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Sandwich-type fiber scaffolds with square arrayed microwells and nanostructured cues as microskin grafts for skin regeneration

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ABSTRACT

The paper reports the fabrication of sandwich-type scaffolds consisting of radially-aligned nanofibers at the bottom, nanofiber membranes with square arrayed microwells and nanostructured cues at the top, and microskin tissues in between as microskin grafts for use in skin regeneration. This class of nanofiber scaffolds was able to confine the microskin tissues in the square arrayed wells and simultaneously present nanotopographic cues to the cultured NIH 3T3 fibroblasts and primary rat skin cells, guiding and facilitating their migration *in vitro*. More importantly, we demonstrated that the sandwich-type transplants exhibited an even distribution of microskin grafts, greatly improved the 'take' rate of microskin tissues, and promoted re-epithelialization on wound *in vivo*. In addition, the void area in the scaffolds was well suitable for exudate drainage in wound. The sandwich-type scaffolds show great potential as microskin grafts for repairing extensive burn injuries and may provide a good solution for the treatment of acute skin defects and chronic wounds including diabetic ulcer, pressure ulcer, and venous stasis ulcer.

1. Introduction

Burn injuries requiring treatment occur in 500,000 patients and cause about 3500 deaths per year in the United States [1]. There are currently over 2000 cases annually involving burns over 50% of the patient's total body surface according to American Burn Association [1]. Mortality and morbidity from burns, trauma, and other skin loss injuries remain significant medical and socio-economic problems estimated to cost more than $1 billion annually in treatment costs and lost productivity [2]. Over the past three decades, extraordinary advances and improved understanding in cell/molecular biology have led to achievements in skin tissue regeneration for wound healing [3]. Skin tissue engineering products involving cultured epithelial autograft (CEA) are believed to be a promising solution to provide permanent wound coverage and achieve healing for extensive burned patients with very limited donor sites [4]. It is now possible to obtain large amounts of cultured epithelium from a small skin biopsy. Within 3–4 weeks, a 3-cm^2 biopsy can be expanded more than 5000–10,000 folds to yield enough skin to cover the body surface of an adult [5–7]. But the detachment of CEAs sheets by Dispase results in a significant loss of clonogenic cells which have significant growth potential and are sole resource of re-epithelialization for wound healing [8]. In addition, the use of CEAs has been generally hampered by delays in obtaining the grafts, handling difficulties due to their extreme friability, significant contraction, varied taking rates, sensitivity to infection, fragility after taking, and high cost [9]. The allografts are associated with the limited abundance and availability of donors, possible transmission of disease, the eventual rejection by the host and its handling storing, transporting and costs of provision [10].

Excision of the burned area and grafting with autologous split thickness skin grafts (STSG) is still the gold standard for the current treatment of burn skin injury over large areas and remains the mainstay of treatment to provide permanent wound coverage and achieve healing [11–13]. Mesh skin grafts and MEEK skin grafts are widely applied STSG techniques to treat burn injuries with limited donor sites around the world [14,15]. However, mesh skin grafts usually exceeds the available unburned skin (donor sites) for burn-injured patients over large areas due to its low expansion ratio (1:6). Although the expansion ratio for MEEK grafts has been increased to 1:9, further increase of expansion ratio is hampered by the size of skin graft ($>3 \times 3$ mm^2) and fixed distances between grafts [16]. MEEK grafts are also required to tailor skin grafts in regular square shape, resulting in partial loss of harvested skins which are very rare for extensively burned patients.

The large expansion ratio for skin grafts is critical to cover wound over large areas with a small area of skin. The practical strategy for STSG skin grafts in extensive burns is to decrease the

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size of grafts (microskin) and to enlarge the distance between adjacent graft islands to a greater degree. Previous studies demonstrated that orientation of microskin tissues has marginal influence on skin grafts 'take' when skin tissue islands are smaller than 1 mm^3 [17–19]. Our recent studies developed two new assemblies of electrospun nanofibers: nanofiber membranes with arrayed microwells and controlled structural cues on the surface and nanofiber scaffolds consisting of radially-aligned nanofibers [20,21]. However, when the distance between the two adjacent metal beads was larger than 3 mm, the aligned nanofibers tended to adhere to the substrate between beads using the collector reported in our previous work [20]. Toward this end, a new collector was designed in this study, which can overcome the above issue and was thus used to generate nanofiber scaffolds with larger distances (>3 mm) between two adjacent microwells as the simple way for increasing expansion ratio is to enlarge the distance between the skin tissue islands.

