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Application of Hydrogels in Heart Valve Tissue Engineering

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Abstract

With an increasing number of patients requiring valve replacement, there is heightened interest in advancing heart valve tissue engineering (HVTE) to provide solutions to the many limitations of current surgical treatments. A variety of materials have been developed as scaffolds for HVTE including natural polymers, synthetic polymers, and decellularized valvular matrices. Among them, biocompatible hydrogels are generating growing interest. Natural hydrogels, such as collagen and fibrin, generally show good bioactivity, but poor mechanical durability. Synthetic hydrogels, on the other hand, have tunable mechanical properties; however, appropriate cell-matrix interactions are difficult to obtain. Moreover, hydrogels can be used as cell carriers when the cellular component is seeded into the polymer meshes or decellularized valve scaffolds. In this review, we discuss current research strategies for HVTE with an emphasis on hydrogel applications. The physicochemical properties and fabrication methods of these hydrogels, as well as their mechanical properties and bioactivities are described. Performance of some hydrogels including *in vitro* evaluation using bioreactors and *in vivo* tests in different animal models are also discussed. For future HVTE, it will be compelling to examine how hydrogels can be constructed from composite materials to replicate mechanical properties and mimic biological functions of the native heart valve.

Keywords

tissue engineering; hydrogels; scaffolds; heart valve; decellularization

I. INTRODUCTION

Valvular heart disease causes significant morbidity and mortality worldwide. The most common treatment for end-stage aortic valve disease is surgical replacement, with the number of these patients worldwide increasing from approximately 290,000 in 2003 to an estimate of over 850,000 by 2050.[1] Surgical replacement of diseased aortic heart valves has been widely performed, primarily with mechanical valves (Fig. 1A) and bioprosthetic heart valves (Fig. 1B). All these current devices have significant limitations with risks of further

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morbidity and mortality. For example, mechanical valves may cause hemorrhage and thromboembolism, and thus they require anticoagulation for the lifetime of the patient.[2] Bioprosthetic valves have relatively poor long-term durability due to degeneration, calcification and fibrosis, and may cause immunogenic complications.[3–5]

In addition, a common problem with these devices for pediatric applications is their failure to grow, repair and remodel following somatic growth. Each year in the US alone, there are approximately 20,000 infants born with congenital heart diseases[6] who will likely require valve replacement sometime during their lifetime. Unfortunately, there is currently no valve treatment that can circumvent the above mentioned limitations, and as such implantation of tissue engineered heart valves (Fig. 1C) comprised of cell-seeded scaffolds is an appealing alternative for this patient population.[1,7–9]

A number of approaches have been developed for heart valve tissue engineering (HVTE) using a range of scaffold types (*e.g.*, natural and synthetic polymers,[10–12] decellularized xenografts and homografts[13–15]) and various cell sources (*e.g.*, valvular cells,[16,17] bone marrow stem cells,[18] and progenitor cells from the peripheral blood or amniotic fluid[19–21]). In this review, we discuss current research in HVTE, while particularly focusing on applications of hydrogels in HVTE.

Hydrogels, composed of hydrophilic polymers that form three-dimensional networks, can be fabricated into various shapes and sizes and engineered to mimic the extracellular matrix environment of human tissues. Thus, hydrogels have been widely used for tissue engineering, medical implants and drug delivery. This paper describes a variety of hydrogels, including natural (*e.g.*, collagen,[10] fibrin,[12,23] and hyaluronic acid (HA)[16,24]), synthetic (*e.g.*, poly(ethylene glycol) (PEG)[11,25] and poly(vinyl alcohol) (PVA)[26]) and composite hydrogels (*e.g.* type I collagen with chondroitin sulfate[27]), their fabrication methods, and their applications for HVTE. *In vitro* conditioning and evaluation with bioreactors and *in vivo* performance of tissue engineered heart valves, especially from hydrogel materials, are also discussed.

II. HEART VALVE TISSUE ENGINEERING

A. Compositions, Structures, and Functions of Heart Valve

The predominant function of heart valves is to maintain the unidirectional blood flow through cyclic opening and closing during cardiac systole and diastole. Human semilunar valve leaflets are normally thinner than 1 mm[28] and stratified into three layers[29] (Fig. 2A): the upper fibrosa layer is dominated by circumferentially oriented type I and III collagen fibers to withstand high pressure loads;[30] the lower ventricularis layer is composed of radially oriented elastic fibers to provide elasticity and preload for stretch and recoil;[30–32] the middle spongiosa layer mainly contains glycosaminoglycans (GAGs) and proteoglycans (PGs) offering compression resistance and lubrication functions. Such unique arrangements of extracellular matrix (ECM) determine the anisotropic material properties of heart valves, *i.e.*, stiff in the circumferential direction while compliant in the radial direction, which provides both sufficient mechanical strength and elasticity during valve opening and closing.

The cellular components of valve leaflets include valvular endothelial cells (VECs) that cover the blood-contacting surfaces and valvular interstitial cells (VICs) that populate all three layers of leaflets (Fig. 2B). Although not fully characterized and understood, VECs are thought to contribute to valve homeostasis indirectly through the regulation of permeability, adhesiveness to immune system cells, and paracrine signaling to local VICs,[33] whereas VICs are believed to be primarily responsible for maintaining the delicate microstructure that is critical to valve function and actively remodeling ECM in valve repair as well as disease progression.[34,35] All mesenchymal cells within valve leaflets are classified as VICs,[36] which have a high proliferation index and frequently turnover ECM components, indicating their continual repair of mechanically induced micro-damage to guarantee long-term durability of valves.[36] VICs are heterogeneous and dynamic in their phenotypes, which include progenitor endothelial/mesenchymal cells, quiescent fibroblasts, activated myofibroblasts, and osteoblast-like cells.[37] Although the complex relations among VIC phenotypes and their origins are still unclear, VIC phenotype switch and ECM remodeling seems to occur with valve maturation and pathological progression. For example, in fetal valves, a large fraction of VICs display the myofibroblast phenotype, which has the characteristics of both fibroblasts and smooth muscle cells, with expression of vimentin and alpha smooth muscle actin (α-SMA). In healthy adult valves, however, more than 95% of VICs are categorized as quiescent fibroblastic cells, characterized by the expression of vimentin but not α-SMA.[34] Moreover, in response to valve injuries induced by mechanical stress, quiescent VICs can be activated into myofibroblasts that become more contractile and secret large amounts of cytokines, chemokines, growth factors, ECM components, matrix metalloproteinases (MMPs) and their tissue inhibitors, which modulate the matrix and release sequestered growth factors by enzymatic degradation.[38] Under disease conditions, VICs may also undergo osteogenic differentiation to form osteoblast-like cells that are associated with valve calcification[39,40]—the formation of calcified mineral deposits analogous to bone formation.[41] However, it remains unclear whether myofibroblast activation is an intermediate stage for VICs to become osteoblast-like cells.[42,43]

B. Heart Valve Diseases and Replacement

Driven by the mechanical forces exerted by the heart and the circulating blood, normal heart valves open and close about 40 million times a year without obstruction or regurgitation.[44] Heart valve diseases are commonly diagnosed clinically as stenosis—outflow obstruction due to incomplete openness, and regurgitation—backward flow resulting from inefficient closure. Specifically, aortic valve disease causes more than 50,000 hospitalizations every year in the United States,[45] with increased incidence with aging. No longer considered as a passive consequence of aging, valve calcification, which is identified as the leading cause of valve diseases, is an active and progressive process.[24] It is intimately related to collagen degeneration, proteoglycan and lipid accumulation, and possibly even cellular changes.[33,46]

However, to date there are no medical agents that are FDA-approved to prevent the valve disease progression,[38] rendering valve replacement as the most common and virtually the only clinical therapy for valve stenosis.[36] Aortic valve replacement as a result of calcific aortic stenosis has become the second most common cardiac operation in the world.[47]

Patients with severe aortic stenosis have a mortality rate of 37% at one year after symptom onset without surgical valve replacement.[48]

The two types of most commonly used valve replacements are mechanical valves and bioprosthetic valves. Mechanical valves are generally composed of metals, pyrolytic carbon, and expanded poly(tetrafluoroethylene) (ePTFE), with various models mainly including caged-ball, tilting disk and bileaflet.[49] Although mechanical valves have evolved significantly toward better fluid mechanics and enhanced durability (>20 years), the risk of infections and thromboembolic complications after implantation is unavoidable because they present foreign materials to the human immune system.[50] All mechanical valve recipients therefore must manage anticoagulant drug therapies for the remainder of their lives; a consequence that carries inherent risks of hemorrhagic complications.[51] On the contrary, bioprosthetic valves from decellularized and glutaraldehyde-fixed biological valves[13] do not require anticoagulation medication. However, the structural deterioration and extensive calcification that are the major causes of their failure are closely associated with the chemical, mechanical and morphological changes of biological valves during decellularization and chemical fixation.[51] First, nonviable cells in bioprosthetic valves are incapable of repairing the cumulative ECM damage, and their fragments serve as nuclei for calcification.[51] Second, chemical fixation increases the flexural rigidity of bioprostheses[52] and locks them into one configuration, which does not allow the dynamic ECM arrangements necessary for normal valve function.[51] Specifically, the risk of valve failure due to progressive tissue deterioration is particularly high for pediatric and adolescent patients who have an active lifestyle; the rate of valve failure is up to 10% within four years after bioprosthetic valve implantation in these patients.[53] The following conclusions can be made when considering the advantages and drawbacks of these two valve replacements: mechanical valves are more suitable for younger patients (<60 years) with higher physical activity levels and able to tolerate blood thinner medication, while bioprosthetic valves serve better the older patients (>60–65 years) who are relatively physically inactive.[36] In a nutshell, an ideal valve substitute has yet to be developed.

C. Design Principles and Current Strategies for HVTE

Although current options for valve replacements generally enhance survival and quality of life, they have severe limitations. Moreover, neither mechanical nor bioprosthetic valves are capable of accommodating somatic growth, which is a problem for children.[54] Ultimately, these therapies result in valve failure and require subsequent valve replacement. The difficulties associated with mechanical and bioprosthetic valve replacements in children have greatly motivated the development of tissue engineering approaches for valve replacement, which aim to construct living valve substitutes with regeneration and growth potential. Autologous cells seeded in such substitutes should sense and respond to the changes in environments and adapt themselves for optimal performance. Towards this goal, many studies have been performed over the past few decades to clarify the desirable characteristics of tissue engineered heart valves and to develop strategies for generating these valve substitutes.

