Living Nano-Micro Fibrous Woven Fabric/Hydrogel Composite Scaffolds for Heart Valve Engineering

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ABSTRACT

Regeneration and repair of injured or diseased heart valves remains a clinical challenge. Tissue engineering provides a promising treatment approach to facilitate living heart valve repair and regeneration. Three-dimensional (3D) biomimetic scaffolds that possess heterogeneous and anisotropic features that approximate those of native heart valve tissue are beneficial to the successful in vitro development of tissue engineered heart valves (TEHV). Here we report the development and characterization of a novel composite scaffold consisting of nano- and micro-scale fibrous woven fabrics and 3D hydrogels by using textile techniques combined with bioactive hydrogel formation. Embedded nano-micro fibrous scaffolds within hydrogel enhanced mechanical strength and physical structural anisotropy of the composite scaffold (similar to native aortic valve leaflets) and also reduced its compaction. We determined that the composite

scaffolds supported the growth of human aortic valve interstitial cells (HAVIC), balanced the remodeling of heart valve ECM against shrinkage, and maintained better physiological fibroblastic phenotype in both normal and diseased HAVIC over single materials. These fabricated composite scaffolds enable the engineering of a living heart valve graft with improved anisotropic structure and tissue biomechanics important for maintaining valve cell phenotypes.

KEYWORDS: Structure anisotropy; nanofiber yarns; textile technique; human aortic valve interstitial cells; fibroblastic phenotype

1. INTRODUCTION

Heart valve-related disease is a major cause of substantial morbidity and death worldwide. Approximately 300,000 patients require heart valve replacement each year, and this number is expected to triple by 2050 [1]. Mechanical or bioprosthetic valves are currently the most common surgical replacement of diseased heart valves, especially aortic valves. However, these valve replacement devices have a number of limitations including the lack of biocompatibility and durability (i.e., thrombo-embolic complications, mechanical failure and extensive calcification), and in particular the inability to grow and remodel in vivo [2-5]. Tissue engineered heart valves (TEHV) are envisioned to have the capacity for growth and adaptation, representing a promising one time surgical solution [6,7].

The complex, striated extracellular matrix (ECM) of aortic valve leaflet is organized into three layers: ventricularis, with radially aligned laminate of collagen and elastin fibers; glycosaminoglycan (GAG) rich spongiosa, and the fibrosa consisting of large circumferentially aligned collagen fiber bundles [8-11]. Two main cell types populate the leaflets: valvular endothelial cells (VEC) and valvular interstitial cells (VIC). VIC are located throughout each layer of ECM and play an important role in maintaining the valvular structure and function due

to their ability to synthesize and remodel the ECM [12-14]. A variety of synthetic and natural polymers have been employed to fabricate porous, fibrous or hydrogel valve scaffolds [18,19]. Though these valved conduits have functioned for upwards of 20 months in vivo, progress to clinical use remains impaired. These materials are limited in their ability to create one or more of these features: native-like anisotropic material architecture [20-22], microenvironments for cellular engraftment and differentiation [23,24], native tissue biomechanics (e.g. stiffer scaffolds or softer hydrogels) [25-27], and/or inferior suturability [28, 29]. These findings motivate the need for a three-dimensional (3D) scaffold that can not only provide physiological support for cell growth and ECM production, but also mimic the anisotropic structure and mechanical properties of the native aortic valve leaflet [15-17].

Our group and others recently fabricated hydrogel scaffolds based on methacrylated hyaluronic acid (Me-HA) and methacrylate gelatin (Me-Gel) to produce TEHV for heart valve disease and tissue engineering research [30-33]. The Me-HA/Me-Gel hybrid hydrogel system can mimic the unique 3D physiological microenvironment of ECM in native aortic valve leaflet. This hydrogel system with transverse stiffness tuned to that of the ventricularis and fibrosa layers of aortic valve leaflet [33] enabled high efficiency for cell attachment, proliferation, and maintenance of a fibroblastic phenotype (normal phenotype) of human aortic valve interstitial cells (HAVIC). However, these hydrogel materials lacked macroscale anisotropic structure and were far weaker in extension than the native aortic valve leaflets. We hypothesized such shortcomings can be addressed and compensated by adding a fibrous component to both reinforce the structure and physical property and to provide contact guidance for internal organization of the cells and protein deposition [34-38].

