ABSTRACT
Electrospun Nanofibrous Scaffold for Skin Tissue Engineering
Dhakshinamoorthy Sundaramurthi
Dr. Swaminathan Sethuraman

Developing a potential skin graft to treat full-thickness skin damages like traumatic wounds, burns and diabetic wounds continues to be a challenging task since the currently available skin grafts does not meet the clinical demands. Autologous skin grafting or split-thickness skin grafting (SSG) is the “gold standard” protocol to treat full-thickness skin injuries. However, SSGs suffer various disadvantages such as limited graft availability, creation of secondary wound site and scar formation. A scan of the literature reveals that there are wide amount of research being carried out to fabricate tissue engineered skin substitutes to replace SSGs. Nanofibers made of biodegradable polymers could be effective in promoting skin regeneration owing to the structural similarity with native ECM, high surface area-to-volume ratio, desirable porosity and mechanical strength. The present study aims to develop electrospun nanofibrous scaffold and to test its in vitro, in vivo and clinical efficacy in promoting skin regeneration. Chitosan (C)–poly(vinyl alcohol) (PVA) blend nanofibers were made using electrospinning technique. Several parameters such as solvent ratio, polymer concentration, applied voltage, flow rate, and tip-to-target distance were optimized to achieve defect-free morphology. C-PVA nanofibers were characterized and compared with 2D C–PVA films. The stability of the C-PVA nanofibers and 2-D films were improved by glutaraldehyde vapor crosslinking. The physico-chemical properties like tensile strength, water contact angle and porosity of the C-PVA nanofibers and 2-D films were analyzed. It was observed that C-PVA
nanofibers were hydrophilic and also possesses tensile strength, porosity suitable for skin tissue engineering. The *in vitro* characterization of the C–PVA nanofibers and 2D films were investigated using mouse 3T3 fibroblast cells. *In vitro* results have demonstrated that C-PVA nanofibers favor the adhesion and proliferation of mouse 3T3 fibroblast cells. Collagen I, collagen III and elastin gene expression of the mouse 3T3 fibroblast cells cultured on C–PVA nanofibers and 2D film were evaluated after one and seven days of culture. Collagen I expression was significantly higher on C-PVA nanofibers after 7 days of culture. Collagen III and elastin expression were down-regulated after 7 days. The *in vitro* results have demonstrated the suitability of C-PVA nanofibers for skin tissue engineering. The *in vivo* potential of C-PVA nanofibers were tested in full-thickness wound created in a rat model. Percentage wound healing was complete in C-PVA nanofibers treated group when compared to positive (bactigras®) and negative control (no treatment) after 7 and 14 days post-surgery. Further, biochemical parameters of the wound bed like collagen synthesis, total protein, catalase activity and superoxide dismutase (SOD) activity were evaluated. Catalase and SOD activity was higher in C-PVA nanofibers treated group while collagen and total protein level were comparable between the groups after 7 and 14 days post-surgery. Human peripheral blood lymphocytes were cultured on C-PVA nanofibers to test its biocompatibility. Inflammatory cytokines production and gene expression were evaluated after 24, 48 and 72 hours of culture. There was higher production of inflammatory cytokines and up-regulation of lymphocyte activation genes after 72 hours of culture. The *in vitro* and *in vivo* results demonstrated that C–PVA nanofibers can be used as dermal substitute.
However, C-PVA nanofibers triggers inflammatory cytokines production in human peripheral lymphocytes and hence not suitable for clinical testing.

Poly-3-(hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) is a biodegradable and biocompatible polymer with sufficient elasticity and oxygen permeability that makes it a suitable skin scaffold. PHBV is a natural, biodegradable polymer with no adverse reactions, degradation rates in between PLLA and PLGA, elastic nature, good oxygen permeability which made it most researched polymer for various biomedical applications.

