Form Follows Function: Advances in Trilayered Structure Replication for Aortic Heart Valve Tissue Engineering

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ABSTRACT

Tissue engineering the aortic heart valve is a challenging endeavor because of the particular hemodynamic and biologic conditions present in the native aortic heart valve. The backbone of an ideal valve substitute should be a scaffold that is strong enough to withstand billions of repetitive bending, flexing and stretching cycles, while also being slowly degradable to allow for remodeling. In this review, we highlight three overlooked aspects that might influence the long term durability of tissue engineered valves: (i) replication of the native valve trilayered histoarchitecture, (ii) duplication of the three-dimensional shape of the valve, (iii) and cell integration efforts focused on getting the right number and type of cells to the right place within the valve structure and driving them towards homeostatic maintenance of the valve matrix. We propose that the trilayered structure in the native aortic valve that includes a middle spongiosa layer cushioning the motions of the two external fibrous layers should be our template for creation of novel scaffolds with improved mechanical durability. Furthermore, since cells adapt to micro-loads within the valve structure, we believe that interstitial cell remodeling of the valvular matrix will depend on the accurate replication of the structures and loads, resulting in successful regeneration of the valve tissue and extended durability.

Keywords: heart valve tissue engineering, trilayered structure replication, microstructural fatigue, long term durability, tissue remodeling, bioreactor

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1. INTRODUCTION

Aortic Valve Disease (AVD) is the third most common cause of cardiovascular disease affecting roughly 5 million people in the United States alone [1–3]. It is estimated that valve disease affects over 25% of people over 65 years of age [3]. Moreover, AVD is expected to become more prevalent in the coming years as the population life expectancy increases. It is estimated that the annual number of patients requiring heart valve replacement will triple from approximately 290,000 in 2003, to over 850,000 by 2050 [4]. Thus, strategies to address AVD are bound to have a global impact.

Currently, the only effective treatment for AVD is total valve replacement. Present valve replacement strategies include use of mechanical heart valves, bioprosthetic heart valves, and allografts [5, 6] which offer excellent quality of life after implantation. Although all devices successfully restore the aortic root hemodynamics and facilitate ventricular remodeling, each strategy has significant limitations. Allografts are excellent choices; however, their supply is limited. Mechanical valves are biologically inert but susceptible to thromboembolic events and thus require lifelong anticoagulation medication. Bioprosthetic heart valves and allografts have poor long-term durability and are often subject to progressive leaflet deterioration and calcification. Finally, all replacement heart valves are relatively sensitive to infection and host tissue overgrowth which ultimately impair normal functioning and require surgical replacement. Taken together, current artificial valves offer excellent short and mid-term solutions that will require additional invasive surgeries to replace failed implants in the long term.

One major limitation of all the aforementioned valve replacement options is their inability to grow, repair, and remodel [7]. These characteristics are generally important for homeostatic maintenance of valve integrity under physiologic loads and especially relevant for the pediatric population. Current valve replacement strategies are effective in treating congenital valve disease. However, they do not accommodate the growing heart and thus, pediatric patients suffering from congenital valve disease require multiple invasive surgeries throughout their childhood. It is estimated that 20,000 children are born with congenital heart disease in the United States alone; thus, there is a great clinical need to develop valve replacements capable of restoring normal hemodynamics, while being non-thrombogenic and capable of repair, remodeling and growth with the patient [7].

Tissue engineering (TE) has emerged as a promising technology for the development of novel replacement valves that address the aforementioned shortcomings [5, 6, 8 – 13]. The general TE working paradigm involves scaffold fabrication, cell integration and bioreactor conditioning before implantation. The scaffold can be generated via many different methods but ultimately serves the purpose of providing a temporary matrix for seeded cells to secrete their own extracellular matrix (ECM) proteins. For tissue engineered heart valves (TEHVs), this matrix must be able to withstand the dynamic mechanical environment of the aortic valve (AV); therefore, scaffold fabrication requires careful consideration and design. Cell integration for TEHVs incorporates seeding of aortic valve interstitial cells (AVICs) inside the scaffolds and aortic valve endothelial cells (AVECs) onto the valve surfaces; other cells sources include endothelial progenitor cells and mesenchymal stem cells (bone-marrow derived) as they represent a clinically feasible strategy [14]. Once the cells are successfully integrated into the scaffolds, a bioreactor is necessary to condition the construct, allowing the cells to migrate,

proliferate, and secrete ECM proteins and matrix-degrading enzymes as a normal homeostatic response to physiologic loads. This step is important as it prepares cells to deal with the 3-D loads immediately after implantation. This *in vitro* procedure may take 3–4 weeks to generate a conditioned construct that contains a large population of viable, functionally adapted cells. A more recent approach in TE involves the implantation of an unseeded scaffold. This approach is termed *in situ* TE and has shown promising results. By utilizing conjugated antibodies, Jordan et al. was able to show good cell accumulation onto the TEHV in an animal model [15].

Ultimately, the goal of TE constructs is to successfully restore hemodynamics with the ability to repair and remodel with time. Ideally, the original scaffold will slowly degrade and will be replaced by the naturally generated ECM. Recently, TEHVs have been an active area of research making much progress in the understanding of successful clinical implementation. However, there are still many issues that must be further addressed before TEHVs can become an effective treatment.

In this review, we will investigate novel methodologies proposed for scaffold fabrication and design of TEHVs. Furthermore, we will present some of our current research approaches that may inspire future investigations for TEHVs.