In the current study, we created a composite biomaterial construct combining a fibrous woven biomaterial fabric and bioactive hydrogel with encapsulated HAVIC. This strategy combines the properties of ECM-mimicking hydrogel and anisotropic woven fibrous biomesh to provide both elasticity and anisotropy. We hypothesized that the composite constructs can mimic the structural and mechanical properties of native aortic valve leaflets while simultaneously support cell growth and tissue formation with controlled micro-architecture. Both normal and diseased HAVIC were isolated, expanded in vitro and encapsulated into composite scaffolds. The HAVIC-seeded woven fibrous scaffolds and HAVIC-laden hydrogel scaffolds were used as control groups. Specimens of each group were examined by their physical and mechanical properties, and cell behaviors, including cell growth, proliferation, ECM production, and gene expression. This approach has the potential for successful translation towards a TEHV replacement in aortic valve leaflet application.

2. Materials and Methods

2.1. Fabrication of nano-micro fibrous woven fabric

A novel modified electrospinning setup designed by our group [39] was employed to continuously fabricate polyacrylonitrile (PAN) nanofiber yarns (NY). The diameters of the PAN NY and inside nanofibers were $136.59 \pm 28.55 \mu\text{m}$ and $550.32 \pm 66.91 \text{ nm}$, respectively. PAN microfiber yarns (MY) were obtained from Weifang Jinyi Yarn Co., Ltd (China). The diameters of the PAN MY and inside microfibers were about $235 \mu\text{m}$ and $20 \mu\text{m}$, respectively. We employed an electronic jacquard machine (Donghua University, China) to manufacture nano-micro fibrous woven fabric ($\sim 250 \mu\text{m}$ in thickness). PAN NY were used as the weft yarns to pass through PAN MY (the wrap yarns) to form the plain weaving structure, as shown in Fig. 1A.

2.2. Fabrication of hydrogel scaffolds and woven fabric/hydrogel composite scaffolds

A hydrogel precursor solution composed with Me-HA (0.5 % w/v) and Me-Gel (1 % w/v) was prepared in cell culture medium with 2-hydroxy-1(4-(hydroxyethoxy)phenyl)-2-methyl-1-propanone (Irgacure 2959, 0.05 % w/v, CIBA Chemicals, Switzerland) [31,33]. The gel precursor was transferred into silicone molds (8 mm, 0.5 mm thickness) and subsequently exposed to 365 nm UV light (EN-280L, Spectroline, USA, 2.0 mW/cm²) for 5 min, to obtain hydrogel scaffolds.

PAN nano-micro fibrous woven fabrics were cut into 10 mm × 10 mm rectangular samples and were put into contact with the precursor hydrogel solution by silicone molds (8 mm, 0.5 mm thickness), as shown in Fig. 1A. The resulting composite was crosslinked using UV light to form woven fabric/hydrogel composite scaffolds.

2.3. Physical characterization of the scaffolds

Field emission scanning electron microscope (FESEM, Tescan Mira3, Czech Republic) was used to characterize surface morphology of PAN nano-micro fibrous woven fabric. For mechanical characterization, uniaxial tensile test was performed by an Instron mechanical tester. The gauge length and crosshead speed were set as 10 mm, 10 mm/min, respectively. PAN nano-micro fibrous woven fabrics were cut into rectangular shapes (L × W= 30 mm × 10 mm). The displacement and load data were converted to strain and stress, respectively, by normalizing to sample thickness and area. We measured and calculated the initial modulus (E Init.), transient modulus E (E Trans.), peak tangent modulus (E Peak), yield strength, and yield elongation from the representative stress-strain curves, and the calculation methods were depicted in Fig.2A, B.

2.4. Cell isolation and cell culture

Normal human aortic valve interstitial cells (nHAVIC) were isolated separately from the aortic valve leaflets of the donor heart from a patient undergoing cardiac transplant for a myocardial

contractility deficiency [31]. Diseased HAVIC (dHAVIC) were isolated from non-calcified areas of calcified aortic valve leaflets from a patient undergoing valve replacement. All the tissues were procured with consent as approved by the Institutional Review Board of Weill-Cornell Medical College in New York City as previously described [40]. nHAVIC or dHAVIC were cultured in HAVIC growth medium (GM) containing MCDB131 medium (Sigma, USA), 10 % fetal bovine serum (FBS, Invitrogen, USA), 1 % penicillin/streptomycin (P/S, Invitrogen, USA), 0.25 µg/L recombinant human fibroblast growth factor basic (rhbFGF, PeproTech, USA) and 5 µg/L recombinant human epidermal growth factor (rhEGF, Invitrogen, USA) at 37 °C and 5 % CO₂ [33,40,41]. Cells were used at passages 4-8.

