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Engineering multi-layered skeletal muscle tissue by using 3D microgrooved collagen scaffolds

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ABSTRACT

Preparation of three-dimensional (3D) micropatterned porous scaffolds remains a great challenge for engineering of highly organized tissues such as skeletal muscle tissue and cardiac tissue. Two-dimensional (2D) micropatterned surfaces with periodic features (several nanometers to less than 100 μm) are commonly used to guide the alignment of muscle myoblasts and myotubes and lead to formation of pre-patterned cell sheets. However, cell sheets from 2D patterned surfaces have limited thickness, and harvesting the cell sheets for implantation is inconvenient and can lead to less alignment of myotubes. 3D micropatterned scaffolds can promote cell alignment and muscle tissue formation. In this study, we developed a novel type of 3D porous collagen scaffolds with concave microgrooves that mimic muscle basement membrane to engineer skeletal muscle tissue. Highly aligned and multi-layered muscle bundle tissues were engineered by controlling the size of microgrooves and cell seeding concentration. Myoblasts in the engineered muscle tissue were well-aligned and had high expression of myosin heavy chain and synthesis of muscle extracellular matrix. The microgrooved collagen scaffolds could be used to engineer organized multi-layered muscle tissue for implantation to repair/restore the function of diseased tissues or be used to investigate the cell–cell interaction in 3D microscale topography.

1. Introduction

Skeletal muscle tissue malfunction or loss can be caused by inflammatory muscle diseases, aging of muscle and sports injuries [1–3]. In skeletal muscle tissue, muscle fibers which are surrounded by basement membrane are highly aligned and assembled into organized muscle bundles to achieve muscle functions [4,5]. To engineer such well-organized tissue for tissue/organ repair, two-dimensional (2D) micropatterned surfaces with periodic features (several nanometer to less than 100 μm) have been fabricated by various methods, such as aligned nanofiber by electrospinning [6–8], groove/ridge micro- and nanopatterns by photolithography or spin coating [9–13], aligned extracellular matrix (ECM) molecules or cell-adhesive polymer by contact printing [14,15]. The 2D micropatterned surfaces can guide alignment of muscle myoblasts and myotubes from fusion of myoblasts and lead to formation of pre-patterned cell sheets [16–19]. However, cell sheets from 2D patterned surfaces have limited thickness, and harvesting the cell sheets for implantation is inconvenient and can lead to less alignment of myotubes. Three-dimensional (3D) micropatterned scaffolds that guide cell alignment and tissue formation are desirable to engineer the organized skeletal muscle tissue.

3D micropatterned porous collagen scaffolds with well controlled pore structures can be prepared via liquid dispensing and freeze-drying [20]. In this study, we used the water dispensing and freeze-drying to prepare 3D microgrooved collagen scaffolds for skeletal muscle tissue engineering (SMTE). With this method, we could use extracellular matrix (ECM) components such as collagen to prepare 3D micropatterned scaffolds for tissue engineering and the concave structure of microgrooves to mimic the

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tubular structure of muscle basement membrane which surrounds each muscle fiber. Muscle basement membrane is mainly composed of ECM molecules such as collagen and laminin and has a tubular shape, which promotes muscle development and myogenesis and maintains muscle integrity [21–23]. While many studies have used scaffolds of synthetic polymer and sharp-edged micropatterns for SMTE [24–30], we tried to use the 3D collagen scaffolds with large concave microgrooves (120–380 μm) to mimic the ECM environment and topographical characteristic of muscle fibers. Another advantage of the 3D micropatterned scaffolds over 2D micropatterned surfaces is that the engineered organized muscle tissue in 3D scaffolds can be directly implanted for tissue repair. By culturing rat L6 skeletal myoblasts in the 3D microgrooved collagen scaffolds we developed multi-layered muscle bundle tissue in which myoblasts aligned and formed myotubes. The formation of aligned muscle bundles from L6 myoblasts was dependent on the size of microgrooves and cell seeding concentration. The 3D structural micropatterns have potential to repair organized tissues or organs which is hard to achieve via traditional methods or be used to investigate cell–cell interaction in 3D microscale topography [26,31–33].

