ABSTRACT

Heart valve related disease is a significant cause of mortality worldwide and in severe cases requires the patient to undergo a heart valve replacement surgery. The available heart valve prostheses on the market are mechanical and biological substitutes. However, both types of available heart valves have negative side-effects because they are fabricated from foreign materials. Mechanical heart valves have proven to be very durable, but are susceptible to thrombosis and thromboembolism and necessitate long-term anticoagulation therapy. On the other hand, bioprosthetic valves require little or no anticoagulation; however, the underlying problem with them is a limited life because of structural changes such as leaflet wear and calcification leading to valve failure.

This study focuses on polyurethane as a potential material for prosthetic heart valves which is biocompatible, flexible and can withstand many cycles of stress and deformation before failure. The main objective of this study was to address the issue of calcification of polyurethane heart valve prostheses which require lower anticoagulation levels than mechanical valves, yet offer the potential for reduced calcification and increased durability when compared to tissue valves. The valve leaflets are fabricated from Angioflex®, a proprietary polyetherurethane material that has been successfully and clinically evaluated by ABIOMED, Inc.

In the first phase of this study, a series of accelerated in vitro experiments were performed on the Angioflex® heart valves when subjected to a synthetic calcification solution. Results showed that these types of polyurethane have the potential to be considered as heart
valve substitute material with significantly lower levels of calcification deposits compared to tissue valves. Another outcome of these studies was to evaluate the calcification resistance of bisphosphonate modified Angioflex® valves. Bisphosphonates are a class of drugs that are considered to enhance the calcification resistance of polymers once covalently bonded to the bulk of the material. However, our test’s results showed that, although bisphosphonate-modified Angioflex® valves showed lower levels of calcification compared to tissue valves, but they are not superior to Angioflex® valves.

It has also been suggested that surface defects, shear and mechanical stresses are contributing factors in the calcification process. In the second phase of this study, a number of experiments were performed to evaluate the effect of surface irregularities (such as roughness and cracks) and flow shear rate on the calcification process in the absence of any source of mechanical stresses. Results showed that even in the absence of mechanical stresses a polymeric surface gets calcified and all the above mentioned factors directly affect the calcification process. Roughness, cracks and low shear rate promote the calcification. Calcification process under steady flow rate and no mechanical stresses occurs mostly on the surface of the polymer rather than being a subsurface phenomenon.
ACKNOWLEDGEMENT

First and foremost I express my sincerest gratitude to my thesis advisor, Professor Hamid Nayeb-Hashemi, who has supported me throughout this research with his patience and knowledge whilst giving me the opportunity to work independently. This work would have never been completed without his constant support and ingenious guidance.

I would like to offer my sincere appreciation to my thesis co-advisor, Professor Ahmet Umit Coskun, who never deprived me from his generous technical and valuable guidance. I also wish to thank Scott C. Corbett for providing me the opportunity to work on such an outstanding research and always helping me out throughout the way. And especial thanks to Professor Hameed Metghalchi for his warmth support whenever I needed his advice.

This acknowledgement would be incomplete, if I miss to mention the support I received from my friends who encouraged and helped me during my graduate studies especially: Amin Ajdari, AmirAli Kia, Mona Hdeib, and Sara Mohammadlou.

Last but not least, I would like to dedicate this work to my family. To my father whose memory was an inspiration for me throughout this research. To my mother for her everlasting love and support and to my sister who paved the way for my excellence. I could have never asked for a better family.
# TABLE OF CONTENTS

ABSTRACT .................................................................................................................................... i

ACKNOWLEDGEMENT ........................................................................................................... iii

TABLE OF CONTENTS ............................................................................................................ iv

LIST OF FIGURES .................................................................................................................... vii

LIST OF TABLE .......................................................................................................................... x

CHAPTER 1 - “BACKGROUND“ ............................................................................................. 1

1.1 Introduction .......................................................................................................................... 2

1.2 The Heart and the Aortic Valve ........................................................................................... 5

1.2.1 The Heart ................................................................................................................ 5

1.2.2 The Aortic Valve ..................................................................................................... 7

1.2.2.1 Aortic Valve Anatomy and Biomechanical Properties ............................... 7

1.2.2.2 Mechanics of Movement ........................................................................ 11

1.2.3 Mechanical Forces on the Aortic Valve .................................................................... 16

1.2.3.1 Diastolic Pressure ................................................................................... 16

1.2.3.2 Bending .................................................................................................. 17

1.2.3.3 Shear Stress ............................................................................................ 18

1.3 Aortic Heart Valve Disease ............................................................................................... 18

1.4 Aortic Valve Treatments and Surgical Interventions ......................................................... 18

1.4.1 Mechanical Valves ................................................................................................ 19

1.4.2 Bioprosthetic Valves ............................................................................................. 20

1.4.3 Allograft Valves .................................................................................................... 22

1.4.4 Autograft Valves ................................................................................................... 22

1.4.5 Tissue Engineered Heart Valves ........................................................................... 23

1.4.6 Polymeric Heart Valves ........................................................................................ 24
CHAPTER 2 - “CALCIFICATION“ ................................................................. 32

2.1 Abstract .............................................................................................. 33

2.2 Details of the Calcification Process ......................................................... 34

2.2.1 Introduction ....................................................................................... 34

2.2.2 Mechanism of Vascular Calcification .................................................. 39

2.2.2.1 Activation of Osteogenic Mechanism in the Vessel Wall ................. 39

2.2.2.2 Loss of Molecular Inhibitors of Calcification .................................. 40

2.2.2.3 Enhanced Bone Turnover ............................................................. 41

2.2.2.4 Abnormalities of Mineral Metabolism ......................................... 42

2.2.3 Bioprosthetic Heart Valve Calcification Mechanism ....................... 43

2.2.4 Polymeric Heart Valve Calcification Mechanism ............................. 46

2.3 References .......................................................................................... 48

CHAPTER 3 - “CALCIFICATION OF POLYMERIC HEART VALVES, MATERIAL DEPENDENCY“ .......................................................... 51

3.1 Abstract .............................................................................................. 52

3.2 Introduction ........................................................................................ 53

3.3 Angioflex® .......................................................................................... 56

3.4 Bisphosphonate Modified Angioflex® .................................................. 56

3.5 Calcification Metastable Solution ......................................................... 57

3.6 Valve Accelerated Life Tester (VALT) .................................................... 58

3.7 Objective ............................................................................................ 59

3.8 Experimental Setup and Method of Analysis ........................................ 59

3.8.1 Experimental Setup .......................................................................... 59

3.8.2 Method of Analysis .......................................................................... 60

3.8.3 Validation of the In Vitro Calcification Experiment ......................... 60

3.8.5 Angioflex® and BP-Angioflex® Calcification Comparison .............. 67
3.8.6 Verification for Superiority of Angioflex® valves ........................................... 73
3.9 Discussion and Conclusion ..................................................................................... 77
3.10 References ............................................................................................................. 80

CHAPTER 4 - “CALCIFICATION OF POLYMERIC HEART VALVES, SHEAR RATE AND SURFACE PROFILE DEPENDENCY” ............................................................................... 82

4.1 Abstract ..................................................................................................................... 83
4.2 Introduction ............................................................................................................... 83
4.3 Objective .................................................................................................................. 85
4.4 Experimental Setup and Method of Analysis ................................................................. 86
   4.4.1 Crack and Material Effect Loop ................................................................. 86
   4.4.2 Surface and Shear Rate Effect Loop 1 ...................................................... 91
   4.4.3 Surface and Shear Rate Effect Loop 2 ...................................................... 98
4.5 Loop 1 and 2 Comparison ...................................................................................... 106
4.6 Discussion and Conclusion ...................................................................................... 108
4.7 References .............................................................................................................. 110

RECOMMENDATIONS FOR FUTURE RESEARCH ...................................................... 111

“APPENDIX” ................................................................................................................ 113
LIST OF FIGURES

Figure 1.1 - Heart anatomy, arrows indicate blood flow direction. Source: www.tmc.edu/thi/anatomy.html................................................................. 6

Figure 1.2 - Anatomic relationship between the aortic valve and surrounding structures. Source: http://cardiacsurgery.ctsnetbooks.org/cgi/content/full/3/2008/825/F2 ......................................................... 8

Figure 1.3 - A schematic diagram of the relationship of the aortic valve leaflets to the structures underlying the commissures. Source: http://cardiacsurgery.ctsnetbooks.org/cgi/content/full/2/2003/791/F2 ........................................... 8

Figure 1.4 - Schematic representation of a cross section through the aortic valve leaflet. Inset illustrates the radial and transverse (circumferential) axes of the valve leaflet and the line of attachment to the aortic wall. Source: http://cardiacsurgery.ctsnetbooks.org/cgi/content/full/3/2008/825/F4 ........................................... 9

Figure 1.5 – “Positions of the aortic valve leaflets at end diastole and end systole and of a single leaflet in profile during ejection as the leaflet moves from the closed position (0) to full opening (26).” Source: http://cardiacsurgery.ctsnetbooks.org/content/vol2/issue2003/images/large/791fig6.jpeg .......... 15

Figure 1.6 - a) caged ball, b) tilting disc, c) bileaflet. Source: J Biomechanics 19:1, 39-51,1986 19

Figure 1.7 – a) Carpentier-Edwards Duralex porcine valve; b) Carpentier-Edwards Perimount pericardial. Source: J Long Term Eff Med Implants 11(3-4):151-61, 2001 ................................. 21

Figure 1.8 - a) ABIOMED trileaflet valve, b) ABIOMED next generation valve ....................... 26

Figure 2.1- Extended hypothetical model for the calcification of bioprosthetic tissue. Schoen and Levy, [c] ........................................................................................................................................ 46

Figure 3.1 – Valve Accelerated Life Tester (VALT) ................................................................... 58

Figure 3.2 - Angioflex® Valve: a) before the test, b) after the test................................................. 61

Figure 3.3 - a) SEM picture b) EDX result confirms the existence of Calcium and Phosphorous on the surface of the polyurethane........................................................................................................ 62

Figure 3.4 - a) old design trileaflet Angioflex® valve, b) new design trileaflet valve ............... 63

Figure 3.5 - SEM results of: a) Angioflex® valve's leaflet, b) BP-Angioflex® valve's leaflet...... 64

Figure 3.6 - EDX result corresponding to: a) figure 3.5a, b) figure 3.5b ................................. 65

Figure 3.7 - ICP results performed on one leaflet of four types of valves.............................. 66

Figure 3.8 - Angioflex® valve: a) before the test, b) after the test................................................. 68
Figure 4.14 - CFD results: a) Shear rate contour for low shear sample, b) Axial velocity contour for low shear sample, c) Shear rate contour for high shear sample and d) Axial velocity contour for high shear sample .................................................................................................................. 101

Figure 4.15 - Shear rate for low (Tube_1.25”) and high (Tube_0.625”) shear samples .......... 102

Figure 4.16 - Average Ca and P weight percentages for the low shear sample...................... 103

Figure 4.17 - Average Ca and P weight percentages for the high shear sample...................... 104

Figure 4.18 - Calcium content comparison between loop 1 (Test 1) and loop 2 (Test 2) for low shear model ................................................................................................................................. 107

Figure 4.19 - Calcium content comparison between loop 1 (Test 1) and loop 2 (Test 2) for high shear model ........................................................................................................................................ 108

Figure A - Structures of some common lipids. Source: www.wikipedia.org ...................... 115

Figure B – a) Phospholipid structure, b) Polar group of the molecule, highlighted in red. The U indicates the uncharged hydrophobic portion of the molecule, highlighted in blue. Source: www.wikipedia.org ......................................................................................................................................... 116

Figure C - a) Pyrophosphate anion, b) Ball-and-stick model of the pyrophosphate anion (P$_2$O$_7^{4-}$). Source: www.wikipedia.org ......................................................................................................................................... 117
LIST OF TABLES

Table 3.1- ICP results for Angioflex®, BP- Angioflex®, Deiwick’s tissue valves and Alferiev’s Biospan and BP-Biospan leaflets........................................................................................................... 71

Table 4.1 - Calcification contents concentration for polyurethane (PU) and polycarbonate (PC) samples in “weight percentage” ........................................................................................................... 88

Table 4.2 - EDX results for low and high shear samples ................................................................................................................................. 96

Table 4.3 - Calcification level comparison between surface defected samples and smooth sample from the low shear model ........................................................................................................... 104

Table 4.4 - Calcification level comparison between surface defected samples and smooth sample from the high shear model ........................................................................................................... 105
CHAPTER 1

“BACKGROUND“


1.1 Introduction

Valvular heart disease is known to be a significant cause of mortality worldwide. In the United States of America, about five million people are diagnosed with heart valve disease annually. Due to heart valve malfunctions, approximately 90,000 valve replacement operations were performed in 2004 [1, 2]. Some major etiologies of valvular heart disease include rheumatic fever or bacterial infection, medication side-effects or congenital abnormalities. The most common of the etiologies though remains valve degeneration due to calcification which tends to weaken the supportive structures of the heart valves. [3].

There are different methods to treat valvular heart disease depending on severity and type of diagnosis. In some cases, careful monitoring is sufficient enough while in other cases, prescribed medications can control the symptoms and slow down or even halt the progression of the disease. However, in severe cases, heart valve replacement surgeries are required. Heart valve replacement procedures, which have been used successfully since the 1960s, are safe and effective even though 10-year survival rate reviews still range from 37-58% [4]. The most commonly replaced of the heart valves, accounting for nearly 50% of all the replacements, is the aortic valve [5]. There are several biological and mechanical options for the replacements of the valves. These include biological types of cryopreserved homografts from cadavers or xenografts from animals, as well as mechanical options such as bileaflet tilting discs [6].

Cryopreserved homografts are categorized into allografts and autografts. Allografts are human heart valves which are obtained from a cadaver. These valves are immediately cryopreserved after being removed from the donor prior to implantation into the recipient.
Allografts are the best option for heart valve replacements; however, their use is limited because of their inadequate supply. In addition, autografts are an alternative to allografts. The use of autograft valves was pioneered by the Ross procedure. In this procedure, a patient’s diseased aortic valve is replaced by his/her pulmonary valve, which is then substituted by a cryopreserved cadaveric pulmonary valve. The drawback to this procedure is that it is highly dependent on the surgeon’s skills and the patient’s condition [6].

Diseased heart valves are commonly replaced by mechanical ones. This type of heart valve is durable but susceptible to blood clot formation requiring anticoagulation therapy. Anticoagulation therapy is necessary to reduce thromboembolic complications. Conversely, xenografts, or bioprosthetic heart valves, are made of either porcine valves or bovine pericardium. These valves are more biocompatible and less thrombogenic than mechanical valves, yet they too undergo tissue degeneration and failure over time due to immune response to the graft [7-9]. Therefore, xenograft valve durability ranges between 5 to 20 years, and can be even shorter in younger patients with more pronounced immune responses. This creates a need to re-operate sooner in these patients.

Although the various replacement valves have their own advantages and disadvantages, they all share a common weakness. They all lack the ability to grow and repair limiting their use especially in the pediatric population, whose organs themselves are still growing. This calls for designing a living valve which can adapt to the surrounding hemodynamics and remodel accordingly.

Recent advances in tissue engineering have encouraged scientists to design a tissue engineered (TE) heart valve. This is accomplished by taking a small biopsy of the necessary
tissue and expanding its cells in a culture process. The cells are then seeded onto a biodegradable scaffold which is designed to help the cells organize and develop into tissue which is then used to make a new heart valve or blood vessel that can replace its diseased counterpart [10]. TE valves should compensate for the shortcomings of the other types of valves. Because the cells are harvested from the recipient, they should be non-immunogenic. Furthermore, since TE valves will eventually be fabricated in a factory scale, there should be no concerns about supply. Most importantly, since these valves are made of living cells, they are able to grow and therefore, overcome the common drawback of the other types of valves. However, it still takes a very long time before a successful clinical trial TE valve, without any complex challenges, is fabricated.

