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## Textile-templated electrospun anisotropic scaffolds for regenerative cardiac tissue engineering

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### ABSTRACT

For patients with end-stage heart disease, the access to heart transplantation is limited due to the shortage of donor organs and to the potential for rejection of the donated organ. Therefore, current studies focus on bioengineering approaches for creating biomimetic cardiac patches that will assist in restoring cardiac function, by repairing and/or regenerating the intrinsically anisotropic myocardium. In this paper we present a simplified, straightforward approach for creating bioactive anisotropic cardiac patches, based on a combination of bioengineering and textile-manufacturing techniques in concert with nano-biotechnology based tissue-engineering stratagems. Using knitted conventional textiles, made of cotton or polyester yarns as template targets, we successfully electrospun anisotropic three-dimensional scaffolds from poly(lactic-co-glycolic) acid (PLGA), and thermoplastic polycarbonate-urethane (PCU, Bionate®). The surface topography and mechanical properties of textile-templated anisotropic scaffolds significantly differed from those of scaffolds electrospun from the same materials onto conventional 2-D flat-target electrospun scaffolds. Anisotropic textile-templated scaffolds electrospun from both PLGA and PCU, supported the adhesion and proliferation of H9C2 cardiac myoblasts cell line, and guided the cardiac tissue-like anisotropic organization of these cells *in vitro*. All cell-seeded PCU scaffolds exhibited mechanical properties comparable to those of a human heart, but only the cells on the polyester-templated scaffolds exhibited prolonged spontaneous synchronous contractility on the entire engineered construct for 10 days *in vitro* at a near physiologic frequency of ~120 bpm. Taken together, the methods described here take advantage of straightforward established textile manufacturing strategies as an efficient and cost-effective approach to engineering 3D anisotropic, elastomeric PCU scaffolds that can serve as a cardiac patch.

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### 1. Introduction

Cardiovascular diseases remain one of the major health problems in the United States. According to the 2012 statistical update of the American Heart Association, 7,900,000 Americans have had a myocardial infarct (MI). Annually, an estimated 610,000 Americans will have a new coronary attack, 325,000 will have a recurrent MI

and 195,000 will experience a silent first MI [1]. MI-associated ischemia leads to regional cell death followed by replacement of the injured myocardial tissue by fibrous scar tissue, which is comprised mainly of collagenous extracellular matrix (ECM) and fibroblasts, and results in discontinuous propagation of the electrical signal and impaired cardiac function [2]. In repairing or replacing the damaged myocardium, a major goal is to engineer

**Abbreviations:** Bionate®, Bionate® thermoplastic polycarbonate urethane (PCU); PLGA, poly(lactic-co-glycolic) acid; CT, cotton fabric template; PE, polyester fabric template; TG, target plate template; PLGA\_CT, PLGA fibers collected on cotton fabric; PLGA\_PE, PLGA fibers collected on polyester fabric; PLGA\_TG, PLGA fibers collected on flat target; Bionate\_CT, Bionate fibers collected on cotton fabric; Bionate\_PE, Bionate fibers collected on polyester fabric; Bionate\_TG, Bionate fibers collected on target plate.

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biomimetic 3-D cardiac scaffolds, which emulate the structural organization and mechanical properties of the healthy native tissue, especially its elasticity and its anisotropy.

The mammalian heart is composed of collagen-based ECM with well-aligned myocytes and fibroblasts and capillary endothelial cells, leading to structural and mechanical anisotropy of the contractile ventricular myocardium [3,4]. One of the central aims of functional cardiac tissue engineering is to generate anisotropic scaffolds that will emulate the intrinsic anisotropy of the myocardium and guide the alignment of cells growing on/in these scaffolds. The desired orientation and elongation of cultured cells can be induced by mechanical and electrical stimulation [4–7] and also by tissue scaffolds engineered to provide “contact guidance” [8], i.e. a permissive environment in which the cells are “guided” to align according the surface topography of the scaffolds. Photolithography [8,9], soft lithography [10], micro-grooving [11], molecular self-assembly [12], micro-fluidics [10], micro-abrasion [13], micro-contact printing [14], micro-ablation [15,16] are some of the techniques used for manufacturing anisotropic scaffolds. For example, Gonnermann and colleagues described a series of geometrically anisotropic collagen-GAG (CG) scaffolds with aligned tracks of ellipsoidal pores, fabricated via directional solidification and freeze-drying technique [17]. Engelmayr and colleagues used micro-ablation of polyglycerol sebacate (PGS) to manufacture accordion-like honeycomb scaffolds, which matched the anisotropy and mechanical properties of native myocardium and guided the alignment of cultured neonatal rat heart cells and C2C12 myoblasts without any external stimuli [15]. However, while elegant, these techniques are tedious, time-consuming and costly in terms of manufacturing [18] and entail potential thermal degradation of bioresorbable polymers and biomaterials [19]. Therefore, one of the goals of this study was to develop a simple, efficient and cost-effective approach to engineering complex 3-D cardiac scaffolds, which emulate the mechanical properties of the native tissue, specifically its elasticity and anisotropy.

Textile engineering and tissue engineering are two distinct disciplines that are rapidly becoming intertwined in providing life-saving solutions to debilitating biomedical problems [20]. Currently, there are a number of textile-based biomedical devices on the market, such as vascular grafts made of Dacron®; (polyethylene terephthalate) and Goretex® (expanded polytetrafluoroethylene) to replace blocked large and medium-sized blood vessels [21], or silk-fibroin based surgical meshes, such as SeriACL™ and SeriFascia, for ACL repair and abdominal surgery, respectively. For cardiac applications, knitted textile structures have been implemented because of their high elasticity, porosity, and micro-scale patterns that promote anisotropy. For example, the Acorn CorCap™ passive cardiac support device is a knitted poly(ethylene terephthalate) (polyester, or PET) mesh that is wrapped around the dilated heart to provide mechanical support [22]. Knitted fabrics are also being used as scaffolds for cartilage and heart tissue engineering [23–26].

Knitted fabrics like the Acorn CorCap™ provide global mechanical support to the heart, but have disadvantages for tissue engineering purposes, because of their macroscopic fiber structure and pore size. Cells seeded onto such macro-scale scaffolds need to deposit their own ECM in order to generate a suitable (nano-scale) microenvironment that promotes adhesion, proliferation and also functional tissue [25,27]. In the past hybrid scaffolds have been designed to provide an ECM-like environment, for example by forming collagen micro-sponges in the openings of a large-pore knitted silk mesh [28].

Textile engineering has provided a number of established platform technologies for biomedical applications, such as weaving, knitting or, more recently, electrospinning. Electrospinning,

invented in 1930s [29], is a versatile textile engineering-based platform technology that is widely used for fabricating non-woven scaffolds with a nano-/micro-fibrous architecture for a variety of applications in tissue engineering, such as wound healing, drug delivery, biosensor, protective clothing, cosmetics and filtration [30]. The major advantages of electrospun scaffolds are high surface area to volume ratio and tunable porosity. The nano-structure of electrospun fibers mimics that of the ECM and facilitates cell adhesion, proliferation, and differentiation [30–32].

Contact guidance and anisotropy have also been investigated in the context of electrospun scaffolds. Several groups have reported the production of electrospun meshes with fibers aligned by post-stretching [33] or by using rotating cylinder collectors, auxiliary electric fields, rotating drums and counter electrodes, and electrostatic lenses [31,34–39]. The alignment of the fibers provided the necessary contact guidance for cellular alignment and anisotropy of the resulting constructs. However, with their densely packed fibers and reduced porosity, fully aligned electrospun scaffolds do not allow for ready cellular penetration throughout the depth of the scaffolds [40]. Previous studies suggested that the surface topography of nanofibrous mats electrospun onto patterned metallic collectors mimicked that of the targets [41]. Neves and colleagues determined that templated scaffolds demonstrate various tensile properties depending on their surface topography [41]. Since textile engineering provides an infinite number of designs and patterns of fabrics, we hypothesized that textile materials might serve as convenient and inexpensive templates for engineering electrospun tissue scaffolds with a wide variety of surface topographies, including anisotropic patterns.

In this study we used ordinary, commercially available knitted textiles as templates for electrospinning anisotropic scaffolds for engineering intrinsically anisotropic tissues, such as the myocardium. We hypothesized that by using knitted textiles as templates, the electrospun scaffold can emulate topographic properties of the knitted fabric, while its ECM-like nanofibrous structure can support cardiac cell adhesion, proliferation and the assembly of functional tissue-like, beating myocardial constructs.

## 2. Materials and methods

### 2.1. Materials

Poly(L-lactide-co-glycolide) (PLGA 80:20 PURASORB® 1.24 kg/l) was purchased from PURAC Biomaterials (Gorinchem, Groningen The Netherlands). Bionate® 80A UR Thermoplastic Polycarbonate Urethane was purchased from DSM Biomedical (Part Number: FP70063, Berkeley, California). Fabrics, made from polyester and cotton were knitted at Philadelphia University, Department of Engineering & Textiles, as previously described [42].