To generate living valve substitutes *in vitro*, the concept of HVTE involves the design of a three-dimensional scaffold and the choice of the right cells. The scaffold should provide temporary structural support and specific biological cues to dictate cell fate until cells produce their own matrix proteins to remodel the scaffold and mature into a valve substitute with composition, structure and mechanical properties analogous to native valves. A significant challenge in tissue-engineered valves is to recapitulate the natural ECM composition and distribution, *i.e.*, the creation of a sufficient and appropriate amount of matrix proteins and their arrangement.[55]

1. Design of Tissue Engineering Scaffolds—Polymeric materials and decellularized valves have been the most widely studied scaffolds for HVTE. A variety of methods have been applied to the fabrication of polymeric scaffolds, including dip casting, film fabrication and injection molding. It should be first noted that the primary principle of scaffold design is to support cell adhesion, spreading and growth in three dimensions, which requires the scaffold to be porous and cytocompatible. Hydrogels, with hydrophilic and crosslinked structures, are attractive scaffold materials for tissue engineering because they can provide a porous, hydrated environment, similar to soft tissues, that allows the exchange of oxygen, nutrients and cellular waste.[56] Another important process involved in a variety of cellular behaviors is the proteolytic degradation of ECM, which is necessary for cell spreading, migration and differentiation.[57] Biodegradable/bioresorbable hydrogels satisfy this requirement when designed with degradable segments. These hydrogels may be degraded through hydrolysis or by enzymes secreted by resident cells, such as MMPs, plasmin, and elastase.[58] To date, several hydrogels explored for HVTE applications have been prepared from natural or synthetic materials, such as collagen,[10] HA,[16,24] fibrin,[12,23] and PEG.[17] A significant challenge in the use of these materials is to obtain a balance between scaffold degradation and new ECM deposition, which is important for creating and maintaining sufficient mechanical properties of the cell-seeded constructs. Generally, scaffold degradation represents polymer chain scission and tends to decrease the mechanical properties of the scaffolds whereas new ECM synthesis by seeded cells functions to enhance the mechanical properties.[59] A more rapid degradation might impair the integrity and mechanical properties of constructs whereas an overly slow rate of degradation might impede tissue regeneration.[58] Thus, an ideal situation requires the scaffold to possess a controlled degradation rate that matches the new ECM deposition and tissue regeneration.[59] Since the course of scaffold degradation is highly dependent on the susceptibility of the cleavage sites and cellular activity, the control of degradation rate and profile can be achieved by either manipulating the susceptible segments[60–63] or regulating cellular activities.[35]

Second, the interactions between cells and their local environment play a critical role in determining cell fate and physiological functions. For this reason, an ideal scaffold should provide informative microenvironments mimicking physiological niches to direct advanced cell behaviors, such as differentiation, proliferation and apoptosis, without inducing pathological outcomes such as calcification. For example, although the characteristics of different VIC phenotypes and the role of ECM on VICs function and dysfunction are still not fully understood, it is generally accepted that activated myofibroblasts demonstrate

wound healing functions by up-regulating cell proliferation and ECM production upon injuries. However, myofibroblast persistence may also cause fibrosis and calcification leading to valve stenosis[64] while osteoblast differentiation portends valve calcification and deterioration. Differentiation of VICs toward myofibroblast or osteoblast phenotype is highly dependent on the interactions between VICs and the ECM components.[40] Thus, myofibroblast activation may be required to promote cell proliferation and matrix protein production at the initial phase and then would be reversed to a quiescent fibroblast phenotype to maintain valve homeostasis after maturation, as opposed to osteoblast differentiation, which should be inhibited or avoided. The balances between cell proliferation and apoptosis, and matrix production and degradation should be considered for the scaffold design in order to achieve optimal performance of valve substitutes. Therefore, an ideal scaffold should encode all these instructions in its composition and structure for cells to interpret and modify their fate accordingly. For example, although the exact molecular mechanisms implicated in VIC differentiation remain unclear, some of the factors that elicit or regulate myofibroblast activation and deactivation include substrate rigidity[65] (*e.g.*, activation on stiff substrates and deactivation upon substrate softening induced by photo degradation[64]) and regulation of growth factors (*e.g.*, basic fibroblast growth factor (bFGF) inhibits activation while transforming growth factor-β1 (TGF-β1) stimulates activation).[66–69]

Third, the natural valve ECM, a layered and heterogeneous structure, provides an ideal model for the design of scaffold structure and architecture. To generate functional valve substitutes, the ultimate construct should replicate the composition, structure and architecture of natural ECM in order to recapitulate the cell-cell and cell-matrix interactions in natural valves. The development of micro-engineered hydrogels (*i.e.*, hydrogels with micro-scaled features in at least one direction) has enabled the fabrication of 3D scaffolds with controlled structure and architecture mimicking biological tissues.[70] Promising micro-fabrication techniques[70] used to engineer such hydrogels include photolithography,[71,72] microfluidics,[73,74] micromolding,[75,76] and tissue printing.[77,78]

Finally, the application of cyclic mechanical forces mimicking the stretch, flexure, and shear stress experienced by valves *in vivo* has been shown to promote the formation and maturation of tissue engineered constructs with regard to enhanced ECM secretion and alignment, leading to the concept of bioreactors.[18,79] The design of bioreactors facilitating the formation and maturation of engineered constructs with desired *in vivo* performance is a fundamental component of HVTE efforts.

2. Choice of Cells—VICs, the predominant cell population in valve leaflets, are known to be responsible for active ECM remodeling in valve repair as well as to contribute to valve disease progression. Thus, it is crucially important to investigate VIC growth, differentiation and ECM production within microenvironments that mimic physiological niches as well as diseased conditions. For these reasons, as well as their ease of isolation, VICs have been widely used in the research of HVTE.[44,59,59] Nevertheless, due to the heterogeneity, source-dependence and plasticity of these cells, it can be difficult to keep consistency between cell batches thus complicating the research. For example, VICs from fetal valves have a significantly higher percentage of activated phenotypes than those from adult valves;[34]

aortic VICs are stiffer and have higher contractility than pulmonary VICs;[80] VICs demonstrate age- and valve-region-specific response to substrate stiffness;[81] and improper differentiation of VICs may contribute to pathological progression. For these reasons, when VICs are used in experimental studies they are often limited to early passages (P5 or earlier). More characterization is needed for a comprehensive understanding of VIC behavior and the molecular mechanisms underlying valve diseases, an understanding that will be beneficial to the future design for HVTE.

Stem cells have increasingly been evaluated as potential cell sources for tissue engineering due to their potential to differentiate into various cell types and their self-renewal properties.[82,83] Yet ongoing ethical concerns limit the use of embryonic stem cells in research and potential therapies. As an alternative, mesenchymal stem cells (MSCs) from bone marrow may serve as a clinically feasible cell source without raising ethical concerns.[83,84] As mentioned previously, a key consideration in the generation of functional valve substitutes is the cellular response to mechanical forces experienced by heart valves. Cells seeded into valve substitutes should mimic the functions of resident cells in valve leaflets. Studies show that MSCs can be induced to differentiate into a phenotype that resembles VICs.[18,86] When exposed to the same stretching profile, MSCs demonstrated responses similar to VICs with regard to collagen synthesis.[18] When implanted into the pulmonary position of sheep with established cardiopulmonary bypass, fibrous scaffolds seeded with autologous MSCs functioned well for more than four months and underwent extensive cellular and ECM remodeling to resemble native valves.[83] Circulating endothelial progenitor cells also have the potential to differentiate into endothelial and interstitial-like cells.[19] The option of creating valve constructs from autologous cells not only eliminates chances of immune rejections but also offers the potential to grow and remodel with recipients,[85] which is especially important for the pediatric population. Other autologous cell sources unique to the pediatric population include amniotic fluid, umbilical cord blood, placenta, and chorionic villi,[36,86–89] which allow autologous replacements ready for use at or soon after birth of infant patients. Combined with the emergence of cell banking technology, these sources may have wider applications toward autologous cell based therapies.

An endothelial cell layer on blood-contacting surfaces is important for valve constructs to maintain anti-thrombogenicity and transmit nutrients, mechanical, and biochemical signals to the underlying VICs.[90] VECs, which are less understood, have been compared with the well-characterized vascular endothelial cells. Although VECs share some similarities with other endothelial cells such as arterial endothelial cells (AECs), hundreds of differently expressed genes have been identified between VECs and AECs, indicating that VECs are a distinct phenotype.[91] Such phenotype difference between VECs and AECs is also shown by applying fluid shear stress—VECs align perpendicularly to the flow direction whereas AECs align in a parallel orientation.[90]

III. APPLICATIONS OF VARIOUS HYDROGELS IN HVTE

Hydrogels are primarily composed of hydrophilic polymeric materials with a large amount of water and generally form three-dimensional macromolecular networks. Hydrogels have a

permeability to nutrients and physicochemical profiles similar to the native ECM, demonstrating tremendous advantages in a variety of tissue engineering applications.[56] For example, recent developments in molding, micro-patterning and three-dimensional (3D) printing have enhanced the promise of hydrogels for HVTE. Moreover, cultures of valvular cells atop or inside hydrogels have been widely developed as models to investigate cell-matrix interactions[10,17,92] and to shed light on valvular cell biology.

Hydrogels can be divided into two major classes, natural and synthetic hydrogels, based on the polymeric material(s) in the hydrogels. Natural hydrogels, including collagens,[10] fibrin[12,23] and hyaluronan,[17,62] are composed of materials found in native ECM. These hydrogels offer good biocompatibility and bioactivity for tissue engineering applications. Synthetic hydrogels (*i.e.*, PEG[17] and PVA[26] based hydrogels) are composed of synthetic polymers. Synthetic hydrogels have some advantages over natural hydrogels, such as tunable mechanical properties, easy control of structural architecture and chemical compositions. Additionally, these hydrogels can be modified with bioactive moieties to elicit specific biological functions. Moreover, composite hydrogels that take advantage of the versatile biological activities of natural materials and the flexible mechanical properties of synthetic materials are of great interest, because they have potential to fulfill the requirements of proper mechanical properties and biological functions for HVTE. Different types of hydrogels for HVTE are further discussed below.

