Gustav Steinhoff *Editor*

Regenerative Medicine - from Protocol to Patient

3. Tissue Engineering, Biomaterials and Nanotechnology

Third Edition

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Foreword: Regenerative Medicine: From Protocol to Patient

Third Edition

The vision to unravel and develop biological healing mechanisms based on evolving molecular and cellular technologies has led to a worldwide scientific endeavour to establish *regenerative medicine*. This field involves interdisciplinary basic and (pre)clinical research and development on the repair, replacement, regrowth or regeneration of cells, tissues or organs in congenital or acquired diseases. Stem cell science and regenerative biology is prompting the most fascinating and controversial medical development of the twenty-first century. It can be envisaged that this development will establish completely new molecular and cellular techniques for medical diagnosis and therapy. The early rush of scientific development was initiated more than one hundred years ago by the physiology of blood regeneration (Hall and Eubanks 1896) and successful vascular surgical techniques for organ transplantation (Carrel and Guthrie 1905). However, the clinical realization of allogenic blood transfusion lasted until the discovery of the blood group antigens (Landsteiner and Levine 1928) and successful routine allogenic organ and bone marrow transplantation towards the end of the last century.

Similar to the field of allogenic cell and organ transplantation, it seems that *regenerative medicine* again condenses mankind's visions, hopes and fears regarding medicine: Hopes of eternal life and effective treatment of incurable disease, as well as fears of the misuse of technology and uncontrolled modifications of life are polarizing the scientific field. The development and public acceptance of new ethical and regulatory guidelines is a necessary process to support further clinical development. Nevertheless, the vision of a new medicine using the regenerative power of biology to treat disease and restructure the organism is setting the aims for scientific, technological and medical development. Viewing the great expectations to restructure and regenerate tissues, organs or even organisms, the current attempts of both scientists and physicians are still in an early phase of development.

The field of *regenerative medicine* has developed rapidly over the last 20 years with the advent of molecular and cellular techniques. This collection of volumes on *Regenerative Medicine: From Protocol to Patient* aims to explain the scientific knowledge and emerging technology, as well as the clinical application in different organ systems and diseases. The international leading experts from four continents describe the latest scientific and clinical knowledge in the field of *regenerative medicine*. The process of translating the science of laboratory protocols into therapies is explained in sections on basic science, technology development and clinical translation including regulatory, ethical and industrial issues.

This collection is organized into five volumes: (1) *Biology of Tissue Regeneration*; (2) *Stem Cell Science and Technology*, (3) *Tissue Engineering, Biomaterials and Nanotechnology*, (4) *Regenerative Therapies I.;* and (5) *Regenerative Therapies II. Biology of Tissue Regeneration (Volume 1)* focuses on regenerative biology with chapters on the extracellular matrix, asymmetric stem cell division, stem cell niche regulation, (epi)genetics, immune signalling, and regenerative biology in organ systems and model species such as axolotl and zebrafish.

Stem Cell Science and Technology (Volume 2) provides an overview of the classification of stem cells and describes techniques for their derivation, programming and culture. Basic properties of differentiation states, as well as their function are illustrated, and areas of stem cell pathologies in cancer and therapeutic applications for these cells are discussed with the emphasis on their possible use in *regenerative medicine*.

Tissue Engineering, Biomaterials and Nanotechnology (Volume 3) focuses on the development of technologies, which enable an efficient transfer of therapeutic genes and drugs exclusively to target cells and potential bioactive materials for clinical use. The principles of tissue engineering, vector technology, multifunctionalized nanoparticles and nanostructured biomaterials are described with regards to the technological development of new clinical cell technologies. Imaging and targeting technologies, as well as the biological aspects of tissue and organ engineering are described.

Regenerative Therapies I (Volume 4) gives a survey of the history of regenerative medicine and clinical translation including regulation, ethics and preclinical development. Clinical state-of-the-art, disease-specific approaches of new therapies, application technologies, clinical achievements and limitations are described for the central nervous system, head and respiratory systems. Finally, *Regenerative Therapies II (Volume 5)* contains state-of-the-art knowledge and clinical translation of regenerative medicine in the cardiovascular, visceral and musculoskeletal systems.