### 2.2. Electrospinning

Bionate scaffolds were electrospun using the NEU Complete Nanofiber Producing Unit (Nanospinner, Kato Tech, Japan) at Philadelphia University, Department of Engineering & Textiles. PLGA scaffolds were generated in a home-made electrospinning device, as previously described [42–46]. Briefly, PLGA and Bionate were dissolved at 5% (w/v) and 8% (w/v), respectively, in 1,1,1,3,3,3 Hexafluoro-2-Propanol (HFP, from Sigma) for 24 h. In the homemade device, polyester and cotton fabrics (8 × 8 cm<sup>2</sup>) were mounted on a polycarbonate frame and stretched 50% in both the x and y directions. The frame was placed in front of a rectangular (10 × 10 cm<sup>2</sup>) copper target. In the NEU Nanospinner unit a 20 × 30 cm<sup>2</sup> fabric was stretched to 50% in both directions on a stationary target. Optimized electrospinning parameters that yielded uniform bead-less PLGA fibers and a scaffold thickness of 20 µm in our homemade system were as follows: The distance between the textile and needle was 15 cm, the syringe pump flow rate was 0.5 ml/h, and the applied voltage was 20 kV. Parameters to obtain uniform bead-less Bionate fibers and a scaffold thickness of ~100 µm in the Nanospinner unit were as follows: The distance between the textile and needle was 18 cm, the syringe pump flow rate was 0.8 ml/h, and applied voltage was 20 kV. Electrospun PLGA and Bionate scaffolds were peeled off (separated) from the knitted textile templates and the flat target, respectively, immediately upon completion of the electrospinning process, and used within one week of manufacturing.

### 2.3. Ultrastructure of scaffolds

For surface analysis of the electrospun mats, we utilized three different tools: Scanning Electron Microscope (SEM), Atomic Force Microscope (AFM) and Laser Scanning Microscope (LSM). For ultrastructural analysis, PLGA and Bionate scaffolds were cut into discs ( $d \sim 0.5$  mm) and coated with Pt/Pd for 40 s at a gas pressure of 0.025 mbar using a Cressington sputter coater 208 (Watford, England) with current set at 40 mA. The samples were visualized by SEM (XL-30 Environmental SEM-FEG or Zeiss Supra 50VP), at an acceleration voltage of 10 kV. For AFM analysis, we used a Bioscope (Veeco, Plainview, NY) mounted on a Nikon TE-3200 using the tapping mode with silicon cantilevers ( $k = 40$  N/m). Surface roughness of the scaffolds was also analyzed via a Laser Scanning Microscope (Violet Laser, 408 nm, VK-9700 Series, Keyence). Fiber and cell alignment was analyzed using a custom-made image analysis software (HÜCRECI) through a user-interface driven input and visual processing as previously described [47]. For details, see [Supplemental data](#).

### 2.4. Mechanical testing

The tensile properties of electrospun PLGA and Bionate mats ( $25 \text{ mm} \times 5 \text{ mm}$ ,  $L \times W$ ) with a thickness of  $\sim 20 \mu\text{m}$  and  $\sim 100 \mu\text{m}$ , respectively were evaluated in an Instron System (Model 5564, Norwood, MA, USA) using a 10 Newton load cell and strain speed of 1 mm/min. The Young's modulus of each sample was determined in triplicate from the slope of the initial linear segment of the strain-stress curves using the manufacturer's software [45].

### 2.5. Cell culture

#### 2.5.1. H9C2 cells

H9C2 rat cardiomyoblasts (ATCC CRL-1446; American Type Culture Collection, Rockville, MD, USA), were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Gland Island, NY) supplemented with 10% fetal bovine serum (FBS, from Gibco), 2 mM L-glutamine, and 1 mM sodium pyruvate, as previously described [3]. The various electrospun scaffolds, textile fabric templates, and control wells were coated with rat-tail collagen-I ( $50 \mu\text{g}/\text{ml}$ , Sigma) for 30 min at  $37^\circ\text{C}$ . The cells were seeded at a density of  $25 \times 10^3$  cells/ $\text{cm}^2$  on the collagen-coated scaffolds and cultured for 3–10 days in a humidified  $\text{CO}_2$  incubator (100% humidity, 5%  $\text{CO}_2$ ,  $37^\circ\text{C}$ ). The medium was changed once every three days.

#### 2.5.2. Primary cardiomyocytes

Neonatal cardiomyocytes with an average yield of  $\sim 30$  million neonatal cardiomyocytes were isolated from ten 1–3-day-old neonatal Sprague–Dawley rats according to an IACUC-approved protocol, using 6–7 cycles (30 min each) of enzyme digestion with collagenase type II ( $95 \text{ U}/\text{mL}$ ; Worthington, Lakewood, NJ, LS004176) and pancreatin ( $0.6 \text{ mg}/\text{mL}$ ; Sigma, P1750). At the time of isolation, the mixed population of cells consisted of  $50 \pm 5\%$  cardiomyocytes, with the remaining cells being fibroblasts and endothelial cells, as also reported by others [48]. For further purification, the mixed populations were pre-plated for 45–60 min to allow for differential adhesion of fibroblasts and endothelial cells. The supernatant was then centrifuged through a discontinuous Percoll gradient using 40.5% and 58.5% Percoll (Sigma) solution, prepared in  $1 \times$  PBS without calcium and magnesium to purify the cardiac myocytes and eliminate non-myocyte cell types, following established methods [49–51]. After centrifugation at 2200 rpm for 20 min at room temperature, the cardiomyocytes had migrated to the interface between the layers [52]. Purified neonatal cardiomyocytes were seeded immediately upon isolation on 0.1% gelatin (ATCC, PCS-999-027) - coated target surfaces, i.e., Bionate scaffolds (with a diameter of  $\sim 1.6$  cm and thickness of  $\sim 50 \mu\text{m}$ ) and 24 well TCP at a density of  $10^6$  cells/ $\text{cm}^2$  and cultured in NS Medium (with high serum, Catalog #M8031, Cellutron) in a humidified  $\text{CO}_2$  incubator for 10 days. The medium was changed every day.

### 2.6. Cell imaging

H9C2 cells on PLGA and Bionate scaffolds and neonatal cardiac myocytes on Bionate scaffolds were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton-X-100 and then stained with  $1 \mu\text{g}/\text{mL}$  DAPI (nuclear stain, from Invitrogen) and 1 unit/ $\text{mL}$  Alexa Fluor<sup>®</sup> 488 phalloidin (F-actin stain, from Invitrogen) in  $1 \times$  PBS for 30 min, as previously described [53]. Following 4 washes in  $1 \times$  PBS for 5 min each the samples were examined in a confocal laser scanning fluorescence microscope (Olympus FluoView 1000), using excitation wavelengths of 488 nm (for phalloidin labeled actin) and 405 nm (for DAPI-labeled nuclei) and the appropriate emission filters. 3-D z-projections of the micrographs were generated using the FluoView confocal software, as previously described [53].

### 2.7. Contraction analysis

In order to evaluate the contractile properties of the cell-seeded scaffolds, videos of spontaneously contracting regions of the cultures were recorded for 20–45 s at 30 frames per second using a Hitachi video camera (KPD-50) connected via FireWire to a Windows-based desktop computer running ProgRes capture software (Jenoptik, Jena Germany). The files were saved as AVI files. For these studies we used both a

Nikon TE 2000U fluorescence microscope and a Keyence Fluorescence Microscope (BZ-9000, Bioevo) and analyzed movements of the cells and/or scaffolds using Motion Analysis Software (VW-9000, Keyence). For the analysis we selected 8–16 anchor points in each experiment ( $n = 3$ ). The software collects the coordinates of each anchor point and calculates the displacement during each contraction ("beat"). Using our own custom-made scripts developed in MatLab [47], we performed three kinds of contraction analyses: Displacement, Beating, and Synchronization Analysis. For details see [Supplemental data](#). For displacement analysis, the average amount of distance that a given anchor point traveled during each "beat" was calculated. For beating analysis, the average number of "beats" per minute was calculated for all anchor points in all video segments. For synchronization analysis, beating synchronization was quantified with a 'synchronization index (SI)' based on the standard deviation of the timing for the first beats across different spatial locations.

### 2.8. Statistics

An omnibus statistical analysis with factorial design was set up to compare all contributing factors to the calculated Young's moduli such as orientation and scaffold type. The test involved three-way ANOVA analysis of the contribution of materials (PLGA and Bionate), templates (PE, CT and TG) and orientation ( $x$  and  $y$ ). For significant main effects and interactions, Tukey–Kramer multiple comparison tests were used. The significance criterion of the test was  $\alpha = 0.05$ . All statistical analyses were performed using the Number Cruncher Statistical Software (NCSST) 2007 ([www.ncss.com](http://www.ncss.com)). Observed effects were taken as statistically significant for \*:  $p < 0.05$  and \*\*: highly significant for  $p < 0.01$ .

