Surface modification of poly (L-lactic acid) to improve endothelial cell-material interaction

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**SURFACE MODIFICATION OF POLY (L-LACTIC ACID) TO
IMPROVE ENDOTHELIAL CELL-MATERIAL
INTERACTION**

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

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INTERACTION**

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School of Materials Science and Engineering

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To my husband Gerry, my son Joshua,
my parents and parents in-law

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Table of Contents

Acknowledgements	i
Abstract.....	viii
Chapter 1 Introduction.....	1
1.1 Coronary heart disease and its treatments	1
1.2 Endothelialization of vascular prostheses	3
1.3 Surface modification of synthetic polymers.....	4
1.4 Motivations of the study	5
1.4.1 Surface modification of PLLA	5
1.4.2 EC-biomaterial interaction	6
1.4.3 Effect of seeding density	7
1.5 Objectives and scope of the study	8
1.6 Organization of the thesis.....	9
Chapter 2 Literature Review	10
2.1 Lactic/glycolic acid polymers	10
2.1.1 Physiochemical properties	10
2.1.2 Biomedical applications	13
2.2 Endothelial cells.....	16
2.2.1 Blood vessel structure	16
2.2.2 Functions of endothelial cells.....	17
2.2.3 EC-matrix interactions	19
2.2.4 Cell-cell interactions	23
2.3 Cell-biomaterial interactions.....	25

2.3.1	Protein adsorption	26
2.3.2	Temporal and spatial sequence of events in cell adhesion	28
2.3.3	Integrin-mediated cell adhesion strengthening	30
2.4	Surface modification for endothelialization.....	31
2.4.1	Topological modification	32
2.4.2	Chemical modification	32
2.4.3	Micro-/nano-patterning	34
2.5	Vaults	35
2.5.1	Vault composition	35
2.5.2	Vault structure	35
2.5.3	Assembly of recombinant vaults	37
2.5.4	Engineering recombinant vaults for biomedical applications	37
2.6	Summary	39
Chapter 3	Surface modification and characterization	40
3.1	Introduction.....	40
3.2	Materials and Methods.....	40
3.2.1	Materials.....	40
3.2.2	Surface modification	41
3.2.3	Surface characterization	44
3.2.4	Statistics	46
3.3	Results and Discussion.....	46
3.3.1	Effect of plasma treatment on the polymer	46
3.3.2	Characterization of surface modified PLLA	49
3.4	Summary	57

Chapter 4	Endothelial cell-biomaterial interaction	58
4.1	Introduction.....	58
4.2	Materials and methods	58
4.2.1	Cells, antibodies and chemical reagents.....	58
4.2.2	Cell adhesion and proliferation	59
4.2.3	Cell retention under shear stress	60
4.2.4	Immunofluorescent imaging	61
4.2.5	Scanning electron microscopy imaging	62
4.2.6	Flow cytometry assessment of vinculin expression	63
4.2.7	Statistic analysis	63
4.3	Results	64
4.3.1	Cell adhesion.....	64
4.3.2	Cell proliferation	67
4.3.3	Cell retention under shear stress	68
4.3.4	Cell adhesion strengthening mechanism	70
4.4	Discussion.....	76
4.4.1	Cell affinity of modified PLLA.....	76
4.4.2	Cell adhesion strengthening mechanism	79
4.5	Summary.....	85
Chapter 5	Seeding Density Matters.....	87
5.1	Introduction.....	87
5.2	Materials and Methods.....	89
5.2.1	Cell adhesion and proliferation	89
5.2.2	Mechanistic study on improved cell attachment in high-density seeding.....	89

5.3	Results and discussion	90
5.3.1	High seeding density masks the surface dependence of cell adhesion.....	90
5.3.2	The influence of surface chemistry on cell morphology, independent of cell density 93	
5.3.3	High seeding density masks surface dependence of cell proliferation.....	97
5.4	Summary	102
Chapter 6	Immobilization of recombinant vaults on solid substrates	103
6.1	Introduction.....	103
6.2	Materials and Methods.....	104
6.2.1	Materials.....	104
6.2.2	Construction of C-terminus modified recombinant plasmids	104
6.2.3	Expression, purification and characterization of recombinant vaults	105
6.2.4	Immobilization of vaults on solid substrates.....	106
6.2.5	Surface characterization of vaults immobilized substrates	108
6.2.6	Cell affinity of recombinant vaults immobilized substrates.....	109
6.3	Results and Discussion.....	109
6.3.1	Structural characterization of C-terminally tagged vaults.....	109
6.3.2	Immobilization of recombinant vaults on APTES-modified glass	113
6.3.3	Immobilization of recombinant vaults on APTES-modified PLLA	123
6.4	Summary	127
Chapter 7	Conclusions and Perspectives	129
7.1	Conclusions.....	129
7.2	Perspectives.....	131
Reference		133

Publications145

Appendix.....146

Abstract

Poly (L-lactic acid) (PLLA) is recognized as one of promising materials for cardiovascular applications, attributed to its mechanical and degradative properties which could match with human artery healing process. However, endothelialization on PLLA appears difficult due to its hydrophobicity and poor cell affinity.

In this work, a mild and easily-controlled surface modification method was developed to immobilize biomolecules (gelatin and chitosan) on PLLA, which is considered as a significant contribution. This three-step surface modification method involved argon (Ar) plasma treatment, acrylic acid grafting polymerization and covalent coupling reaction between amine groups and carboxylic groups. Surface characterization techniques, including X-ray photoelectron spectroscopy (XPS), contact angle measurement and colorimetric methods for surface density of functional groups, proved the feasibility and stability of this surface modification method. Immobilization of these biomolecules is found to increase the surface wettability and to alter the surface chemistry of PLLA.

EC-biomaterial interaction study demonstrated that gelatin and chitosan modified PLLA had better cell affinity and promoted endothelialization. Human umbilical vein endothelial cells (HUVEC) cultured on modified PLLA exhibited improved cell adhesion, proliferation and retention under shear stress. Further study on cell adhesion strengthening mechanism on various PLLA substrates revealed the positive correlation between the cell adhesion strength with cell spreading, focal adhesion size and number-per-cell, as well as fibronectin fibril formation. Surface physiochemical properties were found to modulate the cell adhesion strength through regulating cell-biomaterial interactions. There has been no previous report of a quantitative correlation of these parameters to adhesion strength.

This work pointed out the effect of seeding density on assessing cell-biomaterial interactions, as we demonstrated that high seeding density improved cell adhesion and masked differences in cellular response on different substrates. During the first 2 h incubation, low seeding density enabled us to observe surface chemistry-dependent cell adhesion behavior. It was shown that high seeding density masked differences amongst substrates, and enhanced cell adhesion on all substrates examined. Cell proliferation profile was found to be surface-dependent at low seeding density, whereas surface dependence disappeared at high seeding density.

Another biomolecular nanoparticle, self-assembling recombinant vault, was also successfully immobilized on a solid substrate (glass and PLLA) by either electrostatic adsorption or covalent bonding. Such immobilized vaults were found to retain their barrel-and-cap structure on solid substrates in dry conditions. The *in vitro* study on cell adhesion and proliferation demonstrated the cell affinity of these vaults immobilized substrates, though more optimization is required before the self-assembled vault protein can be made to enhance the desired cellular interactions. This work demonstrated the feasibility of immobilizing recombinant vault nanoparticles on a solid substrate and may open up future applications for selective cellular interactions as well as for localized delivery of bioactive agents.

Chapter 1 Introduction

1.1 Coronary heart disease and its treatments

Coronary heart disease (CHD) is myocardial infarction caused by lipid accumulation on vascular wall and the subsequent narrowing of the coronary arteries and the reducing of blood flow to heart muscles (Van der Zijpp, 2003). It is the main cause of death in most of the developed countries (Kedersha, 1986a). Two main surgical treatment options are available: Bypass surgery is performed in case of more than 70% occlusion; Percutaneous transluminal coronary angioplasty (PTCA) with or without stenting is used otherwise (de Mel, 2008).

PTCA, as illustrated in Figure 1-1 (A) was first developed to treat acute myocardial infarction to restore blood flow in late 1970s (Levy, 1979). A deflated balloon is inserted to the stenosis site in the coronary artery by a guiding wire from femoral artery. When the balloon is progressively inflated, the blood flow was restored by the plaque reduction and vessel lumen enlargement (Virmani, 1994). To address the complications associated with PTCA, such as arterial dissections, vessel recoil and intimal hyperplasia, stents, the expandable metallic mesh tubes made of 316L stainless steel or nitinol, are then introduced as mechanical support during PTCA, as illustrated in Figure 1-1 (B).

However, patients with stent have to be administered anti-platelet agents to overcome subacute stent thrombosis which would occur with 7 to 10 day as the early complication. (Padera, 2004). In addition, 50% of patients with stenting would experience the in-stent restenosis (ISR) within 6 months (Padera, 2004). The most successful approach to overcome ISR is drug-eluting stents (DES), which release single or multi drugs that inhibit smooth muscle cell proliferation and extracellular matrix synthesis (Sousa, 2003a, 2003b). However, application of DES is limited by the drug elution

duration, the inhibition or delaying of stent endothelialization and late thrombosis and restenosis (Pfisterer, 2006; Venkatraman, 2008).

Figure 1-1 (A) Coronary Balloon Angioplasty, and (B) Stent Placement ("How is coronary angioplasty done?," 2010).

Blood vessels undergo a healing process after stent insertion, effectively remodeling themselves. As such, it is not necessary for a permanent vascular prosthesis to be in place beyond this initial period. Its permanent presence in fact could have other negative problems (Colombo, 2000). Accordingly, fully biodegradable stents have become an active area of research as an alternative (Eberhart, 2003). Biodegradable polymeric stents could behave as the physical support only during vessel healing. The mechanical load could be gradually transferred to the tissue as the stent mass and strength decrease over time. Furthermore biodegradable stent could function as long-term drug delivery system to the vessel wall (Zilberman, 2006). The first biodegradable stent made of PLLA was developed by Stack and Clark from Duke University in 1980s (Stack, 1988). Later on, biodegradable polymeric stents made of lactic/glycolic acid polymers with diverse designs have been invented (Yamawaki, 1998; Tamai, 1999; Tamai, 2000; Venkatraman, 2006). Currently, only investigational biodegradable stents are presently

available. Similar to other stents, thrombosis and restenosis are the two major issues to be addressed for biodegradable stents.

For patients requiring bypass surgery, autologous vein or artery graft are their first choices. In the case of lack of suitable blood vessel due to previous usages, patients have to use synthetic grafts (Kannan, 2005). Despite their prevalence, these grafts offer low patency due to compliance mismatch, thrombogenic surfaces, and the tendency to form intimal hyperplasia. They are also limited to use in larger diameter artery applications (>6 to 8 mm) (de Mel, 2008).

1.2 Endothelialization of vascular prostheses

Endothelial cells (EC) form a monolayer layer, namely endothelium, which covers the entire vascular system. It acts as non-thrombogenic barrier to regulate local homeostasis and smooth muscle cell proliferation. Endothelial denudation due to balloon angioplasty and/or stent implantation was found to be the key mechanism associated with restenosis (Kipshidze, 2004). Hence, endothelialization of vascular prostheses is crucial and currently could be achieved by either *in vitro* endothelialization or *in situ* endothelialization.

In vitro endothelialization is an approach in which autologous EC are seeded on vascular stents/grafts before being implanted. The seeded cells are supposed to have physiological functions to inhibit thrombosis and restenosis (Thompson, 1992; Alobaid, 2005; Bhattacharya, 2005). Hence, “an anti-thrombogenic surface which is similar to that possessed by normal vessels” was produced (Dichek, 1989; Neal, 1995; Conklin, 2004; Bhattacharya, 2005). Another major application of EC seeding is on small-diameter prosthetic cardiovascular bypass grafts to avoid high occlusion rates (Van der Zijpp, 2003). In spite of the intensive efforts to seed such surfaces, cell retention after stent

deployment and blood flow exposure was found to be low (Neal, 1995), which suggests cell adhesion strength on implant surface determines the success of this approach.

In situ endothelialization is indeed the natural healing process of blood vessels, whereby the regeneration of the endothelial layer is derived from the migration of EC from adjacent tissue (“trans-mural endothelialization”) and/or attachment of the circulating endothelial progenitor cells (EPC) (Rotmans, 2005; Avci-Adali, 2008). However, available cell density in both situations is considered to be low (Hill, 2003; Q. Lin, 2010). Hence, materials that promote EC adhesion and proliferation would be highly desirable.

1.3 Surface modification of synthetic polymers

In order to achieve endothelialization on synthetic polymers, a broad spectrum of surface treatment methods was developed. The most successful approach is to modify substrate surface to mimic the physiological surroundings of EC, as EC are sensitive to physical and biochemical stimuli.

Most of synthetic polymers are lack of reactive functional groups on their surfaces. Thus, introducing chemically active functional groups on biomaterial surface becomes the first challenge. Plasma surface treatment is the most prevailing practice, since it is a relatively clean and simple method to provide surface modification within nanometer scale depth. The imparted functional groups could be varied by plasma gas selection (Goddard, 2007). For examples, oxygen plasma was utilized to introduce oxygen containing functional groups to polymer surfaces such as polyurethane (PU) (Inn-Kyu Kang, 1997), poly(methyl methacrylate) (PMMA) (I.-K. Kang, 1993) and poly(ethylene terephthalate) (PET) (Y. J. Kim, 2000). Ammonia plasma is used to introduce amine groups to the surface of polytetrafluoroethylene (PTFE) (Lu, 2001) and lactic/glycolic acid polymers (Chu, 1999). Surface activation enables the subsequent chemical reactions

which lead to covalent immobilization of bioactive macromolecules. They include 1) extracellular matrix materials, such as fibronectin (Lu, 2001; Pompe, 2007), collagen (S.-D. Lee, 1996; Yang, 2003; Cheng, 2004), gelatin (Zhu, Gao, He, , 2004) and laminin (Chandy, 2000); 2) adhesion polypeptides such as RGD (Quirk, Chan, , 2001; Tugulu, 2007) and YIGSR (Hersel, 2003; Jun, 2004). These modifications methods were reported to be successful in modifying polymeric materials and enhancing *in vitro* cell affinity with polymer substrates.