Although considerable attention has been given to the making of TE valves, researchers are trying to find new substitute materials for heart valves. This material should require lower levels of anticoagulation compared to mechanical valves and have a longer life span than biologic tissue valves. Historically, polyurethanes are known to be very important when used with implantable devices. They are a family of segmented block copolymers composed of alternating polycarbonates or polyethers known as soft segments and ureas or urethanes often referred to as the hard segment. The ratio and chemistry of the hard and soft segment components can be altered, thus allowing polyurethanes to possess a broad range of physical properties, making these elastomeric polymers an attractive composite material for cardiovascular implants, such as artificial heart devices, and prosthetic heart valves [11].

For applications in prosthetic heart valves, the material used must be able to withstand many cycles of stress and deformation before failure. High tensile strength, abrasion and degradation resistance, flexing life and biocompatibility of polyurethanes have all led to their sustained use as a biomaterial. The use of a stable, durable material, such as polyurethane may
reduce the incidence of re-operation due to calcification and tissue valve failure. Another benefit of using this type of heart valve is the reduction of the cost burden on the health care system and further improvement in the survival rate of patients with heart valve prostheses due to decreased need to perform additional procedures.

Current research is focused on evaluating a specific type of polyetherurethane, Angioflex®, as a possible design material for replacement heart valves. Angioflex® has already demonstrated excellent durability and hemocompatibility in previous clinical evaluations. A thorough literature review discussing anatomic features of the heart itself, the aortic valve, valve replacement options and an overview of previous and present studies in this field is presented in this chapter.

1.2 The Heart and the Aortic Valve

1.2.1 The Heart

Heart is one of the most important organs in a human body (Figure 1.1). The heart consists of four chambers: the right and left atrium, the right and left ventricle (LV). Its primary function is to pump deoxygenated blood to the lungs to be re-oxygenated and then have the newly oxygen-rich blood pumped back to the rest of the body. Blood flow through the heart is regulated by means of four one-way valves: aortic valve, pulmonary valve, mitral and tricuspid valves [12].

Arteries bring the needed oxygen-rich blood to all the body’s organs and tissues. Once oxygen has been taken out of the circulating blood, veins bring this deoxygenated blood back to the heart’s right atrium through the superior and inferior vena cava. The right atrium contracts to
force the blood down to the right ventricle through the tricuspid valve. Once the right ventricle fills, it contracts forcefully to move the blood through the pulmonary semilunar valve into the pulmonary artery and out to the lungs. Then, the lungs re-oxygenate the blood which is then ready to travel back to the heart via the pulmonary veins that spill into the left atrium. Upon filling this chamber, blood is then pushed through the mitral valve into the left ventricle (LV). Finally, filling of the LV causes a forceful contraction needed to release blood through the aortic semilunar valve out to the large aorta that then splits to smaller arteries to deliver the newly oxygenated blood to the body once more to continue the cycle. [13].

Figure 1.1 - Heart anatomy, arrows indicate blood flow direction
Source: www.tmc.edu/thi/anatomy.html
One contraction and one dilation of the heart make a cardiac cycle, which lasts about 860 ms, with a ratio of contraction (systole) to dilation (diastole) of 1:2 [14]. In an average person at rest, the heart pumps out a total of 5 L/min (the cardiac output) with a rate of 70 bpm, and a pressure of 120 mmHg (systolic) / 80 mmHg (diastolic). During an average lifespan of 70 years, considering a heartbeat of 70 bpm, the aortic valve opens and closes three billion times [40].

1.2.2 The Aortic Valve

1.2.2.1 Aortic Valve Anatomy and Biomechanical Properties

The aortic valve (AV) regulates blood flow between the aorta and LV and is located at the aortic root (Figure 1.2). The AV consists of three leaflets, which open and close in a synchronized motion during each cardiac cycle. These leaflets meet at the commissures and based on their anatomical positions are called: left coronary, right coronary and non-coronary leaflets (Figure 1.3). For a normal aortic valve, it appears that the three leaflets are equally sized; however, over 50 percent of patients have one valve leaflet slightly larger than the others. Also, there is a trend that larger-sized hearts have larger valves. There is a bulging cavity behind each leaflet which is known as the Sinus of Valsalva and is defined as the area between the valve leaflet edge and the aorta when the valve opens. On the contrary to non-coronary sinus, which does not feed a coronary artery, the left and right coronary sinuses contain astia opening into the left and right coronary arteries. For a proper function of an aortic valve, these structural components are very important [15-18].

Each valve leaflet is composed of three connective tissue layers of mainly collagen, elastin, and glycosaminoglycans. These layers include the fibrosa or arteriosa, the spongiosa, and
the ventricularis. It can be seen from Figure 1.4 that the ventricular and arterial sides of the aortic leaflet are continuous with those of the ventricular and aortic walls [19].

Figure 1.2 - Anatomic relationship between the aortic valve and surrounding structures
Source: http://cardiacsurgery.ctsnetbooks.org/cgi/content/full/3/2008/825/F2

Figure 1.3 - A schematic diagram of the relationship of the aortic valve leaflets to the structures underlying the commissures. Source: http://cardiacsurgery.ctsnetbooks.org/cgi/content/full/2/2003/791/F2
The ventricular side of each aortic valve cusp contains elastin-rich fibers. These sides are aligned in a radial direction and are perpendicular to the leaflet free margin. The purpose of elastin, which is mechanically coupled with collagen, in the aortic valve leaflet is to maintain a specific collagen fiber configuration. Also, once the external forces of blood flow subside, elastin returns the fibers to their initial state [20].

The aortic side contains a collagen-rich layer referred to as the *corrugated fibrosa*. These fibers are arranged in a circumferential direction and are in a relaxed state. The middle layer, referred to as the *spongiosa*, consists mainly of loose connective tissue. These principal layers of the aortic leaflet provide the necessary biomechanical properties for proper valve function.

On the arteriosa (fibrosa) side of the valve leaflet, endothelial cells are present. In an artery, endothelial cells are aligned in the direction of blood flow because flow creates the most
stress. However, endothelial cells on the aortic valve leaflet are arranged in a circumferential
pattern, or perpendicular to blood flow, causing the major stress in the valve, as opposed to shear
stress across the valve [21].

The mechanical properties of the aortic valve must allow the valve to open with minimal
transvalvular pressure differences and to close completely with minimal flow reversal. Although
these functional requirements might seem simple, durability must be provided by mechanical
properties. Large stresses are generated within the leaflets due to the pressure drop across the
aortic valve. These stresses are too great for the leaflets and must be distributed to the fibrous
skeleton of surrounding structures by the valve anatomy [19]

For an aortic valve to close properly during diastole, leaflet strains should not be equal in
all directions, which causes the valve leaflets to demonstrate anisotropic properties. Differences
in strain properties allow the cusps to stretch during closure and to completely close along the
free edges. Strain distribution is in two directions: circumferential in blood flow direction, and
radial or perpendicular to blood flow. Circumferential stiffness is higher than radial stiffness.
These anisotropic properties allow the valve leaflet to stretch in a radial direction, considering
the fact that moving downward is relatively restricted. This helps leaflet closing and sealing
during diastole. The corrugated property of the fibrosa layer of the valve leaflet (Figure 1.4)
allows it to stretch in a radial direction and enables each leaflet to billow toward the other
leaflets. These properties do not prevent the fibrosa layer from stretching, even though this layer
is considered to be the principal load-bearing layer. The ventricularis layer makes up for the
stiffness in the circumferential direction [19].
The endothelial cells on the fibrosa layer of the leaflets are oriented in a radial direction, same as the principal stressors. Most of the leaflet stress is concentrated at the interface between the two coapting edges of the cusp. Each leaflet reduces the stress of the other by providing a mutual coaptive support. These stresses are then distributed along the leaflet edges to corners of the commissures. Further stress reduction is also achieved by the interaction of the sinus of Valsalva with the leaflet assembly. Changing from systole to diastole reduces the radius of curvature for the sinus of Valsalva by approximately 16 percent [18]. This change in radius of curvature distributes the stresses within the sinus in accordance with the Laplace formula. In diastole, where the valve leaflets close, an inward bending of each sinus at the commissural attachments and an outward bowing of the aortic wall within the sinus between commissures occur. This helps decrease the radius of curvature in the aortic wall of the sinus and verifies that each sinus of Valsalva shares the stresses during diastole [40].

Therefore, valve durability is somehow dependent on the mechanisms of stress reduction. Abnormalities can reduce this stress amelioration by creating imperfect closings of the valve leaflets or or other innate anatomic differences. These abnormalities which decrease stress reduction capability will in turn lead to further damage to the valve leaflet endothelium and progressive valve functional deterioration [40].

1.2.2.2 Mechanics of Movement

The opening and closing of the aortic valve is a response to pressure fluctuations of the cardiac cycle and pressure differences between the ventricular chamber and the aorta comprising a passive mechanism. In other words, the principal component behind the opening and closing of a valve is the pressure difference between the ventricle and the aorta. In normal conditions, the
valve leaflets offer little obstruction to blood flow because of the equality of the leaflets’ specific gravity to that of blood [22].

**Opening:** During diastole, the pressure difference between the ventricle and the aorta puts valve leaflets under stress. These stresses toward the central portion of the aortic opening cause the base of the aortic root to constrict. An additional contributor to this decrease in the base diameter is the elastic property of the aortic root. During late diastole, as blood fills the ventricle, the aortic root expands by 12 percent. This occurs approximately 20 to 40 ms prior to aortic valve opening [23]. This slight expansion in the aortic root minimizes the valve leaflets’ apposition by flattening them. Increasing pressure in the ventricular outflow tract causes a decrease in the tension across the leaflets until a certain point where the pressure difference across the valve leaflets becomes minimal and the tension within the leaflets disappears [24]. At this point, the aortic root expands to allow the valve to open rapidly at the beginning of ejection. Ejection takes place with an upward movement of straightened leaflets, and the angle at their bases becomes more acute [25]. These mechanisms permit the valve to offer minimal resistance to ejection and open quickly.

**Closing:** The closure mechanism of an aortic valve is very elegant and involves the vortex theory [26]. In the vortex theory, the sinus of Valsalva plays an important role in providing a reservoir for small vortices to develop which allow opened valve leaflets to fully expand. However, the vortices maintain a space between the aortic wall and the edge of the leaflet helping the reversal of flow to provide instant closure at the end of systole.

According to Mercer, [25], “as ejection occurs, deceleration of blood at the stream edge creates small eddy currents or vortices. These small vortices along the aortic wall
gradually move toward the base of the ventricular arterial junction to the edge of the leaflet and top of the sinus of Valsalva. As flow declines at end-systole, the pressure difference across the opened aortic valve leaflet also decreases. At the end of ejection and prior to valve closure, the vortices within the sinus of Valsalva balloon the valve leaflets toward the center of the aorta. The angle at the base of the leaflet becomes more obtuse and rounded, in contrast to the very sharp angle at maximal valve opening. This point of flexure begins to move up the valve leaflet and eventually terminates at the free margin of the valve cusp”.

Therefore, during the ejection, the mechanism of valve closure begins while vortices develop within the sinus of Valsalva and enable the valve leaflets to close. When there is no pressure difference between the aorta and the ventricular outflow tract, a small amount of flow begins to reverse, because the ejected blood decelerates, and therefore leaflets close rapidly.

**Rheology:** Unlike a laminar flow through a pipe at constant rate, blood flow across the aortic valve is pulsatile. There are different factors that make the analysis of flow characteristics difficult. Two of these factors include differences in the structural components above and below the aortic valve among patients, and variability in dynamics of the valve mechanism and rate of ejection force. Although such factors limit the flow characteristics analysis, certain characteristics can be described for normal aortic flow rheology.

When the ventricle contracts, blood pressure increases within the chamber. As this pressurized blood moves through the ventricular outflow tract, which acts as a funnel, velocity increases. This increase continues until the blood is ejected at the aortic valve. The flow, which goes from the relatively fixed aortic valve ring to the slightly larger aorta, would not be laminar and causes a ventricular flow profile.
A skewed peak systolic velocity profile is produced by ejected blood with variable location along the aortic wall [27]. At the end of the ejection, this velocity profile becomes blunted. The blood column along the aortic wall has a relatively low velocity, and because the size of the aorta is usually greater than the effective valve orifice, the ejection flow pattern interacts with this column. Turbulence is the result of the interactions between blood flow moving at varying velocities. Turbulence is defined as random velocities in more than one plane. This definition explains the existence of some turbulence; however, the degree of turbulence is two-fold and is directly proportional to the interface between ejected blood and the relatively stagnant blood in the aorta and the velocity of ejected blood.

The position of the leaflets during the process of blood ejection through the valve orifice reduces turbulence. This happens when sinuses dilate to produce an aortic root with uniform diameter (Figure 1.5). Under normal conditions, the effective valve orifice is smaller than the aorta. Turbulence decreases when the flow moving through the valve orifice reaches the aortic wall. At this point the interaction between high-velocity and stagnant blood disappears.
Turbulence occurs when low and high velocity blood interact with each other. Energy transfer is the result of this interaction with the density of up to 14 times greater in highly turbulent blood as compared to normal blood turbulence. The produced energy density transfers to the surrounding structures, especially the endothelium of the aorta. However, damage due to turbulence does not stop at this point and penetrates to intimal surfaces possibly causing platelet deposition, intimal thickening, and subsequent thrombosis [28].
According to Mihaljevic et al., [29], “Throughout systole and early diastole, a bidirectional velocity profile is present. Retrograde velocities present around the wall of the aorta subsequently contribute to closure of the aortic valve leaflets. Bidirectional velocity is present under normal circumstances; however, if it is severe, it may contribute to early systolic closure of the aortic valve”.

1.2.3 Mechanical Forces on the Aortic Valve

Physiologically, the human aortic valve is exposed to a harsh and diverse mechanical environment. The major forces include diastolic pressure, shear and bending stresses which are briefly described in the subsequent sections.

1.2.3.1 Diastolic Pressure

For a normal aortic valve, the average pressure during diastole is about 100 mmHg, which causes tensile stress on the leaflets mainly affecting the collagen fibers in the fibrosa [30]. Although the diastolic pressure is well known, there are no exact values for the resulting stress and/or strain within the leaflet. Thubrikar et al. [15] have estimated a circumferential stress of 2.4 MPa during diastole by means of the radiopaque marker technique and membrane stress analysis.

Numerical simulations on bioprosthetic valves with a 120 mmHg pressure load suggest that the forces along the cusps are transmitted through compressive and tensile forces [31]. In stented bioprosthetic xenografts, compressive stresses up to 0.1 MPa near the base of the stent post and tensile stresses up to 0.5 MPa at the junctions between two leaflets were found. For stentless human allografts, tensile stresses up to 0.5 MPa with no compressive stresses were
observed. By using magnetic resonance imaging (MRI) defined geometry, Grande et al. [32] determined a peak tensile stress of 0.5 MPa and a maximum strain of 15% in human aortic valve.

1.2.3.2 Bending

Bending stresses are the result of leaflet motion during the course of a cardiac cycle [15]. Bending stresses will result in internal shear when the ventricularis and fibrosa layers are deformed. The layer between these two, spongiosa, acts as a lubricant which prevents tissue damage due to their relative motion.

There are two modes of bending in each leaflet. First, during valve opening and closing, the belly of the leaflet undergoes reversal of curvature. Active protein synthesis may be a result of bending in this region [33]. Secondly, the attachment zone acts as a hinge during leaflet motion. A couple of studies have been performed using radiopaque markers. There is a report for circumferential bending strains of 2% in systole and 2.2% in diastole. Also another similar study has estimated bending strains to be about 5.1% [35, 36].