## 3. Results

### 3.1. Scaffold design and characterization

For testing the idea of that the anisotropy and unique surface topography of textile templates can be transferred to the electrospun scaffolds, we initially used, as proof-concept, PLGA [42] as scaffold material. Electrospun PLGA scaffolds have been employed for a variety of tissue engineering applications, including creating cardiac tissue constructs [33]. Subsequently, we focused on Bionate (polycarbonate urethane, PCU), a biostable elastomeric polymer with mechanical properties (e.g. Young's modulus) closer to those of the native myocardium. As seen in low-resolution stereo-macroscopic images in [Fig. 1](#) and the inserts showing stereo-macroscopic images of the same scaffolds at higher magnification, each of three textile templates yielded electrospun scaffolds with distinct surface topography and architecture. For polyester-templated PLGA and Bionate scaffolds (termed PLGA\_PE and Bionate\_PE, respectively), these patterns were very similar to those of the textile template: the 3-D topography of the electrospun scaffolds mimicked the striated surface topography of the polyester knitted fabric ([Fig. 1A and D](#)). By contrast, for cotton-templated scaffolds, the electrospun fibers formed concave circular structures independent of the patterns of the cotton fabric ([Fig. 1B and E](#)). When spun onto the flat target, the topography of both PLGA and Bionate scaffolds was flat without any distinct patterns as they mimicked the flat surface of the metal template ([Fig. 1C and F](#)).

The ultrastructure of the textile-templated fibrous scaffolds was further analyzed by SEM, assessing fiber size, fiber uniformity and surface topography. The optimized electrospinning conditions described above in [Materials and methods](#) yielded PLGA and Bionate uniform bead-less fibrous scaffolds with average fiber diameters of  $280.4 \pm 96.5 \text{ nm}$  for PLGA and  $699.4 \pm 201.1 \text{ nm}$  for Bionate, respectively. Detailed visualization of the striated structures on the polyester-templated PLGA\_PE and Bionate\_PE scaffolds indicated distinct regional fiber alignment ([Figs. 2A and 3A](#), respectively). A unique pattern of fiber alignment and fiber topography was observed in the cotton-templated PLGA\_CT and Bionate\_CT scaffolds, where areas of aligned fibers were detected within regularly spaced concave, well-like circular structures ([Figs. 2B and 3B](#)). In the flat-templated PLGA\_TG and Bionate\_TG scaffolds, fibers were randomly oriented and did not exhibit any

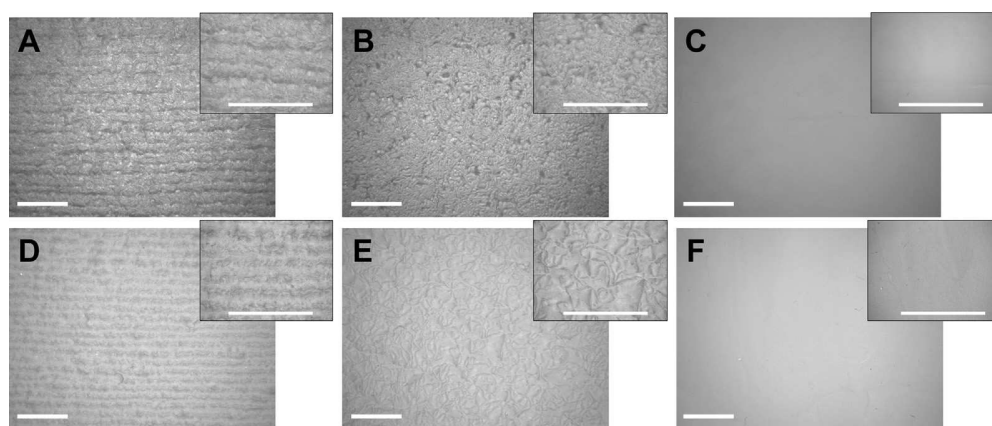


**Fig. 1.** Representative stereo-macroscopic images of PLGA and Bionate scaffolds electrospun on diverse templates, at low and (insert) higher magnifications: (A) PLGA scaffold electrospun on polyester template (PLGA\_PE); (B) PLGA scaffold electrospun on cotton template (PLGA\_CT); (C) PLGA scaffold electrospun on flat copper target (PLGA\_TG); (D) Bionate scaffold electrospun on polyester template (Bionate\_PE); (E) Bionate scaffold electrospun on cotton template (Bionate\_CT); (F) Bionate scaffold electrospun on flat aluminum target (Bionate\_TG). Scale Bars: 5 mm.

noticeable fiber alignment or any pattern formation (Figs. 2C and 3C). Fiber alignment on the various scaffolds was quantified using the HÜCRECI program, a new analysis software that was developed, using Microsoft Visual Studio in C# programming language as stand-alone application, specifically for this work. The method for quantifying fiber alignment is described in the methods section, details of the code for the HÜCRECI program are found in Supplemental data. As seen in the histograms for PLGA\_PE (Fig. 2a) and Bionate\_PE (Fig. 3a), the values for the angles of fiber orientation were narrowly distributed, suggesting that the majority of fibers were aligned along the main axis of the striation, indicating a significant degree of anisotropy in these scaffolds. We observed a similar anisotropic fiber distribution, albeit not as narrow, in the concave circular structures of PLGA\_CT (Fig. 2b) and Bionate\_CT (Fig. 3b). However, for PLGA\_TG and Bionate\_TG scaffolds, the

angles of fiber orientation were evenly distributed along the entire range, indicating random fiber deposition and lack of anisotropy (Figs. 2C and 3C).

Evaluation by atomic force microscope (AFM) provided further information about the 3-D surface topography and fiber alignment of the scaffolds in the scan range of 50  $\mu\text{m}$  (Fig. 4). In line with the SEM images, the fibers in the Bionate\_TG and PLGA\_TG scaffolds were randomly distributed and the surface topography of the scaffolds was flat (Fig. 4C and F), reflecting the topography of the template. Knitted-textile templated scaffolds contained aligned fibers with a specific 3-D surface topography that replicated the surface topography of the template. The 3-D surface roughness of the textile templated scaffolds, however, was too rough for evaluation by AFM. We therefore used laser-scanning microscopy (LSM), which is a more convenient and user-friendly alternative for

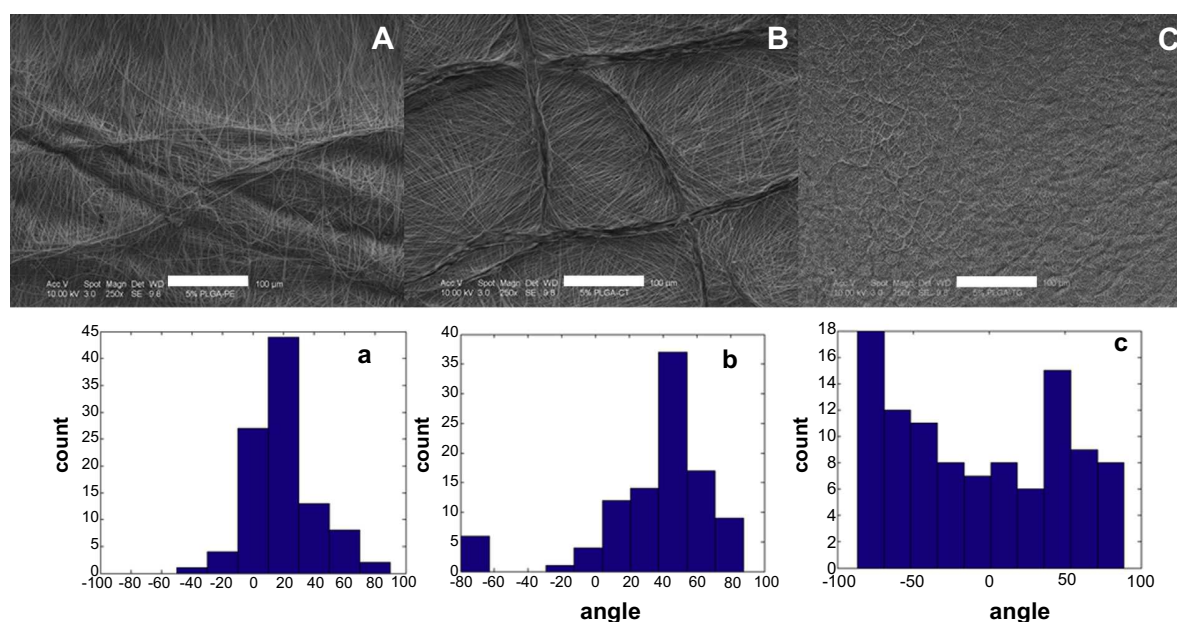


**Fig. 2.** Representative scanning electron micrographs (SEM) of PLGA scaffolds (A–C) and histograms (a–c) of the respective fiber alignment as determined by using HÜCRECI software: (A,a) PLGA scaffolds electrospun on polyester template (PLGA\_PE), (B,b) PLGA scaffolds electrospun on cotton template (PLGA\_CT), (C,c) PLGA scaffolds electrospun on flat copper target (PLGA\_TG). Scale Bar: 100  $\mu\text{m}$ .

