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The potential of anisotropic matrices as substrate for heart valve engineering

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ABSTRACT

Cells environment is increasingly recognized as an important function regulator through cell–matrix interactions. Extracellular matrix (ECM) anisotropy being a key component of heart valves properties, we have devised a method to create highly porous anisotropic nanofibrillar scaffolds and studied their suitability as cell-support and interactions with human adipose derived stem cells (hADSCs) and human valve interstitial cells (hVICs). Anisotropic nanofibrillar scaffolds were produced by a modified jet-spraying method that allows the formation of aligned nanofibres (600 nm) through air-stream diffraction of a polymer solution (poly (ε-caprolactone, PCL) and collection onto a variably rotating drum. The resulting matrices of high porosity (99%) mimicked valve mechanical anisotropy. Dynamically seeded hADSC and hVIC cultured on scaffolds up to 20 days revealed that hADSC and hVIC penetration within the matrices was improved by anisotropic organization. Within 10 days, cells populated the entire scaffolds thickness and produced ECM (collagen I, III and elastin). As a result, mechanical properties of the constructs were improved over culture, while remaining anisotropic. In contrast to isotropic matrices, anisotropy induced elongated hADSCs and hVICs morphology that followed nanofibres orientation. Interestingly, these morphological changes did not induce hADSC differentiation towards the mesoderm lineages while hVIC recovered a physiological phenotype over culture in the biomimetic matrices. Overall, this study indicates that highly porous anisotropic jet-sprayed matrices are interesting candidates for valve tissue engineering, through anisotropic mechanical properties, efficient cell population, conservation of stem cells phenotype and recovery of hVIC physiological phenotype.

1. Introduction

In an organism, tissues are formed using a bottom-up approach where cells first synthesize an optimal extracellular matrix, which subsequently supports cell proliferation and resulting organization into a mature tissue. A promising reverse-engineering approach consists in providing cells with a well-defined three-dimensional fibrillar matrix that mimics the structure and organization of the extracellular matrix and provides a directive template for the formation of a functional tissue [1]. Such a strategy appears of particular interest for specialized tissues as, for instance, heart valves. Indeed, this complex tissue is characterized by the strong polarization of the collagen fibers along the circumferential axis, which provides its specific anisotropic mechanical properties [2,3].

With the aim of providing a template for the formation of various functional tissues, synthetic nanofibrillar matrices have been extensively studied over the past decade [4–7]. In this context, electrospinning has become the prominent method for fabricating nanofibrillar analogs [8–10] owing to a strong versatility in applicable materials, fiber sizes produced or structural organization [11]. Of particular interest is the possibility to produce aligned nanofibers that mimic the natural organization of anisotropic tissues such as myocardium [12,13] or nerves [14] and provide organizational instructions to the cells. Surprisingly, heart valves functional anisotropy has been seldom considered in the design of supporting matrices for valve engineering [15].

A significant limitation of electrospinning is the production of structures composed of closely packed nanofibers and hence small pore sizes [16,17]. As a result, cellular infiltration and colonization are hampered and limited to the surface of electrospun matrices

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[18,19]. The alignment of the nanofibers further aggravates this drawback [20] and reduces the capacities of the matrices to direct the formation of an anisotropic tissue.

To circumvent this limitation, we developed anisotropic nanofibrillar structures using a jet-spraying approach [21] and poly (ε-caprolactone) (PCL). This polymer was selected for its biocompatibility, mechanical properties, slow degradation rate and ability to support a wide variety of cell types [22,23]. We hypothesized that the elevated porosity of the produced matrices combined with nanofibrillar alignment would allow a swift, efficient and homogenous colonization by adipose derived stromal cells while allowing extracellular matrix production, directing cell organization and providing a functional substrate for heart valve tissue engineering.