In this study we presented a class of nanofiber skin grafts which was constructed in the form of a 'sandwich': radially-aligned nanofiber scaffolds at the bottom, nanofiber scaffolds with square arrayed microwells and structural cues at the top, and microskin tissue islands seeded in microwells in between. This nanofiber skin grafts achieved with a combination of tissue engineering strategy (e.g., making use of scaffolds) and current viable clinical approach (e.g., 'gold-standard' autologous skin micrograft) simultaneously presented the following unique features: *i)* nanotopographic cues (direct and facilitate cell migration which is not available in the current bioengineered skin products); *ii)* square arrayed microwells (confine skin islands with a uniform distribution, resulting in better cosmetic appearance after wound healing); *iii)* large expansion ratio (smaller donor sites needed to cover a large wound area); *iv)* permanent (not a temporary coverage), *v)* immediate availability and ease of operation (no delay for the treatment and adhere very well to the wound and thus prevent microskin grafts loss during transplantation which usually occurs in traditional skin grafts on extensive burns); and *vi)* biosafety (biocompatible materials and autologous tissue without immune rejection) [22]. We chose poly(ε-caprolactone) (PCL) for this study because it can provide the desired biomechanical properties and retain a controllable biodegradability *in vivo* from several weeks to months by incorporating some enzymes [23]. The degradation products of PCL are nontoxic and can be eliminated from the body in the form of carbon dioxide and water [24].

2. Materials and methods

2.1. Fabrication of electrospun nanofiber scaffolds

In a typical procedure for electrospinning PCL (M_w = 70–90 kDa, Sigma–Aldrich) nanofibers, we used a solution of 10% (w/v) PCL in a mixture of dichloromethane (DCM) and N, N-dimethylformamide (DMF) (Fisher Chemical) with a volume ratio of 4:1. The nanofibers were spun at 10–17 kV with a feeding rate of 0.5 mL/h, together with a 23 gauge needle as the spinneret. The membranes with square arrayed microwells and structural cues were fabricated using a modified collector which is constructed from stainless steel beads with a diameter of 1.58 mm capped rods which were arranged in a square array and the distances between adjacent beads were 2 mm, 3 mm and 6 mm, respectively. The membranes with square arrayed flat wells and structural cues were fabricated using a modified collector composed of flat surface with a diameter of 1.58 mm capped stainless steel pins. The pins were arranged in a square array and the distances between adjacent pins were 3 mm. Aligned nanofibers were collected using a high speed rotating mandrel with rotating speed of 20,000 rpm. Random nanofibers were collected using a piece of aluminum foil. The nanofiber membranes made of either uniaxially aligned or random nanofibers with square arrayed wells were created on aligned and random nanofibers by gently pressing the bead collector on the surface of nanofiber membranes. Radially-aligned nanofiber scaffolds were fabricated utilizing a collector consisting of a ring electrode (e.g., metal ring) and a point electrode (e.g., a sharp needle) according to our previous study [21]. For *in vivo* study, nanofiber scaffold samples were treated with plasma in air for 5 min using a plasma cleaner (Harrick Plasma, USA). The nanofiber scaffolds were sterilized by soaking in 70% ethanol overnight and left to dry in a biosafety cabinet prior to implantation *in vivo*.

2.2. Characterization of nanofiber scaffolds

The morphologies and structures of nanofiber scaffolds were characterized by SEM (200 Nanolab, FEI, Oregon). To avoid charging, the PCL nanofiber scaffolds were coated with gold using a Sputter coater for 40 s in vacuum at a current intensity of 40 mA after the scaffolds had been fixed on a metallic stud with double-sided conductive tape. The accelerating voltage was 15 kV for the imaging process.

2.3. NIH 3T3 fibroblast cell culture

NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% gentamycin/streptomycin (Invitrogen) at 37 °C in an atmosphere of 95% air/5% CO_2. Cell culture medium was replaced every 2 days.