Some detailed hemodynamic in vitro experiments under physiological pulsatile flow conditions have been performed on trileaflet polyurethane valves. The leaflets were marked with a grid and videotaped during the experiment. Shell bending theory and physical properties of polyurethane have been used to determine the bending strain and stress from leaflet curvature. Maximum bending stress, 1.22 MPA, and maximum bending strain, 14.5%, were observed during the opening phase of the leaflets. During the closing phase, maximum strain of 8.3% and maximum stress of 0.71 MPa were observed. Both bending stress and strain were shown to be minimal at peak systole (6.9% strain, 0.58 MPa stress). Porcine aortic valves which were tested under similar conditions demonstrated a higher degree of curvature [37, 38], but the authors did not address bending stresses since the physical properties of the leaflet’s tissue were unknown.
1.2.3.3 Shear Stress

As blood ejects through the aortic valve during systole, both surfaces of the leaflets experience shear stress. These stresses are different because of the differences in flow profiles. Ventricular side flow profile is pulsatile (only present during systole) and laminar. The flow on the aortic side is also laminar, but its magnitude is much smaller than the ventricular side. The average shear stress on the ventricular surface of the aortic valve during systole was estimated to be 12-40 dynes/cm², with peak values up to 80-100 dynes/cm² [39].

1.3 Aortic Heart Valve Disease

There are numerous diseases and malfunctions of the heart valves that prevent the proper flow of blood. Heart valve diseases fall into two major categories: stenosis (or narrowing) and incompetence (or regurgitation). The stenotic heart valve prevents the valve to open fully, resulting in an obstruction of the flow due to stiffened valve tissue. In this case, more work is required to push blood through the valve. The three major causes for aortic stenosis are calcified aortic stenosis, rheumatic aortic stenosis, and degenerative aortic stenosis. Incompetence (or regurgitation) causes inefficient blood circulation by permitting backflow of blood to the left ventricle during diastole. Some of the causes for aortic valve regurgitation include cusp prolapse, dilation of sinus aorta, cystic medial necrosis, and coaptation failure of the cusps [13, 40].

1.4 Aortic Valve Treatments and Surgical Interventions

Treatment for heart valve disease depends on the type and severity of the diagnosis. On a large scale, medication is the best alternative and can control the symptoms by slowing or halting
disease progression. In some cases, only careful monitoring is needed; however, more serious cases require surgery to repair or replace defective valves in order for the patient to live a normal life. Currently, there are four major options for aortic valve replacement: mechanical, bioprosthetic, allograft and autograft valves [40].

1.4.1 Mechanical Valves

Nearly half of the heart valve replacements are performed with mechanical valves. Dr. Charles Hufnagel implanted the first caged ball valve for aortic insufficiency in 1952. This was the first clinical use of a cardiac valvular prosthesis. Since then, 50 different designs for mechanical valves have originated worldwide. Current mechanical valves, primarily composed of metal or carbon alloys, are classified according to their structure as caged-ball, single-tilting-disk, or bileaflet-tilting-disk valves (Figure 1.6). The bileaflet valve accounts for the majority (about 80%) of implanted mechanical valves [41].

The three types of mechanical valves mentioned are better received than other designs because their central flow pattern is close to that of a natural heart. All mechanical heart valve
prostheses have three major components in common: the occluder (such as a ball, a disc or a hinged leaflet), the housing, and the sewing ring [42].

Artificial implants are known to trigger inflammatory responses in the body that can eventually cause coagulation in the blood stream, which is called blood clot or thrombosis. Mechanical heart valves are widely used because of their lifelong durability; however, their potential for causing thrombosis requires recipients to be under life-long anti-coagulation therapy.

1.4.2 Bioprosthetic Valves

The next important category for aortic valve replacements is the bioprosthetic valve. Bioprosthetic valves are made of biological material, such as a fixed porcine valve or bovine pericardium, and account for about 40% of replacement valves (Figure 1.7). In contrast to mechanical valves, bioprosthetic valves are hemocompatible and hence have lower likelihood of producing thrombi. In addition, their central flow pattern is more similar to natural aortic valves. Yet, because the valves are of animal material means they have the potential to produce an immune response, and hence must be treated to prevent rejection of the graft. Therefore, the valves are pretreated with glutaraldehyde in order to cross-link the surface antigens to prevent triggering immune cells from attacking. This type of treatment though causes changes in the mechanical properties and tissue microstructure of the valves [43].
Currently, there are three major types of glutaraldehyde-pretreated bioprosthetic valves available on the market: stented porcine, bovine pericardial aortic valve, and stentless porcine aortic valve. Out of the three, stentless valves seem to have a major advantage over stented valves by giving the possibility for larger valves to be implanted, and therefore, through enhancement of hemodynamic processes and ventricular remodeling, possibly increasing patient survival [43].

A disadvantage to bioprosthetic valves, compared to mechanical ones, is their limited lifespan on average of 7-15 years. The major cause of the valve’s failure is calcium build-up which stiffens the animal tissue, therefore restricting blood flow or tearing of leaflets. Bioprosthetic valves are more suitable for older patients because of their lower calcium metabolism compared to younger patients. Recent research on bioprosthetic valves has been focused on the following areas: improvement of the fixation methods (e.g. zero fixation or dynamic fixation, using tissue fixatives other than glutaraldehyde); developmental strategies to inhibit calcification such as removal of a calcifiable component of the biomaterial and decellularization; the remodeling of stentless valves [43].
1.4.3 Allograft Valves

Allografts are human aortic valves obtained from cadavers. After being explanted from the cadaver, allografts are sterilized by incubating in antibiotics. This procedure is followed by cryopreservation (without chemical cross-linking) by freezing with protection from crystallization by dimethyl sulfoxide and storing them at -196°C in the vapor over liquid nitrogen. One of the benefits of allografts is that they are implanted directly into the aortic root without a stent. Aortic valve allografts have exceptionally good hemodynamic profiles and are rarely vulnerable to thromboembolism without anti-coagulation. These valves do not degenerate, and replacement is not needed for up to 10-15 years. This superior performance was once attributed to the initial fibroblast viability at implantation [44-46]. However, this theory was disputed when further studies showed that normal structural complexity and cellularity in allograft valves was lost soon after implantation [47].

1.4.4 Autograft Valves

Autografts are an alternative to allografts and originated from the Ross procedure. In this procedure, patient’s aortic valve is replaced by his/her pulmonary valve, for which an allograft pulmonary valve is used to replace it. The Ross procedure has proven to be the best way of valve replacement, which results in a long term survival and freedom from complications for patients with aortic valve disease. After 20 years, only 15% of patients required additional valve procedures. In cases where a human pulmonary artery homograft is used to replace the patients’ pulmonary valve, freedom from failure has been 94% after 5 years, and 83% at 20 years. The tissues of the patients’ pulmonary valve have not shown a tendency to calcify, degenerate, perforate, or develop leakage [43]. This procedure is particularly more beneficial for children
and young adults and offers several advantages over traditional aortic valve replacement with manufactured prostheses. Longer life span of the pulmonary autograft in the aortic position is one of the advantages compared to bioprostheses such as the porcine valves. A drawback of this procedure is that it is highly dependent on the surgeon’s operating skills and patient’s condition in handling two-valve surgery at one time.

1.4.5 Tissue Engineered Heart Valves

Over the past 20 years, physicians and scientists have proposed the tissue-engineered heart valves to be the ultimate solution for valvular heart diseases. The advantage of using this type of valve, compared to the available prosthesis, is its being a living organ and can respond to growth and physiological forces like a native valve. Over the past 10-15 years, two main approaches have been attempted towards making a tissue engineered valve: regeneration and repopulation. “Regeneration involves the implantation of a resorbable matrix that is expected to remodel in vivo and yield a functional valve composed of the cells and connective tissue proteins of the patient. Repopulation involves implanting a whole porcine aortic valve that has been previously cleaned of all pig cells, leaving an intact, mechanically sound connective tissue matrix. The cells of the patients are expected to repopulate and revitalize the acellular matrix, creating living tissue that already has the complex microstructure necessary for proper function and durability [48].” Therefore, though the field is more than 20 years old, it will take more time to develop a successful clinical trial that resolved all the complex challenges.
1.4.6 Polymeric Heart Valves

Polymers are biocompatible materials which have desirable hemodynamic characteristics similar to those found in the human body. This biocompatibility has led to the polymers being more readily integrated into their surrounding with no additional consequences for life-long anticoagulation therapy to prevent rejection. [49].

Starting in 1958, _in vivo_ and _in vitro_ studies have been performed with flexible polymeric heart valves. These valves were among the first prostheses that underwent human implantation. Roe was the first one who implanted a silicon rubber polymeric valve in a human body. However, the study had to be interrupted because of high morbidity and mortality due to embolism [50]. During the period of 1961 to 1963, Braunwald et al. performed aortic heart valve replacements with tricuspid polytetrafluoroethylene (PTFE) prostheses in 23 patients. Again, the rate of mortality was high, valves were severely thickened and leaflets were ripped at explants [51].

Following these attempts, in 1966 Roe tried another type of polymeric material in aortic valve implantation. This time valves were made of Dacron and silicone; however, out of 18 patients only 4 survived between 33 and 61 months [52]. Furthermore, in 1977, Hufnagel used Dacron as the base material for a single leaflet aortic valve replacement. Most valves failed, but some patients survived up to 15 years [53]. Thereafter, more studies have been conducted, but all of them have failed due to complications from embolism, fatigue and calcification.

Among all the polymeric materials used in clinical settings, polyurethanes have paved their way to being the best choice for implant applications. Biocompatibility, high tensile strength, lubricity, abrasion resistance, ease of handling, resistance to thromboembolism and
good flex fatigue life are some of the reasons why polyurethanes are used for a variety of medical devices [49, 54].

Current scientific data related to polymeric heart valves shows their susceptibility to biodegradation and mineralization, which limits the use of these valves, in particular polyurethanes, for long term implants. Typically, polyester or polyether-based formulations have been used as medical polyurethanes. When the ether linkages are oxidized, cellular processes degrade the polyether urethanes by hydrolosis. This degradation eventually leads to failure of the prosthetic valve due to loss of tensile strength as a result of surface cracking and loss of molecular weight [49].

In order to achieve long term durability for polyurethane valves, an adequate design is absolutely necessary. One of the most important factors that affects the functionality and durability of a polymeric valve is the leaflet thickness. Thick leaflets with minimal regurgitation produce high pressure gradients across the valve and result in the failure of the valve due to fatigue [49].

To date, no approved polyurethane heart valve has been developed for use as replacement. ABIOMED, Inc. is one of the leading cardiovascular bioengineering corporations that manufacture a trileaflet polymeric valve, among its other products, for use with cardiac assist and replacement devices. This valve is fabricated from smooth elastomers with no seam between the cusps and conduit walls (Figure 1.8-a). It is designed and configured for use in an artificial device and has demonstrated excellent durability and hemocompatibility in clinical evaluation of the AbioCor replacement heart. The changes necessary for adapting the design of this valve to the requirements of a prosthetic heart valve have been ABIOMED’s concern for years. Currently, they have introduced a prosthesis, inspired by their trileaflet valve design, which is under in vitro and in vivo evaluations (Figure
1.8-b). This valve is fabricated from Angioflex®, proprietary highly purified polyurethane. Angioflex® has already demonstrated excellent durability and hemocompatibility in previous clinical evaluations.

![Figure 1.8 - a) ABIOMED trileaflet valve, b) ABIOMED next generation valve](image)

### 1.5 Objective

Current options for heart valve replacement procedures carry negative points due to the very fact that they are fabricated from foreign materials. This fact may cause risk of infections and thromboembolism. For thromboembolic complications associated with mechanical valve replacements, anticoagulants are necessary. On the other hand, biological substitutes are less thrombogenic than mechanical valves, though they inevitably undergo tissue degradation and valve failure much faster over time.

Besides extensive research regarding tissue engineered heart valves, researchers are trying to find a new material for heart valve prostheses. To be competent, this material should be as durable as mechanical valves and as biocompatible as tissue valves. The present studies presented here will build on prior investigations in the field of aortic heart valve replacement while focusing on ABIOMED’s trileaflet Angioflex® valve. With continuous successful
experiences using this material in cardiac assist devices, it is anticipated that Angioflex® may have the potential to be considered as a base material for polymeric heart valve substitutes. In this project, different experiments have been performed on ABIOMED’s polyurethane valves to test their calcification -, as well as, its comparable life span to that of -mechanical and tissue valves.

The expectation from this polymeric valve is the decrease in calcification levels. In vitro studies have been performed to verify the viability of this statement. Polymeric heart valves have been cyclically loaded by means of a valve accelerated life tester within a synthetic calcification solution. Additional experiments under static conditions (without mechanical loading) have been performed to verify calcification dependency on material, surface profile, and shear rates. The results of these studies are reported in the following chapters.
1.6 References


CHAPTER 2

“CALCIFICATION“
2.1 Abstract

Calcium and phosphorous are the most abundant elements in a human body. 99% of the total calcium content and 85% of the total phosphorous content in a human body reside in the skeleton (bone, teeth). The rest of these elements are distributed through intracellular and extracellular fluids and in soft tissue. Under controlled conditions, calcium is released from the bone into the bloodstream and transported from there into the body. This calcium is either in the form of dissolved ions or bound proteins. Both deficient and excessive amounts of calcium and phosphorous would put one’s health in danger. Vascular calcification is one of the problems accompanied by excessive amount of calcium in human intracellular or extracellular fluids. Vascular calcification can be destructive and lead to organ dysfunction.

In the past, vascular calcification was considered to be a passive and degenerative process but this conception has changed over time and now it is recognized as a regulated active process. Based on this belief, four different mechanisms for initiating vascular calcification have been proposed: 1) Activation of osteogenic mechanisms in the vascular wall, 2) Loss of inhibitory factors, 3) Enhanced bone turnover, and 4) Irregularities in mineral metabolism.

Aortic calcification is a kind of vascular calcification through which calcium deposits build up on the aortic valve leaflets. The calcium deposits thicken and cause narrowing at the opening of the aortic valve. This impairs blood flow through the valve, causing chest pain or a heart attack. In severe cases, patients should undergo surgeries and their aortic heart valve should be replaced with mechanical or bioprosthetic heart valves. Replacement valves, however, are subject to same calcification whether they are made up of tissue, or polymers. Although some
mechanisms have been proposed (as discussed below), the calcification in bioprosthetic or polymeric valves is not yet completely understood. The calcification mechanism for bioprosthetic or polymeric valves is not yet completely understood; however some mechanisms have been proposed for their calcification process.

Bioprosthetic heart valves should be pretreated with glutaraldehyde before being put inside a human heart. This is to make them immunologically inert and improve the tissue durability. It is believed that the glutaraldehyde fixation process leads to calcification of these types of valves. The glutaraldehyde pretreated cells become nonviable and produce the primary sites for calcification. Calcium phosphate is the calcification mineral which deposits through the reaction of the calcium in extracellular fluid with the membrane-associated phosphorus.

Different mechanisms have been suggested for polymeric valves; but the most sensible hypothesis has been proposed by Kou Imachi et al. They believe that calcification of a polymeric heart valve is due to entrapment of blood proteins or phospholipids in the microgaps of the polymer’s molecules. These microgaps are produced due to the repeated stretching stresses and the trapped elements inside them attract calcium ions followed by phosphate ions to make their complexations, as calcium phosphate ($\text{Ca}_3(\text{PO}_4)_2$).

