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# Anisotropic silk fibroin/gelatin scaffolds from unidirectional freezing

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ABSTRACT

Recent studies have underlined the importance of matching scaffold properties to the biological milieu. Tissue, and thus scaffold, anisotropy is one such property that is important yet sometimes overlooked. Methods that have been used to achieve anisotropic scaffolds present challenges such as complicated fabrication steps, harsh processing conditions and toxic chemicals involved. In this study, unidirectional freezing was employed to fabricate anisotropic silk fibroin/gelatin scaffolds in a simple and mild manner. Morphological, mechanical, chemical and cellular compatibility properties were investigated, as well as the effect of the addition of gelatin to certain properties of the scaffold. It was shown that scaffold properties were suitable for cell proliferation and that mesenchymal stem cells were able to align themselves along the directed fibers. The fabricated scaffolds present a platform that can be used for anisotropic tissue engineering applications such as cardiac patches.



## 1. Introduction

The current trend in tissue engineering strives for the recapitulation of native tissue architecture. In the past, tissue engineering focused on matching mechanical properties of engineered materials to that of native tissues. This was particularly important for load-bearing tissues, wherein the materials implanted should be able to withstand the stresses it will be subjected to. Later on, it was discovered that cells responded to the stiffness of materials, even capable of directing mesenchymal stem cells into different phenotypes [1]. This emphasized the importance of matching the stiffness of scaffolds to the native extracellular matrix. Recently, the topography of the environment was shown to direct cells through contact guidance, influencing their migration, morphology and even gene expression [2]. Although the exact regulatory mechanisms as to how cells respond to these stimuli are not yet fully elucidated, these observations collectively point out the importance of matching scaffold properties to the biological milieu. This is vital in cardiac tissue engineering, wherein the goal of regenerating cardiac tissue remains a challenge.

The healthy myocardium is arranged as a network of aligned fibers and sheets, where extracellular matrix (ECM) fibers are arranged longitudinally alongside with cardiomyocytes [3,4]. The ECM is composed of type I and type III collagen that couple cardiomyocytes and maintain the spatial arrangement between the myofilaments and their capillary microcirculation. Healthy myocardium is predominantly composed of type I collagen whereas type III is produced during injury [3]. This stress-tolerant viscoelastic environment optimizes force development and evens out force distribution in the ventricular wall to prevent sarcomeric deformation because as the heart contracts, cardiomyocytes develop a tensile force due to shortening [5]. Cardiomyocytes in vivo have their nuclei aligned in an anisotropic direction, and it has been shown that it is possible to achieve this in vitro with neonatal cardiomyocytes by using patterned ECM [3]. In addition, cardiomyocyte attachment and infiltration was shown to be higher for aligned scaffolds relative to isotropic scaffolds [6].

There has been a lot of previous work done on cardiac tissue engineering, but most have been isotropic scaffolds. As for the synthesis of anisotropic or aligned scaffolds, several methods have been explored, as tabulated in Table 1. Studies using unique technology include the use of a commercial cotton candy machine to generate sucrose templates for PLGA [4], laser microablation to form accordion-like structures [7], and shrink film technology [8]. Electrospinning of aligned fibers and unidirectional freezing have also been recently used in fabricating anisotropic scaffolds for cardiac tissue engineering. The results of these studies reinforce the hypothesis that an anisotropic architecture does result to a significantly better cardiac construct as indicated by different parameters such as electrophysiological assessment and cardiac gene and protein expression. Thus, this merits the pursuit of fabricating anisotropic scaffolds for cardiac patches.

For general tissue engineering applications, including those not specifically for cardiac applications, methods utilized to fabricate anisotropic scaffolds include solid free-form fabrication, modifications of conventional techniques like particle leaching, gas foaming, thermal induced phase separation, electrospinning, particle sintering, and other alternative methods such as direct molding and post-processing [14].

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**Table 1**
Anisotropic scaffolds for cardiac tissue engineering.

| Reference | Material | Technique | Cells |
|---|---|---|---|
| Bursac et al. [4] | Poly(lactic-co-glycolic acid) | Sucrose templates formed using commercial cotton candy machine | Neonatal rat cardiac cells |
| Engelmayr et al. [7] | Poly(glycerol sebacate) | Accordion-like honeycomb scaffolds from laser microablation | Neonatal rat cardiac cells |
| Gonnerman et al. [9] | Collagen-glycosaminoglycan | Unidirectional freezing in a PTFE mold with a copper base | HL-1 cells (AT-1 mouse atrial cardiomyocyte tumor lineage) |
| Lin et al. [10] | Polyacrylonitrile | Electrospinning of aligned fibers | Rat cardiomyocytes with rat endothelial cells |
| Ayaz et al. [11] | Bionate® (polycarbonate urethane) or poly(lactic-*co*-glycolic acid) | Electrospinning of aligned fibers on textile-patterns (polyester and cotton fabrics) | H9C2 rat cardiac myoblasts and neonatal rat cardiac cells |
| Rinaldi et al. [12] | Silk fibroin with ECM from rat hearts, collagen I or fibronectin | Unidirectional freezing in silicon mold | Rat cardiac fibroblasts |
| Mendoza et al. [8] | Polyethylene | Shrink film technology | Human ESC-derived ventricular cardiomyocytes |
| Stoppel et al. [6] | Silk fibroin with porcine derived ECM | Unidirectional freezing using 100% ethanol dry ice bath | HL-1 cells and human ESCs |
| Chen et al. [13] | Polyurethane/ethyl cellulose | Electrospinning of aligned fibers | H9C2 rat cardiac myoblasts |

However, the major limitations include complicated fabrication steps, harsh processing conditions and toxic chemicals involved [15]. This is especially a concern when scaffold materials include natural polymers, proteins and drugs which are sensitive to conditions such as high temperature that could potentially degrade, denature or alter their desired properties. These drawbacks are what have led us to use ice templating to attain anisotropic scaffolds.