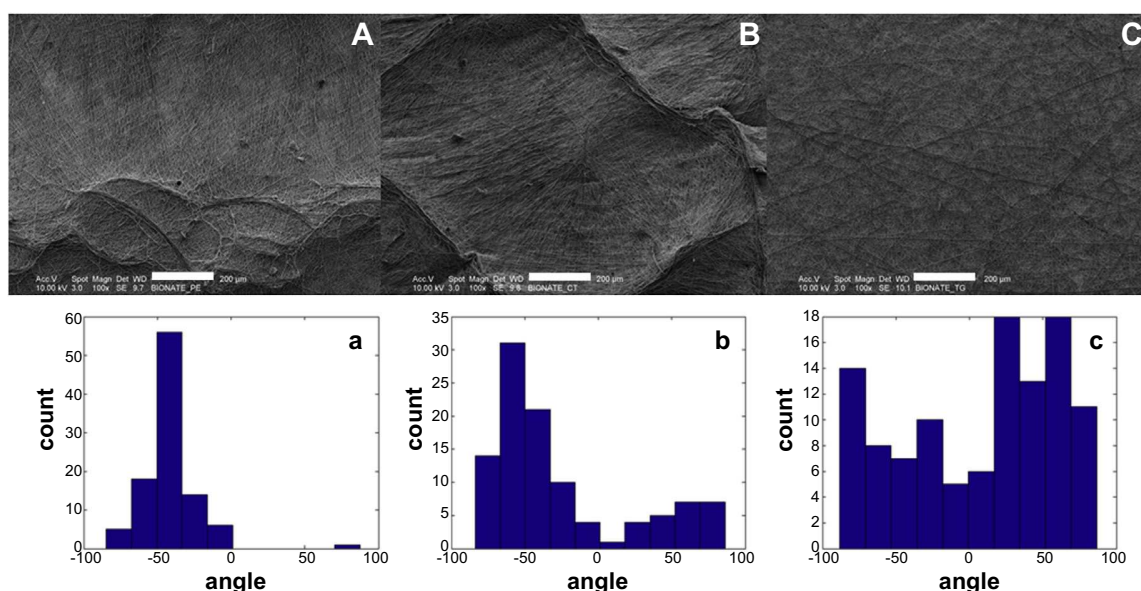


**Fig. 3.** Scanning electron micrographs of Bionate scaffolds and histograms of the respective fiber alignment as determined by using HUCRECI software: (A, a) Bionate scaffolds electrospun on polyester template (Bionate\_PE), (B, b) Bionate scaffolds electrospun on cotton template (Bionate\_CT), (C, c) Bionate scaffolds electrospun on flat aluminum target (Bionate\_TG). Scale Bar: 200  $\mu\text{m}$ .

visualizing differences in the surface features of 3-D scaffolds. Shown in Fig. 5 are color-coded height representations of the PLGA and Bionate scaffolds on all three templates obtained by LSM. The red (in web version) and blue (in web version) colors on the surface topography micrographs represent the highest and lowest peaks and valleys, respectively. Flat metal target-templated PLGA\_TG and Bionate\_TG scaffolds yielded an even and flat surface topography (Fig. 5C and F). In contrast, knitted polyester- and cotton-templated PLGA and Bionate scaffolds demonstrated a rough and wavy 3-D

surface topography (Fig. 5A and D for PE, Fig. 5B and E for CT). These findings further strengthen our notion that electrospun scaffolds emulate the 3-D characteristics and surface topography of their respective templates.

In order to quantify the differences in the surface topographies of flat-metal and textile templated scaffolds, we used the LSM data to determine the surface roughness by calculating vertical deviations within the scanned region. We calculated a roughness parameter,  $R_a$ , which is the arithmetic average of the absolute

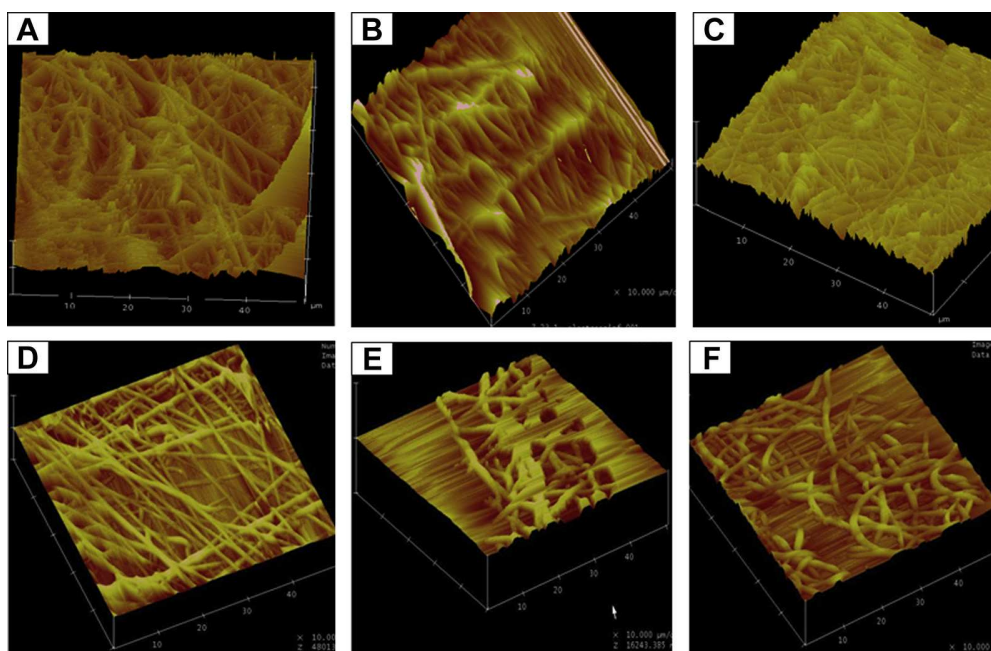


**Fig. 4.** Representative atomic force microscopic (AFM) images of the 3-D surface topography of electrospun PLGA and Bionate scaffolds: (A) PLGA scaffold electrospun on polyester template (PLGA\_PE), (B) PLGA scaffold electrospun on cotton template (PLGA\_CT), (C) PLGA scaffold electrospun on flat copper target (PLGA\_TG), (D) Bionate scaffold electrospun on polyester template (Bionate\_PE), (E) Bionate scaffold electrospun on cotton template (Bionate\_CT), (F) Bionate scaffold electrospun on flat aluminum target (Bionate\_TG). Size of the scanning area 50  $\times$  50  $\mu\text{m}$ .

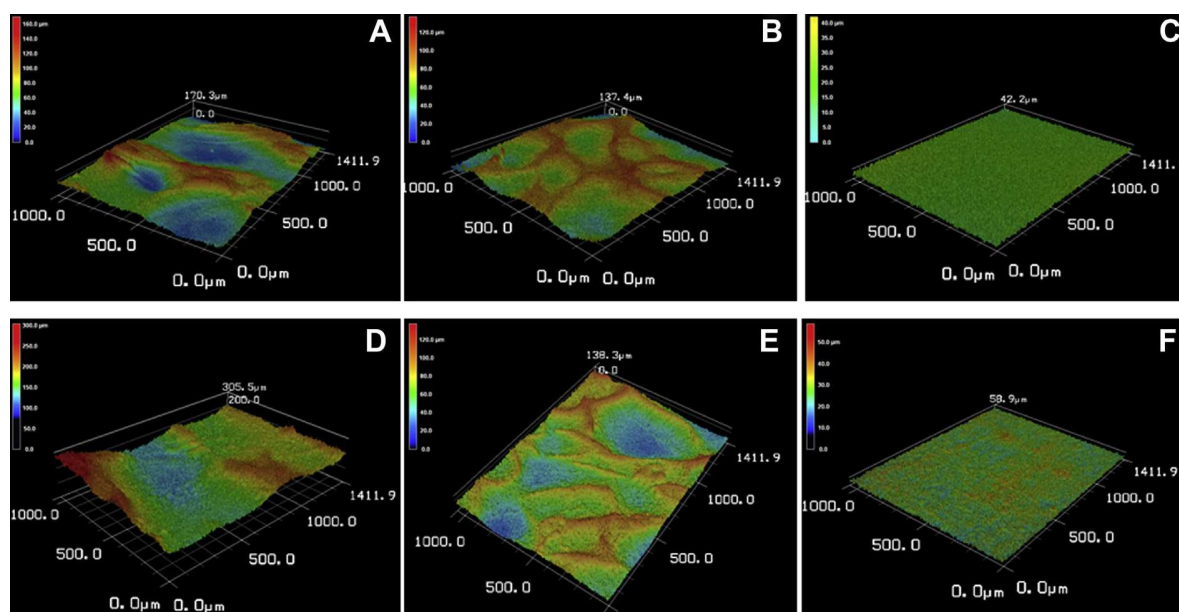


**Fig. 5.** 3-D surface topography of PLGA and Bionate scaffolds visualized by laser scanning microscopy (LSM): (A) PLGA scaffold electrospun on polyester template (PLGA\_PE), (B) PLGA scaffold electrospun on cotton template (PLGA\_CT), (C) PLGA scaffold electrospun on flat scaffold target (PLGA\_TG), (D) Bionate scaffold electrospun on polyester template (Bionate\_PE), (E) Bionate scaffold electrospun on cotton template (Bionate\_CT), (F) Bionate scaffold electrospun on flat aluminum target (Bionate\_TG). Size of the scanning area  $1,000 \times 1,500 \mu\text{m}$ .

