NANOFIBER BASED SCAFFOLD FABRICATION, CHARACTERIZATION AND OPTIMIZATION FOR TISSUE ENGINEERING AORTIC HEART VALVE

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A thesis submitted in fulfilment of the
requirements for the award of the degree of
Doctor of Philosophy (Mechanical Engineering)

Faculty of Mechanical Engineering
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FEBRUARY 2017

To my beloved family

ACKNOWLEDGEMENT

First and above all, I praise God, the almighty for providing me this opportunity and granting me the capability to proceed with this research successfully. Further, there are no proper words to convey my deep gratitude and respect for my thesis and research advisor, Professor Dr. Noordin Mohd Yusof. He has inspired me to become an independent researcher and helped me realize the power of critical reasoning. He also demonstrated what a brilliant and hard-working scientist can accomplish.

My sincere thanks must also go to co-advisory, Professor Dr. Ani Idris for the trust, the insightful discussion, offering valuable advice, for her support during the whole period of the study, and especially for her patience and guidance during the writing process. She generously gave her time to offer me valuable comments toward improving my work.

Besides, I would like to thank the authority of Universiti Teknologi Malaysia (UTM) for providing me with a good environment and facilities. I also greatly appreciate the excellent assistance and spiritual supports of my family and my friends during my PhD study.

ABSTRACT

The four valves in a mammalian heart provide a unidirectional, unobstructed blood flow pathway as a result of synchronic movement of valves' leaflets during cardiac cycle. When one of the valves malfunctions, the medical choice is to replace the original valve with an artificial one. However, the inability to grow or to remodel an artificial valve leads to the innovation of tissue engineering heart valve (TEHV). The previously tissue engineered heart valve tends to be rigid, have low degradation rate and adverse structure which leads to TEHV failure. This study presents the design and fabrication of an aortic heart valve (AOHV) based on tissue engineering (TE) principle via electrospinning method. In TE, a three-dimensional (3D) scaffold with proper design, structure, and mechanical properties that resembles the original tissue is required as an initial template for tissue regeneration. For this purpose, materials' ratio tuning and process optimization as well as the 3D scaffold design were considered. Initially, five different ratios of poly-L-lactic acid (PLLA)/thermoplastic polyurethane (TPU) blends containing 1% (w/v) maghemite (γ-Fe_2O_3) nanoparticles were electrospun and characterized in terms of morphology, degradation rate, biological compatibility and mechanical properties. The existence of three components in the mats was confirmed by Fourier transform infrared and energy-dispersive X-ray spectroscopy. Scanning electron microscopy images illustrated well fabricated nanofibers with smaller diameter distribution for PLLA. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay using human skin fibroblast cell indicates desired proliferation on the samples. Blood biocompatibility results in terms of clotting time, fibrin formation, and hemolysis were almost in the normal range. Samples' degradation rate was investigated over 24 weeks where the PLLA shows 47.15% loss in mass versus 6.7% loss for TPU. High tensile strength and an extremely low elongation-at-break were determined from the stress-strain curve for PLLA, while TPU exhibits high elasticity. Overall, 50:50% of (1% γ-Fe_2O_3) loaded PLLA/TPU mats are the most appropriate. Next, a two-level Taguchi (L8) experimental design followed by the response surface methodology (RSM) were used to optimize the fabrication process where the elastic modulus is the response while the factors investigated were A-flow rate (2-3 ml/h), B-voltage (20-30 kV), C-maghemite% (1-3% w/v), D-solution concentration (10-15 wt.%) and E-collector rotating speed (1000-2000 rpm). From the signal-to-noise ratio values, the influences of the factors were ranked as: D>B>C>E>A. The empirical quadratic model obtained consists of the voltage-B and second order effect of flow rate-$(A)^2$, voltage-$(B)^2$, maghemite %-$(C)^2$ and concentration-$(D)^2$. The optimum elastic modulus of the scaffold was found to be 35.24±0.64 MPa. Finally, an AOHV template was designed and installed as the electrospinning collector to fabricate the 3D scaffold based on the optimum ratio and settings. Later, the human aortic smooth muscles cell migration and proliferation, as well as the elastic modulus loss percent of the optimum 3D scaffold after cell seeding were checked during 34 days of incubation. Overall, the structural, biological and mechanical specifications of the fabricated TEHV have successfully proved that it can be a potential alternative in AOHV replacement surgery.