A. Natural Hydrogels

1. Collagen—Collagen hydrogels have been successfully used for wound healing, drug delivery, and tissue engineering for various tissues.[93–97] Collagen is the most prevalent protein in animal tissue. The structure of the collagen molecule is a triple helix of polypeptide chains primarily stabilized by inter-strand hydrogen bonds between adjacent -CO and -NH groups. In each of the three chains, the amino acid sequence follows a distinctive three amino acid repeat pattern of Gly-Xaa-Yaa, in which Xaa and Yaa are any amino acids. However, proline and hydroxyproline mainly occupy the Xaa and Yaa positions, respectively, playing key roles in collagen stability.

Among the twenty-eight types of collagen that have been identified in vertebrates, type I collagen is the most abundant. It can be found in a wide range of tissues and organs, such as skin, tendons, artery walls, heart valves, and the organic part of bone. Type I collagen has a triple helix heterotrimer structure formed by two identical alpha1(I) chains and one additional alpha2(I) chain.[98] *In vivo*, collagen molecules are synthesized as soluble precursors (procollagen), which are glycosylated and hydroxylated prior to self-assembly of the alpha chains. After secretion, the triple helix is cleaved by procollagen metalloproteinases. The collagen molecules can then self-assemble into periodic cross-striated fibrils, which are stabilized by the inter- and intra-molecular covalent bonds between collagen polypeptide chains.[99] Collagen fibrils further aggregate into collagen fibers that assemble to form the macroscopic structures. Hereafter, the term collagen will represent type I collagen in this article, unless specifically indicated.

Collagen hydrogels can be prepared from purified type I collagen extracted from skin or tendons. Human recombinant collagen is a possible alternative to that extracted from animal

tissue and the protein sequence can be designed based on the chemistry and structure of the collagen molecule, but the cost is relatively high.[100] The fibrillogenesis process is initiated when the acidic collagen solution is neutralized or warmed, and self-assembly of collagen fibrils leads to formation of a hydrogel. The fibril diameter in the hydrogel is determined by solution pH, ionic strength, ion types, and gelling temperature. A correlation between the fibril diameter and mechanical properties of the collagen hydrogel has also been determined.[101]

Because collagen is a predominant ECM component of the heart valve, its use has the potential to maintain distinct valvular mechanical properties and facilitate the interaction between valvular cells and the ECM. Collagen-based scaffolds have in fact been fabricated to promote benign cell-matrix interactions for HVTE[102] with high cell viability observed in this system after long term *in vitro* culture. Another study demonstrated that VICs seeded in collagen hydrogels upregulated expression of α-SMA,[27] indicating the activation from quiescent fibroblastic phenotype into myofibroblastic phenotype. It is worth noting that the interaction between the Arg-Gly-Asp (RGD) peptide sequences in collagen fibrils and the β1 integrin subunits on cell surfaces activates intracellular signal pathways, including a crucial pathway to repress apoptosis[103] and a pathway involved in mediating cell proliferation.[104] Moreover, VICs cultured inside the collagen hydrogel express proteolytic MMPs, such as MMP-1, -8, -13, -14, which have been found to be involved in scaffold degradation.[105–107] Additionally, to investigate the effect of the controlled anisotropic strain on cell behavior, VICs have been cultured in collagen hydrogels with cyclic stimulation using a bioreactor to mimic *in vivo* dynamic physiological environments.[10] This applied cyclic anisotropic strain showed obvious influences on cell phenotype, turnover, and matrix remodeling.

Collagen gels are in general mechanically weaker than native heart valve ECM due to the low protein concentration in these hydrogels.[36] Higher protein concentration renders high strength to the gel, but results in unfavorably dense microstructure, which restricts diffusion of oxygen and nutrients to cells.[108] Chemical modification to increase the covalent crosslinking among collagen fibrils is an option to improve the mechanical durability of collagen gels.[109] Our experience with this crosslinking has been to react collagen with acrylate-PEG-succinimidylvalerate, which can be further photo crosslinked to form a hydrogel. The succinimidylvalerate (SVA), an active ester, was used for the PEGylation of primary amines, forming a stable amide linkage. This reaction was generally performed in DMSO with a base catalyst or was performed in an aqueous solution at pH ~8–9. Seeded VICs demonstrated excellent adhesion to the PEGylated collagen hydrogel (Fig. 3A). Obvious elongation of VICs was found for 3D culture in the PEGylated collagen (Fig. 3B), whereas only rounded VICs were visible in pure PEG hydrogels (Fig. 3C). Importantly, these results indicated that bioactivities of collagen were preserved after the PEGylation reaction.

In vivo, collagen typically has low antigenicity,[110] but the potential for thrombogenicity might be detrimental to its biomedical applications. Collagen can activate the aggregation of platelets, thus triggering the blood coagulation pathway.[111] Therefore, a collagen-based heart valve must be thrombo-resistant. For example, some collagen composites have been developed to withstand blood clot formation.[112] Another feasible solution has been to recruit

VECs to the surface of the collagen construct. A co-culture model of VICs and VECs was reported to activate expression of endothelial nitric oxide synthase (eNOS) by VICs, thereby restricting thrombotic events.[27] Other evidence suggests that co-culture of VICs and VECs in a collagen hydrogel may also attenuate valvular calcification.[113]

2. Fibrin—Fibrin gels have been used for tissue engineering applications due to their attractive bioactivity and availability as an autologous source.[12,23,114] Fibrin, the final product in the coagulation pathway, is converted from the plasma protein fibrinogen in the presence of the activated protease thrombin and calcium. The fibrinogen molecule is a hexamer with a molecular weight of 340 kDa, and contains two sets of three different chains (α, β, and γ), stabilized by disulfide bonds. The polymerization process is initiated by the cleavage of fibrino peptide A in the fibrinogen α-chain and fibrino peptide B in the β-chain. The exposed α- and β-chains bind to the γ-chain and form protofibrils. When the twisted protofibrils are present in a sufficient concentration, they assemble into fibers that form the fibrin network.[115] The microtopology of a fibrin hydrogel can be influenced by the initial fibrinogen, thrombin and calcium formula.[114] The mechanical strength and degradation rate of a fibrin gel can be tuned by controlling the polymerization process.

Many growth factors,[116,117] ECM components (*e.g.* fibronectin[118] and HA[119]), and clot components (*e.g.* von Willebrand factor)[120] can bind to fibrin rendering specific cell-matrix interactions. Two RGD sites in the fibrin molecule enable interaction with many cells through their integrin receptors on the cell membrane. Endothelial cells can bind to the fibrin network through VE-cadherins.[121] Enzymes, plasmin and MMPs, contribute independently to degradation of the fibrin hydrogel,[122] and *in vitro* degradation of fibrin gels can be modulated by the addition of aprotinin.[123–125] Moreover, tranexamic acid can be used to control gel properties by inhibiting the fibrinolysis process.[126] An important advantage of fibrin use is that soluble fibrinogen in blood plasma (normal blood concentration at 1.5–4.0 g/l)[127] can be easily collected from a patients' own blood to create fibrin as an autologous source for tissue engineering applications.

Fibrin gels currently have been used as haemostatic glue for wound healing,[128] and the product has also been modified to deliver drugs and cells.[12,129] Fibrin hydrogels have exhibited great biocompatibility, similar to collagen gels, when used for HVTE. VICs or myofibroblasts seeded inside the fibrin scaffold showed high viability with promotion of proliferation and migration.[125] ECM synthesis and phenotypic markers of activation were observed under mechanical conditioning,[130] indicating the potential application of fibrin hydrogels as scaffolds for HVTE.

Fibrin based heart valve scaffolds can be prepared using an injection molding technique (Fig. 4).[22] In this method cells, such as VICs or myofibroblasts, mixed with calcium and thrombin in a proper buffer were injected into a mold formed by a positive stamp and a negative stamp with heart valve shape. The simultaneous addition of fibrinogen into the mold using a dual syringe system immediately initiated the fibrin polymerization process. The newly polymerized heart valve was removed from the mold, and conditioned in a bioreactor before implantation.[22] This technique can be adapted to fabricate heart valves from other biomaterials as well.

Low mechanical strength is a common limitation for application of hydrogels in HVTE. Like collagen gels, fibrin-based scaffolds display relatively poor initial mechanical strength and are not suitable for direct implantation. Another disadvantage is the shrinkage of fibrin gels caused by cell-mediated contractile forces, resulting in valvular insufficiency. This problem has also been observed within other cell-seeded hydrogels, including collagen. In one study, chemical fixation with a stiffer biomaterial, poly-L-lysine,[131] was attempted to strengthen the fibrin gel and reduce tissue shrinkage. Moreover, bioreactor stimulation after hydrogel molding was performed to reduce the tissue shrinkage and enhance matrix remodeling, and further contributed to the maturation of the fibrin scaffold *in vitro* as well.[23]

3. Hyaluronic Acid—Hyaluronic acid (HA, or hyaluronan) hydrogels have been used for various applications including cell or drug delivery, wound healing and tissue engineering.[132–135] HA is a soluble, linear polysaccharide (molecular weight (MW) ~10^3–10^4 kDa) that contains alternating N-acetyl-D-glucosamine and D-glucuronic acid residues. This natural macromolecule has been widely found in vertebrate tissue and even in some plants and bacteria with high structural homology across the species, making it less immunogenic and relatively biocompatible. Human-grade HA can be produced by genetically modified bacteria, *Bacillus subtilis*, with high yield, stable molecular weight (MW) and purity.[136] As the most abundant GAG in the mammalian body, high concentrations of HA are found in a variety of tissues including heart valves.[137] Cell mobility and adhesion can be modulated by HA through cell membrane receptors, CD44, ICAM-1 and RHAMM,[138] and the macromolecule can be quickly degraded *in vivo* by hyaluronidases through three different pathways. Generally, small HA fragments are signals of inflammation, immune-stimulation and angiogenesis leading to the activation of specific cells, while large fragments are involved in maintaining tissue structural integrity and cell quiescence.[139] However, the mechanisms for these activities are not yet clearly understood.