vertical deviation values. As seen in Fig. 6, the surface roughness of the flat target-templated scaffolds was approximately two times lower than that of the two other scaffolds ( $p < 0.01$ ). These roughness measurements verified the notion of a “smooth” surface of the flat-target-templated scaffolds and provided quantitative proof that the fibers were deposited in same plane, as also shown in the AFM and LSM micrographs (Figs. 4C,F and 5C,5F, respectively). We did not detect any statistically significant differences between the roughness of PLGA and Bionate scaffolds electrospun onto the polyester or cotton templates. Taken together these findings support our contention that the electrospun scaffolds mimic the 3-D structures and surface topography of their respective templates, especially when using polyester as a template. While the surface topography of cotton-templated scaffolds does not exactly resemble the topography of the jersey-knit textile fabric, the surface roughness of cotton-templated and polyester-templated scaffolds are similar (Fig. 6).

We hypothesized that the template-induced differences in scaffold morphology and surface topography might also lead to differences in their mechanical properties. As expected, the Young's moduli of the elastomeric Bionate scaffolds' were about 2 orders of magnitude lower than those of the stiffer PLGA scaffolds and dependent on the target (Fig. 7). The Young's moduli of textile-templated scaffolds were significantly lower than those of flat-metal templated scaffolds for both  $x$  and  $y$  directions, for both PLGA and Bionate ( $p < 0.01$ ). Surprisingly, only PLGA\_PE and Bionate\_CT scaffolds demonstrated some form of mechanical anisotropy. We did not observe any mechanical anisotropy at a macroscopic scale for PLGA\_CT and Bionate\_PE, in spite of regional alignment of the fibers, indicating that the microscopically observable regional anisotropy does not universally translate into a global heterogeneity of the scaffolds. Since only scaffolds made of Bionate exhibited mechanical properties in a range reported for cardiac patches and the native heart [54–56], we subsequently focused on Bionate scaffolds for the contraction analyses described below.

### 3.2. Cell- scaffold morphology

In order to elucidate the effects of scaffold surface topography and structure on cell proliferation and topography, H9C2 rat cardiomyoblasts were seeded onto textile-templated and flat PLGA and Bionate scaffolds and cultured for up to 10 days. Cell proliferation, as monitored using the alamar blue assay [42,44,45] was essentially identical on all electrospun scaffolds (data not shown). Cells seeded onto PLGA and Bionate scaffolds were fixed, permeabilized and stained for F-actin and nuclei and evaluated in a confocal microscope. As seen in Fig. 8 (PLGA scaffolds) and Fig. 9,

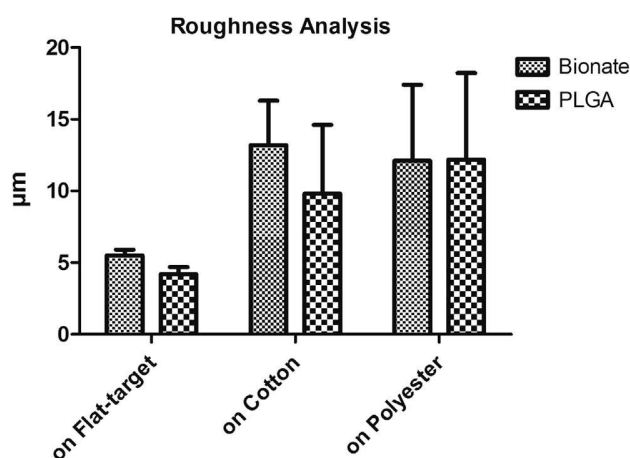


**Fig. 6.** Roughness of knitted textile-templated (templated after polyester and cotton, respectively) and flat-target-templated PLGA and Bionate scaffolds as determined by laser scanning microscopy at  $10\times$  original magnification, scanning an area of  $1 \times 1.5 \text{ mm}$ . The roughness data shown in this figure (Ra in microns, details see text) are the mean values from at least 3 independent measurements performed in triplicate, the error bars represent standard deviations.

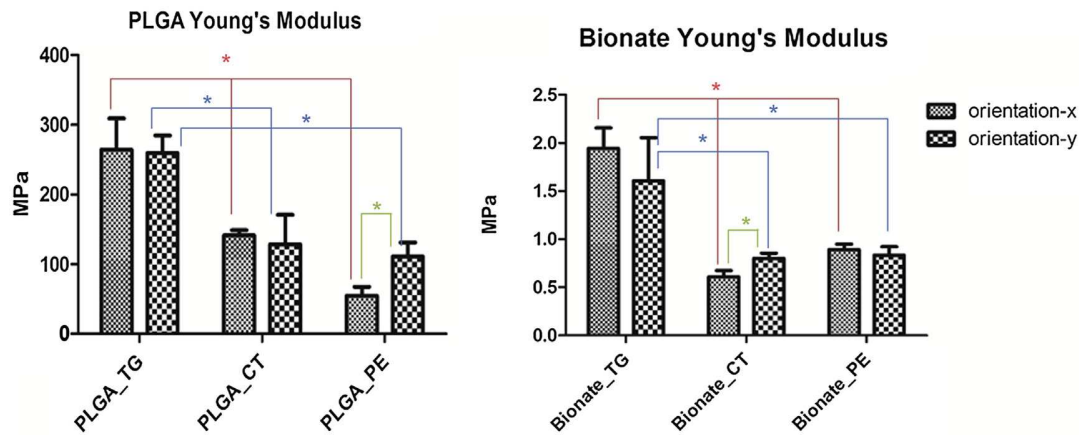


**Fig. 7.** Mechanical properties (Young's moduli) of templated electrospun scaffolds. Left graph: PLGA scaffolds electrospun on flat copper target (PLGA\_TG), on cotton template (PLGA\_CT), and on polyester template (PLGA\_PE). Right graph: mechanical properties (Young's Moduli) of Bionate scaffolds electrospun on flat aluminum target (Bionate\_TG), on cotton template (Bionate\_CT), and on polyester template (Bionate\_PE). All data are expressed as mean  $\pm$  standard deviation.  $n=3$  \*  $p < 0.01$ , comparisons as indicated in the Figure.

(Bionate scaffolds), following 10 days of culture the cells formed confluent monolayers on all scaffolds. Moreover, the intrinsic regional anisotropy of the knitted textile-templated scaffolds was translated into a local anisotropy or differential regional cell alignment. Even though the scaffolds' mechanical properties did not reveal global anisotropy (the Young's moduli in both  $x$  and  $y$  orientation were statistically largely indistinguishable), H9C2 cells detected and responded to the local surface topography and fiber alignment and formed regions of local anisotropy. The alignment of the H9C2 cells in each micrograph was analyzed with the HÜCRECI software (see Figs. 8 and 9) similar to the analyses for fiber alignment (see Figs. 2 and 3). For details see Materials and methods and also Supplemental data. On textile-templated scaffolds, the angles of cell alignment showed a rather narrow distribution range, indicating that majority of the cells were oriented in a cooperative, anisotropic fashion, essentially irrespective of the material properties of scaffolds or the templates (Figs. 8a,b and 9a,b). However, in

PLGA\_TG and Bionate\_TG scaffolds, the angles of cell alignment were distributed equally in an isotropic fashion (Figs. 8c and 9c).

### 3.3. Functional analysis of contractile "beating" scaffolds

Given their elastomeric nature and cardiac tissue-like mechanical properties, we hypothesized that textile templated electrospun Bionate scaffolds seeded with neonatal, contractile cardiomyocytes would be suitable for creating contractile anisotropic patches. We further hypothesized that the observed differences in scaffold anisotropy will significantly affect the capability of the cardiomyocytes to form contractile tissue-like assemblies and, hence result in differences in the contractility of the scaffolds. To test these hypotheses, we seeded electrospun cotton- or polyester-templated Bionate scaffolds with purified primary isolates of neonatal rat cardiomyocytes.

