULTS: The histological analysis of the decellularised bovine pericardium did not show any remaining cells or cell fragments. The histo-architecture of collagen-elastin matrix appeared to be well preserved. Untreated decellularised pericardium was biocompatible in contact cytotoxicity tests with porcine smooth muscle and human fibroblast cells. The glutaraldehyde treated tissues were cytotoxic. There were no significant differences in the mechanical properties of variously treated pericardia. There was an overall trend for the glutaraldehyde treated pericardia to be stiffer and stronger than their untreated counterparts.

DISCUSSION & CONCLUSIONS: This study has developed a successful protocol for decellularisation of bovine pericardium and shown that it is possible to produce biomaterials from acellular bovine pericardium, using different concentrations of glutaraldehyde, with excellent biomechanical properties. The biomaterials now require evaluation in small and large animals in order to determine their potential for clinical use.

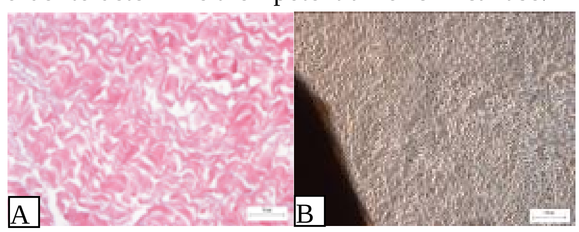


Figure 1: A – *H&E stained decellularised bovine pericardium (200x) showing no cells or nuclei.*

B *- Confluent porcine smooth cells in contact with decellularised pericardium (unstained specimen, 40x).*

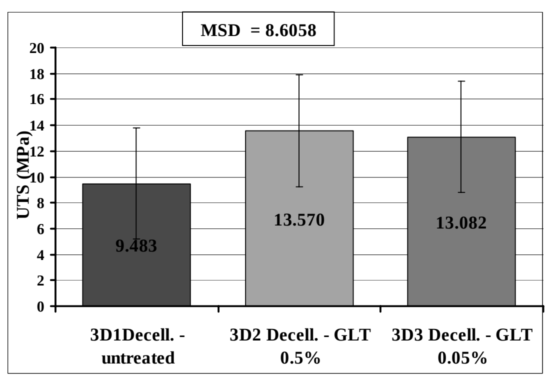


Fig.2: *Average ultimate tensile strength (UTS) of variously treated decellularised bovine pericardia (MSD- Minimum significance difference; p<0.05).*

ACKNOWLEDGEMENTS: This work was supported by a generous grant from Nuffield Hospital, Leeds, UK.

 ISSN 1473-2262

Investigation of Spider Egg Case Silks for Cartilage Tissue Engineering

A C MacIntosh, A Crawford & P V Hatton

Centre for Biomaterials and Tissue Engineering, School of Clinical Dentistry, University of Sheffield,UK

INTRODUCTION: Spider silks are some of the toughest natural materials, and they have shown potential for use as scaffolds for cartilage tissue engineering. Our group previously reported the use of silk harvested from *Nephila edulis* egg cases, and processing methods to improve their efficacy as tissue engineering scaffolds. Here, the distinct regions of the egg case are investigated.

METHODS: The morphological features of the silk fibres taken from the inner and outer portions of egg cases were observed by scanning electron microscopy (SEM). Scaffolds for cartilage tissue engineering were formed from these silk samples and sterilized by autoclaving. Freshly harvested mature chondrocytes obtained from bovine metacarpophalangeal joints were seeded onto the scaffolds using published methods [1]. After 7 days, samples were observed by SEM. Further cell-scaffold constructs were cultured for 40 days, to engineer hyaline-like cartilage. After 40 days, constructs were analysed biochemically[2] and histologically to determine glycosaminoglycan (GAG) content and distribution. Collagen type I and type II were detected immunohistochemically. Poly(glycolic acid) scaffolds were used as comparative materials.

RESULTS: SEM showed scaffolds had similar fibre morphology. Cells had attached to all scaffolds and formed confluent sheets after 7 days. After 40 days in culture, extracellular matrix proteins were detected in all constructs.

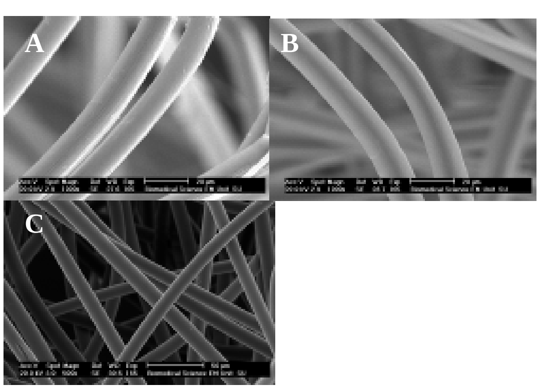


Fig 1 Morphology of tissue engineering scaffolds. A: Nephila edulis outer egg case silk, B: N.edulis inner egg case silk, C: Poly(glycolic acid) non-woven felt

DISCUSSION & CONCLUSIONS: We have demonstrated that different regions of *Nephila edulis* egg case silks support the attachment, proliferation and re-differentiation of bovine articular chondrocytes *in vitro*. We found that all scaffolds supported the formation of a hyaline-like cartilage, and therefore conclude that spider silks have potential as scaffolds for cartilage tissue engineering.