These volumes aim to provide the student, the researcher, the healthcare professional, the physician and the patient with a complete account of the current scientific basis, therapeutical protocols, clinical translation and practised therapies in *regenerative medicine*. On behalf of the sincere commitment of the international experts, we hope to increase your knowledge, understanding, interest and support by reading the book.

After the successful introduction of the first edition in 2011, this publication has been developed and expanded for the third edition into five volumes.

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Chapter 1
Novel Concepts in Design and Fabrication of 'Living' Bioprosthetic Heart Valves: From Cell Mechanosensing to Advanced Tissue Engineering Applications

Maurizio Pesce and Rosaria Santoro

Abstract Despite the use of bio-valve prostheses to replace diseased heart valves dates more than 50 years ago, and the large and increasing need of this type of implants for heart surgery worldwide, definitive solutions to manufacture 'lifetime-long' valve replacements are not yet available. In fact, although various problems in the manufacturing process of these implants have been circumvented compared with the beginnings, these solutions have not yet led to a full biological compatibility in the human system due to long term inflammation, calcification and ultimately structural valve deterioration. Importantly, the more limited duration of the valve bio-prostheses occur in pediatric patients and adults under the age of 65. These are the patients who more often need prosthesis replacement and therefore new invasive surgical interventions with a compromised quality of life.

The present contribution is centred onto the dissection of the valve cells response to mechanical stimuli regulated by the extracellular matrix, and new engineering systems that have been set up to mimic the tissue mechanics in the heart valve leaflets and manufacture the 'living bioprosthetic' valves. This latter goal is being pursued intensely worldwide by exploiting the most advanced technologies in material science and scaffolds design.

Keywords Valve bioimplant • Tissue engineering • Valve interstitial cell • Mechanosensing

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1.1 Introduction – The Current Limitations in Biological/ Bio-Prosthetic Valve Implants Design

Diseased and dysfunctional heart valves are routinely repaired or replaced by surgical interventions. If damage is too severe to enable valve repair, the native valve is replaced by a prosthetic valve. About 300,000 heart valve procedures are performed every year worldwide, and this number is expected to triple by 2050 consequently to the trend of the lifespan to increase. Two types of commercially available heart valve prostheses are used at present: mechanical or biological (David 2013; Kheradvar et al. 2015). Despite having excellent durability and a non-modifiable mechanical performance, the mechanical prostheses are prone to thromboembolic complications requiring lifelong anti-coagulation therapy. Biological valves undergo structural deterioration, and this is still the principal cause of prosthetic valve failure in the mid/long term, affecting significant portions of the patients populations, especially in the young (Forcillo et al. 2013).

The technology employed to produce the commercial bio-valve implants is based on tissues of animal origin. Pericardial membrane and valve leaflets, from bovine and porcine are the most commonly used. In order to increase mechanical resistance, the animal-derived tissues are normally treated with low concentration aldehydes (e.g. glutaraldehyde, GA). This generates covalent bonds between components of the extracellular matrix and prevents acute host immune rejection (Carpentier et al. 1969). Treatment with aldehydes has also major drawbacks concerning pericardial or valve tissue long-term durability. In fact, clinical data from long term follow up of patients receiving pericardium-made bio-prosthetic implants, have indicated severe structural valve deterioration (SVD) and calcification. SVD is primarily caused by a chronic inflammatory condition resulting from a non-complete detoxification of the fixative remnants from the xenograft tissue (Grabenwoger et al. 1996; Siddiqui et al. 2009), and/or by the failure of the fixation protocols to remove major xeno-antigens such as the 1, 3 α-Galactose (Konakci et al. 2005; Naso et al. 2012, 2013; Galili 2005; Hülsmann et al. 2012) (α-Gal). In addition, although it has been demonstrated that bioprosthetic valves are liable to undergo an *in vivo* recellularization process by recruitment of circulating cells (De Visscher et al. 2007), the clinically employed biological implants are not designed to contain living cells, making them prone to infiltration by inflammatory elements of the recipient (Rieder et al. 2005), causing chronic inflammation.