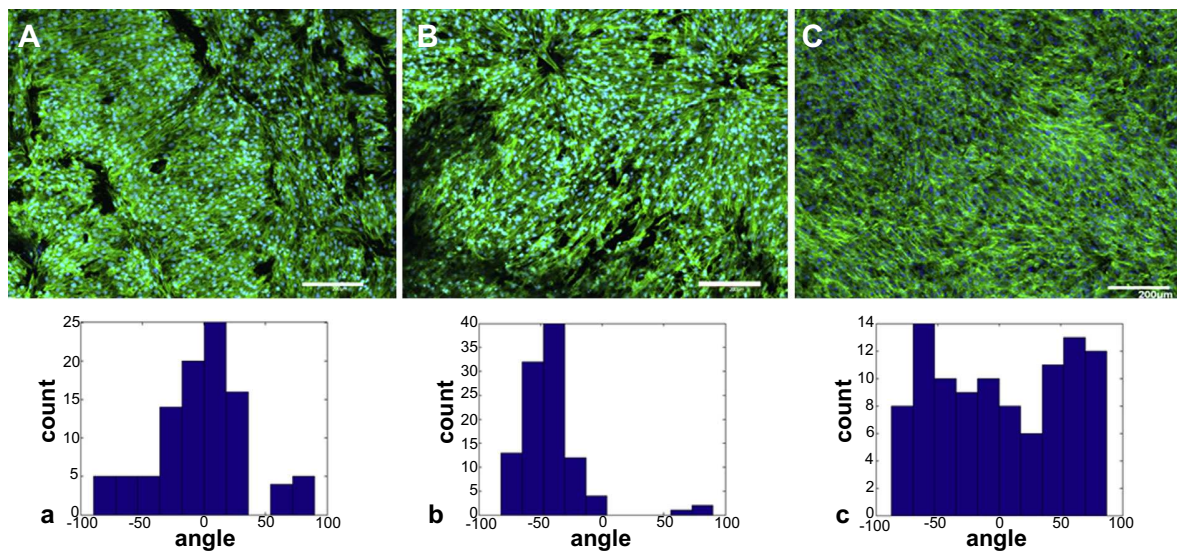


**Fig. 8.** Representative confocal micrographs (A–C) of PLGA scaffolds seeded with H9C2 cardiomyoblasts demonstrating cell alignment and their respective cell alignment histograms (a–c), measured by using the HÜCRECI software (for details see text and Supplemental materials): (A, a) PLGA scaffold electrospun on polyester template seeded with H9C2 cardiomyoblasts (PLGA\_PE+H9C2), (B, b) PLGA scaffold electrospun on cotton template seeded with H9C2 cardiomyoblasts (PLGA\_CT+H9C2), (C, c) PLGA scaffold electrospun on flat copper target seeded with H9C2 cardiomyoblasts (PLGA\_TG+H9C2), green: F-actin (phalloidin), blue: nuclei (DAPI). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

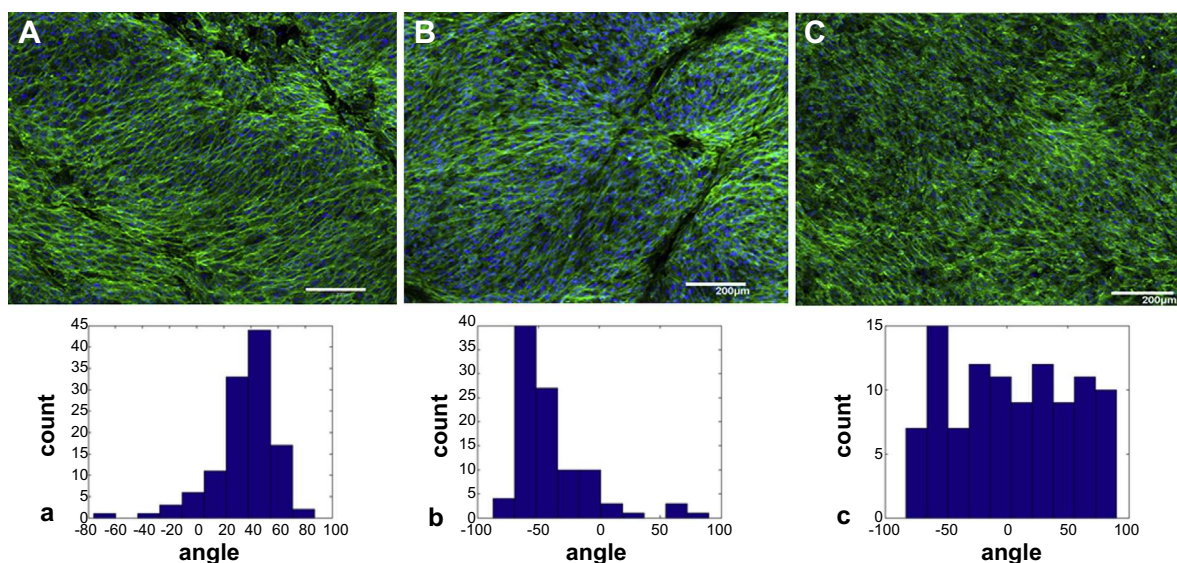


**Fig. 9.** Representative confocal micrographs (A–C) of Bionate scaffolds seeded with H9C2 cardiomyoblasts demonstrating cell alignment and their respective cell alignment histograms (a–c), measured by using the HÜCRECI software (for details see text and [Supplemental materials](#)): (A, a) Bionate scaffolds electrospun on polyester template seeded with H9C2 cardiomyoblasts (Bionate\_PE+H9C2), (B, b) Bionate scaffolds electrospun on cotton template seeded with H9C2 cardiomyoblasts (Bionate\_CT+H9C2), (C, c) Bionate scaffolds electrospun on flat aluminum target seeded with H9C2 cardiomyoblasts (Bionate\_TG+H9C2), green: F-actin (phalloidin), blue: nuclei (DAPI). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Both Bionate\_PE and Bionate\_CT scaffolds induced regional alignment of neonatal cardiomyocytes in a manner similar to what we observed with H9C2 cells. Cell alignment was more pronounced on Bionate\_PE scaffolds, while the cardiomyocytes growing on Bionate\_TG scaffolds were randomly distributed (for details, see [Supplemental data](#)).

Upon adhesion to the scaffolds, individual neonatal cardiomyocytes started contracting spontaneously on all substrates. Video recordings of the constructs confirmed that, depending on the substrate and duration of the culture, contraction of individual cells progressed to encompass synchronously-beating cardiac functional syncytium-like regions on the scaffold and, in some cases, extended to synchronous contraction of the entire scaffold [47] (see videos in [“Supplemental data”](#)). As described in [Materials and methods](#) and detailed in [“Supplemental data”](#), we developed a new image analysis-based software to quantitate the global contractility of the scaffolds. Contractility was analyzed in terms of displacement (distance traveled between contractions), frequency (“beating frequency”), and synchronization (between individual cells/Regions of Interest (ROIs) in a given field).

For all 4 time-points analyzed (3, 5, 7, and 10 days after seeding), displacement was highest on the Bionate\_PE scaffolds. Three days after seeding, cells were beating robustly (~25  $\mu\text{m}$  displacement) and increased their mean displacement to ~35  $\mu\text{m}$  during the duration of the study ([Fig. 10](#)). By contrast, maximal displacement on Bionate\_CT scaffolds never exceeded 5  $\mu\text{m}$ . Bionate\_PE scaffolds were also the only ones that displayed robust contraction of the entire scaffold, while Bionate\_CT scaffolds exhibited some regional cardiac syncytium-like contractility. No significant synchronous contraction was observed on cells growing either on flat Bionate-TG scaffolds, or on TCP controls.

In terms of the beating frequency, neonatal cardiomyocytes seeded on Bionate\_PE scaffolds demonstrated the most stable beating pattern during the entire experiment period. After 5 days the initial beating frequency (~225 bpm) dropped to ~120 bpm and remained essentially constant during the entire study period. By contrast, for cells cultured on Bionate\_CT the beat rate never

exceeded 100 bpm, while it fluctuated significantly between ~175 bpm and 50 bpm for cells cultured on Bionate\_TG scaffolds. For cells cultured on TCP, the beat frequency remained very low (<20 bpm) throughout the duration of the experiments.

Beat synchronization was quantified, using a ‘synchronization index’ (SI), which is essentially inversely correlated with the variation of beating times across different spatial locations within the same sample (see [Materials and methods](#)): the lower the variation (higher SI value) the more synchronized beating. As seen in [Fig. 10](#), SI was consistently high for cells cultured on Bionate-PE scaffolds, indicating significant synchronization of cardiomyocyte beating throughout the entire experimental duration. Synchronization, i.e. synchronous beating in a tissue-like fashion, fluctuated on all other substrates, depending on the substrates/scaffolds and time in culture.