REFERENCES: [1] in *Biopolymer Methods in Tissue Engineering*, AP Hollander & PV Hatton (Eds), Humana Press (2004). [2] RW Farndale, DJ Buttle, AJ Barrett, *Improved quantitation of glycosaminoglycans by use of dimethylmethylene blue.* Biochemica et Biophysica Acta, **883** (1986): 173-177.

ACKNOWLEDGEMENTS: The authors thank Smith & Nephew for the donation of PGA scaffolds, Fritz Vollrath (Oxford University) for the supply of spider silks, Jen Mundy and Chris Hill for technical assistance. We also gratefully acknowledge funding from the European Commission under the Fifth Framework (G5RD-CT-2002-00738 SPIDERMAN). This work was carried out in an EXPERTISSUES laboratory (NOE-500283-2).

 ISSN 1473-2262

3D Culture of Human Intervertebral Disc Cells Within PDLLA/Bioglass® Composite Scaffolds: Assessment of Extracellular Matrix Production

W. Helen & J.E. Gough

Biomaterials and Tissue Engineering Group, School of Materials, University of Manchester, UK

INTRODUCTION: With a view to the repair of damaged human intervertebral disc (IVD), we grew human annulus fibrosus cells (HAF) on 3D composite scaffolds made from the bioresorbable polymer PDLLA with different weight fractions of Bioglass®. These scaffolds were characterised in terms of their structural and biological properties including: glycosaminoglycan (GAG) production, HAF cell attachment, and collagen production.

METHODS: Scaffold fabrication: PDLLA composite scaffolds containing 0wt%, 5wt% and 30wt% Bioglass® were prepared by thermally induced phase separation (TIPS) [1].

Scanning electron microscopy: Cell attachment studies were performed using SEM. The AF cells were seeded within PDLLA/Bioglass® composite scaffolds at a density of 5×10^6 cells/cm^2 and cultured. At pre-determined time points the cells were fixed in 1.5% glutaraldehyde at 4 ºC for 30 min. They were then dehydrated through a series of increasing concentrations of ethanol and dried using hexamethyldisilazane. The samples were sputter coated with gold and viewed using a JEOL 6300 SEM operated at 10 kV.

DMMB assay: GAG production by the HAF cells was studied using the 1,9 dimethylmethylene blue (DMMB) method described by Farndale [2].

Collagen production: The amount of collagen type I and type II were determined using Western blotting [3].

RESULTS: SEM micrographs in Figure 1 show (left) HAF cells bridging pore walls in the interior of the scaffolds and (right) HAF cells on the surface of PDLLA/30wt% Bioglass® composite scaffold after 2 wks in culture. GAG assay in Figure 2 shows the increase of GAG production on the 5wt% Bioglass® scaffold from week 1 to week 4 and indicates the ability of HAF cells to secrete a GAG rich extracellular matrix. Western blotting in Figure 3 shows (left) collagen type I bands at approximately 300 KDa and 120 KDa and (right) collagen type II bands at approximately 130 KDa.

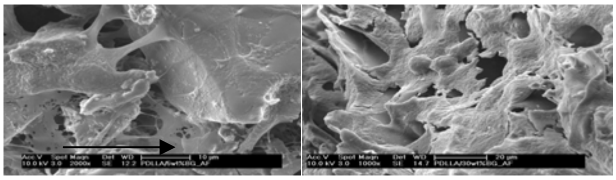


Fig. 1: SEM micrographs show the morphology of HAF cells within PDLLA/5wt% Bioglass® (left) and PDLLA /30wt% Bioglass® (right) composite scaffold..

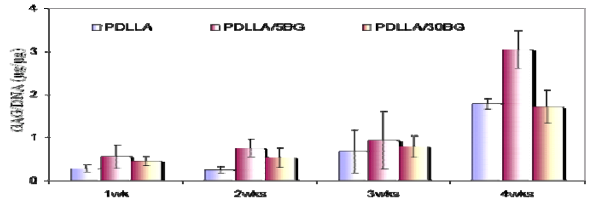


Fig.2: GAG production of HAF cells on PDLLA, PDLLA/5wt% Bioglass® and PDLLA/30wt% Bioglass® over 4weeks.

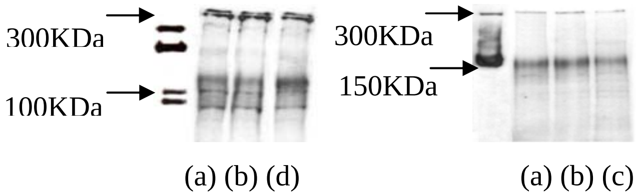


Fig.3:Type I collagen (left) and type II collagen (right) expression by HAF cells cultured on PDLLA foam (a) PDLLA/5wt% Bioglass® (b) and 30 wt% Bioglass® (c) composite scaffolds.

DISCUSSION & CONCLUSIONS: By adding Bioglass® particles to PDLLA scaffolds it is possible to tailor the scaffolds bioactive behaviour and promote extracellular matrix production. These scaffolds have potential for intervertebral disc tissue engineering.

