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Fabrication of Human Serum Albumin Film for Enhanced Hemocompatibility and Mitigation of Intimal Hyperplasia Under Physiologically Relevant Flow Shear Conditions

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FABRICATION OF HUMAN SERUM ALBUMIN FILM FOR ENHANCED HEMOCOMPATIBILITY AND MITIGATION OF NEOINTIMAL HYPERPLASIA UNDER PHYSIOLOGICALLY RELEVANT FLOW SHEAR CONDITIONS

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ABSTRACT

Current endovascular stents and synthetic vascular grafts have poor clinical outcomes in small diameter applications due to high incidence of thrombogenicity and intimal hyperplasia. To address this, we have developed an albumin film for mitigation of the complications of platelet adhesion and smooth muscle cell hyperplasia and hypertrophy.

A human serum albumin (HSA) film is fabricated on an anchoring layer of poly (glycidyl methacrylate) on surfaces of metallic and polymeric substrates. The PGMA and HSA layers are characterized by FT-IR spectroscopy, scanning electron microscopy, energy dispersive X-ray spectroscopy and contact angle analysis. We have confirmed the thromboresistance of albumin film by in vitro measurement of adsorption of human fibrinogen and platelets. We found that albumin film controlled the adsorption of fibrinogen evidenced by measurement of fluorescently labeled protein and adhesion force measurement. Human platelet adhesion was significantly lower on albumin coated compared to uncoated substrates. Smooth muscle cells play a key role in progression of intimal hyperplasia and hence we assessed the proliferation, hypertrophy and contractile state of cells in static and flow conditions. A vascular simulator was employed to provide forces of cyclic strain and flow shear to smooth muscle cells on albumin coated and uncoated ePTFE grafts. It was found that albumin film controlled proliferation and maintained the contractile state of smooth muscle cells on nitinol and ePTFE substrates. We developed a flow circulation loop model to assess the response of fibrinogen and platelets on albumin coated and uncoated e PTFE grafts. A significantly lower adsorption of fibrinogen and platelet adhesion was measured on albumin coated e PTFE grafts compared to uncoated grafts.

Exhibiting strong adhesion strength to polymeric and metallic substrates, human albumin film fabricated using PGMA as an anchoring layer has been shown to shield the surface from adhesive protein fibrinogen and prevents adhesion of platelets thereby providing an anti-thrombogenic layer.

Human albumin has been shown to maintain a controlled proliferation profile and spindle shaped morphology of vascular smooth muscle cells with increased expression of contractile protein smooth muscle alpha actin mitigating the complication of intimal hyperplasia post a percutaneous interventional procedure and bypass surgery.

DEDICATION

This doctoral tenure is dedicated to my parents Mrs. Nishi Khanna and Mr. Surinder Khanna who have been with me at every step of this challenging journey with their eternal, selfless, abundant love and blessings for my success and happiness. All the years of living away from their daughter with unspoken pain only to see her fulfill her dreams and support her tremendously to stand to on her own feet to see her living the life that she desires. My success and career will always be dedicated to their invaluable blessings, love and support. My grandmother Ms. Bimla Bhasin has showered me with her abundant blessings and love and has always prayed for my success and happiness being so far away to see me fulfill my dreams and ambitions. I would like to thank my elder brother Anirudh Khanna with all my heart for always protecting and loving his little sister and wishing for her success, happiness, prosperity and well being. I would like to thank my sister-in-law Charu Khanna for her love and care and my dear niece Anayra Khanna for bringing so much joy in our lives.

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CHAPTER 1

INTRODUCTION

Vascular implants and devices employing metals and synthetic polymers have frequently been associated with adverse clinical outcomes including, thrombosis, cell hyperproliferation and inflammation. The primary causes of the short-term and long-term failure of endovascular stents and small-diameter vascular grafts are thrombosis and intimal hyperplasia. Synthetic polymers such as ePTFE and Dacron have shown long term stability and inertness, however, their hydrophobic nature and low hemo-compatibility limit their success in low-diameter vessels [1-6]. A primary reason behind failure of synthetic polymers used for small diameter grafts has been low flow environments which has limited their application in large diameter vessels [7,8]. Low flow shear stress promotes strong and irreversible binding of adhesive protein fibrinogen with platelets resulting in platelet activation and aggregation, release of PDGF and thrombosis [9-13]. Myointimal hyperplasia has also been observed in those cases. Different surface modification techniques, bioactive coatings and approaches to facilitate endothelialization of surfaces have been proposed as means to prevent thrombosis and mitigate hyperplasia [72-76], however, there is still a need for an ideal vascular graft material alternative to autologous vessels with excellent clinical outcomes and long term patency rates.

We propose that fibrinogen adsorption, platelet adhesion and smooth muscle hyperplasia & hypertrophy will be prevented with the use of human serum albumin coating.

Research Significance

Small diameter grafts fail as a result of intimal hyperplasia and luminal surface thrombosis leading to vessel occlusion [5-8]. Normal blood vessels lined by inactivated endothelial layer maintain the anti-thrombotic, anti-inflammatory and fibrinolytic properties [29]. Autologous blood vessels such as saphenous vein and internal mammary artery lined by endothelial cell layer function well as compared to synthetic conduits [30,31]. However, due to poor vein harvest or poor vein quality in patients undergoing peripheral arterial reconstructive procedures, there is an unavailability of adequate autologous grafts [32]. Vessel injury due to surgerical procedure causes platelets and leukocytes to bind to the surface and anastomosis site. Adherent platelets and leukocytes release growth factors and molecules such as platelet derived growth factor, thrombospondin, b-thromboglobulin and reactive oxygen species [33,34]. These molecules can trigger a thrombotic response as well as promote proliferation and migration of smooth muscle cells leading to intimal hyperplasia at the anastomosis [35-37]. Early failure of the graft results from thrombosis and late failure results from intimal hyperplasia [38]. Intimal hyperplasia results in abnormal thickening of the vessel with lumen narrowing. Owing to the high propensity for intimal hyperplasia and thrombosis in low flow environments, application of synthetic grafts is limited to large diameter vessels with high flow rates [39-41]. Blood-biomaterial interactions play a major role in facilitating a thrombotic response. Upon implantation, the biomaterial surface is encapsulated by an adsorbed protein layer that triggers the activation, adhesion and aggregation of platelets and activates the coagulation cascade resulting in the formation of a thrombus on the surface [42-44]. The biomaterial can activate the complement system resulting in an inflammatory response [45]. Thus, the biomaterial surface, and the adsorbed protein layer, becomes an important determinant of its biocompatibility and long-term success. Adhesive proteins such as fibrinogen and von-Willebrand factor have been shown to facilitate activation, adhesion and aggregation of platelets [46-48].

Lacking amino acid sequences that can bind to platelet receptors, serum albumin has been shown to passivate the surface of a biomaterial against adhesion of platelets [49-52]. However, rapid degradation of the protein from the surface [53-55] and change in secondary structure conformation of albumin has been shown to be critical in facilitating the adhesion of platelets [56].

Research Innovation

Synthetic polymers employed in vascular grafts delay endothelialization and facilitate adhesion of platelets [57-59]. In the present study, we have employed a technique to design human serum albumin (HSA) films on substrates using poly(glycidyl methacrylate) (PGMA) as an anchoring layer (Fig.1). HSA is the most abundant protein present in human blood. It has been shown to passivate material surfaces thus blocking sites for adsorption of other proteins and adhesion of platelets and cells [60-67]. PGMA has been known for its high surface density and affinity for reacting with amines [68]. PGMA binds HSA through an easy nucleophilic addition reaction that results in the formation of strong covalent bond with the surface. Epoxy groups of PGMA act as spacer molecules and bind to albumin, keeping them close to their native secondary structure conformation [69-71].

Aim 1: Fabrication, characterization and assessment of human serum albumin film on poly(glycidyl methacrylate) (PGMA) anchoring layer for enhanced hemocompatibility and mitigation of neointimal smooth muscle cell hyperplasia.

Hypothesis: Human Serum Albumin film will reduce the incidence of thrombosis and restenosis by preventing theadhesion of platelets and mitigating the proliferation and growth of smooth muscle cells on a metallic surface.

Rationale: Human Serum Albumin is the most abundant protein present in human blood. It has been shown to passivate material surfaces thus blocking sites for adsorption of other proteins and adhesion

of platelets and cells [14,15-21]. In this study, PGMA, a polymer with epoxy functionality, is used to form a reactive anchoring polymer layer since the reactions of epoxy groups are quite universal and can covalently anchor PGMA to the substrate surface [22]. The glycidyl methacrylate units located in the "loops" and "tails" sections of the attached PGMA chain could serve as reactive sites for the subsequent attachment of (macro) molecules with complementary functional groups. PGMA is also known for its high surface density and affinity for reacting with amines [23].

Aim 2: Fabrication and characterization of human serum albumin films on ePTFE graft surfaces for enhanced hemocompatibility and adhesion strength under physiologically relevant flow shear stress conditions.

Hypothesis: Human albumin with high adhesion on ePTFE surface will reduce the incidence of thrombus formation.

Rationale: Exposure of ePTFE to blood results in rapid irreversible protein adsorption by hydrophobic interaction [24]. Proteins adsorbed on the surface activate extrinsic pathway of blood coagulation resulting in the formation of fibrin and platelet deposition. It is imperative to develop functional groups on ePTFE surface to enhance surface wettability and reduce rapid substantial protein and platelet adhesion. Strong adhesion strength of a vascular coating is essential for long term beneficial effects in regulating cellular and platelet growth to maintain vascular integrity and clinical patency.

Aim 3: Assessment of potential of albumin film to mitigate intimal hyperplasia in a smooth muscle cell-platelet co-culture model under physiologically relevant forces of flow shear.

Hypothesis: Human serum albumin film on ePTFE surface will reduce hyper-proliferation and hypertrophy of vascular smooth muscle cells under clinically relevant mechanical flow shear forces observed in small diameter blood vessels.

Rationale: Adherent platelets on the hydrophobic ePTFE surface release growth factors such as platelet derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) to stimulate migration and proliferation of vascular smooth muscle cells. Synthetic grafts perform well in large-diameter, high-flow, low resistance locations such as the aorta and the iliac and proximal femoral arteries. ePTFE has not been successful as small-diameter (2-5mm) vascular grafts replacement for the below knee popliteal artery or the tibial vessels [25,26]. Low blood velocity allowing more interaction between blood and biomaterial has been found to a major reason behind low performance of synthetic grafts in small diameter vessels [27,28]. Anastomotic intimal thickening in low shear stress conditions can cause substantial decrease in the luminal diameter of the vessel. This is one of the major obstacles in the development and application of small diameter vascular graft.

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CHAPTER 2

ANTI-THROMBOGENIC COATINGS FOR VASCULAR DEVICES

Abstract

Thrombosis and restenosis remain major complications after bypass graft surgery. Autologous vessels are the gold standard for bypass graft surgeries. However, due to unavailability of healthy autologous arteries, synthetic polymers have been widely employed for the fabrication of vascular grafts. Even though commonly employed synthetic polymers such as Teflon, Dacron and polyurethanes offer the advantages of being stable in biological environments and non-toxic, they have shown poor clinical outcomes as small-diameter vessel replacements because of high rates of thrombosis in low flow environments. Currently, no material has been developed that has the same patency rates as autologous vessels. Many attempts have been made to design coatings that reduce the incidence of thrombosis in vascular graft conduits. This review attempts to provide a summary of the coatings employed to prevent the incidence of thrombosis and improve hemocompatibility of vascular grafts.

Keywords: Vascular grafts, thrombosis, synthetic polymer conduits, anastomotic hyperplasia

Introduction

Atherosclerosis is the major cause of mortality and morbidity in the developed world, causing nearly $300 billion USD annually in revascularization treatments [1]. Atherosclerosis is a chronic, progressive disease of the vessel wall intima. Atheromatous plaque is the characteristic lesion, with a central core of lipid and cholesterol crystals and cells such as macrophages, smooth muscle cells

and foam cells with necrotic debris, proteins and degenerating blood fragments [2,3]. The major complications of atherosclerosis result from progressive obstruction of a vessel lumen or disruption of a plaque followed by thrombus formation.

Percutaneous interventions have increased the longevity of patients suffering from atherosclerosis-affected vessels [4]. Balloon angioplasty involving insertion and inflation of a balloon into a stenosed artery is used to break the plaque and dilate the artery to restore blood flow. A metallic stent implantation is often accompanied by balloon angioplasty to prevent the incidence of elastic recoil and restenosis by the release of an antiproliferative agent. In the case of elderly and diseased patients, bypass or replacement of the stenosed artery is employed to restore blood flow. The patient's own blood vessel such as the saphenous vein or internal mammary artery is the preferred choice for bypass [99,100]. However, due to unavailability of an autologous healthy vessel and/or need for multiple surgeries, synthetic vascular conduits have been employed to bypass stenosed arteries. [5,6,7,8].

Vascular grafts made of Teflon were introduced in 1957 and have proven superior to all other synthetic prostheses due to rapid healing, minimal tissue reaction and gain of strength after implantation. Edwards and collaborators showed that tubular knitted Teflon was easy to suture, inert and biocompatible [9,10]. In late 1960s, a paste extrusion protocol was developed to create a fluorocarbon polymer ePTFE by heating, stretching and expanding Teflon to create a microporous graft with inertness and improved tissue adhesion [11]. ePTFE formed into sheets has solid nodes interconnected by fine fibrils, and the intermodal distance can be changed to alter the graft porosity [10].

In 1958, DeBakey et al, introduced the Dacron (polyethylene terephthalate) (PET) graft, which has demonstrated high strength and stability for over 10 years without deterioration, with 5-year patency rates of 93% for aortic replacements [12] and 43% for small-diameter vessels (below the knee) [98].

Polyurethane (PU) has been used as a vascular conduit owing to its good elasticity and superior compliance [13] However, PU-based vascular grafts have been susceptible to degradation in vivo [14]. Biodegradable polymers polylactic acid (PLA) and poly-glycolic acid (PGA) have been explored as vascular graft conduits because they can biodegrade without leaving toxic residues. However, they have been prone to undergo aneurysmal dilation and rupture prior to healing. PGA has been shown to rapidly degrade due to a hydrolysis reaction [15].

ePTFE and Dacron have been shown to have good mechanical stability to be promising candidates as vascular graft replacements. Dacron has shown to have better compliance than e PTFE which is measured as the ratio of incremental volume change to incremental transmural pressure change (% diameter change/mm mercury). Both PET and ePTFE have been shown to have the similar patency rates for 3 (61%) and 5 years (45%) [16,17] compared to 5 (77%) [105] and 10 year (50%)[106] patency rates of autogenous vein grafts as femoropopliteal vessel replacements.

Synthetic grafts perform well in large-diameter, high-flow, low resistance locations such as the aorta and the iliac and proximal femoral arteries. In contrast, Dacron and Teflon have not been successful as small-diameter (2-5mm) vascular grafts replacements for below the knee popliteal artery or the tibial vessels [18,19,20,21,22]. Low blood velocity allowing more interaction between blood and biomaterial has been found to a major reason behind low performance of synthetic grafts in small diameter vessels [23, 24]. ePTFE has been shown to provide satisfactory performance as a conduit for femoropopliteal reconstruction (in the case of nonavailability of saphenous vein) due to its smoothness, easy care and no required pre-clotting. [25, 26]. However, despite recent advances in developing biocompatible graft materials, the need for a nonthrombogenic, compliant and endothelial-cell-growth-promoting material remains a challenge in tissue engineering and biomaterials research.

Vascular graft pathology

Artery-graft anastomotic sites are the primary areas where neo-intimal hyperplasia is observed. Factors that contribute to loss of patency are vessel injury, adsorption of plasma proteins, neo-intimal hyperplasia, thrombosis and hemodynamic factors.

1. Vessel injury

Graft surgery causes damage to the intima, media and adventitia of the vessel. Injury to the vessel wall and denudation of the endothelial layer triggers the contact of platelets with adhesive proteins such as collagen, von Willebrand factor, fibronectin and laminin resulting in platelet activation and aggregation [27] and contact of smooth muscle cells with the inflammatory and procoagulant blood constituents. Activated platelets release cytokines such as platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF), which facilitate migration and proliferation of smooth muscle cells from the media to the intima. Proliferating smooth muscle cells release cytokines in an autocrine and paracrine fashion and synthesize extracellular matrix, causing formation of neointima.

2. Attachment of plasma proteins

When a synthetic graft is implanted, the luminal surface of the graft becomes coated with a layer of plasma proteins. The most abundantly present albumin is replaced by the heavier adhesive proteins, predominantly fibrinogen, which forms a pseudo-intima [28]. Endothelial cells serve as a nonthrombogenic surface covering the inner layer of a blood vessel called neo-intima. After a graft implantation, only the anastomosis site becomes covered by endothelium, and thus the inner lining of the synthetic grafts remain covered by the pseudo-intima platelet-protein layer even weeks after implantation. Dislodgement of the pseudo-intima layer can cause distal embolization or a flap-valve formation, both causing acute obstruction.

3. Anastomotic hyperplasia

Intimal hyperplasia is a healing response to injury in the vascular wall that promotes abnormal thickening of the vessel with lumenal narrowing. Intimal hyperplasia involves migration and proliferation of vascular smooth muscle cells from the media to the intima with subsequent release of extracellular matrix proteins, which cause narrowing of the lumen. Patency of synthetic grafts becomes impaired due to intimal hyperplasia at the anastomotic regions. While medial cell proliferation begins immediately after injury, peaks in 2 days and declines by 7 days, intimal cell proliferation peaks at 1 week and declines by 2 weeks [29]. The number of smooth muscle cells remains fairly constant after 2 weeks; however, the cells increase in size up to 8 weeks due to accumulation of extracellular matrix [30]. Mismatch in the mechanical properties of the native vessel and synthetic graft alsocause1 hyperplasia of smooth muscle cells [31].

4. Thrombosis

Thrombosis involves activation of the coagulation cascade and complement system and activation of cellular components of blood such as leukocytes and platelets. Together these cause the formation of a blood clot that obstructs blood flow [33].

In the blood coagulation cascade (Figure 2.1), inactive enzymes (zymogens) are enzymatically serially activated due to surface contact or proteolytic cleavage. The cascade is initiated either by intrinsic or extrinsic pathway from the site of endothelial injury. The pathways converge to cause the activation of thrombin from prothrombin, which catalyzes the conversion of fibrinogen to a fibrin clot, which is stabilized to an insoluble fibrin gel upon activation by factor XIII [32].

The complement system is involved in regarding biomaterials as non-self and facilitating an inflammatory response. It comprises of more than 20 plasma proteins and two pathways, classical

and alternate which form C3 convertases and C5 convertases catalyzing formation of C3a (an anaphylatoxins) and C3b and C5a (an anaphylatoxin) and C5b respectively resulting in the formation of membrane attack complex causing cell lysis[33]. Biomaterials activating complement system facilitate the interaction of factor B with surface-bound C3b as opposed to nonactivating surfaces that favor binding of factor H with C3b[34].

Injury to the vessel wall activates endothelium, which causes enhanced secretion of prostacyclin (PGI2), von-Willebrand factor (vWF) and increased expression of cell surface adhesion molecules [32,36] such as PECAM, ICAM-1, P-selectin, V-selectin and VCAM-1, which bind to leukocytes and platelet receptors, causing platelet aggregation.

Leukocytes facilitate platelet recruitment and activation, promoting further leukocyte adhesion, fibrinolysis and thrombin formation [35]. Sticky activated leukocytes bound to the damaged endothelial layer and biomaterial surfaces express high levels of membrane receptors CD11b and PSGL-1, which bind with GP-IIB/IIIA and P-selectin, respectively [33] expressed on activated platelets facilitating the incidence of thrombosis.

The platelet-mediated response resulting in thrombosis on biomaterials comprises plasma protein adsorption, leukocyte adhesion, platelet aggregation and blood coagulation. Thus, minimizing surface-induced platelet adhesion is a central goal in designing hemocompatible biomaterials.

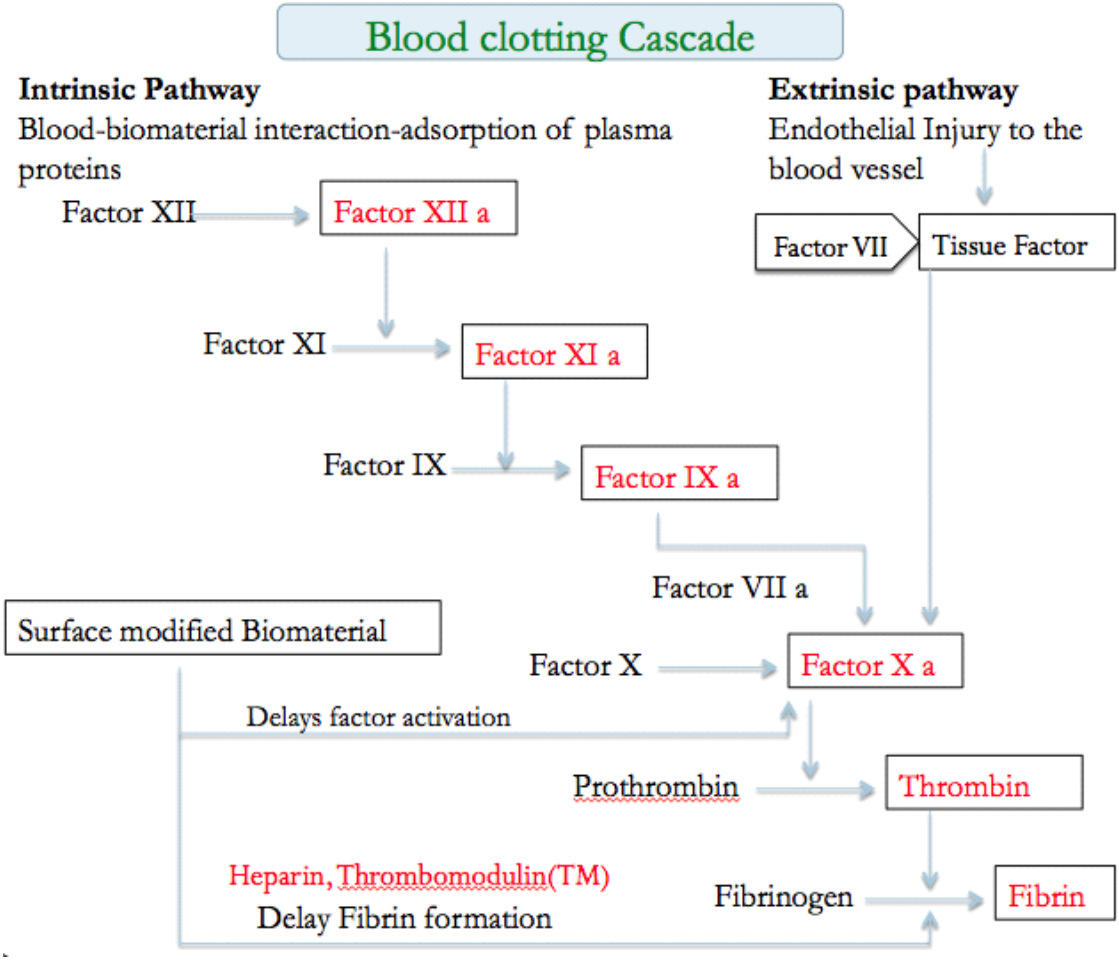

Figure 2.1 Blood Coagulation Cascade

Hemodynamic factors

It has been shown that reduction in shear stress causes neo-intimal hyperplasia [37,133,134]. Reduction in shear stress also causes release of vasoactive substances such as nitric oxide, prostacyclin, mitogens such as platelet derived growth factor (PDGF) and basic fibroblast growth factor (bFGF), activation of vascular cell transcription factors, protein synthesis and rearrangement of cytoskeleton causing vessel thickness [38,135].