Taken together, our results clearly indicate that seeding of Bionate\_PE scaffolds with neonatal rat cardiomyocytes will yield a functional tissue-like assembly, in which the cells exhibit robust, sustained contractility, characterized by significant displacement, physiologically relevant beating frequency and high levels of synchronization. Indeed, cell-seeded Bionate\_PE scaffolds were the only ones, which exhibited global robust and sustained contraction of the entire construct, making them good candidates for engineering cardiac patches that will be tested in the future *in vivo*.

#### 4. Discussion

Functional cardiac patches require anisotropic three-dimensional patterned scaffolds to mimic the anisotropic organization of native myocardium [15]. A major aim of this study was to develop an anisotropic and contractile ‘smart’ scaffold exhibiting the ‘contact guidance’ necessary to induce optimal alignment of cultured cardiomyocytes, which then would result in sustained synchronized contractility (“beating”) at physiological frequencies across the entire scaffold. In this study we implemented a straightforward and cost-effective approach to engineering anisotropic contractile patches using anisotropic textiles as

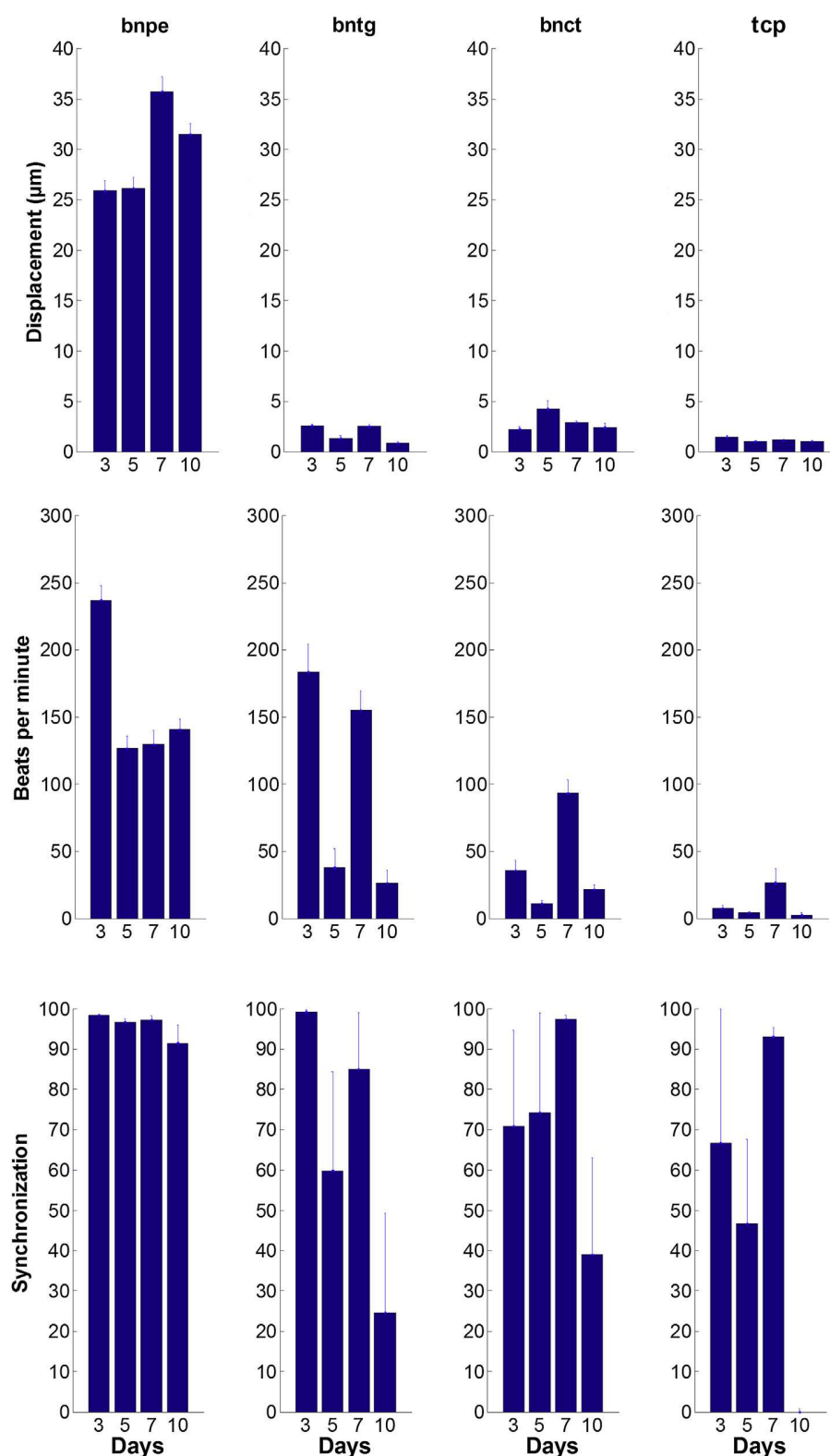


**Fig. 10.** Contraction analysis (displacement, “heart beat” frequency, and synchronization) of contracting Bionate scaffolds seeded with neonatal rat heart cardiomyocytes, as measured using a newly developed, proprietary “Motion Analysis Software”. For details see [Materials and methods](#) and [Supplemental data](#). The rows depict the results from cells cultured for up to 10 days on Bionate scaffolds electrospun on polyester template, Bionate\_PE (bnpe), Bionate scaffolds electrospun on flat aluminum target, Bionate\_TG (bntg), Bionate scaffolds electrospun on cotton template, Bionate\_CT (bnct), Tissue Culture Plastic (tcp). The bars represent the means  $\pm$  standard deviation of at least 3 independent triplicate experiments.

templates for electrospun scaffolds. For the initial proof of concept that the anisotropy and unique surface topography of textile templates can be transferred to electrospun scaffolds, we initially used, as proof-concept, PLGA [42] as scaffold material. Subsequently we focused on Bionate (polycarbonate urethane, PCU), a biostable elastomeric polymer that has excellent oxidative stability. Although not bioresorbable, Bionate has been used for a wide range of biomedical application, such as articulating joint implants, cardiovascular electrostimulation, spinal motion preservation devices, neurostimulation and total disc replacement [57]. In future studies, beyond the scope of this paper, we will also test other oxidation-resistant elastomeric materials that are also biodegradable.

The main motivation for our use of using conventional knitted textile fabrics as templates was that knitted textiles provide a good match for tissue engineering studies. Knitted textile combine tunable mechanical properties and surface topography with 3D porous structures that can promote cell migration and vascular ingrowth [25]. The sizes and shapes of the pores can also be tuned by stretching the knitted textile. By contrast, woven fabrics do not stretch unless elastic fibers are woven into the fabric at the same time. We chose Jersey knit, a conventional clothing fabric, to explore how to implement ordinary 'everyday use' textiles in high-tech tissue engineering applications. Both the synthetic textured polyester yarn (which is also used in the Acorn Cardiac Device [22], see Introduction), and the natural spun cotton yarn, tested in this study, are widely used for manufacturing conventional fabrics, e.g., in the garment industry.

Conventional weaving and knitting techniques result in highly customizable and flexible surface patterns of all kinds of garments and textiles, including those used for biomedical applications. The templates are easy to manufacture and inexpensive. Electrospinning elastomeric polymers onto these templates yields contractile cardiac constructs, whose mechanical properties and surface pattern can be controlled efficiently. Conventional knitted textiles can serve as tunable anisotropic templates with a variable 3-D surface topography. Depending on the material properties of the templates, scaffolds generated by electrospinning onto these templates can replicate the 3-D surface topography and anisotropy of the templates. Our results indicate that the structure of the yarn used for the template is essential for the unique patterning and surface architecture of the diverse textile-templated scaffolds. The topography of the resulting electrospun scaffolds resembled that of the polyester templates and less so the patterning of otherwise matching cotton templates. Both the polyester (synthetic) and cotton (organic) textile templates were Jersey knitted fabrics using "textured" and "spun" yarn respectively. When visualized under a light microscope, textured polyester yarn is made of long filaments and has a smooth surface, whereas cotton spun yarn is made of short filaments and has a fuzzy structure. We therefore surmise that the surface structure of the individual types of yarn will affect the topography and surface patterning of the electrospun scaffold [47].

The electrospinning process onto a stationary flat target typically produces rather flat scaffolds with randomly aligned fiber meshes. Using textured metallic collectors as templates to obtain a patterned structure of electrospun scaffolds, Neves and colleagues demonstrated that the mechanical properties of templated scaffolds can be modulated by manipulating the surface topography of the templates [41]. Specifically, these authors showed a significant decrease in Young's moduli of textured metal templated scaffolds as compared to control scaffolds spun onto a flat target, similar to our results for textile templated scaffolds (Fig. 7). Taken together, our results indicate that fiber alignment as well as surface patterning and surface topography can significantly modulate the

mechanical properties, such as the stiffness, of electrospun cardiac scaffolds and thus modulate their functionality.