New pericardium and valve tissues decellularization strategies have been therefore proposed, based on treatment with ionic/non-ionic detergents and enzymes that remove the genetic content and xenoantigens (Mirsadraee et al. 2006, 2007). While these methods have been found to reduce immunogenicity of the pericardial tissue for xenotransplantation (Vinci et al. 2013), and to favor post-graft recellularization by host cells (Iop et al. 2014), the decellularized tissues employed to manufacture valve prostheses are still devoid of valve competent cells, which may contribute to renew the extracellular matrix and thus repair the tissue over time increasing its longevity (CardioPulse Articles 2015).

Tissue engineering methods to produce 'off the shelf' living valve substitutes have been set up. This approach consists in combining three-dimensional (3D) biodegradable scaffolds produced with different materials and manufacturing methods or decellularized tissues (e.g. porcine-derived aortic valves; pericardial membrane) with cells of various origin and level of potency (Jana et al. 2014; Kheradvar et al. 2015). Apart from advantages and drawbacks depending on the bio-compatibility of the scaffold materials and the cell types which may have a variable, and in several cases still unknown, adaptability to the high degree of mechanical stimulation acting in cardiac valves, there are two major limitations in this approach: ***(i)*** the insufficient structural stability of the tissue-engineered valve leaflets that undergo retraction and thickening thus causing regurgitation and insufficiency in the mid long term (van Vlimmeren et al. 2011), and ***(ii)*** the limited access of cells in the decellularized tissues (Dainese et al. 2012) that probably requires advanced cell seeding methods to achieve good recellularization efficiency. Finally, in this respect it is surprising that the employment of valve-derived cells, and in particular the so called 'valve interstitial cells' (VICs), has been introduced to this aim only recently in the valve tissue engineering scenario, while other cells such as bone marrow-derived mesenchymal progenitors or endothelial cells have been used for longer time.

1.2 Designing the Right Mechanosensing Environment – Anisotropic Structure and Valve Resistance to Mechanical Load

When observed at an ultrastructural level, the valve tissue has a very complex and well-organized structure (Fig. 1.1). A crucial feature is, for example, the specific arrangement of the extracellular matrix components (namely collagen, glicosaminoglycans and elastin), whose specific orientation and prevalent distribution in the small leaflet thickness and width has evolved to make the tissue very resistant to mechanical stress at valve closure during diastole, and soft and pliable to allow the blood flow through the valve at opening during systole (Breuer et al. 2004; Balguid et al. 2007, 2008; Hammer et al. 2014). In three leaflet valves (the aortic and the pulmonary) for example, it comprises three specialized layer (the *Fibrosa*, the *Spongiosa* and the *Ventricularis*; Fig. 1.1) whose distinct cellular and extracellular matrix composition ensures correct absorption of mechanical stress, confined in a thickness of around 500 μm. The presence of non-uniformly arranged collagen bundles in the *Fibrosa* (the layer exposed to the aortic outflow segment; Fig. 1.2a) is, for example, the crucial structural component determining the anisotropic mechanical behavior of the leaflets. This is adapted to ensure a maximal resistance to stress at the leaflets commissures and at the 'belly' portions, where the largest mechanical stresses are normally acting when valve closes (Fig. 1.2b). The presence of elastin bundles in the *Ventricularis* (the part of the tissue located on the ventricular side of the valve) has specifically evolved to support the recoil of the leaflets to their