2. AORTIC VALVE STRUCTURE-FUNCTION RELATIONSHIP

For creation of successful TEHVs, it is necessary to have a clear view of the structure and function of native heart valves. We will focus on the aortic valve since among the four cardiac valves, the aortic valve is the one most diseased, replaced and researched. The aortic valve is located between the left ventricle and aorta and functions to ensure unidirectional blood flow and to prevent regurgitation of blood into the left ventricle. It consists of three semilunar cusps attached to the inner wall of the aorta residing within the sinuses of Valsalva. The cusps or leaflets are the main functioning components of the aortic valve. These delicate structures endure the dynamic opening and closing of the valve 40 million times a year and more than 3 billion times during an average lifetime [16, 17]. The highly dynamic environment of the valve illustrates the complex function of the leaflets and pinpoints the importance of processes involved in maintaining healthy valve function.

The aortic valve leaflet is composed of three layers: fibrosa, spongiosa, and ventricularis [13]. The fibrosa layer is located closest to the outflow area and is composed of densely aligned collagen fibers; this layer is responsible for the mechanical strength and stiffness of the leaflet. The ventricularis is located closest to the left ventricle and is largely comprised of elastin fibers which play an important role of extending and recoiling during diastole and systole, respectively. The middle layer, spongiosa, is mainly comprised of proteoglycans and glycosaminoglycans (GAGs) which act as a cushion and bears the applied stresses of valve function. This tri-layered structure, each layer being composed of different matrix elements, is unique to the cardiovascular system. The three layers are structurally continuous and work in conjunction with one another to fully satisfy the mechanical demands involved in normal valve function.

Although the structural design of the leaflet makes it mechanically suitable for opening and closing, the structure consistently accumulates micro-damages and therefore requires continuous repair. The repair mechanisms are carried out by the resident cells of the aortic leaflet which include AVECs and AVICs [18]. AVECs form

a monolayer on the surface of the AV leaflet and are believed to regulate vascular tone, inflammation, thrombosis, and remodeling. Similar to endothelial cells in other segments of the cardiovascular system, AVECs form a layer around the leaflet which acts as a selective barrier for various components in the blood. AVICs are a heterogeneous cell population residing within the AV leaflet and serve to maintain tissue homeostasis and structural integrity. AVICs continuously secrete collagen types I and III, glycosaminoglycans (GAGs) and other matrix components as well as matrix metalloproteinases (MMPs), their inhibitors (TIMPs), and other matrix degrading enzymes such as GAG-degrading enzymes that mediate remodeling [19–21]. AVICs exhibit a dynamic phenotypic spectrum ranging from quiescent fibroblast-like cells (characterized by expression of vimentin, fibroblast surface antigen and low expression of alpha-smooth muscle cell actin and MMP-13), to activated AVICs, assimilated as myofibroblasts (characterized by proliferation, migration, high expression of vimentin, alpha-smooth muscle cell actin and MMP-13) [13, 22–25]. The activated AVICs are considered the pivotal cells that control valve structure and function [25]. Interactions among mechanical forces, valvular cells and the extracellular matrix influence remodeling potential and therefore durability of heart valves. Duplication of these structures and interactions in a man-made device is truly bioengineers' dream and has the potential to provide tremendous clinical benefits.

3. THE IDEAL TISSUE ENGINEERED HEART VALVES (TEHV)

Generally, in order for a TEHV to successfully function in vivo, it must be biocompatible, durable, and also have the ability to remodel and grow. Design and fabrication of TEHV scaffolds must carefully consider these variables. Biocompatibility is crucial for successful TE and is often dependent on scaffold material [26, 27]. Synthetic and biological materials must thus be evaluated to determine biocompatibility before scaffold design. Decellularized xenografts often encounter biocompatibility issues because of their animal origin and thus need to utilize various decellularization solutions to remove foreign antigens in order to prevent immune responses. Durability considerations are important because of the highly dynamic environment of the aortic valves [17]. The mechanical integrity of the scaffold must not be compromised during the implantation procedure or shortly thereafter. Different scaffold materials exhibit different mechanical properties; therefore scaffold selection requires *in vitro* testing of mechanical properties. Manipulation of the mechanical strength of materials can be carried out through various techniques such as cross-linking of acellular tissues and general design schemes using polymers.

Cell integration and remodeling of the scaffolds are of utmost importance to successful TEHV because cell viability and state of activation determine the continued function of the TEHV *in vivo*. The goal is not simply to seed cells onto a scaffold; rather, it is necessary for the cells to be viable and functional, positioned in the right tissue niche and able to maintain normal tissue homeostasis. Recent studies have indicated that cell function is closely modulated by its substrate environment [28–30]. Therefore, imitating the structural makeup of the native leaflet could improve cell retention and function. The ideal TEHV must be able to be compatible with the *in vivo* environment, be durable enough to withstand the dynamic mechanical environment,

and create an environment that promotes cell attachment, integration and function. In the following sections, we will discuss current studies that represent the strides made toward fabrication the ideal TEHV scaffold.

4. CELL SOURCE AND SEEDING

The cell is clearly an important contributing factor to successful TE. Although the resident cell, AVICs and AVECs, are a logical choice for seeding TEHVs, they may not be economical in the clinical setting. Therefore, other sources must be considered for TEHVs. Bone marrow-derived stem cells (BMSC), umbilical cord-derived stem cells (UCSC), and blood derived endothelial progenitor cells (BEPCs) are a few attractive options for cell source [31–35]. BMSCs are a promising cell source because they can be obtained without surgical interventions, and can be set up as a routine clinical procedure. Hoerstrup et al. used BMSCs and demonstrated viable tissue formation with characteristic extracellular matrix proteins [31]. UCSCs also present an attractive option as a cell source for TE applications. These cells are easily attained by isolation via intact donor samples from newborns. UCSCs have been shown to have excellent growth potential and production of extracellular matrix *in vitro*. BEPCs are also easily attainable as they are circulating in the blood and can be isolated in many minimally invasive manners [36]. BEPCs have been used to endothelialize scaffold prior to implantation to reduce coagulation and inflammatory complications [32].

