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In vivo bone generation via the endochondral pathway on three-dimensional electrospun fibers

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ABSTRACT

A new concept of generating bone tissue via the endochondral route might be superior to the standard intramembranous ossification approach. To implement the endochondral approach, suitable scaffolds are required to provide a three-dimensional (3-D) substrate for cell population and differentiation, and eventually for the generation of osteochondral tissue. Therefore, a novel wet-electrospinning system, using ethanol as the collecting medium, was exploited in this study to fabricate a cotton-like poly(lactic-co-glycolic acid)/poly(ε-caprolactone) scaffold that consisted of a very loose and uncompressed accumulation of fibers. Rat bone marrow cells were seeded on these scaffolds and chondrogenically differentiated in vitro for 4 weeks followed by subcutaneous implantation in vivo for 8 weeks. Cell pellets were used as a control. A glycosaminoglycan assay and Safranin O staining showed that the cells infiltrated throughout the scaffolds and deposited an abundant cartilage matrix after in vitro chondrogenic priming. Histological analysis of the in vivo samples revealed extensive new bone formation through the remodeling of the cartilage template. In conclusion, using the wet-electrospinning method, we are able to create a 3-D scaffold in which bone tissue can be formed via the endochondral pathway. This system can be easily processed for various assays and histological analysis. Consequently, it is more efficient than the traditional cell pellets as a tool to study endochondral bone formation for tissue engineering purposes.

1. Introduction

Bone lesions and defects present a significant problem in clinics due to the limited self-healing capacity of bone tissue. Therefore, the use of a biodegradable scaffold seeded with multipotent cells and biological cues offers a promising approach for bone repair and regeneration [1]. Since the introduction of this concept, experiments to engineer bone tissue have primarily focused on a process resembling intramembranous ossification, i.e. direct osteoblastic differentiation [2]. However, the success of this approach is hindered by poor vascularization inside the entire cell-based construct [3]. In line with skeleton development and bone fracture healing, a new tactic has been reported recently that is based on mimicking endochondral bone formation. In this new approach, the bone is generated after an intermediate cartilage stage instead of direct osteoblastic differentiation [4–7]. The rationale behind this approach is that chondrocytes are able to survive with limited nutrition and oxygen. Secondly, they can secrete vascular endothelial growth factor (VEGF) in the hypertrophic stage, which is beneficial for blood vessel in-growth [8]. As a consequence, this route has the potential to circumvent the problem faced by conventional bone tissue engineering: that, upon implantation, the constructs are cut off from the supply of nutrition and oxygen.

The cell pellet culture system is the technique that is used most frequently to study this endochondral pathway [9]. The cell pellet system provides a three-dimensional (3-D) environment and allows cell–cell interactions, which are similar to pre-cartilage condensation during embryonic development. However, this model is not suitable for the regeneration of bone tissue because of limitations in the size and quantity, and the lack of mechanical stability [10]. As a consequence, a scaffold material must be used for the final clinical application of this endochondral pathway; this can serve as a carrier for the cells to provide a 3-D environment as well as mechanical support. In this way, robust cell proliferation and differentiation can be achieved, which leads to the formation of a proper extracellular matrix (ECM) and eventually functional tissue.

Tremendous efforts have been made in the development of bone supporting scaffolds with different compositions and 3-D configurations using a wide variety of techniques, among which electrospinning has recently gained popularity. Electrospinning, also named electric spinning, is known as a simple and versatile method to produce fibrous polymeric meshes [11]. With the range of tens of nanometers to a few microns in diameter and the form of

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a non-woven structure, electrospun fibers are morphologically similar to natural ECM, which makes them attractive for cells to populate on and to function effectively [12,13]. However, a scaffold made by the conventional electrospinning process is mostly composed of a compact fiber network; thus it is very difficult for the cells to penetrate into the internal part of the scaffold [14,15]. Such a scaffold cannot be considered a 3-D structure as the cells only populate on the superficial surface.

To address the aforementioned issues, the aim of this study was to fabricate a 3-D scaffold using a modified electrospinning technique, and to further test its efficacy in endochondral bone formation. To this end, the newly developed scaffolds were seeded with rat bone marrow cells (RBMCs), followed by in vitro chondrogenic differentiation and in vivo subcutaneous implantation. It was hypothesized that the RBMCs can generate bone in vivo on the 3-D electrospun scaffolds via the endochondral pathway.

2. Materials and methods

2.1. Fabrication of electrospun scaffolds

Poly(lactic-co-glycolic acid) (PLGA, Purasorb® PDLG 5010) and poly(ε-caprolactone) (PCL, LACTEL® Absorbable Polymers, inherent viscosity range: 1.0–1.3 dl g^{-1}) were purchased from Purac Biomaterials BV (Gorinchem, The Netherlands) and DURECT Corporation (Pelham, USA), respectively, and used in the electrospinning process. Organic solvent 2,2,2-trifluoroethanol (TFE) (purity ⩾ 99.8%) was obtained from Acros (Geel, Belgium). The electrospinning solution was prepared by dissolving PLGA/PCL (weight ratio 3:1) in TFE at a concentration of 0.12 g ml^{-1}.