Shear stress has been proven to vary with arterial diameter. To maintain a constant shear force, arterial diameter increases or decreases in response to changes in blood flow[139]. Wall shear stress affects aggregation of platelets [39]. Low shear rates promote platelet aggregation. High shear rates cause reduced size of platelet aggregates, however, shear stress values greater than 70 dynes/cm^2 cause an exponential increase in platelet aggregation [40,41].

Cells in the vessel wall are exposed to three types of mechanical forces, 1) stress caused by pressure of the blood perpendicular to the vessel wall, 2) circumferential wall stress or hoop stress exerted as a result of the stretching of the vessel wall by the transmural pressure gradient, 3) fluid shear stress, the friction at the interface between flowing blood and the apical surface of endothelial cells [140]. In the case of denudation of endothelial cells after the trauma of a bypass surgery, smooth muscle cells and fibroblasts are exposed to blood flow. These stresses regulate the function and dynamic remodeling of the vessel wall and also contribute to development of the pathological conditions. A string correlation between low shear stress and atheroma formation was first postulated in 1969. Numerous preclinical and clinical studies have proved that physiological high shear stress inhibits smooth muscle cell proliferation while low shear stress causes disturbed and turbulent flow, low flow velocity and recirculation flow significantly contributing towards progression of neo-intimal hyperplasia in those areas and also by upregulating PDGF [42,43, 101,102,103].

Effect of shear stress on plasma proteins:

High shear stress conditions facilitate the weak and reversible adsorption of von Willebrand factor and GP-IB/IX/V receptors of platelet, a process known as platelet tethering to slow down platelets under blood flow [44,45]. At low shear stress conditions (<12 dynes/cm^2), surface adsorbed fibrinogen forms a strong complex with GP IIB/IIIA receptors on the platelet membrane [130]. The

adsorption-induced secondary structure conformation change of fibrinogen has been shown to be a critical determinant correlated with the adhesion of platelets [131]. Both fibrinogen and von Willebrand factor work synergistically to promote platelet adhesion after vessel injury and on a biomaterial [125, 126]. Other integrins expressed on the platelet membranes GP-IIA/IB, GP-VA/BI and GP-VIA/BI promote adhesive interactions with ECM proteins such as collagen, fibronectin and laminin, respectively [127, 128, 129].

Methods employed to reduce thrombogenicity

Poor patency rates and clinical outcomes of synthetic conduits in small-diameter vessels have led to intensive research interest in surface modification of biomaterials. These include surface coatings to reduce the incidence of thrombogenicity and neointimal hyperplasia and to improve tissue adhesion and healing. Also, because the required mechanical properties such as suture strength and vessel-wall thickness are provided by existing synthetic conduits, surface modification strategies have been employed to achieve the ideal healing response. Three approaches have been widely employed to reduce the incidence of thrombogenicity — surface modification, endothelialization and bioactive coatings. These coatings include: carbon, poly-ethylene glycol, heparin, nitric oxide, thrombomodulin, hirudin, salicyclic acid, albumin among others.

Carbon

Carbon coating enhances electronegativity of a surface, thus reducing the propensity toward platelet binding. A homogeneous and stable coating of graphite on polyester grafts has shown reduced incidence of platelet activation, adherence and spreading in comparison to noncoated and control grafts [46]. However, graphite-coated polyester grafts were found to be difficult to suture. High density carbon-coated Dacron grafts have shown less thrombosis and thinner fibrin capsule than

noncoated control grafts [47]. However, surface irregularities have caused poor performance in other studies [48]. Carbon-coated grafts have shown short-term mitigation of platelet adhesion; however, overall patency rates were not higher when compared to uncoated controls [49]. Robertson and collaborators demonstrated the efficacy of diamond like carbon coatings for anti-microbial and anti-thrombogenic properties [145]. In clinical trials for below knee popliteal and distal bypass grafting did not show significant improvement in patency rates 2 years after implantation [138].

Hydrophilic coatings

Polyethylene glycol (PEG), polyethylene oxides and polyurethanes are the most frequently employed hydrophilic polymers as coatings as they prevent protein adsorption. PEG can be readily immobilized on surfaces using silane conjugation techniques [141]. The length and degree of branching of these materials affect the degree of platelet repulsion. PEG has been widely used to immobilize protein and other biomolecules such as heparin to render a thromboresistant surface: PEG chains allow conservation of 3D structure and mobility of biomolecules. Long chains of ethylene oxide create a high dipole moment, which causes high degrees of hydration to repel surface proteins. Surface modification with PEO has been researched by investigators by covalent grafting and physical adsorption. PEO- and PEG-coatings on ePTFE grafts have provided short term protection to platelet adhesion and intimal hyperplasia [52, 107, 108]. However, in vivo results have been inconsistent with in vitro results, and no clinical studies have yet been performed to test the efficacy of these coatings in reducing thrombosis incidence [142]. Further, it has been speculated that creating a hydrophilic surface is not sufficient to reduce thrombosis incidence [143]. Thus, the need for bioactive coatings to prevent adhesion of platelets is essential to create a thromboresistant surface.

Heparin

Heparin is a sulfated glycosaminoglycan that has been shown to inhibit blood coagulation by binding to antithrombin III, a serine protease inhibitor that inactivates pro-coagulant thrombin (activated factor IIa) and other proteases involved in the blood clotting process [53, 110]. Lev and collaborators compared rates of thrombosis in heparin-coated (0.8%) and noncoated stents (6.1%) in patients undergoing percutaneous intervention procedures [54]. In this study, Heparin-coated stents showed improved 30-day outcomes as compared to noncoated stents [54]. Lin and collaborators found that heparin-coated small caliber vascular grafts caused mitigation of neointimal hyperplasia and platelet deposition in aortoiliac reconstruction in a baboon model [113]. Nie and collaborators demonstrated heparin as an effective coating for promoting endothelialization and anti-thrombogenic potential [144]. Preclinical and clinical studies on covalently bound heparin have been shown to render surfaces thromboresistant. However, covalently bound heparin has been shown to have limited use due to loss of functional activity as a result of improper immobilization and rapid degradation [55, 112]. Heparinized surfaces have also been shown to be centers for affinity of adhesive proteins such as collagen, fibrinogen and von Willebrand factor [56]. Heparin-bonded Propaten® vascular grafts (W.L. Gore and Associates) are manufactured using Carmeda® Bioactive Surface modification [58, 109]. The graft has shown promising results for lower extremity bypass with lesser rates of thrombosis [59, 111]; however, the clinical trials were non-randomized and retrospective which brings into question the significance of the results.

Nitric oxide-releasing biomaterials

Nitric oxide (NO) has been shown to inhibit smooth muscle cell proliferation and to inhibit platelet aggregation and activation [57, 60]. Materials facilitating the release of NO at the graft site have been

widely employed to reduce the incidence of thrombosis. Hydrophobic polymer films releasing diazeniumdiolate, an NO-donating compound, was shown to facilitate the release of NO for 72 hours, partially inhibiting platelet adhesion [61]. Polyurethane-releasing nitric oxide by a diazeniumdiolate-modified nitric oxide-producing peptide was also shown to reduce platelet adhesion and smooth muscle cell proliferation and promote endothelial cell growth. [62]. NO-releasing polyurethane inhibited thrombus formation in a sheep arteriovenous bridge graft model for up to 21 days [63]. However, none of the studies have been capable of facilitating the release of NO for a sufficiently long time period to prevent incidence of thrombosis.

The effect of nitric oxide is mediated by cyclic 3'5'- guanosine monophosphate (c GMP) secondary messenger pathway. Sildenafil, a phosphodiesterase inhibitor, facilitates accumulation of c GMP, enhancing NO function offering an alternative as anti-platelet adhesive grafts [64]. However, the effect of NO as a platelet inhibitor been shown to be less relevant in mouse models [65].

Thrombomodulin

Thrombomodulin (TM) expressed by endothelial cells forms a complex with thrombin causing the release of protein C (aPC), a potent inhibitor of Factor V and Factor VIII of the blood coagulation cascade [66]. TM has also been shown to inhibit leukocyte adhesion and endothelial cell apoptosis. TM ligated using C-terminal azide moiety on PU-coated PTFE grafts maintained activity for up to 2 weeks [67]. However, the modified surface was not able to inhibit in vivo platelet adhesion using a baboon arteriovenous shunt model [68].

Hirudin

Hirudin, produced by medicinal leeches (Hirudo medicinalis), is a potent inhibitor of thrombin. Hirudin has been an effective therapy for patients with unstable angina. Polyester grafts with covalently bound hirudin via an albumin coating was shown to be capable of inhibiting thrombin activity under dynamic flow conditions [69]. However, the hirudin coating degraded in 7 days. PLLA vascular grafts with immobilized hirudin using a PEG spacer via EDC/NHS chemistry in a rat carotid artery interposition model was shown to maintain better patency at one month (83%) as compared to control grafts (50%) promoting endothelial cell growth and less infiltration of macrophages and monocytes [70].

Salicylic Acid

Salicylic acid and its derivatives have been used as therapeutic agents. Aspirin®, a form of salicylate, inhibits prostaglandin biosynthesis blocking thromboxane formation in blood platelets [50]. Hydrophilic acrylic polymer coatings bearing salicylic acid residues on Dacron grafts showed improved thrombogenicity as compared to uncoated controls [104]. Rodriguez and collaborators created coatings of a polyacrylic derivative of triflusal (2-aqcetyloxy-4trifluromethyl) benzoic acid (a derivative of salicylic acid) that allowed slow release of triflusan, improving platelet anti-aggregation without an anti-thrombotic drug [51].

Endothelial cell-seeding of vascular grafts

Early failure due to thrombosis is more frequent in synthetic grafts compared to vein grafts because of the presence of an endothelial cell lining in vein grafts, which prevents thrombosis and intimal

hyperplasia, thereby improving patency rates. Further, complete regeneration of the endothelial layer does not occur in humans as compared to animals.

Herring, Mansfield and collaborators introduced the idea of extraction and implantation of autogenous endothelial cells (endothelial cell seeding) to decrease the incidence of thrombosis and improve patency of synthetic grafts [71]. The endothelial cell layer regulates vascular tone and permeability, thrombosis, inflammation and fibrinolysis by expression and secretion of anti-inflammatory molecules.

Intact endothelium releases potent vasodilators such as nitric oxide (NO) and prostacyclin (PGI2), which prevent platelet activation, aggregation and adhesion to the endothelium. Therefore, endothelium has been shown to prevent thrombosis formation [71].

A number of investigators have attempted to coat an EC layer on biomaterials. However, the attempts have not been successful due to difficulties in isolation and seeding of endothelial cells in large numbers in a short time frame and under sterile conditions. It has been proven that a confluent layer of ECs displays a nonthrombogenic profile. However, endothelial cells appear to be more thrombogenic at subconfluent densities [72]. The healing process of synthetic prostheses remains incomplete in humans, with formation of a fibrous capsule, minimal capillary ingrowth and sparse lining of endothelial cells in the lumen [73]. Uncoated e PTFE and Dacron have been shown to promote poor endothelial cell attachment and endothelial cell retention [74,114]. This has necessitated the importance of a coating to improve the adherence, growth and morphology of EC culture. Basement membrane proteins such as fibronectin [75], arginine-glycine-aspartate (RGD)-containing peptide (active component of fibronectin) [76], collagen [95], laminin [96] have shown improvement in endothelial cell layer adhesion on untreated graft materials. In a porcine interposition graft model, coating ePTFE with fibronectin improved cell growth and endothelialization [77]. However, coating such basement membranes on the biomaterial increases the incidence of

thrombosis as a result of exposure to blood and/or loss of endothelial layer due to shear [78]. A two-stage seeding process involving extraction of endothelial cells followed by prolonged culture to increase cell number and then seeding onto the graft material (a process called sodding) has been proposed to be better than a single seeding process, which has shown no significant response in clinical trials. A reason behind inefficiency of the single-seeding process is cell loss due to exposure to blood flow. After exposure to blood flow shear, 70% of cell loss occurs within 45 minutes [79]. e PTFE grafts have been shown to have 10+7% endothelial cell attachment and 4+3% endothelial cell retention [80]. Preconditioning of endothelial cells with long-term flow shear has improved retention in in vitro studies [81].

Preclotting of grafts

Porosity (for transfer of nutrients, fluid, ions and ingrowth of tissue) of a graft is an important parameter for healing and long term patency. However, porous grafts are permeable to blood during implantation. Impregnation of graft pores with the patient's blood is employed to prevent initial leak. Investigators have studied biodegradable coatings that will degrade over time to support healing.

Albumin is shown to be advantageous in passivating surfaces. Albumin-coated prostheses are now commercially available in Europe and Canada and are under trials in the US [82].

Gelatin with poor antigenicity has been used under the trade name Gelseal Triaxial graft. It has been shown to promote cellular growth and tissue integration [83]. Collagen has been proven advantageous in promoting faster healing and in endothelial layer formation; however, collagen has been associated with immunogenicity and thrombogenicity issues [97].

Albumin

Albumin is the most abundant protein present in the blood [28]. Because of its abundance, it has been shown to passivate surfaces and is considered a thromboresistant protein. Adsorption of albumin to medical device surfaces has been shown to minimize platelet activation and adhesion and activation of complement [84,85,86].An albumination has been shown to render surfaces nonthrombogenic and noninflammatory by minimizing adsorption of adhesive proteins on the surface and adhesion of platelets and leukocytes [115, 116, 117].

Albumin adsorbs rapidly. However, it is replaced by other adhesive proteins such as fibrinogen, von Willebrand factor and factor XII [87]. Albuminated surfaces have also been shown to go undetected by microorganisms [88, 89], and thus can minimize the incidence of infection.

Attempts to create surfaces coated with albumin by physical adsorption become displaced by other proteins. Investigators have employed methods to either promote albumin adsorption on a surface or coat surfaces with albumin [121,122,123]. However, physical adsorption of albumin on a surface causes rapid degradation and reduced passivation effect. Covalent binding methods have been employed to enhance the longevity of albumin coating on a surface [118, 119, 120]. Immobilization of straight chain 16 or 18- carbon alkyl groups on surfaces was achieved to selectively adsorb albumin for similarity to high-albumin fatty acid binding sites of albumin [90]. This method caused increased affinity of the surface towards albumin; however, it also enhanced adsorption of other proteins such as fibrinogen and subsequently encouraged denaturation of adsorbed proteins and platelet activation.

Grafting cibacron dye on a surface was attempted to selectively adsorb albumin on the surface [91]. CB has structural similarity to bilirubin, which is an endogenous ligand associated with albumin in vivo. This process increased adsorption of albumin on the surface. However, improper presentation

of CB affinity ligand on the surface and inaccessibility of ligand to plasma protein were the major limitations of this process.

Immobilization of a monoclonal antibody (Mab) against albumin on a surface showed increased adsorption of albumin [92]. However, nonuniform immobilization of the ligand caused irregular adsorption of albumin on the surface and reduced functional activity.

Glutaraldehyde cross-linked human albumin-coated polyester knitted grafts have been shown to provide short-term prevention of thrombus formation by reduced platelet and leukocyte adhesion and aggregation.[93,94].Albumin-coated knitted Dacron grafts have been shown to reduce postoperative inflammation[132l However, significant difference from the controls long term was not observed. This might have been because of rapid degradation of albumin from the surface or because of surface adsorption-induced denaturation.

Structural stability of albumin on a surface has been considered an important factor in reducing activation and adhesion of platelets. Beyond a critical degree of unfolding, albumin has been shown to allow platelet adhesion and aggregation [124]. Hence, there is still need of immobilization techniques to coat albumin on a surface in such a way that its structural stability is maintained, which would allow it to retain its native conformation to prevent fibrinogen adsorption and platelet adhesion.

Human serum albumin immobilized on a poly(glycidyl methacrylate) PGMA anchoring layer [136] has been shown to prevent hyperplastic response of rat aortic smooth muscle cells up to 30 days and vascular platelet adhesion under flow shear conditions [137]. PGMA covalently bonded to albumin allowed its secondary structure conformation to remain intact, thereby preserving albumin's functional property of preventing adhesion of platelets and preserving long term stability on the surface.

Conclusions

Developing antithrombogenic coatings for vascular devices is an area of wide interest. Despite extensive research on designing surfaces that reduce the incidence of thrombosis, no one has been able to develop a biomaterial alternative to autologous vein grafts, which have excellent clinical outcome in terms of reducing platelet adhesion and promoting endothelial healing. Integrating our knowledge of blood-biomaterial interactions, tissue engineering and biomaterials, there is a need to develop a new generation of vessel constructs the would be nonthrombogenic and self-endothelializing and mitigate inflammatory and hyperplastic responses of vascular smooth muscle cells for superior biocompatibility and long-term clinical patency rates.

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CHAPTER 3

HUMAN SERUM ALBUMIN FILM FOR ENHANCED HEMOCOMPATIBILITY AND PREVENTION OF INTIMAL HYPERPLASIA

Abstract:

Introduction: Restenosis and thrombosis are two major clinical complications of endovascular stents. In this study, a novel biopolymeric coating made of human serum albumin (HSA) grafted on poly (glycidyl methacrylate) (PGMA) is proposed to reduce neointimal hyperplasia by inhibiting smooth muscle cell (SMC) proliferation and shielding fibrinogen (Fg) adsorption and platelet adhesion, thereby reducing thrombosis incidence.

Materials and Methods: Plasma treated Nitinol (NiTi) discs were surface modified with 0.5% wt/vol PGMA (M_n=176000g/mol) in chloroform to produce an epoxy rich anchoring layer followed by dip-coating of 5% wt/vol HSA. Samples were then annealed at 150℃ vacuum oven for 1 hour. HSA grafted samples were incubated in NHS-Rhodamine- labeled human plasma fibrinogen solution in phosphate buffer for 1 hour at 37℃. Fluorescence was measured at 525nm and 575nm. Change in secondary structure conformation of annealed albumin was assessed by CD spectroscopy. Platelet-rich plasma isolated from fresh human blood was incubated on HSA nanofilms for 1 hour at 37℃. Platelet adhesion was measured by lactate dehydrogenase (LDH) assay. Sprague Dawley rat aortic SMCs were cultured on HSA nanofilms for 5, 15 and 30 days in DMEM with 10% FBS. Cell proliferation was assessed by MTT assay. Expression of contractile marker smooth muscle alpha-actin was assessed by a cell based ELISA assay.

Results: The thickness of the PGMA (18±0.4nm) and HSA coatings (102±4.5 nm) was determined using ellipsometry. There was significantly less human plasma fibrinogen adsorption on HSA nanofilms (90±9.8ng/mm2; p = 0.023) as compared to bare NiTi. LDH assay showed significantly less platelet adhesion on HSA films compared to NiTi under static (p = 0.014) and shear conditions (p = 0.021). HSA films allowed significantly less rat aortic VSMC proliferation over a period of 5, 15 and 30 days as compared to bare NiTi (p =0.027, p =0.031 and p =0.032 respectively). The difference in the smooth muscle alpha actin expression on HSA for 5 and 15 days (p = 0.063) was insignificant as compared to bare NiTi. VSMCs cultured on HSA at Day 30 expressed significantly higher smooth muscle alpha actin content as compared to bare NiTi (p = 0.038).

Keywords: Human serum albumin; thrombosis; restenosis; synthetic bypass grafts

3.1 Introduction

Endovascular stent deployment and bypass graft surgery are the most commonly employed revascularization procedures to treat blood vessels stenosed by atherosclerosis. However, stent and graft implantation are often associated with two major complications, restenosis and thrombosis. Restenosis is the re-narrowing of a revascularized blood vessel due to hyperplastic response of smooth muscle cells (neointimal hyperplasia). Thrombosis causes occlusion of the blood vessel due to platelet adhesion and aggregation at the site of endothelial denudation and on the thrombogenic surfaces of stent struts and grafts. The occlusion is a result of activation of the coagulation cascade, which impedes blood flow [1,2,3]. Anti-platelet treatment has been shown to increase the risk of hemorrhagic complications [4] and ischemic complications [5]. Although rates of restenosis and target vessel revascularization are reduced after deployment of drug-eluting stents releases pharmacological agents [6,7], the incidence of in-stent thrombosis is not decreased when drug-

eluting stents are compared to bare-metal stents [8,9,10,11,12]. Further, it has also been found that agents released from drug-eluting stents not only affect proliferation and migration of VSMCs, but also that of endothelial cells in *in vitro* [13,14], preclinical [15] as well as clinical studies [16]. Further, polymers used for designing drug-eluting stents and grafts have been associated with hypersensitivity reactions [14-17], stenosis and thrombosis [18,19,20]. It has been observed that endothelial cells (ECs) adhere and spread moderately on hydrophilic surfaces while EC adhesion is reduced or even absent on hydrophobic surfaces [21,22]. Because Dacron and ePTFE polymers are hydrophobic, they allow adsorption of adhesive proteins and adhesion of platelets with poor or no EC adhesion. Nitinol and 316SS surfaces commonly used for designing stents have also been shown to inhibit normal growth of endothelial cells [23].

Investigators have attempted to coat biomaterials with endothelial cells. However, the technique has not been clinically successful due to complications associated with the isolation and seeding of ECs in large numbers in a short time under sterile conditions and non-translatable results from animals to humans. ECs have also been shown to display a thrombogenic profile under sub-confluent conditions [24].

Protein-resistant surfaces have been developed by immobilizing hydrophilic molecules such as poly(ethylene oxide) (PEO) and polyethylene glycol (PEG) on synthetic polymers [25], reducing protein adsorption by steric repulsion and creating a hydration layer with low interfacial energy at the surface. Although PEG-coated implants have shown significantly reduced platelet adhesion [26-29], most *in vivo* studies of PEG-coated implants have been unsuccessful at promoting blood coagulation and blood clot formation [30,31,29]. Various bioactive coatings such as heparin [32], nitric oxide [33], thrombomodulin [34,35] and hirudin [36] have been proposed to prevent platelet adhesion. However, their short half-life on a biomaterial's surface decreases their suitability for long-term hemocompatibility. Hence, improvements of currently available systems and introduction of

novel techniques are needed to advance towards the goal of preventing restenosis without the cost of delayed endothelial healing and increased thrombogenicity.