1.4 Motivations of the study

1.4.1 Surface modification of PLLA

1.4.1.1 Immobilization of model biomolecules

Lactic/glycolic acid polymers belong to the most important alpha polyester family. The attractive property of this family is degradation into non-toxic products by hydrolysis in physiological environment. They have been widely used in many biomedical applications, including orthopedic devices (Middleton, 2000), cardiovascular implants (Agrawal, 1992), sutures (Frazza, 1971) and drug-delivering systems (Langer, 1990). PLLA has high mechanical strength and tailored degradation rate and was considered as one of promising vascular materials (Venkatraman, 2003; Venkatraman, 2006). However, PLLA has poor EC affinity due to its hydrophobicity (Kohn, 2004). Although some surface modification methods for PLLA were explored using aminolysis and NaOH etching (Zhu, 2003; Zhu, Gao, He, , 2004; Zhu, Gao, Liu, He, , 2004), those wet chemical reactions is harsh and difficult to control due to their dependence on reaction temperature and duration (Zhu, 2002). Plasma treatment provides a controllable, flexible and uniform surface modification within nanoscale depth, but it is generally confined to non-degradable synthetic polymers modification. Clearly, there is a need to develop a mild

and effective surface modification method for the purpose of promoting endothelialization on biodegradable polymer surfaces.

1.4.1.2 Immobilization of recombinant vaults particles

Vaults are 13 MDa ribonucleoprotein particles, which are highly conserved and distributed in most eukaryotes (Kedersha, 1986b). The recombinant vault particle has a capsule-like structure with a thin shell (ca. 2 nm) surrounding a large hollow interior compartment with a volume of $5 \times 10^4 \text{ nm}^3$, which is capable of accommodating hundreds of proteins (Mikyas, 2004). In addition, the recombinant vault particles are self-assembled. These attributes make vaults very promising vehicles for delivery of therapeutic agents. However, all of the previously reported work to date, which explored the possibility of vaults as a drug/protein delivery system, has used vaults in aqueous solutions. Under these conditions, generally the release of drug/peptide is not controlled or sustained to any great extent. In order to control the release of encapsulated bioactivities, vaults may need to be incorporated into solid matrices or onto substrates.

Moreover, short cell adhesion ligands, such as RGD, could be fused on to C-terminus of recombinant vaults. Once recombinant vaults particles are immobilized on substrate, these cell adhesion ligands may promote cell adhesion. These immobilized particles could also behave as localized delivery system to release bioactive proteins, such as vascular endothelial cell growth factor (VEGF) to promote cell proliferation. To this end, we attempt to express recombinant vaults with RGD C-terminus tag, and to immobilize them on solid substrate with hope of improving endothelial cell adhesion and proliferation.

1.4.2 EC-biomaterial interaction

Cell-biomaterial interaction is a loosely-defined term used for referring to cellular response to biomaterials *in vitro* and *in vivo*. It involves a complex interplay of various

cell activities, including adhesion, proliferation and migration. In the past few decades, a better understanding of cell-biomaterial interaction was achieved from two different approaches. One approach analyzes cellular behaviors from the molecular biology point of view. It has been recognized that the cell-biomaterial interaction starts from cell adhesion which is mediated by the binding of membrane receptor proteins (mainly from the integrin superfamily) with cell adhesion ligands present on the surface (Hynes, 2002). Focal adhesion (FA) and fibrillar adhesion (FB) are formed later on to sense their immediate environment and to remodel their extracellular matrix (Geiger, 2001). The other approach focuses on how biomaterial properties such as surface chemistry (Faucheux, 2006), wettability (Tzoneva, 2007), roughness (Lampin, 1997) and rigidity (Choquet, 1997) influenced cellular behaviors.

After modifying the PLLA surface, the EC-biomaterial interaction arising from the surface modification has to be investigated. In addition, the ability to retain endothelium on biomaterial is essential for blood-contacting devices which are constantly subjected to shear flow. Cell adhesion strength has to be investigated for the purpose of cardiovascular applications.

1.4.3 Effect of seeding density

A wide range of cell seeding densities from 4×10^3 to 2×10^5 cells/cm² have been used for *in vitro* studies on EC-biomaterial interaction (Lu, 2001; Gumpenberger, 2003; Yang, 2003; Miller, 2004; Crombez, 2005; Boura, 2006; Bérard, 2009; Z. G. Chen, 2010). To date, there has been no established standard protocol to define the seeding density, which makes it difficult to compare different studies done on the same substrate. The biocompatibility of certain materials and protocols evaluated using relatively high seeding densities, might not be appropriate for applications whereby the availability of cells is limited. For instance, in the case of *in situ* endothelialization, the density of the

circulating EPC and adjacent EC are considered to be low. Stimuli from neighboring cells via interaction of cell-surface receptors and secreted growth factors/cytokines are strongly dependent on the cell density. When the cell density is low, direct cell-cell contacts are limited and cell-biomaterial interaction is expected to be pre-dominantly influenced by cell-substrate contact. As cell density increases, cell-cell interaction becomes more extensive and is expected to profoundly influence cellular behaviors (Nagahara, 1996). Hence, there is a need to study the effect of seeding density on assessing EC-biomaterial interactions, in order to correctly predict the biocompatibility of biomaterials.

1.5 Objectives and scope of the study

The key objectives of this work were:

1. To develop and characterize feasible methods to modify PLLA surface in order to enhance endothelialization, including the immobilization of recombinant vaults particles.
2. To understand the EC-biomaterial interactions arising from the surface modification, in particular on cell adhesion strength under hydrodynamic flow.
3. To study the effect of seeding density on assessing EC-biomaterial interactions.

In order to achieve the above objectives, the following tasks were carried out.

- 1) A three-step surface modification method was developed to modify PLLA surface with biomolecules such as gelatin and chitosan.
- 2) Surface characterization techniques, including scanning electronic microscopy (SEM), water contact angle, X-ray photoelectron spectroscopy (XPS), colorimetric methods for surface density quantification of carboxylic groups and biomolecules were employed to analyze the surface physiochemical properties.

- 3) Human umbilical vein endothelial cells (HUVEC) were selected for *in vitro* study.
The EC-biomaterial interaction was studied according to four criteria for a successful endothelialization on materials: (a) the expression of cell phenotype with regards to different modified surface; (b) the ability of HUVEC to adhesion and proliferation onto biomaterials; (c) the retention of HUVEC under shear flow; (d) the formation of focal adhesion and fibrillar fibronectin.
- 4) The effect of initial seeding density on assessing EC-biomaterial interaction was studied by seeding HUVEC on PLLA substrates at two different seeding densities, a low seeding density of 5000 cells/cm² and a high seeding density of 50,000 cells/cm². EC-biomaterial interaction was compared in terms of cell adhesion, morphology and proliferation up to 7 days.
- 5) Immobilization of vaults was achieved through both electrostatic adsorption and covalent coupling reaction on solid substrates (glass and PLLA), and was further characterized using XPS, attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) and atomic force microscopy (AFM). In order to induce better EC affinity, RGD tags were fused to C-terminus of recombinant vaults particles for the first time. EC affinity of those vaults coated substrates was also assessed in terms of adhesion, proliferation and morphology.

1.6 Organization of the thesis

This thesis contains seven chapters. Chapter 1 is Introduction. Literature review is summarized in Chapter 2. Surface modification method and surface characterization is reported in chapter 3. Investigation of EC-biomaterial interactions arising from different surface modification of PLLA is described in Chapter 4. The effect of seeding density on assessing EC-biomaterial interaction is described in Chapter 5. Surface immobilization and characterization of recombinant vaults on solid substrates is presented in Chapter 6. Future perspectives are recommended in Chapter 7.

Chapter 2 Literature Review

Lactic/glycolic acid polymers and their copolymers are widely used in biomedical applications attributed to their biocompatibility and biodegradability. Their physiochemical properties and biomedical applications are reviewed in section 2.1. In the view of their cardiovascular applications, the physiological functions of endothelial cells (EC), the interaction of EC with their extracellular matrix and cell-cell interaction will be of considerable relevance; hence these aspects are reviewed in section 2.2. The cascade of events of cell adhesion to a foreign substrate, involving protein absorption, focal and fibrillar adhesion formation as well as cell adhesion strengthening, are discussed in section 2.3. Surface modification techniques employed in the field of biomedical materials have a crucial role in enhancing endothelialization on biomaterials, and as such are reviewed in section 2.4. An introduction of vaults is given in section 2.5 including their composition, structure and self-assembly.

2.1 Lactic/glycolic acid polymers

2.1.1 Physiochemical properties

Lactic/glycolic acid polymers are homo- and copolymers of lactic acid (LA) and glycolic acid (GA) (X. S. Wu, 1995). They are classified as, aliphatic polyester (Pitt, 1981; Dahlmann, 1990), or poly(α -hydroxy acid) (Pfisterer, 2006). Initial research mainly focused on oligomers from direct condensation of LA and GA, until Du Pont scientists found a way to achieve high molecular weights by ring opening polymerization in 1954 (X. S. Wu, 1995). The polymerization reactions are shown in Figure 2-1.

Figure 2-1. Lactic/glycolic acid polymerization by ring opening reaction.

Poly (glycolic acid) (PGA) is the simplest linear polyester. Due to its high degree of stereoregularity, the crystallinity of PGA could reach up to 55%. Its glass transition temperature (T_g) is ranged from 35°C to 40°C, and a melting temperature (T_m) from 225°C and 230°C. The high degree of crystallinity also results in high modulus and tensile strength (Middleton, 1998). LA is a chiral monomer containing a carbon with four different substituents. Hence, two optical isomers are present, namely L-LA and D-LA, from which three types of polymers (PLA, PLLA, PDLA) are generated. The crystallinity of PLLA could reach about 37%. Its T_g ranges from 50°C to 80°C and its T_m is up to 178°C. A polymerization of the racemic mixture produces PDLA, which is amorphous due to the irregular structure. Copolymers with different LA/GA ratio are commonly desired for their intermediate mechanical and degradation characteristics as listed in Table 2-1.

Table 2-1. Properties of common biodegradable polymers. ^aTime to complete mass loss depending on part geometry (Middleton, 1998).

Polymer	T_g (°C)	T_m (°C)	Tensile Modulus (GPa)	Degradation Time (months) ^a
PGA	35-40	225-230	7.0	6 - 12
PLLA	60-65	173-178	2.7	> 24
PDLLA	55-60	Amorphous	1.9	12 - 16
PLGA(85/15)	50-55	Amorphous	2.0	5 - 6
PLGA(75/25)	50-55	Amorphous	2.0	4 - 5
PLGA(65/35)	45-50	Amorphous	2.0	3 - 4
PLGA(50/50)	45-50	Amorphous	2.0	1 - 2

The most attractive property of LA and GA polymer is its degradation into nontoxic products by hydrolysis in physiological environment, which enables their clinical applications.

Biodegradable polymers can be categorized into two groups on the basis of biodegradation mechanisms, namely, surface (heterogeneous) degradation and bulk (homogenous) degradation (Kohn, 2004). Water penetration rate is an important factor in discerning the degradative mechanism as the presence of water promotes the process. In bulk degradation, water penetration rate into the matrix is faster than polymer degradation rate, causing uniform degradation throughout the polymer. Conversely in surface degradation, water penetration is slower; hence the degradative process occurs principally on the surface of the polymer. The LA/GA polymers undergo bulk degradation, during which not only is there degradation at the surface of a device, but the polymer matrix also swells and degrades simultaneously. Proposed four-step hydrolysis process is shown in Figure 2-2 (A) (X. S. Wu, 1995).

The degradation of LA/GA polymers *in vitro* and *in vivo* is through random hydrolysis. The degradation pathways are illustrated in Figure 2-2 (B). The final degradation product of PLA is lactic acid which is a normal product of muscular contraction in animals and humans (Agrawal, 1995). LA could be excreted as water and carbon dioxide through tricarboxylic acid cycle (Wu, 1996). PGA produces glycolic acid by degradation process which could be excreted either in urine or as water and carbon dioxide through tricarboxylic acid cycle (Agrawal, 1995).

Figure 2-2. (A) Degradation mechanism of lactic/glycolic acid polymers in four-step scheme (X. S. Wu, 1995); (B) Degradation pathway of PGA and PLA in the physiological environment (Agrawal, 1995).

2.1.2 Biomedical applications

As reviewed in previous section, a wide range of mechanical and degradative properties is offered by lactic/glycolic acid polymers through manipulating through their ratios of monomers, molecular weights and processing parameters. It makes LA/GA polymers ideal candidates for biomedical applications. Their biomedical applications are reviewed in the five following fields: the temporary support device, the temporary barrier, the drug delivery device, the tissue engineering scaffold and the multifunctional implant (Kohn, 2004).

Temporary support devices are commonly used in circumstances whereby the implants are initially required to provide mechanical supports. Then they are gradually degraded and weakened when the natural tissue heals. Some of these examples are sutures used in surgery wounds, bone fixations in bone fractures, cardiovascular stent in damaged

blood vessel. Hence, matching the degradation rate of these temporary implants with the healing rate of the natural tissues is crucial in the device design process (Kohn, 2004).

Suture is the first and most successful example of biodegradable synthetic polymers used in surgery. The first totally absorbable suture was made of PGA under the trade name “Dexon” in 1970 (Frazza, 1971). Later on LA is copolymerized into PGA to slow down the degradation rate. For example, the widely used “Vicryl” suture is made of a 90:10 copolymer of GA/LA (Kohn, 2004).

Orthopedic fixation devices, such as fracture fixation, interference screws, suture anchors, craniomaillofacial fixation and meniscus repair, are the secondary major applications for biodegradable polymers. Biodegradable polymers offers several advantages over traditional metallic devices through (1) eliminating the subsequent removal surgery, (2) avoiding stress shielding of recovering tissues and (3) offering tremendous potential as drug delivery system to speed the healing process (Middleton, 2000). Orthopedic fixation requires polymers of exceptionally high mechanical strength and stiffness, hence semicrystalline PGA and PLLA are usually used in fixation applications. Commercial examples are self-reinforced PLLA (SR-PLLA) fracture fixation devices under the trade name of “SmartPins” and SmartScrew” from Bionx Implants (Ambrose, 2004). According to Frost and Sullivan Report, the sale of bioabsorbable suture made up over 95% of \$300 million revenues from absorbable polymer products in 1995 (Middleton, 2000).

Biodegradable stents for implantation into coronary arteries are currently being investigated (Colombo, 2000). They are designed to mechanically prevent the collapse and restenosis of arteries that have been opened by balloon angioplasty and eventually disappear through degradation after vascular healing. Currently, only investigational devices are available. One of them is Igaki-Tamai stent made of PLLA monofilament and

designed as a zigzag helical coil (Tamai, 1999). The 6-month human clinical test suggested that this PLLA stent was feasible, safe, and effective in humans, although the long-term efficacy was yet to be investigated (Tamai, 2000). Venkatraman *et al.* also reported one helicoidal bi-layered stent made of PLLA and PLGA. This bi-layered stent could self-expand at body temperature due to polymer elastic memory property (Venkatraman, 2006).

The idea of stenting then extends to other applications. A biodegradable self-reinforced stent made of PLLA was reported to use in 16 male rabbits after urethrotomy, and was considered as a promising material for a urethral stent to prevent urethral restenosis (Kemppainen, 1993). Fry and Fleischer used a PLLA stent for benign esophageal strictures (Fry, 1997; S.-G. Kang, 2010).