REFERENCES: [1]V. Maquet (2004) *Biomaterials* **25**:4185-4194. [2]R.W. Famdale, et al (1982) *Connect Tissue Res* **9**:247-248. [3]J. Kumagai, et al (1994) *J Anat* **185**:279-284.

ACKNOWLEDGEMENTS: Financial support from Back Care is gratefully acknowledged. The authors would like to thank Mr Jonny Blaker (Imperial College London) for the gift of the PDLLA/Bioglass® foams and Novamin for the gift of bioactive glass powder.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 79) ISSN 1473-2262

Development and Characterisation of a Full-thickness Engineered Human Oral Mucosal Model for *In Vitro* Studies

K. Moharamzadeh, R. Van Noort, I. M. Brook, and A. M. Scutt

Centre for Biomaterials and Tissue Engineering, University of Sheffield, England.

INTRODUCTION: Restorative materials and oral health care products come into direct contact with oral mucosa and can cause adverse reactions. To obtain an accurate risk assessment, the *in vitro* test model must reflect the clinical situation as closely as possible. A three-dimensional full-thickness engineered human oral mucosal model for biological assessment of dental materials is reported.

METHODS: Oral keratinocytes were isolated from the biopsies from patients having canine exposures with their written informed consent and cultured in Greens medium. Fibroblasts were isolated from the connective tissue layer of the biopsy and cultured in serum containing DMEM. Fibroblasts were seeded onto a Collagen-Glycosaminoglycane-Chitosan scaffold and cultured for 7 days to produce an engineered lamina properia. Oral Keratinocytes were then seeded on the top of this connective tissue and grown at the air/liquid interface for 2 weeks to produce a full-thickness engineered human oral mucosa. The tissue was frozen sectioned and stained by haematoxylin and eosin and tiny pieces of engineered oral mucosa were cut, fixed, and processed for Transmission Electron Microscopy examination.

RESULTS: An engineered oral mucosa with a well-differentiated stratified epithelium and viable fibroblasts in the connective tissue layer was reconstructed (Fig. 1). Ultrastructural examination showed numerous desmosomes joining keratinocytes throughout the epithelium especially in the spinose layer and cytoplasmic keratin increasing towards the cornified layer. Newly synthesized ECM produced by fibroblasts present in sub-epithelial layer.

Fig. 1: Engineered oral mucosa on collagen-GAG-chitosan matrix

CONCLUSIONS: The optimised oral mucosa reconstruct resembled native human oral mucosa and it has the potential to be used as an accurate and highly reproducible test model in biocompatibility evaluation of dental materials

 ISSN 1473-2262

RACK 1 associated with focal adhesions alters the cells sensitivity to contact guidance.

A.Hart, S.J. Yarwood, M.J. Dalby

Centre for Cell Engineering, Infection and Immunity, Institute of Biomedical and Life Sciences, Joseph Black Building, University of Glasgow, Glasgow, G12 8QQ, UK.

INTRODUCTION: Through the use of firstly microtopography and then nanotopography, it has been shown that all cell types tested experience contact guidance. Typically, the most extreme cases have been demonstrated through the use of grooves, along which the cells align.

Filopodial sensing (a nanoscale form of contact guidance) and whole cell guidance are mechanisms of cellular motility and are thus likely to involve focal adhesions and the cytoskeleton. In this report, we focus on RACK1, a part of the focal adhesion. Using nanodepth grooves to align cells of three types: NEO (normal), RACK1 overexpressing (RACK$^{+)}$ and neo cells treated with an antisense oligo fro RACK1 (hence express little RACK 1, RACK^{-}), we have observed the effects of RACK 1 on contact guidance.

METHODS: Grooved substrates were fabricated in silicon by photolithography. The grooves were 10 µm wide and 200 nm deep. The master substrates were used as a stamp to hot-emboss the grooves in to polycaprolactone (PCL) sheets.

As described in the introduction, NEO, RACK^{+}) plasmid transfection) and RACK^{-} (scrape loading) MCF-7 cells were cultured on the grooves and planar PCL for 3 days before fixation. After fixation, cells were stained for vinculin, actin and tubulin and observed by fluorescence microscopy.

As well as morphological observation of adhesions and cytoskeletons, percentage of aligned cells was calculated in order to measure degree of contact guidance.

RESULTS & DISCUSSION: Compared to NEO, RACK^{+} cells had numerous focal adhesions, well defined actin cytoskeleton and were poorly aligned. Compared to NEO, RACK^{-} cells had few focal contacts, only peripheral actin, but were well aligned.

It appears that increasing adhesion formation through up-regulation of RACK 1 leads to decreased motility and hence contact guidance.

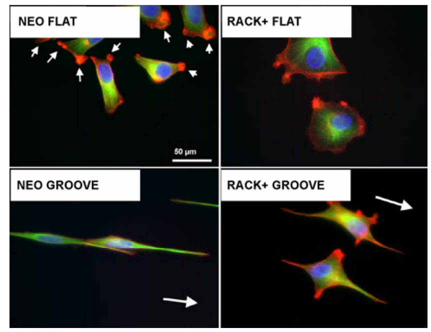


Fig. 1: RACK^{+}cells on flat and grooved materials – comparison to NEO.