ABSTRAK

Empat injap yang terdapat di dalam hati mamalia menyediakan laluan aliran darah yang searah, tidak terhalang disebabkan pergerakan daun injab yang diselarikan semasa kitaran jantung. Apabila salah satu daripada injap rosak, pilihan perubatan adalah menggantikan injap asal dengan injap tiruan. Walaubagaimanapun, injap tiruan tidak mempunyai kemampuan untuk tumbuh atau dimodel semula. Ini telah membawa kepada inovasi injap jantung kejuruteraan tisu (TEHV). Injap jantung kejuruteraan tisu sebelum ini adalah tegar, mempunyai kadar penurunan yang rendah dan struktur yang tidak sesuai yang membawa kepada kegagalan TEHV. Kajian ini membentangkan reka bentuk dan fabrikasi injap jantung aortik menggunakan prinsip kejuruteraan tisu (TE) melalui kaedah elektropintal. Dalam TE, perancah tiga dimensi (3D) dengan reka bentuk yang sesuai, struktur dan sifat-sifat mekanik yang boleh menyerupai tisu asal akan digunakan sebagai pencontoh permulaan untuk pertumbuhan semula tisu. Untuk tujuan ini, penalaan nisbah bahan-bahan utama yang digunakan dan pengoptimuman proses serta reka bentuk perancah 3D dipertimbangkan. Buat permulaan, lima nisbah berbeza poli-L-laktik asid (PLLA)/poliuretana termoplastik (TPU) dicampurkan dengan 1% (w/v) maghemite (γ-Fe_2O_3) nanopartikel. Campuran ini telah melalui proses elektropintal dan pencirian dibuat dari segi morfologi, kadar penurunan, keserasian biologi dan sifat-sifat mekanik. Kewujudan tiga komponen dalam lapisan serat nano telah disahkan oleh jelmaan inframerah Fourier dan serakan-tenaga X-ray spektroskopi. Imej imbasan mikroskopi elektron menunjukkan bahawa serat nano yang baik terhasil dengan garis pusat yang lebih kecil untuk PLLA. Kajian MTT menggunakan sel fibroblas kulit manusia dan ia menunjukkan percambahan yang baik ke atas sampel. Keputusan bio-keserasian darah dari segi masa pembekuan, pembentukan fibrin, dan hemolisis hampir dalam julat normal. Kadar penurunan sampel telah diselidiki selama 24 minggu yang mana PLLA menunjukkan penurunan jisim sebanyak 47.15% berbanding dengan penurunan 6.7% bagi TPU. Kekuatan tegangan yang tinggi dan kadar pemanjangan sebelum putus yang amat rendah ditentukan dari lengkung tegasan-terikan untuk PLLA, manakala TPU mempamerkan keanjalan yang tinggi. Secara keseluruhan, lapisan serat nano PLLA/TPU yang mengandungi 50:50% daripada 1% (γ-Fe_2O_3) adalah yang paling sesuai. Seterusnya, reka bentuk eksperimen dua aras Taguchi (L8) diikuti dengan kaedah gerak balas permukaan (RSM) telah digunakan untuk mengoptimumkan proses di mana modulus elastik merupakan sambutan manakala faktor yang dikaji ialah A- kadar alir (2-3 ml/h), B-voltan (20-30 kV), C-maghemite % (1-3% w/v), D-kepekatan larutan (10-15wt.%), dan E-kelajuan puteran pengumpul (1000-2000 rpm). Dari nilai nisbah isyarat-kepada-hingar (S/N), pengaruh faktor adalah: D>B>C>E>A. Model kuadratik empirikal yang diperolehi terdiri dari voltan-B dan kesan peringkat kedua kadar alir-$(A)^2$, voltan-$(B)^2$, maghemite %-$(C)^2$ dan kepekatan-$(D)^2$. Modulus elastik optimum perancah yang diperolehi adalah 35.24±0.64 MPa. Akhir sekali, AOHV telah direka dan dipasang sebagai pemungut kepada elektropintal untuk menghasilkan perancah 3D berdasarkan kepada nisbah optimum dan tetapan. Kemudian, penghijrahan dan perkembangan aortik sel-sel otot licin manusia serta peratusan kehilangan keanjalan modulus daripada perancah 3D optimum selepas pembenihan sel diperiksa semasa 34 hari pengeraman. Secara keseluruhan, spesifikasi struktur, biologi dan mekanikal bagi TEHV yang telah difabrikasi berjaya membuktikan yang ia boleh menjadi alternatif yang berpotensi untuk pembedahan penggantian AOHV.

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LIST OF ABBREVIATIONS

2D	-	Two-dimensional
3D	-	Three-dimensional
Adj-R^2	-	Adjusted R-Square
ADSCs	-	Adipose derived stem cells
AFM	-	Atomic force microscopy
ANOVA	-	Analysis of variance
AOHV	-	Aortic heart valve
AV	-	Atrioventricular valve
BMSCs	-	Bone marrow stem cells
C	-	Carbon
CAD	-	Computer aided design
CCD	-	Central composite design
CLSM	-	Confocal laser scanning microscopy
CT	-	Computed tomography
DCM	-	Dichloromethane
DMF	-	Dimethylformamide
DMEM	-	Dulbecco's modified Eagle's medium
DMSO	-	Dimethyl sulfoxide
DOE	-	Design of experiments
DSC	-	Differential scanning calorimetry
ECM	-	Extracellular matrix
EDX	-	Energy dispersive X-ray
EGFP	-	Enhanced green fluorescent protein

FBS	-	Fetal bovine serum
FDA	-	Food and drug administration
Fe	-	Iron
FE-SEM	-	Field-emission scanning electron microscopy
$FeCl_2$	-	Ferric (Iron (II) chloride)
$FeCl_3$	-	Ferrous (Iron (III) chloride)
FTIR	-	Fourier transform infrared spectroscopy
GAG	-	Glycosaminoglycan
HASMCs	-	Human aortic smooth muscles cells
HBSS	-	Hank's buffered salt solution
HCl	-	Hydrochloric acid
HNO_3	-	Nitric acid
HRBCs	-	Human red blood cells
HSF-1184	-	Human skin fibroblast
HA	-	Hydroxyl apatite
LV	-	Left ventricles
MRI	-	Magnetic resonance image
MTT	-	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NH_3	-	Ammonia solution
O	-	Oxygen
P4HB	-	Poly-4-hydroxybutyrate
PAN	-	Polyacrylonitrile
PBS	-	Phosphate buffer saline
PCL	-	Polycaprolactone
PF	-	Polyfumaroles
PGA	-	Polyglycolic acid
PGS	-	Polyglycerol sebacate
PLA	-	Polylactid acid
PLGA	-	Poly (lactic-co-glycolic) acid