### 2.2 Details of the Calcification Process

#### 2.2.1 Introduction

Calcium in the form of ion ($\text{Ca}^{2+}$) has an important role in the biochemistry and physiology of organisms and is essential for the normal function of cells. Calcium is the most abundant element in the human body. About 99% of the human body’s total calcium content
resides in the skeleton (bone, teeth) and the other 1% is distributed through intracellular and extracellular fluids and in soft tissues. Structural integrity of the bone is provided by calcium salts, which exist largely in the form of hydroxyapatite (Ca$_{10}$(PO$_4$)$_6$(OH)$_2$) [1, 2]. “The primary functions of the calcium in the body include:

1. To mineralize teeth mineral components
2. To facilitates interaction between proteins or between proteins and phospholipids (refer to appendix) in cell membrane
3. To participate blood clotting, nerve impulse transmission, muscle contraction, enzyme regulation, and membrane permeability
4. To be the major structural element in bones and teeth. The mineral component of bone consists mainly of nerve impulse transmission, which contains large amounts of calcium and phosphate. Bone is a dynamic tissue that is remodeled throughout life
5. To mediate the constriction and relaxation of blood vessels, and the secretion of hormones” [2].

In mammals, bone is considered to act as the main storage site for minerals. Because of this characteristic of the bone, it tightly regulates the calcium levels. Under controlled conditions, Ca$^{2+}$ is released from the bone into the bloodstream and transported from there into the body. This calcium is either in the form of dissolved ions or bound proteins such as serum albumin (the most abundant plasma protein in mammals). There are also other factors that can affect the calcium levels, one of which is parathyroid hormone. Parathyroid gland secretes the parathyroid hormone, which regulates the resorption of calcium ion from bone [3].

Phosphorous (P) is the second most abundant element in the human body. About 85% of the total human body’s phosphorous content resides in the skeleton while the remainder of it is
associated with organic substances of soft tissues [3]. The bone phosphorous is in the form of a compound, calcium phosphate and the crystal hydroxyapatite, which are involved with the bone formation process. Other phosphorus is found in extracellular and intracellular fluid [2]. There are always organic phosphate compounds present in every animal cell’s structural unit, while the calcium associated component in bone and teeth is the inorganic phosphate [1].

Above discussion demonstrated the need for calcium and phosphorous in a human body. Insufficient amounts of these elements in the body can bring up unwanted symptoms. For instance, calcium insufficiency in the blood causes hypocalcemic tetany, a symptom manifested by intermittent muscle contractions, muscle pain, muscle spasm and osteoporosis in adults. Phosphorous deficiency is a rare situation; however if in any case happens, it may lead to myopathy, weakness, and cardiomyopathy [2].

Not only calcium and phosphorous deficiencies in the human body puts one’s health in danger, but also their excess amount is not good either. For example, if calcium enters a cell in excessive amount, it may damage the cell and even cause it to die by necrosis. In order to maintain a normal body function, these elements as well as the rest of the nutrients in the body should cooperatively work together [2, 3].

Having a brief knowledge about the calcium and phosphorous contents in the human body makes it easier to describe the “Calcification” process. Calcification is a process through which calcium or calcium salts, such as hydroxyapatite (Ca_{10}(PO_{4})_{6}(OH)_{2}), gets accumulated in a body tissue.

There are two descriptions for calcification phenomenon. When calcification occurs in functional soft tissues, it is considered to be pathologic or “abnormal”. On the other hand if it
occurs in skeletal tissues, it is called physiologic or “normal”. Abnormal calcification is categorized as “Dystrophic” and “Metastatic”. Dystrophic calcification occurs in case of necrotic, damaged tissues in hosts with normal calcium concentrations in their blood [3-5]. On the other hand, metastatic calcification takes place in normal tissues by deposition of calcification elements as a result of elevated serum levels of calcium and phosphorous in blood and tissue fluids [1]. According to Srivatsa et al., [6], “Dystrophic calcification has been characterized as haphazard or unregulated and is proposed to be an undesired but common feature of degenerative or inflammatory tissue changes throughout the body”.

Cardiac calcification is one of the common types of calcification and can be either dystrophic or metastatic based on the situation. Cardiac calcification occurs in different parts of the heart and can cause any of the: pericardial, myocardial, left atrial, annular, coronary (artery) and vascular calcification [7].

A number of diseases are associated with vascular calcification such as End Stage Renal Disease (ESRD) and cardiovascular disease. There are three different locations for vascular calcification: in intima, in the tunica media (also known as Monckeberg’s medial sclerosis) and in the cardiac valves. Atherosclerotic lesions are susceptible to intimal calcification, whereas medial calcification is related to vascular stiffening and arteriosclerosis observed with age, diabetes, and ESRD. Intimal and medial calcification may occur independently or together, such as in patients with ESRD. Calcium phosphate deposition is the sign for vascular calcification. The deposit is in the form of bioapatite and can accumulate in the blood vessels, myocardium and cardiac valves. Calcified deposits in blood vessels are found in distinct layers and are associated with underlying pathology [8, 9].
Blood vessel calcification has been historically considered as a benign disease that is commonly seen with ESRD, aging, diabetes and atherosclerosis. However, developments in noninvasive measurement techniques for vascular calcification, has changed our conception about the risks of this disease [8].

Vascular calcification can be destructive and lead to organ dysfunction; however, it depends on its extent and the organ which has been affected by that. In the heart, heart valve’s leaflet calcification in either native or bioprosthetic valve, is recognized as a major mode of failure [10, 11]. In dialysis patients, vascular medial calcification is responsible for calcific uremic arteriolopathy, a necrotizing skin condition associated with extremely high mortality rates [12]. According to Giachelli, [8], “Finally, a genetic deficiency in pyrophosphate (refer to appendix) levels cause idiopathic infantile arterial calcification, a disease characterized by arterial calcification, fibrosis, and stenosis that leads to premature death in affected neonates”.

In the past, vascular calcification was considered to be a passive and degenerative process associated with atherosclerosis and aging [9, 14]. However, according to Shanahan et al., [14], “vascular calcification is now recognized as a pathobiological process sharing many features with embryonic bone formation”. It has been argued that there is a universal mechanism for the calcification of native human tissue, porcine tissue, and synthetic biomaterials. This mechanism is independent of substrate which is susceptible to modulation by host and biomechanical factors [6].

The focus of this study is mostly on valvular calcification, as part of the vascular calcification, especially in the aortic position. Although artificial heart valves and a variety of anticalcification therapies have developed over the years, but prosthetic heart valve calcification
is still poorly understood and remain the main obstacle for a life-long function of these valves which makes it hard to develop treatment strategies and effective therapies [6].

2.2.2 Mechanism of Vascular Calcification

Unlike the old conception of vascular calcification as a passive process, it is currently considered to be an actively regulated one. Vascular calcification may arise by several different and non–mutually exclusive mechanisms [8, 13].

Generally, these active processes recapitulate osteogenesis as in bone. For simplification and a better understanding, four different mechanisms for initiating vascular calcification have been proposed: 1) Activation of osteogenic mechanisms in the vascular wall, 2) Loss of inhibitory factors, 3) Enhanced bone turnover, and 4) Irregularities in mineral metabolism [8, 9].

2.2.2.1 Activation of Osteogenic Mechanism in the Vessel Wall

In the process of Vascular Calcification (VC), the cells and proteins are either directly or indirectly involved. These cells and proteins normally maintain the balance in bone and mineral metabolism [14, 15]. According to Dellegrottaglie et al., [9], “Many of these regulators, such as osteonectin, osteopontin (OPN), parathyroid hormone (PTH), bone morphogenic protein (BMP)-2, matrix γ-carboxyglutamic acid protein (MGP), are expressed in atherosclerotic plaques as well as in sites of medial arterial calcification". At sites of vessel wall calcification and in the circulation, osteoblast-like cells (cells that make bone) as well as tartrate resistant acid phosphatase (TRAP), which contains cells resembling osteoclasts (cells responsible in bone resorption) can be found. This observation further confirms the commonality of the cellular
determinants of bone formation, either in the vessel wall or in bone. Therefore, factors involved in pathologic VC can be appreciated if the underlying mechanism in the regulating of bone metabolism is understood [9].

2.2.2.2 Loss of Molecular Inhibitors of Calcification

VC, like most biological processes, may result because of the lack of modulators that control the movement of cells and minerals involved in calcification. It is a fact that the calcium and phosphate levels in plasma under normal conditions are above their theoretical solubility limit. In addition to specific proteins, plasma also contains some key player chemicals, such as citrate and magnesium that maintain mineral in the solution. These constituents may prevent ectopic calcification. Fetuin-A, matrix G1a protein (MGP) and (OPN) are considered to be amongst key negative regulators of calcification [9].

*Fetuin-A* is a glycoprotein (a molecule that consists of a carbohydrate plus a protein) which is abundantly found in plasma. Fetuin-A is able to prevent undesirable calcification by inhibiting the precipitation of apatite precursor mineral; however it does not have an effect on bone mineralization [17].

*Matrix Gla protein (MGP)*, as described by Dellegrottaglie et al., [9], “is a small-size protein and its functional activity is dependent on γ-carboxylation of glutamic acid residues through a vitamin K-dependent γ-carboxylase. MGP is found in normal arterial wall and appears to be up-regulated in atherosclerotic plaques”. Some studies suggest that MGP limits the mineralization within vessel wall.
**Osteopontin (OPN)** is another important inhibitor of calcification. OPN binds to $\alpha_\text{v}\beta_3$ present in osteoclasts and cause this cell to become active. This activation is presumed to be done by decreasing the cellular calcium levels [18]. OPN is also involved in the osteoclastic bone resorption mechanism through the expression of carbonic anhydrase II. Anhydrase II is a key enzyme in creating an acidic microenvironment which is critical for bone resorption [19]. In addition, OPN binds to apatite crystals, which through this binding the subsequent mineralization of calcification deposits is inhibited. This can be considered as a direct calcification inhibition [19, 20].

Some studies have been performed to see the effect of OPN and MGP in aortic calcification in mice. Less calcification was observed in mice deficient only in MGP rather than the mice with deficiencies in both OPN and MGP. If recombinant OPN is used, it would result in ectopic bone resorption [21, 22].

2.2.2.3 **Enhanced Bone Turnover**

Bone remodeling, or turnover, is a natural process during which a microscopic amount of bone tissue is removed (bone resorption) and then replaced with new bone in the same location. Bone turnover can be the cause of osteoporosis if the rate of bone resorption exceeds that of formation [23].

Osteoporotic patients are frequently diagnosed with VC or vice versa. Many risk factors are shared in these two diseases, factors such as aging, estrogen deficiency, chronic kidney disease, inflammatory disease, glucocorticoid use [24, 25]. In post-menopausal women, the enhanced bone turnover that leads to release of circulating nucleational complexes, has the
potential to be proposed as a link between VC and osteoporosis [26]. Osteoporotic patients are more susceptible to arterial calcification [24]. In adverse, osteoporosis is elevated if the patient is diagnosed with atherosclerosis. It is believed that in animal models, agents known to diminish bone loss and inhibit osteoporosis may also prevent arterial calcification. Agents such as bisphosphonates and osteoprotegerin (OPG), a soluble molecule found in the circulation as well as the vessel wall and is able to inhibit osteoclastic differentiation. Therefore, it has been suggested that calcium is allowed to move from bone to vascular wall if there is an imbalance in its allocation [9, 27].

2.2.2.4 Abnormalities of Mineral Metabolism

To determine VC, mineral milieu is a critical factor. Minerals may be the initiator of calcification through the coordinate expression of molecules related to bone, differentiating them into osteogenic cells and forming mineralized structures resembling bone. For a long time the strong belief about phosphate levels was that they only have influence on mineralization through physico-chemical means; however, it was later observed that vascular smooth muscle cells absorb extra cellular phosphate by means of a sodium-dependent phosphate co-transporter called Pit-1. This will increase the intracellular phosphate level in smooth muscle cells, which induces genes that are related to mineralization such as Cbfa-1, osteocalcin and OPN and at the same time loses smooth muscle cell phenotype [28-30]. According to Dellegrottaglie et al., [9], “Primary cultures of human vascular smooth muscle cells express bone proteins and mineralize when treated with β-glycerophosphate, which serves as an inorganic phosphate donor in the presence of alkaline phosphatase. Similarly, elevated levels of calcium in the culture media leads to enhanced mineralization and phenotypic transition of vascular smooth muscle cells. This is
postulated to occur via enhanced phosphate sensitivity of smooth muscle cells, thereby increasing the functionality of Pit-1.”

The key players in preventing calcium deposition are the inorganic pyrophosphate and Nucleotide Pyrophosphatase Phosphodiesterase (NPP), which is the enzyme regulator of pyrophosphate levels. In addition to being a biophysical chelator, which prevents calcium from being deposited and nucleated, pyrophosphate also acts as an inhibitor for vascular smooth muscles to differentiate and become an osteogenic phenotype. Patients who are in lack of NPP levels are diagnosed with a disorder called "infantile idiopathic arterial calcification". This disorder is characterized by medial calcification and results in cardiovascular disease which causes early death [9].

2.2.3 Bioprosthetic Heart Valve Calcification Mechanism

Porcine or Bovine bioprosthetic heart valve implantation is considered to be a form of live-tissue transplantation or xenotransplantation. In xenotransplantation, usually a very aggressive form of humoral and cellular immune system rejection occurs. In order to decrease this type of immunogenicity of bioprosthetic valves, glutaraldehyde fixation is used. Through this fixation process, cross-linking of antigens happens, which is supposed to make the valves immunologically inert and improve tissue durability [31].

Valves which have undergone glutaraldehyde fixation have the propensity to calcify. It is believed that the glutaraldehyde fixation process leads to calcification of the valves [32]. Calcification is a major contributor in tissue and bioprosthetic heart valve failure. Glutaraldehyde pretreatment devitalize the cells and these residual cells become the primary sites for
calcification. Calcium phosphate is the calcification mineral which deposits through the reaction of the calcium in extracellular fluid with the membrane-associated phosphorus. Recent studies have suggested a similarity between pathologic calcification and physiologic bone mineralization, which is regulated by inductive and inhibitory factors. Many aspects of the pathophysiology of calcification process have been elucidated through \textit{in vitro} and \textit{in vivo} pathological analysis on bioprosthetic heart valves \cite{33}.

Factors such as host metabolism, implant structure and mechanical stresses determine the mineralization in bioprosthetic heart valves or other biomaterials. Calcification mineralization is further enhanced at the sites that are under intense mechanical stresses, such as commissures in heart valves \cite{33}.

In the cusps of bioprosthetic heart valves, the mineralization process is dominantly initiated in connective tissue cells that are no longer viable due to glutaraldehyde pretreatment procedures. These cells have become devitalized but not removed from the structure of the valve. As stated above, dystrophic calcification of the cells happens due to reaction of calcium in extracellular fluid with membrane-associated phosphorus. This is likely because the glutaraldehyde pretreated cells have become nonviable and their calcium ion expulsion has been disrupted. Calcium concentration in the extracellular plasma in normal condition is 1 mg/mL (approximately $10^{-3}$ M) and since the calcium is pumped out by membranes of healthy cells, calcium concentration in the cytoplasm becomes 1,000 to 10,000 times lower (approximately $10^{-7}$ M). However, this normal mechanism for elimination of calcium from the cells does not happen in tissues that have been pretreated with glutaraldehyde. Phosphorous constitutes a large amount of the cell membranes and other intercellular structures (as phospholipids, especially phosphatidyl serine, and the phosphate backbone of the nucleic acids). They can bind with
calcium and provide a nucleation site. Further calcification deposits accumulate in these initial nucleation sites and eventually combine and become larger. As a result of this mineralization, the tissue becomes stiff and therefore weak and causes malfunction in bioprosthesis performance [33].