A drug delivery device is a temporary implant, as the matrix is not desirable when the drug run out or the disease is treated. Hence, degradable polymers, especially LA/GA polymers based on their extensive safety profile used as sutures, become widely investigated for the development of implantable drug delivery systems (Langer, 1990). Amorphous polymers PLGA gain popularity when considering for drug delivery application, as a homogeneous dispersion of the drugs within the matrix is important. Several implantable and controlled drug delivery products made of PLGA have already become commercially available. One successful example is goserelin acetate implants under the trade name of Zoladex, in which PLGA is used as drug loading matrix to release *gonadotropin releasing hormone super-agonist* (GnRH agonist) in the treatment of breast and prostate cancer.

A temporary barrier has its major medical use to prevent surgical adhesion between two tissue sections by blood clotting (Kohn, 2004). Limited success was reported

to use PLGA nonwoven nanofibrous membranes for prevention of postsurgery-induced abdominal adhesion (Zong, 2004).

Tissue engineering scaffold describes a degradable artificial implant which is able to induce cell proliferation and tissue regeneration (Kohn, 2004). One interesting example could be biodegradable heart patch which is capable to restore heart functions after myocardial infarction. PLGA-collagen micro sponge patch was reported to show acceptable *in situ* cellularization in a dog model for the pulmonary artery reconstruction (Iwai, 2004).

Multifunctional devices, as the name implies, combine several of the functions mentioned above within one single device. For examples, biodegradable bone nails and screws made of ultrahigh strength PLLA is capable of both mechanically holding the fractured bones and also capable of slowly releasing bone growth factors to stimulate the new bone tissue growth during its degradation process (Kohn, 2004). Drug-eluting biodegradable stent is another good illustration of combining a mechanical support function with *in situ* drug delivery. In addition, stents could be surface treated to facilitate endothelialization, behaving as tissue engineering scaffold in some extent.

2.2 Endothelial cells

2.2.1 Blood vessel structure

The large blood vessels, such as arteries and veins, consist of layers of thick and tough wall of connective tissue and smooth muscle cells (SMC), as illustrated in Figure 2-3 A (Alberts, 2002). A monolayer of endothelial cells (EC), known as the endothelium, lines along the inner lumen of the wall, and is separated from the surrounding outer layers by a basal lamina. The thickness of the blood vessel varies depending on the location and function of the vessel, whereas the endothelium lines the entire circulating system. The basal lamina is a thin mat with 40-120 nm in thickness, and mainly consists of mesh-like

type IV collagen, laminin, and heparin sulfate proteoglycan as illustrated in Figure 2-3 B (Alberts, 2002). The basal lamina supports EC adhesion, proliferation and migration, also provides a platform whereby cells can interact with each other (Alberts, 2002).

Figure 2-3. (A) The structure of blood vessel, and (B) A model of the molecular structure of a basal lamina (Alberts, 2002).

2.2.2 Functions of endothelial cells

Endothelium lines the entire vascular system and participates in various physiological (Michiels, 2003) and pathological processes (Garg, 1989; Kipshidze, 2004). EC function as “gatekeepers”. They separate blood from the underlying tissues and control the transfer of blood proteins and permeability of white blood cells (Dejana, 2004). EC have mechanoreceptors that allow them to sense the hemodynamic forces and regulate the vascular tone by a diverse array of biochemical responses (Michiels, 2003). One profound discovery was the relaxation of vascular SMC by endothelium-derived relaxing factors known as the free radical gas nitric oxide (NO). NO was later exploited in the treatment of acute angina (Michiels, 2003). The growth-inhibitory effect of NO was capable of maintaining the mitogenic quiescence of SMC (Garg, 1989). In addition, EC

also regulate SMC proliferation by synthesizing several growth factors, including fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) (Kipshidze, 2004).

Under normal condition, EC provide a non-thrombogenic surface to resist platelet adhesion and coagulation through producing vasoprotective and anti-thrombotic molecules. Prostacyclin (PGI₂) and NO are two important molecules in suppressing platelet activation. Thrombomodulin, heparin-like molecules and protein S are the anti-coagulant molecules expressed on EC surface (Wu, 1996). In the case of injury to endothelium and the accompanied EC loss, the haemostatic balance is tilted toward thrombosis by loss of protective molecules and expression of adhesive ones (Wu, 1996). The increased pro-coagulant activities promotes thrombosis, SMC migration and proliferation (Lowe, 2002).

It was found that endothelial denudation is one of the most important mechanisms associated with restenosis after balloon angioplasty with/without stenting (Kipshidze, 2004). The longer it takes to re-endothelialization the longer the patient is on anti-thrombotic drugs, which are very expensive and have side effects. Many approaches have been attempted to promote endothelialization following coronary angioplasty, including low-power laser irradiation (Basford, 1995; Kipshidze, 1998), and local delivery of growth factors (van Belle, 1997; van der Zee, 1997). Another interesting concept is endothelialization of vascular stent, also known as “EC seeding” (Dichek, 1989; Thompson, 1992; Conte, 1994). In this approach, EC are seeded and allowed to grow into a confluent monolayer on vascular stent before being implanted. It was initially investigated on bare or polymeric coated metallic stents during or after coronary intervention. However, the major limitation is marginal cell retention after subjected to blood flow. This problem remains with biodegradable stent made of LA/GA polymers. It

is thus important to modify these polymer surfaces and to study EC responses to these modified polymers so as to promote endothelialization.

2.2.3 EC-matrix interactions

Extracellular matrix (ECM) is described as “the glue holding EC together”, providing texture, strength and integrity to the tissue. In addition, ECM transmits environmental signals to cells and influences cell migration, differentiation, and death (Geiger, 2001). It is well established that EC-matrix interactions are mediated by the integrin superfamily through integrin-based adhesion complexes (Geiger, 2009). Due to the bi-directional nature of integrins, EC-matrix interactions are bi-directional. EC behaviors are regulated by ECM, while EC are able to remodel ECM components by protein synthesis or breakdown (Sottile, 2002).

2.2.3.1 Integrin-mediated cell-matrix adhesion

Integrin receptor superfamily have become the best-understood transmembrane receptors for cell adhesion to ECM proteins since their first recognition in 1980s (Hynes, 1987). Integrins are heterodimers consisting of α and β subunits. Each subunit crosses the cell membrane once: the majority of each subunit is located in the extracellular space with two short cytoplasmic domains (20-50 amino acids) (Hynes, 2002). As shown in Figures 2-4, there are totally 8 β subunits and 18 α subunits to form 24 distinct integrin types (Hynes, 2002). As connections between ECM and cytoskeleton, integrins activate many intracellular signaling pathways which are crucial in development, immune response, leukocyte traffic, homeostasis and tumor growth (Hynes, 2002).

Integrins are bi-directional signaling machineries which transmit mechanochemical information across the cell membrane in two directions. Via outside-in signaling pathway, adherent cells sense their growing environment and regulate intracellular activities which are important in cell spreading and cell migration (Berrier,

2007). Conversely, via inside-out signaling pathway, cells are able to induce integrin conformation and to alter ligand-binding activity (Ginsberg, 2005; Luo, 2007).

Figure 2-4. The integrin receptor family. They are classified into several subfamilies based on evolutionary relationship and ligand specificity. The subunit β_2 and β_7 integrins are expressed only on leukocytes (Hynes, 2002).

Cell-matrix adhesion structure consists of more than 50 cytoplasmic proteins (Lo, 2006), and classified into three basic categories according to Berrier *et al.*: (1) integrin-binding proteins, (2) adaptors and/or scaffolding proteins that lack intrinsic enzymatic activity, and (3) enzymes (Berrier, 2007). Talin is a structural adhesion protein that directly binds to integrin cytoplasmic domains; it regulates integrin activation and signaling (Calderwood, 2004). Vinculin and α -actinin, acting as “adaptors”, link cytoskeletal stress fibers (actin filaments) with integrin-associated proteins. Focal adhesion kinases (FAK) is one examples of enzymes, acting as signaling proteins, to regulate adhesion protein recruitment (S. Li, 2005).

On 2-D matrix, two main classes of matrix adhesion are focal adhesions (FA, also termed as focal contact) and fibrillar adhesions (FB, also termed as ECM contact). Though integrins and actin are commonly present in both classes of adhesion, FA is markedly different from FB as depicted in Figure 2-5. In terms of composition, FA

contains relatively high levels of $\alpha_v\beta_3$ integrin, paxillin and vinculin, and only modest levels of $\alpha_5\beta_1$ integrin and tensin. In contrast, only $\alpha_5\beta_1$ integrins are found in FB in addition to the characteristically enriched tensin. In terms of cellular location and shape, FA usually binds to fibronectin-free ECM and is found about 10-20 nm located at the ends of F-actin at the cell periphery, whereas FB is commonly found ≤ 100 nm associated with fibronectin fibrils close to the cell centre (Zamir, 2000). In terms of their functions, FA is considered as the major site of integrin signaling due to its high level of tyrosine phosphorylation (Zamir, 1999). In contrast, FB is believed to participate binding to fibronectin fibrils and fibronectin fibrillogenesis through its $\alpha_5\beta_1$ integrins (Zamir, 2000).

Figure 2-5. Comparison of focal adhesion and fibrillar adhesion (Berrier, 2007).

2.2.3.2 Remodeling of ECM proteins

ECM remodeling is important for numerous different processes in the adults, including neovascularization and repair processes (Sottile, 2002). Two distinguished pathways are present: the synthesis of ECM components and the catabolism of ECM structures (Streuli, 1999). The balance between both pathways is controlled by ECM feedback, and regulates many normal and pathological processes such as homeostasis, neovascularization, wound healing and tumor growth (Sottile, 2002). Fibronectin (FN), having a high molecular mass of 450-500 kDa, is one abundant and ubiquitous glycoprotein, either distributed in a soluble form in plasma and most body fluids, or

organized into fibrillar network through direct interaction with cell receptors (Mao, 2005). During ECM remodeling, FN is deposited and polymerized into fibrils which was reported to strengthen the cell-material adhesions (Sottile, 2002). In the view of project scope, FN fibrillogenesis, the synthesis and deposition of FN is reviewed. ECM breakdown/destruction could be found in recent review publications (Birkedal-Hansen, 1993; Davis, 2005).

FN fibrillogenesis consists of three major steps as depicted in Figure 2-6 (Mao, 2005). The first step involves binding of compact soluble FN to cell surface at the FA sites, which is mainly mediated by $\alpha_5\beta_1$ integrins (Geiger, 2001). A critical event in this first step is to expose the cryptic self-association sites of FN so that to initiate the subsequent FN polymerization. The FN conformation change induced by binding to integrins is believed to be one possible mechanism for exposing FN polymerization sites (Schwarzbauer, 1999). Another requirement for FN unfolding is the static tension generated at FA driven by contraction of actin stress fibers (C. Wu, 1995). Furthermore, the translocation of ligand-bound $\alpha_5\beta_1$ integrins from FA to FB discovered by Pankov was believed to provide the dynamic tensile force to stretch FN and the subsequent fibrillogenesis (Pankov, 2000). Thus the synthesis and elongation of FN fibrils is associated with the translation of $\alpha_5\beta_1$ which leads to the segregation of FB from FA (Zamir, 2000; Geiger, 2001). The FN fibrils are usually observed through FN immunofluorescent imaging and characterized as fibrous FN positive stains (Ilic, 2004).

Figure 2-6. Major steps involves in FN fibrillogenesis. (A) Compact soluble FN binds to $\alpha_5\beta_1$ integrin via its cell binding domains. (B) FN conformational changes are induced by binding to integrins and the contraction of actin stress fibers. (C) the formation of FN fibrils is associated with the translocation of $\alpha_5\beta_1$ and FN-FN interactions (Mao, 2005).

2.2.4 Cell-cell interactions

The endothelium separates blood from underlying tissues and controls the infiltration of blood proteins and cells into the vessel wall by the coordinating the opening and closure of cell-cell junctions (Dejana, 2004). Cell-cell junctions also functions as signaling machinery that communicate cell position as well as regulate cell growth and apoptosis. Disruption of the junctional organization might lead to the breakdown of homeostasis balance and the remodeling of blood vessel (Dejana, 2004). On the basis of morphological and functional characteristics, endothelial cell-cell junctions are classified into four types (Dejana, 1995).

Tight junctions (TJ). TJ regulate paracellular permeability and maintain cell polarity through forming a very close contact between neighboring cells (Bazzoni, 2004). Figure 2-7A is the schematic representation of TJ formed by transmembrane proteins, including occludin, claudins, and small junctional immunoglobulins (SJI; e.g. JAM-A).

Their cytoplasmic tails bind various cytoplasmic and cytoskeletal proteins, which in turn are involved in many cellular functions such as signaling, transcriptional regulation, and membrane trafficking (Bazzoni, 2004).

Adherent junctions (AJ). The cadherin family is involved in AJ formation (Bazzoni, 2004). Vascular endothelial (VE)-cadherin is uniquely expressed in EC and is considered as the “signature” of EC (Dejana, 1995). Figure 2-7B shows the molecular organization of adherens junction. VE-cadherin, through its cytoplasmic tail, binds β -catenin and plakoglobin, which in turn link α -catenin and actin microfilaments (Bazzoni, 2004). This association of VE-cadherin with actin microfilaments creates a lateral tension, which acts as an opposition to the forces generated by cell contact with the substratum and thus counteracts the cell spreading and motility (Underwood, 2002). Thus the strength of AJ may control the cell shape and the cytoskeletal organization in the establishment of vascular endothelial integrity, and may also influence cell growth and differentiation (Dejana, 1995).

Figure 2-7. Molecular organization of (A) tight junctions and (B) adherens junctions (Bazzoni, 2004).

Gap junctions (GJ). GJ form hydrophilic channels through which ions and small molecules are exchanged directly between adjacent cells (Dora, 2001). GJ are important in EC for the establishment of homotypic (EC to EC) or heterotypic (EC-SMC, EC-macrophages) communications (Polacek, 1993).

Syndesmos or complexus adherents. These type intercellular junctions contain the transmembrane protein desmoplakin and usually are distributed with the cadherins in the adherent junctions (Dejana, 1996).

2.3 Cell-biomaterial interactions

In this section, cell-biomaterial interactions studied *in vitro* environment are reviewed. Protein adsorption on implants or scaffold always precedes cell recruitment. Surface physical and chemical properties affect protein adsorption, which in turn mediates cell adhesion. For adherent cells like EC, cell adhesion to solid substrate is known as a dynamic process. A diverse array of cell-matrix adhesion proteins that interplay with each other in a “spatially and temporally regulated manner” (Cohen, 2004). Cell adhesion regulates the subsequent cell spreading and cytoskeletal reorganization. During cell spreading and morphological development, adhesion strength between cell and substrate increases. Meanwhile, cells migrate, proliferate and interact with neighboring cells. Eventually a monolayer of EC covers the entire biomaterial surface, a process referred to as “endothelialization” (Figure 2-8).