PLLA	-	Poly-L-lactic acid
PDLLA	-	Poly-DL-lactide
Pred-R^2	-	Predicted R-Squares
PT	-	Pro-thrombin time
PTT	-	Partial thrombin time
PVA	-	Polyvinyl alcohol
RSM	-	Response surface methodology
SBF	-	simulated body fluid
SD.	-	Standard deviation
SFF	-	Solid free form fabrication
S/N	-	Signal to noise
SV	-	Semilunar valve
TCP	-	Thricalcium phosphate
TE	-	Tissue engineering
TEHV	-	Tissue engineering heart valve
TEM	-	Transmission electron microscopy
TPU	-	Thermoplastic polyurethane
Tris	-	Tris hydroxymethyl amino methane
TT	-	Thrombin time
UTM	-	Universal testing machine
VICs	-	Valve interstitial cells
XRD	-	X-ray diffraction spectroscopy
γ-Fe_2O_3	-	Maghemite

LIST OF SYMBOLS

A	-	Flow rate – First factor
B	-	Voltage – Second factor
C	-	Maghemite content – Third factor
D	-	Solution concentration – Fourth factor
E	-	Collector rotating speed – Fifth factor
EI	-	Stiffness index
F	-	Force
H	-	Valve height
HP	-	Hemolysis percent
K	-	Number of factors
L	-	Gap length between grids
Ms	-	Mean of squares
S	-	Square area
SS	-	Sum of squares
T	-	Thickness
v/v	-	Volume/Volume
w/v	-	Weight/Volume
%	-	Percent
°C	-	Degree Celsius
γ	-	Gamma
χ_c	-	Crystallinity
ΔH_m	-	Melting enthalpy
W_f	-	Weight fraction of reference polymer

ΔH_m°	-	Melting enthalpy of the reference polymer
$\sigma_{(\varepsilon)}$	-	Tensile stress
ε	-	Tensile strain
β	-	Regression coefficient
δ	-	Depth of indentation
δ_{max}	-	Maximum depth
ρ	-	Density
ΔL	-	Change in length
ΔX	-	Step size
$\hat{Y}$	-	Regression response
R^2	-	R-square
A_0	-	Cross section area
C_d	-	Commissure diameter
C_h	-	Commissure height
d_f	-	Degree of freedom
D_b	-	Base diameter
D_c	-	Absorbance value of control
D_{NegC}	-	Absorbance value of negative control
D_{posC}	-	Absorbance value of positive control
D_s	-	Absorbance value of sample
D_t	-	Top diameter
E_e	-	Elastic modulus
E_r	-	Relative elastic modulus
L_0	-	Origin length
L_f	-	Leaflet free-edge
L_h	-	Leaflet height
M_1	-	Average of counted cells via hemocytometer
M_2	-	Number of cells to be seeded
P_r	-	Porosity

R_a	-	Surface roughness average
R_e	-	Contact surface area between ball probe and sample
R_{pv}	-	Peak to valley
R_q	-	Root-mean square of surface roughness
R_z	-	Five lowest valley and five highest peak averages
T_g	-	Glass transition temperature
V_c	-	Required volume of cell suspension
V_t	-	Total required solution
V_1	-	Known volume
V_2	-	Total of new volume after immersion of sample
V_3	-	Total of new volume after sample removal
V_f	-	Poisson's ratio
W_d	-	Dry weight
W_o	-	Original weight of sample
W_t	-	Mass change
W_w	-	Wet weight
$\hat{Z}$	-	Predicted response

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CHAPTER 1

INTRODUCTION

1.1 Overview of the Research

The four valves represented in the mammalian hearts are responsible to maintain unidirectional, non-hinder blood flow from heart to the other parts of body. The heart valves synchronically open and close approximately 40 million times a year and more than 3 billion times during the life (75 years average life expectancy) (Aagaard, 2004; Rabkin-Aikawa *et al.*, 2005). The four valves are namely; (I): Aortic, (II): Pulmonary, (III): Mitral (bicuspid) and (IV): Tricuspid. The aortic and pulmonary valves are in the arteries leaving the heart and known as semilunar valves (SV), the mitral and tricuspid are between the atria and ventricles which known as atrioventricular valves (AV) (Gallyamov *et al.*, 2014; Saito *et al.*, 2016). Valvular heart dysfunction is a significant cause of morbidity and mortality around the world. The prevalence of heart disease in adult US population in the early of 21st century has been estimated at more than 5 million (Schoen, 1997; Basso *et al.,* 2013). In the meantime; heart valves (especially aortic and mitral) dysfunction is a significant part of heart disease, which leads to death of approximately 20,000 people around the world annually. Approximately more than 290,000 heart valve surgeries is required around the world each year and according to the increase in average age of the population, it is estimated to reach 850,000 by the year 2050 (Yacoub and Takkenberg, 2005; Sewell-Loftin *et al.,* 2011). A heart valve consists of two or three semicircular shape moving flaps which are called leaflets and comes together in the center of the valves to close it. These leaflets are attached to the walls of a cylindrical conduit which is called the valve root. The histology of the valve leaflets (cusps)

exhibits an extracellular matrix (ECM) structure of three distinct layers: fibrosa, spongiosa and ventricularis. The fibrosa, the surface away from the blood flow, compose of parallel, dense collagen which is associated with the mechanical properties such as stiffness and strength of the leaflets. The spongiosa, the middle surface, compose of proteoglycans and lower abundance of collagen which facilitate the movement, and finally, the ventricularis, the adjacent surface, compose of aligned fiber of elastin interspersed and short collagen fiber, which provides the elasticity properties of leaflets. The aortic valves leaflets comprise of 45% collagen (types, I (74%), III (24%) and V (2%)), 20% elastin in dry weight and 35% of glycosamino acid (Gross and Kugel, 1931; Garcia-Martinez *et al.,* 1991; Cox, 2009; Falk *et al.,* 2011; Gallyamov *et al.,* 2014; Roberts *et al.,* 2015) Figure 1.1 illustrates the anatomy/position of the four valves and the structure of ECM of leaflets.