At physiological pH, the carboxyl group attached to each disaccharide unit of HA is ionized ($–COO^–$), resulting in a highly negatively charged HA molecule. The negatively charged molecule may attract positive ions, absorb water, and expand in volume up to 1000 times because of the osmotic gradient.[140,141] Chemical modification of HA can be used to make spontaneous or crosslinkable hydrogels. The carboxylic group in the glucuronic acid moiety or the C-6 hydroxyl group in the N-acetyl glucosamine sugar is amenable to various chemical modifications, including the addition of disulfide or vinyl groups or PEGylation. After modification, HA can be crosslinked to form hydrogels, decreasing the degradation rate of the polymer and increasing its mechanical strength.[135,142] Recently HA has been used for heart valve scaffolds because HA constitutes up to 50% of the total GAGs in human heart valve leaflets,[143,144] providing compressive resistance in the cardiac cycle[145] and playing an essential role in valvular cell-matrix interactions. Additionally, HA-based signaling pathways, particularly through CD44 receptors on the cell membrane, were found to influence VIC adhesion, migration, and proliferation.[24]

In addition, researchers have found that methacrylated HA could be photo crosslinked to form hydrogels,[16] which led to high viability of encapsulated VICs and facilitated a significant increase in elastin synthesis through the stimulation from degraded lower MW HA fragments. Furthermore, the immobilization of CD34 antibodies on the surface of

gelatin methacrylate modified HA hydrogels was reported to attract endothelial progenitor cells (EPCs) and control cell spreading and elongation.[146] This strategy could potentially promote endothelialization and enhance the biocompatibility of tissue engineered heart valves.

Although the chemical modifications of HA could possibly pave the way for implantation of these hydrogels, less is known about how to control cell-HA interaction in a precise manner —a critical control since improper signaling may elicit a pathological cascade of events. A previous study revealed that there is a correlation between the abundance of HA and the calcified nodules on diseased aorticvalves.[147] In addition, depletion of HA or disruption of cell-HA interactions by blocking the CD44 receptor was found to increase nodule formation.[24] As mentioned previously, the activation of VICs from the quiescent state is the prerequisite for the remodeling of matrix in HVTE, and proper expression of myofibroblastic phenotypic markers, such as α-SMA, is essential for this remodeling process. Ongoing research focuses on regulation and control of cell activation and matrix remodeling by HA, as well as on determining the microstructure of HA hydrogels that is suitable for heart valve scaffolds in terms of mechanical properties and biological activities.

4. Other Natural Hydrogels—Gelatin gels have often been used as the basis for 3D tissue scaffolds and drug delivery systems, either alone or together with other materials.[148,149,150] Gelatin is derived by partial hydrolysis of collagen, which can form a solution in water above the melting point (~35°C) and set to a gel upon cooling. Methacrylated gelatin (GelMA) has most commonly been synthesized by reaction of methacrylic anhydride with a gelatin solution, and can be further crosslinked by UV light to form a hydrogel. GelMA hydrogels can be micro-patterned with varying geometrical features (~50–150 μm height) to guide 3D endothelial cord formation.[151] In another study, microgrooved GelMA hydrogels were fabricated to build muscle-like fibrous structures using cultured murine C2C12 myoblast cells.[148]

Similarly, porous hydrogels formed by photo crosslinking of GelMA have been employed for investigation of VIC function *in vitro*. VICs grown in these hydrogels exhibited key characteristics of their native morphology within 2 weeks of seeding. The cells achieved a more spread morphology when the hydrogels were treated with collagenase, indicating degradation of GelMA network. Moreover, the addition of TGF-β1 was found to increase VIC spreading and process extension, corresponding to greater cell activity.

Alginate-based hydrogels are also applied in a variety of biomedical applications, including cell encapsulation, drug delivery and tissue engineering.[152–155] Alginate (or alginic acid) is a linear anionic polysaccharide primarily derived from the cell walls of brown algae. Alginate is composed of (1-4)-linked repeating or alternating β-D-mannuronic acid (M units) and α-L-guluronic acid (G units) monomers.[156] The ratio between M units and G units affects the alginate hydrogel properties, such as swelling and viscoelasticity.[157] Alginate is not just extracted from algae, but can also be biosynthesized by two bacterial genera, *Pseudomonas* and *Azotobacter*, creating stable batches of alginate for biomedical applications. The anionic alginate molecules have high affinity for divalent cations, *i.e.* Ca^{2+}, resulting in the formation of soft hydrogels in the presence of these ions. The

mechanical properties of the alginate hydrogels are mainly determined by alginate MW and relative content of G units,[158] though covalent crosslinking can be used to stabilize and strengthen the hydrogels. In general, the mechanical properties of alginate hydrogels are tunable with a wide distribution of compression, shear and tensile moduli, however, like other hydrogels, they are orders of magnitude weaker than the natural ECM.[159] Under physiological conditions, alginate hydrogels are negatively charged and highly hydrophilic, making them resistant to protein absorption. *In vivo*, an immune response will not be triggered by alginate with greater than 50% G-content. In addition, alginate hydrogels are biodegradable. Although no enzyme recognizes natural alginates, *in vivo* degradation and depolymerization still occurs spontaneously via alkaline β-elimination at the glycosidic linkages of alginate molecules.

For tissue engineering applications, cells seeded inside the unmodified alginate scaffolds were found to persist in spherical shapes due to lack of bioactive recognition sites in alginate in its natural state. To address this deficiency, the hydroxyl and carboxyl groups along the alginate backbone can be easily modified to couple bioactive moieties, *e.g.* peptides. Alginate hydrogels coupled with the RGD sequence have been shown to enhance the attachment of a variety of cells.[160,161] A recent study employed computer-aided 3D printing to directly fabricate cell-seeded alginate/gelatin and its crosslinker calcium into a desired shape and structure, such as that of heart valves. The mechanical properties of the alginate hydrogel can be adjusted by modulating the alginate or calcium concentration.[149] This technique may allow the development of a biologically and mechanically functional alginate-based scaffold for HVTE.

Hydrogels prepared from chitosan have been employed for several biomedical applications such as drug delivery, gene therapy, wound dressing and tissue engineering.[162–167] Chitosan, a deacetylated derivative of chitin, is a linear polysaccharide that consists of randomly distributed β(1-4)-linked 2-acetamide-2-deoxy-b-D-glucopyranose and 2-amino-2-deoxy-b-D-glucopyranose residues, which assemble into a three dimensional α-helix stabilized by intramolecular hydrogen bonds.[168,169] This polysaccharide is extracted from the exoskeleton of crustaceans, usually shrimp and crab, through demineralization and deproteinization.[170] Chitosan is absent from the mammalian body, however, nonspecific lysosomal proteases can hydrolyze this molecule into non-toxic and antigenic fragments,[171] making it biodegradable in mammals. Further, the structural similarity of chitosan to mammalian GAGs makes chitosan a relatively biocompatible polymer.

Chitosan hydrogels can be prepared by non-covalent or covalent cross-linking strategies. Reversible hydrogels form by electrostatic, hydrophobic, and hydrogen bonding interactions among chitosan molecules. Covalent, irreversible crosslinking via various linking chemistries can be used to generate chitosan hydrogels with robust mechanical properties as well as controllable degradation rates and pore sizes.[162] A few attempts at applying chitosan for HVTE have been conducted, which mainly focused on the biocompatibility of chitosan as a heart valve scaffold and/or usage as mechanical reinforcement for other materials.[172] Experiments have shown that natural chitosan supports VEC growth and morphology better than the synthetic polymer polyhydroxyalkanoate (PHA), which is more commonly used for HVTE.[173] Additionally, 3T3 fibroblasts were observed to attach to chitosan hydrogel

surfaces with high viability and polygonal morphology.[171] However, it remains unclear whether purified chitosan is biologically suitable for VICs.

B. Synthetic Hydrogels

1. Poly(ethylene glycol) Hydrogels—Poly(ethylene glycol) (PEG) hydrogels are of great interest for tissue engineering applications because PEG is biocompatible and non-immunogenic and has been approved for internal use by the FDA.[174] The structure, mechanical behavior, and degradability of PEG hydrogels can be tuned by controlling chemistry and processing conditions.[175,176] Additionally, the biological functions of PEG hydrogels can be modified by incorporation of bioactive molecules.[177–179]

PEG is a hydrophilic molecule with either linear or branched structures (Fig. 5). The basic PEG structure is PEG diol with two hydroxyl end groups that can be replaced by other functional groups, such as methyloxyl, carboxyl, amine, thiol, azide, vinyl sulfone, azide, acetylene, and acrylate.[180] The most common method employed to prepare PEG hydrogels is photo crosslinking. To be photo crosslinkable, poly(ethylene glycol) diacrylate (PEGDA) is synthesized by reaction of PEG with acryloyl chloride. PEG hydrogels are then prepared by crosslinking a PEGDA solution using either UV light[71] or white light.[181] For UV light crosslinking, the photoinitiator 2-Hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959) is added to the PEGDA solution, whereas triethanolamine, Eosin Y, and N-vinyl pyrrolidone are mixed into the PEGDA solution for white light crosslinking.

The PEG hydrogels have tunable mechanical properties based on their fabrication methods; various reports of their mechanical behavior in tension and compression are summarized in Table 1.[182] Given that flexure represents a major mode of deformation of heart valve leaflets, a method to measure the bending properties of PEG hydrogels has also been developed.[11,25] Photodegradable PEG hydrogels exhibiting a wide range of elastic moduli, similar to various soft tissues, have been used to study the effect of substrate modulus on the activation of VICs.[64] This study found that high-modulus PEG hydrogels generally promoted VIC activation towards the myofibroblast phenotype whereas low-modulus hydrogels suppressed such activation. The results of that work also showed that deactivation of myofibroblasts could be achieved solely by decreasing the modulus of the underlying substrate.

PEG hydrogels are bioinert primarily due to their non-adhesive characteristics. However, PEG hydrogels can be modified to be bioactive by incorporation of proteins,[177] peptides,[178] and/or polysaccharides[179] into the polymer network. As example, an MMP-degradable PEG hydrogel system that includes the enzyme-susceptible peptide KKCGGPQGIWGQCKK was developed.[183] The addition of fibronectin-derived pendant RGD into this hydrogel system was found to promote cell extension and migration of encapsulated VICs. These results demonstrated that PEG hydrogels can be readily modified for the culture and characterization of VICs.

More recently, micro-structured PEG hydrogels have been developed for use in HVTE. Trilayer hydrogel quasilaminates containing two “stiff” outer layers and one “soft” middle layer were fabricated to mimic the structure of aortic valve leaflets using PEG hydrogels

(Fig. 6).[11] It was demonstrated that these scaffolds did not fail at the interface (delaminate) during tensile or bending tests, thus demonstrating the potential for formation of integrated layers with different mechanical properties such as the layers in heart valve leaflets. In another study, anatomical heterogeneous valve conduits were produced by 3D printing of PEG hydrogels with controlled photo crosslinking (Fig. 7); VICs cultured in these conduits showed high viability after 21 days.[77] This study demonstrated that complex geometries mimicking native and axisymmetric aortic valve anatomy could be accurately reproduced by 3D extrusion printing and curing of PEG hydrogels.