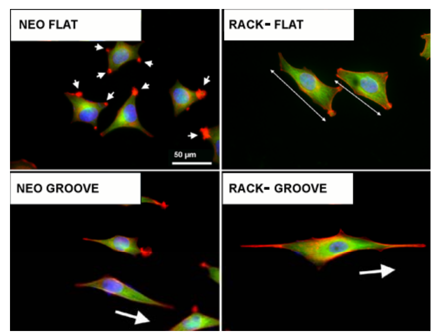


Fig. 2: RACK^{-} cells on flat and grooved materials – comparison to NEO.

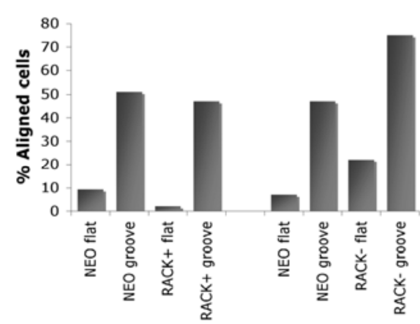


Fig. 3: % Aligned cells for each treatment.

ACKNOWLEDGEMENTS: This work was funded by MJD's BBSRC David Phillips Fellowship.

 ISSN 1473-2262

Direct current influences Ca^{2+} signalling in chondrocytes

O.O.Akanji, D.L.Bader, D.A.Lee

Medical Engineering Division, Department of Engineering, Queen Mary, University of London, London, UK

INTRODUCTION: It is well established that there exists a coupling of the electric fields detected within cartilage tissue to the mechanical deformation to which the tissue is subjected [1,2]. The mechanism by which this coupling occurs is yet undetermined. In neural and muscle cells electric fields instigate the opening of voltage operated channels causing Ca^{2+} ion influx into the cytosol. The present study investigates the effect of electric current on Ca^{2+} signalling in chondrocytes.

METHODS: *Cell Preparation:* Full depth bovine articular chondrocytes were isolated from the metacarpophalangeal joint and seeded at 10^7 cells/mL in agarose[3]. Following a 24 h equilibration period, the constructs were labelled with 5 µM Fluo-4 AM in bicarbonate free (BF) medium for 1 h at 37°C. Thereafter, constructs were washed for 10 min in BF medium. Selected constructs were treated with 10 µM verapamil in BF medium during the 10 min wash period. *Electric stimulation*: Each construct was mounted in a Perspex chamber containing its corresponding wash medium and connected by agarose saltbridges to platinum electrodes in dilute KCl solutions (Figure 1). A current density of 4 mA/cm^2, supplied by an electrophoresis power pack, was passed through the construct for 10 min. Cell viability was maintained at > 95%. *Calcium imaging*: Images of 10-30 cells per field of view per construct were visualised using a confocal microscope associated with an inverted microscope and captured with a x 20 plan apo lens every 4 s for the 10 min imaging period.

RESULTS: The application of (direct current) dc did not alter the percentage of cells exhibiting a Ca^{2+} response. For cells that were not exposed to dc, verapamil significantly reduced the percentage of cells showing a response. By contrast, verapamil did not influence the response when cells were exposed to dc.

DISCUSSION & CONCLUSIONS: These data suggest that dc did not affect overall Ca^{2+} reponse. However, the application of dc did appear to alter the mechanism of the response. In the absence of dc the response could be markedly reduced by verapamil suggesting a mechanism dependent on voltage operated channels. During the application of dc, verapamil did not affect the response, implying that Ca^{2+} influx is independent of voltage operated channels. This suggests that some mechanism other than the opening of voltage operated Ca^{2+} channels may influence chondrocyte response to electrical stimulation.

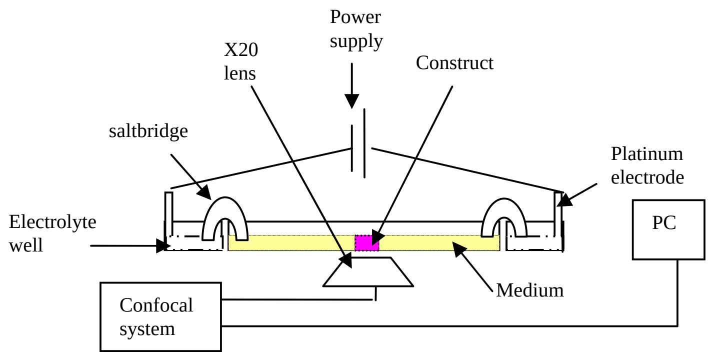


Fig. 1: Schematic of the electrical stimulation chamber

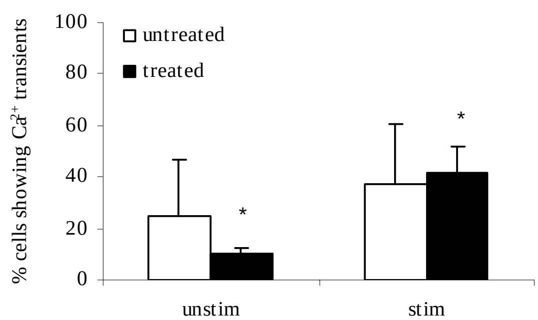


Fig. 2: The effect of dc and/or verapamil on Ca^{2+} response in chondrocytes. Data represents the mean ± SD of 2 cell isolations