Cell seeding strategies for TE are well summarized in a review by Villalona et al. [37]. In general, there are two main types of approaches for cell seeding: static seeding and dynamic seeding. The most widely used techniques are static seeding which involves the pipetting of a cell suspension directly onto the scaffold. Although simple, this method has many disadvantages including difficulty in uniform seeding, poor seeding efficiency, and poor proliferation [38]. Other static approaches include seeding with biological glues. This method allows the cells to stay in close contact with the scaffold thereby improving seeding efficiency [39]. Dynamic seeding methods are designed to address the limitations of the static methods. Dynamic methods include perfusion systems, rotational systems, vacuum systems, electrostatic seeding, and even magnetic seeding methods [40–46]. These methods possess many advantages and have been combined to yield desirable results as well. However, there has yet to be an established method for optimal cell seeding of TE constructs. Many groups are currently developing new strategies to provide adequate cell seeding for TE constructs.

There has been a push towards *in situ* TE which involves minimal to no *in vitro* seeding and aims to utilize the *in vivo* environment for cell accumulation. Jordan et al. showed that conjugating their scaffold with an antibody was all that was necessary to get endothelialization of their scaffold *in vivo* [15]. This study presents new questions to the TE paradigm and represents an area for further investigation.

5. SCAFFOLD MATERIALS

5.1. Decellularized Allografts and Xenografts

Acellular tissues have been widely investigated as a scaffold material for TE applications [47–56]. The advantage of this technique is that the overall structural composition of the leaflet is retained; however, the decellularization process alters some

of the ECM components and leads to weaker mechanical properties and decreased durability [57, 58]. The conventional route to overcome this limitation has been utilizing cross-linking agents to increase mechanical strength and durability after decellularization [26, 59–61]. However, many cross-linking agents such as glutaraldehyde are toxic to cells and therefore not ideal for TE applications. Efforts have also been focused on detoxifying harmful cross-linking agents as well as proposing new agents to enhance acellular tissue mechanics [62, 63]. The Simionescu group proposed use of penta-galloyl glucose (PGG), a naturally derived, non-toxic polyphenol, to stabilize acellular scaffolds [59, 64, 65]. The PGG-treated scaffolds exhibited many desirable properties and thus PGG is considered a promising stabilizing agent for decellularized scaffolds. Other approaches utilize decellularization protocols that are less harsh and induce less damage to the ECM. Booth et al. investigated the effects of various chemicals (sodium dodecyl sulfate, Triton X-100, sodium deoxycholate, MEGA 10, TnBP, CHAPS, and Tween20) on decellularization and concluded that a certain combination of the chemicals led to an optimal result which allowed for retention of major structural proteins [66].

Due to the scarcity of allografts, decellularization of xenogeneic valves (e.g., porcine valves) also attracted considerable research interest [67, 68]. When acellular xenogeneic valves are used as scaffolds, the challenges are not only the complete removal of cells and cell debris, but also the thorough elimination of the alpha-Gal epitope, the most potent xeno-antigen responsible for transplant rejection [52, 69–72]. Despite the fact that optimized decellularization protocols apparently maintained the 3-D ECM arrangement, some cell-seeded scaffolds encouraged calcification and thickening upon implantation [73], indicating the need for more biological tests including resistance to inflammation and calcification to be added to the armamentarium of scaffold selection tools.

There have been some exciting studies illustrating the potential of xenogeneic tissues for TE. Recently, Tedder et al. suggested use of acellular porcine pericardium for construction of TEHVs; the scaffolds exhibit outstanding mechanical properties and can be fashioned into cusp-shaped structures that mimic the human aortic valve [65, 74]. Matheny et al. also demonstrated the feasibility of utilizing small intestinal submucosa (SIS) as a potential scaffold for TE [75]. This study revealed successful endothelialization of the SIS construct placed in the pulmonary position.

The search for the ideal decellularization procedure continues [76]. It is anticipated that, ultimately, a combination of acceptable decellularization methodology and cross-linking or stabilizing agents would lead to a promising scaffold for TEHVs. Present questions include the following: Which decellularization process is the most suitable? What is the best method to retain acellular valve mechanics and at the same time create good porosity for cell reseeding/infiltration? What is the appropriate seeding method for successful cell infiltration?

5.2. Polymeric Scaffolds

Polymeric scaffolds can be derived from biological or synthetic sources, each having advantages and disadvantages. Biological polymers possess intrinsic cell compatibility as most are structural components familiar to cells, but also tend to be mechanically weak and difficult to manipulate. Synthetic sources are easily tailored and can be reproduced

readily, allowing for novel structural designs that can control mechanical properties, surface topography, and porosity. Some limitations include uncertainties regarding the degradation rate and products as well as cell compatibility. A short description of studies investigating the feasibility and optimization of polymer scaffolds for TEHVs follows.

5.2.1. Biological Sources

For biological polymer scaffolds, collagen, fibrin, and hyaluronic acid have been actively investigated [27, 77–87]. Many of the studies have shown acceptable cellular growth into these materials and desirable degradation control, but these scaffolds are too weak for the aortic valve environment and require more development to increase their mechanical characteristics.