2.5. HAVIC seeding on nano-micro fibrous woven fabric and encapsulation within hydrogels and composite scaffolds

PAN fibrous woven scaffolds were cut into 10 mm × 10 mm rectangular samples and sterilized in 70 % (v/v) ethanol overnight, and then washed twice in phosphate buffered saline solution (PBS). The nHAVIC or dHAVIC were seeded onto the fibrous scaffolds, and incubated for 3 h to allow the cells to attach. The cell-laden-fibrous scaffolds were maintained in GM, and the GM was changed every 2 days. For HAVIC encapsulation into hydrogels and composite scaffolds, HAVIC were resuspended within hydrogel precursor solutions. The hydrogel or composite scaffolds were fabricated as previously described with HAVIC encapsulation after photocrosslink. These nHAVIC or dHAVIC-laden hydrogels and composite scaffolds were washed with PBS and maintained in GM, and the GM was replaced every 2 days.

2.6. Cell viability and area shrinkage ratio test

The viability of nHAVIC within the scaffolds was determined on days 7 and 14 of culture by Live/Dead assay (Invitrogen) as previously described [33] and fluorescence images were

obtained using a confocal laser scanning microscopy (CLSM, LSM 710, Carl Zeiss, Germany). Zeiss Discovery v20 stereomicroscope (Spectra Services, Inc.) was employed to observe and record the area change of nHAVIC-laden scaffolds (n=6 per group). The scaffold area was determined on day 0 (3 h after cell seeding or encapsulation) and 14 for each type of scaffold sample. Image J software was used to assess the area shrinkage ratio.

2.7. Biochemical assays

DNA content was quantified on nHAVIC-laden fibrous, hydrogel and composite scaffolds at day 7 and 14 by using a PicoGreen dsDNA quantification kit (Invitrogen, USA). Dimethylmethylene blue (DMMB) assay was performed to quantify the sulfated GAG content that was synthesized at each specific time point [33]. The DNA, collagen and GAG content (n=6 per group) were measured according to manufacturer's instruction using a microplate reader (Bio-Tek Instruments, USA).

2.8. Immunofluorescent staining

The protein expression of α -SMA and vimentin was visualized by immunofluorescent staining after 14-day culture. The nHAVIC-laden scaffolds were fixed in 4 % paraformaldehyde and then blocked with 1 % bovine serum albumin (BSA) overnight at 4 °C. The constructs were then treated with primary antibodies to monoclonal anti- α -smooth muscle actin (α SMA)-Cy3 antibody (1:200, Sigma, USA, mouse monoclonal), vimentin (1:100 Sigma, USA, clone V9 in mouse). Secondary fluorescent antibodies were incubated for 2 h and nuclear counterstaining (via Draq 5, 1: 1000, Biostatus, UK) were performed for 30 minutes at room temperature. The stained samples were imaged with Zeiss 710 CLSM.

2.9. Osteogenic differentiation induction

HAVIC (nHAVIC or dHAVIC) laden constructs were conditioned in GM for 1 day, and then were induced into osteogenic differentiation medium (ODM) for another 13 days. The ODM consisted of MCDB131 medium, 10 % FBS, 1 % P/S, 100 nM dexamethasone (Sigma), 10 mM β -glycerophosphate (Sigma) and 50 μ M ascorbic acid (Sigma). The ODM was replaced every 2 days.

2.10. Alkaline phosphatase (ALP) staining and activity

ALP staining and activity quantification was performed after 14-day culture. Alkaline phosphatase leukocyte kit (Sigma) was used according to the manufacturer's protocol for ALP staining. ALP activity quantification was performed as previously described [33]. The total protein content was determined using BCA assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard and the ALP activity was expressed as μ mol of p-nitrophenol formation per minute per milligram of total proteins (μ mol/min/mg protein).

2.11. RNA isolation and quantitative real time polymerase chain reaction (PCR)

Total RNA was extracted from three different constructs after 14-day culture in GM or ODM using QIA-Shredder and RNeasy micro-kits (QIAGEN, USA) according to the manufacturer's instructions. Total RNA was synthesized into first strand cDNA in a 20 μ L reaction using iScript cDNA synthesis kit (BioRad Laboratories, USA). Real-time PCR analysis was performed in a CFX Connect Real-Time PCR detection system (Bio-Rad, USA) using SsoAdvanced SYBR Green Supermix (Bio-Rad, USA). All primers used in this study are listed in Supplementary Table S1. cDNA samples were analyzed for gene of interest and for the housekeeping gene 18s rRNA. The level of expression of each target gene was calculated using comparative Ct method (also known as $2^{-\Delta\Delta C_t}$ method).