Figure 2-8. The progressive process of endothelialization on biomaterial surface.

2.3.1 Protein adsorption

Proteins are omnipresent in body fluids and in culture media. Protein adsorption onto implant surfaces always occurs before cellular adhesion hence is an important issue when considering cell-surface interaction. As illustrated in Figure 2-9, water molecules are the first molecules to reach the implant surface and form a shell which affect the subsequent protein adsorption. Depending on the surface wettability and polarity, protein adsorbed is in either native or denatured confirmation. That implies that the surface properties determine the characteristics of protein adsorption, which in turn mediates the cell-surface interaction when cells reach the surface (Kasemo, 2002).

The hydrophobic effect and the electrostatic effect are the two main thermodynamic interactions between a protein and a substrate upon which it adsorbs. Hydrophobic effect takes place when the hydrophobic surfaces adsorb hydrophobic amino acid residues on the protein to avoid interaction with water. Electrostatic effect results from the interaction between a protein and a surface carrying opposite charges. (Elbert, 1996).

Thus, protein adsorption may induce protein conformational change to minimize the overall energy. Based on this principle, hydrophilic surfaces were designed to reduce platelet and fibronectin absorption for non-thrombogenic vascular applications (Desai, 1991; Jeon & Andrade, 1991; Jeon, Lee, , 1991; Francois, 1996).

Figure 2-9. Protein adsorption influences the subsequent cell adhesion (Kasemo, 2002).

In cell adhesion studies under serum-presenting condition, the role of pre-adsorbed proteins must be considered. The interaction of serum proteins with the polymer surface was demonstrated to consist of consecutive adsorption and displacement of plasma proteins (Vroman, 1986). The *in vitro* studies indicated that the poor EC adhesion to serum pretreated PET and fluoroethylenepropylene copolymer appeared to correlate with poor displacement of adsorbed serum proteins (Kirkpatrick, 1994). Recently, using self assembled monolayers (SAMs) presenting well-defined surface chemistries, surface chemistry is found to modulates the conformation of adsorbed FN which in turn regulates the integrin binding and cell adhesion behavior (Keselowsky, 2003).

In summary, surface properties of an implant determine the protein adsorption which in turn influences cellular responses to the implant. Sometimes serum-free medium is used to avoid the influence of protein adsorption during *in vitro* study of cell-biomaterial interaction.

2.3.2 Temporal and spatial sequence of events in cell adhesion

As mentioned earlier, cell adhesion to solid substrate involves a diversity of cell-matrix adhesion proteins that interplay with each other in a “spatially and temporally regulated manner”(Cohen, 2004). In this section, the hierarchical process of cell-substrate adhesion consisting of several sequential molecular events is reviewed.

Hyaluronan-mediated adhesions. Most of vertebrate cells are covered with a micrometer-thick pericellular coat which consists of hyaluronan (HA) (Rilla, 2008). This pericellular coat was shown to mediate initial stages of cell-substrate adhesion before the formation of integrin-mediated adhesions (Zimmerman, 2002; Zaidel-Bar, 2004). As proposed by Cohen *et al.*, when cell membrane is several microns away from surface, cell initiates binding through its HA pericellular coat at the first contact (Figure 2-10 b). HA-mediated adhesion ensures cell anchorage. The integrin-RGD binding could be achieved through three possible mechanism: 1) diffusion/translocation of HA: 2) collapse of HA and 3) degradation of HA. Once integrin-RGD contacts have been established, adhesion sites started to develop.

Figure 2-10. Proposed sequence of cell adhesion initiated with a hyaluronan brush of several micron thickness (Cohen, 2004).

Integrin-mediated adhesions. The HA-mediated cell adhesion is a transient process and replaced by integrin-mediated adhesion as fast as within a few second (Zaidel-Bar, 2004). The very first integrin-mediated adhesions are called focal complexes (FX) located under the protrusive lamellipodia of migrating cells. During the forward movement of the lamellipodia, the cycle of new FX formation and previous FX dissociation persist as long as the lamellipodia advance until the lamella retracts, or stops protruding. Then FX disappear and transform into definitive focal adhesions (FA). The FX-FA transition was found to depend on actomyosin-driven contractility generated from stress fibers. Further application of this contractile force results in the formation of fibrillar adhesions (FB) which are associated with FN fibrillogenesis (Zamir, 2000). In addition to actomyosin contractility, the formation of FB depends also on matrix pliability (Katz, 2000).

Evolution of cell adhesion on time and distance scales. It takes hours from the first cell-surface recognition to full development of cell spreading. During that time, cell membrane approaches the surface from micrometers to 10-20 nm. Meanwhile, cell-substrate contact area increases up to thousands of μm^2 (Cohen, 2004). Figure 2-11 summarizes the progression of cell-substrate adhesion in terms of separation distance and contact area as function of time (Cohen, 2004).

Figure 2-11. Progression of cell-substrate adhesion in terms of separation distance and contact area as a function of time (Cohen, 2004).

2.3.3 Integrin-mediated cell adhesion strengthening

During the progression of cell adhesion, both formation of cell-substrate adhesion complexes and cytoskeletal reorganization with progressive spreading of the cell on the substrate increase cell adhesion strength (Schoen, 2004). The generally accepted model for adhesion strengthening was proposed by McClay and Erickson (Lotz, 1989). The cell adhesion strengthening arises from three aspects during the progression of cell adhesion: (1) the increase in cell spreading area increases the cell-substrate contact; (2) integrins are recruited to the adhesion sites through clustering; and (3) the formation of various cell-substrate adhesions increases the local membrane stiffening (Gallant, 2005).

Recently, combining micro-contact printing techniques, fibroblast cell spreading area was restricted to defined adhesion islands. Gallant *et al.* have showed that, independent of cell spreading, cell adhesion strength improved dramatically upon integrin recruitment and FA formation. And vinculin recruitment to FA was found to contribute 30% of overall adhesion strength (Gallant, 2005). Based on their experimental results, a model of force balance at both macroscopic and microscopic level was deduced as shown in Figure 2-12 (Gallant, 2007). In their model, cell detachment was assumed to occur by peeling of the leading cell edge. The bond force of FA at the periphery of the cell-substrate contact area provided the dominant force resisting this peeling force. Once the maximum peripheral bond force is exceeded, cell could detach from substrate like “the tent in the storm”.

Figure 2-12. Model of cell adhesion strengthening on micropatterned island (radius a). (A) Force balance at macroscopic level for cell (radius R) under hydrodynamic shear flow. (B) The non-uniform distribution of adhesive patches at microscopic level. Segment 1 represents the clustered focal adhesion at farthest point of attachment (Gallant, 2007).

To summarize, cell adhesion plays a vital role in cell-biomaterial interactions. During first few hours of cell adhesion, cells reorganize cytoskeleton to develop adherent morphology. Through formation of cell-substrate contacts (FX, FA and FB), cells establish and strengthen the physical attachments, as well as signaling transmission pathways to regulate almost every aspect of cell fate, including migration, proliferation and apoptosis.

2.4 Surface modification for endothelialization

Hemocompatibility is an important consideration in designing blood-contacting implants. To reduce short-term thrombogenicity, the implant surface could be modified by protein/cell repelling layers, such as polyethylene glycol (PEG) hydrogels, or active anticoagulant agents such as heparin or plasminogen activator (Sefton, 2004). Artificial vascular grafts, stents and cardiac valve leaflets are devices that remain in the body for long-term, or even permanently and thus require a surface that is able to retain its hemocompatibility throughout its service life. Endothelialization of device surface meets

this long-term requirement, as explained previously. However, the fundamental problem with most of synthetic polymers is poor endothelial cells affinity. Hence, a broad spectrum of surface modification methods was developed to promote EC adhesion and proliferation (Goddard, 2007; de Mel, 2008). Generally, these are classified into three categories: topological modification, chemical modification and patterning.

2.4.1 Topological modification

Topological modification is mainly to change the surface roughness, which is believed to modulate cell affinity of certain substrates (Tzoneva, 2007). For instance, enhanced cell adhesion and migration was reported by increasing surface roughness of a polymethylmethacrylate (PMMA) using sand-blasting with aluminum grain of size 50-150 μm (Lampin, 1997). Surface topology could also be modified using solvent casting/particle leaching (SCPL). This method was able to create a wide range of surface porosity as well as to construct a three-dimensional scaffold (Mattioli-Belmonte, 2008; Sin, 2010). Surface hydrolysis of polyester by NaOH represented another topological modification strategy via chemical etching. It was reported surface hydrolysis by NaOH created nanoscale roughness and increased the wettability of PLLA films, and significantly improved the adhesion and growth of fibroblast (Wang, 2007).

2.4.2 Chemical modification

A wide range of modification strategies targeted to improve cell affinity through impartation of surface functions groups, immobilization of ECM molecules, or/and incorporation of cell adhesion ligands.

Impartation of functional groups. Studies have demonstrated that physiochemical properties of biomaterial surface, such as surface hydrophilicity/hydrophobicity (Webb, 1998), surface charge density (J. H. Lee, 1997), free energy (Hallab, 2001) and specific chemical groups (Arima, 2007) greatly affect cell affinity. Gaseous plasma treatment

could alter these surface properties in a well-controlled manner. Many types of gases have been studied for better cell affinity, including ammonia (Chu, 1999; Lu, 2001), argon (J. Y. Chen, 2007), air (Pratt, 1989), oxygen (Ryu, 2005), helium (De, 2005), and water vapor (Pompe, 2007). Functional groups such as hydroxyl ($-OH$) and amine ($-NH_2$) were imparted onto the polymeric surface to improve EC compatibility. It was believed these functional groups changed the wettability of the polymeric surface, and sequentially influenced cell adhesion. Plasma polymerization/grafting are alternative ways to introduce functional groups on surfaces, such as lactic acid grafting (Hsu, 2000) and acrylamide polymerization (C.-L. Li, 2006). Wet chemical reaction is relatively difficult to control, but success of introducing primary amines to PLA and PLGA by aminolysis using 1, 6-hexanediamine was reported (Croll, 2004; Zhu, Gao, Liu, He, , 2004). Impartation of functional groups only improves cell attachment marginally by increasing hydrophilicity or protein adsorption. Therefore, it is usually used as the very first step to provide chemically active function groups for sequential modification.

Immobilization of ECM molecules. It has become evident since that certain molecules in the basal lamina are responsible for EC adhesion. Researchers have attempted to immobilize them onto polymeric surfaces to mimic ECM environment (Eisenbarth, 2007). One of the most widely studies of these is FN (James, 1988; Heilshorn, 2003; Wittmer, 2007) which accounts for approximately 15% of the protein synthesized and secreted by EC. Other commonly studied ECM proteins are collagen (Yang, 2003; Cheng, 2004), gelatin (Zhu, Gao, He, , 2004; Ma, 2005) and laminin (Chandy, 2000).

Incorporation of cell adhesion peptides and ligands. Cell adhesion, both on natural tissue and on synthetic substrates, is mediated by cell adhesion ligands interaction with cell-surface receptor. These cell adhesion ligands can be oligopeptides, saccharides,

or glycolipids (Alberts, 2002). Immobilization of them on synthetic polymer surface could improve cell-surface interaction. Certain short amino acid sequences appear to bind to receptors on cell surfaces and mediate cell adhesion, for example, RGD and REDV found in FN (Massia, 1990; Hersel, 2003; Gabriel, 2006; Larsen, 2006; Tugulu, 2007), YIGSR (Jun, 2004) and IKVAV (X. Lin, 2006) found in laminin.

The general method of chemical surface modification usually involves several steps. Most of the polymeric surface is chemically inert. In order to introduce active functional groups, plasma treatment and wet chemistry are the common strategies. In some surface modification, cell adhesion promoting molecules or motifs are covalently immobilized on surface (I.-K. Kang, 1993). Another popular strategy is layer-by-layer (LBL) coating using opposite polyelectrolytes, as illustrated in Figure 2-13 (Zhu, 2003).

Figure 2-13. The schematic representation of aminolysis and layer-by-layer self-assembly process with oppositely charged polyelectrolytes on an aminolyzed PLLA membrane surface (Zhu, 2003).

2.4.3 Micro-/nano-patterning

Micro-contact-printing (μ CP) is a technique whereby patterns of self-assembled monolayer (SAM) of alkanethiols on gold surfaces can be produced. The μ CP techniques are widely applied in the study of cell-biomaterial interactions, including cell spreading (Lehnert, 2004), adhesion strength (Gallant, 2005) and focal adhesion development (Balaban, 2001). As indicated in Figure 2-14, micropatterns printed on substrates were

found to regulate/control EC adhesion and function (Christopher, 1998; Ito, 1999; Kumar, 2003; Gauvreau, 2005; Satomi, 2007; Yamamoto, 2007).

Figure 2-14. Micropatterned substrate showed the dependence of cell behaviour on pattern size and interspacing (C. S. Chen, 1997; Kumar, 2003).

2.5 Vaults

2.5.1 Vault composition

Vaults, first described in 1986, are 13 MDa ribonucleoprotein particles, which are highly conserved in most eukaryotes (Kedersha, 1986b). There are between 10^4 and 10^6 vault particles in the cytoplasm of most eukaryotic cells. Native vaults, purified from rat liver, consist of a small untranslated RNA (Kickhoefer, 1993), multiple copies of main vault protein (MVP 110 kDa), vault poly-ADP-ribose polymerase (VPARP, 193 kDa) and telomerase associated protein 1 (TEP 1, 290 kDa) (Kickhoefer, Siva, , 1999; Kickhoefer, Stephen, , 1999).

2.5.2 Vault structure

Reconstruction images, taken on the cryoelectron microscope (cryoEM), revealed that the vault was a “hollow, barrel-shaped particle” with two protruding caps and an invaginated waist (Kong, 2007), as shown in Figure 2-15A. Its maximum dimensions are

about $42 \times 42 \times 75 \text{ nm}^3$, larger in size and mass than some icosahedral viruses. Coupled with the cryoEM analysis, a $9 \text{ \AA}$ draft crystal structure of an empty vault built from a cysteine-tagged construct of MVP (CP-MVP) indicated that each vault is composed of two identical halves (Figure 2-15B). 48 copies of MVP are believed to make up each half with 6 MVPs corresponding with each “flower petal” in eight-fold symmetry (Mikyas, 2004). All of the MVP N-termini non-covalently come together around the barrel waist in the lumen and extend toward the interior of the particle, whereas the C-termini of the MVP are collected at the barrel caps (48 on each cap) (Anderson, 2007). Recently, Tanaka *et al.* reported the structure of rat liver vault at $3.5 \text{ \AA}$ resolution, revealing that vault cage structure consists of a dimer of half-vaults, with each half-vault comprising 39 identical MVP chains (H. Tanaka, 2009). Nevertheless, vaults have a capsule-like structure with a thin shell (ca. 2 nm) surrounding a large hollow interior compartment with a volume of $5 \times 10^4 \text{ nm}^3$, which is capable of accommodating hundreds of proteins (Mikyas, 2004).