2. Materials and methods

2.1. Scaffold preparation

Perfluoroalkoxy film (PFA film, Universal Co., Ltd) was wrapped on a copper plate which was cooled by liquid nitrogen. Dispensing nozzle of a jet dispenser (MJET-3-CTR, Musashi Engineering Inc.) which was controlled by a SHOT mini 200α (Musashi Engineering Inc.) moved back and forth over the PFA film-wrapped copper plate and ejected water droplets that sequentially fell onto the cooled plate. The water droplets were immediately frozen and formed continuous frozen ice lines (Fig. 1a,b). CAD programs were used to control the movement of dispensing nozzle which enabled formation of aligned frozen ice lines on the plate. The width of frozen ice lines was controlled by the pressure (0.001 MPa) that pumped water out of the nozzle and different types of nozzles (32-35G). The temperature of the copper plate bearing frozen ice lines was balanced to −5 °C, and the plate was covered with a silicon frame (thickness: 500 μm). Cooled type I collagen aqueous solution (1 (wt/v)% in a mixture of ethanol and pure water (10:90 v/v, pH 3.0), −5 °C) (Nitta Gelatin Inc.) was cast onto the frozen ice lines and covered with a glass plate. The whole construct was frozen in liquid nitrogen and freeze-dried to get a 3D microgrooved scaffold. Different scaffolds (G120, G200 and G380) were prepared by using frozen lines of different widths. Scaffolds with a flat surface as a control group were prepared without using ice lines. All scaffolds were cross-linked using a solution of 50 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and 20 mM N-hydroxysuccinimide at room temperature for 12 h [34], rinsed with pure water and freeze-dried.

2.2. Scanning electron microscopy and porosity measurement

Scaffold samples were cut using sharp blades, fixated using carbon tape, sputter-coated with platinum and observed with a scanning electron microscope (SEM) at the acceleration voltage of 10 kV (JSM-5610, JEOL, Ltd.). The SEM images were imported in ImageJ software to analyze the widths and depths of scaffolds. 4 images of each type of samples were analyzed and at least 6 microgrooves in each image were analyzed. The porosity of different kinds of scaffolds was measured using the method as reported previously [35].

2.3. Cell culture in scaffolds

Rat L6 skeletal muscle myoblasts (American Type Culture Collection) were subcultured in Dulbecco's Modified Eagle's Medium (D8437, Sigma–Aldrich) supplemented with 10% fetal bovine serum and 10% horse serum and 1% penicillin/streptomycin. Microgrooved scaffolds were punched into disks (diameter: 10 mm), sterilized in 70% ethanol, rinsed in phosphate buffered

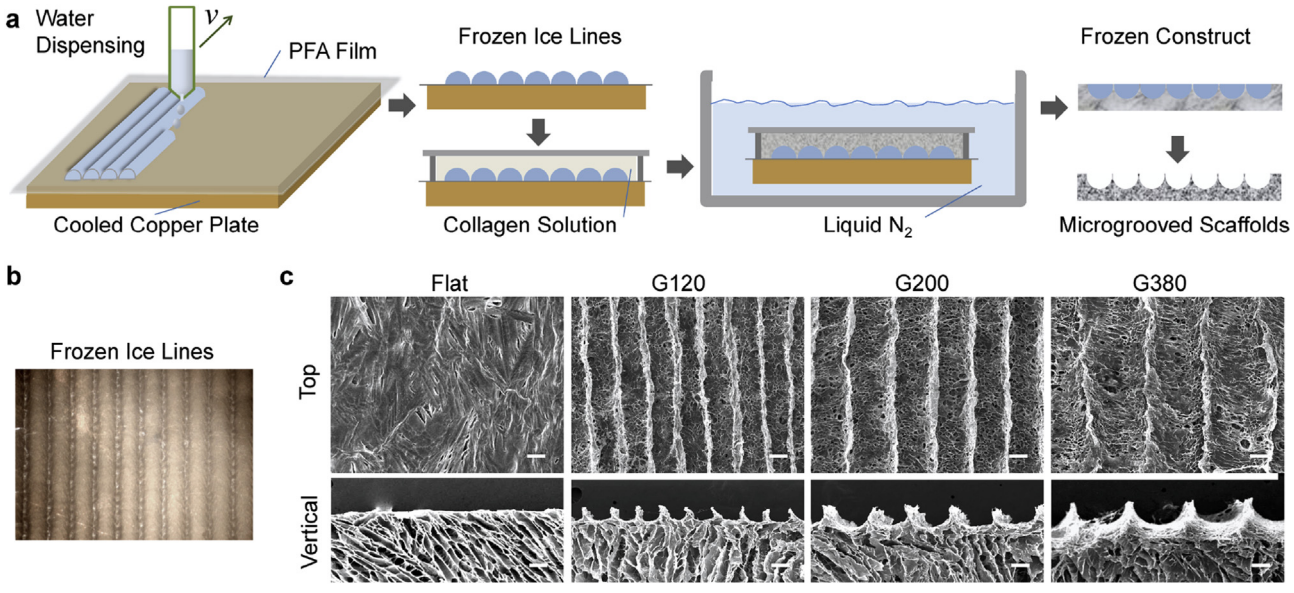

Fig. 1. Preparation of microgrooved collagen scaffolds. (a) A schematic for the preparation of microgrooved collagen scaffolds. (b) Image of frozen ice lines prepared from water dispensing. (c) SEM images of different microgrooved collagen scaffolds. Flat: control collagen scaffolds with a flat surface; G120, G200, G380: collagen scaffolds with mean microgroove widths of 120, 200, 380 μm, respectively. Upper images show the top view and lower images show the vertical cross-sectional view of different scaffolds. Scale bar = 100 μm.