2. Poly(vinyl alcohol)—Poly(vinyl alcohol) (PVA) is an attractive polymer scaffold for tissue engineering applications due to its large availability, good biocompatibility and biodegradation, and absence of toxicity.[184–186] Due to the instability of the monomer vinyl alcohol, PVA is generally produced by hydrolysis of poly(vinyl acetate), which is formed by polymerization of vinyl acetate. Different methods have been explored for fabrication of PVA hydrogels. First, PVA hydrogels can be formed through crosslinking using different chemical agents, such as glutaraldehyde, acetaldehyde and formaldehyde. Second, electron beam and γ-irradiation have been used for chemical crosslinking of PVA to avoid elution of toxic crosslinking agents. The physical properties of the resulting PVA hydrogels were found to be influenced by the γ-irradiation dosage.[187] However, crosslinking by γ-irradiation can result in bubble formation.

The third PVA hydrogel fabrication mechanism is physical crosslinking as a result of crystallite formation. Crystallite formation was found after the repeated freezing and thawing of PVA solutions,[188] therefore, this technique has been adapted for the preparation of PVA hydrogels.[189] In addition, PVA physical hydrogels can also be assembled via a “salting-out” technique, which is a non cryogenic method developed for micro- and nano-scale material design (Fig. 8).[190] Thus, PVA materials can be prepared in a manner that allows nano- to micro- to macro- translation of control.[184] The resulting PVA-based hydrogels have been widely used for biomedical applications due to their good mechanical properties and high level of biocompatibility.[184,191–193]

PVA hydrogels can be further modified by incorporation of other molecules to improve cell interactions. For example, by covalently attaching fibronectin onto the PVA hydrogel surface, attachment and proliferation of NIH 3T3 fibroblast cells was found to be significantly improved compared to pure PVA hydrogels.[185] A photo crosslinkable and degradable hydrogel scaffold prepared from poly(lactic acid)-g-PVA multifunctional macromers was also developed for HVTE.[186] The rate of mass loss from this degradable scaffold could be controlled by modulation of the composition of the macromers, their solution concentration and other factors; it was found that the adhesion of VICs was improved by increasing the network’s hydrophobicity. Finally, PVA hydrogel materials has been fashioned into a novel one-piece tricuspid valve by integrating the PVA hydrogel into a stent and sewing ring.[26] These prototype heart valves were formed in a mold from a PVA solution through processing with freeze/thaw cycles. The design criteria of these valves included minimization of the central orifice to prevent regurgitation, minimizing curvature of the free edge, and promoting circumferential expansion of the stent. This PVA heart valve prototype was demonstrated to open and close successfully.

C. Composite Materials

As discussed above, the two major concerns for HVTE scaffold design are mechanical properties and bioactivity. In general, natural hydrogels mainly comprised of ECM components show adequate bioactivity and can provide a proper microenvironment for cell-matrix interactions, but demonstrate poor mechanical strength. Synthetic hydrogels, on the other hand, have tunable mechanical properties but their bioactivity must be adjusted for HVTE applications. Composite hydrogels, which can take advantage of the versatile functions of their components, have thus been developed in attempts to meet the requisite needs for HVTE mechanical properties and bioactivity.

A previous study showed that composite scaffolds for HVTE can be prepared from collagen-elastin or collagen-GAG mixtures.[27] The addition of other biological materials, elastin or GAGs (namely chondroitin sulfate), into collagen gels modified the microstructures of these scaffolds such that they permitted oxygen and nutrient diffusion and preserved the basic bioactivity to promote cell adhesion and proliferation. As a result, these composite scaffolds showed enhanced ECM synthesis and formation of an endothelial cell monolayer, indicating a strong VEC-VIC interaction in driving matrix remodeling. In other examples, aortic valve conduits were fabricated by 3D printing of composite hydrogels.[76,149] As shown in Fig. 9, gelatin/alginate composite scaffolds were printed based on the anatomical information collected from microCT imaging of aortic valve.[181] The significance of this approach was that it was possible to conduct a precise fabrication of tissues with multiple components, such as different cells and biomaterials.

In another study, a fibrin gel was coated on knitted polycaprolactone (PCL) to generate a composite scaffold that provided additional bioactivity for HVTE applications.[194] The knitted PCL was designed to exhibit the requisite mechanical strength for the scaffold. This fibrin-PCL composite remained intact even after 10 million loading cycles *in vitro* and also showed a good performance under conditions of simulated physiological flow. A concern for the future application of this fibrin-PCL material centers on whether fibrin and PCL are capable of synchronous degradation and remodeling. Fibrin may detach from the pores of the PCL mesh, resulting in leakage and *in vivo* pressure drop.

IV. BIOREACTORS

The dynamic mechanical environment in which native heart valves exist influences the behavior of valve cells as they experience shear, compressive, and tensile strains throughout the cardiac cycle. Engineering a living replacement valve must include consideration of the biological responses to these various forces, including their influence on cell proliferation, migration, and extracellular matrix remodeling. Bioreactors are an important tool for HVTE as they can provide control over the mechanical stimuli for cells and scaffolds during the initial development of the engineered valves.

A. Bioreactor Design Principles and Categories

A variety of bioreactors have been used in the HVTE field to seed cells onto scaffolds, apply mechanical stimulation to tissue engineered constructs, and evaluate the performance of potential scaffold materials. These bioreactors are designed to fit inside standard tissue

culture incubators to enable sterile conditions, physiological temperature control, and gas exchange. Bioreactors have been used primarily for non-hydrogel polymer tissue engineered heart valves, but the same principles apply no matter which scaffold material is used.

The most common type of bioreactor reported employs pulsatility to produce fluid flow and pressure gradients similar to what the native adult heart valve would experience. The motivation behind these types of bioreactors is that physiological flow conditions will stimulate embedded cells to remodel the scaffold into engineered heart valves that resemble native tissues. The earliest design was developed by Hoerstrup et al.[195] in order to grow heart valves from cells seeded on polymer scaffolds. This bioreactor was used both to seed cells onto polymer scaffolds and to condition engineered valves for up to 4 weeks before they were implanted into sheep.[85] Other researchers have also described variations of bioreactors that supply physiologically similar flow rates and pressures to full size, tri-leaflet tissue engineered heart valves.[196–200] An example of a full scale bioreactor is shown in Fig. 10A, B.

While these physiological designs target a preclinical path for tissue engineered heart valve development, simplified mechanical conditioning bioreactors have also been designed to help understand how cells respond to the forces placed on them, as mediated through the scaffold design. The full scale, physiological bioreactor designs have less control over—and difficult quantification of—the strain field applied to the leaflet sections. Simplified bioreactor designs can reduce the uncertainty of the local stress and strain applied to scaffolds and thus provide more exact information about how valve cells respond to mechanical signals. One type of simplified bioreactor used for HVTE is a parallel plate flow chamber to apply shear stress to cells. The effects of shear stress on valve leaflets has been investigated with both constant and pulsatile flow rate.[201] Other bioreactors have applied cyclic tensile stress[18] or flexure[202] to potential tissue engineered heart valve scaffolds. Finally, the combination of flexure with shear stress from fluid flow was obtained in a specially designed flow-stretch-flow (FSF) bioreactor.[203] While only a few bioreactors have been used with hydrogel based scaffolds for HVTE, they offer well defined mechanical stimulation in systems that are better suited for hydrogel scaffolds than the full scale physiological designs.

B. Performance of Hydrogel Scaffolds in Bioreactors

Hydrogels are typically much weaker than other scaffold materials and therefore have not been subjected to bioreactor evaluation as frequently as valves made with thermoplastic polymers, which can easily be fabricated into the valve leaflet shape. The main limitation of pursuing hydrogels as scaffolds for HVTE is their lack of mechanical strength. However, it has been hypothesized that conditioning cell-seeded scaffolds in a bioreactor will stimulate cells to synthesize their own extracellular matrix, thereby strengthening the scaffold so that it can be implanted *in vivo*. A limited number of studies have pursued hydrogel scaffolds to this end. A fibrin based tissue engineered heart valve was reported[22], which was seeded with cells and shaped into a valve through an injection molding process. The fibrin valves were placed in a bioreactor and conditioned for 12 days at a low pulse rate and low pressure difference across the valve, reportedly to mimic fetal development conditions. After

conditioning, the valves were evaluated by immunohistochemical analysis for extracellular matrix proteins and compared to statically conditioned controls. The results showed that the mechanically conditioned valves exhibited healthy and active cell phenotype with aligned ECM synthesis, whereas the control valves had rounded, inactive cells without ECM synthesis. Type I and type III collagen were abundantly expressed in the conditioned valves along with laminin and fibronectin, demonstrating extensive remodeling of the conditioned valve. These proteins were all absent from controls. Later work by the same lab furthered this research by implanting conditioned fibrin valves into an ovine model.[204] The fibrin valves were seeded with fibroblasts and smooth muscle cells harvested from the carotid artery and were conditioned for 28 days in the same bioreactor and conditions used for the *in vitro* study. The valves were surface seeded with autologous endothelial cells and then implanted in series within the pulmonary valves of sheep for 3 months. Explant analysis again revealed positive ECM remodeling due to the mechanical stimulation applied to the cell-seeded scaffold.

Other research into the effects of dynamic culture on fibrin-based scaffolds for HVTE subjected the cell-seeded scaffolds to controlled cyclic stretching, rather than using a physiological bioreactor approach. In one study, tubular fibrin constructs seeded with porcine VICs were subjected to cyclic distension to produce defined amounts of strain to the scaffolds.[205] This study compared constant mechanical strain to increasing strain over time for up to 3 weeks. The results showed that the mature collagen content and therefore the mechanical properties of the tubular fibrin constructs increased with mechanical stimulation, and that increasing the amount of strain over time yielded stronger tissue than did the application of constant strain. The research was later expanded to apply cyclic stretch to fibrin-based molded tissue engineered heart valves with the bioreactor depicted in Fig. 10C.[79] The application of increasing strain to the scaffold over 3 weeks led to not only increased tissue strength over controls, but also resulted in circumferentially oriented collagen fibers. The aligned collagen fibers are promising as they caused the tissue to have anisotropic material properties similar to native pulmonary valves.