Figure 2-15. (A) TEM images of purified vaults. The scale bar is 100 nm (Kedersha, 1986a). (B) Assembly of the CP-MVP vault shell model purposed by Anderson *et al.*. The half vault consists of 48 copies of MVP and the whole-vault model consists of two halves (Anderson, 2007).

2.5.3 Assembly of recombinant vaults

MVP (99 kDa) makes up more than 70% of total vault mass (Kedersha, 1991). The expression of MVP alone in the insect cells, which are infected with a baculovirus containing an MVP cDNA, can result in self-assembly of the MVP into the distinctive barrel-and-cap structure (Stephen, 2001). Thus multimerization of this protein is sufficient to form the exterior shell of the particle. Although variations in particle morphology were observed, most recombinant vaults were in the similar shape and size comparing with that of purified rat vaults (Figure 2-16). These particles varied from 32-37 nm across to 59-65 nm in length and displayed the distinctive bifold symmetric vault central barrel with dual caps (Stephen, 2001).

Figure 2-16. Vault-like particles formed in Sf9 cells (Stephen, 2001).

2.5.4 Engineering recombinant vaults for biomedical applications

Although many different functions have been proposed for the vaults, including roles in nucleo-cytoplasmic transport, multidrug resistance and innate immunity, their normal cellular function remains undetermined (Chugani, 1993; Scheffer, 1995; Kowalski, 2007). However, their attributes, such as the nano-cage structure, natural occurring protein and capability of self assembly, make vaults the very promising vehicles for delivery of therapeutic agents.

Several strategies were used to engineer vault nanoparticles with additional properties to increase their versatility as in drug delivery system. One of them was

through a vault targeting peptide found in VPARP at its C-terminus (aa 1563-1724), which is known to bind to MVP in the vault lumen (van Zon, 2002). Different mapping has shown that the mINT domain binds to the inside of the recombinant vaults at two locations, above and below the waist of the vault particles. This vault-targeting peptide sequence has been designated as mINT, minimal interaction domain. Fluorescent or enzymatic proteins, fused to mINT, were directed to the lumen of recombinant vaults, while retaining their native properties (Kickhoefer, 2005). Another way was to introduce protein tags at MVP C-terminus. It was reported that 11 amino acid epitope tag (VSVG), a 33 amino acid IgG-binding peptide, and the 55 amino acid epidermal growth factor (EGF) were fused onto the MVP C-terminus, and provided the ability to target vaults to specific cells as delivery vehicles (Kickhoefer, 2009). The third way to engineer recombinant vaults is to introduce protein tags at MVP N-terminus. Recently, recombinant vault has been shown to encapsulate a semiconducting polymer, and pH lability of cross-linked vaults at N-terminus may be useful for controlled release of encapsulated materials (Ng, 2008; Yu, 2008).

However, all of the previously reported work to date, which explored the possibility of vaults as a drug/protein delivery system, has used vaults in aqueous solutions. With the view of cardiovascular applications, our group would like to see the feasibility of incorporating vaults particles into solid matrices or onto substrates (such as PLLA). By doing this, the release of encapsulated bioactive agents (such as vascular endothelial cell growth factors, anti-restenosis drugs) could be better controlled and sustained. In addition, short cell adhesion ligands, such as RGD and REDV, could be fused on to C-terminus of recombinant vaults. These cell adhesion ligands expressed on immobilized particles may be able to provide beneficial cell-substrate interaction.

2.6 Summary

After reviewing the properties and applications of biodegradable LA/GA polymers, the need to modify these polymeric surfaces to enhance endothelialization is revealed to be one of challenges for cardiovascular applications. To this end, the various aspects of the cell-biomaterial interactions, the current strategies to promote endothelialization on synthetic polymers and the need to immobilize the recombinant vaults particle were also reviewed. This project is aimed to promote endothelialization through understanding EC-biomaterial interaction on biomolecules modified PLLA.

Chapter 3 Surface modification and characterization

3.1 Introduction

PLLA is considered a promising material in cardiovascular stent design due to its mechanical properties (Tamai, 1999; Venkatraman, 2006). However, the poor EC affinity of PLLA mars its potential of being exploited as part of a blood-contacting device. This is a critical flaw, since rapid endothelialization is important for a successful outcome of cardiovascular stenting. The EC covering increases the effectiveness of the stent by protecting the vessel from thrombosis and restenosis. Furthermore, it will also prevent degradation debris from entering the bloodstream and causing further complications (Deutsch, 2009). The aim of the study described in the following chapter is to overcome the EC affinity limitations of PLLA through surface modification. The experimental procedure should allow the PLLA surface to be modified in order to influence the bio-interaction, without any detriment to the key physical properties of biomaterial.

This chapter describes a three-step surface modification method to immobilize biomolecules (gelatin and chitosan) on PLLA surface using argon (Ar) plasma treatment and grafting polymerization. The plasma condition was optimized by measuring molecular weight degradation after plasma treatment. The feasibility of the surface modification method was evaluated by various surface characterization techniques, such as X-ray photoelectron spectroscopy (XPS), contact angle measurement and colorimetric method for functional group quantification.

3.2 Materials and Methods

3.2.1 Materials

PLLA (intrinsic viscosity = 8.24) was purchased from Purac (Singapore). Organic solute dichloromethane (DCM) and chloroform were purchased from Sigma-Aldrich.

Chemicals used in surface modification, including 2-(N-Morpholino) ethanesulfonic acid (MES, low moisture content, >99%), acrylic acid (AA, anhydrous, 99%), sodium metabisulfite (ACS reagent, 99%), potassium persulfate (ACS reagent, 99%), N-hydroxysuccinimide (NHS), gelatin (Porcine skin, Type A), chitosan oligosaccharide lactate ($M_n=5000$) and Rhodamine 6G were from Sigma-Aldrich. 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and coomassie plus (Bradford) protein assay were purchased from Thermo Scientific.

3.2.2 Surface modification

3.2.2.1 PLLA film preparation

PLLA (IV=8.4, $M_w = 1.9 \times 10^6$ Dalton) was dissolved in DCM and cast into films by in-house built film applicator. These films were put into vacuum oven at 80°C until no trace of DCM detected by thermogravimetric analysis (TGA). The final thickness of PLLA film was around 40 μm . The film was cleaned by immersing in 70% ethanol/water solution for 30 min to remove any oily dirt, and then was rinsed with large amount of deionized water. The cleaned PLLA films were dried in 37 °C vacuum oven for future use.

3.2.2.2 Effect of plasma treatment on polymer bulk properties

PLLA films were subjected to Ar plasma under three different power values, 80w, 120w and 160w, with the combination of durations varied from 60s to 180s. The weight average molecular mass (M_w) of these plasma-treated polymers were determined using an Agilent series 1100 size exclusion chromatography (SEC). A mixed bed column (PLGel Mixed Bed C, 5 μ , 300 x 7.5 mm, Polymer Laboratories), along with a guard column (50 x 7.5 mm, Polymer Laboratories), was employed. The mobile phase was chloroform. The molecular weights of samples were obtained relative to polystyrene standards. The flow

rate was 1 ml/min and the temperature (both the column compartment and the flow cell of the refractive index detector) was 35 ± 0.1 °C.

The effect of plasma treatment on crystallinity of PLLA was studied using a modulated differential scanning calorimetry (DSC 2920, TA Instruments, USA) equipped with a refrigerated cooling system under a constant flow of nitrogen (50 ml/min). Heat flow of each plasma treated sample was recorded between -50 and 230°C at a ramp rate of 3°C/min, and an integral of the melting endotherm, ΔH_m , was obtained to calculate the degree of crystallinity, χ_c using the following equation 3-1

$$\chi_c = \frac{\Delta H_m}{\Delta H_c} \quad (\text{equation 3-1})$$

where ΔH_c is the heat of fusion (93.3J/g) of a PLLA α -form crystal with an infinite size (M. Tanaka, 2006). The χ_c value was determined from averaging three replicated measurements.

3.2.2.3 Three-step surface modification protocol

Surface modification protocol was designed to have three steps as illustrated in Figure 3-1: (I) Ar plasma treatment, (II) AA grafting polymerization and (III) biomolecules immobilization.

The Ar plasma surface treatment was carried out in a March PX-500 cleaning system. The plasma power was set at 150 w at 13.56 MHz. The PLLA film was placed between the two parallel plate electrodes and subjected to the glow discharge for 120 s at an Ar pressure of 0.4 Torr. The surface activated PLLA films were then exposed in air for 30 min, to allow the formation of surface peroxides and hydroperoxide, followed by immersion in a solution of AA in Milli-Q water with redox agents (sodium metabisulfite and potassium persulfate) for 5 h at room temperature. The AA-grafted PLLA, termed as PLLA-gAA, was rinsed once with a 0.1wt% Triton X-100 aqueous solution, in an

ultrasonic cleaner for 15 min, and twice with MilliQ water. The biomolecules immobilization was carried out in water soluble carbodiimide (WSC) which was composed of MES buffer solution (pH=6.5) in the presence of EDC and NHS. The PLLA-gAA films were kept in the WSC solution at room temperature for 2 h to pre-activate the carboxylic groups introduced by AA polymerization. Then, gelatin or chitosan was added to initiate the covalent coupling reaction between carboxylic groups on PLLA-gAA and amine groups present on the biomolecules. The reaction was carried out for 12 h at room temperature. After immobilization, PLLA-gAA-gelatin and PLLA-gAA-chitosan were rinsed with 0.1% Triton X-100 aqueous solution and subsequently with MilliQ water.

Figure 3-1. The three-step biomolecules immobilization protocol. Step I: Ar plasma treatment of PLLA. Step II: AA grafting polymerization on the activated PLLA film. Step III: Covalent coupling of amine groups on biomolecules with carboxylic groups on PLLA-gAA in WSC solution.

3.2.3 Surface characterization

3.2.3.1 XPS

The surface chemical composition of various PLLA films was analyzed by XPS (Kratos AXIS Ultra) with monochromatic Al K α (1486.71 eV) X-ray radiation (15 kV and 10 mA). 160 eV pass energy was used for survey scans whereas 40 eV was used for the high-resolution scans.

3.2.3.2 Contact angle measurement

The advancing contact angle (θ_{adv}) was measured by a dynamic contact angle system (FT Δ 200) with the FT Δ μ -tip $\text{\textcircled{R}}$ needles (ID= 5 μ m, OD = 1 mm). The pump flow rate was programmed at 0.1 μ L/sec to achieve a relatively low expansion or contraction volume in a pseudo-equilibrium condition. The spreading of the drops was monitored, from the moment they detached from the needle, by a video recorder connected to a CCD camera and a microscope. Images of the growing drop were then recorded by the computer with FT Δ 32 software at a rate of a picture every 2 s. An advancing contact angle was defined as the one measured when the sessile drop had the maximum volume allowable for the liquid-solid interfacial area.

3.2.3.3 Surface density of carboxylic groups

The surface density of carboxylic groups gained from AA polymerization on PLLA was determined by the rhodamine-carboxyl interaction method (I.-K. Kang, 1993). Rhodamine dye changes its color from yellow to pink by interacting with carboxylic groups. Hence, higher absorbance measurement at 530 nm is associated with higher carboxyl concentration in a linear relationship. PLLA-AA film was dissolved into an equal volume of benzene-dye reagent and the absorbance of the solution at 530 nm was measured using a spectrophotometer (Pharmaspec UV-1700 Shimadzu). The absorbance of unmodified PLLA was measured as background control. A standard curve (seen Figure

A-1 in Appendix) was constructed using known concentrations of AA solution as described above. The concentration of carboxylic groups in the solution could then be calculated from the standard calibration curve.

3.2.3.4 *Surface density and stability of immobilized gelatin*

The surface density of gelatin was measured by the Bradford method, in which maximum absorbance shift from 465 nm to 595 nm of coomassie blue was used to indicate the presence and the amount of certain protein (I.-K. Kang, 1993). In this study, gelatin solutions of known concentration were added to the dye solution. The gelatin-dye solutions were incubated for 10 min at room temperature and the resulting protein-coomassie complexes were precipitated by centrifugation at 15000 rpm for 20 min. The absorbance of the supernatants at 465 nm was used to construct the standard calibration (Figure A-2 in Appendix). Incubation of PLLA with dye solution was considered as the background control. During the incubation of PLLA-gAA-gelatin with dye solution, protein coomassie complexes were formed on the PLLA surface. The absorbance of the supernatant dye solution was measured at 465 nm. The actual absorbance of PLLA-gAA-gelatin was calculated as the difference between the measured absorbance from sample and the background absorbance. The gelatin concentration immobilized on the PLLA surface was measured from the actual absorbance based on standard curve.

The stability of the covalent immobilization of gelatin on PLLA was compared to gelatin dip-coated on to PLLA (termed PLLA-gelatin), produced by immersing plasma-treated PLLA into 4% gelatin solution in 4% acetic acid for 12 h. Both PLLA-gAA-gelatin and PLLA-gelatin were immersed in phosphate buffer saline (PBS) at 37°C for 7 days. Gelatin coated on PLLA surface might dissolve into PBS due to hydrolysis or

unstable absorption. The surface stability of gelatin was assessed by comparing the initial and the residual surface density.

3.2.3.5 Surface topology analysis

Surface topology of modified sample was observed using either a field emission scanning electron microscope (FESEM, JEOL JSM-6340F) or scanning electron microscope (SEM, JEOL JSM-6360) at 5.0 KV. The samples were mounted on a metal holder using double-sided carbon tape, and then coated with gold particles using a pulse plasma system before observation. At least 7 pictures were taken for each experimental sample.

3.2.4 Statistics

The results were presented as the means $\pm$ SD (n=3). Student's t-test was conducted to compare the surface density of gelatin before and after 7 days PBS immersion. Statistical significance refers to $p < 0.05$.

3.3 Results and Discussion

3.3.1 Effect of plasma treatment on the polymer

Ar plasma treatment is a versatile technique for polymer surface modification. Successful polymer surface modification requires only seconds to minutes of exposure to Ar plasma. During exposure to Ar plasma, PLLA film surface interacts with the Ar ions and electrons. These reactive species cut polymer chains by the C-H and C-C bonds scission, and produce in carbon radicals on PLLA surface (Inagaki, 2002). Once removed from the plasma reactor, these carbon radicals are oxidized into oxygen functional groups such as hydroxyl, carbonyl, carboxyl (Inagaki, 2002).

In addition to the oxidation at the carbon radicals, degradation reactions initiate from the carbon radicals at the end points of the polymer chains to yield small molecules.