Surface modification techniques such as plasma treatment are used to increase surface hydrophilicity; however, these surfaces do not allow selective adsorption of nonadhesive albumin protein. Cellular and platelet response largely depends on the adsorbed protein layer on the biomaterial [37-41]. The exposure of a biomaterial to blood results in competitive adsorption of plasma proteins [42,43]. While higher levels of surface-adsorbed adhesive proteins such as fibrinogen, von Willebrand factor and vitronectin have been shown to correlate with adhesion and aggregation of platelets[44], the albumin layer has been shown to minimize adhesion and aggregation of platelets, thus preventing subsequent thrombus formation [39,45-51,56]. Researchers have immobilized molecules such as straight chain 16-18 alkyl chains, dextran, dextran-cibacron-blue (CB) dye conjugate and monoclonal antibodies specific to albumin to generate albumin-binding surfaces[49,50,54]. However, these molecules have not been efficient in allowing the adsorption of specifically albumin on biomaterials. Crosslinking agents such as glutaraldehyde and carbodiimide have also been employed to bind albumin on polymers such as Dacron [55,32]. However, these crosslinking agents have caused intense inflammatory reactions such as formation of foreign body giant cells both *in vitro* and *in vivo* and have demonstrated short-term antithrombogenicity and hemocompatibility properties due to rapid resorption of crosslinked albumin [56-58]. Denaturation of albumin after adsorption has also been shown to allow adhesion of platelets because of exposure of hidden RGD sequences [33].

In the present study, human serum albumin films were anchored on metallic substrates using poly (glycidyl methacrylate) (PGMA). PGMA has been known for its high surface density and affinity for reacting with amines [59]. PGMA binds HSA through a nucleophilic addition reaction that results in the formation of strong covalent bonds with the surface. Epoxy groups of PGMA act as spacer

molecules and bind to albumin, keeping them close to their native secondary structure conformation. The effect of HAS on fibrinogen adsorption, human platelet adhesion and vascular smooth muscle cells was assessed.

3.2 Materials

Nitinol (NiTi) sheet (0.25mm thick) was purchased from Johnson Matthey Inc. (Part#83975;Wayne, PA).Using wire electrical discharge machining (EDM), 15mm diameter discs were cut from the NiTi sheet keeping a square lip to facilitate sample handling. NiTi discs were cleaned with 100% acetone for 20 minutes in an ultrasonic cleaner. Discs were then placed in a 1% (by vol) detergent solution (Liquinox brand, Alconox Inc.) and ultrasonicated for another 20 minutes. The discs were then put through three 15-minute ultrasonic rinse cycles in ultrapure de-ionized water, where the water was completely changed after each rinse. Air-dried samples were then sterilized by a validated ethylene oxide sterilization process according to ISO-11135-2014 [60].

Highly polished single-crystal silicon wafers (1cm x 1.2cm) were purchased from Semiconductor Processing Co. (Boston, MA) and were used as a substrate for surface morphology and roughness analysis. Silicon wafers and hollow glass beads of 25 mm diameter used for fibrinogen adhesion force measurements were first cleaned in a hot (80ºC) piranha solution (3:1 conc. H_2SO_4: 30% H_2O_2) for an hour, then in an ultrasonic bath for 30 minutes and then rinsed three times for 10 minutes with high purity DI water and dried under a nitrogen stream. 6mm diameter silicone membranes were cut from biomedical grade silicone sheets (SMI, 0.15" NRV G/G 40D; SimPore, NY) and used as a control.

Human albumin (Sigma-Aldrich Corp., St. Louis, MO; CAS # 7024-90-7) (25% wt/vol) in phosphate buffered saline was freeze dried and stored at -20C. Potassium phosphate buffer (25mM,

pH 7.4) was prepared by combining 4:1 monobasic:dibasic salts (Sigma-Aldrich Corp., St. Louis, MO) to maintain a pH of 7.4. Poly (glycidyl methacrylate) (PGMA) ((Polymer Source, Dorval, QC) was prepared via free radical polymerization following the procedure of Luzinov et al. [88]. A number-average molecular weight M_n = 58,000 g/mol and polydispersity index (PDI) =1.57 were determined using size-exclusion chromatography (SEC) and a separations module (Waters Corp., Milford, MA, Alliance 2965 separations module) equipped with three PLgel 5mm MIXED-C columns 7.5x300 mm (Polymer Labs, Amherst, MA, Part no. PL1110-6500) in series and a refractive index detector (Waters Corp., Milford, MA, Waters Model 2414).

Differential scanning calorimetry (DSC) (TA instruments, New Castle, DE; Model 2920) was performed to determine the denaturing temperature and safe processing temperature window of human serum albumin at a heating rate of 20C min^{-1}. Thermogravimetric analysis (TGA) (TA instruments, New Castle, DE; TGA 2950) was carried out under N_2 purge (40mL min^{-1}) to study thermal stability.

3.3. Methods and Characterization

3.3.1 Substrate coating of PGMA and HSA

Plasma treated substrates functionalized with hydroxyl groups were modified with dip-coating (Mayer Feintechnik, Gottingen, Germany; D-3400) using a 0.5% wt/vol PGMA (M_n = 176000 g/mol) solution in chloroform for 20 minutes followed by annealing at 110℃ for 10 minutes to produce an epoxy-rich anchoring layer. PGMA- modified substrates were dip coated with 5% wt/vol HSA in phosphate buffer for 30 minutes, followed by annealing at 150℃ for 1 hour (Fig 1). PGMA-HSA

coated substrates were rinsed in fresh phosphate buffer three times for 30 minutes to remove unbound protein and were sterilized using ethylene oxide sterilization [88].

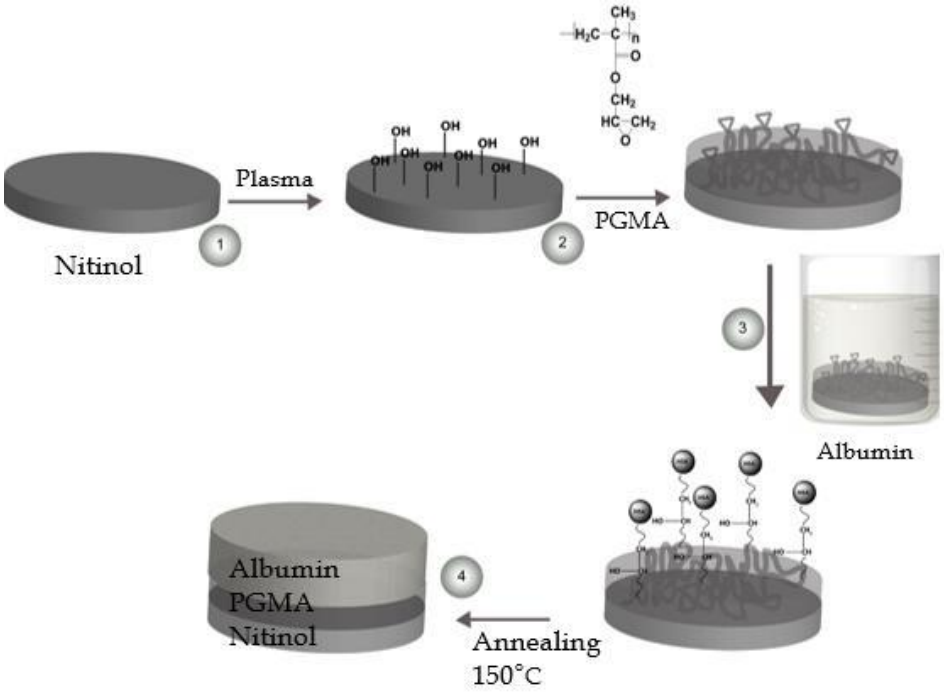


Figure 3.1: Schematic of the fabrication of human serum albumin film on nitinol.

3.3.2. Surface Analysis

An atomic force microscope (VECCO Probes, Santa Barbara, CA; Dimensions 3100 Atomic Force Microscope) equipped with a NanoScope IIIa controller was used to image the morphology of the HSA film on silicon wafers in tapping mode at different annealing times. Samples were scanned using a silicon cantilever and silicon tips with spring constants of 50 N/m. Imaging was done at scan rates in the range 1-2 Hz.

Swelling studies were conducted in contact mode with HSA films prepared under different annealing times using a Multimode AFM (VECCO Probes, Santa Barbara, CA; Dimensions 3100 Atomic Force

Microscope) with a NanoScope IIIa controller and a liquid cell and silicon nitride cantilevers (VECCO Probes, Santa Barbara, CA; DNP-20) with a force constant of 0.24Nm^{-1}.

Thickness measurements were made using a variable angle ellipsometer (Beaglehole Instruments, Wellington, New Zealand), which utilizes a He-Ne laser light source (l= 632.8nm). Measurements were conducted at integral values of incident angle ranging from 80℃ to 35℃. The thicknesses reported are averages of three ellipsometric measurements from selected areas near the center of a modified silicon wafer. The refractive indices used for each layer were as follows: N_{PGMA}=1.525, N_{HSA}=1.5.

The binding between PGMA and HSA was confirmed by Fourier Transform Infrared (FT-IR) Spectroscopy using an FTIR Spectrometer (Thermo Nicolet, Walpole, MA; Continuum 6700). A total of 16 scans at a resolution of 4 cm^{-1} were collected for the HSA-PGMA sample and compared against a background collected of bare NiTi disc.

Static water contact angles were measured using sessile drop method using a Drop Shape Analyser (Kruss Optronic, Germany; DSA10-MK2). A water droplet (1 uL) was placed onto the sample automatically using a syringe. The contact angle values reported are averages of three drops along the center of HSA-coated and bare-nitinol surfaces.

3.3.3 Fibrinogen Adhesion Force Measurement

The Adhesion force of fibrinogen to HSA films was examined using an AFM colloidal probe technique (Figure 3.2). Force constant 0.093± 0.005Nm^{-1} of AFM cantilevers (N-P series, Veeco Inc.) was determined by cantilever vs. cantilever calibration method. Cleaned 20μm hollow glass beads (as described in section 1.1) were stirred in a 1% wt/vol solution of PGMA (M_n = 170000 g/mol) in chloroform for 24 hours and rinsed three times with pure chloroform. This treatment creates a thin (about 2nm) PGMA film on the glass surface. The beads were glued to the cantilevers, applying

the procedure described elsewhere [89]. Modified cantilevers were immersed in 1mg/mL solution of human fibrinogen (FIB-3; plasminogen, von Willebrand factor, and fibronectin depleted; Enzyme Research Laboratories, South Bend, IN) in phosphate buffer for 30 min and were rinsed in pure buffer solution and DI water and dried at room temperature. PGMA has a high affinity to fibrinogen and the protein is adsorbed irreversibly on the polymer's surface. Force distance measurements were carried out by AFM contact mode. The deflection of the cantilever with fibrinogen-coated beads on HSA nanofilms corresponds to the adhesion force between fibrinogen and the surface. Thin films (10-20nm) of carboxy-terminated poly(ethylene glycol) (PEG) methyl ester (P14170, Polymer Source, Inc)[61,62] and polystyrene (PS) grafted on to a surface modified with PGMA were used as negative and positive control, respectively.

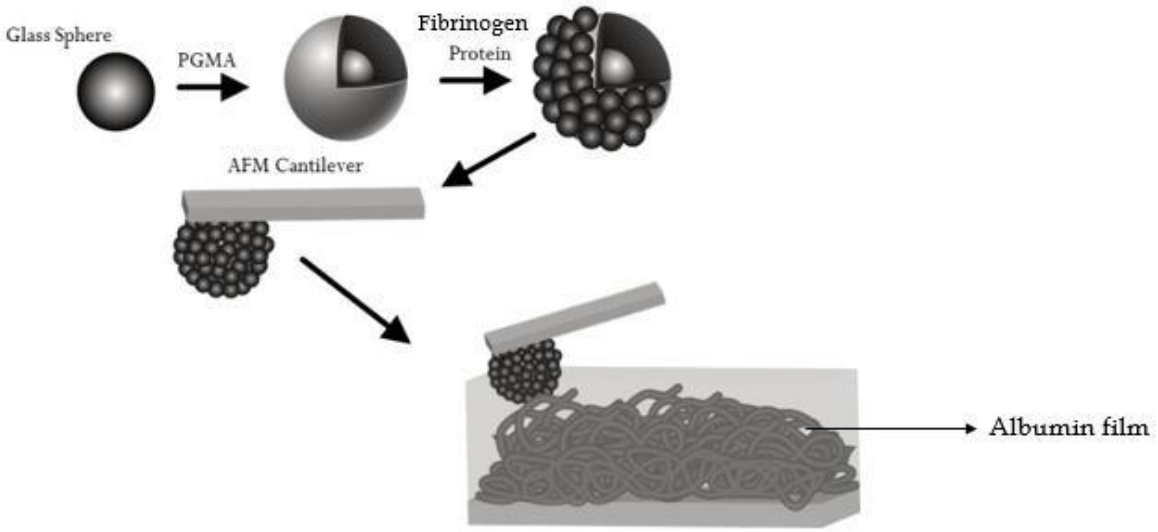


Figure. 3.2. Schematic for measuring the adhesion force of fibrinogen to HSA films.

3.3.4 Human Albumin Secondary Structure Confirmation

The secondary structure conformation of annealed human albumin was determined by circular dichroism (CD) spectroscopy. Quartz slides (0.375"x 1.625" x 0.0625", Chemglass) were cleaned in piranha solution for 30 minutes, followed by a Radio Corporation of America (RCA) basic wash (1:1:5 v/v $NH_4OH/H_2O_2/H_2O$, Sigma-Aldrich, St Louis, MO; CAS no 1336-21-6, 7722-84-1). The slides were then rinsed with 100% ethanol (Pharmco-Aaper; Catalog No. 11100200, City, State), followed by nanopure water and then dried under a stream of nitrogen gas. HSA films were prepared on quartz slides as described in 2.1. CD spectropolarimetry was performed using a JascoJ-810 spectropolarimeter (Jasco, Inc., Easton, MD) to determine the native and adsorbed secondary structures of human albumin. High transparency quartz cuvettes (Starna Cells, Inc., Atascadero, CA) were used for determining the native solution structure while the adsorbed structure of HSA on quartz slides was determined using a cuvette custom-designed [63] for maximizing signal-to-noise-ratio. The CD spectra (molar ellipticity vs. wavelength) obtained were deconvoluted using the sp22x algorithm and analyzed using the SELCON and CONTIN/LL software packages [90] to quantify the percentage of alpha-helix and beta-sheet content of native/adsorbed protein.

3.2.5 Fibrinogen adsorption

Human plasma fibrinogen fluorescently labeled with rhodamine-NHS dye ester (CAS no. 1352809-17-6, Sigma-Aldrich, St. Louis, MO) was dialyzed for 48 hours to remove unbound dye. Labeled human fibrinogen was incubated on HSA films coated on NiTi discs along with controls for 1 hour at 37ºC with gentle shaking. Fluorescence was measured at excitation-emission wavelengths of 575 and 525nm.

3.2.6 Human Platelet Isolation and Adhesion

Platelet rich plasma (PRP) from fresh human blood was isolated by centrifuging the acid-citrated dextrose (ACD) anticoagulated blood (225g, 15 min, 25C) using a Beckman Coulter Allegra 6R centrifuge (Beckman Coulter, Fullerton , CA). All protocols for participation of human blood donors were approved by the Institutional Review Board at Clemson University. Platelet concentration was measured using a Beckman Coulter Z2 Particle count and size analyzer (Bechman Coulter, Fullerton, CA). The platelet concentration was adjusted to 10^6 platelets/mL with platelet suspension buffer (137mM NaCl, 2.7mM KCl, 5.5mM Dextrose, 0.4 mM sodium phosphate monobasic, 10 mM HEPES and 0.1U/ml apyrase)[84]. Platelet suspension buffer was added with 2.5 mM $CaCl_2$ and 1.0mM $MgCl_2$ to add metal ions. The platelet solution was allowed to rest for 30 minutes at 37ºC before being added to the HSA coated and the uncoated disc controls in a 24 well and then allowed to adhere for 1 hour at 37ºC under static or shear condition. At the end of platelet adhesion time, the suspension was aspirated from each well, and each well was rinsed with phosphate buffer to remove unbound platelets. Orbital shear was provided using an orbital plate shaker (VWR, S-500) calculated using the following equation,

$$Tmax = a \sqrt{\eta\rho} (2\pi f)3$$

(a- radius of gyration of the shaker (cm), ρ- density of solution (g/mL), η- viscosity at 37°C (poise) and f- frequency of rotation (rps)). Frequency of 250 rpm, as per the above equation corresponded to a maximal shear stress of 12.7 dynes/cm^2, comparable to arterial shear levels.

Platelet adhesion was quantified by measuring the lactate dehydrogenase (LDH) released when adherent platelets were lysed with Triton-PSB buffer (2% v/v Triton-X-100 in PSB), using a CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega Corporation, Madison, WI). Absorbance was measured at 490nm using a UV/Vis spectrophotometer (Bio-Tek Instruments Inc.,Vinooski,

Vermont). A calibration curve was constructed with known number of platelets, counted using a Beckman Coulter Z2 Particle Count and Size Analyser (Beckman Coulter, Fullerton, CA), and the platelet adhesion on the HSA films were determined using the calibration curve.

3.2.7 Bioactivity of Vascular Smooth Muscle Cells (VSMCs)

Using an approved protocol by Clemson University Institutional Animal Care and Use Committee, abdominal aortas were harvested from 6-10 weeks old female Sprague-Dawley rats. Adventitia, fat layers and endothelium were removed. VSMCs were isolated from the vascular medium using collagenase type II (Worthington, Biomedical, Lakewood, NJ) and elastase (Worthington Biomedical, Lakewood, NJ) [64]. The cells were maintained in Dulbecco's modification of Eagle Medium (DMEM, 10-013-CV; Mediumtech, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (F-4135; Sigma-Aldrich, St. Louis, MO) and 1% antibiotic-antimycotic (A5955, Sigma-Aldrich, St. Louis, MO) under standard cell culture conditions (37C, 5%CO_2, 95% relative humidity).

Experiments were carried out using VSMCs between passages 4-8. For experimental testing, cells were trypsinized using 0.25% trypsin containing 0.2mg/mL EDTA (Sigma Chemical Company, E6511, St. Louis, MO, USA).

The experiment was divided into HSA films and bare NiTi experimental groups, and polystyrene (PS) surfaces served as the control (N=4 samples/group). Cells were cultured for 5 days, 15 days and 30 days. After each time period, the culture medium was removed, and the cells were evaluated using the test methods described below.

3.2.8 Characterization of Smooth Muscle Cells

Smooth muscle cells were characterized by immunofluorescent staining for contractile markers smooth muscle α-actin (SM α-actin) and calponin. VSMCs were fixed with cold 4% formaldehyde

and blocked for 45 minutes at room temperature with a blocking solution that consisted of 40mg/ml bovine serum albumin in PBS, 6% FBS, and 0.05% Triton-X. Cells were then treated with primary mouse anti-α-actin antibody (Abcam) and primary rabbit monoclonal anti-calponin antibody, both diluted 1:500 overnight at 4C. Cells were then treated with fluorescent tagged goat anti-mouse secondary antibody and goat anti-rabbit secondary antibody, both diluted 1:200 in the blocking solution, for 3 hours respectively at room temperature. The cells were then stained with DAPI (Molecular Probes, D-1306, Eugene, OR) and imaged using fluorescent microscopy (Nikon LV-UDM, Nikon Instruments Inc., Melville, NY).

3.2.9 Cell Morphology

After each time period, culture medium was removed, and cells were fixed in 4% paraformaldehyde solution for 20 minutes. Cells were then permeabilized using 0.25% Triton-X detergent in phosphate buffer for 10 minutes. The cytoskeletal F-actin and cell nuclei were stained using 25 μg/ml rhodamine-phalloidin (Invitrogen, R415) and by 4', 6-diamidino-2-phenylindole (DAPI) (Molecular Probes, D-1306, Eugene, OR) respectively. The cells were then imaged using fluorescent microscopy (Nikon Inc., Diaphot 30).

3.2.10 Cell proliferation

VSMC proliferation was analyzed using a methylthiazol tetrazolium (MTT) (Sigma-Aldrich, MO, USA) assay after 24 hours. A total of 3-(4,5-dimethylthiazol -2-YL)-2,5-diphenyltetrazolium bromide was dissolved in 1x DPBS at a concentration of 2mg/mL. VSMCs were rinsed with 1x DPBS and then placed in 1mL DMEM solution with no FBS. A total of 240 μl of MTT was added to the well, and the solution was incubated for 4 hours. After incubation, the MTT-DMEM solution was removed and 1mL of DMSO was added to the well. Using a plate reader, absorbance was then read at 570nm.

3.2.11 Expression of alpha-actin

α-actin expression in smooth muscle cells cultured on HSA, bare NiTi and PS surfaces was analyzed using a modified cell-based ELISA assay [65]. Briefly, at indicated times, the medium was removed, and the cells were fixed in cold methanol. They were then incubated with a blocking solution that consisted of 40mg/ml bovine serum albumin in PBS, 6% FBS, and 0.05% Triton-X for 45 minutes at room temperature. Primary mouse anti-α-actin antibody diluted 1:200 in the blocking solution was then added to the metal discs for 2 hours at room temperature followed by a 2hr treatment with biotin conjugated bovine anti-mouse IgG, also diluted 1:200 in the blocking solution. After extensive washing, the cells were incubated with horseradish peroxidase (HRP) conjugated anti-mouse secondary antibody. Chromogenic substrate (3 mM p-nitrophenyl phosphate, 0.05 M Na_2CO_3, and 0.05 mM $MgCl_2$) was then applied to produce color, and the absorbance was read at 405 nm with a microplate reader (Beckman Instruments, Inc., DTX 880, Fullerton, CA). The cells were then DAPI stained and imaged to facilitate cell counting. Lastly, absorbance due to nonspecific binding was subtracted from that of the experimental groups, with the net absorbance normalized against cell count on each disc giving a number directly related to the α-actin content per cell.

3.2.12 Adhesion Strength of HSA coating

Adhesion strength of HSA coating on NiTi discs was evaluated by ASTM D1002 procedure [91]. An HSA-coated NiTi disc was attached to bare NiTi disc using an epoxy glue. A dynamometer (Instron 4204), to which the NiTi discs were glued, was used to calculate the failure load and displacement of the interfaces. The tests were performed with a 1kN load cell and cross bar speed of 1.3mm/min. The load measured at the moment of detachment was divided by the adhesion overlap

surface between metal and protein film to obtain shear stress value. Presence of HSA film after displacement of interfaces was assessed by FT-IR spectroscopy.

3.2.13 Degradation of HSA film

HSA-coated discs were immersed in phosphate buffer in an orbital shaker maintained at 37°C with a rotation of 250rpm (N=5) for 120 days. The buffer was collected to measure the HSA released from the discs using a bicinchoninic acid (BCA) test for protein estimation.

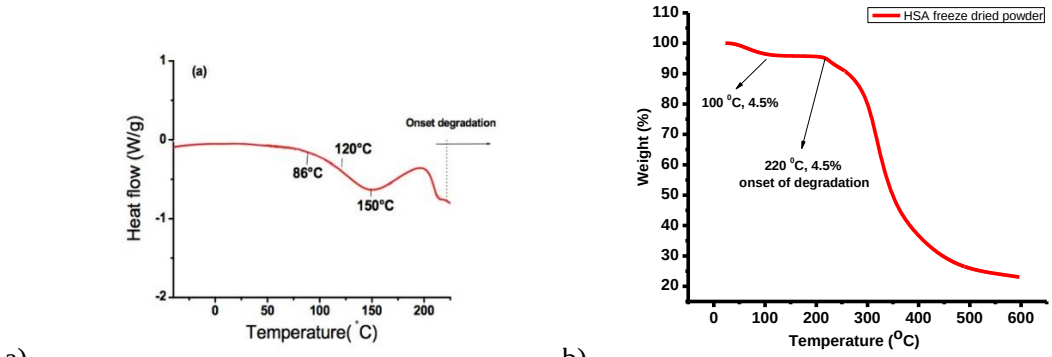
3.2.14 Statistical Analysis

The results presented are the mean values with 95% confidence intervals (CI). The statistical significance of differences between mean values for different samples and conditions was evaluated using a student's t-test, with $p \leq 0.05$ considered as statistically significant.