Although fibrin is the only type of hydrogel scaffold to be used to create full scale valve shaped constructs, collagen gels have also been investigated as a potential hydrogel scaffold material for HVTE. A small number of studies have examined the response of valve cells to mechanically stimulated collagen gels. It was found that application of cyclic biaxial strain to valve cells grown in collagen gels increased the synthesis of GAGs compared to static culture.[206] The results demonstrated that cyclic biaxial strain could be applied anisotropically to cell seeded collagen gels to influence cell orientation and extracellular matrix remodeling alignment.[10] A diagram of this stretching bioreactor is shown in Fig. 10D. Taken together, these studies reinforce the notion that mechanical stimulation has an important impact on valve cell behavior, especially matrix synthesis and remodeling, while also demonstrating the utility of hydrogels to study these effects.

Despite these studies in fibrin and collagen gels, mechanical conditioning of synthetic polymer hydrogel scaffolds for HVTE has been absent from published literature. However, mechanical conditioning of synthetic hydrogels for other applications has been reported. In one example, cyclic compressive strain was applied to stem cells encapsulated in PEGDA

hydrogels leading to chondrogenesis.[207] Valve cell behavior in synthetic hydrogels scaffolds under static conditions has been extensively studied, but future work will need to include the effect of mechanical stimulation in order to fully evaluate the utility of synthetic hydrogels for HVTE.

V. *IN VIVO* EVALUATION

In vitro cell culture and conditioning in dynamic bioreactors provide important information about mechanical and biological properties of tissue engineered heart valves, which can be used to optimize the product design for further *in vivo* tests. A variety of animal models have been used to evaluate the *in vivo* performance of tissue engineered heart valves prepared from decellularized valve grafts as well as those constructed from natural and synthetic polymers (including hydrogels).

With respect to hydrogels for HVTE, pilot *in vivo* studies were performed to evaluate the structure and mechanical strength of fibrin gels seeded with ovine arterial-derived cells after implantation in an ovine pulmonary trunk model.[204] These fibrin-based tissue engineered heart valves maintained sufficient mechanical durability after implantation for 3 months and showed obvious tissue remodeling. Compared with the reported *in vivo* performance of synthetic polymer scaffolds, such as poly(lactic-co-glycolic acid) (PLGA) woven meshes with non-woven poly(glycolic acid) (PGA),[208,209] and non-woven PGA scaffolds coated with poly-4-hydroxybutyrate (P4HB),[85,210] the fibrin-based tissue engineered heart valves showed enhanced bioactivity and tissue remodeling.

Decellularization can be used to obtain cell-free heart valve grafts with preservation of the native ECM components and structures, which are desirable characteristics for HVTE scaffolds. Previous studies have shown degradation of decellularized porcine aortic valve leaflets[14] or conduits[211] and evidence of tissue remodeling after implantation. However, a unique combination of decellularized tissue and hydrogels has shown improved performance *in vivo*. Decellularized porcine aortic valves filled with PEG hydrogels (seeded with goat bone marrow mesenchymal stem cells) were implanted in a goat abdominal aorta model;[212] the addition of the PEG hydrogels improved the integration of seeded cells to the decellularized scaffold compared to those without PEG hydrogels.

VI. SUMMARY AND FUTURE PERSPECTIVES

Heart valve tissue engineering is garnering increasing interest, especially considering the limitation of current devices for heart valve replacement. Key factors for current HVTE strategies include scaffold materials, cell sources, fabrication methods, and pre-clinical evaluation. In this review, we focused on applications of hydrogels for HVTE, and discussed the factors involved in these applications.

A number of hydrogel scaffolds have been developed for HVTE, including natural, synthetic and composite hydrogels. As mentioned previously, the scaffolds for HVTE should recapitulate the mechanical properties and biological functions of heart valves—a task that is difficult to obtain with a single material. As such, composite materials that can fulfill both mechanical and biological requisites for HVTE would be ideal for this purpose. Specifically,

composite hydrogels reinforced with biocompatible fibers to enhance durability, and incorporated with bioactive proteins/peptides/polysaccharides to elicit specific biological functions, may be suitable scaffolds for future HVTE.

Different cells, including fibroblast cells, endothelial cells, valvular interstitial cells, stem cells (*e.g.*, bone marrow mesenchymal stem cells) and other progenitor cells (*e.g.*, progenitor cells from the peripheral blood or from the amniotic fluid) have been used for HVTE. Autologous valvular cells would be the best choice for future clinical applications. However, due to the limited supply of these cells, application of stem cells or progenitor cells for HVTE is still of great interest.

Various fabrication techniques have been developed for HVTE, including molding, bioprinting, decellularization, and recellularization. In some studies, molds were fabricated from Teflon or polyoxymethylene and used for casting hydrogels to form valve shapes. 3D bioprinting of different tissues and organs from cells was attempted through a computer-aided fabrication method used to create heterogeneous aortic valve hydrogel scaffolds. Molding and bioprinting represent direct and fast fabrication processes. However, most hydrogels utilized in these strategies have poor mechanical durability. Thus, novel composite hydrogels with requisite mechanical strength may be applied to these two methods for future HVTE. Decellularized heart valve grafts with preserved ECM components and structures were demonstrated to have mechanical properties close to native tissues, but filling these decellularized scaffolds with a hydrogel, *e.g.* PEG, allowed greater cell persistence, fulfilling both mechanical durability and biological functions of native heart valves.

Evaluation of engineered heart valves has been conducted using a number of dynamic bioreactors (*in vitro*) and animal models (*in vivo*). Due to the poor durability of most hydrogel scaffolds used for HVTE, these studies were performed in a relatively short period. Future tissue engineered heart valves should be designed for long-term evaluation in bioreactors and animal models as pre-clinical evidence.

In summary, a variety of hydrogels have been fabricated to engineer artificial heart valves with or without cells by different advanced techniques. Many hydrogel systems were also developed as models to investigate valve cell biology. Overall, the development of novel tissue engineered heart valves that can meet the challenge of requisite mechanical behavior and biological functionality for future clinical applications is still ongoing.

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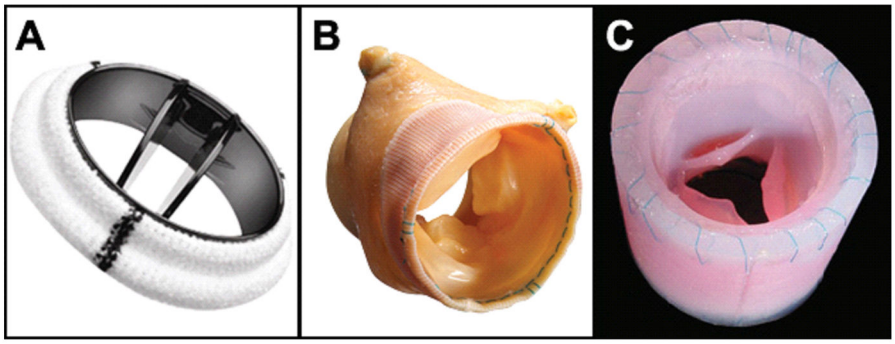

FIGURE 1.

Examples of heart valve prostheses: (A) mechanical heart valve (Medtronic Open Pivot AP360®), (B) biological heart valve (Freestyle® Aortic Root Bioprosthesis), (C) living, fibrin-based tissue engineered tri-leaflet heart valve. Reproduced with permission from Flanagen et al.[22]

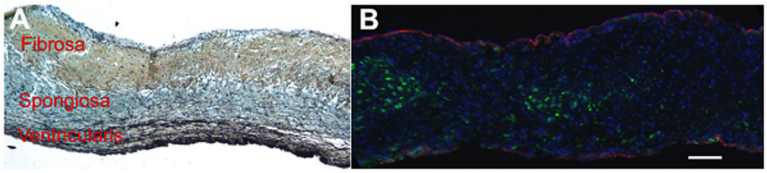

FIGURE 2.

(A) Movat's pentachrome stain (collagen = yellow, PG/GAG = blue, elastin = black, cell nuclei = purple) of heart valve leaflets. The separate layers of the leaflet can be distinguished by the matrix stain in Movat's: the fibrosa is mostly yellow from collagen; the spongiosa is blue with GAGs/PGs; and the ventricularis contains fine black elastic fibers. Reproduced with permission from Tseng et al.[29] (B) Immunohistochemical staining of a section of aortic valve leaflet with CD31 (red), α-SMA (green) and DAPI (blue) to show the VECs line on the surfaces of the leaflet while VICs resident deep to the surfaces and through all layers. Scale bar = 200 μm.

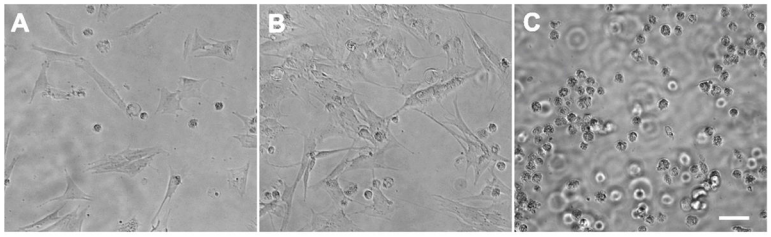

FIGURE 3.

VICs cultured (A) 2D on top of PEGylated collagen gel (0.15% w/v), (B) 3D in PEGylated collagen gel (0.15% w/v), and (C) 3D in PEG hydrogels (5% w/v), for 1 day. Cell density for 2D culture is ~10^4cells/cm^2. Cell density for 3D culture is~10^6 cells/ml. Scale bar = 50 μm.