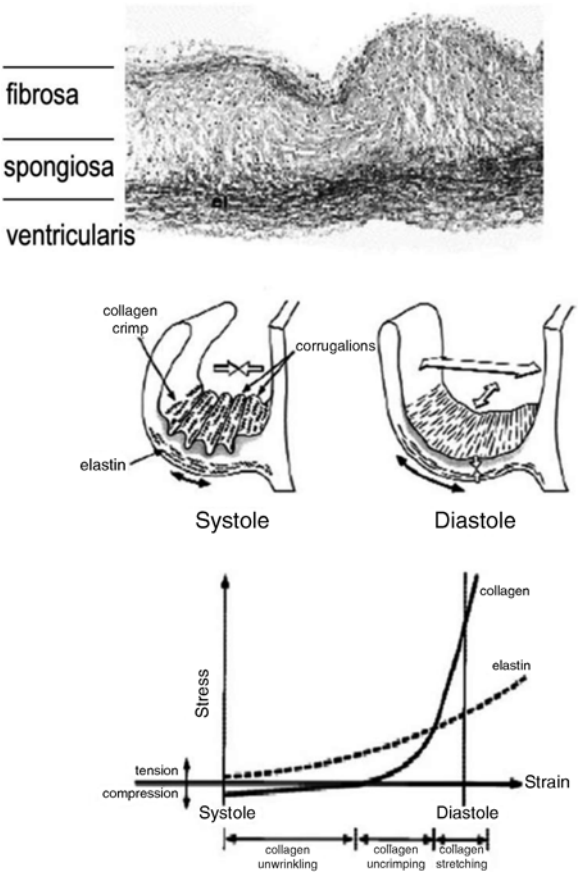

Fig. 1.1 Structure and mechanical behaviour of the aortic valve tissue throughout the cardiac cycle (Adapted from van Vlimmeren et al. 2011). The picture on *top* shows the three layered structure of the leaflet (split in *fibrosa*, *spongiosa* and *fibrosa* layers from the aortic through to the ventricular side). The drawing in the *middle* describes the modifications of the leaflet structure in the diastole/systole transition and the main changes in the strain of the collagen and elastin fibres in the *fibrosa* and *ventricularis*. The *graph* on the *bottom* shows the strain/stress relationship in the transition from systole (valve closed) to diastole (valve closed) and the main structural components in the leaflet layers that are involved (From Breuer et al. (2004)

crimped initial state after diastolic loading (Breuer et al. 2004) (Fig. 1.1). Finally, the spongiosa layer (located in the middle portion between the two other has a lower structural organization, it is mainly composed of Glicosaminoglycans (GAGs) that function as a 'cushion' absorbing mechanical solicitations caused by leaflet motion. Leaflet complexity is increased by a non-homogeneous cell composition and distribution in the valve. This consists in valve endothelial cells (VECs), which line the inflow and outflow valve surfaces, and valve interstitial cells (VICs), a plastic fibroblast/myofibroblast phenotype, that provide the necessary ECM components renewal into a tissue undergoing, in its average lifetime, three billion load/unload cycles (Sacks et al. 2009). Mechanical forces, acting especially during embryonic shaping of the heart valves, give a primary contribution to differentially align and determine different shapes of VECs on the two leaflet surfaces, and are crucial to induce differential strain-dependent maturation of the valve fibrillar matrix structure by modulating the function/phenotype of VICs in the three presumptive layers (reviewed in (MacGrogan et al. 2014)).