The degradation reactions involve a depropagation reaction in which monomer fragments split off stepwise along the polymer chain, and a transfer reaction of the carbon radicals to yield fragments with a higher molecular weight than that of the monomer takes place. Surface modification and degradation reactions occur simultaneously on exposing polymer film surfaces to Ar plasma. Degradation reactions cannot be avoided whenever Ar plasma is utilized for surface modification (Inagaki, 2002). Therefore, it is necessary to study the polymer molecular weight loss after Ar plasma treatment.

In the present study, three plasma operation powers, 80w, 120w and 160w, were employed in combination with five different treatment durations from 60s to 180s. After plasma treatments, film samples were measured using SEC. Figure 3-2 shows the weight average molecular mass (M_w) profiles of PLLA films subjected to different plasma treatment parameters. Upon 60s treatment, M_w dropped about 20% at 80w, 50% at 120w and 60% at 160w. It implied that the magnitude of plasma treatment power has strong influence on polymer degradation rate within 60s treatment. Stronger plasma power induced greater polymer degradation within 60s treatment. When the treatment duration increased to 90s and 120s, the molecular mass kept decreasing all three levels of plasma power. However, M_w reached plateau around 600KD at 120s regardless of the magnitude of plasma power. And further prolonged plasma treatment showed little impact on molecular mass. In a summary, polymer degradation took place during plasma surface treatment. In this study, the weight average molecular mass of PLLA film with thickness of 40 μm dropped from 1900KD to 600K, a two third decrease of the original molecular weight upon 120s treatment. This effect was independent of the applied plasma power. Therefore, plasma duration of 120s is recommended when using the plasma powers from 80w to 160w in order to avoid processing variation.

Figure 3-2. The effect of plasma treatment conditions (operation power and duration) on weight average molecular mass (M_w) of PLLA film with the thickness of 40 μ m. M_w was determined by SEC.

To study the impact of plasma treatment on crystallinity of PLLA, the crystallinity of PLLA before treatment was compared with these after treatments. As listed in Table 3-1, crystallinity of PLLA is 0.30 before plasma treatment (M. Tanaka, 2006). After plasma treatments, the crystallinity was in the range of 0.34-0.42. It implies that plasma treatments increased the crystallinity of PLLA by 10% to 40%. Taking together with the observations from the polymer molecular mass profiles in Figure 3-2, it is conceivable that polymer degradation occurred predominantly at the amorphous regions of PLLA under current experimental conditions. Crystalline regions consist of well-packed polymer chains with much higher density compared with amorphous regions. As a consequence, the C-C and C-H bonds on polymer chains in the crystalline region are less susceptible to plasma particles. Therefore, one can expect less surface modification in crystalline region than in amorphous region.

Table 3-1. The crystallinity of PLLA films subjected to different plasma treatment condition.

χ_c	60(s)	90(s)	120(s)	150(s)	180(s)
80W	0.38	0.36	0.40	0.34	0.36
120W	0.41	0.41	0.37	0.38	0.40
160W	0.37	0.41	0.42	0.38	0.39
χ_c of unmodified PLLA (IV =8.4) is 0.30 (M. Tanaka, 2006)					

In conclusion, plasma surface treatment is accompanied by surface degradation. Most of degradation took place in the amorphous region of the polymer. When the plasma treatment exceeds 120s, the molecular mass showed a plateau around 600K. This effect was independent of the applied plasma power. Therefore, plasma duration of 120 s is recommended when using the plasma powers from 80 w to 160 w.

3.3.2 Characterization of surface modified PLLA

3.3.2.1 Surface Topology

The surface topology of gelatin and chitosan modified PLLA film was observed by SEM. The images were shown in Figure 3-3 in comparison with unmodified PLLA and plasma treated PLLA. Alteration of surface topology was merely observed after plasma treatment and immobilization reaction. It demonstrated that plasma surface treatment provided a controllable and uniform surface modification with sub-surface alteration occurring only to a limited depth.

Figure 3-3. Scanning electron microscopy images of (A) unmodified PLLA, (B) Ar plasma treated PLLA, (C) PLLA-gAA-gelatin and (D) PLLA-gAA-chitosan.

3.3.2.2 Surface chemical composition

The surface chemical composition of the modified PLLA films was analyzed by XPS. Element survey spectra are plotted in Figure 3-4. The measured oxygen-to-carbon (O/C) ratio and nitrogen-to-carbon (N/C) ratio are tabulated in Table 3-2. The measured O/C ratio of unmodified PLLA surface was 0.59, which was in a good agreement to its theoretical value of 0.67 (Quirk, Davies, , 2001; Ding, 2004). The O/C ratio remained similar after AA grafting polymerization at 0.52, as poly(acrylic acid) (PAA) has the same theoretical O/C ratio of 0.67. No nitrogen peak was detected on PLLA, plasma-treated PLLA and PLLA-gAA. The appearance of nitrogen was found only after immobilization of gelatin or chitosan on PLLA-gAA, indicating the successful immobilization of those biomolecules on PLLA surface. The N/C ratio is directly associated with the surface density of the immobilized gelatin or chitosan on PLLA substrate. From Table 3-2, it was noted that both PLLA-gAA-gelatin and PLLA-gAA-chitosan showed N/C ratios much smaller than their respective theoretical values. As the sampling depth of XPS in polymer matrix usually is about 7 nm (Ding, 2004), it was

concluded that the thickness of the immobilized gelatin and chitosan layers do not exceed 6-7 nm.

Table 3-2. The atomic ratios with corresponding theoretic values indicated in parentheses and the percentage of functional groups on various surfaces by deconvoluted high-resolution C1s spectra of (A) PLLA, (B) PLLA-gAA, (C) PLLA-gAA-gelatin and (D) PLLA-gAA-chitosan.

Surface	Atomic ratio		Contribution of C1s components for surface modified PLLA			
	O/C	N/C	C-C	C-O (-N)	N-C=O	O-C=O
A	0.59 (0.67)	0 (0)	38.5	31.0	-	30.5
B	0.52 (0.67)	0 (0)	40.4	29.2	-	30.4
C	0.35 (0.38)	0.12 (0.31)	33.8	26.1	24.4	15.7
D	0.47 (0.67)	0.03 (0.17)	38.7	36.4	-	24.9

Figure 3-4. XPS survey spectra of (A) PLLA, (B) PLLA-gAA, (C) PLLA-gAA-gelatin and (D) PLLA-gAA-chitosan.

The C1s spectra were resolved into four characteristic peaks at 289.0, 288.1, 286.5, 285 eV, indicating the functional groups of O-C=O, N-C=O, C-O (C-N) and C-C (C-H) respectively, as shown in Figure 3-5. The percentage of individual peak contribution is listed in Table 3-2. As expected from the monomer structure, unmodified PLLA gives a C1s core spectrum consisting of three peaks of comparable area, which was consistent with results from other research groups (Quirk, Davies, , 2001; Ding, 2004). After AA grafting polymerization on PLLA, the C-O peak dropped slightly and C-C increased accordingly as expected from differences in monomer structure between acrylic acid (AA) and lactic acid (LA). The O-C=O peak was attributed to the carboxylic side groups of PAA. It was noted that C-O peak took up 29.2% contribution in PLLA-gAA C1s spectrum, which was not expected from the AA monomer structure. That implied the thickness of grafted PAA also does not exceed 6-7 nm. With the immobilization of gelatin onto the surfaces, the carboxyl peak (O-C=O) decreased significantly and the amide peak (N-C=O) increased to 24.4% accordingly. The increment in amide peak (N-C=O) was mainly attributed to the contribution of amide bond found in the amino acid backbone of gelatin (Hou, 2003). On PLLA-gAA-chitosan, a significant increase of C-O (C-N) peak coupled with a decrease in O-C=O peak was observed compared with the other PLLA substrates, which was expected from the structure of D-glucosamine unit found in chitosan (Ding, 2004).

Figure 3-5. The C1s spectra of (A) PLLA, (B) PLLA-gAA, (C) PLLA-gAA-gelatin and (D) PLLA-gAA-chitosan, resolved into four characteristic peaks at 282.2, 288.1, 286.5, and 285eV indicated the functional groups of O-C=O, N-C=O, C-O (C-N) and C-C respectively.

3.3.2.3 Surface density of carboxylic groups on PLLA-gAA

The carboxylic group concentration standard curve, shown in Figure A-1 in Appendix, was established by measuring the absorbance of an array of dye solutions mixed with known AA concentrations. The surface density of carboxylic group of PLLA-gAA was calculated to be $4.17 \pm 0.15 \mu\text{mol}/\text{cm}^2$, and was comparable with previously published results. Kang *et al.* reported that their concentration of carboxylic acid density on AA-grafted PMMA (treated by oxygen plasma for 30 seconds) was 0.47-9.48 $\mu\text{mol}/\text{cm}^2$, depending on the pressure of the plasma chamber during the discharge treatment (I.-K. Kang, 1993).

3.3.2.4 Surface density and stability of immobilized gelatin

In order to evaluate the efficiency of covalent immobilization, the surface density of gelatin of PLLA-gAA-gelatin was measured and compared with that of PLLA-gelatin in which gelatin was physically adsorbed on PLLA. As shown in Figure 3-6, gelatin surface density on PLLA-gAA-gelatin was $4.8 \mu\text{g}/\text{cm}^2$, which was considerably greater than that of PLLA-gelatin ($1.5 \mu\text{g}/\text{cm}^2$), thus demonstrating the advantage of covalent immobilization over physical coating. The stability of immobilized gelatin was investigated by immersing PLLA-gAA-gelatin and PLLA-gelatin in PBS over 7 days. The surface density of gelatin on both surfaces was measured before and after immersion. As shown in Figure 3-6, the gelatin surface density on either PLLA-gelatin or PLLA-gAA-gelatin did not show significant loss after 7-day immersion in PBS (N.S. refers to $p>0.05$). However, this may not be directly representative of the physiological environment, since the stability of the physically adsorbed gelatin coating may be reduced, especially in the presence of enzymes and at different pHs, from which the covalent immobilization provides a degree of protection.

Figure 3-6. Surface density and stability of gelatin immobilized on PLLA, comparing physical coating of (A) PLLA-gelatin with covalent coupling of (B) PLLA-gAA-gelatin. N.S. refers to no significant difference by Student's t-test.

3.3.2.5 Surface wettability

The advancing contact angles (associated predominantly with surface wettability) of PLLA samples are shown in Table 3-3. Unmodified PLLA is hydrophobic, having a measured advancing contact angle of 82.3°. After gelatin and chitosan immobilization, the contact angle was decreased to 52.8° and 30.7° respectively. The better wettability of PLLA-gAA-chitosan was attributed to the presence of primary amide group (-NH₂) in each unit of D-glucosamine, which is positively charged in water. In contrast, gelatin contains a large amount of glycine (Gly) and proline (Pro) which are hydrophilic amino acids with neutral non-polar side chains. Therefore gelatin-modified PLLA exhibited a moderate wettability. The result suggests that biomolecules immobilization improved the PLLA surface wettability.

Table 3-3. Advancing contact angle of unmodified and modified PLLA films. * $p < 0.05$ refers to statistically significance in comparison with PLLA by Student's t-test.

Samples	Advancing angle (°)
PLLA	82.3 ± 2.7
Plasma-treated PLLA*	35.5 ± 5.0
PLLA-gAA-gelatin*	52.8 ± 3.8
PLLA-gAA-chitosan*	30.7 ± 2.6

In this study, PLLA surface was treated by Ar plasma followed by AA grafting polymerization. This procedure facilitated the incorporation of carboxylic groups onto the PLLA surface, which then enabled subsequent biomolecules immobilization through covalent coupling.

Gelatin and chitosan were selected to modify the PLLA surface in this study because of their chemical and structural similarities with type IV collagen and glycosaminoglycans (GAGs) respectively, which are the main components of the basal

lamina. It is well-known that the incorporation of gelatin can enhance cell affinity of materials, because gelatin contains cell adhesion amino acid sequences such as the RGD peptide motif that can bind cell surface receptors (Liu, 2010). Chitosan is derived from one of the common naturally-occurring biomolecules, chitin, and it is known to enhance endothelial cell affinity (Yan Huang, 2005). Furthermore, gelatin and chitosan are cost-effective and readily available model biomolecules. The surface modification method has the potential to utilize other extracellular matrix proteins, such as collagen IV and laminin (Eisenbarth, 2007) , and cell adhesion motifs, such as RGD (Hersel, 2003) and REDV (Massia, 1992).

The results from surface characterization analyses, including XPS, contact angle measurement and surface density of function groups lead to the conclusion that the three-step surface modification method developed in this study was able to immobilize biomolecules onto PLLA surface, resulting in different surface properties while preserving the bulk physical properties. The observations that allowed the formation of this conclusion are in summary: After step II of AA grafting polymerization, the incorporation of carboxylic groups was quantified by rhodamine-carboxyl interaction method, and the surface density was calculated to be $4.17 \pm 0.15 \mu\text{mol}/\text{cm}^2$. After step III of immobilization gelatin/chitosan, nitrogen peaks appeared in XPS spectra of PLLA-gAA-gelatin and PLLA-gAA-chitosan, while being absent in that of PLLA and PLLA-gAA, indicating successful immobilization was achieved. The approach of covalent coupling of biomolecules on PLLA-gAA surface was proved to produce a surface with higher gelatin surface density than that by physical adsorption of gelatin. The amount of immobilized gelatin on PLLA-gAA-gelatin as shown in Figure 3-6 was twofold of that on PLLA-gelatin. Moreover, negligible loss of gelatin was observed on PLLA-gAA-gelatin, proving stability of the surface modification method. Contact angle measurement showed in Table 3-3 indicated that immobilization of gelatin and chitosan increased surface

wettability, which has been shown to be one of important surface properties affecting cell-substrate adhesion (Tzoneva, 2007).

3.4 Summary

The surface of biodegradable polymer PLLA was modified through a mild and easily controlled three-step method, which involves sequentially (1) Ar plasma treatment, (2) AA grafting polymerization and (3) covalent immobilization of biomolecules. Polymer degradation studies on Ar plasma treated PLLA found that degradation occurred mostly in amorphous region. The Ar plasma treatment duration was recommended to be less than 120 s in order to have the polymer weight loss less than 30% of initial weight.

It was also found that immobilization of gelatin and chitosan on PLLA altered the physicochemical surface properties. Contact angle study showed that gelatin and chitosan improved the wettability of PLLA surface. Chemical composition of modified PLLA was analyzed using XPS in comparison to unmodified PLLA. The results showed evidence to prove that surface modification method was successful. Colorimetric methods were used to quantify the surface density of carboxylic group and immobilized gelatin. Their results were comparable to these results reported in literature. Gelatin immobilized on PLLA using the three-step chemical modification was significantly more stable in comparison to that using physical adsorption.

In conclusion, a feasible surface modification method of PLLA was developed and the modified PLLA surfaces were well characterized in accordance to the first objective of the work (Chapter 1).