2. Materials and methods

2.1. Materials

Poly (ε-Caprolactone) (PCL, molecular weight 80,000 g/mol), obtained from Solvay Caprolactones (Warrington, United Kingdoms) and chloroform of analytical grade, acquired respectively from Sigma Chem. corp. (St. Louis, United States of America), were used as received. The spraying device was a standard commercial sprayer. Collagenase A was acquired from Roche (Mannheim, Germany). CellCrown inserts were supplied by Scaffdex (Tampere, Finland). Low glucose Dubbelco's modified eagle's medium (DMEM), L-glutamine, penicillin and streptomycin, trypsin, phosphate buffered saline, type 2 collagenase, DNA quantification kit, 4′-6-Diamidino-2-phenylindole (DAPI) and paraformaldehyde were obtained from Sigma. Fetal bovine serum (FBS) was acquired from PAA Laboratories GmbH (Pasching, Austria). Tissue Tek O.C.T. was supplied by Sakura (Zoeterwoude, The Netherlands). Immunohistochemistry peroxidase staining kit was purchased from DAKO (Via Real, CA, USA) and antibodies were obtained from Novotec (Lyon, France) for collagen I and III, Abcam (Cambridge, United Kingdom) for tropoelastin, Serotec (Oxford, United Kingdom) for CD44 and osteopontin, R&D systems (Minneapolis, U. S. A.) for Stro-1and sox 9, Santa cruz (California, U. S. A.) for core binding factor alpha and peroxisome proliferator-activated receptor gamma, DAKO for smooth muscle alpha actin, calponin, myosin and vimentin, and Dianova (Hamburg, Germany) for fibroblast surface antigen. Secondary antibodies (alexa goat anti mouse IgG for mouse monoclonals antibodies and Goat anti rabbit for rabbit polyclonals antibodies) and rhodamine-conjugated phalloidin staining were acquired from Invitrogen (Paisley, United kingdom).

2.2. Algined nanofibrillar matrices by jet-spraying

Aligned nanofibers were prepared using a jet-spraying method. A PCL solution in chloroform (0.1 g/ml of chloroform) was placed in a reservoir connected to a spraying device by a tube. The spraying device (paintbrush) consisted of a conic nozzle containing a needle of adjustable position (nozzle opening of −0.5 mm). The tip of the nozzle was positioned in front of a hollow shaft dispensing an air stream (air pressure of 6 bars). This airflow drove the polymer solution from the reservoir to the nozzle, where it was diffracted on the needle and projected (spraying distance of 30 cm) onto a 15 cm diameter voltage-driven drum rotating at various speeds (0, 5.8, 11.6, 17.5, 23.3 m/s). Fibers are formed in transit by cohesion of the polymer phase and solvent evaporation.

2.3. Characterization of matrices

Fiber diameters and fiber orientation of jet-sprayed materials were investigated by scanning electron microscope (SEM, JSM-6010LA, JEOL, Tokyo, Japan). Fibers produced after a few seconds of spraying to obtain a thin layer were collected on a carbon stub and gold sputtered prior to observation (Emitech K550X, Quorum Technologies Ltd, Kent, UK). For each sample, five SEM pictures were taken randomly at two different magnifications (×700 and ×1000) and fiber diameters were measured by randomly selecting three diameters along each available fiber. An average of 860 values was obtained for a given sample. Quantification of fiber alignment was computed using the NIS-Elements software (Nikon Instruments Europe B.V., Badhoevedorp, The Netherlands) "orientation" routine. Briefly, after thresholding of the SEM images, the software automatically separated each selected fibers into smaller objects of comparable sizes while excluding non-linear objects. Longitudinal orientation relative to the picture bottom (referred as 0°) of each object was then computed. 150 values were obtained on average for a given picture.

Mechanical properties of the nanofibrillar mats (5 mm × 1 cm, 500–1000 μm thick) were measured by tensile testing (Bose Electroforce TestBench, Minnesota, USA) at a speed of 0.1 mm/s. For each condition, 5 samples, cut longitudinally and orthogonally to the drum rotational direction, were measured.

Porosity of the matrices was evaluated from the dry weight and volume of 5 cylindrically cored samples (12 mm in diameter, 800–1000 μm thick). The thickness of each sample was determined using a micro-caliper and resulting porosity was then deduced from the following equation:

$$p = 1 - (\text{sample weight}/(\text{sample volume} \times \text{polymer density}))$$

Polymer density was obtained similarly, from the weight and volume of dense polymer cylinders obtained by film-casting.

Median pore diameter and total pore area of the matrices were evaluated using mercury intrusion porosimetry (Autopore IV 9500, Micromeritics, Norcross, U.S.A.) as previously reported [17]. Briefly, each measured sample was weighted prior to being filled with mercury from an initial pressure of 0.33–60,000 pound per square inch absolute (psia). The pore diameter distribution was then calculated from the Washburn equation.

