## Tissue Engineering

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### 3D Culture of bone-derived cells following light-triggered gelation of alginate by $Ca^{2+}$-release from photosensitive liposomes

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We have recently been investigating a system in which gelation of alginate is triggered through the release of $Ca^{2+}$ from photosensitive liposomes upon stimulation of an integral photochromic phospholipid with light of an appropriate wavelength. Ultimately it is anticipated that such a system may provide for the selective crosslinking of alginate/cell mixtures and the means to create tissue engineering constructs of specific morphology with cells entrapped in precise locations. We have previously demonstrated the feasibility of alginate gelation via this route (Smith et al 2005) and now report that this method of phototriggered gelation can be used to immobilise bone derived cells in a three-dimensional (3D) alginate matrix, with no detrimental effect on cell viability for periods of up to 10 days. Photosensitive interdigitation fusion vesicles (IFV) were prepared from dipalmitoylphosphatidylcholine (DPPC) 90% and the photosensitive lipid Bis Azo PC 10% (previously described by Bisby et al 2000), encapsulating 0.2 M $CaCl_2$ using the method described by Smith et al (2005). Initially the effect of lipid presence upon cell viability was examined by combining 100 $\mu$l of liposomes suspended in buffer (total lipid weight: 20mg/ml) with a suspension of rat bone marrow stromal cells (rBMC) at three initial seeding densities of $2.5 \times 10^6$, $1.25 \times 10^6$ and $5.0 \times 10^5$ cells/ml. Cells were immobilised within a light-triggered alginate matrix by adding 100 $\mu$l of photosensitive $Ca^{2+}$-IFV to a mixture of 0.5 ml rBMC suspension and 0.5 ml 4% alginate solution. Mixtures were irradiated with light at 375 nm from an array of three light emitting diodes (LED) for time periods equating to doses of up to 9 mJ/cm$^2$. A Live/Dead Viability/Cytotoxicity Kit (Molecular Probes, Invitrogen, USA) was employed in conjunction with fluorescence microscopy to assess rBMC survival at 3, 6 and 10 days. rBMCs immobilised within an alginate construct retained in excess of 97% viability for up to 10 days throughout the gelled structure. The depth at which cells were immobilised did not appear to influence viability, although constructs were limited to thicknesses of 2 mm in this study and future work on thicker constructs is planned. The use of wavelengths centred at 375 nm (within the UVA range) to trigger gelation did not appear to compromise rBMC viability however the DNA was not analysed following irradiation so it is impossible to indicate whether any DNA damage occurred. Additional bilayer excipients (e.g. alcohols and sterols) are currently under investigation to shift the photo-trigger to the blue light range of the spectrum (as demonstrated by Bisby et al (2000)) and avoid any potential for damage to DNA. Small deformation rheological analysis of the crosslinking mechanism is presently underway using a rheometer fitted with a quartz geometry to facilitate analysis of flow with simultaneous irradiation. It is anticipated that these studies will allow analysis of the rate of gelation on stimulation with light, accurately illustrating the onset of gelation and the mechanical properties of the resulting gel. Investigations on liposome stability and the seeding of different cell types in different positions through crosslinked alginate network are ongoing.

Bisby, R. et al (2000) *Biochem. Biophys. Res. Comm.* **276**: 169–173
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### In-vitro differentiation of mouse embryonic stem cells into pancreatic insulin producing cells

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Embryonic stem cells can differentiate into various cell types and have a strong potential to be used in cell transplantation. The in-vitro differentiation from embryonic stem cells into insulin-producing cells has been reported successfully, which offers potential for the treatment of type 1 diabetes. The aim of our study is to investigate mouse ES cells' ability to differentiate into mature islet-like clusters and further optimize the cells differentiation strategy. In our experiments, mouse ES cells were cultured on fibroblast feeder layer to maintain a un-differentiation state. They were then treated with a variety of supplements to achieve differentiation into pancreatic islet-like clusters. Differentiated cells were analyzed and characterized by various techniques. Zinc-chelating dithizone stain and immunofluorescence were employed to assess the protein expression; ELISA was used to study the insulin released by differentiation cells under various glucose stimulation conditions. RT-PCR demonstrated the gene expression during cell differentiation and flow cytometry offered the percentage of mature differentiation cells inside the whole cells cluster. Radioactive immuno-assay was finally carried out to demonstrate C- Peptide synthesis by differentiated cells in order to assess in-vitro functionality of cells. Our results demonstrate that differentiated embryonic stem cells formed pancreatic islet-like clusters, which release insulin upon glucose challenging. The dose of released insulin is dependent on the glucose challenging time and glucose concentration. Analysis of protein and gene expression showed differentiated ES cells gave positive results to insulin antibody and also the expression of endocrine genes, such as insulin, CK19 and Nkx-6.1. By using our current differentiation strategy, approximately 43% of cells were positive to insulin antibody under flow cytometry conditions. In the absence of designed supplements, the response was only approximately 12%. Each supplement involved in our differentiation protocol was assessed in order to optimize the differentiation process. Our results indicated that differentiated cells can be stored in-vitro for at least thirty days, and continually express insulin protein. Repeat glucose challenges in-vitro achieve similar insulin responses. We further investigated C-Peptide, qualitatively and quantitatively. Results showed that differentiated ES cells express C-Peptide protein after challenged with glucose. We conclude therefore that mouse embryonic stem cells can differentiated into pancreatic insulin producing cells in vitro.