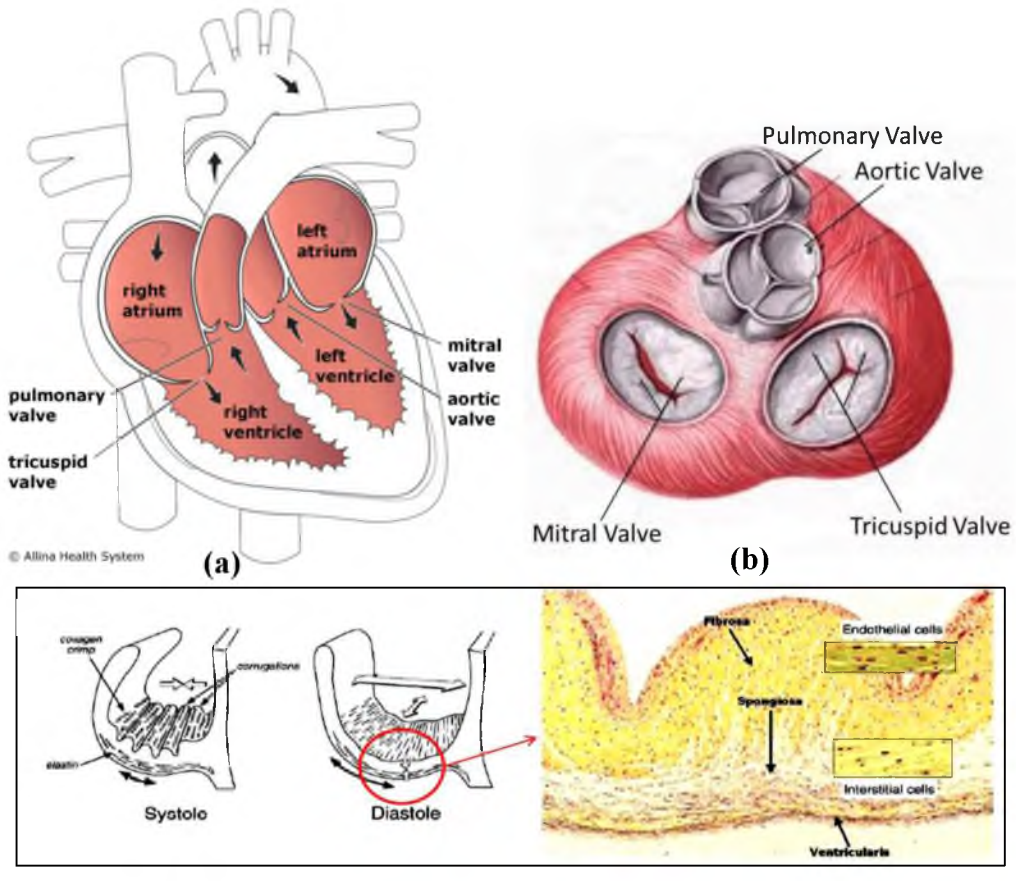
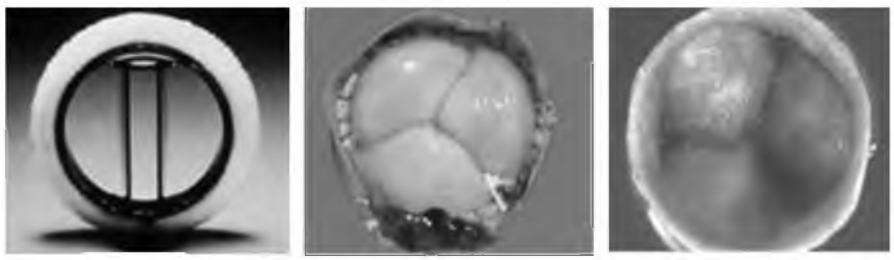


Figure 1.1: Schematic of (a) heart anatomy (b) valve position (c) single valve leaflet and its ECM structure (Schoen, 1997)

Heart valve dysfunctions may arise due to development regulation, mechanical properties shortage (such as leakage and overlaps of the leaflets), absence or abnormality of tissue in congenital cases and even calcification causes by the deposition of mineralized calcium and genetic defect in the matrix protein structure (Nasuti *et al.*, 2004; Ng *et al.*, 2004). When one of the valves malfunctions, the end stage of medical choice may be to replace the original valves with an artificial one. Generally, the cardiac surgeries to replace the heart valves are common around the world and improve the life expectancy. Currently, the mechanical valves and the biological (glutaraldehyde xeno-grafts or cryopreserved homo-grafts) are used clinically as the state-of-the-art of artificial valves, despite the occurrence of prosthesis side effects such as the need to anticoagulation treatment and durability of valves (Iung *et al.*, 2003; Nkomo *et al.*, 2006; Thom *et al.*, 2006). In order to resolve the drawbacks of prostheses, the tissue engineering concept is introduced (Tanaka *et al.,* 2005; Jain *et al.,* 2010; Lueders *et al.,* 2014; Cheung *et al.,* 2015). Figure 1.2 depicts the artificial heart valves.



(a) (b) (c)

Figure 1.2: Artificial heart valves (a) mechanical (b) biological (c) tissue engineering heart valve (Fallahiarezoudar *et al.,* 2015c)

Tissue engineering (TE) is an integrated science between the engineering principle and life science to overcome the limitation of prostheses. The principle of TE is to provide a three-dimensional (3D) scaffold (that resembles the original tissue properties) for a specific tissue to develop the neotissue from their cellular components. The scaffold provides an initial environment for cell attachment and tissue growth. The cell can be either seeded onto the scaffold matrix *in vitro* (pre-implementation) or *in vivo* (post-implementation) to develop a neotissue for replacement or repair the damaged tissue. The fabrication of a neotissue from cellular

combination (which depicts most of the characteristics of the original tissue such as non-inflammation and non-immunogenic reaction, adequate mechanical properties and durability) is the ultimate goal of TE. The concept of tissue engineering heart valve (TEHV) was introduced by Shinoka *et al.* (1995). The TEHV principle can be summarized in three main phases: (I) 3D biocompatible scaffold fabrication, (II) cell cultivation and seeding over the scaffold, and (III) development conditions of the TEHV before implantation (Sheridan *et al.*, 2000; Teebken *et al.*, 2000). Figure 1.3 illustrates the concept of TEHV.