Schoen and Levy, [33], have summarized the current understanding about tissue calcification determinants, mechanisms and regulation as shown in figure 2.1.

Figure 2.1 shows a model in which host factors, implant factors, and mechanical damage are considered to be involved in the tissue calcification process. This figure indicates that there is a relation between the intracellular calcium, which have been increased in bioprosthetic residual cells and cell fragments, and initial sites of mineral nucleation. Growth of calcification deposition and gross mineralization results in valve failure with tearing or stenosis.

In this model, the role of phosphorus, which exists in membrane phospholipids and nucleic acids, has been emphasized in determining the initial sites of crystal nucleation. As illustrated and discussed before, mechanical deformation generated by motion which induce
intensive stresses on flexion points in the heart valve, probably have an effect on accelerating both nucleation and growth of calcific crystals [33].

2.2.4 Polymeric Heart Valve Calcification Mechanism

Using biomaterial such as polyurethane have become the center of attention as a heart valve substitute because of the shortcomings of available mechanical and biological prosthesis on the market. Mechanical valves are durable but require the patient to undergo anticoagulation therapies. On the other hand, biological prosthesis are biocompatible and do not need the anticoagulation therapies; however their life-span is limited due to tissue degeneration and
calcification. The calcification mechanism of bioprosthesis has been elaborated in section 2.3. However, the mechanism for polyurethane calcification is not yet completely understood.

There are different hypothesis related to polyurethane calcification. It has been hypothesized that polyurethane calcification is due to deposition of cellular debris and thrombi on the surface of the material [34, 35]. However, Alferiev et al., [36], mentioned that there is no need for thrombus formations in order for a polyurethane surface get calcified. This has been verified by rat subdermal implants [37, 38]. Other’s findings have shown that, like what was seen for bioprosthesis calcification, calcium deposits were mostly localized to flexing regions of polyurethane heart valves. It has also been shown that cracks and tears of the polyurethane trap more calcium deposits; however it is not yet understood whether these surface irregularities cause the calcification or the calcification itself causes the polyurethane surface to tear or crack. [36].

Furthermore, as hypothesized by Kou Imachi et al. [39], when segmented polyurethane receives repeated stretching stresses, it would be extended and some loosening between the polymer’s molecules would occur to make microgaps, especially in the soft segment domains. Blood proteins and/or phospholipids, major component of all cell membranes which are lipids containing one or more phosphate groups, would invade into these microgaps between the loosened polymer molecules and be trapped and then would attract Ca ions followed by phosphate ions to make their complexations, as calcium phosphate (Ca$_3$(PO$_4$)$_2$).

Chapter 3 is focused on some bench-top calcification studies that have been performed on polyurethane heart valves.
2.3 References


reverses osteoporosis by inhibiting endosteal osteoclasts and prevents vascular calcification by blocking a process resembling osteoclastogenesis, J. Exp. Med., 192: 463- 474


CHAPTER 3

“CALCIFICATION OF POLYMERIC HEART VALVES, MATERIAL DEPENDENCY“
3.1 Abstract

Heart valve disease is a common type of cardiac disease that causes large number of mortalities worldwide. Patients with severe heart valve problems are required to undergo heart valve replacement surgeries. Mechanical and bioprosthetic heart valves are the current available prostheses for patients in need of a heart valve replacement surgery; however, both types of valves have their own drawbacks. Mechanical heart valves have proven to be very durable, but the underlying problem with them is their susceptibility to thromboembolism and thrombosis. Because of this problem, patients should be under anticoagulation therapies for their whole life. On the other hand, bioprosthetic valves require little or no anticoagulation; however, the underlying problem with them is a limited life-span because of structural changes such as leaflet wear and calcification leading to valve failure.

The search for implantable hemocompatible materials has been a concern for scientists for more than 50 decades. Polyurethanes, which have historically been used in medical devices, have proven to be a suitable implant biomaterial. It is believed that polyurethanes have the potential to be used as a heart valve material, which require lower anticoagulation levels than mechanical valves, yet offer the potential for reduced calcification and increased durability when compared to tissue valves.

This chapter presents the results of a series of accelerated in vitro experiments on the Angioflex® heart valves when subjected to a synthetic calcification solution. Results showed that Angioflex® have the potential to be considered as heart valve substitutes with significantly lower levels of calcification deposits compared to tissue valves. In addition, the calcification resistance
of bisphosphonate modified Angioflex® valves was evaluated. Bisphosphonates are a class of drugs that are considered to enhance the calcification resistance of polymers once covalently bonded to the bulk of the material. However, results showed that -bisphosphonate-modified Angioflex® valves -are not superior to Angioflex® valves in calcification.

3.2 Introduction

Heart valve related disease is a significant cause of mortality worldwide. In USA, approximately 90,000 valve replacement operations were performed in 2004 because of valvular heart disease [1]. Heart valve substitution procedures, which have been used successfully since 1960, are safe and effective even though 10-year survival rates still range from 37-58% [2]. The valves being used include mechanical and biological substitutes [3].

However, both types of available heart valves have negative side-effects because they are fabricated from foreign materials. Therefore, these valves are associated with increased risks of immune response and thromboembolism. Anticoagulation therapy is necessary to reduce thromboembolic complications [4, 5]. Today’s biological substitutes are less thrombogenic than mechanical substitutes, but they undergo tissue degeneration and valve failure over time [4, 6]. Durability of bioprosthetic valves range from 5 to 20 years, which leads to reoperation for the recipient and because of a more pronounced immune response, their life-span is even shorter in younger individuals. Current heart valve prostheses all lack the ability to grow and repair, which limits their use, especially in pediatric patients.

Currently, researchers are trying to find new substitute materials for heart valves which require lower levels of anticoagulation compared to mechanical valves and have a longer life-
span than tissue valves. Historically, polyurethanes have been very important for use with implantable devices. They are a family of segmented block copolymers composed of alternating polycarbonates or polyethers known as soft segments and ureas or urethanes often referred to as the hard segment. The ratio and chemistry of the hard and soft segment components can be altered, thus allowing polyurethanes to possess a broad range of physical properties, making these elastomeric polymers an attractive composite material for cardiovascular implants, such as artificial heart devices, and prosthetic heart valves [7].

For applications such as a prosthetic heart valve the material must be able to withstand many cycles of stress and deformation before failure. Mechanical properties and biocompatibility of polyurethanes have all led to their sustained use as a biomaterial. The use of a stable, durable material, such as polyurethane, may reduce the incidence of re-operation due to calcification and tissue valve failure. Another benefit of using this type of heart valve is the reduction of the cost burden on the health care system and further improvement in the survival rate of patients with heart valve prostheses.

Bioprosthetic heart valves should be pretreated with glutaraldehyde before being implanted in a human body. This is to make them immunologically inert and improve the tissue durability; however it is believed that glutaraldehyde pretreatment causes calcification, which is a major contributor in tissue and bioprosthetic heart valve failure. Glutaraldehyde pretreatment devitalize the cells and these residual cells become the primary sites for calcification. Calcium phosphate is the calcification mineral which deposits through the reaction of the calcium in extracellular fluid with the membrane-associated phosphorus mineral [8].
The mechanism for polyurethane calcification is not yet completely understood. There are different hypothesis related to polyurethane calcification. It has been hypothesized that polyurethane heart valve calcification is due to deposition of cellular debris and thrombi on the surface of the material [9, 10]. However, it has been shown that there is no need for thrombus formations in order for a polyurethane surface get calcified. This has been verified in rat subdermal implants [11, 12].

Furthermore, as hypothesized by Kou Imachi et al., [13], when segmented polyurethane receives repeated stretching stresses, it would be extended and some loosening between the polymer’s molecules would occur to make microgaps, especially in the soft segment domains. Blood proteins and/or phospholipids (refer to appendix), major component of all cell membranes which are lipids containing one or more phosphate groups, would invade into these microgaps between the loosened polymer molecules and be trapped and then would attract Ca ions followed by phosphate ions to make their complexations, as calcium phosphate (Ca$_3$(PO$_4$)$_2$).

Alferiev et al., [14], have found a novel method to covalently bond bisphosphonate and diethylamino groups to the hard segment of polyurethane material (refer to appendix). This new material has comparable physical and biomechanical properties to unmodified polyurethane. They have hypothesized, based on their findings, that bisphosphonate-modified polyurethane is more calcification resistant than the unmodified polyurethane.

Angioflex®, ABIOMED's proprietary polyurethane, and bisphosphonate-modified Angioflex® are the subjects of calcification study in this chapter. Heart valves made from these two materials were cyclically loaded on a valve accelerated life tester and their calcification process has been investigated.
3.3 Angioflex®

Polyurethanes have been very important for use with implantable devices. High tensile strength, abrasion and degradation resistance, flexing life and biocompatibility have all led to their sustained use as a biomaterial. Angioflex® is ABIOMED's proprietary polyether-based polyurethane plastic which has been developed and clinically evaluated by ABIOMED to be a dependable substance and safe for contact with blood.

3.4 Bisphosphonate Modified Angioflex®

Bulk-modification of Angioflex® with bisphosphonate and diethylamino groups would result in a new type of polymer that is postulated to have a better calcification resistance than Angioflex®. Nitrogen containing bisphosphonates are used to treat osteoporosis and other fragile bone conditions. They are digested by osteoclasts when attached to bone tissue which inhibits the osteoclastic function [15].

Bisphosphonates have different effects in regards to calcification. They bind to and stabilize calcium-phosphates which make them effective in preventing mature bone osteoporosis. On the other hand, they act as crystal inhibitors for calcium-phosphate crystal formation and prevent nucleation of most calcium phosphates. In addition, bisphosphonates are also inhibitors of the enzyme alkaline phosphatase, which is a key enzyme in biomineralization [16]. This is why it has been hypothesized that bisphosphonate-modified polymers can be used as a suitable material for implantable prosthesis while reducing the calcification levels.
3.5 Calcification Metastable Solution

Two different calcification solution compounds have been identified for in vitro experiments in this study. Golomb and Wagner’s compound [17] is the one used here, while Starcher and Urry’s [18] compound was previously used by Deiwick et al., [20], for in vitro calcification studies on bioprostheses.

**Golomb and Wagner’s Compound:** The calcification metastable solution consists of 3.87 Millimole (mM) CaCl₂, 2.32 mM K₂HPO₄, yielding a ratio of Calcium to phosphate (Ca/PO₄)=1.67, and 0.05 M Tris Buffer (in this study C₄H₁₁NO₃) solved in Reverse Osmosis (RO) water [17].

**Starcher and Urry’s Compound:** Solution consists of 20mM barbital buffer PH 7.41 containing 55 mM KCl, 1.25mM KH₂PO₄ and 1.5 mM CaCl₂ (yielding a Ca/PO₄ ratio of 1.2) [18].

Although there are substantial differences in the contributing chemicals and their molarities in these compounds, it is believed that samples tested within each of them can be compared to each other. The main purpose of using any synthetic calcification solution is to obtain calcium-phosphate compounds which can precipitate out of the solution and deposit on a sample. Both above mentioned compounds have proven to have this characteristic and can be used as a calcification metastable solution; however, Golomb and Wagner’s compound seems to be more aggressive than the other because of its greater amount of calcium content (calcium to phosphate ratio (Ca/PO₄) ratio of 1.67 vs. 1.2). Also, the Ca/PO₄ ratio for Golomb and Wagner’s compound is more physiologically representative of hydroxyapatite (10:6=1.67 from its chemical formula Ca₁₀(PO₄)₆(OH)₂). Hydroxyapatite is a semicrystalline plaque and is the most often form
of calcium minerals in the vascular calcification process. Therefore Golomb and Wagner’s compound has been chosen to be used in this study.

### 3.6 Valve Accelerated Life Tester (VALT)

The Valve Accelerated Life Tester (VALT) is designed to assess heart valve durability. It consists of a test module, a controller and monitoring software (Figure 3.1). The module consists of 6 test chambers and a unique Scan-Valve for rapid monitoring of pressures across the valve (upstream and downstream of the valve). Valves should be mounted on pistons in cylinders filled with fluid. A shaker drives the pistons at a selectable frequency. Variable restrictors provide control of the fluid recirculation path within each chamber. The controller provides the power for driving the module and includes trigger signals for data acquisition and stroboscopy of valve operation [19].

![Figure 3.1 – Valve Accelerated Life Tester (VALT)](image)
3.7 Objective

Based on the extensive use of polyurethanes in medical devices as well as their biocompatibility and mechanical properties, they have been considered to be a suitable material for heart valve prosthesis. One of the major shortcomings of this type of valves is their propensity to calcify. Although there are a number of hypothesis regarding the calcification mechanism for polymeric valves, it is not yet completely understood [9-13].

A number of experiments have been performed to: 1) validate the in vitro calcification process setup, 2) evaluate different materials’ response to calcification solution, prepared according to Golomb and Wagner’s compound [17], 3) evaluate the calcification levels for Angioflex® and bisphosphonate modified Angioflex® valves and 4) verify if the results are repeatable and find the superior material between Angioflex® and bisphosphonate modified Angioflex® valves. In the latter two experiments, results are compared with Deiwick et al.’s, [20], in vitro calcification study for tissue valves.

3.8 Experimental Setup and Method of Analysis

3.8.1 Experimental Setup

Valves were placed on the VALT with chambers filled with calcification metastable solution. Each chamber was de-aired before the beginning of the test. Valves were cyclically loaded at a frequency of 1000 cycles per minute at 37°C. A period of one month (about 50 M-cycles) was considered for each set of experiment. For the last two set of experiments (sections 3.8.5 and 3.8.6), the calcification solution was changed weekly during the experiment. For the
same two sets of experiments, valves were rinsed weekly with RO water to remove excess solution and loosely attached deposits.

3.8.2 Method of Analysis

Two different spectrometry methods as well as a microscopy method were performed to characterize the level of calcification deposits on the surface or in the subsurface of the material: ICP (Inductively Coupled Plasma) spectrometry, EDX (Energy Dispersive X-ray) spectroscopy and SEM (Scanning Electron Microscopy). ICP is a technique for elemental analysis which is applicable to most elements over a wide range of concentrations. Advantages of using ICP include its ability to identify and quantify all elements except Argon. EDX spectroscopy is used for the elemental analysis and/or chemical characterization of a sample. Although EDX does not quantify the exact amount of the element present in a particular sample, it can be used as a relative comparison technique to show the surface deposition differences between two samples.

3.8.3 Validation of the In Vitro Calcification Experiment

The main purpose of this test was to validate the in vitro experimental method for calcification studies. The goal of this experiment was to verify the existence of calcium-phosphate deposits on the surface of the valves after a certain amount of time using a specific experimental setup. Angioflex® valves were used for this investigation.

**Materials and Methods:** In 1990, Golomb and Wagner developed a model for in vitro studies on implantable polyurethane calcification. They examined polyurethane films incubated in vials filled with metastable calcium and phosphate solution. Vials were placed in a shaker with
100 rev/min at 37°C. They validated their model by examining the calcification of both highly calcifiable biomaterial (bioprosthetic tissue) and a non-calcifiable biomaterial (charge-modified tissue and polyurethane containing anticalcification agent) and comparing the results with what they acquired from their *in vitro* polyurethane film calcification studies [17]. The same concept was used in the present study benefitting from recent technological advances, such as using VALT. VALT is mainly designed for valve durability evaluation; however, it is beneficial to test its potential to be used for calcification studies. Two Angioflex® valves were tested on the VALT with calcification solution prepared according to Golomb and Wagner’s compound, [17]. The solution was not changed during the experiment. Valves were cyclically loaded at 1000 cycles per minute at 37° C till they reached 52 million cycles. SEM was performed on the leaflets of each valve. Figure 3.2 shows one of the valves before and after the experiment.