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### Development of a non-viral delivery scaffold for tissue engineering applications.

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Gene therapy approaches to tissue engineering have been widely explored (Fang et al 1996; Whittlesey & Shea 2006). In tissue engineering polymer scaffolds are used to temporarily support new tissue formation. They can also serve as delivery vehicles for viral and non-viral vectors, to give a local expression of tissue inductive factors. Additionally, the scaffold can protect the DNA from the biological system until it is released. In this study we investigated the release of plasmid DNA from a poly(DL-lactic acid) ($P_{DL}LA$) polymer matrix made by using supercritical carbon dioxide ($scCO_2$) technology. To increase transfection efficiencies the plasmid was condensed with a non-viral vector, a polyamidoamine cationic polymer (PAA) made from methylene-bisacrylamide and dimethylethylenediamine monomers. There is a need to lyophilise the complex in order to make the bioactive porous scaffold using $scCO_2$. However, following freeze-drying a significant loss of transfection efficiency was observed. Levels could be restored when the cryoprotectant trehalose was added to the complex-solution prior to freeze-drying. A549 cells were seeded onto the scaffolds and cultured for 48 h. Staining the scaffold with toluidine blue and propidium iodide showed that A549 cells were evenly distributed along the scaffold. In initial studies plasmid (8 $\mu$g) encoding the firefly luciferase reporter gene (gWIZLuc) was complexed with PAA. Luciferase detection was performed 48 h after seeding of the scaffold with cells. The control conditions included blank scaffolds (no delivered DNA), scaffolds encapsulating plasmid DNA (non-condensed i.e. naked) and a scaffold containing a Lipofectamine complexed plasmid. This study showed the plasmid was still active after $scCO_2$ processing and able to transfect cells, although the levels were low. Cell seeding density was optimised to increase transfection levels. According to the literature higher amounts of plasmid should be incorporated into the polymer scaffold to construct a controlled release system. A long-term study was performed to look at the delivery of the plasmid from the $P_{DL}LA$ scaffold. Transfection levels from the PAA/DNA containing scaffolds were sustained over a 60-day study period and were higher than the naked DNA control. Transfection levels of the Lipofectamine complexed plasmid were higher than the PAA complex but levels dropped to 0 after day 40. The results reported in this study showed the successful incorporation of a non-viral DNA delivery system into a $P_{DL}LA$ scaffold made by $scCO_2$ processing. The incorporated plasmid is released and capable of cell transfection over a 60-day period.

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Whittlesey, K. J., Shea, L. D. (2006) *Biomaterials* **27**: 2477–2486

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## Development of an injectable scaffold for application in regenerative medicine to deliver stem cells and growth factors

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The clinical need for synthetic scaffolds challenges tissue engineering to develop suitable materials for bone regeneration and their integration with surrounding tissue. The properties of the scaffold such as porosity and mechanical strength have to meet the needs of the implant site. The compressive strength properties of cancellous bone vary with density 10–80MPa (Gibson & Ashby 1997). Scaffold implants usually require invasive surgery for placement at the desired site and sculpting to fit the defect void. We propose a particulate scaffold delivery system compatible with arthroscopic minimally invasive surgical techniques, to inject a mixture of structural components, cells and growth factors to fill the defect. We hypothesize that the in situ assembly of the components can be triggered by normal body temperature forming a self supporting, porous biodegradable 3-dimensional scaffold. Our approach uses a two component system comprising of temperature insensitive microsphere and temperature responsive microparticle based on poly(DL-lactic acid) (PLA) and copolymer poly(DL-lactic-co-glycolic acid) 50:50 (PLGA), under normal body conditions. The microsphere surfaces act as the transplant vehicle and function to anchor cells, promote spreading and deliver growth factors. Microspheres are made using water in oil emulsion solvent evaporation (Jeyanthi et al 1996) method utilizing poly(vinyl alcohol) to improve hydrophilicity. Temperature sensitive microparticles function as adhesive components and are manufactured by heat fusion with poly(ethylene glycol) (PEG). By manipulation of composition we can control temperature induced changes below and above body temperature. Modulated differential scanning calorimetry with a TA 2920 instrument was used to identify thermal transitions in the region of interest. Modulation was conducted at 1°C heating rate for a period of 60 s and amplitude ± 0.159°C. Oscillatory rheological techniques were conducted on an Anton Paar Physica MCR 301 rheometer with parallel plates and Peltier temperature controlled hood over the temperature range 4–90°C at a heating rate of 1°C per minute to monitor the bulk physical response of the material to thermal changes. The loss modulus was used to determine the glass transition region and Tan delta the physical viscoelastic response of the material. The strength of the scaffold formed was determined on a TA.XT plus texture analyzer. Using these techniques a range of compositions were evaluated for temperature sensitivity and the strength of association formed. We can reproducibly initiate sintering of the scaffold from 25 ± 3°C maintaining mild conditions for the protection of functional proteins and cells. Scaffold strength was demonstrated to increase with lower PEG molecular weight. The increased viscoelastic behavior correlated with increased strength. We demonstrate that optimization of the components can produce scaffold strengths compatible with cancellous bone and has implications for the method of application.

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