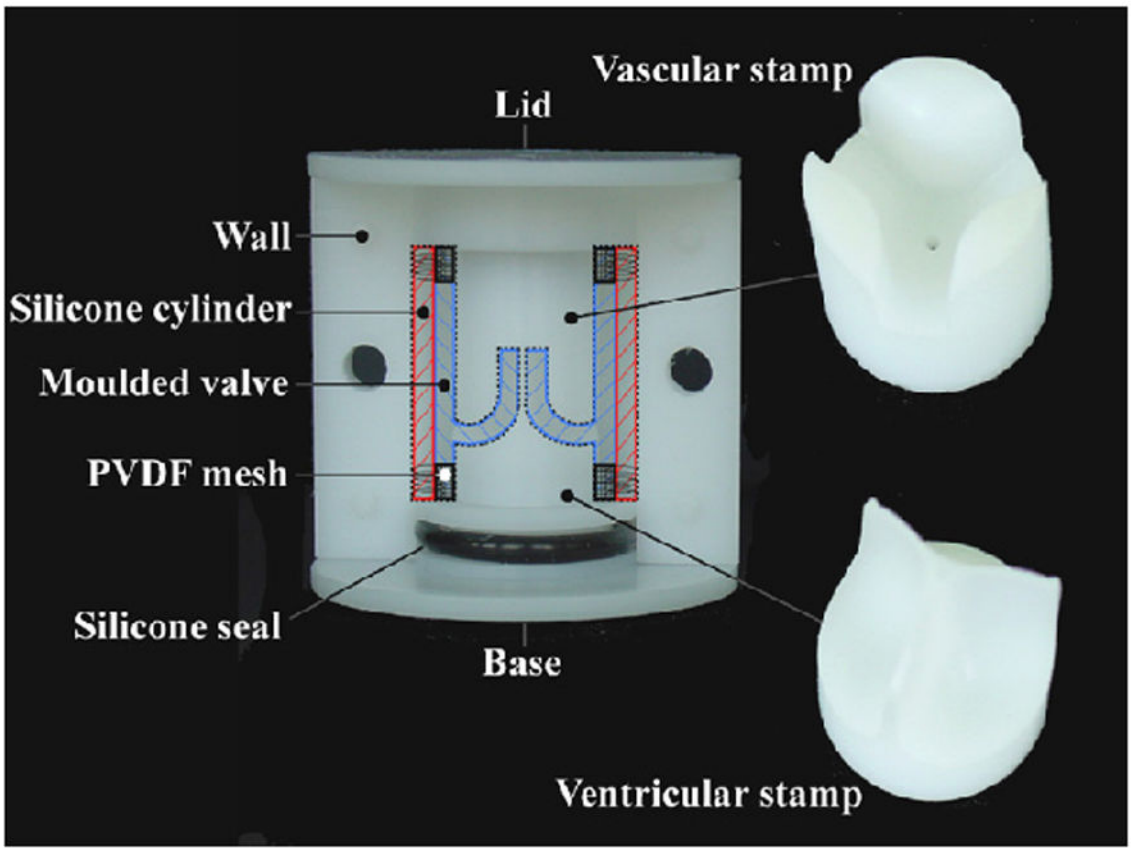

FIGURE 4.

This valve mold consists of vascular and ventricular stamps machined from polyoxymethylene (POM), which are positioned against each other in a customized housing (also POM), consisting of two wall components, a lid and a base. The inner surface of the mold housing consists of a silicone cylinder. A polyvinylidene fluoride (PVDF) support mesh is sutured to each end of the silicone cylinder in order to prevent longitudinal compaction of the molded fibrin gel conduit. Reproduced with permission from Flanagan et al.[22]

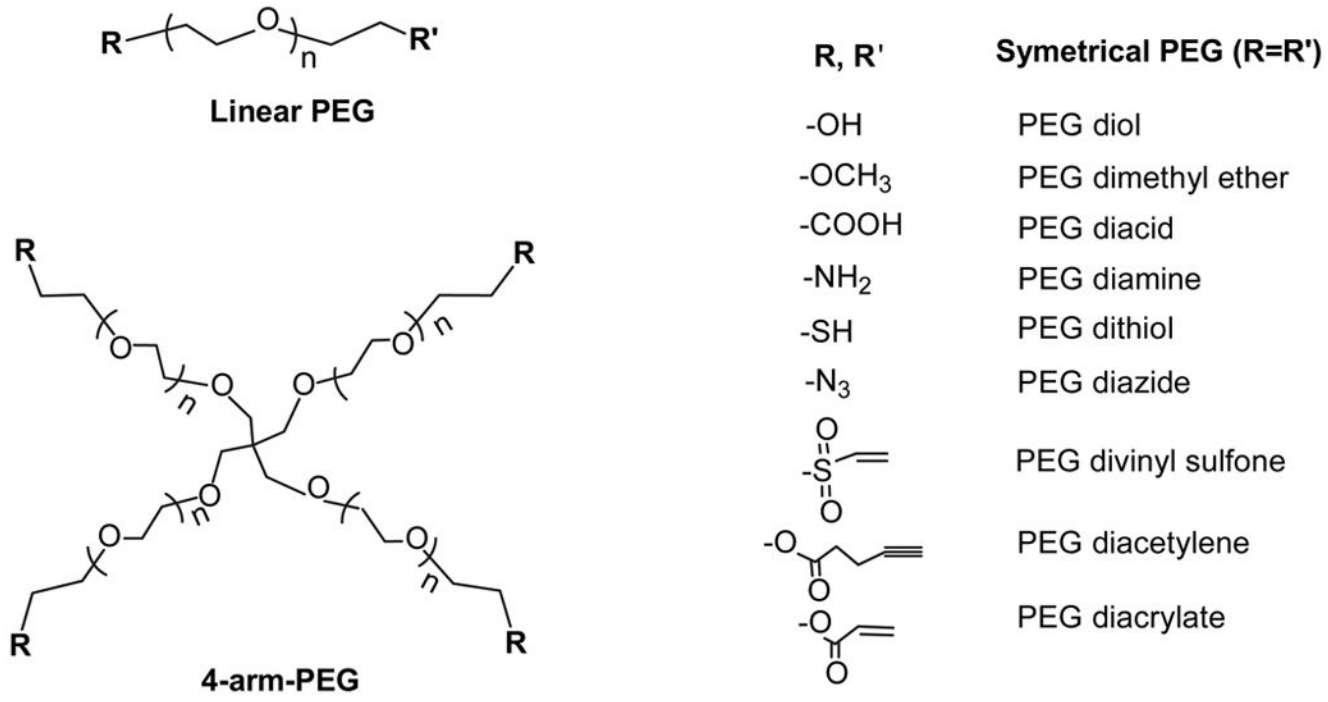

FIGURE 5.
Structures of the PEG family of molecules. Reproduced with permission from Zhu.[180]

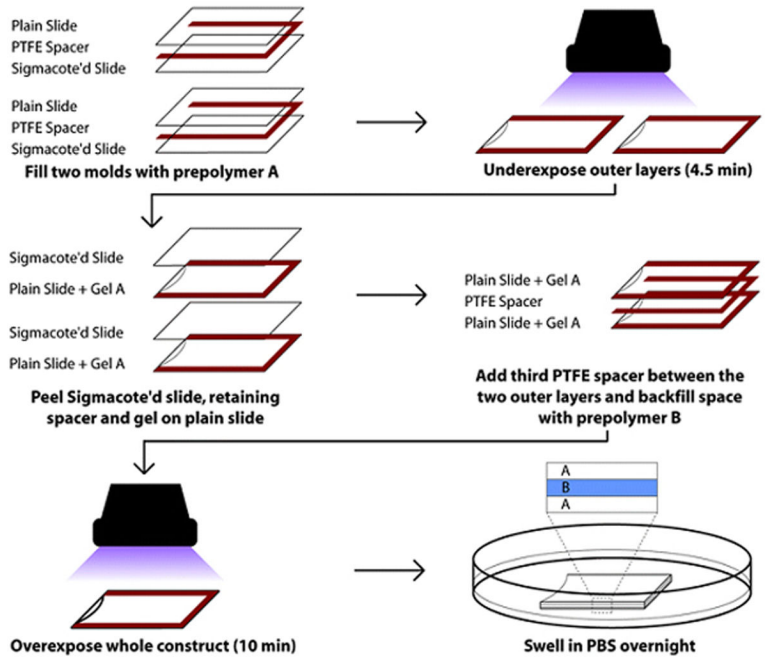

FIGURE 6.

Schematic depicting the fabrication of trilayer quasilaminates with an A-B-A composition. Gel A is 12.5% 3.4 kDa PEGDA, gel B is 10% 6 kDa PEGDA. This fabrication technique can be used to generate scaffolds with different stiffnesses and cellularity in each layer. Reproduced with permission from Tseng et al.[11]

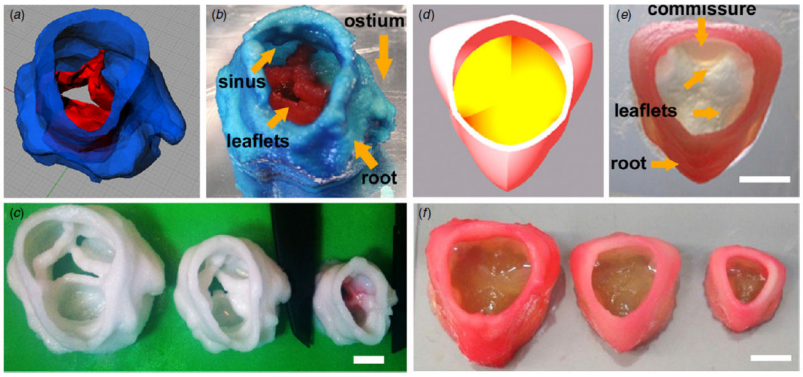

FIGURE 7.

Printing heterogeneous valve and scaled valve constructs. (a) Porcine aortic valve rendered model was (b) printed, where root was formed with 700 MW PEG-DA hydrogel and the leaflets were formed with 700/8000 MW PEG-DA hydrogels. Key features such as the coronary ostium and sinuses were present (c) Scaffolds were printed with 700 MW PEG-DA at different scale for fidelity analysis, where the inner diameters (ID) were 22, 17, and 12 mm. (d) Axisymmetric valve model was (e) printed with two blends of hydrogels (f) and at 22, 17, and 12 mm ID. Scale bar = 1 cm. Reproduced with permission from Hockaday et al.[77]

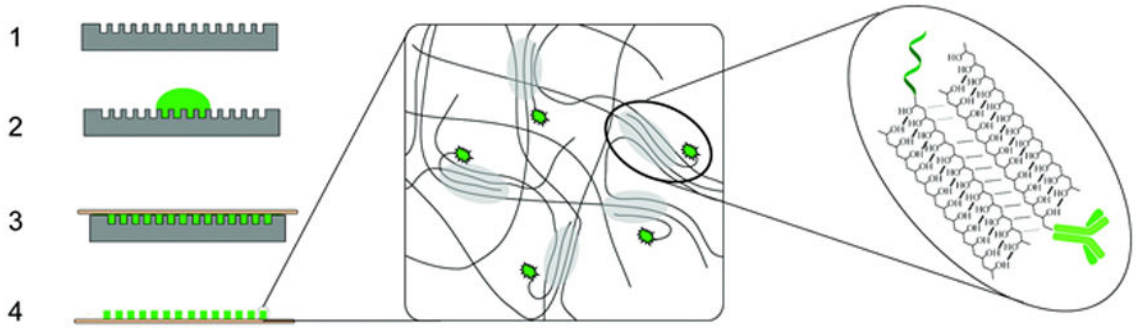

FIGURE 8.