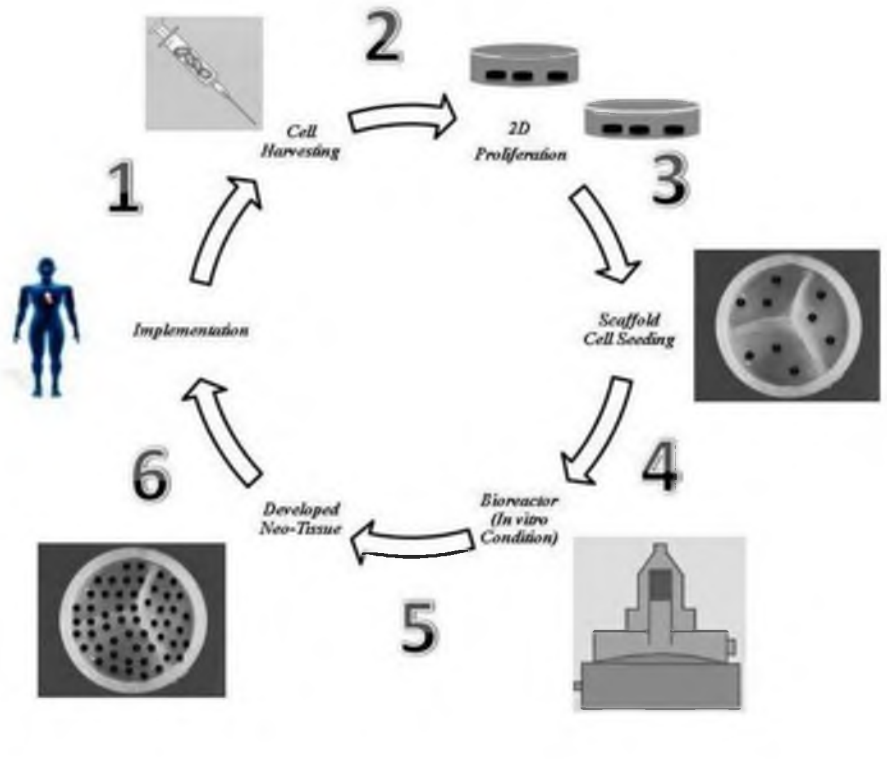


Figure 1.3: Concept of tissue engineering heart valve (TEHV) (Fallahiarezoudar *et al.*, 2015c)

In order to design and fabricate a proper 3D heart valve scaffold, the initial and probably one of the most important phases of TEHV concept is to investigate the valve structure and function. Each layer (fibrosa, spongiosa and ventricularis) which has a particular property, forms the valves structure. In the scaffold fabrication phase, the appropriate materials selection and fabrication techniques are debatable. The scaffold architecture (matrix) is very important as it is the basis of TEHV concept. In order to ensure a successful scaffold: (I) The utilized materials should be biologically

compatible, biodegradable and cover the requirements for mechanical properties, (II) the structure should provide a hierarchical extensive network of interconnecting pores to facilitate the cells attachment and provides the oxygen and nutrients to those cells which are far away from the surface (usually more than 1 mm) and (III) the shape and the size of the scaffold should resemble the native tissue (Aikawa *et al.*, 2006; Baaijens *et al.*, 2010).

The usable materials for scaffolds fabrication purpose can generally be classified into two groups: the polymer based and the natural based materials. The basic of scaffold mechanical properties and hemocompatibility with *in vivo* are highly dependent on the materials selection. The required properties for TEHV scaffold can be translated into biocompatibility, biodegradability, thermo-plasticity, elasticity and stiffness characteristics (Argento *et al.,* 2012; Chen *et al.,* 2013; Alves *et al.,* 2014). The main advantages of a synthetic polymer-based scaffold are the fact that biomechanics and degradation properties can be chemically controlled according to the requirements. Against the cytotoxicity, low degradation rate and inflammation are the main drawbacks of the synthetic polymeric scaffold. Although no biodegradable polymeric materials have been proven to be a desirable substitute for the native valves, work continues to be promising (Zhai *et al.,* 2010; Eckert *et al.,* 2013; Masoumi *et al.,* 2013a). Furthermore, the utilization of the natural materials such as collagen has also been reported as the raw material for scaffold fabrication. Collagen is the main extracellular matrix protein of the origin heart valves. The fibrosa is considered to be the main load carrying structure and is primarily composed of circumferentially arranged densely packed bundles of collagen fibres and a micro-fibrillar network of elastin. The use of collagen regarding to low antigenicity and less immunogenicity can be the advantages of collagen based scaffold. However, poor handling, low mechanical properties, and less controlled biodegradability are the defects of collagen based scaffold (Chevallay and Herbage, 2000; Levy *et al.,* 2004; Balguid *et al.,* 2007; Chen *et al.,* 2013).