3.3 Results

3.3.1 Thermal Analysis

The DSC thermogram in Figure 3.3a indicates that the human albumin protein denatures at a temperature of 130–150°C. However, this temperature is clearly not the protein degradation temperature, which begins around 220°C, as observed in the TGA thermogram in Figure 3.3b



a) b)

Figure 3.3. a) Differential scanning Calorimetry (DSC) thermogram of human serum albumin powder. b) Thermogravimetric analysis (TGA) of human serum albumin powder.

3.3.2 Surface Roughness Analysis

Figure 3.4 shows the surface morphology and roughness of HSA annealed at 150°C for an hour. The average roughness of the HSA layer is 0.307±0.07nm. The morphology and roughness values indicate a homogeneous and smooth surface of HSA films.

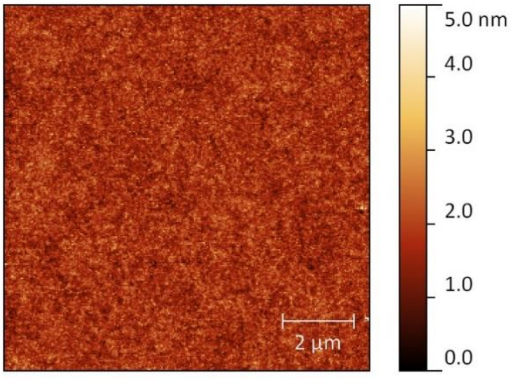


Figure 3.4. Surface morphology and surface roughness value of human serum albumin (HSA) assessed by atomic force microscopy.

3.3.3 Fourier Transform Infrared (FT-IR) Spectroscopy

Binding between PGMA and HSA on nitinol surface was confirmed using FT-IR spectroscopy as shown in Figure 3.5.

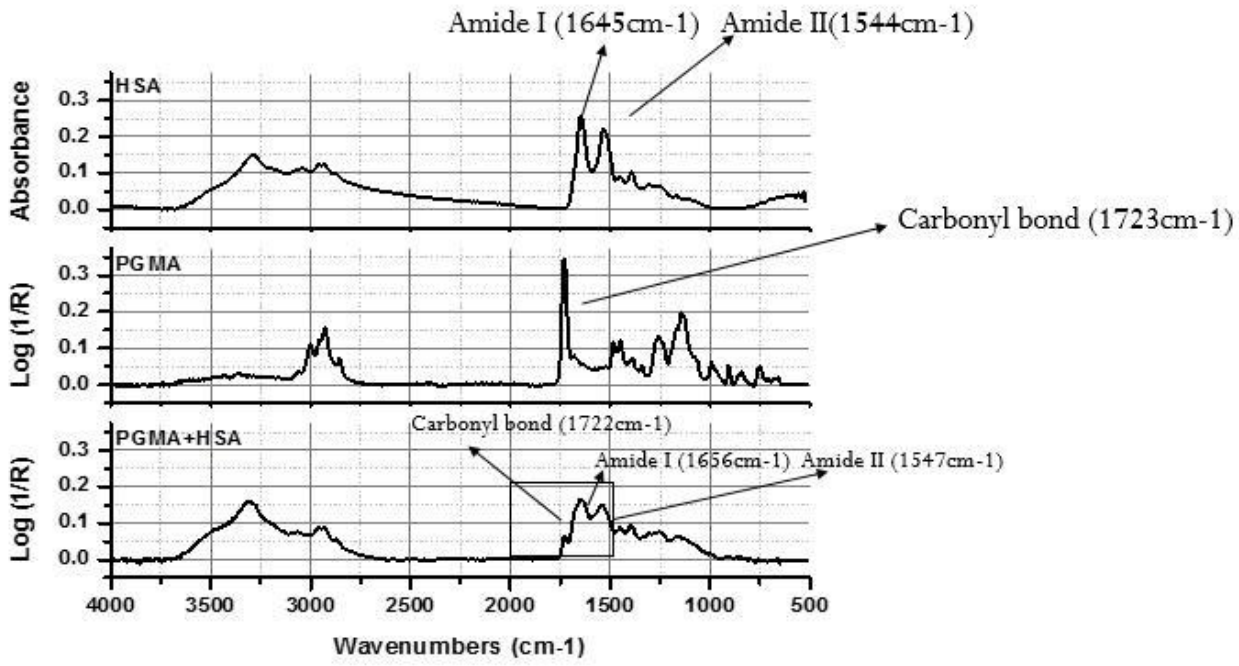


Figure 3.5: IR spectra of HSA and PGMA with characteristic carbonyl and amide bonds.

3.3.4 Circular Dichroism (CD) Spectroscopy

A 15.3% decrease in the α-helix secondary structure was observed for annealed albumin as compared to its native form (Figure 3.6). HSA films showed a significant decrease in the adsorption of fluorescently labeled human fibrinogen as compared to NiTi ($p = 0.023$).

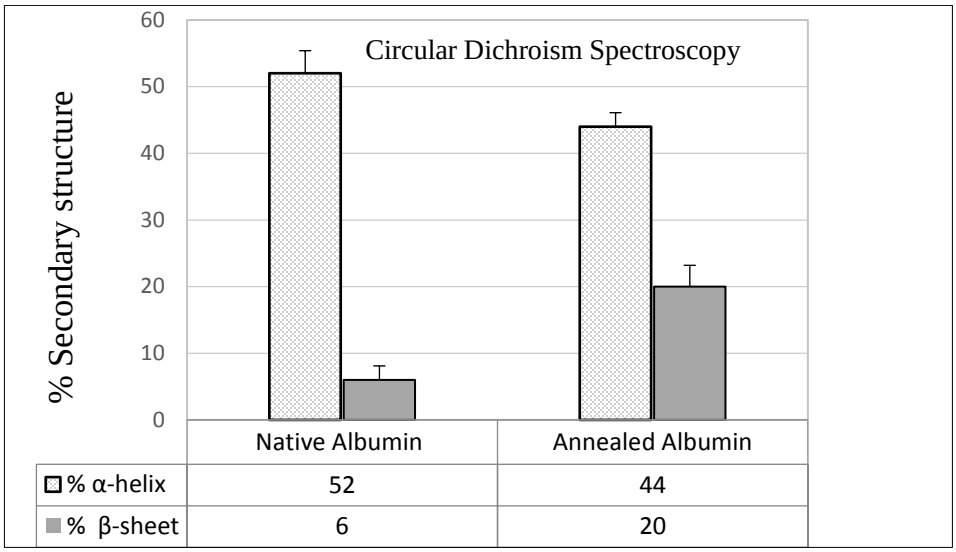


Figure 3.6: Circular dichroism spectroscopy analysis to determine the secondary structure of native and annealed human albumin on quartz slide.

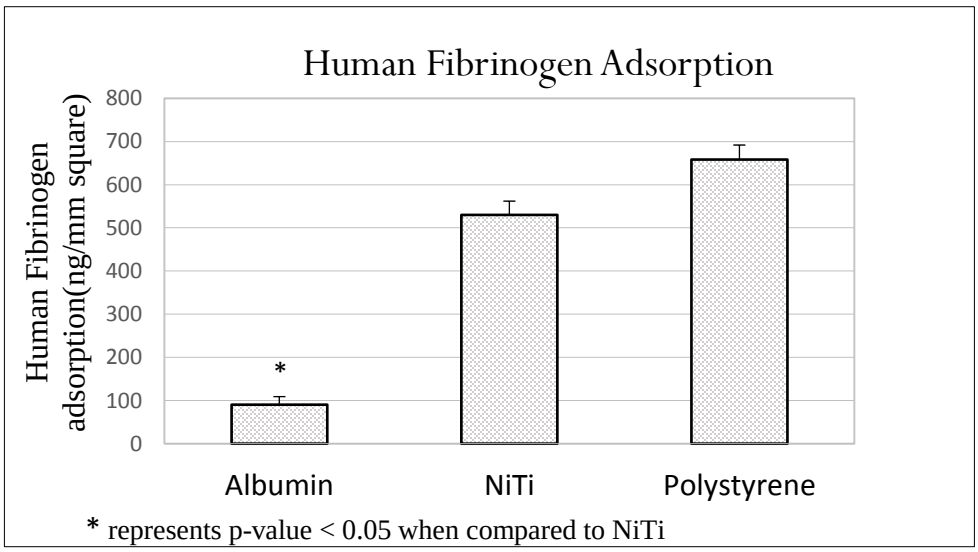


Figure 3.7: Adsorption of fluorescently labeled human fibrinogen on different surfaces.

3.3.5 Adhesion Force measurement of fibrinogen

As shown in Figure 3.8, a minimal adhesion force 0.3±0.1nN/m (mean±SD; N=50 points) between fibrinogen and human serum albumin film was observed using AFM as compared to adhesion of fibrinogen and PGMA surfaces fibrinogen and silicone used as a negative control.

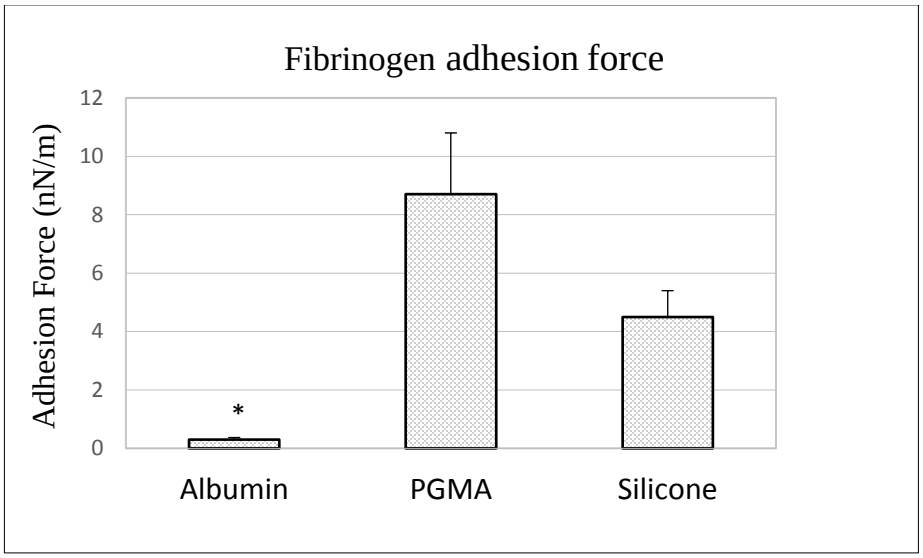


Figure 3.8: Adhesion force measurement between human fibrinogen and different surfaces using AFM colloidal probe microscopy.

3.3.6 Human Platelet Adhesion

HSA films allowed 6.54±3.3% platelet adhesion under static conditions and 4.59±2.4% adhesion under shear conditions (12dynes/cm^2) significantly less than NiTi under static (32.3% adhesion) (p-value =0.014) and shear (21.7% adhesion)(p-value = 0.021) conditions (Fig. 3.9).

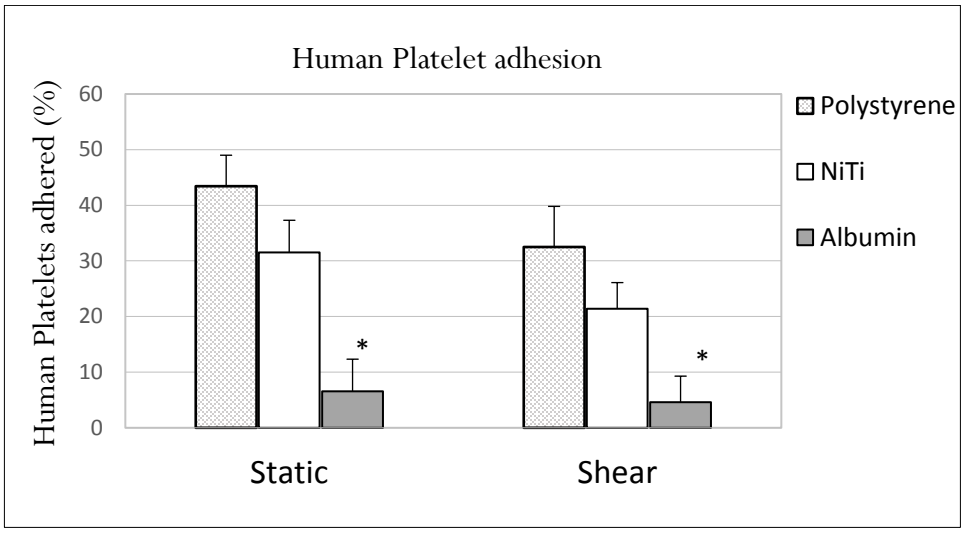


Figure 3.9: Assessment of adhesion of human platelets on HSA, NiTi and PS surfaces under static and shear conditions.

3.3.7 Smooth muscle cell characterization, proliferation, phenotype and morphology

Smooth muscle cells were characterized for F-actin fibers by Rhodamine-Phalloidin staining and for contractile markers α-actin and calponin by immunofluorescent staining. HSA films allowed significantly less proliferation of rat aortic smooth muscle cells, assessed over a period of 5, 15 and 30 days compared to bare NiTi (p-value 0.027, p-value 0.031, and p-value 0.032 respectively) assessed by MTT cell proliferation test (Fig.11). MTT dye (yellow color) was reduced to formazon dye (purple color) by active reductase enzymes, thus permitting quantification of the metabolically active, healthy and proliferating cells (materials cytotoxicity) via colorimetric measurements. The cell-based ELISA assay developed for this application resulted in a number directly proportional to smooth muscle α-actin content per cell.

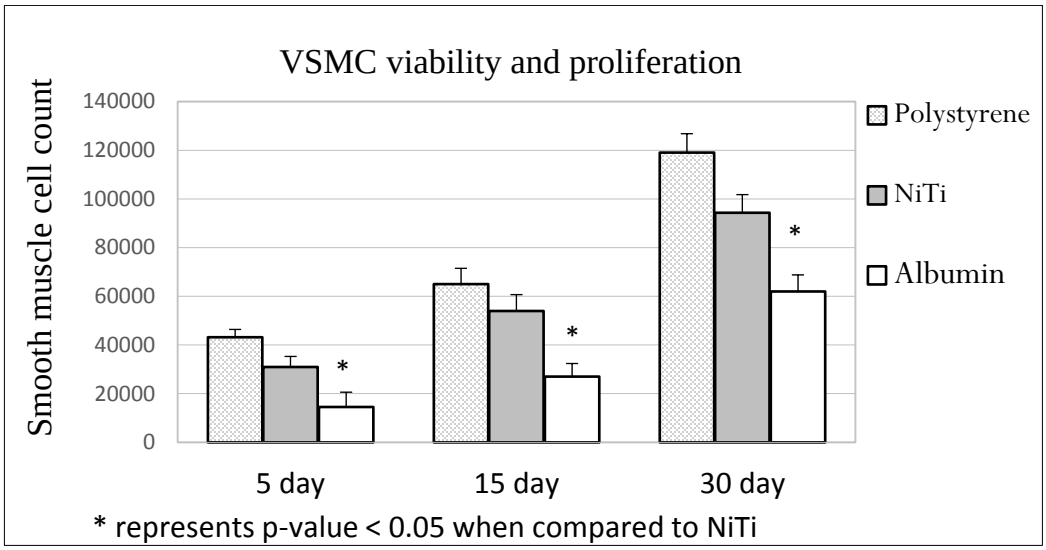


Figure 3.10: MTT cell proliferation assay for assessment of smooth muscle cell viability and proliferation.

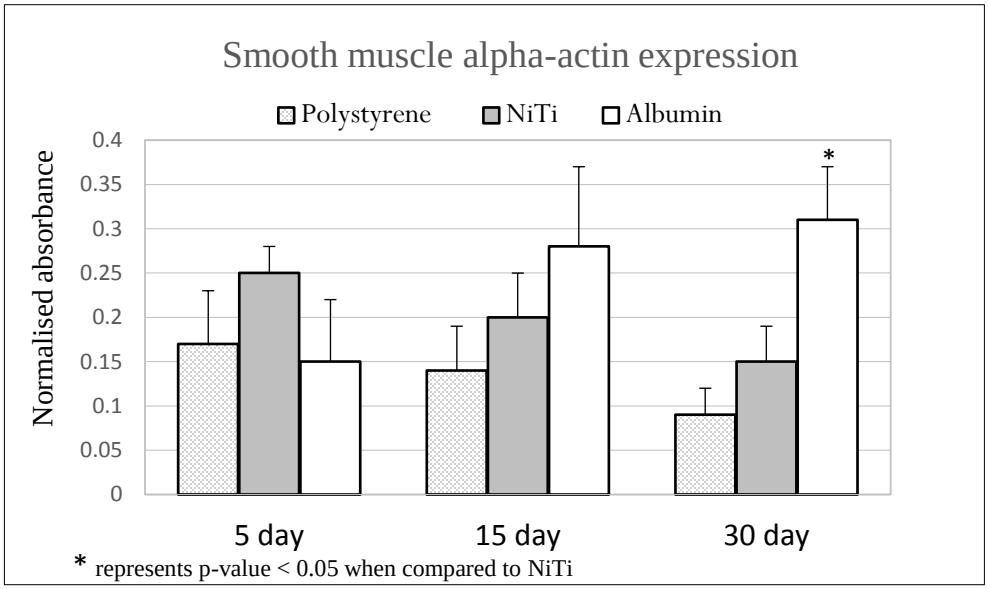


Figure 3.11: Smooth muscle alpha actin expression of VSMCs cultured on HSA, NiTi and PS for 5, 15 and 30 days. * represents $p<0.05$ compared to NiTi.

Rhodamine-phalloidin staining demonstrates more elongated and spindle-shaped morphology of VSMCs cultured on HSA films as compared to NiTi and PS surfaces assessed by confocal microscopy.

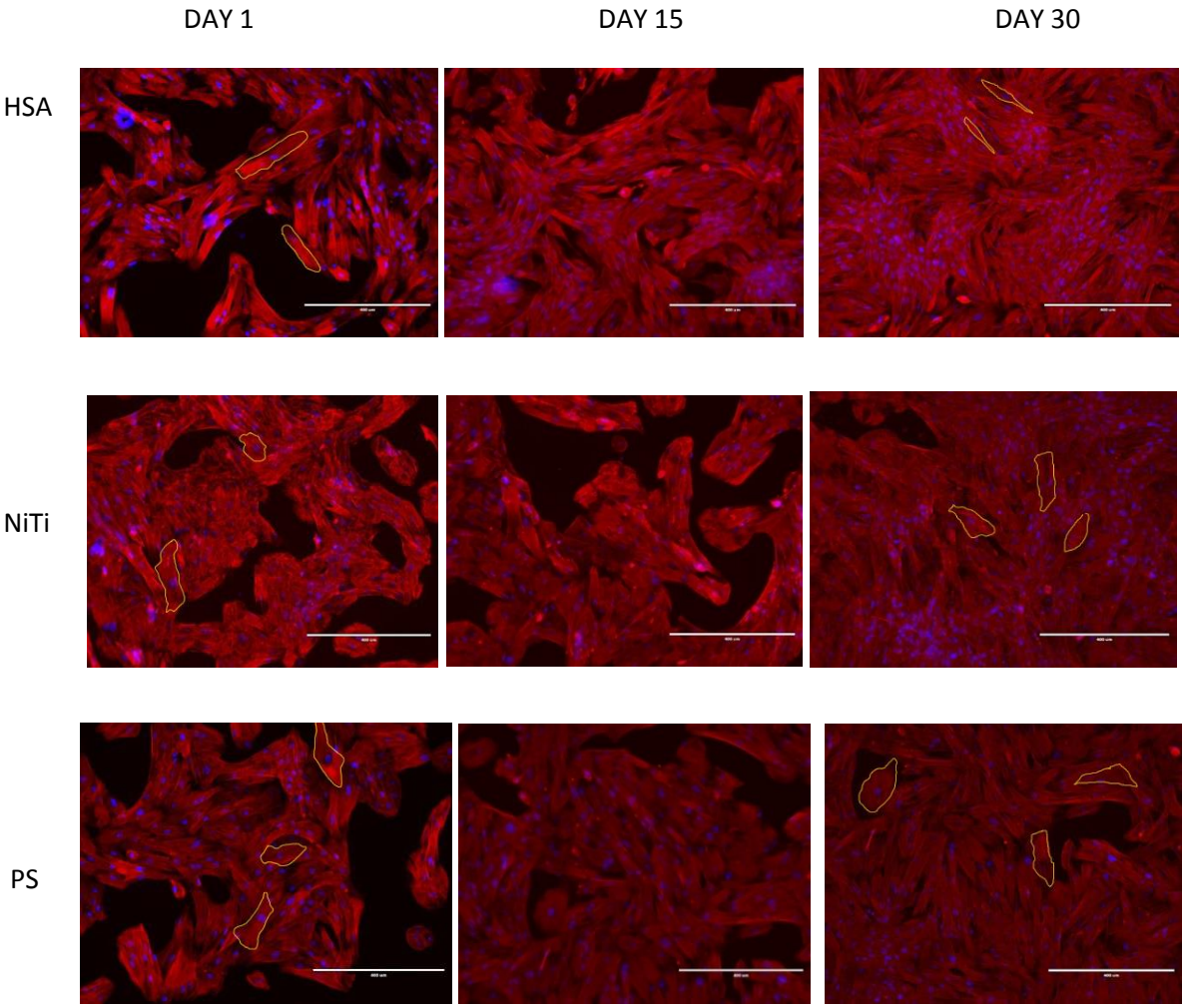


Figure 3.12: Rhodamine-phalloidin staining of rat aortic vascular smooth muscle cells cultured on HSA, bare NiTi and PS surfaces. Red: F-actin; Blue: Cell nuclei: 10X; Bar represents 400 µm.

3.3.8 Adhesion Strength of HSA film

The presence of albumin film sheared at 5.48MPa was confirmed by FT-IR spectroscopy. Albumin film on a nitinol surface after application of shear stress, which is three times the stress applied during a balloon angioplasty procedure (~2.02MPa) [92,93], confirms the strong binding of the albumin film on the surface.

Max load (N)	True Strain at maximum load (mm/mm)	True stress at maximum load (MPa)
525.575	0.1017	5.48

Table 3.1: Strain and stress values obtained after application of load on albumin coated NiTi.

3.3.9 Degradation of HSA film

HSA covalently bound to PGMA was fully degraded in 120 days from the NiTi surface in phosphate buffer subjected to a rotation of 250 rpm, equivalent to a flow shear of 12.7 dynes/cm^2.