Collagen comprises a large percentage of the dry weight of the aortic valve leaflet and therefore is a logical choice for TEHV applications [88]. Flanagan et al. utilized collagen gel constructs for TE applications and showed good growth of AVECs and AVICs; however, the mechanical strength of the construct was not thoroughly investigated [87]. Shi et al. used collagen fiber-collagen gel composites to manipulate the mechanics of their scaffold and enhance its mechanical properties to a stiffness of 5 MPa and an extensibility of 14% [83].

Fibrin is a biodegradable, biocompatible matrix protein involved in wound healing and can be produced from a patient's blood and therefore does not initiate inflammation, tissue necrosis, or fibrosis [79, 89–91]. Jockenhoevel et al. tested molded fibrin gels as potential scaffolds for TE [79], and showed their good cell growth and ECM production capability, but poor mechanical stiffness and scaffold shrinkage. Grassl et al. investigated the response of neonatal aortic smooth muscle cells (SMCs) in the presence of fibrin and collagen [92], and found that fibrin was able to induce ECM production, a desirable trait for TE.

Hyaluronic acid (HA) makes up 90% of the total GAGs in human heart valves, thus making it a possible candidate for TEHV scaffolds [20, 93]. HA does not initiate immune or thrombogenic events and also possesses viscoelastic properties [5, 94]. Masters et al. demonstrated that HA can be cross-linked into a hydrogel, and HA degradation products generated a four-fold increase in matrix production by VICs cultured for 6 weeks [94]. Flanagan et al. demonstrated the feasibility of fibrin scaffolds through dynamic conditioning [95] and also conducted a 3 month *in vivo* study in sheep model revealing cell viability and remodeling [96]. However, an insufficient leaflet structural profile led to a decrease in functionality.

In general, biological materials possess many desirable characteristics but are often limited by their xenogeneic origin or poor mechanical properties of individual components [5, 97]. Further studies into fabrication techniques are necessary to improve their mechanical properties.

5.2.2. Synthetic Sources

Synthetic scaffolds are easily reproduced and manipulated by various fabrication techniques. Poly(glycolic acid) (PGA), poly(lactic acid) (PLA), poly(caprolactone) (PCL) and poly(hydroxyalkanoate) [98] are some commonly studied polymers for TE [31, 99]. Each polymer possesses certain properties that can be altered with the

combination of other polymers. Utilizing blends and copolymers such as poly (4-hydroxybutyrate) (P4HB) allows development of materials with very precise characteristics. Shinoka et al. pioneered TEHV designs in the late 1990s and investigated PGA and PLGA nonwoven scaffolds in a sheep model [100–102] with favorable results, but the constructs became thicker and stiffer probably due to inflammation [100]. More recent efforts have been on enhancing cellular integration and regulating mechanical properties. Cell infiltration has been a concern for these nonbiological material; however, significant progress has been made. Ozawa et al. [103] and Nuttelman et al. [104] demonstrated that seeded cells could produce ECM proteins and remain viable, but proliferation rates were not optimal. *In vitro* conditioning studies have shown enhanced cell viability and ECM production on polymer scaffolds [105, 106]. Structural design of the scaffold is important to the improvement of cell integration and mechanical properties. Many studies have investigated various fabrication techniques to generate novel structures for TE applications [97, 107–112]. Porous designs [99, 113, 114], nonwoven and woven designs [31, 36, 99–102, 105], and electrospun designs [98, 115–118] have been studied.

As techniques and approaches become better established, detailed design issues need to be addressed. Most scaffolds have yet to consider the trilayered structure of native leaflets. The structural environment is important to optimal cell function as well as mechanical properties [96]. Recent studies reveal that VICs are sensitive to their substrates, implying that the structural components to which cells attach could be important for maintaining normal cell function [28]. Furthermore, a trilayered scaffold can preserve the mechanical duties of each layer thus reducing added stresses and strains. The remainder of this review will discuss new insights and future direction for trilayered scaffolds.

6. TRILAYERED STRUCTURE IN NATIVE LEAFLETS: IMPLICATION FOR TEHVs

Internal shear is believed to be an important factor underlying valvular bending properties [119–121]. In both systolic and diastolic phases, shear movement develops in native aortic valve leaflets when the fibrosa, spongiosa, and ventricularis layers slide and readjust their positions to minimize the internal stresses [121]. In the absence of optimal shear properties, tissue buckling will take place in response to compression caused by bending deformation [121]. Apparently, in the native aortic valve leaflet, the existence of the spongiosa as a stress-mitigating cushion layer renders optimal leaflet bending behavior and reduces the likelihood of collagen fiber buckling and thus fatigue accumulation.

The effectiveness of nature-designed trilayered structure in valvular flexure can be evidenced by the following study, in which we investigated the flexural responses at microstructure-level in the native, decellularized (0.1% sodium dodecyl sulfate [SDS] treatment), and glutaraldehyde fixed (0.625% Glut) porcine aortic valve leaflets. Briefly, a tissue strip (15 mm × 5 mm) was cut from each of the native, decellularized, and glutaraldehyde fixed groups along the circumferential direction. The leaflet strips were then folded into a “U” shape towards the fibrosa side in a phosphate-buffered saline (PBS) bath. The “U” shape leaflet strips were then

clamped at two edges and fixed with 4% glutaraldehyde for later histological study. The Movat's pentachrome staining and polarized light imaging showed that various microstructural responses existed in the native, decellularized, and glutaraldehyde fixed leaflets (Figure 1). We found that the native leaflet was able to well cope with the extreme flexure with the coordination of the three layers. As revealed by