Poly-L-lactic acid (PLLA) is one of the preferred biomaterials that are widely used in different fields of TE. PLLA due to high tensile strength, good compatibility *in vivo* and biodegradability is considered to be used in this research. However, the

rigidity and non-flexible matrix of PLLA limited the development of soft tissue by this polymer (Kang *et al.,* 2009; Sakai *et al.,* 2013; Qiao *et al.,* 2016). The PLLA scaffold was fabricated through electrospinning technique for bone tissue engineering purpose that indicated elastic modulus value of 35 MPa in such a way that the tensile strain was around 5-10% (Luu *et al.,* 2003). Application of pure PLLA scaffold (fabricated by different techniques) was mostly reported as a template for bone tissue engineering purpose. The biocompatibility and tissue formation using nanofiber based PLLA scaffold was confirmed *in vivo*. PLLA scaffold was well colonized with cells after implantation, but only showed marginal ossification (Schofer *et al.,* 2011). Besides, bio-grade thermoplastic polyurethane (TPU) exhibits superb elasticity (more than 220% of strain) with non-inflammation behaviour *in vivo* (Chen *et al.,* 2010; Jing *et al.,* 2016). The fabricated electrospun scaffold using pure TPU exhibited the samples were deformed easily in a low stress value which may not be appropriate in this case (Chen *et al.,* 2009a; Jing *et al.,* 2016). Fabricated TPU electrospun nanofibers indicated super hydrophobicity that made difficulties for cell culturing (Wang *et al.,* 2011). Therefore, the mixture of these two polymers can lead to a composite with desired tensile strength, elasticity and biocompatibility for soft TE purpose (Mi *et al.,* 2013; Jing *et al.,* 2014; Marycz *et al.,* 2016).

Maghemite (γ-Fe_2O_3) nanoparticle, which is a novel biocompatible material, has recently been used in biomedical applications such as magnetic cell seeding, cell expansion and drug delivery and the results are quite promising. The outstanding bio-behaviours (mechanical and biological) of maghemite have been reported in previous researches (Tartaj *et al.*, 2003; Wei *et al.*, 2011; Ngadiman *et al.*, 2015). Maghemite nanoparticles filled nanofibers such as polyvinyl alcohol (PVA) were used previously for composite reinforcement purpose (Fallahiarezoudar *et al.,* 2015b). Furthermore, maghemite filled polyvinyl alcohol was reported as a potential materials for bone tissue engineering purpose which resulted in higher tensile strength and better cell proliferation (Ngadiman *et al.,* 2017).

Scaffold fabrication can be classified into two main techniques. Conventional techniques such as solvent casting in combination with particulate leaching and phase separation in combination with freeze drying, and fashionable techniques such

as electrospinning and solid-free-form (3D printing) fabrication. The ECM structure and subsequently the mechanical properties and biological compatibility are highly dependent upon the parameters which can be modified in fashionable fabrication methods. The microstructure parameters such as the fiber diameter, interconnectivity, porosity properties and stiffness, which ultimately shape the macrostructure properties of the scaffold, should be investigated. In perspective of scaffold manufacturing, each technique has its own pros and cons (Hutmacher and Cool, 2007; Hoque *et al.,* 2012).

Electrospinning is a versatile and straightforward technique for cardiovascular scaffold fabrication. In the electrospinning setup, a high electric field is responsible for transforming the emerging solution supplied via syringe pump into the fibers. Figure 1.4 depicts a schematic diagram of the electrospinning setup. High surface area to volume ratio and high porosity are the advantages of electrospinning process. In fashionable fabrication techniques such as electrospinning the microstructure can be modified according to the requirements by varying the parameters involved such as types of polymers and solvents, flow rate, voltage, needle-collector distance and others. The fibrous scaffold matrix prepares an auspicious layout for the cell attachment, migrant and growth. However, the lack of mechanical properties, time consuming and problems with residual solvent which may stimulate the possibility of toxicity are the drawbacks (Ahmadipourroudposht *et al.,* 2015; Fallahiarezoudar *et al.,* 2015a).

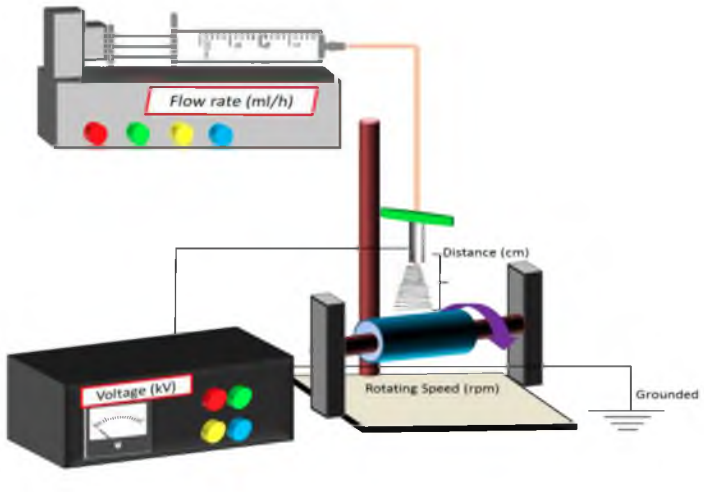


Figure 1.4: Schematic diagram of the electrospinning setup

The second phase in tissue engineering concept is the cell cultivation and seeding over the scaffold. The successful cell seeding process is highly related to the scaffold architecture and biocompatibility of materials that were used for the 3D scaffold fabrication. The inter-related, hierarchical scaffold structure will stimulate the cell proliferation/migration and subsequently shape the ECM. Three types of cells are reported to be useful in TEHV: (i) The vascular smooth muscles cells (Colazzo *et al.,* 2011), (ii) the valves interstitial cells (Masoumi *et al.,* 2013b) and (iii) the bone marrow stem cells (Hadju *et al.,* 2011). Two methods of cell seeding are proposed as dynamic and static. In dynamic method the scaffold is in constant motion during cell seeding in the incubator an opposed to the static environment. Various types of cell adhesion molecules such as integrin have been used to coat the scaffold matrix prior to cell seeding. Interaction between the integrins on the cell membrane and the receptors on ECM are largely responsible for cell attachment. The integrin consist of α and β chains (18 subunits for α and 8 subunits for β) which have the responsibility for cell attachment to ECM and signal translation from the ECM to the cells (Lam *et al.*, 2002; Taylor *et al.*, 2006).