2.12. Statistical analysis

All quantitative data were expressed as mean $\pm$ standard deviation (SD). Pairwise comparisons between groups were conducted using ANOVA with Scheffé post-hoc tests in statistical analysis. A value of $p < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Composite scaffold formation and morphology

The textile weaving approach was first employed to manufacture PAN nano-micro fibrous constructs as depicted in Fig. 1A. PAN nano-micro fibrous woven fabrics possessed notable anisotropic geometric features with controlled pore size (from several micrometers to several hundred micrometers). PAN NY were used as the weft yarns (indicated as nano direction) to pass through PAN MY (the warp yarns, indicated as micro direction) to form the plain weaving structure (Fig. 1B, C). The obtained PAN woven fabric was mechanically stable, since it can be easily manipulated during and after weaving. The PAN MY and PAN NY in the bio-mesh replicated the circumferential and radial anisotropy of native aortic valve leaflets, respectively. Following the textile weaving technique, the Me-HA/Me-Gel bioactive hydrogel precursor was added to the PAN nano-micro fibrous woven fabrics and photocrosslinked by UV light to form the composite scaffolds. The Me-HA/Me-Gel hydrogels can fully penetrate the pores of the woven scaffolds to enable encapsulated cells to be homogeneously distributed. The hydrogel component is stable without delamination during the whole cell culture period. 3D fabric/hydrogel composite scaffolds were successfully fabricated using PAN nano-micro fibrous woven fabrics and bioactive Me-HA/Me-Gel hydrogel system.

3.2. Composite scaffold design mimics native heart valve tissue mechanics

Me-HA/Me-Gel hydrogels possessed very weak tensile mechanical characteristics and could hardly be tested in the tensile testing system. We thus evaluated the uniaxial tensile testing

results of the other two TEHV scaffolds: i.e. PAN nano-micro fibrous woven fabric and 3D composite construct. Both woven fabric and composite scaffold demonstrated mechanical behavior characteristics that mimic native aortic valve leaflets, expressing the initial toe, transient, and peak tangent regions under loading in a stress-strain curve (Fig. 2A, B) [42,43]. Within the initial toe region, stress slowly increased with applying tensile strain, likely resulting from initial PAN NY or MY reorientation. Once past this initial region, the transient region appeared, where the PAN NY or MY were completely aligned and elongated in the direction of tensile loading. With further strain, the tensile curves began to display a steeper slope (known as peak tangent region), where higher force was required to further deform and stretch the TEHV scaffolds. The tangent moduli of TEHV scaffolds in these three regions were also calculated in both micro and nano direction in Fig. 2C-E. Taking composite scaffold as an example, in micro direction, $E_{\text{Init.}}$, $E_{\text{Trans.}}$, and E_{Peak} were calculated to be 0.6 ± 0.4 MPa, 10.2 ± 4.7 MPa, and 21.8 ± 8.4 MPa, respectively, while in nano direction, the values were 0.4 ± 0.2 MPa, 2.5 ± 0.9 MPa, and 6.1 ± 2.2 MPa. Fig. 2F and G showed yield stress (stress at which the material begins to deform plastically, 4.5 ± 0.6 MPa for micro direction and 1.8 ± 0.3 MPa for nano direction) and yield strain (strain representing yield stress, 44.6 ± 5.9 % for micro direction and 61.2 ± 5.0 % for nano direction) of the composite scaffold. These results demonstrate that both the woven fabric and composite scaffolds exhibit biomechanical anisotropy similar to native aortic valve leaflets. The tensile mechanical properties of the micro- and nano- fiber directions of the composite scaffold were comparable to the circumferential and radial directions, respectively, of native aortic valve leaflets [42-47]. The incorporation of the ME-HA/ME-Gel hydrogel component to the woven fabric did not significantly change the overall mechanical properties, which was

expected because of the significantly lower mechanical stiffness of Me-HA/Me-Gel hydrogels compared to PAN nano-micro fibrous woven fabrics.

3.3. Fiber reinforced scaffolds enhanced cell viability and oriented spreading within engineered valve tissues

Cell viability and morphology were measured based on live/dead assays as shown in Fig. 3A. After 7-day culture, nHAVIC cultured on PAN woven fabrics showed high viability (>95%) in GM. The nHAVIC adhered, elongated and aligned along the direction of PAN NY and MY on woven fabrics. After encapsulating the cells in Me-HA/Me-Gel hydrogels, the nHAVIC were shown to be homogenously distributed and maintained their viability (>90%) throughout 14-day culture. The nHAVIC exhibited polarized spindle morphology on the surface of the hydrogel, but became less spread with greater circularity towards the center of the gel thickness. The nHAVIC distributed within the composite scaffolds showed an obvious layered architecture in concert with the embedded fibrous network. Interestingly, in the layer near the woven fabric, nHAVIC spread and aligned along the direction of underlying yarns (both PAN NY and MY). The cell viability (>95%) and elongation within composite scaffolds were remarkably enhanced compared to the hydrogel counterparts. The area shrinkage ratio of three TEHV scaffolds was determined to evaluate the supportive effect of the woven fibrous fabrics (Fig. 3B). The shrinkage ratio of the composite samples significantly decreased compared to the hydrogels ($p < 0.01$) after 14-day culture. This demonstrated that the incorporation of PAN woven fibrous fabrics successfully overcame the compaction of Me-HA/Me-Gel hydrogels.