Chapter 4 Endothelial cell-biomaterial interaction

4.1 Introduction

As reviewed in Chapter 2, the cell-biomaterial interaction starts with cell adhesion, which involves multitude of cell-matrix adhesion molecules interplaying with each other in “a spatially and temporally regulated manner”. During the process of cell adhesion, EC attach on and interact with biomaterials through focal adhesions (FA) and fibrillar adhesions (FB) formation. Meanwhile EC spread out on the surface and undergo a reorganization of their cytoskeletal structures. All these activities are part of a process that results in increased cell adhesion strength. In addition, EC migrate, proliferate and interact with neighboring cells. Eventually one monolayer of EC covers the entire biomaterial surface, a process referred to as “endothelialization”.

The promotion of endothelialization is important for the vascular acceptance of a biomaterial; hence, the aim of this chapter is to study the EC interaction with modified PLLA described in previous chapter. Cell-biomaterial interactions, involving cell adhesion, cell proliferation, morphological development and cell adhesion strength, are the focus of this investigation.

4.2 Materials and methods

4.2.1 Cells, antibodies and chemical reagents

Cryopreserved Clonetics[®] human umbilical vein endothelial cells (HUVEC) in endothelial growth medium (EGM[®]), Clonetics[®] EGM[®] BulletKit[®] supplemented with 0.4% bovine brain extract (BBE), Hepes buffered saline solution (HBSS), and 0.025% Trypsin-EDTA were purchased from Lonza. PBS was from Gibco. WST-8 cell counting reagent kit was from Dojindo, Japan. Paraformaldehyde (PFA, 95%) and glutaraldehyde

(25%, Grade I) were from Sigma-Aldrich. Triton X-100 (ultrapure grade) was from USB Biochemicals.

Monoclonal anti-human vinculin (clone h Vin-1), anti-human FN and goat anti-mouse IgG FITC conjugate were purchased from Sigma-Aldrich; Monoclonal anti-human paxillin, Hoechst 33342 and 7.5% bovine serum albumin (BSA) from Invitrogen; Rabbit anti-mouse IgG Cy3 from Millipore; Alexa Fluor[®] 568 phalloidin and 4',6-diamidino-2-phenylindole (DAPI) from Molecular Probes. Goat serum and fluorescence mounting medium were purchased from DAKO, Denmark.

4.2.2 Cell adhesion and proliferation

HUVEC were cultured in T25 or T75 flasks using BBE supplemented EGM in an incubator with 95% air / 5% CO₂ at 37°C. The EGM was replaced every two days. Cells were harvested by trypsinization upon 90% confluence. Passage four to six was used.

Unmodified and modified PLLA films were sterilized by immersing into 70% ethanol for 15 min, and then were rinsed with deionized water three times followed by PBS rinse three times. Each sample was then placed on the bottom of each tissue culture plate well for cell seeding at density of 10⁴ cells/cm². Cells were allowed to attach for 12 h. The unattached cells were rinsed by PBS. The number of attached cells was quantified using the WST-8 assay. Cell proliferation was monitored by WST-8 on day 1, 3, 5 and 7. The cell number at each time-point was calculated according to standard curve established according to manufacturer's protocol. Briefly, HUVEC were incubated with WST-8 reagent for 4 h, and the absorbance at 450 nm was measured by micro-plate reader (Tecan). A standard curve was established by measuring absorbance of 450nm at different cell density (Figure A-3 in Appendix). Cell doubling time (DT) of an exponential proliferation was calculated according to the equation 4-1:

$$DT = \log(2)/\beta \quad (\text{equation 4-1})$$

where β is the slope of proliferation curve plotted in logarithmic scale and $\log()$ is the natural logarithm (D. Kim, 1995).

4.2.3 Cell retention under shear stress

PLLA substrates were incubated with HUVEC at density of 10^4 cells/cm² for 24 h before mounted into a shear flow system. As illustrated in Figure 4-1A, the flow circuit was constructed by connecting (1) a cell culture medium reservoir, (2) a parallel plate flow chamber (GlycoTech, USA) and (3) a digital peristaltic pump (Masterflex, Cole Palmer, IL) using silicon tubing with 0.89 mm inner diameter. The parallel plate chamber is the core component of this flow system. As shown in Figure 4-1 B, it consisted of a cast acrylic top deck of 35 mm in diameter, and a silicon gasket which has flow width at the centre. PLLA substrate with cells grown on it was sandwiched between the parallel flow chamber and a 35 mm Petri dish. Once this was connected to the flow circuit through the inlet/outlet of top deck, the opening in the gasket facilitated the development of the flow on adherent cells grown on PLLA substrate. The peristaltic pump regulated the shear stress through adjusting the flow rate.

The shear stress τ (dyn/cm²) is calculated using the momentum balance for a parallel-plate geometry and Newtonian fluid, according to the equation 4-2:

$$\tau = 6Q\mu/wh^2 \quad (\text{equation 4-2})$$

where Q is volumetric flow rate, μ is the viscosity of the medium (~ 0.01 dyn/cm²), w is the gasket flow width and h is the gasket thickness. The parallel flow chamber and the medium reservoir were placed in humidified incubator attached onto the stage of Olympus IX81 inverted fluorescence microscopy at 37°C. Cells were subjected to shear flow for 30 min at shear rate of 3 dyn/cm² representing venous system, or 15 dyn/cm² and 25 dyn/cm² representing the arterial system. HUVEC were stained with Hoechst 33342 before mounted to the flow system. Cell adhesion strength was quantified as cell retention

percentage (R%) calculated using $N_{\text{after}}/N_{\text{before}} \times 100\%$, where N_{after} and N_{before} refer to cell count after and before subjected to shear flow. The cell count was calculated from at least 7 images taken by fluorescence microscopy along the flow channel.

Figure 4-1. (A) Shear flow circuit. (B) Parallel flow chamber consists of the top deck (transparent), gasket with opening at the center (green), PLLA film (blue) with EC (light yellow) growing on it and the 35 mm petric dish (red).

4.2.4 Immunofluorescent imaging

Immunostaining. HUVEC were fixed for 15 min with 4% PFA, permeabilized with 0.1% triton X-100 for 10 min and subsequently incubated with 10% goat serum in PBS for 30 min at room temperature. The cells were then incubated with primary antibodies for 4 h, followed by a further incubation with the appropriate secondary antibodies for 30 min. F-actin and nuclei were labeled with Alexa Fluor[®] 568 and DAPI respectively.

Imaging. Cell images were captured by either Olympus IX71 inverted fluorescent microscope (SpectraTECH, Singapore) or Leica TCS SP5 laser-scanning spectral confocal microscope (CLSM, Leica Microsystems, Singapore). At least 7 images or images of 40 cells were taken for each experimental sample.

Morphometric analysis. Morphometric analysis method was adopted from Treiser *et al.* (Treiser, 2007). Original fluorescent images were loaded to Image Pro Plus Version 6 software (SpectraTECH, Singapore). First of all, image enhancement optimization,

including image contrast, brightness, and γ -value adjusting, was applied to each image to equalize image intensity histograms. A pseudo-automated adaptive thresholding process was then employed allowing the whole cell and individual cytoskeletal proteins to be separated using segmentation function based on the image intensity histogram. Once either the whole cell or individual cytoskeletal elements were segmented, shape descriptors were calculated utilizing the count object menu in Image Pro Plus on the segmented objects. Multiple cells were examined for each polymer substrate and used to calculate a population distribution for each descriptor. The descriptors are (i) cellular morphological parameters: area, the length of major and minor axis; (ii) FA parameters: FA size and FA-per-cell; and (iii) FN fibrillogenesis parameters: length of FN fibril. The detailed definition of each parameter is explained in Table 4-1.

Table 4-1. Morphometric descriptors quantified from the immunofluorescent images of EC on various PLLA substrates.

Descriptor	Descriptor Definitions
Area*	Contiguous are of each object minus any holes.
Axis (Major, Minor)*	Length of the major/minor axis of object-equivalent ellipse.
FA size	Length of individual vinculin rich structure
FA-per-cell	The counts of vinculin rich structure observed in each cell
Length of FN fibril	Length of individual FN positive stain fibrillar structure
*The descriptor definitions were adapted from those available in Image Pro Plus.	

4.2.5 Scanning electron microscopy imaging

Endothelialization after 7 days incubation was observed by scanning electron microscopy (SEM) imaging. Cells grown on various PLLA substrates were fixed by 2.5% glutaraldehyde for 30 min and dehydrated by graded ethanol solutions. After gold

coating, cells were observed by SEM (JEOL, JSM-6360). At least 7 pictures were taken for each experimental sample.

4.2.6 Flow cytometry assessment of vinculin expression

Vinculin expression of EC grown on different PLLA substrates was studied by flow cytometry (Boura, 2005; Michael, 2009). Adherent cells were treated by 0.025% trypsin-EDTA and rinsed in serum-free medium. Then cells were suspended in 200 μ l of fixation solution made of 1% PFA in PBS and incubated for 30 min. Fixed cells were then permeabilized in 0.2% trypsin for 15 min under gentle agitation and incubated in 2% BSA for 30 min. Then cells were incubated in primary and secondary antibodies in 1% BSA in PBS for 30 min, washed, and analyzed by flow cytometry. The blank control was measured by incubating EC only with the secondary antibody to evaluate the non-specific binding. Fluorescence intensity was measured and analyzed by FACscan Becton Dickinson, France. 10,000 events were collected and the medium fluorescence intensity was determined as determined by forward and sideward scattering.

4.2.7 Statistic analysis

Box plot. Each box encompasses 25-75 percentiles, with extending-lines covering the 95th and 5th percentiles, the middle line in the box representing the median (50th percentile), and the shorter line representing the mean values. Values outside the 95th and 5th percentiles were treated as outliers and were represented by dots. At least 40 cells of each experimental group were analyzed to establish the box plot of cell spreading area.

Histogram. Focal adhesion size is defined as the length of major axis of vinculin localized structure. FA size distribution histogram on different PLLA substrates was plotted using Matlab software Version 6. FN fibril length distribution histogram on different PLLA substrates was plotted using Microsoft Excel 2007. At least 40 cells of each experimental group were analyzed to establish the box plot.

Others. The results are expressed as means $\pm$ standard deviation (SD) if not specified (n=3). Statistical significance test and correlation coefficient were calculated using Microsoft Excel 2007. Statistical significance was evaluated using Student's t-test and analysis of variance (ANOVA), and was inferred by a *p* value less than 0.05 ($p<0.05$).

4.3 Results

4.3.1 Cell adhesion

Cell adhesion is the initial cell-biomaterial interaction and is mediated by cell membrane receptors (mainly from the integrin super family). Upon cell adhesion, FA formation takes place to provide cell anchorage points on substrate. FA are characterized by a multitude of cytoskeletal and signaling molecules (Geiger, 2009). Paxillin is one of the characteristic proteins accumulated at FA. It regulates cytoskeletal reorganization and gene expression through recruiting regulatory and structural proteins (Deakin, 2008). Depending on the physical and chemical properties of substrates, cells exhibit different adhesion behavior. In this study, HUVEC were seeded on various PLLA films at a density of 10^4 cells/cm² and allowed to attach for 12 h. Then, unattached cells were rinsed off. The remaining adhered cells were evaluated for cell adhesion behavior, in term of quantity of adhered cells, cell spreading area and FA formation.

The number of adherent cells was assessed using the WST-8 assay. The absorbance of WST-8 reagent solution, following incubation with attached cells for 4 h, was linearly proportional to the cell number, as demonstrated by the standard curve shown in Figure A-3 in Appendix. The absorbance measured from different PLLA surface was plotted in Figure 4-2. The cell adhesion on tissue culture plate made of polystyrene (TCPS) was considered as a reference. When compared with unmodified PLLA, both PLLA-gAA-gelatin and PLLA-gAA-chitosan facilitated enhanced cell adhesion ($*p<0.05$). Moreover, PLLA-gAA-gelatin was found to be superior than PLLA-

gAA-chitosan (** $p<0.05$). This study suggests that cell adhesion was greatly influenced by the surface type (Figure 4-2). Hence biomolecules-modified PLLA does improve cell surface adherence.

Figure 4-2. Cell adhesion observed on (A) TCPS, (B) unmodified PLLA, (C) PLLA-gAA-gelatin and (D) PLLA-gAA-chitosan after 12 h incubation at 37°C in humidified air with 5% CO₂. * $p<0.05$, statistical significance compared to PLLA. ** $p<0.05$, statistical significance compared to PLLA-gAA-gelatin.

Cell adhesion behavior was further evaluated by the extent of cell spreading on a substrate, *i.e.* the cell spreading area, measured from CLSM immunofluorescent images using Image Pro Plus software. After 12 h of incubation, cells seeded on PLLA-gAA-gelatin and PLLA-gAA-chitosan exhibited the average spreading area of 851 μm^2 and 711 μm^2 respectively, as indicated in Figure 4-3. In contrast, the spreading area found on PLLA (about 515 μm^2) was significantly less than that of both modified PLLA substrate (Student's t-test, $p<0.05$). These findings imply that biomolecules immobilized PLLA substrates promote the spreading of HUVEC.

FA formation is another criterion to evaluate the quality of cell adhesion. After 12 h incubation, cells were fixed and paxillin was immuno-labeled. Images captured by CLSM on different PLLA substrates are shown in Figure 4-4. A limited number of FA (pointed by red arrows) was found in HUVEC grown on PLLA. However, more FA

formation was observed in HUVEC seeded on both biomolecules immobilized PLLA substrates, especially on PLLA-gAA-gelatin as shown in Figure 4-4 (C). The abundant and well-organized focal adhesions observed throughout these cells suggested the matured and strong cell-substrate adhesion points had developed on PLLA-gAA-gelatin.

Figure 4-3. Cell spreading area observed on (A) PLLA, (B) PLLA-gAA-gelatin and (C) PLLA-gAA-chitosan at 12 h post seeding.

Figure 4-4. FA formation, as denoted by red arrows, exhibits surface-dependence. Cells were seeded on (A) PLLA, (B) PLLA-gAA-chitosan and (C) PLLA-gAA-gelatin at seeding density of 10^4 cell/cm². After 12 h culture, unattached cells were removed by PBS rinsing. The remaining cells were fluorescently stained to label paxillin (green) and nuclei (blue). The images were captured by Leica TCS SP5 CLSM. Scale bar = 20μm.

In conclusion to this study, surface modification with the immobilization of gelatin and chitosan on PLLA both enhanced the number of cells adhered on substrates and improved cell spreading and FA formation.