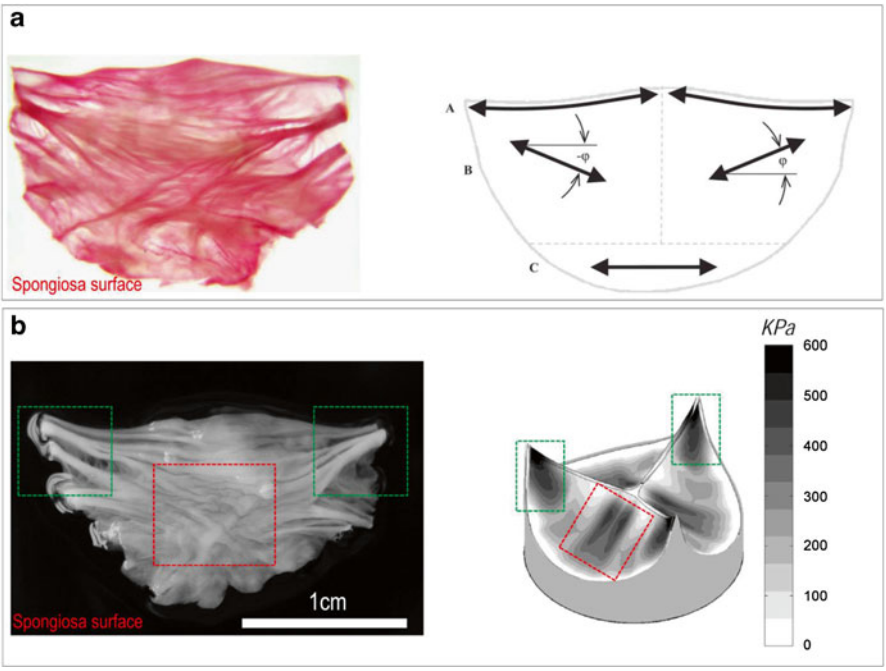

Fig. 1.2 Structure of the aortic valve *fibrosa* (Adapted from Dainese et al. 2012; Breuer et al. 2004; Balguid 2008). (**a**) Sirius Red staining of the aortic valve spongiosa reveals a high degree of anisotropy in collagen fibres deposition. The scheme on the *right* indicates the main direction of tensile strength oriented along the main fibre deposition pattern, resulting in an anisotropic loading distribution. (**b**) The image on the *left* indicates a polarized light picture of the collagen bundles in the *fibrosa. Green squares* indicate the two commissural areas and the red square shows the 'belly' portion. These areas are those where the maximal loading stress is applied at valve closure according to mathematical modelling of stress/strain patterns (From Hammer et al. 2014 and Balguid et al. (2007, 2008)

1.3 Mechanical Load, Mechanosensing and Cellular Responses

The extracellular matrix (ECM) not only provides a passive scaffold to maintain cells in a confined architecture, but it is also an active source of stimuli to the cells that are not just limited to a humoral control of tissue homeostasis. In fact, the ECM is also deputed to transduce mechanical cues that translate into geometric-, positioning- and motion-sensing information by the cells. These powerful forces are thought to be particularly active in the cardiovascular system, including heart valves, where motion is part of the physiologic functions, and positioning information is likely a crucial feedback signal orchestrating correct heart/heart valve patterning. The ability of the cells to sense the environment through mechanical activation of intracellular pathways is intrinsic in the developmental process of multicellular

organisms. For example, the recent implication of the mechanosensing-activated pathway Hippo in the pre-implantation development, has allowed to connect the old concept of cellular 'polarity' acquired by blastomeres undergoing the first embryonic divisions (Johnson and Ziomek 1981) to the events segregating the embryonic (pluripotent) cells from the extraembryonic (trophectoderm) lineages depending on cell positioning (Biggins et al. 2015; Nishioka et al. 2009). This applies also to adult-derived stem cells, e.g. mesenchymal stem cells, in which cellular lineage identity and basic features such as proliferation and differentiation are affected by discrete geometric patterning into cell colonies (Vunjak-Novakovic 2008; Nelson et al. 2005) and intracellular signalling eliciting by shape-dependent discrete cytoskeleton tensioning (Kilian et al. 2010). Finally while geometrical modelling of the environment is strictly associated to normal (stem) cell fate and functions into tissues, alterations of positioning cues can also lead, at least theoretically, to abnormalities in stem cells proliferation and telomeric size (Blagoev 2011).

The ECM composition is complex and highly adapted to regulate the tissue functions. For example, the variable content of adhesive proteins, fibrous proteins and proteoglycans is crucial to generate anchorage and modulate mechanical stimuli that are known to have potent inductive functions on tissue-resident cells. In this regard, one of the most important characteristics of the ECM is its stiffness, described by the Young's elastic modulus (E[Pa]). ECM stiffness is relevant either for passive loading to the cells, or as a stimulus regulating critical cellular activities, such as induction of cytoskeleton tensioning and the associated intracellular signalling pathway. The physiological stiffness changes significantly between different tissues, ranging from the very soft brain tissue (0.1–1 KPa), to the very stiff precalcified bone (25–40 KPa), while intermediate ranges (8–17 KPa) are typical of the skeletal muscle (Engler et al. 2006).