3.4. Cell proliferation and ECM remodeling maintained by woven fiber reinforced constructs.

As shown in Fig. 4A, DNA content increased in woven fabric and composite scaffolds during the 14-day culture, confirming nHAVIC proliferation. The incorporation of PAN woven fabrics significantly improved the cell proliferation rate of Me-HA/Me-Gel hydrogels at day 14 (406.4 ± 33.0 ng for the composite scaffold vs. 249.0 ± 21.5 ng for the hydrogel scaffold, $p < 0.01$). Both GAG and collagen content considerably increased with increasing culture time (Fig. 4B, C). The highest GAG content was found in the PAN woven fabrics after 14-day culture ($p < 0.01$). The GAG content in composite scaffolds was slightly higher than that in Me-HA/Me-Gel hydrogels at day 14 (Fig. 4B). Collagen content in composite scaffolds was significantly higher than that obtain from PAN woven fabrics ($p < 0.01$), but slightly lower than that from Me-HA/Me-Gel hydrogels ($p < 0.05$), as shown in Fig. 4C. These results support that the combination of PAN woven fabrics and Me-HA/Me-Gel hydrogels provide an inductive microenvironment for cell growth, proliferation and ECM remodeling.

3.5. Effects of microenvironment of scaffolds on HAVIC phenotype

HAVIC are major cell population in the valve leaflets and they are highly heterogeneous with different cell phenotypes, i.e. quiescent fibroblasts (vimentin+), activated myofibroblasts (α SMA+), and osteoblastic phenotypes (Runx2+, ALP+), depending on various conditions [14]. We examined and compared the cell phenotype change of both normal and diseased HAVIC (nHAVIC and dHAVIC) in three different TEHV scaffolds conditioned in GM and ODM for a period of 14 days.

Immunofluorescent staining showed that vimentin and α -SMA were expressed by nHAVIC all the three TEHVs scaffolds when conditioned in GM, with the highest α -SMA expression was found in Me-HA/Me-Gel hydrogels (Fig. 5A). qPCR results also confirmed that nHAVIC showed highest levels of vimentin and α -SMA gene expression in Me-HA/Me-Gel hydrogels,

and the lowest level in PAN woven fabrics (Fig. 5B). Moreover, the expression of calponin and MMP1 in PAN woven fabrics group was significantly higher than the other two groups, indicating that the nHAVIC had more myofibroblastic phenotype when conditioned on the fabric alone scaffold.

Under pro-osteogenic (ODM) culture conditions, nHAVIC encapsulated in Me-HA/Me-Gel hydrogels were remarkably positive to ALP staining and had statistically higher ALP activity comparing to nHAVIC in the other two groups (Fig. 6A, B). The qPCR results showed that nHAVIC in Me-HA/Me-Gel hydrogels had the highest expression of vimentin and lowest expression of α -SMA (Fig. 7A, B). However, the osteoblasts-specific gene expression (i.e. ALP, Runx2, OCN) of nHAVIC in Me-HA/Me-Gel group were also significantly upregulated comparing to the other two groups (Fig. 7C-E). Together, these findings indicated that Me-HA/Me-Gel hydrogels permitted osteogenic differentiation of nHAVIC.

For dHAVIC cultured in GM, the gene expression of vimentin and α -SMA was highest in PAN woven fabrics, and lowest in composite scaffolds based on qPCR test (Fig. 8A). dHAVIC significantly down-regulated levels of calponin and MMP1 gene expression in composite scaffolds (Fig. 8A), which indicated that composite scaffolds suppressed transdifferentiation of dHAVIC into myofibroblasts, compared to the other two TEHV scaffolds. Similarly, the composite scaffold also restrains dHAVIC differentiation towards osteoblastic phenotype by downregulating Runx2 and ALP expression when cultured in ODM (Fig. 8B).

4. DISCUSSION

The choice of materials and the fabrication strategy for engineered tissue scaffolds are of critical importance to influence local cellular behaviors including proliferation, differentiation, and remodeling while simultaneously providing structural resilience against mechanical loading

[48,49]. Many scaffold configurations have been proposed to reproduce one or multiple structural/compositional characteristics of native heart valve tissues [50,51]. As we have discussed previously, hydrogel system can be photopolymerized under favorable conditions for cell encapsulation and chemically functionalized to form 3D microenvironment for controlling cellular activities [30-33]. The main obstacle to the use of pure hydrogel scaffolds as the sole material in heart valve tissue engineering is their low strength, isotropic material behavior and non-fibrous architecture. Several researchers have augmented 3D hydrogels by embedding a second material, fibrous scaffold produced by electrospinning, to improve scaffold strength and introduce fibrous shape [43,52,53]. Therefore, a composite scaffold consisting of fibrous scaffold sheets and hydrogels is potentially implemented in preclinical models due to their capability of mimicking the physical and biological features of heart valve tissue.