2.4. Mechanical characterization of cultured nanofibrillar matrices and human valves

Mechanical properties of nanofibrillar mats seeded with hADSC and cultured for 20 days ($n = 5$ longitudinally and orthogonally) and human pulmonary and aortic valves from 3 deceased male patients (57, 59 and 60 years old) were obtained after informed consent and ethical approval from Royal Brompton and Harefield NHS Trust Ethical Committee. Leaflets were dissected and equally split radially in two parts. Each part was subsequently subdivided in 2 or 3 equal strips, either in the radial or circumferential direction. Mechanical properties were then measured as abovementioned in wet conditions (saline).

2.5. Cell isolation and culture

Human bone marrow mesenchymal stem cells over-expressing human telomerase reverse transcriptase (hMSC–TERT) kindly donated by Professor Moustapha Kassem (Department of Endocrinology, University Hospital of Odense, Denmark) were plated at 50,000 cells/cm^2 in DMEM supplemented with 10% FBS, 100 UI/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (complete medium), in 5% CO_2-95% air at 37 °C. The medium was changed three-times a week until cells reached between 90 and 100% confluence and were passaged. hMSC–TERT at passage 40 were used in preliminary seeding experiments.

To isolate adipose derived stromal cells (hADSC), abdominal subcutaneous adipose tissue from 3 female patients (average of 43 years old) undergoing abdominal liposuction was used after informed consent and ethical approval from Royal Brompton and Harefield NHS Trust Ethical Committee. hADSCs were isolated by extensive washing of adipose tissue with equal volumes of 0.9% NaCl saline followed by digestion at 37 °C for 1 h with 0.075% collagenase A. The digested tissue was then successively centrifuged at 400 *g* for 5 min, resuspended in saline, filtered through a 250 nm nylon mesh, centrifuged again at 400 *g* for 5 min, resuspended in saline and finally filtered through a 100 μm and a 40 μm cell strainer. The resulting cell suspension was then plated at 50,000 cells/cm^2 in complete medium and incubated in 5% CO_2-95% air at 37 °C. The medium was changed three-times a week until cells reach confluence and were passaged. Cell expansion was continued till second passage, where cells were seeded on nanofibrillar matrices.

Multipotency of hADSCs at passage 2 was confirmed in adipogenic, osteogenic and chondrogenic conditions using histochemical staining. Cell phenotype was analyzed using FACS for the following marker: presence of CD44, CD73, CD 90 and lack of CD14, CD31 and CD45. (data not shown).

Human valve interstitial cells (hVIC) were isolated from mitral valve leaflets of 3 patients (52, 58 and 66 years old, deceased from intracranial bleeds) after relatives consent and ethical approval from Royal Brompton and Harefield NHS Trust Ethical Committee. Leaflets were excised and washed in PBS, placed in a type II collagenase solution (1000 u/ml) and agitated for 10 min at 37 °C to remove endothelial cells. The tissue was removed, washed in PBS, minced and digested for a further 45 min to remove the hVIC. The cell suspension was then centrifuged and the resulting pellet resuspended in DMEM prior to being plated on tissue culture flasks. The hVIC were grown and maintained in complete medium until confluent. The resulting cells were used between passage 3–6 and individual isolates were not pooled.

2.6. Characterization of cell fate within anisotropic nanofibrillar scaffolds

To determine a suitable cell seeding method, hMSC–TERT were either top-seeded on non-aligned nanofibrillar or added in the culture medium prior to rotating the samples at 12 rpm. The matrices were prepared as aforementioned using drum rotational speeds of 0 m/s and samples were cored from the created mats ($n = 2$ per seeding condition, 1 cm in diameter, 700–900 μm thick) and sterilized by incubation overnight in 70% alcohol prior to rinsing three times for 1 h in sterile PBS. The structures were then fixed on CellCrown inserts. Top-seeding was performed by adding a suspension of 600,000 cells in 20 μl of complete medium on the matrices top surface. After 2 h of incubation (37 °C, 5% CO_2 humidified atmosphere) to permit cell adhesion, the constructs were placed in 12 ml tubes containing 10 ml of complete medium and rotated at 12 rpm.

For cellular tests, mats of different nanofibrillar alignment were prepared as aforementioned using drum rotational speeds of 0 (non-aligned), 11.6 (intermediate alignment) and 23.3 m/s (aligned). Samples cored from the created mats (1 cm in diameter, 700–900 μm thick) were sterilized and fixed on CellCrown inserts as aforementioned, prior to be placed in 12 ml tubes containing 9 ml of complete medium. hADSC and hVICs isolated from three different patients ($n = 3$) were then separately suspended in complete medium (600,000 cells/ml). After adding 1 mL of