REFERENCES: [1] Biosynthetic response to mechanical and electrical forces in *The Biology of Tooth Movements* (eds L.A. Norton and C.J. Burstone) CRC Press, Inc pp 335-347. [2] N. Szasz *et al.*, (2003) *Meet Orthop. Res Soc.* [3] D.A. Lee and D.L. Bader (1995) *In vitro Cell Dev Biol Anim* 31(11):828-835.

ACKNOWLEDGEMENTS: This work was funded by the EPSRC

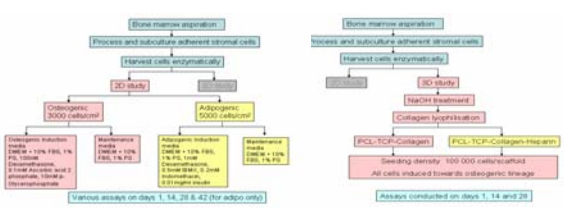
European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 82) ISSN 1473-2262

Characterisation and differentiation of porcine bone marrow mesenchymal stem cells in 2D and 3D mPCL-TCP-collagen scaffolds

Chum Zhi Zhen[1] M.A. Woodruff [1] Suman Lal Chirammal Sugunan[1], S. M. Cool [1,2,] V. Nurcombe [1,2] D.W. Hutmacher[1,2]
[1]*Division of Bioengineering, 9 Engineering Drive 1, National University of Singapore 117576.*
2 Department of Orthopaedic Surgery, National University of Singapore 119260

INTRODUCTION: This study has several objectives; to study the attachment, growth and differentiation potential of porcine bone marrow mesenchymal stem cells (PBMSC) in 2D and 3D environments, and to investigate the effects of heparin (Hep) on proliferation and osteogenic differentiation in medical grade poly (ε-caprolactone) tri-calcium phosphate-collagen (mPCL-TCP-Col) scaffolds. Our results show that scaffolds of mPCL-TCP-Col support multilineage differentiation of PBMSC. Osteogenic differentiation (calcium and collagen I (col I) immunostaining) was evident in cultures stimulated with osteogenic supplements, and areas contained numerous bone nodules (SEM). Furthermore, adipogenic differentiation was also confirmed following stimulation with adipogenic supplements by oil red O staining. Cell attachment and proliferation was also observed in all groups. Notably, stimulation with Hep had no significant effect on the proliferation and osteogenic differentiation of PBMSC cultured on the mPCL-TCP-Col scaffolds. In conclusion, this work showed PBMSC have the capacity to differentiate along osteogenic and adipogenic lineages and that mPCL-TCP-Col scaffolds are suitable supports for cells with potential for tissue engineering applications.

METHODS: Porcine bone marrow was extracted from the iliac crest, plated and cultured in DMEM (low glucose), 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. PBMSC were then sub-cultured until passage 3, then plated into culture wells for the 2D study, or seeded into Col I-lyophilised mPCL-TCP scaffolds for the 3D study and induced with DMEM containing either osteogenic or adipogenic supplements.



RESULTS: *2D Osteogenic Differentiation.* H&E staining showed that following osteogenic supplementation, the cellular morphology changed from a fibroblastic state to a flat, cuboidal form. Furthermore, Alizarin Red staining showed increased calcium deposits in induced samples that by day 28 covered most of the cell layer that was also positive for Col I. SEM analysis showed mineral nodules in only the induced cultures.

2D Adipogenic Differentiation. H&E staining showed cellular morphology change from fibroblastic to flat, oval forms in induced cultures only. Lipid droplets appeared only 28 days post induction; confirmed by Oil Red O staining. SEM analysis and confocal microscopy showed PBMSC attached and spread well on scaffold surfaces, forming cell sheets and bridges. Viability was high in both groups (mPCL-TCP-Col, mPCL-TCP-Col-Hep).Upon induction, ALP activity peaked at day 14 (Fig 1) before decreasing. No significant difference was seen between the two groups ($P>0.05$). Alizarin Red staining using cryo-sectioning showed staining of calcium deposits for both groups (Fig 2). Picogreen Assay showed an increase in cell numbers from day 1-14, demonstrating good cell proliferation on the scaffolds. No significant difference in proliferation was observed between the groups ($P>0.05$).(Fig 3).