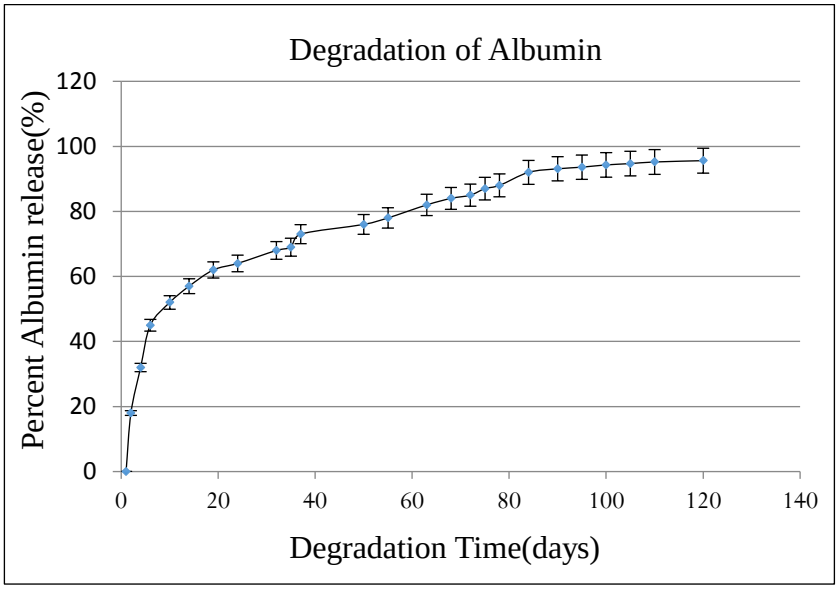


Figure 3.13. *In-vitro* degradation of HSA film from nitinol surface.

3.4 Discussion

This study highlights the potential of HSA as a coating to mitigate the complications of inherent thrombogenicity and smooth muscle hyperplasia. After decades of research on hemocompatibility and intimal hyperplasia, thrombosis and restenosis remain two major complications resulting in failure of endovascular stents. Polymers used in designing stents increase the incidence of thrombus formation by providing a surface for adhesion and aggregation of platelets [18-20] Drugs used in drug-eluting stents for controlling smooth muscle cell proliferation delay re-endothelialization, which is essential as a barrier between the vessel lining and blood components [66]. Therefore, a successful endovascular device must be designed to prevent adhesion of platelets and hyperplasia of smooth muscle cells without delaying healing. In this study, we developed a method for coating substrates with human serum albumin using PGMA as an anchoring layer.

Human serum albumin is the most abundant protein present in our blood. It has been shown to passivate material surfaces, thus blocking sites for adsorption of other proteins and adhesion of platelets and cells [39,45-51]. PGMA was used to form a reactive anchoring polymer layer. A polymer with epoxy functionality was chosen, since the reactions of epoxy groups are quite universal and can covalently anchor PGMA to the substrate surface [67]. The glycidyl methacrylate units located in the “loops” and “tails” sections of the attached PGMA chain could serve as reactive sites for the subsequent attachment of (macro) molecules with complementary functional groups. PGMA is also known for its high surface density and affinity for reacting with amines [68].

The advantageous aspect of covalent immobilization is the ability to provide a stable bond between the biomolecule and the functionalized polymer. Covalent bonding ensures integrity of the layer, promoting prolonged and/or continuous activity of implanted devices.

Binding between PGMA and HSA was confirmed by FTIR spectroscopy. From the IR results, the presence of a peak at 1720cm^{-1} depicting C=O stretch in PGMA, an amide I peak at 1650cm^{-1} and amide II bond peaks at 1540 cm $^{-1}$ in HSA on the surface confirmed that HSA binds to the PGMA anchoring layer. Smooth and homogeneous surface morphology of the HSA layer was studied using atomic force microscopy. For cardiovascular application, a smooth device surface coating can significantly decrease injury to blood vessels. In addition, surface roughness is often correlated to cell adhesion and migration and, in particular, to human endothelial cells growth [68,69]. Furthermore, a smooth stent surface is believed to reduce platelet activation and aggregation, consequently leading to less thrombus formation and neo-intimal proliferation [70]. The developed-coating stent showed a reduced surface roughness when compared to bare metal stents, even in dry conditions, that could lead to less vascular injury at the moment of angioplasty procedures.

Dry layer thicknesses of PGMA and HSA films on silicon wafers measured by ellipsometry were 26±3.2 and 92±9.6nm respectively. In agreement with results from Zdyrko et al. [71], a static water contact angle of 60±2.3º was measured for PGMA on the silicon surfaces. When an epoxy group reacts with a primary amine, a secondary amine and secondary alcohol are formed. HSA binding to PGMA increased the thickness of the surface layer, which suggests that primary amines readily penetrate and react throughout the PGMA film. A water contact angle of 66º was measured for the HSA layer bound to PGMA, which is consistent with the value of 62º reported for the water contact angle of amines [72].

A notable correlation between the secondary structure conformation of albumin and adherence of platelets has been studied. Beyond a critical point of unfolding (>34%), human albumin has been shown to undergo a change in structural orientation facilitating adhesion of platelets on albumin even though it has no amino acid sequences to bind to platelet receptors [33]. PGMA has been used as an

anchoring layer because it has epoxy groups that immobilize and stabilize protein molecules by preventing their surface-adsorbed denaturation [73]. Change in the secondary structure conformation of annealed albumin was studied by CD spectroscopy. A 15.3% decrease in the alpha-helix content of the annealed albumin depicted that HSA covalently bound to PGMA retained most of its native secondary structure conformation.

Shear strength of HSA coating on NiTi was assessed by ASTM D1002. Scanning electron microscopy was used to evaluate the integrity of HSA film after stent expansion. HSA coating appeared to be uniform over the entire length of the stent after application of strain. No cracks in the HSA coating were found along the length of the stent. The presence of Nitrogen on the surface after strain application by EDX spectroscopy confirmed the presence of HSA on the stent surface. Coating ruptures and cracks have been proved to facilitate restenosis and thrombogenic events. If the stent has webbings and bridges between the struts, the coating may break off from the stent when the stent is dilated by a balloon catheter during the deployment, and thrombus and vascular smooth muscle cell (VSMC) proliferation can occur at the site where the coating breaks off [85].

Fibrinogen adsorption and platelet adhesion are considered to be major processes dictating hemocompatibility of a biomaterial. In our study, we measured the adsorption of fluorescently labeled fibrinogen on HSA films. Significantly less adsorption of fibrinogen occurred on HSA films as compared to bare NiTi (p -value 0.023). The number of platelets adhered to different surfaces was quantified by measuring LDH activity. The sensitivity and reliability of the LDH method has been confirmed by researchers, revealing a linear correlation between LDH activity and platelet number [86,87]. Platelets are activated by a foreign stent material and the artery wall gets denuded of endothelial layer post-surgery [74]. Thus, stent implantation or vascular graft surgery results in a highly prothrombotic surface exposed to the blood stream. Stent struts are covered with thrombus

within a few hours or days following implantation. Previous studies on the thrombogenicity of polymeric arterial graft materials have demonstrated high platelet deposition on these surfaces immediately after implantation [18-20]. In vitro biocompatibility studies have revealed that albumin-coated surfaces substantially decrease the platelet adhesion and activation of an untreated or pre-clotted polyester graft [56,57]. An in vivo study by Rumisek et al. [58] demonstrated that an albuminated graft implanted in the canine iliofemoral artery allowed fewer platelets to adhere in the first 3-days compared to the pre-clotted polyester control. We measured platelet adhesion under static and shear conditions since thrombus formation *in vivo* occurs under flow conditions. An orbital shaker system was used to study influence of shear on platelet interactions with the substrate [75,76]. Requiring low volumes of platelet suspension, the setup was used to model disturbed flow patterns *in vivo*. Our results confirm the earlier findings and show that a significantly lower number of platelets adhered on HSA films as compared to bare NiTi in static (p-value 0.014) and shear conditions (p-value 0.021).

Prevention of early thrombosis by anti-thrombogenic therapies does not guarantee long-term vessel patency [77]. The need for an anti-thrombogenic therapy that can prevent the incidence of thrombosis long-term is imperative. We found the HSA film covalently bound to PGMA with slow degradation in *in vitro* conditions. Although we have not reported the long-term effect of the HSA film on adhesion of platelets, we have measured strong binding of HSA film for 120 days rendering an anti-thrombogenic surface facilitating long-term patency of vascular devices.

Contractile smooth muscle cells are characterized by low cell proliferation, elongated spindle-shaped morphology, and high expression of contractile markers such as α-actin. These three characteristics of smooth muscle cells cultured on HSA were evaluated and compared against cells grown on bare NiTi. A possible explanation for low proliferation of smooth muscle cells on HSA films could be that HSA does not allow adsorption of other proteins such as fibronectin, which has been shown to

be important in cell adhesion, ECM synthesis and proliferation [82]. F-actin staining of smooth muscle cells by rhodamine-phalloidin and DAPI revealed that VSMC cultured on HSA films exhibited a more elongated and spindle-shaped morphology, an indication of a contractile state. An elongated smooth muscle cell morphology decreases SMC proliferation [83]. To quantitatively assess the contractile state of VSMCs, a cell based ELISA assay was performed to measure the expression of contractile marker smooth muscle alpha-actin. At Day 5, VSMCs cultured on NiTi exhibited higher amounts of alpha actin as compared to HSA. However, at Day 15 and Day 30, smooth muscle alpha actin content on VSMCs cultured on NiTi decreased, and the actin content of VSMCs cultured on HSA increased. Although the difference was insignificant at Day 15 (p-value =0.063), at Day 30 the alpha-actin content of VSMCs cultured on NiTi significantly decreased as compared to HSA (p-value = 0.038). Contractile healthy smooth muscle cells are characterized by low proliferation, elongated and spindle-shaped morphology and high expression of contractile markers. Nickel ions from nitinol cause decrease in alpha-actin expression of smooth muscle cells [78-81]. HSA film on nitinol surfaces significantly reduces the proliferation and maintains the contractile state of vascular smooth muscle cells.

This study has demonstrated the potential of human serum albumin as a vascular-device coating for metallic biomaterials by shielding the adsorption of adhesive proteins such as fibrinogen and preventing the adhesion of platelets. Human albumin, because of its anti-adhesive and surface passivating effects, has the potential to control the proliferation of smooth muscle cells and maintain their contractile phenotype, possibly obviating the need for an anti-proliferative drug. Thus, HSA coating could be used for endovascular stents and grafts for its potential to prevent the incidence of thrombosis and intimal hyperplasia.

3.5 Conclusions

In this study, a novel procedure to bind human albumin on substrates using PGMA as an anchoring layer is designed to impart hemocompatibility to materials by preventing fibrinogen adsorption and platelet adhesion. In parallel, human albumin coating described in this research can control the complications of neo-intimal hyperplasia by maintaining low proliferation and contractile expression of smooth muscle cells, thus validating its potential as a vascular-device coating. The efficacy of this coating to accelerate endothelialization of vascular grafts would be the focus of further work.

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CHAPTER 4

ASSESSMENT OF POTENTIAL OF HUMAN ALBUMIN TO MITIGATE THE INCIDENCE OF THROMBOSIS AND INTIMAL HYPERPLASIA UNDER PHYSIOLOGICALLY RELEVANT FLOW SHEAR CONDISTIONS.

Abstract: Synthetic graft prostheses such as ePTFE have shown significantly low clinical patency rates as compared to autogenous vessel substitutes such as saphenous vein and internal mammary artery. Intimal hyperplasia and thrombosis remain the major obstacles to the development and application of synthetic conduits. Surface modification strategies to alter surface properties of ePTFE are imperative to enhance its biocompatibility. Our proposed study examined the potential of a human albumin film on ePTFE for enhanced hemocompatibility and adhesion strength. Human albumin film prevents adsorption of adhesive human fibrinogen protein, evidenced by experiments with fluorescently labeled human fibrinogen and adhesion force measurements using atomic force microscopy. In physiological conditions, biodegradation of human albumin from the ePTFE surface shows the film's high adhesion strength. Human albumin coating on ePTFE does not affect the material's superior mechanical property of longitudinal tensile strength. Human albumin film shows significantly less human platelet adhesion on the surface as compared to bare ePTFE, making human albumin film a potential vascular coating for enhanced hemocompatibility with strong adhesion strength. In a platelet-smooth muscle cell co-culture model, smooth muscle cells exhibited controlled proliferation and increased contractile protein expression on albumin coated ePTFE material as compared to bare ePTFE.

Key words: Human serum albumin, GORE-TEX, adhesion strength, expanded polytetrafluoroethylene (ePTFE).

4.1 Introduction

Autologous vessels, by reducing the incidence of thrombosis and restenosis, remain the gold standard for bypass graft surgeries. The standard is challenged, however, by the unavailability of healthy autologous arteries. Synthetic polymers have been widely employed in fabricating vascular grafts, offering the advantages of biocompatibility and stability in biological environments. However, they have shown poor clinical outcomes for small-diameter vessel replacement because of high rates of thrombosis in low flow environments [1-3]. The synthetic surface of the graft material is associated with a high thrombogenicity and increased intimal hyperplasia [4-6].

Blood-biomaterial interactions play a major role in facilitating a thrombotic response. Upon implantation, the surface of a biomaterial is rapidly encapsulated by an adsorbed protein layer that triggers the activation, adhesion and aggregation of platelets and activation of the coagulation cascade, resulting in the formation of a thrombus on the surface [7-9]. The biomaterial can activate the complement system, resulting in an inflammatory response [10]. Thus, the biomaterial surface and the adsorbed protein layer become an important determinant of biocompatibility and long-term success.

Expanded-polytetrafluoroethylene (ePTFE) has been widely used in a variety of applications due to its excellent chemical stability and mechanical properties. However, due to its highly hydrophobic nature, ePTFE has been shown to allow adsorption of adhesive proteins [11], which facilitates adhesion of platelets contributing to higher incidence of thrombosis [12,13]. The inert nature of ePTFE prevents the growth of vascular cells, particularly endothelial cells, further increasing the surface's propensity to attract platelets and increasing thrombogenicity [14,15]. Due to chemical resistance of ePTFE, functionalization with other groups to alter biocompatibility is also challenging. Thus, surface modification strategies to introduce new functional groups to ePTFE becomes imperative to make it an ideal material with high strength and biocompatibility.

Several approaches have been employed to improve long term patency of synthetic grafts [18-21]. Unmodified small diameter ePTFE grafts exposed to blood flow have shown adsorption of fibrinogen and gamma-globulin and subsequent platelet adherence and thrombus formation in a canine model. Covalently grafted molecules such as heparin and prostacyclin have been employed to prevent the incidence of thrombosis [16,17]. However, these attempts failed because of short-term retention of the molecules on the surface and difficulty in covalent binding on the chemically stable ePTFE surface. The heparin-bonded Propaten vascular grafts designed by W.L. Gore and Associates using Carmeda Bioactive Surface modification [22,23] have shown promising results for lower extremity bypass with lesser rates of thrombosis [24,25]; however, the clinical trials are nonrandomized and retrospective, which brings into question the significance of the results.

Injury due to a percutaneous coronary interventional procedure (PCI) causes endothelial denudation, exposing smooth muscle cells to growth factors. This promotes a phenotypic shift in the morphology of smooth muscle cells from contractile to synthetic with increased proliferation and migration from the medial layer to the neointimal layer and increased extracellular matrix formation [26]. Phenotypic modulation has been considered to be a key factor responsible for progression of neointimal hyperplasia and restenosis. Hemodynamic factors such as wall shear stress and cyclic strain from blood flow control the phenotype and function of vascular smooth muscle cells [27]. Physiological levels of cyclic strain promote quiescent, contractile morphology of smooth muscle cells [28], whereas high levels promote SMC proliferation and dedifferentiation [29-31]. Endothelial denudation or VSMC migration causes exposure of the later to blood flow [27]. Shear stress have been known to have a direct effect on smooth muscle cell proliferation with physiological shear stress reducing proliferation and maintaining contractile morphology [32-33] and low shear stress conditions in small diameter blood vessels and grafts promoting smooth muscle cell proliferation and synthetic phenotype [63] and with upregulation of growth factors such

as PDGF [35]. In vivo and clinical studies have demonstrated the occurrence of thicker neointima under low wall shear stress [36-37].

The proposed technology involves coating ePTFE with a human serum albumin (HSA) film using poly(glycidyl methacrylate) (PGMA), an anchoring layer, to improve cellular and blood compatibility under low flow shear stress conditions. HSA exhibits a strong binding strength to ePTFE with an enhanced shield to fibrinogen adsorption and platelet adhesion. Vascular smooth muscle cells cultured on albumin coated ePTFE retained controlled proliferation and contractile state mitigating the complication of restenosis.

4.2. Materials and Methods

4.2.1 Cleaning and Sterilization of ePTFE grafts: ePTFE (Gore-Tex, W.L. Gore and Associates, Inc.) sheet was cleaned in 1% liquinox (vol/vol) detergent solution for 30 minutes and DI water for 30 minutes in an ultrasonic cleaner. The cleaned ePTFE discs were sterilized in an autoclave at 121℃ for 1 hour.

4.2.2 Fabrication of Human Serum Albumin (HSA) film: ePTFE discs are air plasma treated (6.8W for 10 minutes) for functionalization with hydroxyl, carbonyl, carboxyl groups and nitroxide groups. Functionalized ePTFE surface was modified with 0.5% wt/vol PGMA (Polymer Source, Dorval, QC) (M_n =176000 g/mol) in chloroform to produce an epoxy-rich anchoring layer followed by dip-coating in 5% wt/vol HSA (Sigma-Aldrich Corp., St. Louis, MO; CAS # 7024-90-7) solution. HSA adsorbed ePTFE samples were then annealed at 150℃ in a vacuum oven for 1 hour (Figure 4.1) [55] followed by sterilization using standard procedure of ethylene oxide sterilization ISO-11135-2014 [56].

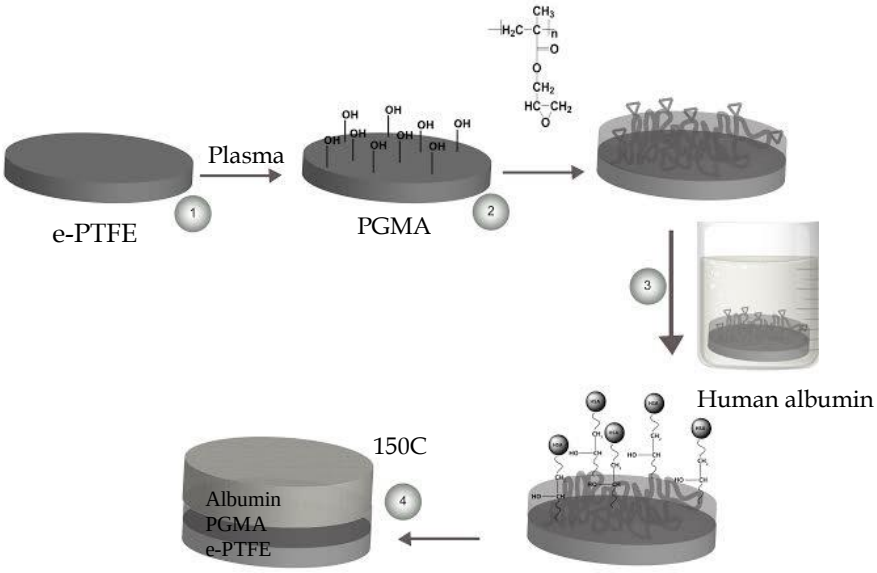


Figure 4.1: Schematic of fabrication of human albumin coating on ePTFE surface.

4.2.3 Fourier-transform Infrared (FTIR) Attenuated Total Reflectance (ATR) spectroscopy: The binding between PGMA and HSA was confirmed by ATR-FTIR spectroscopy (Nicolet SX-60 FTIR spectrometer) performed to collect IR spectra for albumin protein, PGMA and albumin-PGMA coated ePTFE surface pressed on a diamond crystal stage. Absorption spectra over the range 400-4000cm^{-1} was collected at a resolution of 8 with 128 scans using OMNIC software (Thermo Fisher Scientific Inc., UK).

4.2.4 Adhesion strength measurement of albumin film by tensile testing: Tensile strength of albumin coated ePTFE and bare ePTFE was measured to assess the effect of albumin coating on ePTFE. HSA and bare ePTFE graft material samples (length 30mm, thickness 1mm, width 14mm) were elongated at a cross-head speed of 10mm/min, and tensile force was measured using a 100N

load cell in static and fluid state (phosphate buffer, 37°C, pH 7.4) conditions. Binding of HSA film on ePTFE material was assessed using Energy Dispersive X-ray spectroscopy (EDX) at 20Kv to assess the elemental composition of albumin coated ePTFE after longitudinal tensile force application as described below following SEM preparation.

4.2.5 Scanning Electron Microscopy (SEM): Scanning electron microscopy SEM (SU-6600, Hitachi-HTA, Inc.) was used to investigate the morphology of albumin coated and bare ePTFE grafts. Prior to imaging, the samples were cut into 1cm x 1cm pieces and coated with a 10nm layer of platinum to make them electrically conducive. SEM measurements were performed at an accelerating voltage of 5.0kv and a working distance 10.6mm. A set of three images at 250x, 500x and 1000x were taken for each sample.

4.2.6 Surface Wettability: Measurement of static water contact angle (goniometer model 260-F4-VWR) between albumin-coated and bare ePTFE surface and DI water was collected using the sessile drop method. A water droplet (5-10µL) is dispensed onto the sample from a syringe, and measurements are recorded after 30 seconds. The contact angle values reported are averages of three drops along the center of HSA-coated and bare ePTFE surfaces.

4.2.7 Thermal analysis by Thermo-gravimetric analysis (TGA): The degradation temperatures of human albumin coated and uncoated ePTFE were determined using thermo-gravimetric analysis (TA instruments, New Castle, DE; TGA 2950) by heating a 5-15mg sample on a clean platinum pan at a heating rate of 10°C/min under nitrogen.

4.2.8 Fluorescent labeling of albumin: Human albumin (1mg/mL in phosphate buffer) was labeled with 4µg of Alexa Fluor 488 fluorescent dye (CAS no. 1352809-17-6, Sigma-Aldrich, St. Louis,

MO) in 200µL of dimethylformamide (DMF) and dialyzed for 48 hours to remove the unbound dye. Fluorescently labeled albumin was adsorbed on PGMA modified ePTFE samples (10mm thick and 45mm long) and submerged in phosphate buffer in an orbital shaker to qualitatively assess the presence of albumin on the surface using a fluorescent microscope for a period of up to 3 months.

4.2.9 Albumin release quantification: Albumin coated ePTFE discs were immersed in phosphate buffer solution at 37°C in an orbital shaker for a period of up to 4 months at a frequency of 220rpm. The albumin released was quantified using Bradford assay. A standard curve was created using several dilutions (25µl, 75µl, 200µl, 400µl and 800µl) of albumin in phosphate buffer mixed with 200µL of Bradford reagent. The albumin concentration in each sample was assessed by measuring and extrapolating absorbance at 670nm on the standard curve.