![Figure 3.2 - Angioflex® Valve: a) before the test, b) after the test](image)
**Results and Conclusion:** As shown in figure 3.2, valves looked cloudy on the surface after the test. SEM as well as EDX results also confirm the existence of calcium and phosphorous aggregates on the surface of the polyurethane (Figure 3.3).

![SEM and EDX results](image)

*Figure 3.3 - a) SEM picture b) EDX result confirms the existence of Calcium and Phosphorous on the surface of the polyurethane*

The results confirmed the existence of calcium and phosphorous elements on the valves’ leaflets tested on the VALT. Therefore, the calcification solution as well as the experimental
setup using VALT can be used for *in vitro* studies. Results obtained from this test paved the way for further bench-top experiments regarding calcification of the polyurethane heart valves.

3.8.4 Calcification Quantification for Different Types of Polymers The purpose of this test was to compare the level of calcium and phosphorous on different polymeric trileaflet valves. More importantly to compare the reaction of bisphosphonate modified Angioflex® (BP-Angioflex®) valves to calcification solution with respect to Angioflex® ones. Based on Alferiev et al.’s, [14], results for Biospan and bisphosphonate modified Biospan, it was expected that the level of calcium and phosphorous would decrease on BP-Angioflex® surface.

**Materials and Methods:** In this experiment, two types of designs have been used for making a polymeric heart valve. 1) old design and 2) new design. In the old design, which is used for ABIOMED’s Abiocor heart implants, valve’s material solvent is poured inside a conduit with sinuses and no stent. Leaflets of this type of valve coincide at the pointed tips in the center of the valve. In the new design, valve is fabricated using a casting-solvent process. These Valves have no conduits and are formed on a polyurethane stent and a stainless steel mandrel by a dipping process. Leaflets of this type of valve coincide at the commissures and have flat edges at that point. Figure 3.4 shows these two kinds of designs for polymeric valves.

![Figure 3.4 - a) old design trileaflet Angioflex® valve, b) new design trileaflet valve](image)
Two low molecular weight Heparin (LMWH), 1 Angioflex® (with the old design), 2 Angioflex® (with the new design) and 1 BP- Angioflex® (with the old design) valves were tested on the VALT with calcification solution prepared according to Golomb and Wagner’s compound, [17]. The solution was not changed during the experiment. Valves were cyclically loaded at 1000 cycles per minute at 37° C till they reached 56 million cycles. SEM, EDX and ICP were performed on the leaflets of each valve.

**Results and Conclusion:** After 56 million cycles, valves were taken off the VALT with surfaces looking completely calcified. SEM and EDX were performed on 2 leaflets, one from the new design Angioflex® valve and one from the BP-Angioflex® valve. Results are shown in figures 3.5 and 3.6.

![SEM results of: a) Angioflex® valve's leaflet, b) BP-Angioflex® valve's leaflet](image)
ICP was also performed for 4 of the valves: old and new design Angioflex® valves, BP-Angioflex® old design valve and one of the LMWH valves. This was done in order to determine the exact amount of calcium and phosphorous deposition in the bulk of the material. Figure 3.7 graphically summarizes these results.
As can be seen, all valves showed close concentrations for calcium and phosphorous differences were within the measurement error of ICP. In contrast to what was expected from a BP-Angioflex® valve, it did not show a significant difference from the Angioflex® valves in the amount of calcification deposits.

The three types of material used in this test showed about the same reaction towards calcification process. Although it was expected that bisphosphonate reduce the calcium and phosphorous deposition on heart valve leaflets significantly, results showed that there is not much of a difference between BP-Angioflex® valves and unmodified Angioflex® ones. Therefore, none of the mechanisms mentioned in section 3.3 seems to be effective in reducing calcification by modifying the Angioflex® with bisphosphonates; however, before dismissing the
whole idea about bisphosphonates being ineffective in reducing calcification levels, more studies have been performed to substantiate this finding

3.8.5 Angioflex® and BP-Angioflex® Calcification Comparison

The purpose of this test was to compare the calcification levels between Angioflex® and BP-Angioflex® valves when subjected to weekly changed calcification solution. ICP results for calcification deposits were compared with Deiwick et al.’s, [20], in vitro calcium content for tissue valves (205±64.87 µg/mg dry weight). These results were also compared on a different basis with Alferiev et al.’s, [14], in vivo calcium contents results for Biospan and bisphosphonate modified Biospan (0.6±0.46 µg/mg and 0.17±0.02 µg/mg respectively) to evaluate the effectiveness of bisphosphonate modification on the calcification process.

*Materials and Methods:* Three Angioflex® and three BP-Angioflex® valves were tested on the VALT with calcification solution prepared according to Golomb and Wagner’s compound, [17]. The solution was weekly changed during the experiment. Valves were cyclically loaded at 1000 cycles per minute at 37° C till they reached 50 million cycles. At the end of each week, valves were rinsed with RO water three times to remove excess solution and loosely attached deposits.

SEM, EDX and ICP were performed on the leaflets of each valve. Figures 3.8 and 3.9 show valves made from each material before and after the test.
As can be seen in figure 3.9, BP-Angioflex® valve’s leaflets look cloudier than the Angioflex® valve. This is due to the high percentage of BP-Angioflex®‘s water uptake with respect to Angioflex® (20% to 1–2%).

**Results and Conclusion:** Valves were taken off the tester after 50 M-cycles (about 14.5 months in real life), and they all looked calcified. SEM images of the polymeric valve leaflet surfaces displayed non-homogenous patterns of exposed polyurethane combined with deposits in the form of spherical aggregates (Figure 3.10). EDX confirmed the existence of calcification deposits on the surface of the leaflets (Figure 3.11).
ICP was another tool for measuring the calcification levels which was performed for each valve. Results obtained from ICP were compared to Deiwick et al.’s *in vitro* calcium results for tissue valves (205±64.87 µg/mg dry weight).

Deiwick et al.’s calcification results have been obtained from testing four frame-mounted porcine bioprostheses (St Jude Medical Bioimplant; St Jude Medical, Inc, St Paul, Minn). These valves were cyclically loaded at a frequency of 300 cycles per minute at 37°C and within a rapid
synthetic calcification solution. This solution, which is prepared according to Starcher and Urry’s, [18], compound, consists of 20mM barbital buffer PH 7.41 containing 55 mM KCl, 1.25mM KH₂PO₄ and 1.5 mM CaCl₂ (yielding a Ca/PO₄ ratio of 1.2).

Although total number of cycles and solution compounds between the present experiment and Deiwick et al.’s in vitro test are different, results obtained here can be compared to Deiwick’s because:

- Solution in this experiment, is more aggressive than Deiwick et al.’s with a more dominant calcium content as discussed in section 3.5 (Ca/PO₄ ratio of 1.67 vs. 1.2)
- Ca/PO₄ ratio (=1.67) in this experiment is more physiologically representative of hydroxyapatite (10:6=1.67 from its chemical formula Ca₁₀(PO₄)₆(OH)₂) rather than Deiwick’s, which is 1.2
- The duration of this experiment was 50 M-cycles while for Deiwick et al.’s was 19 M-cycles. With longer experiment duration, it is expected to have higher levels for calcification; however, it was shown that even though our experiment’s period was longer, less calcification levels were detected

Comparing chemical solutions used and experimental duration for these two tests, the results indicate that this experiment was running in a worse case than Deiwick et al.’s and more calcification levels were predicted. Alferiev et al., [14], on the other hand, have investigated the effect of bisphosphonate modification of a different type of polymer, Biospan, on calcification process. They did this by replacing a single pulmonary leaflet of a juvenile sheep with Biospan and bisphosphonate modified Biospan (BP-Biospan). Their results for a 150 days in vivo study, showed that bisphosphonate modification of Biospan, increased polymer’s calcification resistance.
Table 3.1 and figure 3.12 summarize all the average values for calcium and phosphorous contents of Angioflex®, BP-Angioflex®, Deiwick’s tissue valves and Alferiev’s Biospan and BP-Biospan leaflets.

<table>
<thead>
<tr>
<th></th>
<th>Ca (µg/mg)</th>
<th>P (µg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Deiwick’s Tissue Valves</strong></td>
<td>205.285</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>BP-Angioflex®</strong></td>
<td>2.852</td>
<td>1.175</td>
</tr>
<tr>
<td><strong>Angioflex®</strong></td>
<td>1.613</td>
<td>0.430</td>
</tr>
<tr>
<td><strong>Alfriev’s Biospan</strong></td>
<td>0.6</td>
<td>0.5200</td>
</tr>
<tr>
<td><strong>Alfriev’s BP-Biospan</strong></td>
<td>0.17</td>
<td>0.1200</td>
</tr>
</tbody>
</table>

Results show that both Angioflex® and BP-Angioflex® valves have significantly lower levels of calcification deposits compared to Deiwick’s tissue valves (p-values less than 0.005). Based on Alferiev et al.’s findings, it was expected to have lower levels of calcification for BP-Angioflex® valves compared to untreated Angioflex® ones; however, results showed that untreated Angioflex® valves are more resistant to calcification process.

The contradictory results obtained for bisphosphonate modification of Angioflex® to Alferiev et al.’s findings could be due to the high water absorption characteristic of the present BP-Angioflex® (about %20). The water uptake of BP-Angioflex® was reduced to 3-5% and further investigation was made with this new material. Results are presented in section 3.8.6.
Figure 3.12- a) Calcification levels for Angioflex®, BP-Angioflex®, St Jude's tissue; b) Calcification levels for Biospan and BP-Biospan leaflets
3.8.6 Verification for Superiority of Angioflex® valves

The purpose of this test was to confirm the results obtained from 3.8.5 for Angioflex® valves and closely compared them with tissue valves. Another outcome was to verify if changing the amount of water absorption of bisphosphonate modified Angioflex® has any effect on the calcification level or not. It was expected that this change would decrease the amount of calcification deposits on BP-Angioflex® valves.

Materials and Methods: Three Angioflex® and three BP-Angioflex® valves were tested on the VALT. For this experiment, bisphosphonate modification was done by covalently bonding bisphosphonates with the polymer and adding nearly equimolar amount of unbound tetrabutylammonium cations. This modification was done to reduce the water absorption of the BP-Angioflex® from 20% to almost 3%. Calcification solution was prepared according to Golomb and Wagner’s compound, [17], and was changed weekly during the experiment. Valves were cyclically loaded at 1000 cycles per minute at 37° C until they reached 50 million cycles. At the end of each week, valves were gently rinsed with RO water three times to remove excess solution and loosely attached deposits.

As a tool for comparison between the latter two experiments, ICP was performed here and results are presented in the subsequent section.

Results and Conclusion: Valves were taken off the VALT at 50 M-cycles and they all looked calcified on the surface. Figure 3.13 and 3.14 show two valves representing each material before and after the test. As can be seen, BP-Angioflex® valve looks cloudier than the Angioflex® one even before the test. Besides the fact that calcification levels for BP-Angioflex® were higher than the Angioflex® material, it seems that the cloudiness is due to silicon
contamination of the material in valve making process and to some extent due to its water absorption.

![Figure 3.13 - Angioflex® valve: a) before the test, b) after 50 M-Cycles](image)

![Figure 3.14 - BP-Angioflex® valve: a) before the test (valve looks cloudier compared to figure 3.13 a), b) after 50 M-cycles](image)

ICP was performed for all six valves and the average calcium and phosphorous contents were obtained. For Angioflex® valves, the average calcium and phosphorous contents were 2.367±1.112 µg/mg and 1.393±0.556 µg/mg, respectively. On the other hand these values for
BP-Angioflex® valves were significantly higher compared to untreated Angioflex®, 9.533±0.471 µg/mg for calcium and 3.52±0.163 µg/mg for phosphorous.

In order to simplify calling the last two experiments, the test performed in section 3.8.5 is called “VALT 3” and the one presented in the current section is called “VALT 4”. Comparing calcification contents for the two mentioned tests shows that the same pattern, though with different values, has repeated between Angioflex® and BP-Angioflex® valves. BP-Angioflex® valves still show more amount of calcification deposits than the Angioflex® ones even though their water absorption was less than what it was before (3% vs. 20%). Furthermore, calcification levels for BP-Angioflex® valves in VALT 4 were higher compared to the results from VALT 3 (p-value < 0.005). This could be either due to silicon contamination of the material in the process of making the valves or lowering the water absorption of the BP-Angioflex® material, unlike what was expected. This matter should be investigated more precisely in future studies avoiding any source of possible contamination. On the other hand, the difference between the calcification levels for Angioflex® valves between VALT 3 and 4 is not significant (p-value = 0.4) which confirms the repeatability of the test for Angioflex® valves. Figure 3.15 graphically demonstrates the differences between VALT 3 and 4 for Angioflex® as well as BP-Angioflex® valves.
Results from VALT 4 again showed that both Angioflex® and BP-Angioflex® valves have lower levels of calcification compared to Deiwick’s tissue valves (p-value < 0.005). This confirms the repeatability of the *in vitro* calcification experiments for Angioflex® and BP-Angioflex® valves. Cross-sectional views of the polyurethane leaflets, after 50 M-cycles, revealed surface calcification (evidenced by dark staining); however, calcium deposits deep to
the surface are not evident (Figure 3.16a). Deiwick et al.’s cross-sectional view of a tissue valve leaflet after 19 M-cycles of testing is provided as a comparison (Figure 3.16b). Some surface calcification is evident, but this is dominated by massive and extensive subsurface calcification deposits.

In the beginning of the test, it was expected to obtain lower levels of calcification for bisphosphonate modified valves in VALT 4, by decreasing BP-Angioflex®’s water absorption; however, results showed that this amount has even increased. Therefore, water absorption is not an issue for justifying the excess amount of calcification in BP-Angioflex® valves. However, another factor which should be taken into consideration is avoiding any source of contamination while preparing BP-Angioflex® solution, which in case of VALT 4 was silicon. There is not enough evidence that silicon contamination has triggered a mechanism for BP-Angioflex® valves to absorb more calcification deposits than VALT 3 but this should be further investigated.