In the present study, we first prepared nano-micro fibrous woven fabrics by textile weaving technique, and the fabrics were immersed in Me-HA/Me-Gel precursor solution to generate hydrogel network within the nano-micro fibrous woven fabric. To mimic the fibrous structure of native tissues, electrospinning, wet spinning and melt spinning techniques have been used to create fibrous scaffolds [54]. Woven scaffolds combine tunable mechanical properties, structural anisotropy and ECM-mimicking fiber texture with porous construction that can promote cell migration and vascular ingrowth, and enhance the transport of nutrients and oxygen to cells and remove the metabolic waste in time. In addition, the woven fabric can be manufactured from different polymer yarns with controlled strength, porosity, morphology, and geometry to satisfy various requirements of different tissues [55]. In the present weaving technique, two distinct sets of warps (PAN MY) and wefts (PAN UANY) are interlaced at right angles to form nano-micro fibrous woven fabrics, which can effectively mimic the fiber architecture and physical anisotropy

of native ECM of aortic valve leaflet. Although PAN is not a widely-used material, it has been demonstrated by our group and others to be a biocompatible material for both in vitro and in vivo applications [34,56,57]. With the additional capacity for textile processing and cellularization, PAN based scaffolds have unique advantages for fabricating engineered valve conduits. Our nano-micro yarn fabrication process and fibrous weave strategy are also adaptable to other biodegradable polymers, like polycaprolactone (PCL) [34]. The nano-micro fibrous woven fabric/hydrogel composite scaffolds developed in this study could combine the advantageous physiological properties of 3D Me-HA/Me-Gel bioactive hydrogel with anisotropic PAN woven fibrous scaffold.

One of the fundamental requirements for scaffolds in TEHV application is to provide appropriate biomechanical and physiological functions, while allowing for cellular in-growth and tissue formation [58]. Native heart valve leaflet tissues have complex 3D anisotropic architectures with three connected layers whose structure determines their anisotropic mechanical characteristics and functionality [59]. Mechanical properties of native aortic valve leaflet are non-linear and different in circumferential and radial directions [60]. TEHV scaffolds could be employed for the replacement of the native valves if their mechanical features could be made to coincide. A recent report from our group determined that the Me-HA/Me-Gel hydrogels had comparable stiffness (4.27 ± 0.18 kPa) to the ventricularis and fibrosa layers of aortic valve leaflet (0.25 to 5 kPa) [33]. However, the uniaxial tensile properties of these hybrid hydrogels were markedly weak. This study embedded micro-nannano-micro fibrous woven fabric into 3D Me-HA/Me-Gel bioactive hydrogel, which increased the overall scaffold tensile strength and imparted anisotropic mechanical behavior. The nano-micro fibrous woven fabric/hydrogel

composite scaffolds demonstrated mechanical behavior characteristic of native aortic valve leaflet, expressing a bilinear stress-strain curve [42].

nHAVIC were encapsulated into the composite scaffolds to test the suitability of this composite construct for heart valve tissue engineering. The combination of a hydrogel-based cell carrier to a fibrous scaffold enabled the cells to be more homogeneously distributed within the entire 3D volume of the composite scaffold without compromising the mechanical properties of the whole scaffold. nHAVIC showed more spreading morphology within the hydrogel layer of stiffer composite scaffold, confirming the previous studies that stiffer substrates could promote the cell spreading and elongation [61,62]. In addition, nHAVIC showed predominant cytoskeletal organization along with the directions of underlying PAN NY and MYs in the layer near the woven structure. This also confirmed that aligned topography guided cell alignment and organization [63-65]. Circumferential HAVIC alignment, collagen secretion, and collagen fibril formation by HAVIC in heart valve leaflets are regulated in part by the anisotropic mechanical stresses experienced during function [66,67]. In our current study, embedment of woven fabric into hydrogel system not only enhanced the mechanical properties of composite scaffolds but also partially aligned the attached cells toward the direction of fibers in woven structure. The initial polymer fiber architecture in the woven component may therefore accelerate and guide subsequent collagen fibril formation along these directions [68] prior to mechanical loading. Furthermore, the incorporation of woven scaffold substantially reduced the area shrinkage of the scaffold. This is a crucial feature to prevent the compaction and regurgitation of TEHV.