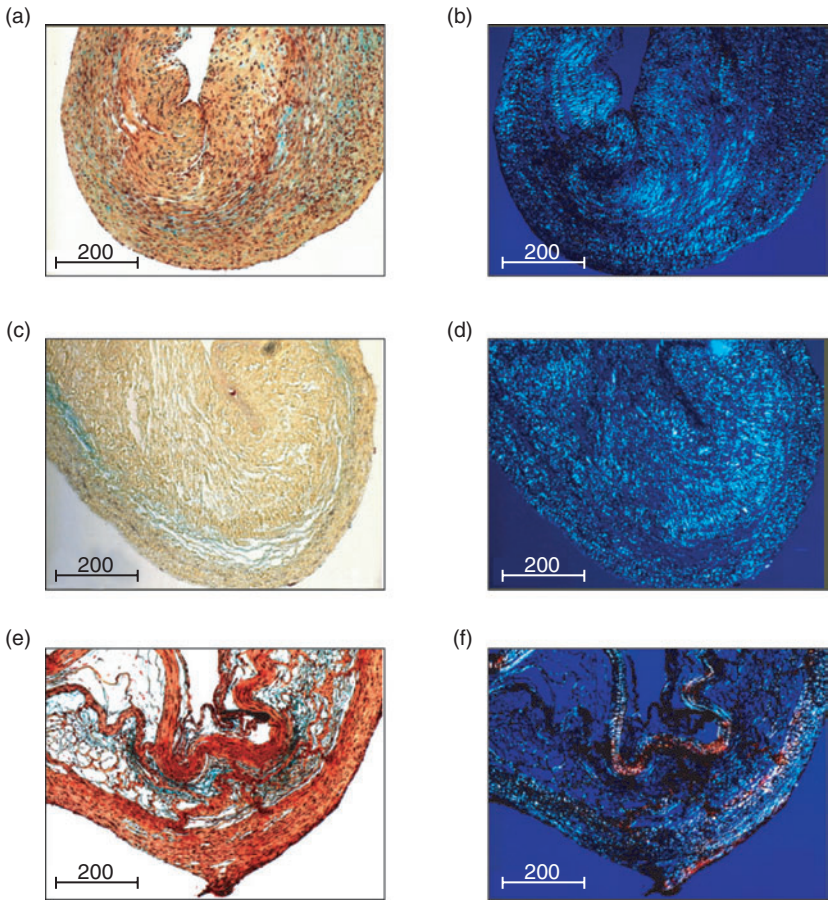

Figure 1. (a) Movat's pentachrome staining and (b) polarized light imaging of the native porcine aortic valve leaflet; (c) Movat's pentachrome staining and (d) polarized light imaging of the decellularized (0.1% SDS treatment) aortic valve leaflet; (e) Movat's pentachrome staining and (f) polarized light imaging of the glutaraldehyde fixed (0.625% Glut) porcine aortic valve leaflet. Sample preparation: The leaflet strips were folded into a "U" shape towards the fibrosa side in a PBS bath; the "U" shape leaflet strips were then clamped at two edges and fixed with 4% glutaraldehyde for histology. Scale bar unit: μm.

histology, the fibrosa layer was able to readjust the fiber crimps to compressive stress without causing fiber buckling; the ventricularis layer experienced uncrimping of fibers due to tensile stress; and the spongiosa seemed to smoothly mediate the layered structure across the leaflet thickness (Figure 1-a, b). For the decellularized leaflet produced by 0.1% SDS treatment, the trilayered structure was found relatively preserved; however, microstructural disruptions such as collagen crimp organization and loss of GAGs were observed [122]. It was thus understandable that the decellularized leaflet was able to cope with the extreme flexure relatively well, i.e., no noticeable fiber buckling in the fibrosa (compressive region), but overstretch of fibers observed in the ventricularis (tensile region) (Figure 1-c, d). For the glutaraldehyde treated leaflet, the crosslinking stiffened the whole fiber network, and the spongiosa layer lost the cushion functionality. Consequently, the lack of a functioning trilayered structure caused difficulty in coping with the extreme flexure introduced by the "U" shape folding; notably, serious fiber buckling was revealed by both histological and polarized light images (Figure 1-e,f).

We thus believe that the design of engineered composites should include mechanisms of coping with the complex bending deformations. This is important as the scaffolds are exposed to such loads immediately after implantation and thus their durability will be tested in every cardiac cycle thereafter.

6.1. Single-layered Design versus Trilayered Structure Design

Due to the challenges associated with the fabrication a trilayered scaffold, most TEHVs are based on a single layer design. Potential of tissue remodeling has been shown in a few *in vivo* studies [99, 113, 123]. Sodian et al. [113] implanted TEHV fabricated from polyhydroxyalkanoate scaffolds (pore size 180 to 240 microns) seeded with vascular cells into pulmonary position of a lamb model. Sodian et al. mentioned that histology demonstrated "laminated fibrous tissue with predominant glycosaminoglycans as extracellular matrix" [113]. In another study, Hoerstrup et al. [123] seeded poly-4-hydroxybutyrate (P4HB)-coated PGA constructs with vascular myofibroblasts and endothelial cells, conditioned the TEHV in a pulsatile bioreactor for 14 days, and implanted the TEHV into pulmonary position of a lamb. It was shown that after 16 weeks of implantation, TEHV leaflet evolved into "layered cellular fibrous tissue, which is denser near the outflow surface" [123]. These studies indicate that scaffold remodeling is possible and that physiologic loading leads to layering of *de novo* synthesized tissues.