A simple method to investigate the stiffness effects on cell behaviour is to manufacture culture surfaces with a defined elastic modulus. These substrates can be easily generated using various technologies and materials such as the polydimethilsiloxane (Gray et al. 2003), polyethilenglicol (Khatiwala et al. 2009), or polyacrylamide (Pelham and Wang 1997). In these bi-dimensional (2D) culturing environments, whose biophysical features can be accurately detected by nanoindentation methods or atomic force microscopy (Engler et al. 2007), cell attachment is mediated by coating the surfaces with specific ECM components such as, for example, collagen (Engler et al. 2006; Quinlan and Billiar 2012), fibronectin (Peyton and Putnam 2005) or specific integrin-binding adhesion peptides (Wang et al. 2012; Gould et al. 2012). This enables the manufacturing of adhesion surfaces transducing discrete mechanical information to the cells *via* the activation of specific receptor signalling (Balaoing et al. 2015; Ramos et al. 2010). Culturing cells onto substrates of known and controlled stiffness has offered the chance to describe for the first time the correlation between the intracellular machinery regulating cell rigidity (e.g. that dependent on the small-GTP binding protein RhoA and its downstream target ROCK (Zhou et al. 2011)) and progenitor cells differentiation. When cultured on high stiffness substrates, various cell types, such as embryonic fibroblasts (Kim et al. 2012), cardiac fibroblasts (Xie et al. 2014), and mesenchymal

progenitors (Engler et al. 2006) were reported to show increased substrate contact areas and focal adhesions, and this correlated with an induction of genes involved in osteogenic commitment.

How does the matrix rigidity affect the biology of the heart valves? As shown in Fig. 1.1, the valve tissue is a composite structure in which cells are embedded into extracellular matrix layers with distinct elasticity. For example, the fibrosa layer contains an anisotropic arrangement of Collagen fibres whose mechanical compliance is higher compared with that of the amorphous-structured spongiosa, in which Glycosamino-Glycans (GAGs) are the most represented matrix components (Schoen 2008). Although a comprehensive dissection of the VICs phenotype with respect to the mechanical compliance of the three valve tissue layers has not yet been performed, the susceptibility of these cells to differentiate into pro-osteogenic cells (Chen et al. 2009), especially when cultured onto surfaces with high stiffness (Yip et al. 2009), along with their mechanical adaptability to the compliance of the surrounding environment (Wyss et al. 2012; Liu et al. 2013), makes them likely potent mechanical 'sensors' in the valve. These cells in fact, are not only able to fulfil the leaflets tissue turnover and to adapt to the leaflets mechanical complexity, but evolve toward pathologic phenotypes depending on local perturbations of the valve matrix compliance. In keeping with this hypothesis, the regions in the Aortic Valve leaflets that are most subject to mechanical load, i.e. the commissures and the 'belly' portions (regions highlighted in red and green, respectively in Fig. 1.2b), are those into which preferentially calcific lesions are first detected (Hinton and Yutzey 2011), probably as a result of stiffness-dependent calcific nodules deposition by VICs (Bouchareb et al. 2014; Bertazzo et al. 2013). The above considerations demonstrate the ability of the cells in general to 'feel' the mechanical environment and, restricted to valve biology, may have implications for tissue pathologic evolution. This suggests that VICs mechanosensing ability will have to be taken into consideration in future tissue engineering approaches aiming at reconstructing durable valve bioprostheses architecture using these cells or other mechanosensing-susceptible cells.

1.4 From Cell and Tissue Mechanics to Tissue Engineering

As well highlighted in other contributions available in the literature (e.g. reference (Kheradvar et al. 2015)), the design concept of off-the-shelf tissue engineered heart valve bioprostheses is based on the ability of *ex vivo* cultured cells (Weber et al. 2012) to colonize 3D scaffolds manufactured with various biologically compatible polymers, and induce maturation of leaflet-like tissues by *in vitro* mechanical loading, before implantation into suitable animal models (e.g. sheep). In early attempts, the adopted engineering strategy was to employ preformed three-leaflet tubular constructs manufactured with non-woven scaffolds made of biodegradable materials (e.g. Poly-Glycolic-Acid, PGA and Poly-L-Lactic-Acid, PLLA) as 3D seeding substrates for cells (Hoerstrup et al. 2002; Schmidt et al. 2010). Unfortunately, despite the initial success of cell seeding and *in vitro* maturation of the tissue constructs,