4.2.10 Fibrinogen adhesion force measurements: The adhesion force of human fibrinogen (FIB-3; plasminogen, von Willebrand factor, and fibronectin depleted; Enzyme Research Laboratories, South Bend, IN) to HSA films was examined using AFM (VECCO Probes, Santa Barbara, CA; Dimensions 3100 Atomic Force Microscope) colloidal probe technique (Figure. 4.2). Hollow glass beads (25µm) were cleaned in piranha solution (3:1 solution of concentrated sulphuric acid/30%hydrogen peroxide) for 1 hour and in DI water for 30 minutes each. 20-30µm hollow glass beads (Potters Industries Inc. Valley Forge, PA) are modified in 0.5% wt/vol PGMA (M_n = 76,000 g/mol) in methylethyl ketone (MEK) overnight and rinsed with pure MEK for 30 minutes. Modified beads were glued to cantilevers using a protocol described by Jones and collaborators [36]. Modified cantilevers were immersed in freshly prepared 1mg/mL fibrinogen solution in phosphate buffer for 30 minutes and rinsed in pure buffer before performing the adhesion experiment on albumin films. The deflection (force) of a cantilever bound with fibrinogen-adsorbed bead on albumin coated and

uncoated ePTFE corresponds to the adhesion force between fibrinogen and the surface. Force-distance measurements were carried out by AFM contact mode.

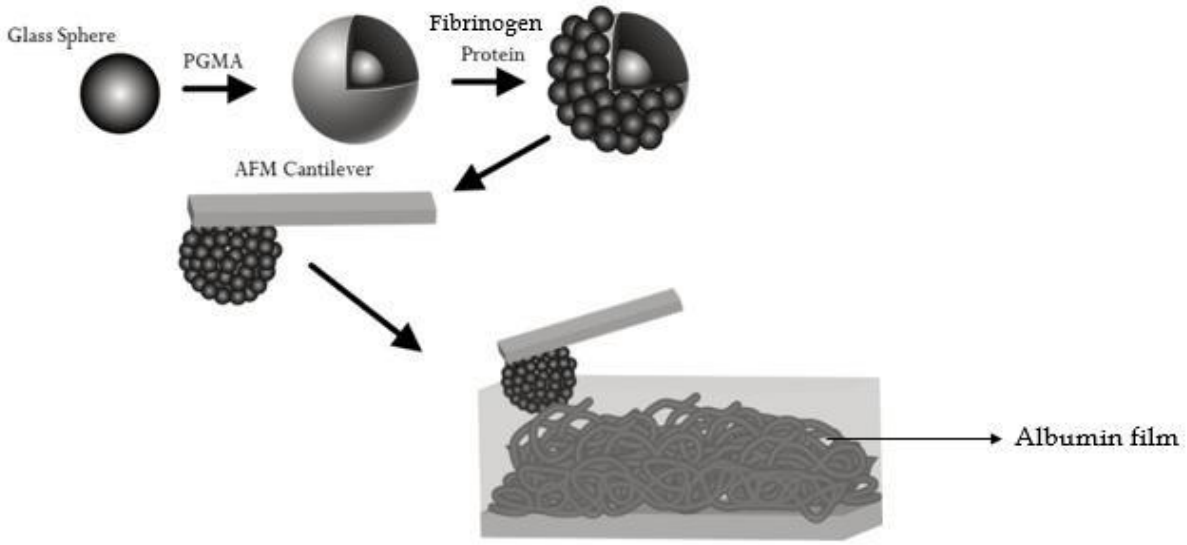


Figure 4.2: Schematic of the procedure to measure adhesion force between human fibrinogen and human albumin coated ePTFE and bare ePTFE surface.

4.2.11 Fluorescent labeling of fibrinogen: Human plasma fibrinogen (1mg/mL in phosphate buffer) was fluorescently labeled with labeled with 4µg Rhodamine-NHS dye in 200µL of dimethylformamide (Fisher) and dialyzed for 48 hours to remove unbound dye. Labeled human fibrinogen [105] is incubated on HSA coated and uncoated ePTFE samples (15mm diameter and 10mm thick) with polystyrene controls for 1 hour at 37°C in an orbital shaker. Fluorescence was measured at 575 and 525nm wavelengths to quantify and compare the adsorption of fluorescently labeled fibrinogen.

4.2.12 Human Platelet Isolation and Adhesion: Heparin anticoagulated fresh human blood was centrifuged (225g, 15 min, 25°C) to obtain platelet-rich plasma using a Beckman Coulter Allegra centrifuge (Beckman Coulter, Fullerton, CA). All protocols for participation of human volunteers for

blood isolation were approved by the Institutional Review Board (IRB) at Clemson University. A platelet concentration of 10^6 platelets/mL in platelet suspension buffer (137mM NaCl, 2.7mM KCl, 5.5mM Dextrose, 0.4 mM sodium phosphate monobasic, 10 mM HEPES and 0.1U/ml apyrase) was used. Platelet suspension buffer was added with 2.5 mM $CaCl_2$ and 1.0mM $MgCl_2$ for activation of platelets.

The platelet solution was allowed to rest for 30 minutes at 37°C before being added to the HSA coated ePTFE and the uncoated ePTFE controls in a 24-well plate and then incubated for 1 hour at 37°C under static and shear conditions. At the end of the 5-day incubation period, the suspension was aspirated from each well, and each well was rinsed with phosphate buffer to remove unbound platelets. Orbital shear was provided using an orbital plate shaker (VWR, S-500) calculated using the following equation,

$$Tmax = a \sqrt{\eta\rho} (2\pi f)^3$$

where, a- radius of gyration of the shaker (cm), ρ- density of solution (g/mL), η- viscosity at 37°C (poise) and f- frequency of rotation (rps)). A frequency of 60rpm, as per the above equation, corresponded to a shear stress of 1.2dynes/cm^2 respectively, comparable to arterial low flow shear levels.

Platelet adhesion was quantified by measuring the lactate dehydrogenase (LDH) released when adherent platelets were lysed with Triton-PSB buffer (2% v/v Triton-X-100 in PSB), using a CytoTox 96^R Non-Radioactive Cytotoxicity Assay (Promega Corporation, Madison, WI). Absorbance was measured at 490nm using a UV/Vis spectrophotometer (Bio-Tek Instruments Inc.,Winooski, VT). A calibration curve was constructed with a known number of platelets, counted using a Beckman Coulter Z2 Particle Count and Size Analyzer (Beckman Coulter, Fullerton, CA), and the platelet adhesion on the HSA films was determined using the calibration curve.

4.2.13 Effect of flow shear on treated ePTFE

A custom-made and validated vascular simulator (Figure 4.3) was used to characterize the effect of combined cyclic strain and flow shear on treated ePTFE [82]. For each experimental simulation, 6mm diameter silicone membranes were cut from biomedical grade silicone sheets (SMI, 0.15" NRV G/G 40D, SMI, MI). The silicone membranes were cleaned as described above and steam sterilized in an autoclave at 121°C for 1 hour. A sandwich was formed using sterile station rings and silicone membrane. The simulator accommodates three such assemblies on each base plate (three loading stations per base plate) providing a total sample size of 6 using 2 baseplates. The baseplates were UV-sterilized for 12 hours followed by attachment of albumin coated and uncoated ePTFE grafts using an epoxy resin (Devcon, MSC part # 00261115). Sterile Teflon rings (2.75 cm^2 inner cross-sectional area and 1.2 cm height) were centered on the ePTFE grafts on the silicone membrane above the loading post.

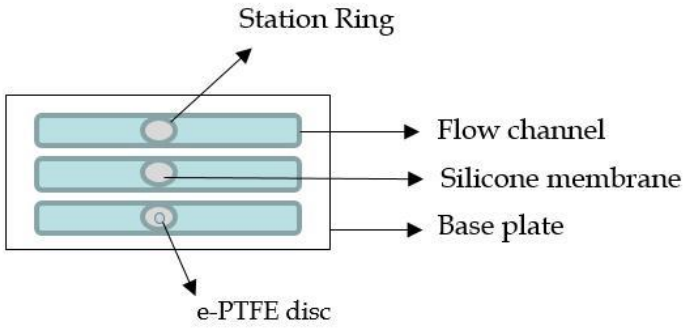


Figure 4.3**:** Schematic for placement of ePTFE disc on the simulator base plate for assessment of cellular response.

4.2.14 Smooth muscle cell culture and characterization

Using a protocol approved by Clemson University Institutional Animal Care and Use Committee, abdominal aortas were harvested from 6-10 week old female Sprague-Dawley rats. Adventitial fat

layers and endothelium were removed. VSMCs were isolated from the vascular medium using collagenase type II (Worthington, Biomedical, Lakewood, NJ) and elastase (Worthington Biomedical, Lakewood, NJ) [64]. The cells were maintained in Dulbecco's modification of Eagle Medium (DMEM, 10-013-CV; Mediumtech, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (F-4135; Sigma-Aldrich, St. Louis, MO) and 1% antibiotic-antimycotic (A5955, Sigma-Aldrich, St. Louis, MO) under standard cell culture conditions (37°C, 5%CO_2, 95% relative humidity).

Experiments were carried out using VSMCs between passages 4 to 8. For experimental testing, cells were trypsinized using 0.25% trypsin containing 0.2mg/mL EDTA (Sigma Chemical Company, E6511, St. Louis, MO, USA).

Smooth muscle cells were characterized by immunofluorescent staining for contractile marker smooth muscle α-actin (SM α-actin). VSMCs were fixed with cold 4% formaldehyde and blocked for 45 minutes at room temperature with a blocking solution that consisted of 40mg/ml bovine serum albumin in PBS, 6% FBS, and 0.05% Triton-X. Cells were then treated with primary mouse anti-α-actin antibody (ab5694, Abcam) diluted 1:500 overnight at 4°C. Cells were then treated with fluorescent tagged goat anti-mouse secondary antibody and goat anti-rabbit secondary antibody, both diluted 1:200 in the blocking solution, for 3 hours respectively at room temperature. The cells were then stained with DAPI (Molecular Probes, D-1306, Eugene, OR) and imaged using fluorescent microscopy (Nikon LV-UDM, Nikon Instruments Inc., Melville, NY).

Experimental groups consisted of albumin coated (15mm diameter; N=6) and uncoated ePTFE discs (15mm diameter; N=6). The cells were maintained in Dulbecco's modification of Eagle Medium (DMEM, 10-013-CV; Mediumtech, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (F-4135; Sigma-Aldrich, St. Louis, MO) and 1% antibiotic-antimycotic (A5955,

Sigma-Aldrich, St. Louis, MO) under standard cell culture conditions (37C, 5%CO_2, 95% relative humidity) and subjected to forces of cyclic strain of 0-7% and flow shear of 0.2-0.5 dynes/cm^2 with a flow rate of 225mL/min for 7 days.

4.2.14.1 Effect of epoxy glue treatment

In order to determine cellular response to the epoxy glue used to affix the ePTFE specimens to the silicone membrane in the vascular simulator, vascular smooth muscle cells between passages 4-8 were cultured on 250 µL of epoxy glue **(**Devcon, Danvers, MA) and non-epoxy treated polystyrene culture wells for assessment of cell proliferation over periods of 7 and 15 days.

4.2.14.2 Co-culture of platelets and smooth muscle cells

Vascular smooth muscle cells were cultured and allowed to adhere to albumin coated and bare ePTFE discs for 72 hours. Platelet rich plasma (PRP) isolated from fresh human blood was incubated on adhered vascular smooth muscle cells for 12 hours. Platelet supplemented smooth muscle cells on ePTFE were subjected to application of forces of cyclic strain of 0-7% and flow shear of 0.2-0.5 dynes/cm^2 with a flow rate of 225mL/min for 7 days; cells were evaluated using the test methods described below.

4.2.14.3 Cell Morphology

After 7 days of culture, cells were fixed in 4% paraformaldehyde solution for 20 minutes and permeabilized using 0.25% Triton-X detergent in phosphate buffer for 10 minutes. The cytoskeletal F-actin and cell nuclei were stained using 25 µg/ml rhodamine-phalloidin (Invitrogen, R415) and 2-(4-amidinophenyl)-1H -indole-6-carboxamidine (DAPI) (Molecular Probes, D-1306, Eugene, OR) respectively. The cells were then imaged using fluorescent microscopy (Nikon Inc., Diaphot 30).

4.2.14.4 Cell proliferation

VSMC proliferation was analyzed by assessing the reduction of methylthiazole tetrazolium (MTT) (Sigma-Aldrich, MO, USA) dye after 24 hours. 3-(4,5-dimethylthiazol -2-YL)-2,5-diphenyltetrazolium bromide salt was dissolved in 1x DPBS at a concentration of 2mg/mL. VSMCs were rinsed with 1x DPBS and then placed in 1mL DMEM solution with no FBS. A total of 240 µl of MTT was added to the well, and the solution was incubated for 4 hours. After incubation, the MTT-DMEM solution was removed and 1mL of DMSO was added to the well. Using a plate reader, absorbance was then read at 570nm.

4.2.14.5 Expression of SMC alpha-actin

α-actin expression in smooth muscle cells cultured on albumin coated and uncoated ePTFE grafts was analyzed using a modified cell-based ELISA assay [83]. Briefly, at indicated times, the medium was removed, and the cells were fixed in cold methanol. They were then incubated with a blocking solution that consisted of 40mg/ml bovine serum albumin in PBS, 6% FBS, and 0.05% Triton-X for 45 minutes at room temperature. Primary mouse anti-α-actin antibody diluted 1:200 in the blocking solution was then added to the discs for 2 hours at room temperature followed by a 2 hr treatment with biotin conjugated bovine anti-mouse IgG, also diluted 1:200 in the blocking solution. After extensive washing, the cells were incubated with horseradish peroxidase (HRP) conjugated anti-mouse secondary antibody. Chromogenic substrate (3 mM p-nitrophenyl phosphate, 0.05 M Na_2CO_3, and 0.05 mM $MgCl_2$) was then applied to produce color, and the absorbance was read at 405 nm with a microplate reader (Beckman Instruments, Inc., Model #DU®640B, Fullerton, CA) normalized against cell count on each disc giving a number directly related to the α-actin content per cell. The cells were then DAPI stained and imaged to facilitate cell counting.

4.2.15 In-vitro Flow Loop circulation model

A flow experiment was designed for albumin coated ePTFE small diameter grafts to assess the adhesion of bovine platelets to the albumin coated and bare ePTFE grafts (Figure 4.4). A flow rate of 225mL/min was employed to simulate the physiological relevant low flow conditions. Silicone tubes were connected to the ePTFE graft using brass connectors. The tubing and graft were connected to a pulsatile pump to provide the required flow rate. A closed loop system was designed to allow fabrication of albumin film and assessment of response of fluorescently labeled fibrinogen and platelet-rich plasma to the albumin coated ePTFE grafts under controllable hemodynamic conditions.

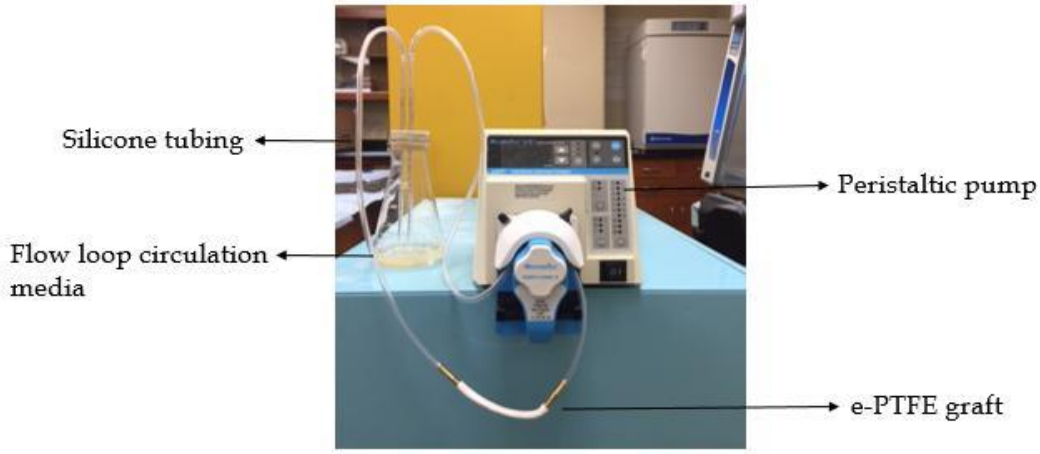


Figure 4.4: Setup of the flow loop circulation model.

4.2.16 Fabrication of albumin film coated small diameter ePTFE tube: ePTFE tubes of 3mm inner diameter and 4mm thickness (Aeos™ ePTFE; Extruded Sub-Lite-Wall®; 0.096+/-.003 ID, Zeus, Orangeburg, SC) were plasma treated before functionalization with surface active hydroxyl, carbonyl, carboxyl and nitroxide groups. Surface activated ePTFE tubes were modified with 0.5% wt/vol PGMA (M_n =176000 g/mol) in chloroform to produce an epoxy-rich anchoring layer

followed by 5% wt/vol HSA solution by circulation of PGMA and HSA solution with a flow rate of 225mL/min. HSA coated tubes were then annealed at 150℃ in a vacuum oven for 1 hour [34] followed by ethylene oxide sterilization by ISO-11135-2014 [35].

4.2.17 Fourier-transform Infrared (FT-IR) Attenuated Total Reflectance (ATR) spectroscopy: The binding between PGMA and HSA was confirmed by ATR-FTIR spectroscopy. FTIR-ATR spectroscopy was performed using a Nicolet SX-60 FTIR spectrometer to collect IR spectra for albumin, PGMA and albumin-PGMA coated ePTFE graft cut open and inner surface pressed on a diamond crystal stage surface pressed on a diamond crystal stage. Absorption spectra over the range 400-4000cm^{-1} was collected at a resolution of 8 with 128 scans using OMNIC software.

4.2.18 Scanning Electron Microscopy (SEM): Scanning electron microscopy was done to visualize the morphology of albumin coated and bare ePTFE surface. Prior to imaging, the grafts were cut open and coated with a 10nm layer of platinum. A scanning electron microscope model SU-6600 with accelerating voltage of 5.0kV and working distance 10.6mm was used to obtain images at 250x, 500x and 1000x.

4.2.19 Energy dispersive X-ray spectroscopy (EDX): Electron dispersive x-rays (EDS, Model S-3400N, Hitachi, Japan) were used to gain a quantitative breakdown of elements on the albumin coated and uncoated surfaces.

4.2.20 Human Fibrinogen adsorption: Human fibrinogen (FIB-3; plasminogen, von Willebrand factor, and fibronectin depleted; Enzyme Research Laboratories, South Bend, IN) was fluorescently labeled using the protocol described in section 2.11 and circulated through the albumin coated and uncoated ePTFE tubes (n=4) for 1 hour followed by measurement of fluorescence at excitation-emission wavelengths of 575 and 525nm.

4.2.21 Bovine Platelet Isolation and adhesion

Heparin-anticoagulated fresh porcine blood was centrifuged (225g, 15 min, 25C) to obtain platelet-rich plasma using a Beckman Coulter Allegra 6R centrifuge. A platelet concentration of 10^6 platelets/mL in platelet suspension buffer (137mM NaCl, 2.7mM KCl, 5.5mM Dextrose, 0.4 mM sodium phosphate monobasic, 10 mM HEPES and 0.1U/ml apyrase) was used. Platelet suspension buffer was added with 2.5 mM $CaCl_2$ and 1.0mM $MgCl_2$ to activate the platelets.

The platelet-rich plasma solution was allowed to rest for 30 minutes at 37°C before being circulated through the HSA coated ePTFE and the uncoated ePTFE tubes (n=4) with a flow rate of 225mL/min for 24 hours in an incubator at 37°C, 5%CO_2, 95% relative humidity. At the end of the 24 hour incubation period, the grafts were rinsed with phosphate buffer to remove unbound platelets. Platelet adhesion was assessed using the protocol described in section 4.2.12.

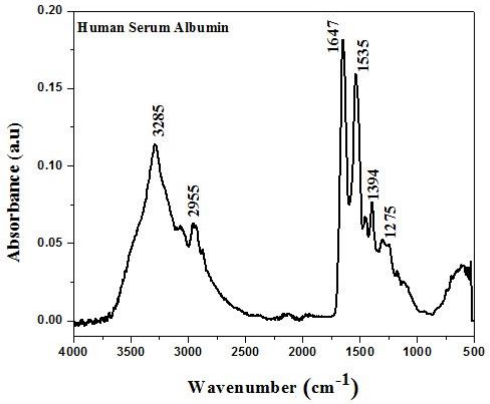
4.2.22. Statistical Analysis

The statistical significance of differences between mean values reported with 95% confidence intervals (CI) for different samples and conditions was assessed using a student's t-test, with p-value ≤ 0.05 as significant.

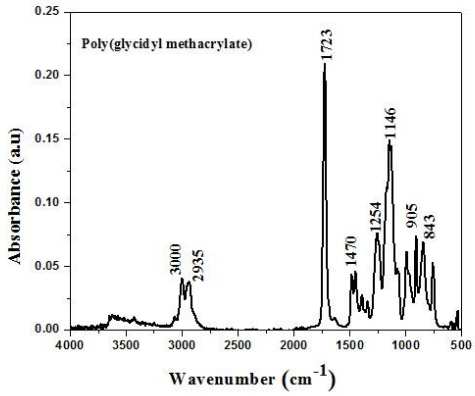
4.3 Results

4.3.1 Attenuated Total Reflectance (ATR) Fourier-transform Infrared (FT-IR) spectroscopy:

ATR-FTIR spectroscopy confirmed the presence of human albumin and PGMA on ePTFE. Wavenumbers of amide I (1655cm^{-1}) and amide II (1541cm^{-1}) bonds depict stability of the alpha-helical secondary structural conformation of human albumin on the ePTFE surface (Figure 4.5).



a)



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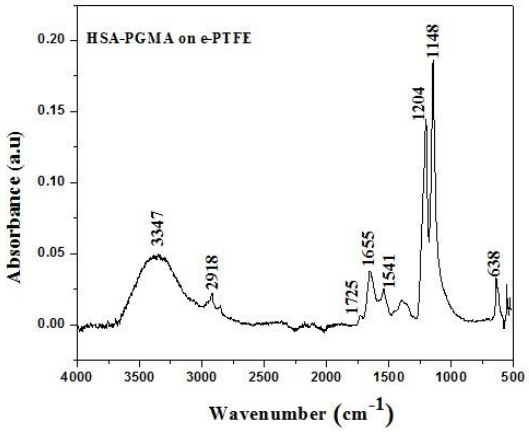
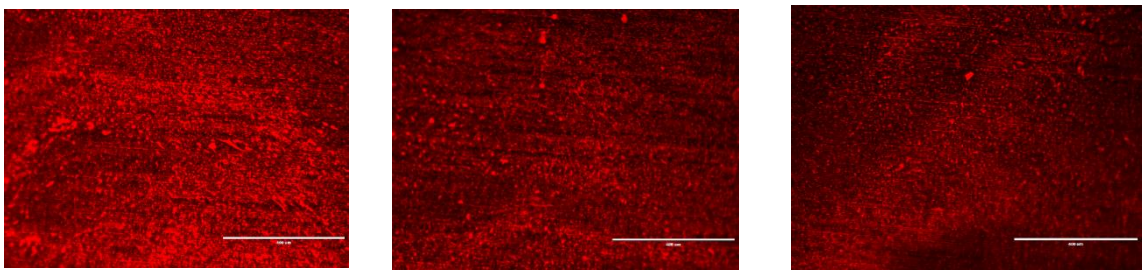


c)

Figure 4.5: IR spectra of a) human serum albumin, b) poly (glycidyl methacrylate) (PGMA) and c) IR spectra of human serum albumin bound to poly(glycidyl methacrylate)(PGMA) on ePTFE surface with characteristic amide I (1655cm^{-1}) and amide II (1541cm^{-1}) bands and carbonyl band (1725cm^{-1}).