Ice templating is a technique based on the use of ice as a template for material fabrication. It involves the freezing of a water-based solution or suspension, and subsequent removal of the ice through sublimation. Ice templating can be considered as a subclass of thermally-induced phase separation (TIPS), which is the phase separation of a homogeneous polymer solution into a polymer-rich and solvent-rich phase due to changes in thermal energy [16]. As the ice grows, the solid phase is pushed out by the ice crystals and concentrated into areas in between the crystals. Once the ice is removed by sublimation using freeze-drying, the scaffold architecture left is a direct negative of the ice structure [17,18]. The conscious utilization of ice templating for tissue engineering scaffolds only started within the last decade, with most publications confined to the last 5 years. Interest in the technique gained momentum due to the advantages offered by the process, as summarized in Table 2 [15,17,19,20,21,22,23,24].

The attainment of anisotropic scaffolds using ice templating is dependent on the solvent's property to be able to achieve dendritic crystal growth [19]. This was shown in several studies wherein at certain situations, water formed into anisotropic hexagonal ice crystals, parallel to the direction of the temperature gradient [21,25,26,27]. As a consequence, an aligned structure is left behind after sublimation of the ice. But given that ice crystals don't always grow in a dendritic way, the key to achieving anisotropic structures through ice templating is the control of the nucleation process - which is the transformation from a liquid to a solid phase. In order to achieve anisotropic structures, nucleation should start and be confined on a planar front, followed by the preferential crystal growth along the direction of heat conduction [21,28]. Strategies usually employed to achieve this include the exposure of one side of the solution to liquid nitrogen and the use of specially-designed molds with one thermally conductive surface among the other insulating surfaces. It is worth noting that other ice templating manipulations have been explored such as making scaffolds with funnel shaped pores [29], multidirectional pores [15], and radial pores [30]. In this light, the ice templating technique to be used in this study will be henceforth termed as "unidirectional freezing.

**Table 2**
Advantages of ice templating.

| Advantage | Description |
|---|---|
| Simplicity | Straightforward and is physical in nature. |
| Biocompatibility | Does not employ heat and is thus compatible with proteins and drugs. Furthermore, ice is removed by sublimation and thus lessens material lost due to the usual aqueous wash required by other processing techniques. In addition, the use of water as the solvent/carrier implies compatibility to most substances, as well as making the process environmentally-friendly. |
| Versatility | Any type of material (e.g. ceramic, polymer) can be used, as the resulting structure is independent of the material. |
| Tunability | Pore orientation and size, and thus material properties, can be controlled using a few key parameters such as freezing temperature, cooling rate and mold design. By controlling the freezing regime of water, it is possible to control the final structure. In addition, the process is not sensitive to freeze-drying paramaters. |
| Scalability | Potentially scalable and is thus more promising in terms of industrial and clinical translation. |

The objective in this study is to fabricate and characterize aligned silk fibroin/gelatin scaffolds using unidirectional freezing. Silk fibroin, a natural polymer from *Bombyx mori* cocoons, was chosen as the base material because of its versatility, biocompatibility, tunable mechanical properties and biodegradability. It can be processed using mild aqueous conditions and has been even shown to stabilize enzymes and other therapeutics processed at high temperatures [31]. Gelatin, another natural polymer derived from the partial hydrolysis of native collagens, was added to improve cell adhesion to silk because of its integrin-binding sites [32]. Silk fibroin and gelatin has previously been shown to be thermodynamically compatible and thus blends homogeneously with each other. The addition of gelatin improved the hydrophilicity of the composite and also reportedly resulted to a more stable non-crystal structure in silk fibroin [33,34]. The scaffolds fabricated in this study were characterized in terms of their mechanical, chemical and cellular compatibility properties and are foreseen to be an attractive option for cardiac tissue engineering in terms of the simpler and more biocompatible way they are fabricated.

## 2. Method

### 2.1. Materials

All reagents were obtained from Sigma-Aldrich, unless otherwise noted.

### 2.2. Mold fabrication

A polytetrafluoroethylene (PTFE) mold with a copper base plate was designed and fabricated to achieve unidirectional freezing (Supplementary Information Fig. S1). Rectangular PTFE molds were screwed down on a copper plate, wherein the copper plate was always touching the freezing plate during freezing. The appropriate freezing temperature was determined by trying 3 different settings, −20 °C, −30 °C and −80 °C and using scanning electron microscopy (SEM) to verify the structure of the scaffold achieved. Isotropic (non-aligned) scaffolds were fabricated by using the same mold with a poly(methyl methacrylate) base (PMMA) instead of a copper base plate.