3.9 Discussion and Conclusion

The main purpose of experiments cited in this chapter can be listed as:
• Validating the experimental setup for *in vitro* calcification studies

• Evaluating the reaction of different type polymers to calcification solution in the validated experimental setup

• Evaluating calcification levels for Angioflex® and BP-Angioflex® valves with respect to Deiwick et al.’s tissue valves, [20]

• Evaluating the effect of material property changes on calcification levels for BP-Angioflex®. Changes were made by attaching cationic diethylamino groups to the bisphosphonate-modified polyurethane and reducing the level of water absorption

• Checking the repeatability of calcification process for Angioflex® valves

After performing the first experiment on the VALT, the *in vitro* setup for calcification studies was validated with the existence of calcium and phosphorous elements on the surface of the valves after the test period. Although the calcification solution compound used in this study (Golomb and Wagner’s compound) is different from what Deiwick et al. used for their tissue valves (Starcher and Urry’s compound), comparisons can still be made between these two because: 1) the duration of the experiment here was almost 2.5 times of Deiwick et al.’s, 2) calcium is more dominant in Golomb and Wagner’s solution (3.87 mM vs. 1.5mM), 3) Ca/PO₄ ratio in Golomb and Wagner’s compound is more physiologically representative of hydroxyapatite (1.67 compared to 1.2 for Starcher and Urry’s compound). Therefore, more experiments were performed knowing that the results can be compared with calcification levels from Deiwick et al.’s calcification studies on tissue valves. Results obtained from VALT 3 and 4 are summarized as follows:

• Both Angioflex® and BP-Angioflex® valves have lower levels of calcification compared to Deiwick et al.’s tissue valves
• Angioflex® valves showed better calcification resistance compared to BP-Angioflex® valves; unlike Alferiev et al.’s findings, [14], that bisphosphonates enhanced the calcification resistance of Biospan

• Reducing water absorption of the BP-Angioflex® from 20% to almost 3% did not reduce the amount of calcification deposits. On the contrary, this amount was higher in VALT 4 but it could have been due to silicon contamination of the BP-Angioflex® material during the valve production process. Silicon might have increased the BP-Angioflex® tendency in absorbing calcification deposits. So far results show that bisphosphonate modification of the Angioflex® does not make it more resistant to calcification, like it did for Alferiev et al.’s Biospan. This issue should be further investigated by cautiously avoiding any source of contamination

• On the contrary to tissue calcification, which is a subsurface phenomenon, calcification of polymeric surfaces mostly occurs at the surface of the material and does not penetrate to the subsurface
3.10 References


[13] Kou Imachi, Tsuneo Chinzei, Yusuke Abe, Kunihiko Mabuchi, Hiroyuki Matsuura, Tatsuo Karita, Kiyotaka Iwasaki, Shuichi Mochizuki, Yam-pin Son, Itsuro Saito,


CHAPTER 4

“CALCIFICATION OF POLYMERIC HEART VALVES, SHEAR RATE AND SURFACE PROFILE DEPENDENCY “
4.1 Abstract

It has been suggested that surface defects, shear and mechanical stresses are contributing factors in the calcification process. In this study, a number of experiments were performed to evaluate the effect of surface irregularities (such as roughness and cracks) and flow shear rate on the calcification process in the absence of any source of mechanical stresses. Results showed that even in the absence of mechanical stresses a polymeric surface gets calcified and all the above mentioned factors directly affect the calcification process. Roughness, cracks and low shear rate increase the calcification levels. Under steady flow rate and no mechanical stresses, Calcification occurs mostly on the surface of the polymer indicating that it is being a surface phenomenon rather than subsurface phenomena.

4.2 Introduction

While heart valve prostheses have been used successfully since 1960, 10-year survival rates still range from 37-58% [1]. Mechanical or bioprosthetic heart valves are the current available valves for patients in need of a heart valve replacement surgery; however, both types of valves have their own drawbacks. Mechanical heart valves have proven to be very durable, but the underlying problem with this type of heart valve is the presence of a centrally located leaflet, or occluder. It propagates high velocity jets, turbulence and areas of stagnation. All these flow disturbances make the valves prone to thrombosis and thromboembolism, and necessitate long-term anticoagulation therapy [2]. On the other hand, bioprosthetic valves require little or no anticoagulation; however, the underlying problem with them is a limited life because of leaflet wear and structural changes such as calcification, leading to valve failure [3-6].
The search for implantable hemocompatible materials has taken a new meaning after discovering the effect of calcification. It has been understood that long-term implants will fail or become severely impaired due to this phenomenon [7, 8].

According to Golomb and Wagner, [9], “the implantable factors affecting the rate of calcification which have received the most attention are the effects of local stress concentrations, calcium-binding serum protein absorption, the presence of surface defects and surface adhered organic or cellular debris”.

Golomb and Wagner have examined the role of several factors associated with polyurethane film calcification: factors such as experiment duration, polymer thickness, polymer porosity, serum and strain effects. They realized that increasing the film’s thickness and porosity significantly increased the level of calcification. Also, the longer the experiment duration, the higher the level of calcification was. On the other hand pre-soaking the polyurethane films in serum and elongating them (to produce strain) did not significantly change this level. They have hypothesized that strain might affect the propensity of the polymer to calcify but this could be detected only at initial crystallization rather than at the propagation phase of the mineralization. Another explanation was that only dynamic stress is more important in promoting calcification [9]. It has also been hypothesized that there is a close relation between the mechanical stress applied to a heart valve and the process of calcification; but it is not clear which one is the cause of failure for a heart valve. Whether mechanical stress initiates calcification and results in the failure of the valve or mechanical stress increases due to calcification and causes valve failure. Porosity was one of the factors which had a direct effect on calcification level of polyurethane films. Calcification level increase due to porosity of the material is likely because of the increased diffusion via surface pores, rather than surface voids serving as preferred sites for
mineralization [9]. Colman et al., [11], suggested that microbubbles or surface fractures may act like porous sites to induce mineralization of smooth elastomers.

Polyurethanes and specifically Angioflex®, ABIOMED Inc. proprietary polyurethane, are the subject of study in this chapter. Surface roughness and cracks have been considered to be a surface defect and comprehensive attention has been drawn towards them in this study. Another important factor which has been investigated is the effect of shear rate in the calcification process.

4.3 Objective

Polyurethanes have historically been used in implantable devices. They have also been considered to be a novel material for heart valve replacements avoiding the calcification mechanism associated with glutaraldehyde fixation in tissue valves. However, polyurethanes would still need to demonstrate acceptable calcification performance in long-term applications. Some factors have been recognized to have an important role in calcification process of polyurethanes. Factors such as experiment duration, porosity, thickness of the material and mechanical stresses [9].

In this study, surface roughness and cracks are considered as surface defects (like porosity of the material) and their effect on the calcification process of polyurethanes, and more specifically on Angioflex®, is investigated. The purpose herein is to characterize the effect of surface irregularities on the calcification process of polyurethane, or Angioflex®, in the absence of mechanical stresses. In addition, calcification shear dependency of this material in the absence of mechanical stresses has been investigated.
4.4 Experimental Setup and Method of Analysis

The base setup for calcification studies under static conditions was a simple loop consisting of polyurethane tubes or mandrels covered with polyurethane, using a solvent-casting process, and a roller pump. The loop was subjected to a synthetic calcification solution and the flow rate was selected in such a way to avoid any turbulence along the solution’s path. The solution used in this study was prepared according to Golomb and Wagner’s compound consisting of 3.87 (Millimole) mM CaCl₂, 2.32 mM K₂HPO₄, yielding a calcium to phosphate ratio of 1.67, and 0.05 M Tris Buffer (C₄H₁₁NO₃) solved in Reverse Osmosis (RO) water [9].

Two different spectrometry methods as well as a microscopy method were performed to characterize the level of calcification deposits on the surface or in the subsurface of the material: ICP (Inductively Coupled Plasma) spectrometry, EDX (Energy Dispersive X-ray) spectroscopy and SEM (Scanning Electron Microscopy). ICP is a technique for elemental analysis which is applicable to most elements over a wide range of concentrations. Advantages of using ICP include its ability to identify and quantify all elements except Argon. EDX spectroscopy is used for the elemental analysis and/or chemical characterization of a sample. Although EDX does not quantify the exact amount of the element present in a particular sample, it can be used as a relative comparison technique to show the surface deposition differences between two samples.

4.4.1 Crack and Material Effect Loop

The purpose of this study was to evaluate the effect of cracks on the calcification process of polyurethane material as well as material dependency of this process using two different polymers, polyurethane (PU) and polycarbonate (PC).
**Materials and Methods:** Four pieces of polyurethane tubes were used in this experiment: polycarbonate tube as a control, crazed polycarbonate tube (the inside surface of the tube was crazed by means of methanol and ethyl acetate), polyurethane tube as a control and a cracked polyurethane tube (cracks were induced randomly inside the tube using a sharp blade). Tubes were selected long enough (average 15 inches each) to avoid any circulation and flow disturbances around the sampling region when arranged in a loop. The loop was filled with calcification solution, which was prepared as described in section 4.4. The calcification solution was circulated in the loop by means of a roller pump set to 0.5 L/min flow rate. Figure 4.1 shows the experimental setup. The experiment duration was one month, and the solution was changed every week. Between repeated solution changes and also at the end of the experimental period, loop was filled and drained 3 times with RO water to remove excess solution and loosely attached deposits.

After the one month period of the experiment elapsed, samples were selected from the middle portion of each tube and were coated by carbon using a Physical Vapor Deposition (PVD) method. SEM and EDX were performed for each of the coated samples. EDX was done at recommended analysis settings (20 KV beam voltage, 15 Amps, 15 mm working distance).
**Results for Material Effect:** Total number of four samples were selected from all of the tubes. PU-Control, PU-Cracked, PC-Control and PC-Cracked. 10 points were selected randomly on each sample and EDX was performed for each of the 10 spots. Table 4.1 along with figure 4.2 shows the results obtained for the samples’ calcification contents.

<table>
<thead>
<tr>
<th>Table 4.1 - Calcification contents concentration for polyurethane (PU) and polycarbonate (PC) samples in “weight percentage”</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Table</strong></td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td><strong>Ca</strong></td>
</tr>
<tr>
<td><strong>P</strong></td>
</tr>
<tr>
<td><strong>Ca</strong></td>
</tr>
<tr>
<td><strong>P</strong></td>
</tr>
<tr>
<td><strong>Ca</strong></td>
</tr>
<tr>
<td><strong>P</strong></td>
</tr>
<tr>
<td><strong>Ca</strong></td>
</tr>
<tr>
<td><strong>P</strong></td>
</tr>
</tbody>
</table>
Results show that there is a significant difference (p-value less than 0.05) in the amount of calcium content between PU-Control and PC-Control samples (p-value = 0.001). This difference is also present for calcium content between PU-Cracked and PC-Cracked with p-value of 0.049. The results show that calcification depends on the material and surface condition.

**Results for Crack Effect:** In order to evaluate the effect of cracks in the calcification process, PU-Control and PU-Cracked samples were compared with each other. For a more accurate judgment, sample size of 20 points were selected for the rest of the analysis (with standard deviation of 3). Figure 4.3 schematically shows how 20 random points were selected on each sample for EDX analysis.
The average values obtained for calcification concentration from EDX analysis for each of the above mentioned samples are shown in figure 4.4. This graph shows that with 95% confidence, there is a significant difference between the amount of calcification deposits for PU-Control and PU-Cracked samples (p-value < 0.005). Therefore, cracks increase the calcification levels on a polymeric surface. This could be explained such that the calcification deposits get trapped in the cracks and provide nucleation sites for further calcification. Therefore, cracked surfaces are more likely to trap calcification deposits than a smooth surface.

Comparing the results for both types of polymers, it is evident that polyurethane showed higher levels of calcification deposits than polycarbonate. As described before, materials that are used for heart valve prosthesis should be flexible and able to withstand many cycles of stress and deformation before failure. These characteristics make polycarbonate to be an unsuitable material for use in heart valve prosthesis.
4.4.2 Surface and Shear Rate Effect Loop 1

A new series of experiments were performed in order to characterize the effect of surface irregularities and shear rate on the calcification process of polyurethane. Specific custom designed samples were used in this study to create uniform flow around the sampling region and to avoid gravity effect, if any.

**Materials and Methods:** Two 8.5” long stainless steel mandrels, with 0.216” outside diameter, covered with Angioflex®, using a solvent-casting process (average thickness of 0.015”), were fixed in two transparent PVC tubes. One with OD=1.375” and ID=1.25” for producing low shear stress region and the other one with OD= 0.75” and ID= 0.625” for producing high shear stress region. A schematic picture of these samples is shown in figure 4.5.
Half of the Angioflex® surfaces over the mandrels were rubbed with sand paper to make their surfaces 2µm rough (A Zygo Newview 6000 with 1nm height resolution was used to measure the surface roughness). Cracks, using a sharp blade, were induced on both rough and smooth regions. These assemblies were subjected to calcification solution at a constant flow rate of 0.5 L/min. Figure 4.6 shows one of the samples with marked cracked regions as well as the experimental setup itself. The loop was assembled vertically to avoid the possible gravity effect on the deposition process.

The solution was prepared according to Golomb and Wagner’s compound, [9], as previously stated in section 4.4. The experiment duration was one month, and the solution was changed every week. Between repeated solution changes and also at the end of one month, samples were rinsed with water three times to remove excess solution.
At the end of the one month period of the test, samples were chosen from all surface profiles. SEM, EDX and ICP were performed for each sample.

**Results and Conclusion:** After the one-month period, the polymer on the mandrels looked completely calcified on the surface. The ICP results indicated very small amount of calcification deposits in each sample. This was likely because calcification of polyurethanes occurs mostly on the surface of the material rather than being a subsurface phenomenon, especially when subjected to no mechanical stresses; and the small thickness of the deposits on the surface of the polymer with respect to original sample thickness diluted to ICP results (Figure 4.7).
This could be a proof that calcification elements will not penetrate through the surface of the polymer in the absence of mechanical stresses. Therefore, in this study, ICP is not an appropriate method to evaluate the amount of calcification deposition on the samples.

On the other hand, SEM and EDX, which were performed for smooth, rough, cracked and cracked-rough samples, showed high peaks of calcium and phosphorous resembling hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$). Figure 4.8 shows SEM and EDX results for low shear samples on smooth and rough regions.
Figure 4.8 - a) SEM picture for low shear smooth sample, b) SEM picture for low shear rough sample, c) EDX result corresponding to part a, d) EDX result corresponding to part b

**Note:** based on all previous results in calcification process studies, it has been seen that phosphorous follows the same pattern with respect to calcium concentration; therefore, it is reasonable to use only calcium contents as a comparison tool for calcification studies. This can be better understood from the summarized data in table 4.2. Values listed in this table are representative of the average weight percentage measured by EDX between 20 random points on each low and high shear sample with different surface profile.
Table 4.2 - EDX results for low and high shear samples

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Ca (wt%)</th>
<th>P (wt %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS_Smooth</td>
<td>17.69</td>
<td>6.39</td>
</tr>
<tr>
<td>LS_Rough</td>
<td>28.57</td>
<td>9.02</td>
</tr>
<tr>
<td>LS_Cracked</td>
<td>24.75</td>
<td>7.59</td>
</tr>
<tr>
<td>LS_Cracked + Rough</td>
<td>30.77</td>
<td>9.44</td>
</tr>
<tr>
<td>HS_Smooth</td>
<td>23.48</td>
<td>8.99</td>
</tr>
<tr>
<td>HS_Rough</td>
<td>24.14</td>
<td>8.11</td>
</tr>
<tr>
<td>HS_Cracked</td>
<td>18.93</td>
<td>7.03</td>
</tr>
<tr>
<td>HS_Cracked + Rough</td>
<td>24.57</td>
<td>8.94</td>
</tr>
</tbody>
</table>

EDX had controversial results for samples from both low and high shear rates. For low shear samples, the weight percentages for calcium and phosphorous were significantly higher on all samples compared to the smooth one. On the contrary, all samples from the high shear model had about the same or even less calcification deposition compared to the smooth sample (Figure 4.9).
Results show that there is an interaction between flow shear rate, surface defects and calcium deposition. As the flow shear rate decreases, surface defects (roughness or crack) will act in favor of calcium deposition. On the other hand, as flow shear rate increases, surface defects do not have a significant effect on calcification deposition.

What is evident from the results is that both rough and cracked surfaces have more calcification deposits; however, the effect of cracks is not as dominant as the roughness but it could be just because EDX might not have detected the deposits possibly accumulated inside the cracks.

This study experimentally demonstrated that surface irregularities as well as shear rate affect the amount of calcium and phosphorous deposition on the surface of a polyurethane material in the calcification process. Higher levels of calcification deposits were detected on both rough and cracked surfaces compared to a smooth one. Also, low shear samples seemed to absorb more calcification deposits than the high shear ones. There is also an interaction between all these three factors. In the case of low shear rate, irregular surfaces trap more calcification deposits than the smooth ones; however, it is not the case for high shear samples. Additionally, this study demonstrated that even in the absence of mechanical stresses, a polymeric surface, such as a polyurethane heart valve, can become calcified. Furthermore, EDX, though unable to quantify the exact amount of an element, is a reliable tool to make relative comparisons of calcification deposits on the surface of a polymer. However, in case of surface cracks, EDX did not detect trapped deposits inside the cracks or shadowing effect occurred. Therefore it is not recommended to use EDX for calcification studies on cracked samples. In conclusion, when considering polyurethanes as a possible material for heart valve replacement, attempts should be made to produce smoother leaflet surfaces in order to reduce calcification deposition.
4.4.3 Surface and Shear Rate Effect Loop 2

To achieve more reliable results about the effect of surface irregularities and shear rate on the calcification process, a modified version of the previous test setup, section 4.4.2, was designed. Some minor changes have been made to the design setup and more systematic methods have been used for inducing surface defects which will be described in the subsequent sections.