4.3.2 Fluorescent labeling of albumin: Presence of fluorescently labeled albumin on ePTFE surface under physiological conditions of flow shear (phosphate buffer, pH 7.4, 37°C) for a period of 30 days confirmed the strong binding strength of albumin to the surface (Figure 4.6).



Day 1 Day 15 Day 30

Figure 4.6: Images of fluorescently labeled albumin on ePTFE surface assessed for up to 30 days.

4.3.3 Tensile strength of albumin film: Albumin coating on ePTFE surface does not cause a significant change in the tensile strength of ePTFE in dry- or fluid-state conditions after application of longitudinal tensile force (Figure 4.8); thus, the ePTFE surface retains its mechanical strength to withstand stresses during flow shear. Presence of albumin before and after tensile application by IR spectroscopy indicates strong binding to the surface (Figure 4.7).

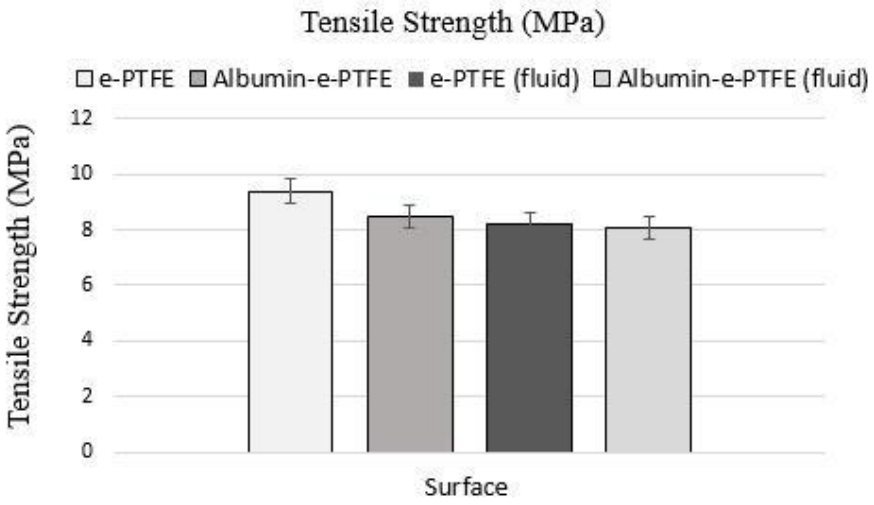
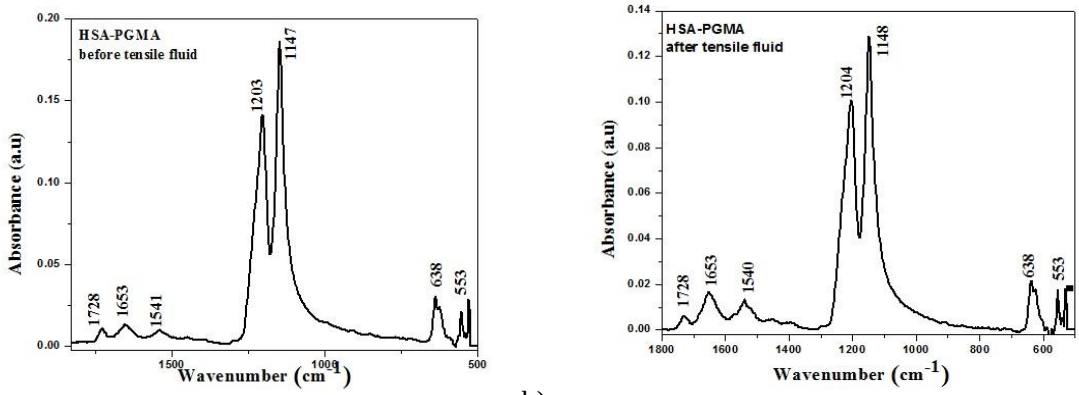


Figure 4.7**:** Measurements of tensile strength of albumin coated ePTFE and bare ePTFE in dry- and fluid-state conditions. Data represented as mean ± standard deviation.

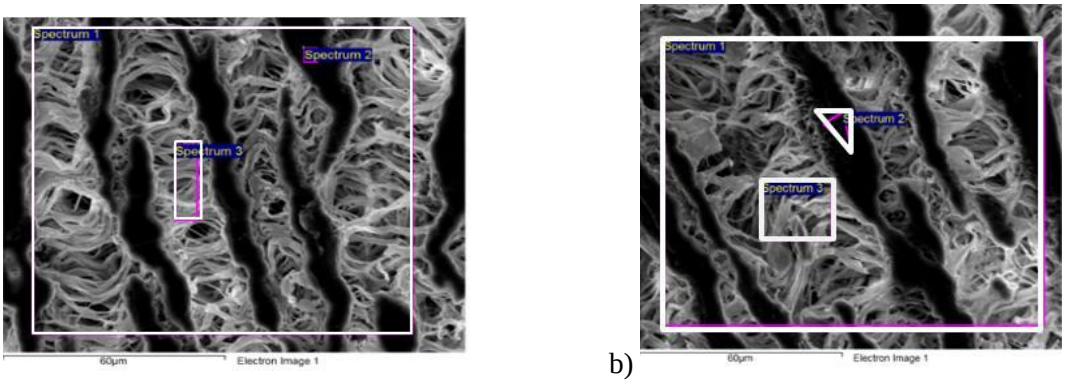


a) b)

Figure 4.8: IR spectra of human serum albumin bound to poly(glycidyl methacrylate)(PGMA) on ePTFE surface a) before and b) after tensile force application.

4.3.4 Scanning Electron Microscopy (SEM) and Energy Dispersive Spectroscopy (EDX).

SEM-EDX analysis revealed the presence of a nitrogen element, signifying the presence of albumin after application of tensile force (Figure 4.9 and 4.10) indicating a strong binding strength of albumin film on ePTFE surface in dry and physiological fluid state conditions (phosphate buffer, 37°C, pH 7.4).



a) b)

Figure 4.9: SEM images of albumin coated ePTFE surface after application of tensile force in a) dry and b) fluid state at 1000x magnification.

Spectrum	C (wt%)	F (wt%)	N (wt%)
Spectrum 1	23.87	73.17	2.95
Spectrum 2	24.95	73.72	1.33
Spectrum 3	25.79	69.58	4.63

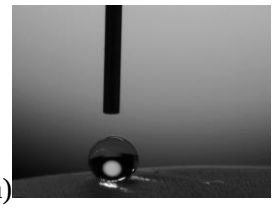
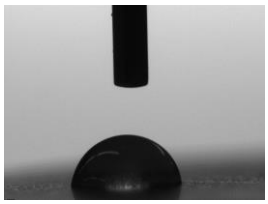
After tensile force in dry state

Spectrum	C (wt%)	F (wt%)	N (wt%)
Spectrum 1	21.73	74.50	3.77
Spectrum 2	21.59	76.92	1.49
Spectrum 3	24.10	70.12	5.78

After tensile force in fluid state

Figure 4.10: Elemental composition of albumin coated ePTFE before and after application of tensile force at 1000x magnification.

4.3.5 Surface Wettability: Human albumin significantly enhanced the surface wettability of the ePTFE surface, as evidenced by measurement of a lower water contact angle of 78±3.7º on albumin coated ePTFE as compared to 125±4.8º for uncoated ePTFE (Figure 4.11).

a)  b) 

Surface	**Contact angle**
ePTFE	125±4.8
Albumin coated ePTFE	78±3.7

Figure 4.11**:** Measurement of surface wettability of albumin coated ePTFE and bare ePTFE by contact angle analysis. Values represented as mean ± SD (error bar) with n=6 for each sample group.

4.3.6 Smooth muscle cell culture

Cell proliferation measurements reveal controlled smooth muscle cell count on albumin coated ePTFE compared to bare ePTFE assessed for a period of 7 days (Figure 4.12) under low flow shear stress conditions of 0.5 dynes/cm square. While no significant difference was observed in proliferation of smooth muscle cells on albumin coated and bare ePTFE, SMCs co-cultured with platelets were significantly fewer in count on albumin ePTFE compared with bare ePTFE. This was revealed by reduction of MTT salt to a formazon product by active reductase enzymes, which permitted quantification of metabolically active, healthy and proliferating cells (materials cytotoxicity) via colorimetric measurements.

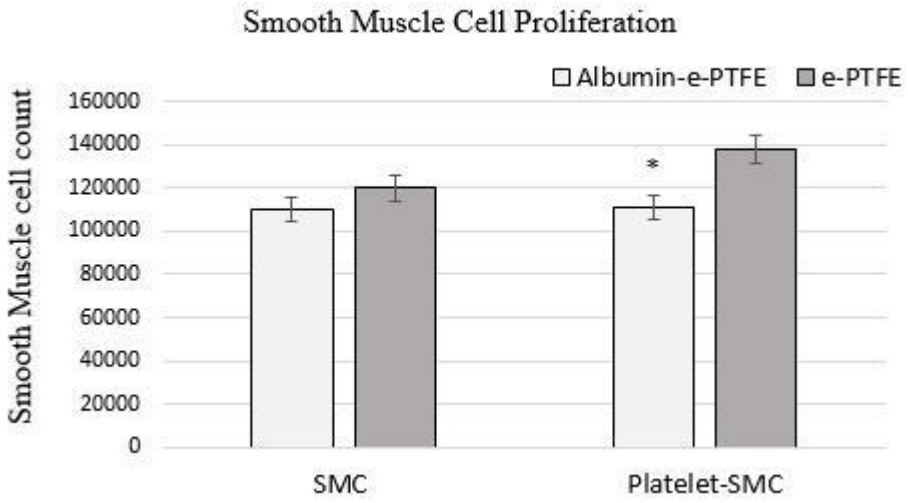


Figure 4.12**:** Measurement of smooth muscle cell proliferation on albumin coated and bare e-PTFE. Values represented as mean ± SD (error bar) with n=6 for each sample.

Morphology (Figure 4.13) and aspect ratio (Figure 4.14) measurement of smooth muscle cells cultured on albumin coated and bare ePTFE reveal a spindle shaped and contractile morphology on

albumin coated ePTFE as compared to bare ePTFE, evidenced by fluorescent staining with rhodamine-phalloidin and DAPI.

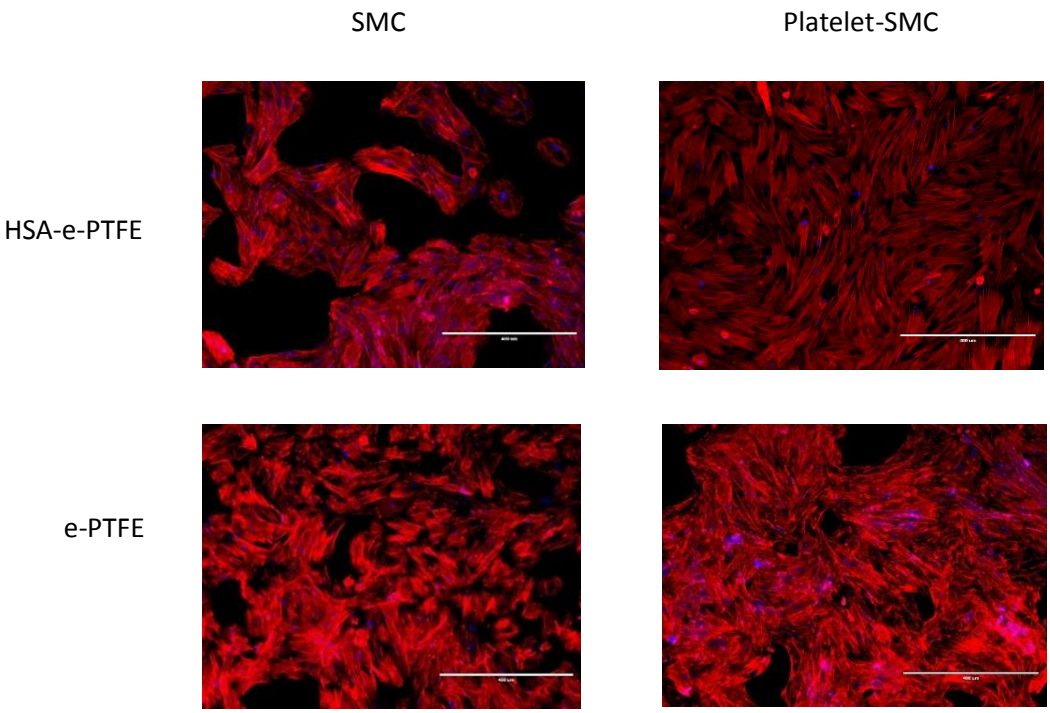


Figure 4.13**:** Morphology of smooth muscle cells cultured on albumin coated and bare e-PTFE. Values represented as mean ± SD (error bar) with n=6 for each sample.

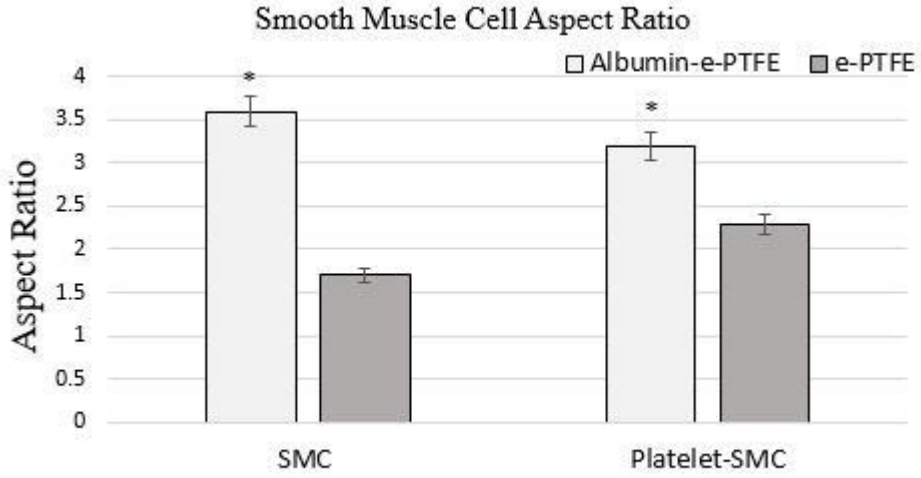


Figure 4.14**:** Aspect ratio of smooth muscle cells cultured on albumin coated and bare e-PTFE. Values represented as mean ± SD (error bar) with n=6 for each sample.

A significant difference (p=0.012) was observed in the expression of contractile marker smooth muscle alpha actin on smooth muscle cells cultured on albumin coated ePTFE compared to bare ePTFE (Figure 4.15).

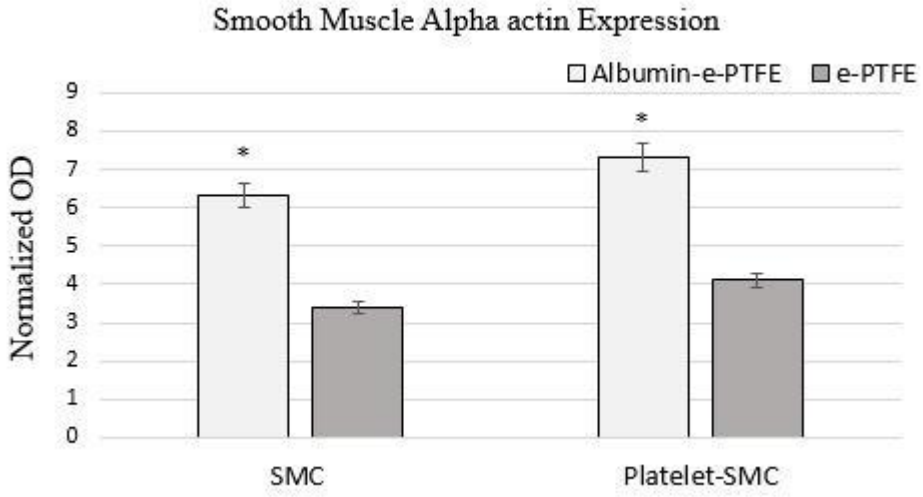


Figure 4.15**:** Measurement of expression of contractile marker smooth muscle alpha-actin by smooth muscle cells cultured on albumin coated and bare e-PTFE. Values represented as Mean ± SD (error bar) with n=6 for each sample.

4.3.7 Fibrinogen adhesion force measurements: Adhesion force of human fibrinogen was significantly less (p=0.01) to albumin coated ePTFE surface compared to bare ePTFE (Figure 4.16).

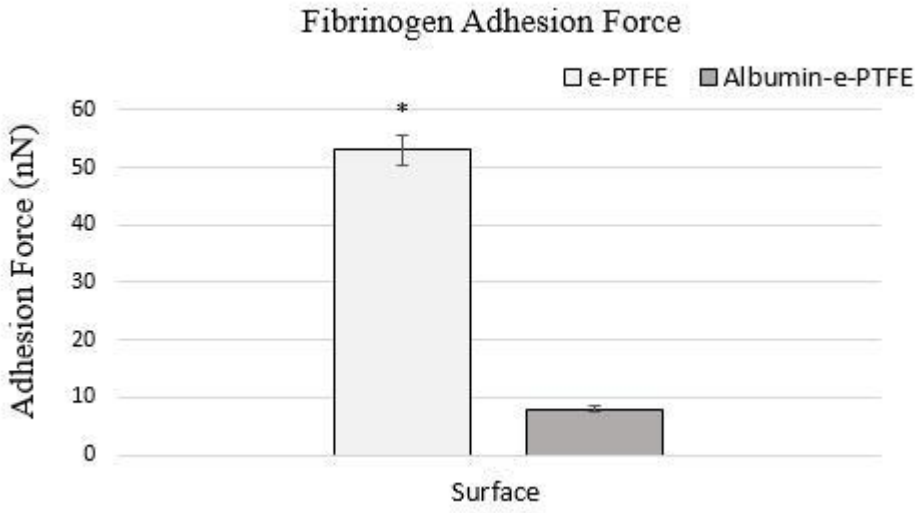


Figure 4.16**:** Measurement of adhesion force of fibrinogen to albumin coated ePTFE and bare ePTFE. Values represented as mean ± SD (error bar) with n=6 for each sample.

4.3.9 Response of epoxy-treated smooth muscle cells

No significant difference (p=0.06) between the smooth muscle cell proliferation on epoxy treated and untreated surface was measured (Figure 4.17).

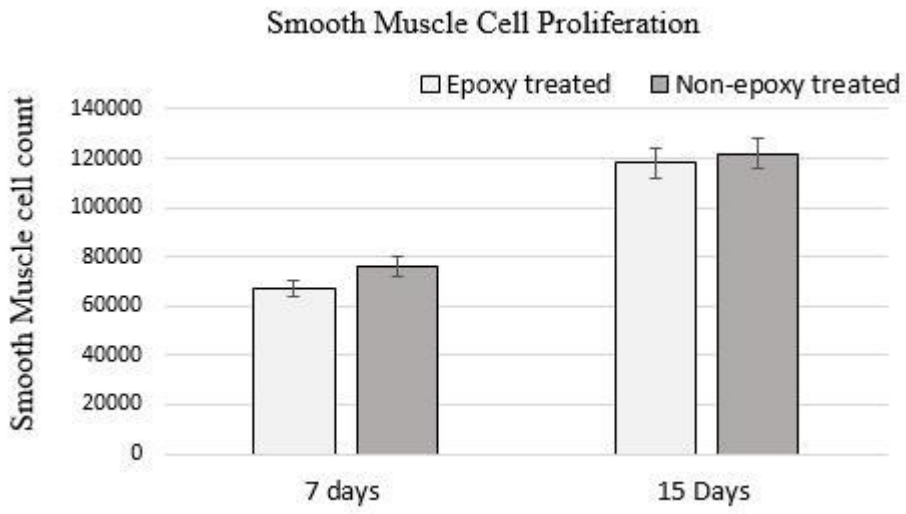


Figure 4.17**:** Measurement of smooth muscle cell proliferation on epoxy-treated and untreated e-PTFE. Values represented as mean ± SD (error bar) with n=6 for each sample.

4.3.10 Fluorescent labeling of fibrinogen: As depicted in Figure 4.18, human albumin film significantly reduced (p=0.03) the adsorption of human fibrinogen as compared to bare ePTFE surface and silicone.

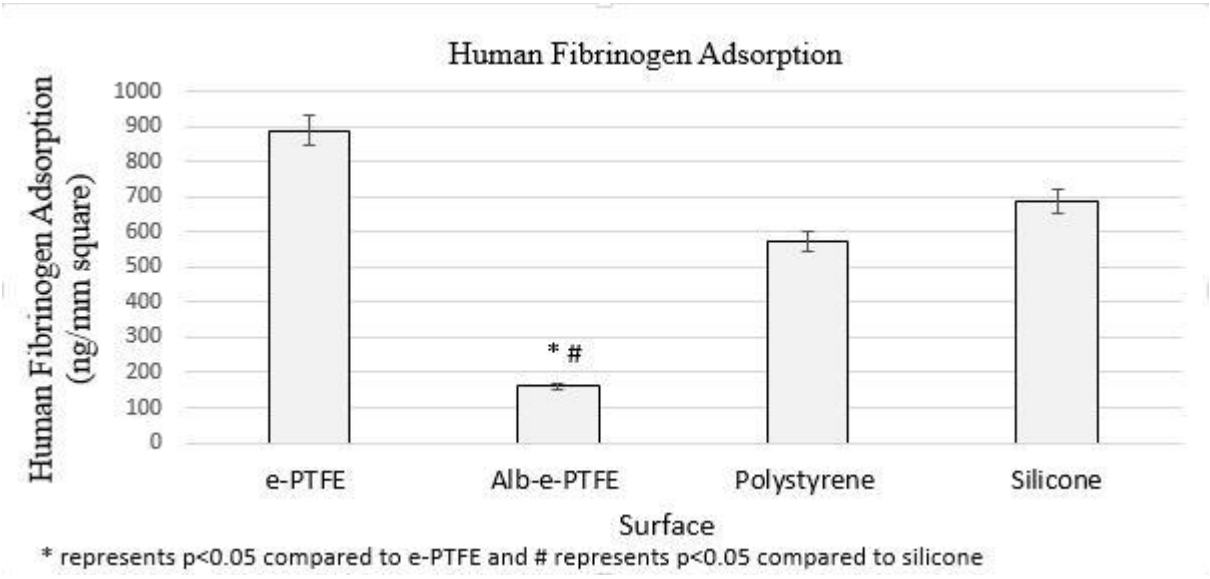


Figure 4.18: Measurement of adsorption of human fibrinogen on albumin coated ePTFE (161 ± 23ng/mm square) and bare ePTFE (562 ± 34 ng/mm square). Polystyrene and silicone were used as positive controls. Values represented as mean ± SD (error bar) with n=4 for each sample.