In short, cyclic bending may cause harsher internal shear and buckling in single layer TEHV, and thus long-term durability will be questionable. If it turns out that valve loading during cardiac cycling is able to remodel a "single-layer" scaffold into "three layers" after implantation, it is possible that valve regeneration is feasible in a bioreactor or *in vivo*. There are still critical issues to be addressed such as whether the TEHV remodeling will be fast enough before destructive buckling and damage occur, how robust the adopted laminated structure will be, and how it will benefit long-term fatigue resistance. Design of a very robust trilayered structure before implantation might therefore offer a better solution. Two approaches to achieving this design are discussed in the following sections.

6.2. Trilayered Design in Acellular Valves: Benefits and Challenges

One option of appraising trilayered designs is using acellular aortic valves. Recently, da Costa et al. [124] reported a study evaluating the early and midterm results of decellularized aortic valve allografts (DAVA, 0.1% SDS treatment) as an aortic valve replacement (41 patients, implanted between 2005 and 2010). They observed that the early and midterm results demonstrated stable structural integrity, low rate of calcification, and adequate hemodynamics of the valves, even in the absence of seeded cells [124]. The reported clinical mid-term results are very promising, possibly indicating that preservation of the trilayered structure translates into aortic root functionality and fatigue resistance. As reported by Liao et al., 0.1% SDS treatment of aortic valve leaflets appeared to maintain critical mechanical and microstructural properties of the scaffolds [122]. The 0.1% SDS treatment preserved the gross morphology, leaflet dimensions, and trilayered structure, with a layered structure similar to the native leaflet. However, microstructural disruptions such as collagen crimp organization and increased extensibility along the radial direction were also observed [122]. The milestone study by da Costa et al. [124] will likely reignite the enthusiasm for TEHVs that was dampened by the failure of the first "tissue engineered" decellularized porcine heart valve (Synergraft) [57].

In a DAVA sample obtained from a reoperation after 18 months, a few cells with fibroblast appearance were believed to migrate superficially into the aortic media [124]; thus the major challenge associated with acellular valves is insufficient cell reseeding/infiltration due to their dense ECM network. For example, in an *in vitro* study, Rieder et al. [52] compared recellularization potential of acellular porcine valves prepared using various treatments, and observed poor recellularization of SDS and trypsin treated valves, with only tert-octylphenyl-polyoxyethylen/SDS combinations showing some potential for human cell recellularization. Lichtenberg et al. [125] showed that a complete endothelialization of decellularized scaffolds can be achieved using a bioreactor that mimics physiological circulating condition. Karim et al. [68] also showed that in a bioreactor, good recellularization took place on the surface of the decellularized porcine AV, with efficiency of adherence higher with myofibroblasts than with ECs. However, the main challenge still lies in the complete recellularization with valve interstitial cells. Liao et al. observed using SEM that acellular collagen network in leaflets treated with SDS, trypsin, or Triton X-100 all showed a dense fiber network that was lack of interconnected pores, suggesting a challenge for cell infiltration [122].

In summary, naturally preserved trilayered structures such as those found in acellular aortic valves are excellent mechanical alternatives; however, these scaffolds are too dense to be populated with cells. The absence of valvular interstitial cells in valve scaffolds may constitute a threat for the long-term durability of the constructs due to lack of tissue remodeling, maintenance and repair potential.

6.3. Trilayered Designs based on Collagen Scaffolds

Our current approach to creating a viable trilayered aortic valve prosthesis was described by Tedder et al. [74]. Briefly, fibrous collagen scaffolds to be used as fibrosa and ventricularis layers were created from decellularized porcine pericardium. Decellularized pericardium exhibits excellent mechanical properties and has been

shown earlier to be a promising substrate for heart valve tissue engineering [126]. Porous spongiosa scaffolds were also created from decellularized and elastase-treated porcine pulmonary arteries. Fibrosa and ventricularis layers were then stabilized with PGG, a collagen stabilizer that reduces biodegradation without chemically crosslinking collagen. Trilayered scaffolds composed of one layer of spongiosa entrapped between two layers of collagen showed good shearing properties that approximated those of porcine aortic valves. Silicone molds of porcine aortic valves were then made and used to shape the valve construct. For bio-functional testing, a spongiosa scaffold was seeded with human-bone-marrow-derived stem cells inserted between the two fibrous pericardial layers and glued together to finalize the construct. The resulting valve-shaped TEHV proved to be biocompatible and able to immediately function under physiological pressures exerted by a heart valve bioreactor [74]. Moreover, the seeded stem cells changed their phenotype into cells resembling valvular interstitial cells in the absence of any added growth hormones or other chemical differentiation stimuli. These results suggest that mechanical loads specific to the aortic valve (stretching, bending, and extension) alone can induce stem cell differentiation into desired valve cell types. Based on the notion that "form follows function", current research is underway to develop a patient-tailored valve molding process with the intent of reducing patient-prosthesis mismatch. Data obtained from computed tomography will be processed digitally to produce an exact three-dimensional model of each patient's heart valve anatomy. This model will then be printed on a rapid prototyper as a mold. The mold will be used to shape the trilayered scaffold for an exact patient fit.

7. BIOREACTOR CONDITIONING OF TEHVs

As mentioned above, bioreactor conditioning of TEHV constructs prior to implantation is useful for ensuring the integrity of the valve under physiological stresses and for inducing phenotypical changes in resident cell populations. Hoerstrup et al. conditioned a cell-seeded, synthetic, tri-leaflet valve in a pulse duplicating bioreactor prior to implantation in lambs [123]. These valves showed similar mechanical properties and ECM production to native tissue. However, there was no development of a tri-layered structure [123]. Another use of bioreactor testing is for identifying and addressing structural faults in valve design prior to lengthy animal studies. Wang et al. tested a novel polymer trileaflet heart valve in a sheep model [127]. The new valve design failed due to leaflet cracking and tearing. Bioreactor testing prior to an animal study would likely have exposed this design's fault. Mechanical stimuli have been shown to travel through ECM and cells and induce a variety of cellular responses [128]. More recently, mechanical stimulation alone has been shown to affect stem cell differentiation [129]. Specific mechanical forces that have been shown to induce differentiation include shear

forces [130], compression [131], and stretch [132]. Heart valves are exposed to a wide variety of forces at every beat of the heart. We anticipate that bioreactor conditioning of stem-cell-seeded trilayered valves in a pulsatile flow bioreactor (Figure 2) will support stem cell differentiation and induce the onset of construct remodeling as a response to the appropriate loads [64]. We believe that partially remodeled valves stand a better chance of long-term survival after implantation.