PHBV was electrospun to form defect-free nanofibers. The diameters of the PHBV nanofibers were in the range of 583-724 nm. PHBV was also solvent-cast to form 2-D films, and its mechanical properties, porosity, and degradation rates were compared with PHBV nanofibers. Results demonstrated that PHBV nanofibers exhibited higher porosity, increased ductility, and faster degradation rate when compared with PHBV 2-D films (p < 0.05). Freshly isolated peripheral blood lymphocytes were cultured on PHBV nanofibers and the inflammatory cytokines production, expression of lymphocyte activation genes like TRA 1, nRap 2, RAD 51, β-Tim and HLA-DRB were evaluated. T Lymphocyte activation and IL-2 mediated lymphocyte activation genes were down-regulated after 48 and 72 hours of culture. After 24, 48 and 72 hours of culture there was no inflammatory cytokines production by the cultured lymphocytes. Thus, these in vitro results confirm the biocompatibility of PHBV nanofibers and suggest that consideration can be given to the use of PHBV nanofibers for skin tissue engineering applications.

*In vitro* studies with PHBV nanofibers and 2-D films were carried out to evaluate the adhesion, viability, proliferation, and gene expression of human skin fibroblasts. Cells
adhered and proliferated on both PHBV nanofibers and 2-D films. However, the proliferation of cells on the surface of PHBV nanofibers was comparable to tissue culture polystyrene (TCPS, control). The gene expression of collagen I and elastin were significantly up-regulated when compared with TCPS control, whereas collagen III was down-regulated on PHBV nanofibers and 2-D film after 14 days in culture. The less ductile PHBV 2-D films showed higher levels of elastin expression. Native morphology and gene expression was better in PHBV nanofibers when compared to PHBV 2D film. Hence, PHBV 2D-film was skipped for further analysis. The potential of PHBV nanofibers to support the human keratinocytes (HaCaTs)(epidermal cells of skin) was investigated in vitro. PHBV nanofibers favor HaCaT adhesion and proliferation. After 14 days of culture, loricrin and keratin-1 gene expression were significantly higher when compared to 3 and 7 days (p <0.05).

Furthermore, the PHBV nanofibers were evaluated for their full-thickness wound healing capacity in a rat model. Percentage wound healing was higher in PHBV nanofibers treated group after 14 days post-surgery (p < 0.05). Furthermore, the presence of nanofibers promoted an increase in collagen and aided re-epithelialization. Thus these results demonstrate that the nanofibrous topography and mechanical stimuli have a pronounced influence on the cell proliferation, gene expression, and wound healing.

The clinical efficacy of PHBV scaffolds in treating full-thickness skin damages like traumatic wounds, surgical wounds, diabetic wounds and burns were investigated through an interventional clinical trial after obtaining the necessary ethical clearance (Approval number: 02/2013/TMC/IEC/TNJ) and trial registration (Registration number:}
CTRI/2013/11/004141). To the best of our knowledge, we are the first research group to conduct an interventional clinical trial to investigate the potential of PHBV nanofibers in the reconstruction of various skin damages requiring autologous graft. A total of 52 patients who satisfied the inclusion and exclusion criteria of the trial protocol were recruited for the clinical trial. Among the 52 subjects recruited for the trial, 43 had traumatic wounds, 2 burn injuries, 2 diabetic wounds, 3 autologous graft failures, 1 non-healing ulcer and 1 wound due to amputated toe (Thromboangiitis obliterans). None of the subjects treated with PHBV nanofibers reported to have any adverse reactions (itching, burning, swelling/inflammation) in the wound bed. PHBV nanofibers exhibited comfort to the wound bed after the application of PHBV graft. No patient had trauma while the PHBV nanofibers were removed from the wound bed during the study period. Traumatic wounds treated with PHBV nanofibers exhibited more than 90% healing within 40 days of treatment. Histopathology results confirmed normal re-epithelialization with abundant angiogenesis. PHBV nanofibers also promote skin regeneration in burn wounds, diabetic wounds, non-healing ulcers and wounds with poor vasculature (thromboangiitis obliterans). More importantly, PHBV nanofibers were also found to accelerate skin regeneration in open surgical wounds and traumatic wounds that failed to respond to autologous graft. Interestingly, there was minimal scar formation in all the treated wounds. Percentage healing in PHBV treated wounds were significantly higher when compared to povidone-iodine treatment (p<0.05). In conclusion, the different studies that have been carried out in this thesis to study the suitability of PHBV nanofibers have shown that this nanofibrous scaffold possess excellent prospects for skin
tissue engineering. Overall, this next generation biomaterial may be a promising skin graft in the armamentarium to treat skin damages.