The 3-D scaffolds were fabricated using a so-called wet-electrospinning technique as depicted in Fig. 1 in a commercial available electrospinning set-up (Esprayer ES-2000S, Fuence Co., Ltd, Tokyo, Japan). The optimal processing parameters for stable formation of electrospun fibers were selected based on an earlier pilot study. Briefly, the prepared polymer solution was fed into a syringe and delivered to an 18G nozzle at a feeding rate of 50 μl min^{-1}. A high voltage (20 kV) was applied at the nozzle to generate a stable polymer jet by overcoming the surface tension of the polymer solution [16]. Unlike the conventional electrospinning setup, a grounded bath filled with 100% ethanol was used to collect the fibers, located 15 cm under the nozzle. To control the size of resulting fiber meshes, the process was stopped every 10 min for fiber mesh collection. Subsequently, the wet-electrospun scaffolds were washed thoroughly in MilliQ water and freeze-dried for 3 days. For comparison, electrospun fibers were also collected by the conventional electrospinning method on top of flat aluminum foil, using the same processing parameters and collecting time.

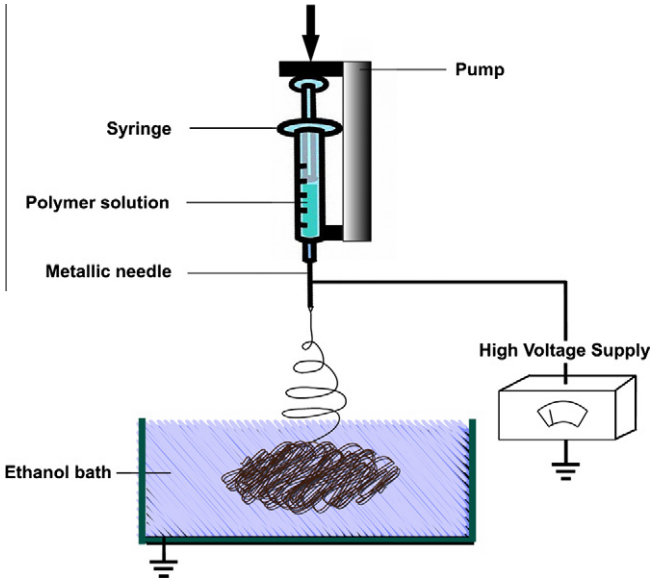

Fig. 1. Illustration of 3-D scaffold generation by the wet-electrospinning method. A polymer solution was fed into a syringe and a high voltage was applied at the nozzle to generate the polymer jet. A grounded bath filled with 100% ethanol was used as the collector. As ethanol is a wetting agent for both PLGA and PCL, the resulting fibers formed a loose, cotton-like mesh in the bath.

2.2. Characterization of the scaffolds

The morphology of the wet- and conventional electrospun scaffolds was observed by scanning electron microscopy (SEM; JEOL6340F, Tokyo, Japan) after being sputter-coated with gold–platinum. The fiber diameters were measured from SEM micrographs that were obtained at random locations ($n = 30$) using Image J software (National Institutes of Health, Bethesda, USA).

The porosity of scaffolds was evaluated by a gravimetric measurement [17]. In brief, the electrospun scaffolds ($n = 4$) were punched into disk-shaped forms (with a diameter of 6 mm for the wet-electrospun scaffolds and 15 mm for the conventional scaffolds) and their dimensions measured to calculate the volume of the scaffolds. The weight of the scaffolds was also measured to determine the apparent density of the scaffolds (ρ_{ap}). The porosity was then calculated according to the following equation:

$$\text{porosity} = (1 - \rho_{ap}/\rho_m) \times 100\%$$

where ρ_m is the density of the blend PLGA/PCL, calculated as 1.215 g cm^{-3} based on the weight ratio and respective densities of PLGA ($\rho = 1.24$ g cm^{-3}, Purac MSDS database) and PCL ($\rho = 1.145$ g cm^{-3}, Sigma–Aldrich MSDS database).

2.3. Cell isolation, seeding and culture

RBMCs were isolated from 7-week-old male Fischer rats after approval from Radboud University Nijmegen Animal Ethics Committee (Approval no: RU-DEC 2011–142). Briefly, two femora of each rat were removed and the epiphyses were cut off. RBMCs were flushed out of the remaining diaphyses using the proliferation medium through an 18G needle (BD Microlance, Drogheda, Ireland). The proliferation medium consisted of alpha minimal essential medium (Gibco, Grand Island, USA) supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, USA), 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin (Gibco), 50 μg ml^{-1} L-ascorbic acid (Sigma) and 10 nM dexamethasone (Sigma). The flushed-out rMSCs were cultured for 2 days in a humidified incubator (37 °C, 5% CO_2), after which the medium was refreshed to remove non-adherent cells. The cells were cultured for an additional 3 days before being detached using trypsin/ethylenediaminetetraacetic acid (EDTA) (0.25% w/v trypsin, 0.02% w/v EDTA; Sigma) and counted.

Disk-shaped scaffolds with a diameter of 6 mm and a thickness of about 2.5 mm were punched out (biopsy punch; Kai medical, Gifu, Japan) from each wet-electrospun mesh. They were subsequently sterilized in 70% ethanol for 2 h and soaked in the proliferation medium overnight having been washed with phosphate-buffered saline (PBS). During cell loading, the scaffolds were incubated in the cell suspension at a concentration of 1×10^6 cells ml^{-1} (4 scaffolds per 1 ml of cell suspension) and gently rotated for 3 h. The scaffolds were then placed in non-adherent tissue culture plates and the unattached cells from the suspension were collected, centrifuged and reseeded onto the scaffolds to ensure a high cell loading efficiency. The cell–scaffold constructs

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