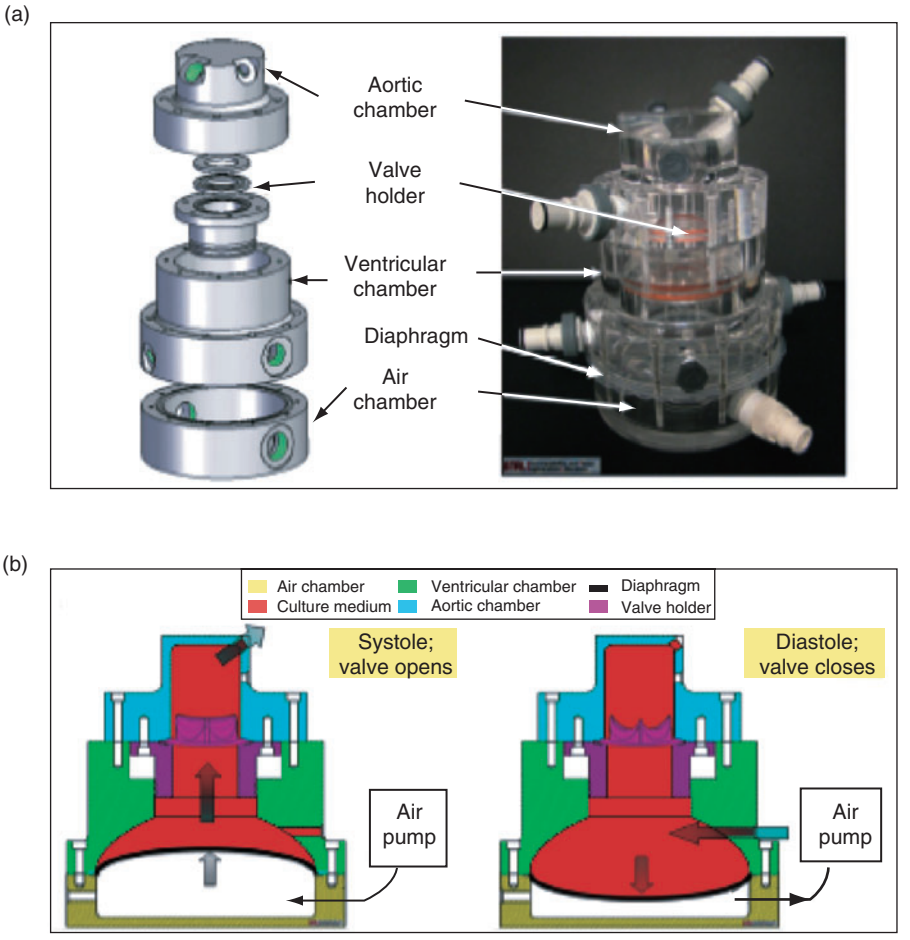

Figure 2. (*Continued*)

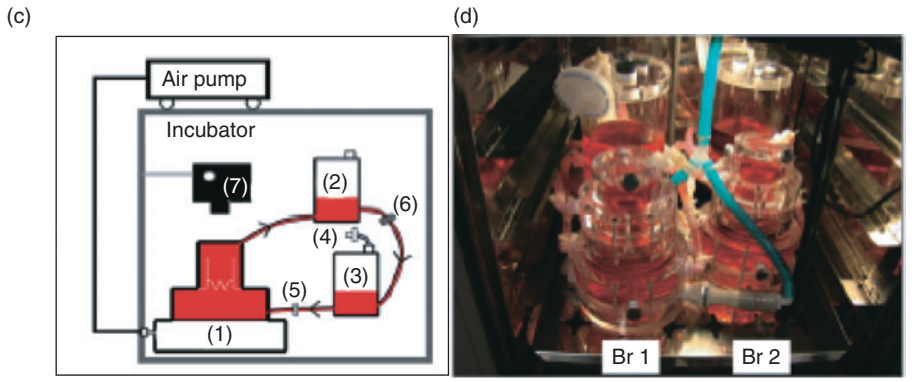

Figure 2. Heart valve bioreactor and conditioning system. (a) computer-aided design (left) and manufactured heart valve bioreactor (right) showing the four main components. A transparent silicone membrane diaphragm is mounted between the air chamber and the ventricular chamber. (b) Air and media flow through the system during systole (left) and diastole (right). Color coding aids identification of the components, and black arrows indicate direction of air and media flow. (c) Schematic overview of the entire conditioning system: a three-chambered heart valve bioreactor (1), an optional pressurized compliance chamber (2), a reservoir tank (3) with sterile filter (4) for gas exchange, one-way valve (5), pressure-retaining valve (6), a webcam (7), and a ventilator pump (air pump). The entire setup fits within a standard cell culture incubator. (d) Two identical bioreactor systems (Br 1 and Br 2) with endothelial cell-seeded, functioning valves inside an incubator; the bioreactors are in the front row while their corresponding reservoirs are in the back row. The webcams normally mounted onto the top viewing windows of the aortic chamber have been removed to reveal bioreactor details. Figure reproduced with permission [64].