Once the fabricated scaffold was seeded, the neo-tissue starts to develop. The environment in which the scaffold grows is one of the criteria for successful TEHV. The scaffold development environment will influence the formation of the ECM. Two perspectives have been proposed by the researchers: (I) the black box approach where the scaffold is implanted *in vivo* shortly after cell seeding and used as cell delivery to the native tissue (Vacanti *et al.,* 1988; Fong *et al.,* 2006), (II) bioreactor approach such as pulse duplicator which provides the physiological pressure and flow to the developed TEHV and promotes both cell function and mechanical properties (Sodian *et al.,* 2001; Engelmayr *et al.,* 2003; Mol *et al.,* 2005).

1.2 Research Problem Statement

Currently the usable clinical prostheses of heart valves (mechanical or biological) have a non-viable structure and have no capacity to grow, remodel or

repair. As a result, almost 50-60% of patients experienced problems with artificial valves and require reoperation (Chevallay and Herbage, 2000; Aagaard, 2004; De Heer *et al.,* 2013; Grzymala-Lubanski *et al.,* 2016). Mechanical valves as a foreign material may cause inflammation, infection and thromboembolic complication due to high shear stresses of blood flow which requires an anti-coagulation medication such as a vitamin *K* antagonist (e.g. warfarin) along the life. Although warfarin could be efficacious to alleviate coagulation, it has the risk of hemorrhagic and also the embryo toxicity in fertile women (Lip *et al.,* 2015; Roberts *et al.,* 2016). On the other hand, despite the lower thrombotic risk of biological valves compared to the mechanical one (0.87% to 1.4% per annum, respectively) and no need to undergo anti-coagulation treatment for the biological valves, the durability of these prostheses are approximately 10-15 years due to the progressive tissue deterioration and this is almost half the lifetime of the mechanical valves (20-30 years) (Potter *et al.*, 2004; Pibarot and Dumesnil, 2009; Tillquist and Maddox, 2011). These limitations forced scientists to further investigate of other possible methods of creating a neo-tissue similar to the original tissue that can fully solve the drawbacks of the artificial valves. However, TEHV principle is introduced as a possible method of resolving these limitations (Tanaka *et al.,* 2005; Jain *et al.,* 2010; Lueders *et al.,* 2014; Cheung *et al.,* 2015). A biodegradable and biocompatible 3D scaffold with adequate characteristics is fabricated, seeded with the appropriate cells source and developed *in vitro* in a bioreactor to create a biomimetic tissue which resembles the original tissue characteristics (Argento *et al.,* 2012; Cui *et al.,* 2016).

Previous researches on TEHV were performed using poly lactic acid (PLA) (Armentano *et al.*, 2013; Sakai *et al.,* 2013), polyglycerol sebacate (PGS) (Masoumi *et al.*, 2013a; Sanz-Garcia *et al.,* 2015), polyglycolic acid (PGA) (Matsumura *et al.*, 2003; Frese *et al.,* 2016), polycaprolactone (PCL) (Vaz *et al.*, 2005; Marei *et al.,* 2016) to fabricate the scaffold. Most of these researches result in a non-viable structure or toxicity due to the use of inorganic solvent. In addition, the degradation rate and mechanical properties of polymers also plays a critical role in TE concept. Fabrication of scaffold using PLA indicated a desirable biocompatibility and biodegradability (around 6 months), but much thicker and less flexible (roughly 1-2% tensile strain) which was in conflict with dynamic mechanism of original tissue

(Armentano *et al.*, 2013; Sakai *et al.*, 2013). Despite good mechanical properties of PCL (3.7 MPa tensile stress and 90% tensile strain), low degradation rate (more than 2 years) causes the failure of TE concept (Vaz *et al.*, 2005; Yao *et al.*, 2016). Also the fabricated PGS-based heart valve scaffold exhibited very fast degradation rate (2-3 weeks) with 0.5 MPa of elastic modulus but it could not provide the required elastic modulus for heart valves (Masoumi *et al.,* 2013a; Lin *et al.,* 2015). On the other hand, the scaffold adverse structure (that can be the result of inappropriate fabrication method) may result in the aggregation of seeded cells just over the scaffolds surface. As reported by Taylor *et al.* (2002) an extremely low expansion of valvular interstitial cells was observed on micromould injection PLGA scaffold. Colazzo *et al.* (2011) reported that the fabricated PLA scaffold using freeze drying technique resulted in cell adhesion only on the surface that can be attributed to disconnected pores. Furthermore, scaffold overgrowth, leakage and rupture before implementation (Van Lieshout *et al.,* 2006; Muylaert *et al.,* 2014) or inflammation due to low degradation rate after implementation (Choi and Park, 2002; Loger *et al.*, 2016) can happen due to the improper selection of polymeric materials source. Thus, the selection of a proper source of polymeric based biomaterials in terms of biocompatibility, biodegradability and mechanical properties as well as an applicable fabrication technique that can satisfy the heart valve characteristics is the major concern.

1.3 Research Questions

(i) What is the optimum ratio of polymer solutions blend according to the required characteristics of an aortic heart valve?

(ii) What is the biological and mechanical effect of maghemite nanoparticles presence in nanofibers matrix?

(iii) What are the biomechanical and structural characteristics of native aortic heart valve leaflets?

(iv) What are the required specifications to design and fabricate a three-dimensional scaffold that can resemble the origin tissue characteristics?

(v) How to perform the cell seeding procedure and evaluate the mechanical performance after cell seeding?