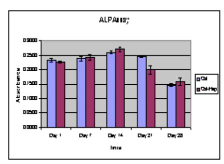


Fig 1

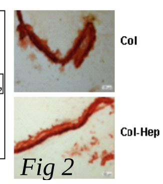


Fig 2

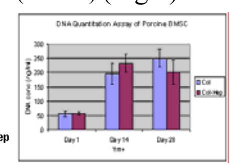


Fig 3

DISCUSSION & CONCLUSIONS: Few studies have characterised the differentiation of PBMSC apart from the study by Ringe (2002). Our results support this study and demonstrate the potential of PBMSC to undergo osteogenesis and adipogenesis following the addition of suitable induction media. Hausser (2004) reported that Hep had a biphasic effect on Saos-2 cells, promoting and inhibiting proliferation and osteogenic differentiation at high (≥5 µg/ml) and low (5-500 ng/ml) concentrations respectively. However this study was limited to an osteosarcoma cell line. Our results could be due to the osteoconductive TCP/Col I together with the osteogenic induction strongly favouring an osteogenic fate that obscured the effects of additional Hep making differences hard to detect. We are now investigating the use of more specific Hep variants to support the osteogenic process.

REFERENCES: [1]Ringe J.(2002) Cell Tissue Res. 2002 Mar; 307(3):321-7. [2]Hausser HJ, (2004). J Cell Biochem 91:1062-1073

ACKNOWLEDGEMENTS: Thanks to Chum Zhi Zhen for experimental contributions.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 83) ISSN 1473-2262

Micelle Forming Hydrogels for Topical Delivery of Ibuprofen and Sodium Ibuprofen

A. Waddon & S. NacNeil

Kroto Research Institute, University of Sheffield, England.

Thermo-responsive hydrogels based on the amphiphilic triblock copolymer [poly ethylene glycol]-[polypropylene oxide]-[polyethylene glycol] (Pluronics F68) are used to prepare a drug delivery system for the anti-inflammatory drugs ibuprofen and sodium ibuprofen. In aqueous solution, the surfactant copolymers form micelles on the size of 10-100nm. The hydrophobic PPO forms the inner core of the micelle and the hydrophilic PEG forms the outer corona. The most common form of ibuprofen is extremely hydrophobic and insoluble in water; however, it is shown that it can be dissolved in the hydrophobic core of the micelle to form “nano-packets” of drug up to ~2.5 % . In contrast, the polar form of ibuprofen, sodium ibuprofen, is shown to be readily soluble in the hydrophilic matrix material surrounding the micelle.

These co-polymer solutions undergo a sharp transition from a flowing, viscous liquid to a self-supporting clear and transparent gel on heating. This transition is a classical sol-gel transition. The transition temperature decreases with copolymer content. Importantly, the transition can be made to occur at the physiologically important temperature of 37^{o}C by suitable adjustment of composition.

The gelation temperature is affected by incorporation of the drugs. Dissolution of non-polar ibuprofen into the hydrophobic micelle core is shown to decrease the gelation temperature. In contrast, incorporation of polar sodium ibuprofen into the hydrophilic matrix is shown to increase the gelation temperature.

.

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 84) ISSN 1473-2262

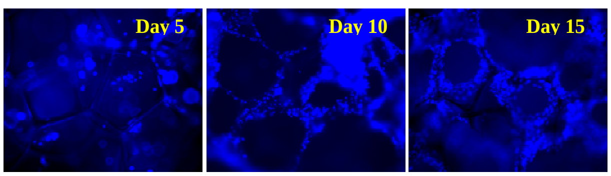
A 3-dimensional, in vitro model to study the effects of compressive loading on osteoblastic cells.

Anuphan Sittichokechaiwut [1], Anthony J. Ryan [2] and Gwendolen Reilly [1]

[1] Department of Engineering Materials, [2] Department of Chemistry University of Sheffield, UK

INTRODUCTION: Mechanical force is an osteoinductive factor that plays an important role in bone growth and repair in vivo[1]. Our aim was to design a model system by which bone cell responses to mechanical load could be examined in vitro in a 3D environment. Our previous study has shown that osteoblastic cells survive well and proliferate in our polyurethane (PU) open cell foam scaffolds. Cell number after 15 days of culture was four times that after 5 days of culture. Examination of cell distribution, under fluorescence microscopy, showed that cells were clearly adherent and spread out along the sides of the pores of the PU and were seen in the centre of a 25 mm diameter, 10mm high scaffold (*Fig.1*).

Fig 1: Cell distribution on PU scaffold by using Fluorescence microscopy (DAPI staining).



METHODS: MLO-A5 osteoblastic cells were seeded at densities of 250,000 cells per scaffold in cylindrical polyurethane (PU) scaffolds, 10 mm thick and 10 mm diameter. The cell seeded PU scaffolds were dynamically loaded in compression at 1Hz, 5% strain in a sterile fluid-filled chamber (*Fig.2*). Loading was applied for 2 hours per day at days 5, 7 and 9 of culture. Between loading cycles, scaffolds were cultured in an incubator in standard conditions. Cell seeded scaffolds were assayed at day 3 and day 11 for cell proliferation by MTS assay of cell viability and collagen by Sirius red.