2.4. Isolation and culture of primary rat skin cells

Skin cells were isolated from skin tissues explanted from Lewis Rats (Hilltop Lab Animals, Inc., USA). One full-thickness, 2-cm diameter circular skin excision wounds were created on each side of the dorsal surface using a 2-cm diameter circular skin biopsy punch and then using an Iris scissor. Panniculus carnosus was removed from harvested skin tissues. Part of the harvested skin tissues was fragmented into 1-mm diameter microskin by a 1-mm diameter skin biopsy punch and transplanted to contralateral wound. The skin cells were isolated from the left skin tissues. Specifically, the dermis was first isolated from the epidermis with scalpels and scissors. Then dermis specimens were fragmented into 4 mm^2 skin pieces. These skin pieces were cultured in a 100-mm^2 petri dish containing 10 mL of DMEM with 20% FBS, 1% penicillin/streptomycin. The culture dish was maintained in a humidified incubator at 37 °C in an atmosphere of 95% air/5% CO_2 and the culture medium was changed every two days till reaching confluence. Skin cells were then plated in 75 cm^2 flasks and expanded (subpassaged no more than five times).

2.5. NIH 3T3 fibroblast and primary rat skin cell migration and repopulation

Prior to cell seeding on fiber scaffolds, cells were trypsinized and counted. Cells were suspended in a desired density in DMEM supplemented with 10% calf serum and 1% penicillin and streptomycin. One μL of cell suspension was carefully seeded into each microwell of scaffolds fixed in a culture dish. Then, the culture dish was placed in the incubator for 2 h followed by adding of culture medium. For sandwich-type scaffolds, the radially-aligned nanofibers were placed on the surface of microwell scaffolds after cell seeding and then fixed by a sterilized polypropylene ring placed on the top. The culture dish was maintained in a humidified incubator at 37 °C in an atmosphere of 95% air/5% CO_2. The cells were cultured for 3, 7, 14, and 21 days, stained with FDA and imaged with fluorescence microscope. Fluorescent images were taken using a QICAM Fast Cooled Mono 12-bit camera (Q Imaging, Burnaby, BC, Canada) attached to an Olympus microscope with OCapture 2.90.1 (Olympus, Tokyo, Japan). The area fraction defined by the ratio between the surface area occupied by cells and the surface area of scaffolds was quantified using Image J software (National Institute of Health).

2.6. Rat skin injury model and sandwich-type nanofiber scaffold implantation

Male Lewis rats (Hilltop Lab Animals, Inc., USA) weighting 250–300 g were used for this study. All surgical procedures and perioperative care measures were performed in strict accordance with the National Institutes of Health Guidelines and were approved by Marshall University Animal Studies Committee. Nine animals were randomized into three groups ($n = 3$) for three time points (7, 14, and 21 days). Anesthesia was performed with an intraperitoneal injection of ketamine (75 mg/kg) and xylazine (25 mg/kg). The hairs on back were removed using an electric shaver. The surgical site was washed with povidone–iodone (Betadine) soap and solution. The area was draped in an aseptic fashion. One 2-cm diameter circular full-thickness skin excision extending through the panniculus carnosus was created on each side of the dorsal surface using a 2-cm diameter circular skin biopsy punch and an Iris scissor. The harvested skin tissues were fragmented into 1-mm diameter microskin tissues and then seeded into each microwell of membranes. Radially-aligned nanofibers were laid on the surface of microskin tissue-seeded membranes to form sandwich-type nanofiber skin grafts. These grafts were applied to the wound with radially-aligned fibers facing the wound bed. Wounds were covered by Gauze Pads (Johnson & Johnson Consumer Products Companies, Inc., USA) which were fixed by Rat Jackets (Harvard Apparatus, USA). Self-Adherent Gentle Wrap (CVS Pharmacy, Inc., USA) was additionally applied to prevent the removal of dressings. The wounds covered with petrolatum gauze were taken as control. Post-operative antibiotics (Neosporin) and analgesic (Buprenex 0.03 mg/kg, administered subcutaneously) were given to minimize the chance of infection and discomfort experience.