Schematic illustration of microtransfer molding technique employed to produce microstructured PVA hydrogels (left) and illustration of internal organization and structure of PVA hydrogels on a macromolecular (right) and supramolecular (middle) levels. Reproduced with permission from Jensen et al.[190]

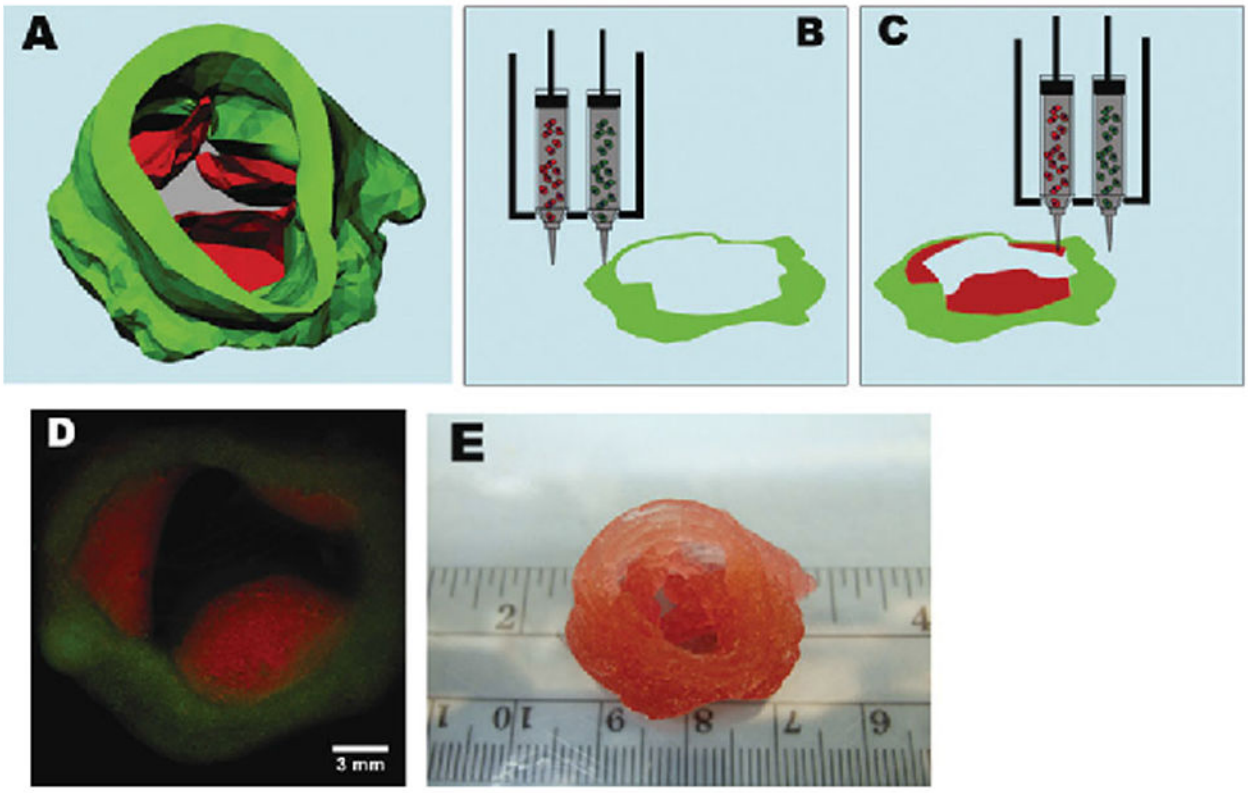

FIGURE 9.

Bioprinting of aortic valve conduit. (A) Aortic valve model reconstructed from micro-CT images. The root and leaflet regions were identified with intensity thresholds and rendered separately into 3D geometries into STL format (green color indicates valve root and red color indicates valve leaflets); (B, C) schematic illustration of the bioprinting process with dual cell types and dual syringes; (B) root region of first layer generated by hydrogel with SMC; (C) leaflet region of first layer generated by hydrogel with VIC; (D) fluorescent image of first printed two layers of aortic valve conduit; SMC for valve root were labeled by cell tracker green and VIC for valve leaflet were labeled by cell tracker red. (E) printed aortic valve conduit. Reproduced with permission from Duan et al.[149]

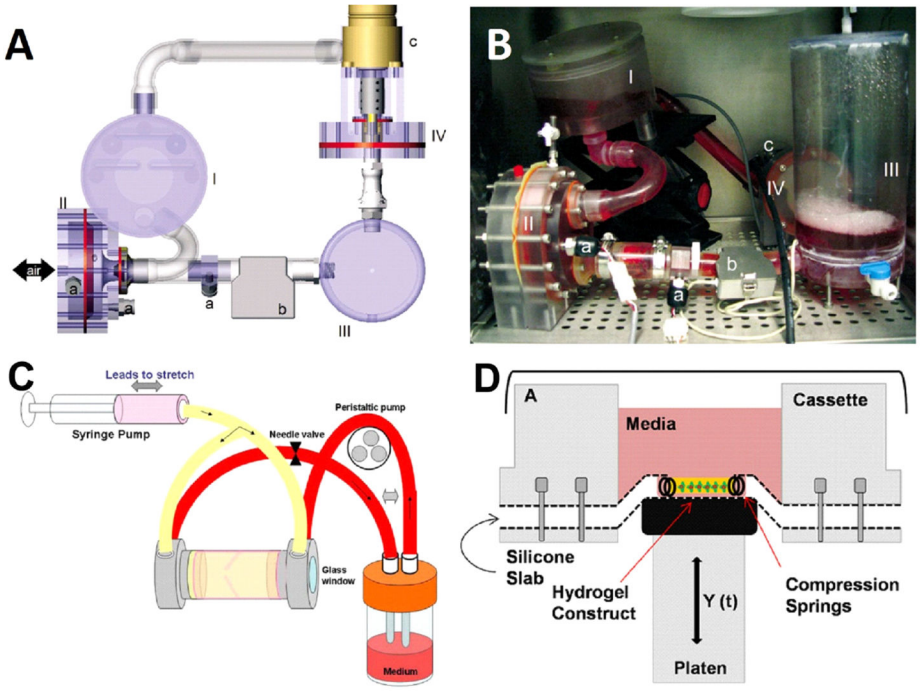

FIGURE 10.

(A, B) Example of a full scale, pulsatile bioreactor for heart valve culture. Top view diagram (A) and pictured operationally inside incubator (B). Different parts of the bioreactor are labeled (I) atrium; (II) ventricle with air/liquid diaphragm to provide pulsatile flow; (III) compliance chamber; (IV) variable resistor. Valve is located in test section between pressure sensors (a). Reproduced with permission from Hildebrand et al.[199] (C) Diagram of cyclic stretching bioreactor for fibrin based tissue engineered heart valves. Syringe pump is cycled to pressurize chamber and stretch valve tissue. Peristaltic pump provides pulsatile media flow through valve. Reproduced with permission from Syedain & Tranquillo.[79] (D) Diagram of stretch bioreactor used for studying response of VICs to cyclic tensile strain. Cyclic strain applied by moving platen up and down, causing hydrogel sample to be pulled in tension by silicone slabs. Reproduced with permission from Gould et al. 2012.[10]

TABLE 1

Mechanical properties of PEG-based hydrogels in compression and tension. Reproduced with permission from Elsevier, Copyright 2012.[182]

PEG formulation	Concentration [%]	Molecular weight (Da)	Methods	Mechanical property determined	Results
PEGDM	10	3000	15% unconfined compression at constant rate of 0.2; oscillatory compression of 15%, 1 Hz	Tangent modulus, maximum stress from dynamic compression	0.06 MPa, 0.0084 MPa
	20	3000			0.67 MPa, 0.12 MPa
O-PEG-F (OPF)	75	1000	Constant extension at 10 (lk and 4k) or 25 mm/min (10k) until failure	Tensile modulus, stress and strain at fracture	0.09 MPa, 0.025 MPa, 0.31
	75	4000			0.023 MPa, 0.013 MPa, 0.52
	75	10,000			0.016 MPa, 0.013 MPa, 0.77
PEG-DA and $PDMS_{star}$	10	3400	Constant extension at 1 mm/min	Tensile modulus (E)	0.09 MPa
	10	6000			0.07 MPa
PEG-DM	10	3400	Unconfined compression at constant rate of 40 mN/min	Compressive modulus	0.034 MPa
	20	3400			0.36 MPa
	30	3400			0.94 MPa
	40	3400			1.37 MPa
PEGDA	20	3000	Constant extension at 0.15/min until failure	Quasi-static modulus, ultimate stress and strain	0.4 MPa, 0.2 MPa, 0.37
	65	508			22 MPa, 2.2 MPa, 0.12
	80	508			27 MPa, 1.9 MPa, 0.1
PEG-DM and PEG-LA	10	3000	Unconfined compression at a constant rate of 40–100 mN/min	Compressive modulus	0.06 MPa
	15	3000			0.17 MPa
	20	3000			0.49 MPa
PEG-DA	15	2000	Unconfined compression at constant rate of 0.0005 mm/s until failure	Shear modulus, Young's modulus, stress and strain at failure	0.01 MPa (G), 0.036 MPa (E), 0.36MPa, 0.71
PEG-b-PLA	25	4600	Unconfined compression at constant rate of 400 mN/min	Compressive modulus	0.25 MPa
	50	4600			0.7 MPa
	70	4600			0.9 MPa

PEG formulation	Concentration [%]	Molecular weight (Da)	Methods	Mechanical property determined	Results
PEG-DM	10	4600	Equilibrium unconfined compression at 5–20%; oscillatory compression of 1%, 0.01–10 Hz at 10% offset	Equilibrium compressive modulus and storage modulus	0.05 MPa, 0.04–0.05 MPa
	15	4600			0.19 MPa, 0.15–0.19 MPa
	20	4600			0.27 MPa, 0.25–0.55 MPa

PEG-diacrylate (PEG-DA), PEG-dimethacrylate (PEG-DM), oligo-(PEG)-fumarate (O-PEG-F), PEG poly (lactic acid) (PEG-b-PLA), PEG-urethane dimethacrylate (PEG-UDM).