in several of these attempts, ‘compaction’ and ‘retraction’ of tissue engineered leaflets were observed. This effect altered the geometry, the compliance and, ultimately, compromised the mechanical performance of the tissue engineered valve prostheses. These effects occurred especially at long term after implantation in animals due to a non-optimized arrangement of the structural elements associated with the cells and inflammatory cells infiltration. In addition, while at least in principles, the presence of a three-dimensioned environment should confine the cells to stay into an ‘instructive’ leaflet geometry, the employment of biodegradable materials does not support a native-like structural and mechanical maturation of the engineered valve tissue, thereby causing loss of structural and mechanical coherence. Models of tissue compaction and retraction have been set to explain this limitation in TEHV design (van Vlimmeren et al. 2011, 2012; van Loosdregt et al. 2014). These investigations have clearly indicated that passive and cell-mediated forces are involved in leaflets retraction. The passive shrinkage is essentially caused by failure of the newly formed tissue to withstand cellular traction forces as the scaffold degrades; a further active retraction is then caused by cellular traction forces that compensate for the hemodynamic loading as well as by cell-mediated remodelling of the ECM components (van Loosdregt et al. 2014; van Vlimmeren et al. 2012).

While the introduction of an anisotropic design in TEHV scaffolds manufacturing may reduce the impact of the cell-mediated shrinkage (Loerakker et al. 2013), the problem of passive compaction due to the rapid reabsorption of the scaffold remains essentially unaddressed. One possibility to circumvent this problem may be in the future to invest into novel ‘hybrid’ approaches, which may take advantage of the physical properties of non-degradable materials to manufacture scaffolds with a specific anisotropic design and mechanical behaviour, and of cell-seeding/depositing techniques to cellularize the anisotropic 3D environment and achieve maturation of the tissues through mechanical stimulation. Another approach, as recently suggested (Kural and Billiar 2014; Hjortnaes et al. 2015), may be to modulate the cell-mediated tensile strength of the tissue directly in the 3D environment by employing materials with defined stiffness to reduce the propensity of VICs to evolve towards myofibroblasts/osteogenic cells and humoral signals (e.g. treatment with TGF-β), and/or to induce VICs to deposit ECM components (e.g. Collagen) with an anisotropic deposition pattern, resembling that present in the native tissue (MacGrogan et al. 2014).

Evolved scaffold fabrication criteria have been finally introduced to achieve a more complex bio-artificial leaflets design with the aim at reproducing the architecture and the mechanical behaviour of the native valve tissues. Examples of these new techniques are electrospinning (Masoumi et al. 2014a), 3D printing (Mosadegh et al. 2015) and stereolithography (Morsi 2014). These methods have been employed with different classes of biocompatible artificial materials (Morsi 2014) and, in some instances, have been combined together (Masoumi et al. 2014b) in order to generate complex scaffolds reproducing the natural layering of the valve leaflets tissue with a specific degree of structural anisotropy. These fabrication methods can be interfaced with computer-added-design (CAD) tools that make possible to

include in the fabrication process the mechanical parameters of the tissue during its motion and loading, thus empowering the manufacture of bio-artificial leaflets with a pre-determined resistance to stress (Lueders et al. 2014).

1.5 Conclusions

Compared with the early and inefficient approaches stemming from application of basic principles in materials/cells interactions, the engineering of 'off-the-shelf' valve substitutes has become a sophisticated process involving an interdisciplinary integration of various techniques and manufacturing strategies. Although advancements in material science allows the employment of various polymers with different chemical composition, different degrees of reabsorption and biophysical properties, the evolution of tools to operate tailored scaffold fabrication makes possible the manufacture of 3D environments where cells might be placed in the right mechanical environment. This will lead to the necessary improvements of the valve bioartificial leaflets manufacturing process to achieve a realistic translation.

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