Flat anisotropic metallic collectors, such as rotating mandrels, can be used for creating global anisotropy, such as found in fibers aligned circumferentially, e.g. in tube-like scaffolds for vascular grafts [58], but these templates generally will not yield electrospun scaffolds with tunable patterns and regional anisotropy, which is critical for use in cardiac tissue engineering [15]. While previous studies demonstrated a significant effect of fiber alignment on the mechanical properties of electrospun scaffolds [32,35], the tubular scaffolds with their fibers aligned circumferentially did not exhibit any local surface patterning, regional anisotropy or 3-D structures, as our scaffolds do.

Another important feature in designing a cardiac patch is the optimization of scaffold stiffness, which affects morphology and functionality of the cells seeded *in vitro*, as well as functional integration of the patch with the host tissue *in vivo*. The mechanical properties of the engineered scaffolds should be compatible with those of the native myocardium. There are various biomaterials and scaffold configurations that satisfy the mechanical requirements of a cardiac patch. For instance, poly-glycerol sebacate (PGS) sheets (engineered by a sheet-forming mold) produced at three different temperatures yielded scaffolds with Young's moduli of 0.056 MPa, 0.22 MPa and 1.2 MPa [59]. These Young moduli cover the reported range of stiffness of the rat and human heart muscle [59]. In another study, flexible electrospun scaffolds, generated by using PGS/gelatin core/shell blends with a Young's modulus of 6.08 MPa, were proposed as a cardiac patch [60]. The Young's modulus of our Bionate\_PE scaffolds (ca. 0.75 MPa) is quite close to that of the human left ventricle at end of systole.

We noted the differences in surface topography/patterning of the scaffolds templated after polyester and cotton. According to an established definition, "pattern" is "a regularly repeated arrangement of shapes, colors, or lines on a surface, usually as decoration" [61]. In line with this definition scaffolds templated after PE exhibit global patterning of repeated/multiple striation (in the order of many millimeters, see Fig. 1A and D), which cannot be detected by the high-resolution AFM (maximal  $50 \times 50 \mu\text{m}$  window, see Fig. 4A and D) and is barely hinted at in the Laser Scattering Microscope (with a  $1 \times 1.4 \text{ mm}$  window, see Fig. 5A and D). Similarly, for the CT-templated scaffolds we see regularly repeated circular structures (again in the multiple mm range, see Fig. 1B and E), which we again interpret as patterns. By contrast no patterning whatsoever is seen on the flat target templated scaffolds (see Fig. 1C and F). These different patterns lead in turn to different surface topographies. Remarkably, in terms of the functional outcomes, only the aligned striated patterns and surface topographies of the Bionate\_PE scaffolds provide the necessary contact guidance for the alignment of cardiomyocytes in a manner that leads to the sustained contraction described in Fig. 10 and shown in the supplemental videos.

Supplementary videos related to this article can be found online at <http://dx.doi.org/10.1016/j.biomaterials.2014.06.029>.

In order to promote an anisotropic structural organization mimicking that of cardiac muscle, engineered scaffolds must provide "contact guidance" for cells seeded onto them. For electrospun scaffolds, contact guidance is provided by the alignment of the individual fibers. However, as one of the key findings of this study, we found that the unique fiber organization of textile-templated scaffolds translated into a corresponding cellular organization, but not necessarily yielded functionality (synchronous beating) of the scaffolds. Even though Bionate\_PE did not demonstrate global mechanical anisotropy (Fig. 7), cells detected the local anisotropy, organized themselves accordingly and exhibited the most robust synchronous spontaneous beating characteristics (Figs. 9 and 10).

By contrast, Bionate\_CT scaffolds demonstrated anisotropy in terms of fiber-alignment and, to a lesser extent, also in terms of cell-alignment and global mechanical properties. However, analysis of spontaneous cell/scaffold “beating” demonstrated that the unique well-like 3-D alignment of the cells on Bionate\_CT scaffolds did not promote robust synchronous contractions. We posit that for similar (Jersey) knitted patterns of the textiles, the individual fiber type (synthetic or natural) and 3-D patterning will play a significant role in determining the overall functionality of engineered cardiac-like constructs comprising textile-templated anisotropic scaffolds.

The most robust, sustained “beating”, as assessed by displacement, frequency and synchrony, was found with neonatal rat cardiomyocytes cells seeded onto Bionate\_PE scaffolds (Fig. 10). According to the literature, hearts of 2 days-old rats beat at 135–155 bpm *in vivo*, while the beat frequency of neonatal cardiomyocytes cultured on TCP is ~60–100 bpm [62]. In this study, we found that under optimized conditions, i.e. on Bionate-PE, the beating frequency was near- physiologic and synchronous throughout the experimental duration, commencing at ~200 bpm and stabilizing at ~120 beats/min. These results are in good agreement with the beating frequencies of neonatal hearts *in vivo*. By contrast, under suboptimal conditions, e.g., when neonatal cardiomyocytes were cultured on Bionate\_TG, Bionate\_CT and on TCP, we observed much weaker (in terms of displacement) and irregular beating patterns (Fig. 10). We surmise that the unique topography of Bionate\_PE scaffolds may have enabled optimum cell–cell communication that is required to keep the beating stable and in a physiological range for duration of the study (10 days). Taken together our results clearly favor the development of Bionate\_PE scaffolds as potential candidates for cardiac patches.

In a previous study individual cardiomyocytes were found to beat asynchronously in different areas of porous cardiac scaffolds; occasionally, the magnitude of contraction was sufficient to move the entire elastic scaffolds with each beat [63]. In another study, using aligned electrospun scaffolds cardiomyocytes showed coordinated beating upon electrical stimulation. Yet, this synchronous beating pattern was limited to individual cell clusters rather than the entire scaffold [64]. Rhythmic contraction of entire cardiac patches generally requires relatively thin elastomeric scaffolds concomitant with high seeding densities of neonatal cardiomyoblasts [65,66]. None of the above cited studies [63–66], however, demonstrated macroscopic cellular alignment through contact guidance of the scaffold. We presume that the contraction data found in the above studies [63–66] is in line with our results for rat neonatal myocytes seeded onto flat-target templated Bionate scaffolds.

Some xenogeneic scaffolds used a cell sheeting technique [67] to attain spontaneous contractions. As a drawback, engineering functional myocardial heart patches by stacking several cell sheets is limited in thickness due to lack of sufficient supply of oxygen and nutrients across more than 3–4 layers of cell sheets [68]. This drawback could be ameliorated by enhancing neo-vascularization of the multilayer cell-sheets using angiogenic growth factors and/or endothelial precursor cells as part of the cell sheet make-up.

In extending their previous studies on accordion like anisotropic scaffolds, Park and colleagues recently manufactured multi-layered elastomeric polyglycerol sebacate scaffolds with controlled pore microarchitectures by laser ablation and seeded these scaffolds with heart cells using an interstitial perfusion technique. After 7 days of culturing cells *in vitro*, these scaffolds contracted in response to electrical field stimulation and could be paced at frequencies of 1–2 Hz. Accordion-like scaffolds are promising, yet technically demanding candidates for cardiac patches. By contrast, in focusing on engineering a “simple” cardiac patch, which

structurally recapitulates the anisotropy of the myocardium, we found that the unique yarn structure and surface topography of knitted polyester textile templates promoted robust global contractility of Bionate\_PE scaffolds. Given the ease of manufacturing of textile-templated electrospun scaffolds and the significant regional alignment of the cardiomyocytes cultured on them (leading to sustained physiological contractions of the entire patches), Bionate\_PE scaffolds may be a favorable choice for engineering contractile cardiac patches.

## 5. Conclusions

Designing contractile anisotropic scaffolds is critical for engineering a functional myocardial patch. For clinical applications, bioactive cardiac patches should not only physically support (girdle) the failing heart, but rather provide functional contractile matrices that can participate in the repair and regeneration of damaged myocardium upon implantation. In this study, we demonstrated that electrospun scaffolds templated according to ordinary textiles, especially woven polyester, exhibit macroscopic three-dimensional patterning as well as fiber alignment, which induce alignment, elongation and cardiac-like organization of rat cardiomyoblasts and neonatal cardiomyocytes. The functionality, i.e. physiologic contractility of electrospun anisotropic, polyester-templated Bionate scaffolds was demonstrated by seeding them with neonatal rat cardiomyocytes *in vitro*. Taken together and in comparison to recently published approaches, our platform technology of electrospinning nanofibrous scaffolds onto patterned textile templates allows for faster, less expensive, scalable, and more efficient manufacturing of smart anisotropic scaffolds for use as cardiac patches or tissue repair. Future studies are aimed at testing our electrospun textile-templated cardiac patches (Bionate\_PE scaffolds) *in vivo* by implanting them epicardially into a large animal MI model. We reason that our patches will also serve as a promising tool for the delivery of cells and/or growth factors for the repair and regeneration of the infarcted myocardium.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found online at <http://dx.doi.org/10.1016/j.biomaterials.2014.06.029>.

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