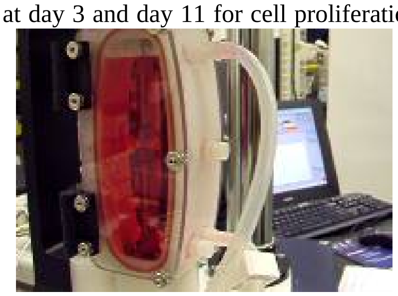


Fig. 2 Bose ElectroForce 3200 with biodynamic chamber

RESULTS: Osteoblastic cells survived in loaded scaffold, final cell number was slightly but not significantly lower in loaded samples compared with unloaded at day 11 (*Fig 3*). In contrast, collagen content increased in loaded scaffolds. Microscopy of Sirius red stained scaffolds showed much more staining in the loaded group on day 11 *(Fig 4).* Sirius red quantification indicated that in loaded samples collagen content increased by 66% between days 3 and 11, compared with only 44% in unloaded controls. The scaffold stiffness (Young's modulus) also increased in loaded samples over time (*Fig 5*).

DISCUSSION & CONCLUSIONS: The goal of this study was to develop a model to analyse the effects of mechanical stimulation on osteoblastic cells in a 3-D system, to understand how mechanical stimulation can enhance bone tissue engineering. Although the number of viable cells decreased under our loading regimen, the amount of collagen and scaffold stiffness increased, indicating increased matrix production by cells. This model has the potential to answer questions about cell survival, distribution, matrix production and stiffness in 3-D, in response to mechanical signals.

Fig 3: Cell viability over 11 days (MTS assay)

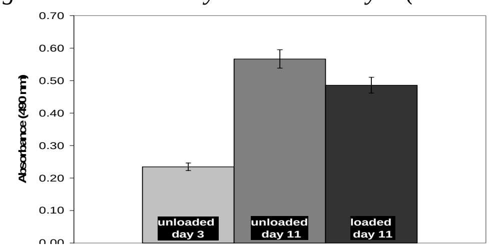


Fig. 4: Collagen on PU scaffold by using light microscopy (Sirius red staining) on day 11.

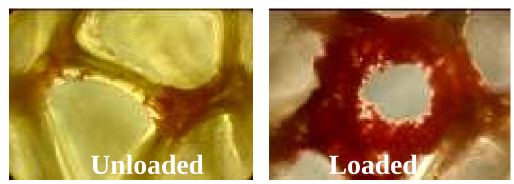
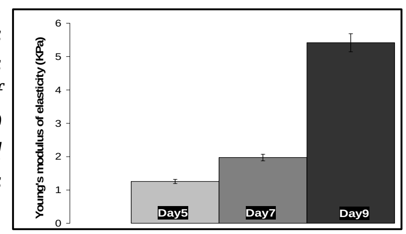


Fig 5: Stiffness (Young's modulus) of Scaffolds over 9 day on loaded specimens



REFERENCES: [1] Carter, D.R et al (1988), J. Orthop. Res. 6(5):736-748. [2] Y. Kato et al (2001), J Bone Miner Res. 16(9):1622-33.

ACKNOWLEDGEMENTS: Dr. L. Bonewald, University of Missouri at Kansas City, UA. kindly donated MLO-A5 osteoblastic cell. Bose provided use of the ElecroForce 3200 for an evaluation period. Funding for this project was provided by the Royal Society of London, The University of Sheffield and the Thai Government.

Endothelial and smooth muscle cell interactions with a PCL-PU composite vascular scaffold with potential for bioactive release.

Matthew R. Williamson[a,b], Richard Black[a,d], Cay Kielty[a,b,c]

[a] *UK Centre for Tissue Engineering*
[b] *Wellcome Trust Centre for Matrix Biology*
[c] *School of Life Science, University of Manchester, Manchester, UK*
[d] *Department of Clinical Engineering, University of Liverpool, Liverpool, UK*

INTRODUCTION: We have developed a polycaprolactone (PCL) – polyurethane (PU) composite scaffold material (Figure 1) which is a promising alternative for small diameter vascular grafts. This scaffold could provide increased compliance and thus reduce mismatch with native blood vessels. The scaffold also provides a favourable luminal surface for endothelial cell (EC) attachment, as well as a porous anti-luminal surface for smooth muscle cell (SMC) attachment and migration.

In this study, we examined how human umbilical venous endothelial cells (HUVECs) adhered to the luminal surface of the prosthesis and, for an improved functional vascular graft, SMCs were seeded to the anti-luminal surface of the prosthesis.

METHODS: The morphology and phenotype of HUVECs and SMCs was assessed on the scaffolds using environmental scanning electron and immunofluorescence microscopy. Endothelial functional behavior assays were used to assess the release of nitric oxide and von Willebrand factor (vWF) under stimulation.

The demonstration that sustained release of therapeutic proteins is possible has stimulated the development of new implantable polymers and devices which controlled delivery of growth factors (3). The scaffolds were loaded with 0.67% and 0.5% (w/w) lyophilised trypsin (25kDa) (a “model” growth factor) to assess protein release and activity in vitro over a period of 4 days.

RESULTS: The HUVECs demonstrated strong cell attachment to the scaffold, approx. 60% compared to tissue culture plastic (TCP). After 7 days of culture, the cells exhibited endothelial markers and had become confluent on the luminal surface of the scaffold. When stimulated, the HUVECs released levels of nitric oxide and vWF that were comparable TCP standard conditions in vitro.

SMC showed strong cell attachment to the PU (or anti-luminal) surface of approx. 50% this could be increased slightly by the absorption of fibronectin (FN) or fibrillin-1 RGD fragment PF8.