Another important responsibility of HAVIC is to synthesize and remodel the ECM in order to maintain sufficient strength and durability for sustaining the unique dynamic blood circulation [69]. GAG, collagen, and elastin are known as the main ECM components of the aortic valve

leaflet [42]. Since elastin has limited effects on valve cell phenotypes in vitro, we mainly focused on GAG and collagen content changes. We quantitatively evaluated and compared the secretion and remodeling of GAG and collagen when cultured in three different TEHV scaffolds. We observed that nHAVIC were able to dynamically produce and remodel their own matrix within the three different TEHV scaffolds. Prior studies have demonstrated that scaffold structure and mechanical properties affect cell-cell and cell-matrix interactions, which may further affect the cellular functionality in terms of ECM deposition and tissue formation [70-72]. Our biochemical assay results showed that nHAVIC seeded woven scaffold had highest GAG and lowest collagen contents, while nHAVIC laden Me-HA/Me-Gel hydrogel possessed lowest GAG and highest collagen contents. As a comparison, nHAVIC encapsulated composite scaffold exhibited moderate values of both GAG and collagen content. Dysregulated ECM composition is a major determinant of calcific valve disease initiation and progression [73, 74]. In diseased valves, dense fibrosis is usually first observed, with significant collagen and GAG accumulation and thickening of the valve leaflets [75, 76]. Therefore, the balanced composite scaffold consisting of Me-HA/Me-Gel hydrogel and woven scaffold may be the preferable option for TEHV applications.

Healthy HAVIC exhibit a quiescent, fibroblast-like phenotype, expressing vimentin and a lesser degree of α -SMA (myofibroblast marker) [77]. In injured or diseased valves, HAVIC transition from quiescent fibroblasts in normal valves into activated myofibroblasts with significant decrease in expression of vimentin and increase in smooth muscle cells-related markers, such as α -SMA, calponin and MMP1 [78-80]. Our immunofluorescent staining and qPCR results determined that the 3D composite scaffolds in current study better maintained fibroblastic-like phenotypes of nHAVIC without obvious spontaneous smooth muscle cells

phenotype differentiation and with limited activation in GM. In contrast, the nHAVIC seeded on the woven fibrous scaffolds dramatically upregulated calponin and MMP1 expression, resembling highly activated myofibroblasts due to the remarkable increase of scaffold stiffness [30,81].

Other studies have shown that various heart valve diseases, such as rheumatic heart valve disease, senile degenerative heart valve disease, etc., will be accompanied with different degrees of valve calcification phenomena [82,83]. Moreover, the amount of calcification in the valve is thought to influence the rate of progression of aortic stenosis and could be an independent risk factor for death [84,85]. Valve calcification is usually characterized by the expression of osteoblast-related markers on the aortic surface of the valve [86,87]. Our ALP staining and activity assay together with qPCR results showed that nHAVIC laden into Me-HA/Me-Gel hydrogels presented robustly highest osteoblast- specific gene expression, while the woven fibrous structure enhanced Me-HA/Me-Gel hydrogels exhibited sparse osteoblast expression, under the induction of ODM. Apart from nHAVIC, dHAVIC, isolated from non-calcified areas of calcified aortic valve leaflets, were also employed to test the cell phenotype maintenance under different culture medium (i.e., GM and ODM). Our results indicated that the composite scaffold could suppress the transformation of dHAVIC into myofibroblastic phenotype in GM and osteoblastic phenotype in ODM compared with the other two TEHV scaffolds. This is the first demonstration to our knowledge of a TEHV scaffold design exhibiting intrinsic calcification protection.

5. CONCLUSIONS

In summary, anisotropic 3D composite scaffolds consisting of Me-HA/Me-HA bioactive hydrogel and PAN nano-micro fibrous woven fabric were designed, fabricated and characterized

for the purpose of heart valve tissue engineering. We determined that the fibrous scaffold manufactured from textile weaving technique significantly increased the overall mechanical properties of hydrogel and impart native-like anisotropic features. These composite scaffolds provided appropriate support and microenvironmental cues for ECM deposition and cell proliferation, while maintaining the fibroblastic phenotype of nHAVIC and restraining the pathological differentiation of dHAVIC into myofibroblasts and osteoblasts compared with pure hydrogels or woven fabrics. Thus, nano-micro fibrous woven fabric/hydrogel composite scaffolds have great potential for living engineered replacement of heart valves.

Notes

The authors declare no competing financial interest.