8. FATIGUE, DAMAGE, AND LONG-TERM DURABILITY OF TEHVs

The current TEHV efforts are still focused on fabrication of a viable heart valve construct which is biomechanically and structurally robust, being able to perform in the dynamic environment of aorta, as well as with effective cell reseeding that can elicit tissue remodeling and adaptation. As we discussed above, there are many approaches and design philosophies in TEHVs. Other than optimizing scaffolds and cell reseeding of a chosen design, it will be very beneficial to address long-term durability issues at the designing stage. For instance, it is important to understand the potential fatigue and damage mechanisms of a specific TEHV design, and how different designs will affect long-term durability of TEHV differently. Without solid bioengineering considerations in long-term durability of TEHV, one can only rely on the expectation that “tissue remodeling and adaption” will occur after implantation,

which is a trial-and-error approach with numerous potential risks. A flawed design will be an inherent risk factor and likely cause TEHV failure along with a possibility of the accumulation of structural damages surpassing the repairing capability of integrated cells. However, there is still lack of knowledge on mechanisms of TEHV fatigue, damage, and repair; there is also absence of data on long-term durability of TEHVs both *in vivo* and *in vitro*.

Due to decades of experience in clinical applications and research in allograft and bioprosthetic heart valves (BHVs), a good amount of knowledge has been obtained on the damage pattern and mechanisms of the structural deterioration of those valve implants. We believe that applying the knowledge gained from allograft and BHV durability tests might shed light on damage mechanisms and durability of TEHVs. Sabbah et al. showed that in porcine BHV leaflets, the sites of calcification and tissue disruption have close association with the sites of high stress concentration [133]. Haziza et al. studied pure leaflet tear failures in both porcine and pericardial BHVs (in the absence of calcification), and reported that damages often took place at sites with intense stress concentrations, for instance, at the mounting sites or portions of leaflet that experienced higher bending stresses [134]. The glutaraldehyde-fixed single-layer pericardium and porcine leaflet demonstrated inferior bending behavior due to either lack of trilayered structure or poor internal shear resistance caused by glutaraldehyde fixation [121]. These observations suggest the importance of the design of the trilayered structure in TEHVs that closely mimics the native heart valve leaflet (fibrosa, spongiosa, and ventricularis), with a goal to eliminate potential structural damages (likely tissue buckling and tear) induced by cyclic fatigue.

Grabenwoger et al. [135] compared BHVs fabricated from autologous pericardium and heterologous pericardium (average implant period was 33 ± 8 months). The autologous pericardium valves (briefly immersed in glutaraldehyde) were found to have very good biocompatibility, evidenced by the absence of calcium deposits, macrophages, and the presence of endothelialization; however, severe disintegration of collagen fibers was observed, indicating lack of fatigue resistance due to inadequate crosslink stabilization [135]. On the other hand, heterologous pericardium valves failed due to calcification resulted from low biocompatibility, but exhibited a superior preservation of collagen network [135, 136]. Aortic valvular allografts, due to non-viability, were also reported to undergo progressive noncalcific structural degeneration such as tearing, sagging, or retraction, with disruption of the normal valve architecture [137]. The above studies [135–137] suggest that, even with good biocompatibility and optimal structure design in heart valve replacements, without cellular maintenance such as damage repairing and tissue remodeling/adaptation, structural damage at the ultrastructural level will likely accumulate with time and impair the long-term durability of TEHVs. In an *in vitro* fatigue mechanism study, Liao et al. [138] found that the fatigue pattern of the acellular leaflets included an unfolded and thinned morphology, straightening of the locally wavy collagen fiber structure, and disruption of elastin network. Although the durability of the acellular leaflets cannot be extrapolated from this type of *in vitro* fatigue mechanism, this mechanistic study [138]

reveals that, in the absence of cellular maintenance, decellularized valve leaflets experience structural deterioration in cardiac cycling (~2.4 million cardiac cycles) due to lack of exogenous stabilizing crosslinks as well as irreversible and cumulative structural disruption. A well-designed cell integration, which reflects the heterogeneous functional roles of valvular cells (VICs and VECs), is thus of great importance to the long-term durability of TEHVs [74, 139].

9. CONCLUDING REMARKS

Many efforts have been made in the last 50 years to develop the ideal heart valve substitute that will last a lifetime. The task is formidable because of the particular hemodynamic and biologic conditions present in the native aortic heart valve [140]. Tissue engineering an aortic heart valve requires extensive interdisciplinary efforts that combine scaffold development, matrix chemistry and biochemistry, biomechanical analysis, cell biology, mechanotransduction, bioreactor engineering, and animal studies.

The existence of a trilayered structure in the native aortic valve is an overlooked aspect of heart valve physiology and should be considered in future tissue engineering endeavors. The current review shows that single layered scaffolds have a low chance of success because of the daunting mechanical requirements specific to the aortic valve. However, even the best trilayered scaffolds might not resist billions of the repetitive bending, flexing and stretching cycles in the absence of viable cells capable of repairing and remodeling the scaffolds on a sustainable basis. Thus, equally important are cell integration efforts focused on integrating the right number of cells of the appropriate cell phenotype at the right place within the valve structure and driving them towards homeostatic maintenance of the valve matrix. It is our hope and expectation that combinations of these new approaches with “old-fashioned” heart valve design would yield novel treatments for thousands of patients needing heart valve replacement.

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CONFLICT OF INTEREST

The authors indicated no potential conflicts of interest.

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