**Materials and Methods:** Same samples, as in loop 1, with the same dimensions were used in this experiment with some differences in the setup. The new setup consisted of a few more connecting tubes with more systematic procedures in producing surface defects. Figure 4.10 shows the new setup designed for this experiment.

![Modified setup for the second surface effect loop](image)

**Inducing Roughness:** A new method was used to induce roughness on the surface of the polymer. This method was utilized different than what was done in section 4.4.2 using sand paper to: 1) prevent embedding any particles from the sand paper into the polymer and 2) avoid tearing the thin polymer by rubbing the sand paper over its surface multiple times.

Stainless steel mandrels, half smooth and half with average 10.8 µm roughness were covered with Angioflex®, using a solvent-casting process. In order to induce roughness on the
surface of the polymer for calcification studies, Angioflex® covering the rough portion of the mandrels were stripped off and put back on the mandrels inside out.

This procedure provided a more uniform roughness for the polymer rather than what was done in loop 1 with sand paper. Figure 4.11 shows 9 steps performed in order to strip the polymer off of the mandrel and put it back inside out by means of soap and a hook.

Figure 4.11 - Procedure of stripping the Angioflex® off of the mandrel and putting it back inside out: 1) cut the rough portion of the covering Angioflex®, 2-3) strip off the rough Angioflex® of the mandrel, 4) make both inside and outside surfaces of the Angioflex® tube slippery by means of soap, 5-8) bring the Angioflex® tube inside out and 9) put the Angioflex® tube back on the mandrel
With this procedure at least 85% of mandrel’s roughness (~10.8 µm) was transferred to the polymer surface (~9.2 µm roughness). Figure 4.12 shows the surface profile of the mandrel and the covering Angioflex® measured by Ambios XP-2 (Northeastern University, Boston).

![Figure 4.12- Surface profilometer output for: a) rough mandrel, b) rough polymer](image)

**Inducing Cracks:** Cracks were induced on the polymer’s surface using a sharp blade in both radial and circumferential orientations of the mandrel. The density of the cracks were 2.5 (crack length / surface area). Figure 4.13 shows how the cracks were induced on the polymer’s surface.

![Figure 4.13 - Crack orientation](image)
**CFD Analysis:** in order to make sure that the samples are taken from portions with constant shear rates, CFD analysis were performed using FLUENT 6.2.16 (Northeastern University, Boston) with GAMBIT 2.2.30 mesh generator (Northeastern University, Boston). 2D-double precision axisymmetric steady state flow model with water as the media was used. Figure 4.14 shows the shear rate and axial velocity contours for the low shear and high shear samples.

![Shear Rate and Axial Velocity Contours](image)

The low shear sample (Tube_1.25”) had an average of 9 sec\(^{-1}\) shear rate while the high shear sample (Tube_0.625”) had an average of 82 sec\(^{-1}\) shear rate. Figure 4.15 summarizes the
results obtained for shear rates for the two shear models. According to this figure, samples were taken from those portions with constant shear rates.

![Graph showing shear rate for low (Tube_1.25") and high (Tube_0.625") shear samples](image)

**Figure 4.15 - Shear rate for low (Tube_1.25") and high (Tube_0.625") shear samples**

After all the precautions made to prepare the samples, the loop was assembled according to figure 4.10 and in vertical position. The loop was filled with calcification solution, prepared as described in section 4.4, and the flow rate was set to 0.5 L/min. The experiment duration was one month, and the solution was changed every week. Between repeated solution changes and also at the end of one month, the loop was rinsed and drained with RO water three times to remove excess solution and loosely attached deposits. SEM and EDX were performed for total number of 8 samples taken from the low and high shear models. EDX was performed on 21 random points for each sample. All values presented hereafter are the average of these measurements.
**Results and Conclusion:** EDX was performed with recommended settings (20 KV beam voltage, 15 Amps, 15 mm working distance) for all samples. Samples for both low and high shear model followed the same pattern as loop 1 but with different weight percentages. This difference was because of the device calibration. As discussed before, since there are some limitations for the values obtained from EDX, only relative comparisons are made in this study, such as looking at the patterns of the results.

For low shear model, all samples with defected surfaces showed significant difference in the calcification level compared to the smooth sample (p-value < 0.005 for all comparisons). Figure 4.16 and table 4.3 summarize the results obtained for this model. Comparisons listed in table 4.3 are made using 2-sample t-test tool in Minitab 15 software (ABIOMED Inc., Danvers).

![Figure 4.16 - Average Ca and P weight percentages for the low shear sample](image)

Figure 4.16 - Average Ca and P weight percentages for the low shear sample
Table 4.3 - Calcification level comparison between surface defected samples and smooth sample from the low shear model

<table>
<thead>
<tr>
<th></th>
<th>2 tests</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca Smooth vs. Rough</td>
<td>&lt;0.0005</td>
<td></td>
</tr>
<tr>
<td>P Smooth vs. Rough</td>
<td>&lt;0.0005</td>
<td></td>
</tr>
<tr>
<td>Ca Smooth vs. 2-Way Cracked</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>P Smooth vs. 2-Way Cracked</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Ca Smooth vs. 2-Way Cracked+Rough</td>
<td>&lt;0.0005</td>
<td></td>
</tr>
<tr>
<td>P Smooth vs. 2-Way Cracked+Rough</td>
<td>&lt;0.0005</td>
<td></td>
</tr>
</tbody>
</table>

For high shear model also, all surface defected samples showed significant difference in the calcification level compared to the smooth sample (p-value < 0.05 for all comparisons); however, the difference between the cracked and smooth samples in this model is on the contrary to other comparisons. Figure 4.17 and table 4.4 summarize the results obtained for this model. Again comparisons listed in table 4.4 are made using 2-sample t-test tool in Minitab 15 software (ABIOMED Inc., Danvers).

![Figure 4.17 - Average Ca and P weight percentages for the high shear sample](image-url)
As can be seen, just like loop 1, both types of surface defects (roughness and cracks) as well as the flow shear rate have an effect on this process; i.e. roughness and shear rate having the maximum and crack having the minimum effect.

This experiment showed that the patterns obtained from loop 1 for calcification concentration for different surface profiles at different shear rates are repeatable but with different percentages. As discussed before, EDX results for this study cannot be reliable for quantification purposes but they can be relied on for relative comparisons. Here, unlike loop 1, calcification level for defected surfaces was significantly different than the smooth surface in the high shear model; however the pattern looked the same. This difference is more visible wherever roughness is involved. Samples in this experiment were intentionally made extremely rough (average 9.2 µm roughness), which made them more susceptible to calcify. Therefore, the effect of roughness is more dominant than the effect of shear rate. With presented results in this experiment in both low and high shear rates, irregular surfaces trapped more calcification deposits than the smooth ones; however the calcification concentrations were higher for low shear samples.
In order to evaluate the effect of shear rate on calcification process by itself, calcium content concentration for smooth surfaces from the low and high shear models were compared with each other. For low shear model the average calcium content concentration, in weight percentage, was 11.94 ± 3.32 (%weight) while this value for high shear model was 7.23 ± 2.28 (%weight). Therefore in the absence of mechanical stresses, with 95% confidence (p-value < 0.005), low shear model has higher level of calcification than the high shear model.

Furthermore, EDX results did not seem to be reasonable for cracked samples and varied from sample to sample. This is because of EDX limitations in detecting elements inside the cracks and the possible shadowing effect. Therefore it can be more confidently stated that EDX is not a suitable tool for calcification analysis on cracked samples in these kinds of experiments.

4.5 Loop 1 and 2 Comparison

Some differences were observed between the results of loop 1 and 2. Patterns obtained from EDX results for calcification levels were the same between these two experiments, although with different weight percentages. It is believed that this is due to calibration problems with the device and the fact that measurements for these loops were done at different days.

The major difference between these two was the results for high shear samples. In loop 1, high shear samples, with defected surfaces, did not show a significant difference in calcification levels with respect to the smooth sample but it was not the same case in loop 2. High shear samples in loop 2, with defected surfaces, showed a significant difference in calcification levels with respect to the smooth sample. This major difference was more vividly present wherever the roughness was involved. This difference is most probably due to the roughness differences
between the samples of the two loops (2 µm for loop 1 and 9.2 µm for loop 2). Therefore, it can be concluded that the roughness has a more dominant effect than the shear rate in the calcification process of a polyurethane surface. For a better understanding of the differences between loop 1 and 2, figures 4.18 and 4.19 graphically compare the results for calcium contents between these loops for low and high shear models.

As can be seen, the pattern repeated in loop 2 for the low shear model with about the same weight percentage differences (Figure 4.18); however, for high shear model the differences between smooth sample’s calcification contents with the rest of the samples were significant except for the cracked sample.

![Calcium Concentration Comparison b/w First and Second Tests](image)

**Figure 4.18 - Calcium content comparison between loop 1 (Test 1) and loop 2 (Test 2) for low shear model**
4.6 Discussion and Conclusion

Main purposes of performing the experiments cited in this chapter can be listed as:

- Evaluating calcification process dependency on surface cracks
- Evaluating calcification process dependency on polymer’s material
- Evaluating calcification process dependency on polymer’s surface profile at different shear rates
- Checking the repeatability of calcification process for polymers with surface defects at different shear rates
After performing the first test, it was evident that polyurethane tubes got calcified even though there were no mechanical stresses involved; however, the calcification was limited to the surface of the material and no subsurface calcification was noted. Results obtained from this test showed that the calcification process, even in the absence of mechanical stress, is dependent on the type of the polymer, in this case polyurethane or polycarbonate. Another outcome of this test was the effect of cracks in calcification process of a polymeric surface. Although some initial findings showed that calcification levels significantly increased for cracked surfaces, but a more systematic procedure was required to characterize and quantify the differences it might have compared to a smooth surface.

Based on the findings from the first experiment, a new setup was designed to further investigate the effect of shear rate and surface defects in the calcification process. Results showed that all three factors (roughness, shear rate and surface cracks) have direct effect on the calcification process. Utilizing EDX as a measurement tool, roughness seemed to have the most dominant effect compared to the other two; however, the effect of cracks should be further investigated due to the device limitations in detecting elements inside the cracks. Shear rate also affected the calcification process. Polyurethane surfaces absorbed more calcification deposits in lower shear rates (9 sec⁻¹ vs. 82 sec⁻¹). This difference was even more evident wherever roughness was involved. Finally, the last experiment confirmed the results obtained from the previous test regarding the main factors affecting the calcification process with their interactions.
4.7 References


RECOMMENDATIONS FOR FUTURE RESEARCH

The focus of the present study was on the calcification process of polyurethanes, specifically Angioflex®, as a possible heart valve replacement material. Accelerated in vitro studies performed on Angioflex® heart valves, have shown that they have the potential to be used as a replacement heart valve with more resistance to calcification compared to available tissue valves on the market.

The effects of various parameters such as leaflet surface roughness, presence of cracks on the leaflet surface and flow condition, low shear and high shear flow, on calcification were further investigated. For the shear rate effect and in the absence of mechanical stresses, it was observed that polymers in lower shear rates were more susceptible to calcify. On the other hand, from studies on the VALT, which is performed under pulsatile flow and at high shear rates, calcification levels were higher than what was seen in the loops. It can be suggested that mechanical stresses, which are present on VALT studies, have caused the valves to become more calcified even though that it is expected to see lower levels of calcification in higher shear rates.

Based on the experiments performed so far, future studies can fall into the following categories:

Effects of mechanical stresses on calcification process of polyurethane material: one possible design could be using the same setup as in surface effect loop 1 or 2 with netted hollow mandrels covered with Angioflex® and connected to a pulsatile air flow. In each cycle of the pulsatility, polymer covering the holes undergoes mechanical stresses. Results from this test can be compared with what is available from loops 1 or 2.
**In vivo calcification studies with Angioflex® valves:** results from the *in vitro* testing on the valve accelerated life tester can be compared with the *in vivo* studies to evaluate the reliability of the *in vitro* calcification studies.

**Bisphosphonate-modification of Angioflex® valves:** Although satisfactory results were not obtained for bisphosphonate modified Angioflex® valves, it is advised to continue modifying Angioflex® with bisphosphonate, reducing their water absorption and negative charge by attaching cationic diethylamino groups to the bisphosphonate-modified polyurethane and evaluate its propensity to calcify both *in vitro* and *in vivo.*
“APPENDIX”
Schematic Bisphosphonate Modification of Polyurethanes

1. Bromobutylation of polyurethane (PU) [1]:

   Urethane site
   \[
   \text{[PU} - \text{NCOO-PU]}_m
   \]

   Bromobutylated urethane site
   \[
   \text{[PU} - \text{NCOO-PU]}_n - \text{[PU} - \text{NCOO-PU]}_{m-n}
   \]

   Intact urethane site

   PU = inactive parts of PU macromolecule

2. Simultaneous attachment of bisphosphonate (BP) and \textit{tert}-amino groups to the bromobutylated PU [1]:

   Bromobutylated urethane site
   \[
   \text{[PU} - \text{NCOO-PU]}_n - \text{[PU} - \text{NCOO-PU]}_{m-n}
   \]

   \[
   \text{[Bu}_4\text{N}_2\text{B}_4\text{O}_6, \text{DMAc, 0 }^\circ\text{C}}
   \]

   Intact urethane site

   R = Et, i-Pr
   \(n:k \text{ should be near 3:1}\)
**Lipids**

“The lipids are a large and diverse group of naturally occurring organic compounds that are related by their solubility in non-polar organic solvents (e.g. ether, chloroform, acetone & benzene) and general insolubility in water” [2]. There is a great structural variety among the lipids which includes fats, waxes, sterols, fat-soluble vitamins, monoglycerides, diglycerides, phospholipids, and others. Figure A shows the structures of some common lipids.

![Figure A - Structures of some common lipids. Source: www.wikipedia.org](https://www.wikipedia.org)
Phospholipids

Phospholipids are a class of lipids and are the main constituents of cell membranes. Most phospholipids contain a diglyceride, a phosphate group, and a simple organic molecule such as choline [2].

![Phospholipid structure](image)

Figure B – a) Phospholipid structure, b) Polar group of the molecule, highlighted in red. The U indicates the uncharged hydrophobic portion of the molecule, highlighted in blue. Source: [www.wikipedia.org](http://www.wikipedia.org)

Pyrophosphate

“In biochemistry, the term pyrophosphate (PPi or P$_2$O$_7^{4-}$) is used to refer to chemical compounds that encompass the esters, salts, and anion of pyrophosphoric acid. The latter, being a negatively charged anhydrous acid of phosphate, becomes reactive when heated. However, when suspended in water, the anion of pyrophosphoric acid also readily triggers the division of water molecules into hydrogen and hydroxide ions in a process called pyrophosphorolysis, which yields inorganic phosphate. Specifically, this involves the conversion of cellular adenosine triphosphate (ATP) to adenosine monophosphate (AMP), (ATP → AMP + PP$_i$) [3].
Figure C - a) Pyrophosphate anion, b) Ball-and-stick model of the pyrophosphate anion (P$_2$O$_7$$^-$).
Source: www.wikipedia.org

References:

[1] Personal communication with Robert Levy and Ivan Alferiev, our collaborators at Children’s Hospital of Philadelphia