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Figure Captions

Figure 1. Fabrication process for cell-laden nano-micro fibrous woven fabric/hydrogel composite scaffolds by using textile weaving technique and bioactive hydrogel. (A) Fabrication of PAN nano-micro fibrous woven fabric. PAN MY were pre-stretched as warp yarns (i.e., micro direction) and PAN NY (weft yarns) were woven across in the perpendicular direction (i.e., nano direction). The fabricated woven fabric was implemented to reinforce the hydrogel and generate composite scaffold. A silicone mold was placed on the top of PAN nano-micro fibrous woven fabric and Me-HA/Me-Gel bioactive hydrogel precursor (with HAVIC) were loaded and photocrosslinked to generate cell-laden hydrogel with fibrous woven fabric reinforcement. (B), (C) FESEM images of the obtained PAN nano-micro fibrous woven fabric. Scale bars: 200 μm for (B) and 50 μm for (C), respectively. The solid arrows and hollow arrows indicate PAN NY and PAN MY respectively.

Figure 2. Uniaxial tensile testing of PAN nano-micro fibrous woven fabric and 3D composite construct. (A) Representative stress-strain curves for fibrous woven fabric and composite scaffold both in the micro direction (along with the PAN MY in the scaffold) and nano direction (along with the PAN NY in the scaffold). The yield points which could be used to calculate the yield strength, and yield elongation were obtained as shown in graphs. (B) The typical calculation methods of initial modulus (E Init.), transient modulus E (E Trans.), peak tangent modulus (E Peak) from the stress–strain curves. (C) E Init. (D) E Trans. (E) E Peak (F) yield strength and (G) yield elongation. (n=6; *p<0.05, ** p<0.01).

Figure 3. (A) Live/Dead images for three different nHAVIC-laden TEHV scaffolds (i.e., PAN woven fabric, Me-HA/Me-Gel hybrid hydrogel, and composite scaffold) conditioned in GM for 7 days and 14 days, which demonstrated that the scaffold structure guided the cell alignment

toward the preferred direction in the fibrous woven fabric layers. Solid arrows and hollow arrows indicate PAN UANY and MY respectively. Scale bars = 100 μm . (B) Area shrinkage ratio testing for three different TEHV scaffolds conditioned in GM for 14 days, demonstrating that the incorporation of PAN woven fibrous fabric successfully overcame the compaction of Me-HA/Me-Gel hydrogel. (n=6; ** p<0.01).

Figure 4. DNA and ECM contents of different TEHV scaffolds with nHAVIC cultured in GM for 7 days and 14 days. (A) DNA content. DNA content increased with time for all three scaffolds. The incorporation of PAN woven fabric can effectively improve the cell proliferation rate of Me-HA/Me-Gel hydrogel. (B) GAG content. PAN woven fabric could promote the GAG production of nHAVIC. (C) Total collagen content. Me-HA/Me-Gel hydrogel was beneficial for the collagen production of nHAVIC. As a comparison, nHAVIC encapsulated composite scaffold exhibited relatively moderate GAG and collagen contents among the three TEHV scaffolds. (n=6; *p<0.05, ** p<0.01).

Figure 5. Composite scaffolds better maintained nHAVIC fibroblastic phenotype in GM. (A) Immunofluorescent staining for α -SMA (red), vimentin (green) and nuclei (blue) within three different nHAVIC-encapsulated TEHV scaffolds in GM after 14-day culture. Solid arrows and hollow arrows indicate PAN UANY and MY respectively. Scale bars = 100 μm . (B) Real-time PCR analysis of vimentin, α -SMA, calponin and MMP1 in three different nHAVIC-encapsulated TEHV scaffolds conditioned in GM for 14 days. Relative gene expression is presented as normalized to 18S. (n=3; *p<0.05, ** p<0.01).

Figure 6. (A) ALP staining and (B) ALP activity test for three different nHAVIC-carried TEHV scaffolds. Solid arrows and hollow arrows indicate PAN UANY and MY respectively. Scale bars = 500 μm . (n=3; *p<0.05).

Figure 7. Osteoblast specific gene expression of three different nHAVIC-laden TEHV scaffolds in ODM after 14-day culture. (A) Vimentin, (B) α -SMA, (C)ALP, (D) Runx2, (E) OCN. Relative gene expression is presented as normalized to 18S. (n=3; *p<0.05, ** p<0.01).

Figure 8. Comparative gene expression demonstrates that composite scaffolds protected encapsulated dHAVIC by preventing their myofibroblastic and osteogenic differentiation. The dHAVIC-laden TEHV scaffolds were conditioned in GM (A) and OGM (B) for 14 days. Relative gene expression is presented as normalized to 18S. (n=3; *p<0.05, ** p<0.01).

Fig. 1

Fig. 2

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Fig. 5

Fig. 6

Fig. 7

Fig. 8

A

B

Woven Fabric
 Hydrogel
 Composite