4.3.11 Adhesion of Human Platelets: Human albumin significantly (p=0.023) reduces the adhesion of human platelets as compared to bare the ePTFE surface (Figure 4.19).

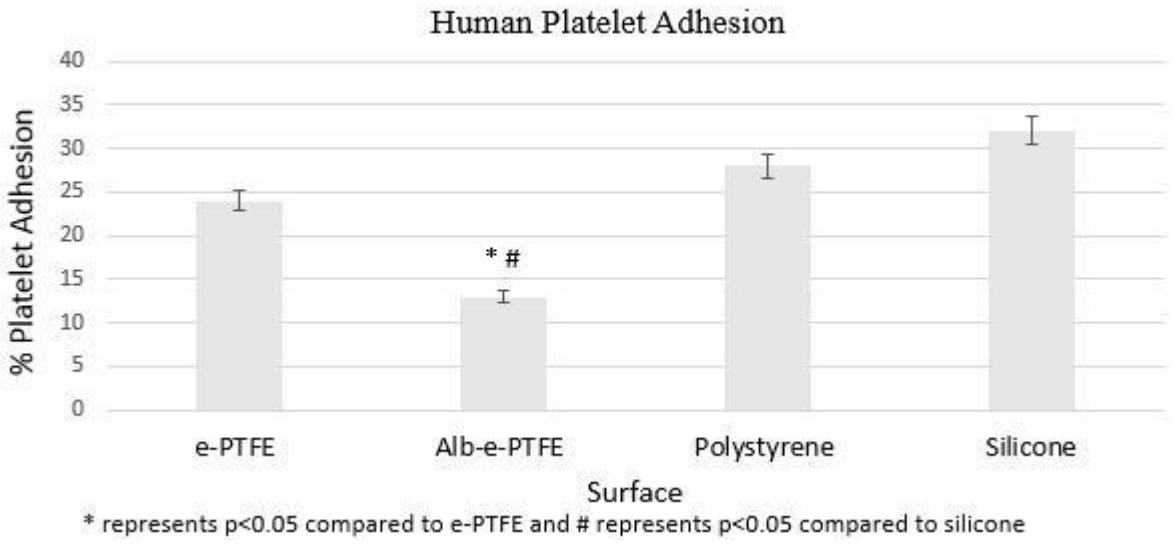


Figure 4.19: Measurement of adhesion of human platelets on albumin coated ePTFE (11.2 ± 1%) and bare e–PTFE (23 ± 2.1%). Polystyrene and silicone were used as positive controls. Values represented as mean ± SD (error bar) with n=4 for each sample.

4.3.12 Albumin release quantification (Biodegradation): Long term stability of albumin on the ePTFE surface is evident from the controlled degradation profile of albumin, indicating strong binding strength. Strong binding strength of albumin to the ePTFE surface as evident from long term stability promises a potential vascular coating (Figure 4.21).

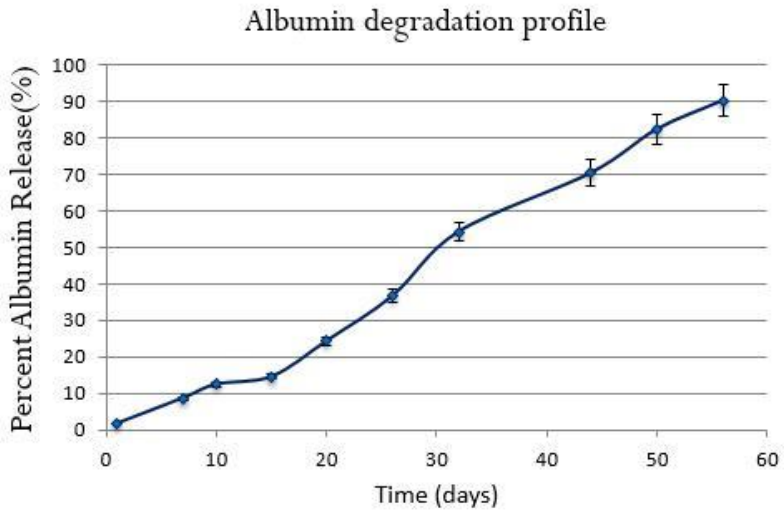
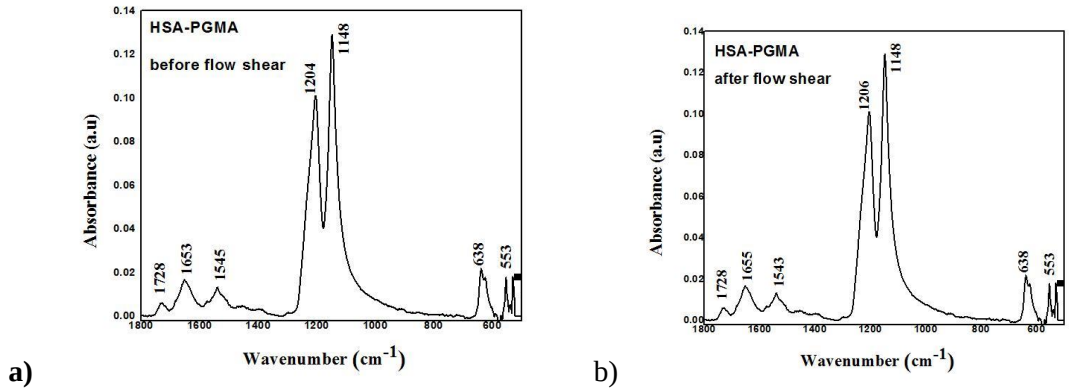


Figure 4.20: Degradation profile of human albumin from ePTFE.

4.3.13 Albumin binding strength

Strong binding strength of albumin on the ePTFE surface was assessed by ATR-FTIR analysis of albumin coated ePTFE exposed to mechanical forces of flow shear for 20 days in phosphate buffer (pH 7.4 and 37°C) in a vascular simulator [82].



a) b)

Figure 4.21: Infrared spectra of albumin-coated ePTFE before a) and after b) application of flow shear for 20 days.

4.3.14 Human Fibrinogen Adsorption

A significant difference in the adsorption of fluorescently labeled human fibrinogen was measured on albumin coated and uncoated ePTFE graft.

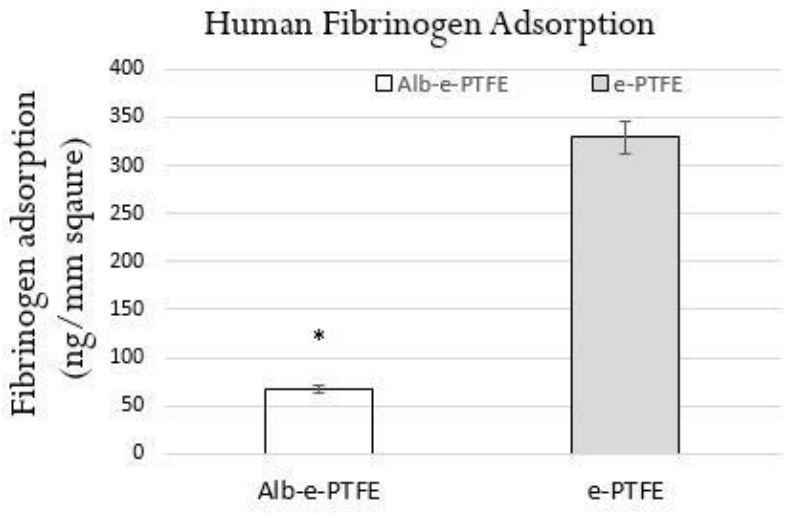


Figure 4.22: Measurement of adsorption of human fibrinogen on albumin coated ePTFE and bare ePTFE. Values represented as mean ± SD (error bar) with n=4 for each sample.

4.3.15 Platelet adhesion

As depicted in Figure 4. 23, albumin coated ePTFE (3.4±0.1%) allows significantly less adhesion of bovine platelets compared to bare ePTFE graft (12.1±0.7%).

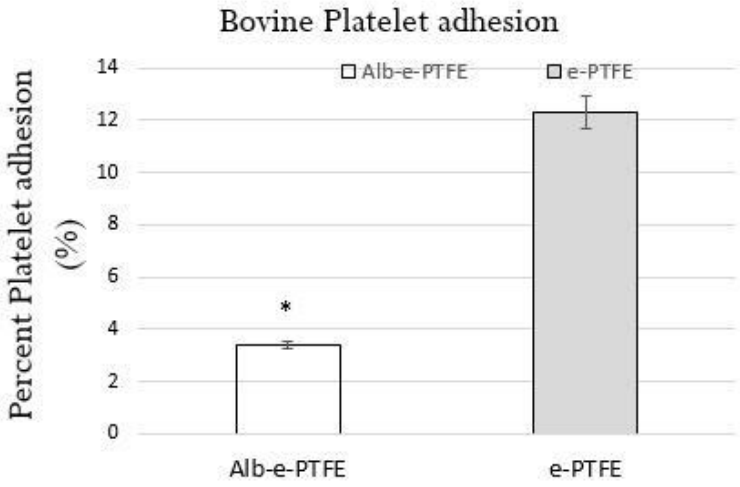


Figure 4.23: Measurement of adhesion of human platelets on albumin coated ePTFE and bare ePTFE. Values represented as mean ± SD (error bar) with n=4 for each sample.

4.4 Discussion

Designing surface modification strategies for mitigating the two major complications of vascular grafts, thrombosis and intimal hyperplasia, is an extensive area of research. High hydrophobicity, a major limitation of expanded-polytetrafluoroethylene (ePTFE), allows significant adsorption of adhesive proteins and subsequently platelets facilitating incomplete healing increasing the propensity of thrombosis and intimal hyperplasia [37-39].

HSA is the most abundant protein present in human blood and has been shown to passivate material surfaces, thus blocking sites for adsorption of other proteins and adhesion of platelets and cells by blocking surface active sites [22-29, 40-42]. Several techniques to fabricate an albumin film on the ePTFE surface have been attempted using cross-linking agents such as carbodiimide and glutaraldehyde [43-45]. However, none of the techniques has allowed long-term stability of

albumin on the surface to for prolonged protection from platelet adhesion and hyperplasia of smooth muscle cells.

In the proposed technology, we employed PGMA as an anchoring layer to covalently bind amine groups in albumin to the ePTFE surface. The glycidyl methacrylate units located in the "loops" and "tails" sections of the attached PGMA chain could serve as reactive sites for the subsequent attachment of (macro) molecules with complementary functional groups [30-32]. PGMA is also known for its high surface density and affinity for reacting with proteins [33].

Epoxy groups in PGMA act as reactive sites for attachment with functional groups on graft surfaces and for immobilization of non-thrombogenic coatings such as albumin. Structural stability of albumin has been shown to be critical to prevent adhesion of platelets. Lacking receptors for binding platelets, albumin has been shown to attract platelets beyond a critical degree of unfolding when it exposes its hydrophobic residues. Epoxy groups in PGMA render albumin in its native conformation, thus preserving its secondary structure conformation, as shown in our previous study [46].

Characterization of albumin coated ePTFE was performed using attenuated total reflection-fourier transform infrared spectroscopy (ATR-FTIR) for confirmation of binding of albumin to PGMA bound ePTFE, energy dispersive spectroscopy (EDX) for chemical composition analysis and scanning electron microscopy (SEM) for morphological analysis, contact angle analysis for estimation of surface energy changes.

Binding between PGMA and albumin on the ePTFE surface was assessed by ATR-FTIR analysis. Presence of a characteristic carbonyl group of PGMA (1723 cm^{-1}) and amide I (1655 cm^{-1}) and amide II (1541 cm^{-1}) bands of albumin confirms PGMA and albumin coating on the ePTFE surface. Secondary structure confirmation of albumin bound to PGMA was assessed by the wavenumbers of the amide I and amide II bands (1655 cm^{-1} and 1541 cm^{-1}, respectively) correspond to the alpha-

helical structural range, confirming the native secondary structure conformation of albumin on PGMA polymer [47].

The degradation temperature of ePTFE was assessed by thermo-gravimetric analysis to assess stability of the material at 150°C albumin coating annealing temperature. Insignificant mass loss of ePTFE at 150°C for 1 hour confirms its stability at the annealing conditions.

Antithrombogenic activity of albumin was assessed by measuring fibrinogen adsorption, adhesion force and human platelet adhesion on albumin coated ePTFE surfaces. Fibrinogen is known to be critical in attracting platelets and subsequently facilitating thrombus formation [48,49]. There was a significant decrease in the adsorption of fluorescently human fibrinogen on the albumin coated ePTFE surface under flow. Adhesion force between human fibrinogen and the albumin surface was significantly low as compared to bare ePTFE surface assessed by AFM colloidal probe microscopy. Albumin coated ePTFE surface significantly reduced the adhesion of platelets assessed by LDH activity for a period of 7 days under physiologically relevant low flow shear stress conditions of 0.5 dynes/cm^2. These results are promising as compared to attempts to bind albumin on Dacron surfaces using crosslinking agents, which have been able to provide only short-term stability and protection to platelets [43-45]. Hydrophilic surfaces have been shown to improve biocompatibility, providing platelet adhesion and activation [50]. With a contact angle of >125, ePTFE is highly hydrophobic. Albumin coating significantly enhances the surface wettability (θ=78±2.3) of the ePTFE vascular graft surface as assessed by contact angle analysis.

Vascular smooth muscle cells play a critical role in facilitating the progression of anastomotic hyperplasia. Smooth muscle cell proliferation, hypertrophy and expression of contractile expression marker smooth muscle alpha actin were studied to assess the potential of albumin film on mitigation of intimal hyperplasia. Injury due to a percutaneous coronary interventional procedure (PCI) causes endothelial denudation, exposing smooth muscle cells to growth factors, which promotes the

phenotypic shift in the morphology of smooth muscle cells from contractile to synthetic with increased proliferation and migration from medial layer to neo-intimal layer and increased extracellular matrix formation [55]. Phenotypic modulation has been considered to be a key factor responsible for progression of neo-intimal hyperplasia and restenosis. Hemodynamic factors such as wall shear stress and cyclic strain from blood flow modulate the phenotype and function of vascular smooth muscle cells [56]. Physiological levels of cyclic strain promote quiescent, contractile morphology of smooth muscle cells, [57] whereas high rates promote SMC proliferation and dedifferentiation [58-60]. Endothelial denudation or VSMC migration causes exposure of the latter to blood flow [56]. Shear stress is known to cause a direct effect on smooth muscle cell proliferation with physiological shear stress reducing proliferation and maintaining contractile morphology [61-62] and low shear stress conditions especially in small diameter blood vessels and grafts promoting smooth muscle cell proliferation and synthetic phenotype [63] and with up-regulation of growth factors such as PDGF [64]. In vivo and clinical studies have demonstrated the occurrence of thicker neo-intima under low wall shear stress [65-66].

In vitro systems have been designed to evaluate cellular response to altered shear stress. Shun et al. designed a parallel flow chamber designed to culture cells on a glass slide sandwiched between polycarbonate plates [67] with a small gap between the plates allowing circulation of cell culture medium. A cone and plate system with the cone rotating at an angular velocity is another system designed. For small angles, the shear rate (shear stress) is constant throughout the flow field [67]. However, none of the models has been able to concurrently provide the cyclic strain and flow shear stress forces to accurately assess the effect of smooth muscle cells for developing effective anti-restenotic therapies.

In this study, we employed a custom designed vascular simulator [68] to provide the physiologically relevant conditions of cyclic strain and low flow shear to assess the response of vascular smooth

muscle cells at the site of a bypass graft anastomosis. The simulator provided a parallel plate flow chamber with a computer-controlled vacuum pressure to strain a silicone membrane fixed on a stationary post to provide forces of cyclic strain and flow shear. Studies highlighting the effect of fluid shear and cyclic strain on endothelial cells have been shown [69]; little study has been given to the effect of the forces on smooth muscle cells. Acampora et. al. studied the combined effect of strain and fluid shear (4% and 0.5 dynes/ cm^2, respectively) and reported a 75% increase in smooth muscle cell proliferation and a 19% decrease in alpha-actin expression [82]. We studied the response of smooth muscle cells on albumin coated and uncoated ePTFE surfaces exposed to forces of cyclic strain and flow shear using the vascular simulator. Smooth muscle cells on albumin coated ePTFE showed a significant decrease in proliferation and a hypertrophic response as compared to bare ePTFE. A significant increase in the expression of smooth muscle alpha actin protein was measured in smooth muscle cells cultured on albumin coated ePTFE compared to bare ePTFE.

After intimal injury, cells such as platelets and inflammatory cells release growth factors and cytokines that cause accelerated migration and proliferation of VSMCs from media to intima and a phenotypic change from a contractile to a synthetic state [81]. In this study, a co-culture of platelets and smooth muscle cells was studied to assess the potential of albumin film to reduce intimal hyperplasia in the presence of growth factors released from platelets. Smooth muscle cells on albumin coated ePTFE conditioned with and without platelets and subjected to physiologically relevant mechanical forces of cyclic strain and flow shear for 7 days were found to have significantly less proliferation and increased expression of contractile marker smooth muscle alpha actin than uncoated ePTFE.

In vitro models employed to facilitate perfusion of blood through polymeric conduits have analyzed many physiological endpoint-associated with blood contacting surfaces. Slee et. al. designed an ex vivo chandler loop apparatus to assess blood and cell interactions to recCD47 bound polyurethane

tubing [68-69]. However, the major disadvantage of these systems have been the requirement to include air in the tube, which might cause leukocyte and platelet aggregation and protein denaturation, [70-72] interfering with physiological end points. Also, air remains at the highest point of the circuit, limiting the blood circulation rate [73]. The perfusion model used in this study to assess the response of fibrinogen adsorption and platelet adhesion to albumin-coated 2.4mm diameter and 10cm long ePTFE tubes allowed full contact with fluid. Adhesion of bovine platelets under perfusion flow on albumin coated ePTFE was lower than bare ePTFE grafts. This is consistent with the data obtained for adhesion of platelets under conditions of flow shear in an orbital shaker. These results are in agreement with low platelet adhesion measurements on albumin coated Dacron grafts gathered by Kottke-Marchantand collaborators in vitro [43]. While albumin has been shown to prevent adhesion of platelets, adhesive protein fibrinogen has been proved to play a key role in facilitating adhesion of platelets to biomaterial surfaces [74-77]. In our flow model, we observed significantly lower adsorption of fluorescently labeled fibrinogen on albumin coated ePTFE compared to uncoated ePTFE evidenced by fluorescence values obtained at 525nm excitation and 575nm emission wavelengths.

Adhesion strength of a vascular coating is crucial to provide long term blood-biocompatibility and graft patency. Albumin coating on Dacron grafts using glutaraldehyde as a cross-linking agent has shown short term stability for only 7-10 days [78-80]. We studied strong binding of albumin on ePTFE surface bound to a PGMA anchoring layer over a 45 day period in vitro under physiological conditions with no significant change in the albumin surface density with time. Binding strength of the albumin film was also assessed by application of longitudinal tensile force to the coated and bare ePTFE surface. There was no significant change in the adhesion strength of the ePTFE material after albumin coating. Confirmation of albumin film on the node-fibril structure of ePTFE was assessed by energy dispersive x-ray (EDX) spectroscopy for chemical analysis of nitrogen element in amine

groups of albumin after application of longitudinal tensile force. We showed that albumin on ePTFE surface can be coated without any change in mechanical properties or node-fibril microstructure.

4.5 Conclusions:

It is expected that the results of this study will be relevant in understanding albumin as a potential coating with strong binding strength to prevent the complications of thrombosis and intimal hyperplasia. Moreover, data from this research could be used by endovascular device manufacturers for development of biocompatible vascular grafts and the appropriate testing protocols for assessing their efficacy. Assessment of the effect of albumin film on mitigation of thrombogenicity and neointimal hyperplasia in an in vivo model would be the focus of future work.

4.6 Acknowledgements

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CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

A technique to create a human albumin film on metallic and polymeric materials was designed using poly-glycidyl methacrylate as an anchoring layer to mitigate the complications of thrombogenicity and intimal hyperplasia. Human albumin film was characterized by FT-IR spectroscopy, scanning electron microscopy, energy dispersive x-ray spectroscopy, atomic force microscopy and contact angle analysis. Potential of albumin film to mitigate thrombogenicity was assessed by measurement of human fibrinogen adsorption and human and bovine platelet adhesion. Response of vascular smooth muscle cells on albumin film was assessed by measurement of proliferation, morphology, cell aspect ratio and contractile protein marker smooth muscle alpha-actin. Several conclusions can be drawn from this doctoral research.

1. Human albumin film exhibits strong adhesion strength to metallic and polymeric substrates using PGMA as an anchoring layer.
2. Human albumin film shields the surface from adsorption of human fibrinogen which plays a key role in facilitating the adhesion and aggregation of platelets on a surface.
3. Human albumin film prevents the adhesion of human and bovine platelet adhesion on the surface thereby conferring anti-thrombogenicity to metallic and polymeric materials.

4. Vascular smooth muscle cells maintain a controlled proliferation profile, spindle shape morphology and increased expression of smooth muscle alpha-actin on albumin coated surfaces thereby mitigating complications of intimal hyperplasia.

Results how that human albumin film on metallic and polymeric surfaces can shield the adsorption and adhesion of mediators facilitating the formation of a thrombus and restenosis. This research, therefore, demonstrated the potential of a human albumin film on a PGMA anchoring layer to mitigate the complications of thrombogenicity and intimal hyperplasia.

RECOMMENDATIONS

1. **In vivo model**: An animal model could be the next step for this research to study the effect of albumin film to enhance hemocompatibility in terms of reducing adhesion of platelets and fibrin formation on the luminal surface of a synthetic graft. The animal model will simulate the exact flow shear conditions in a low diameter graft in the presence of growth factors and proteins present in the bloodstream. Parameters such as compliance mismatch, suturability and dilatation could be studied accurately in an animal model for any required improvements in the mechanical properties of the graft.

2. **Effect of inflammation**: Albumin film on stent and graft surface could be studied for its potential to reduce inflammation in terms of modulating the response of macrophages and complement system which cause adverse reactions post a stent implantation and a bypass surgery in terms of secretion of growth factors to facilitate proliferation of vascular smooth muscle cells.

3. **Efficacy to promote endothelial growth**: Endothelial healing of a vascular graft is essential to protect the luminal surface from platelet adhesion and hyperplasia of smooth muscle cells as endothelial cells release factors such as nitric oxide (NO), prostacyclin (PGI_2) and heparin sulfate to inhibit the growth of smooth muscle cells and prevent platelet activation and deposition for homeostasis and maintenance of vascular integrity.

4. **Drug delivery**: Release of drugs or growth factors for promoting endothelial cell healing or anti-platelet molecules could be facilitated using the human albumin film for further improving its potential to promote healing in a vessel or synthetic conduit post surgery.

5. **A potential coating for diverse applications**: Human albumin film could be used for other vascular or non-vascular applications such as pacemakers, heart valves, ventricular assist devices, catheters, guide wire for its efficacy to be biocompatible and blood-compatible and for coatings featuring slow degrading drug release profile such as orthopaedic implants, sutures and meshes.

6. **Anti-bacterial film**: Human albumin has been known to have anti-bacterial properties enhancing its applications in a diverse array of fields. To validate its efficacy as anti-bacterial film could be tested in in vitro and in vivo models.