1.4 Research Hypothesis

Design of complex structures such as heart valves need to be simplified prior to fabrication and modelling. It this case some assumptions are applied to the design procedure to make it tractable.

First, it is assumed that the valves cusps have identical characteristics in dimensions and mechanical properties. Since the access to the human heart valve information and dimension is difficult, in this research the specifications are extracted from other previous works (Swanson and Clark, 1974; Labrosse *et al.,* 2006). Second, the leaflets lie at 120° from each other in the 3D circle plate. Third, it is assumed that the top and bottom of the cylinder with the valve inside of it (aortic root) are parallel. The final and noticeable hypothesis in designing the valve is to consider that the valves component's dimensions are not changed significantly during the cardiac cycle.

1.5 Research Aim and Objectives

The aim of this research is to fabricate, characterize and optimize a nanofiber-based scaffold of aortic heart valve with the extracellular matrix (ECM) structure and appropriate biological and mechanical characteristics to assist the aortic smooth muscles cell adhesion, migration and proliferation.

Objectives of this research are:

(i) To characterize different ratios of poly-L-lactic acid/thermoplastic polyurethane polymers to tune the ratio that proposes the best performance for tissue engineering heart valve.

(ii) To investigate the effect of maghemite nanoparticles on chemical, biological and mechanical properties of electrospun mat.

(iii) To optimize the fabrication process (electrospinning) in terms of elastic modulus that tailors the scaffold structure and mechanical properties.

(iv) To design and fabricate a three-dimensional nanofiber-based aortic heart valve scaffold.

(v) To evaluate the migration and proliferation of aortic smooth muscles cell over the scaffold and dwindle of mechanical properties as a function of incubation time.

1.6 Research Scopes

The left heart typically achieves a peak pressure about six times of the right over the cardiac cycle. So, the two valves on the left side of the heart are subjected to much higher loads than those on the right heart (Hasan *et al.*, 2014). Since the majority of valve diseases involve the valves of the left heart, therefore, the scope in this research is limited to investigating the anatomy and design microstructure of the tissue engineering aortic heart valves. The heart valve tissue engineering concept can be split into three main steps: (i) Fabrication of 3D scaffold with proper design and properties, (ii) cell seeding over the 3D scaffold, and (iii) development of neo-tissue in bioreactor prior to implementation. In this research the scope is limited to the characterization of the novel mixture of poly-l-lactic acid (PLLA), thermoplastic polyurethane (TPU) and maghemite (γ-Fe_2O_3) nanoparticles as the potential biocompatible and biodegradable materials for heart valve scaffold fabrication. A 3D template associated to the semilunar heart valves was designed to fabricate a 3D scaffold and the biological and mechanical properties of the fabricated scaffold are characterized as well.

In order to fabricate a biomimetic scaffold of aortic heart valve with adequate properties that can resemble the original tissue, two parameters of usable materials and fabrication techniques are among the available options considered.

(i) The utilized materials are PLLA, TPU and (γ-Fe_2O_3) nanoparticles which are categorized in the synthetic, polymeric materials group and their optimum proportions were determined.

(ii) The fabrication method was limited to the electrospinning technique.

(iii) The controllable parameters involved (which may have significant effect on the microstructure) such as flow rate, voltage, maghemite %, solution concentration and rotating speed, as well as noise parameters were selected as the variables to optimize the microstructure properties.

(iv) The characterization of electrospun mats in terms of morphology, porous properties, surface roughness, hydrophilicity, cytotoxicity assay, degradation rate, blood hemocompatibility, mechanical behaviour, cells attachment, migration and proliferation over the samples were investigated during this research.

(v) The characteristics mentioned with emphasis on the mechanical properties (tensile stress, tensile strain and elastic modulus) of the aortic heart valve leaflets were the major scope.

(vi) The optimization of scaffold fabrication technique was performed (based on uniaxial tensile properties) in terms of elastic modulus using Taguchi experimental design and response surface methodology.

(vii) The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay using human aortic smooth muscles cells (HASMCs) were used to verify the cell viability.

(viii) Blood hemocompatibility in terms of clotting time, fibrin formation and hemolysis were investigated.

(ix) The biomechanics behaviours of seeded scaffold as a function of incubation time were characterized using macro-indentation bending/flexural tests.

1.7 Significance of Research

A novel combination of material PLLA/TPU containing maghemite is useful in the design and fabrication of a synthetic biodegradable scaffold. The unique material developed has both the extracellular matrix structure of the aortic heart valve with interconnected networks (to provide the oxygen and nutrients to the cells which are far away from the surface) and the required mechanical properties such as tensile strength and flexural to resist against the blood pressure. In addition, the material has the stiffness and elastic characteristics that can be useful on biomaterials for scaffold development. Information on the electrospinning process parameters that produces nanofibers with optimum fiber diameter distribution and porosity with excellent mechanical properties and structure were also disclosed for the unique material. The findings of the research can also increase the life expectancy of patients experiencing valvular heart dysfunction. The developed synthetic bio-polymeric TEHV has the possibility of reducing the number of times a patient needs to undergo valve replacement surgery and this can reduce the complications after surgery and also cost.

1.8 Organization of Thesis

In the first Chapter of this thesis, general information of the research, objectives and scope are provided. In Chapter 2, the literature review on tissue engineering and particularly tissue engineering heart valves is described including the previous investigation on the microstructure, function and mechanical properties of the origin porcine and/or human tissue. Chapter 3 presents the research framework and detailed explanation of each phase to show the methods of experiments conducted and initial finding is provided. Chapter 4 provides the results and a detailed discussion on the findings of this research. This chapter is divided into three main sections; materials ratio tuning, electrospinning process optimization using Taguchi experimental design and 3D scaffold fabrication/characterization. In Chapter 5, the conclusion was made according to the assumptions made and the results obtained.

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