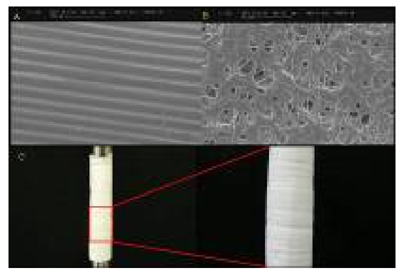


Figure 1 Scanning electron microscopy (SEM) of the 2-ply scaffolds luminal (A) and anti-luminal (B) surface topography. Luminal surface (50x) shows originated PCL fibres with a fibre to fibre gap of approx. 1-5µm. The Anti-luminal surface (400x) shows a highly porous topography with a pore size ranging from 10-30µm. (C) Digital picture of the tubular 2-ply scaffold

Release of total trypsin was only detectable at 24 hours, with a release of approximately 55%. However active trypsin release was confirmed at 24 and 48 hours due to an increase of absorption at 258nm.

DISCUSSION and CONCLUSIONS: The favourable luminal surface for HUVECs attachment coupled with the functionality of the cells and the high attachment of SMCs on the anti-luminal surface, combined with the possibility of active local release of growth factors to the newly regenerating tissue, recommend this scaffold in vascular and other soft tissue engineering.

REFERENCES: [1] Williamson et al. Biomaterials 2004;25:5053-60 [2] Kakisis et al. Journal of Vascular Surgery 2005;41:349-354 [3] Williamson et al. Biomaterials 2006;27:3608-3616

ACKNOWLEDGEMENTS: This work was funded by the UK Centre for Tissue Engineering (MRC, BBSRC, EPSRC). CMK is a Royal Society-Wolfson Research Merit Award holder.

Matthew.williamson@manchester.ac.uk

European Cells and Materials Vol. 11. Suppl. 3, 2006 (page 86) ISSN 1473-2262

On-line monitoring of cell activity in cultured tissue explants

Z. Li[1], O.A. Boubriak[2], J.P.G. Urban[2], Z.F. Cui[1]

[1] *Department of Engineering Scinece, University of Oxford, Oxford, England*
[2] *Physiology Laboratory, University of Oxford, Oxford, England.*

INTRODUCTION: Back pain is a common problem which is associated with intervertebral disc degeneration [1]. One of treatment strategies is the tissue-engineering approach, which aims to replace the damaged disc with an artificial grown disc [2]. Hence a vital problem arisen is how to on-line monitoring the cell and tissue status throughout the whole tissue during the culture period. Here we adopt a new approach to *in situ* monitor cellular activity within cultured tissue explants using the principle of microdialysis.

METHODS: Bovine caudal intervertebral discs were cultured in a perfused bioreactor [3]. Briefly the disc were clamped between two porous plates which were perfused with serum-free DMEM with static load (0.2MPa) to prevent disc swelling. Perfusion culture was continued for up to 7 days. At the start of culture, a microdialysis probe (3000 kDa cut-off) was inserted into the nucleus pulposus. The probe was perfused with HEPES to maintain pH and containing Dextran 40kDa (fluorescein) for relative recovery determination and probe perfusate was collected using a cooled fraction collector. Perfusion rate was 1µl/min. The dialysate was sampled for determination of phenol red to assess probe permeability, Dextran 40kDa for relative recovery, and lactate and glucose measurement to determine energy metabolism. The remaining dialysate was pooled daily and the samples were assayed by FPLC, SDS-PAGE electrophoresis and Western blotting to identify target soluble markers. At the end of the culture period, the discs were sectioned and cell viability determined using a live/dead assay kit. Besides, different static loads were employed to investigate the effect on the expression of target soluble markers.

RESULTS: Lactate concentration fell by 3 times but glucose concentration slight fluctuated during the first 20 hours of perfusion but then remained relatively constant for up to 7 days. Measurement of Phenol red and Dextran 40kDa indicated that there was no significant fouling of the probe during the culture period and the recovery was 8%. A few protein peaks and bands were evident on FPLC and SDS-PAGE respectively. The main peak in FPLC and the main band evident on gel was identified as bovine gp-39 using Western blotting (antibody kindly supplied by Dr. Recklies, Montreal, Canada).

DISCUSSION & CONCLUSIONS: Microdialysis probe of high MW cut-off are suitable for continuous monitoring of cell activity in disc explants, especially for high MW protein species released during the tissue metabolism. Gp-39 is a good candidate marker for monitoring disc cell protein metabolism. We were able to monitor this protein in bovine discs using microdialysis probe.

REFERENCES: [1] J.P.G. Urban, S. Roberts (2003) *Arthritis Research & Therapy* **5(3)**: 120-130. [2] H. Mizuno, A.K. Roy, C.A. Vacanti, et al (2004) *Spine* **29(12)**: 1290-1297. [3] H. Ohshima, J.P.G. Urban, D.H. Bergel (1995) *J Orthop Res* **13**:22-29.

ACKNOWLEDGEMENTS: We thank Dr A. Recklies for kindly supply the gp-39 antibody.