4.3.2 Cell proliferation

The attached cells were allowed to proliferate in BBE-supplemented EGM for 7 days. Figure 4-5 shows the cell proliferation profile on various PLLA substrates as a function of culture duration. The doubling time calculated from the proliferation curve on each substrate is presented in Table 4-2. Cell proliferation profile on TCPS was considered as a reference. HUVEC grown on TCPS and PLLA-gAA-gelatin proliferated with doubling time of about 16-18 hours. Then cell proliferation slowed down after 72 h with a much longer doubling time of 50 hours. It is believed that contact inhibition took place approximately as cell density increased to about 6×10^4 cell/cm² on both surfaces at 72 h (Dejana, 2004). In contrast, cells grown on PLLA-gAA-chitosan and PLLA proliferated with a constant doubling time until 168 h of incubation. The doubling time on PLLA-gAA-chitosan was marginally shorter than that on PLLA. The cell proliferation study suggests that cell proliferation rate was considerably affected by the surface chemistry. Gelatin immobilization shortened the doubling time by a factor of two. Only marginal improvement on proliferation was found on PLLA-gAA-chitosan.

Table 4-2. Doubling time observed on different surfaces.

	TCPS	PLLA	PLLA-gAA-gelatin	PLLA-gAA-chitosan
12h -72 h	15.8	31.1	17.9	28.5
72 h-168h	49.3	29.9	48.5	28.1

Figure 4-5. Proliferation profile for HUVEC cultured over 7 days at 37°C in humidified air with 5% CO₂. Cell seeding density is 10⁴ cells/cm². The y-axis is a log scale (base 2).

After 7 days incubation, cells grown on different PLLA substrates were fixed and observed by SEM (Figure 4-6). Complete endothelialization was found on PLLA-gAA-gelatin and PLLA-gAA-chitosan. HUVEC displayed “spread out” morphology and formed monolayer similar to that of endothelium. However, such endothelialization was absent on PLLA.

Figure 4-6. Cell morphology observed on (A) PLLA, (B) PLLA-gAA-gelatin and (C) PLLA-gAA-chitosan by SEM at day 7. Complete endothelialization was observed on both modified PLLA substrates, but not on unmodified PLLA. Scale =100μm.

4.3.3 Cell retention under shear stress

Endothelium, the monolayer of EC, lines the entire blood vasculature, and is constantly exposed to a hydrodynamic shear stress of 8-12 dynes/cm² (Tugulu, 2007). In view of the potential application of the biomolecules modified PLLA in blood-contacting

devices, it is crucial to determine whether EC adhered to modified surfaces are able to withstand these shear forces. Hence, cell adhesion strength on different PLLA substrates was evaluated using a parallel flow chamber system (Tugulu, 2007). Figure 4-7 displays the cell retention percentage (R%) based on the cell counts from nuclei-stained images of HUVEC on biomaterial surfaces before and after shear stress. At the lowest shear stress of 3 dyn/cm², cell loss was negligible on all the three types of PLLA substrates. Cell retention percentage of PLLA dropped to 38% when shear stress increased to 15 dyn/cm². In contrast, 83% cells were able to remain on PLLA-gAA-gelatin and 64% on PLLA-gAA-chitosan. When the shear stress was further increased to 25 dyn/cm², 60% of the cells were detached from PLLA, while 50% on PLLA-gAA-chitosan. PLLA-gAA-gelatin proved the best pro-adherence surface by maintaining 80% cell retention post stress. This study demonstrated a significant and noticeable increase in cell adhesion strength on both modified PLLA than that on unmodified PLLA, especially at high shear stress levels. Cells were proved to make firmer adhesion on PLLA-gAA-gelatin than that on PLLA-gAA-chitosan.

Figure 4-7. Cell retention (%R) on various PLLA substrates was plotted in the function of shear stress. HUVEC were incubated on various substrates for 24 h before shear stress exposure.

4.3.4 Cell adhesion strengthening mechanism

As reviewed in Chapter 2 section 2.3.3, cell spreading, integrin clustering and FA formation are the three major mechanisms contributing to the increase of cell adhesion strength during the process of cell-substrate adhesion (Gallant, 2007). Hence, we speculate that the difference in cell adhesion strength observed on various PLLA substrates arises from the difference in the characteristics of cell-biomaterial interactions, which are associated with cellular morphology, FA formation and FN fibrillogenesis. In order to correlate the findings of cell adhesion strength with cell-biomaterial interaction characteristics, HUVEC were seeded and incubated with various PLLA substrates in the same experimental conditions as described in section 4.2.4.

4.3.4.1 Contribution of cell morphology

Cell morphology on various PLLA substrates was observed through F-actin (red) and nuclei (blue) staining, and imaged using CLSM. As shown in Figure 4-8 A, HUVEC seeded on all three PLLA substrates showed distinctly matured cytoskeletal structures, *i.e.* defined F-actin stress fiber bundles, stretching from nuclei to cell membranes crossing the entire cytoplasm. Classic cobblestone morphology of EC (Dejana, 2004) was observed only on PLLA-gAA-gelatin, whereas cell membranes appeared concave on PLLA and PLLA-gAA-chitosan. These findings are corroborated by others (Yan Huang, 2005). Those observations indicated compromised cell morphology as well as poor cell affinity on PLLA and PLLA-gAA-chitosan.

Cell spreading area of at least 10 cells on each substrate was measured using Image Pro Plus software. The results are compared in Figure 4-8 B. The average cell spreading area was 1604 μm^2 on PLLA-gAA-gelatin, 1508 μm^2 on PLLA-gAA-chitosan and 1063 μm^2 . Spreading area on PLLA was significantly less than that on gelatin (* $p=0.0272$) and chitosan (* $p=0.0418$) modified PLLA. Cell spreading area is known to

correlate with substrate physiochemical properties, since cells tend to have larger spreading area on higher affinity substrates (Boura, 2006).

Figure 4-8. (A) Cell morphology observed on various PLLA substrates after 24 h incubation. The HUVEC were fluorescently stained to label nuclei (blue) and the F-actin (red). The images were captured by Leica TCS SP5 CLSM. Scale bar = 20μm. (B) Cell spreading area was observed on various PLLA after 24 h incubation. *Significant difference in cell spreading area was observed using student's t-test in comparison to PLLA.

4.3.4.2 Contribution of focal adhesion formation

FA are one type of integrin-mediated cell-matrix adhesions through which cells sense their environmental cues, regulate other cellular responses, and establish adhesion strength to withstand physiological forces (Vogel, 2006; Geiger, 2009). Mature FA are elongated structures, predominantly orientated in the direction of the cytoskeletal stress

fibers (Balaban, 2001). They usually occur within the range of 2-6 μm in length and are distributed along the cell periphery (Goffin, 2006). Vinculin is one of the key structural proteins in FA complexes, whose N-terminal connects to integrin through talin while the C-terminal binds to F-actin (Humphries, 2007). Thus, FA formation on three different PLLA substrates was considered as the localization of vinculin rich structures. In this study vinculin proteins were immuno-stained. As indicated in Figure 4-9 A, vinculin localization to FA was observed on all three substrates and FA appeared to be “characteristic discrete spear-like structures”. It suggests that HUVEC were able to form FA on PLLA substrates regardless of the surface properties.

The size of FA and FA-per-cell observed on each type of PLLA substrate were studied as well. At least 7 cells were measured on each substrate. Histogram distributions of FA size were plotted in Figure 4-9 B. On PLLA, about 50% of measured FA size ranged from 1 to 2 μm , 30% from 2-3 μm , and 10% from 3-4 μm . On PLLA-gAA-chitosan, 40% of measured FA size ranged from 1 to 2 μm , 40% from 2-3 μm and 15% from 3-4 μm . On PLLA-gAA-gelatin, 20% of measured FA size ranged from 1 to 2 μm , 50% from 2-3 μm and 20% from 3-4 μm . The distribution peak of FA size shifted to a larger value by immobilizing chitosan or gelatin on PLLA surface. It implied that FA formation was modulated by surface properties. Cells on PLLA-gAA-gelatin and PLLA-gAA-chitosan had almost twice FA (averaging 46 FA-per-cell and 40 FA-per-cell respectively) compared to cells on PLLA substrate (averaging 22 FAs/cell). It suggests that immobilization of gelatin and chitosan on PLLA substrate improved FA formation.

Figure 4-9. (A) Vinculin localization to focal adhesions was observed on various PLLA substrates as indicated by red arrows. HUVEC were incubated with the substrate for 24 h and vinculin was immuno-labeled (green). The images were captured by Olympus IX71 fluorescent light microscopy. Scale bar = 20μm. (B) The histograms show the distribution of FA size observed on various PLLA substrates. The x-axis represents the FA size in the unit of μm and the y-axis represents the fractional frequency of occurrence.

After HUVEC were harvested from various PLLA substrates, the fluorescent intensity of FITC labeled vinculin was measured using flow cytometry (Figure 4-10). Vinculin expression on PLLA-gAA-gelatin ($p=0.0406$) and PLLA-gAA-chitosan

($p=0.0473$) was significant higher than that on unmodified PLLA. The measured vinculin includes the vinculin deposited at FA as well as that diffused in cytoplasm.

Figure 4-10. Vinculin expression by HUVEC seeded on (A) TCPS, (B) PLLA, (C) PLL-gAA-gelatin and (D) PLLA-gAA-chitosan. * $p<0.05$ refers to statistical significance with respect to PLLA.

When combining these findings, it may be inferred that FA formation, its size and number-per-cell as well as FA protein expression were all influenced by substrate physiochemical properties. PLLA-gAA-gelatin showed the best ability in promoting FA formation and clustering, followed by PLLA-gAA-chitosan, ahead of unmodified PLLA.

4.3.4.3 Contribution of fibronectin fibrillogenesis

Fibronectin (FN) fibrils are a prominent component of FN fibrillogenesis and ECM remodeling (Ilic, 2004). According to Pankov, FN fibrils were polymerized when elongated fibrillar adhesion (FB) was formed through FN-bound integrin $\alpha_5\beta_1$ moving along actin stress fibers (Pankov, 2000). To study how surface properties modulate FN fibrillogenesis, FN matrix deposited by HUVEC on various PLLA substrates was examined using immunofluorescent imaging and the length of FN fibrils was measured using Image Pro Plus. At least 7 cells were measured on each substrate. FN strands

smaller than 1 μm were not considered as fibrils. On PLLA substrate (Figure 4-11 A), most of FN were short fibrils with a granular appearance (Ilic, 2004). The majority of FN fibril length was less than 5 μm . On PLLA-gAA-chitosan substrate (Figure 4-11 B), abundant FN, organized in punctuated short fibrils, was observed. These complexes occurred mostly along cell boundaries. These organized FN patterns appeared to be thicker. Although the length of these fibrils was predominately within 5 μm , approximately 20% of FN fibrils were between 5 to 10 μm in length. On PLLA-gAA-gelatin, the FN fibrils displayed thick and elongated formations (Figure 4-11 C). In this case, most of these fibrillar FN (~85%) were greater than 5 μm in length.

Figure 4-11. FN fibril formation (as indicated by green arrows) observed on (A) PLLA, (B) PLLA-gAA-chitosan, and (C) PLLA-gAA-gelatin. Scale bar = 20 μm . (D) Length distribution of FN fibrils observed on these three substrates. Length was measured from CLSM images and using Image Pro Plus software.

4.4 Discussion

4.4.1 Cell affinity of modified PLLA

The cell affinity of modified PLLA was evaluated at both early stage (cell adhesion) and later stage of cell-biomaterial interaction (cell proliferation and endothelialization). Results suggested that immobilizing gelatin and chitosan on PLLA

improved cell adhesion (Figure 4-2), spreading (Figure 4-3) and FA formation (Figure 4-4) after 12 h incubation, furthermore both cell proliferation (Figure 4-5) and complete endothelialization were enhanced (Figure 4-6). PLLA-gAA-gelatin improved cell adhesion by almost 50% compared with unmodified PLLA, and shortened the cell doubling time by 10 h. The cell spreading and FA formation were improved significantly by gelatin-modified PLLA as well. At initial seeding density of 10^4 cells/cm², endothelialization was achieved on PLLA-gAA-gelatin within 7 days.

The surface properties are not the only factors influencing the quality of the cell interactions. Other factors play important roles in the process as well. Firstly, protein adsorption from body fluids *in vivo* or from cell culture medium *in vitro* on the biomaterial surfaces occurs prior to cellular contact. Secondly, cell-substrate interaction is mediated by affinity binding of integrins to cell adhesion ligands. Both spontaneously protein adsorption and intentionally bio-molecules immobilization upon the material surface could introduce such ligands so that to facilitate the cell adhesion.

In the case of unmodified PLLA, cell adhesive proteins such as FN and vitronectin, from the serum component of the medium, were adsorbed on the surface. However, the stronger binding and unfolding of protein on the hydrophobic PLLA surface may cause conformational changes in the protein molecules, thus lead to a decreased accessibility of specific epitopes of adsorbed protein on PLLA (Tzoneva, 2007) . Consequently, only a small number of integrins were able to activate through binding with cell adhesion domains, leading to the formation of less focal adhesions on PLLA than that of the two modified PLLA substrates (Figure 4-4).

In the case of PLLA-gAA-gelatin, immobilized gelatin containing integrin-binding domains was present on surface with density of $4.8 \mu\text{g}/\text{cm}^2$, providing cell adhesion sites. Moreover, PLLA-gAA-gelatin showed a contact angle of 52.8° (Table 3-3), which was in the wettability range favorable for cell adhesion. Studies have shown

that cells effectively adhere onto polymer surfaces presenting moderate wettability with water contact angles of 40-70° (Tamada, 1993; Arima, 2007). In contrast to hydrophobic PLLA, adsorbed serum proteins were loosely bound. The accessibility of specific domains was increased through remodeling of these adsorbed proteins (Tzoneva, 2007). The presence of both immobilized gelatin and adsorbed proteins were believed to improve cell affinity, resulting in better cell spreading and abundant focal adhesions observed on PLLA-gAA-gelatin in Figure 4-3.

Unlike gelatin containing cell adhesion domains, chitosan is considered as positively charged molecule. Hence, PLLA-gAA-chitosan appeared to be the most hydrophilic surface with contact angle of 30.7° (Table 3-3). The enhanced cell adhesion in the first 12 h might be due to the electrostatic interaction between cell surfaces, which are usually negatively charged, with the positively charged PLLA-gAA-chitosan surface. However, proliferation rate (*i.e.* doubling time) was improved only marginally on PLLA-gAA-chitosan. The positive charged surface is able to attract cell during the cell adhesion stage, however the electrostatic force between cell surface and substrate may reduce cell motility and hinder cell proliferation. Although proliferation rate of PLLA-gAA-chitosan was similar to that of PLLA, endothelialization was achieved within 7 days in this study on PLLA-gAA-chitosan but not on PLLA. It was largely attributed to the improved cell adhesion achieved at initial stage. This implies that initial cell adhesion is of considerable importance in achieving successful endothelialization. Other groups have reported similar observations in their study of immobilization of gelatin and chitosan to improve polymer cytocompatibility by other means (Zhu, Gao, Liu, He, , 2004).

In summary, by introducing biomolecules on PLLA surface, surface treatment not only changed the surface chemistry but also improved surface wettability. Both factors contributed to better EC affinity on modified PLLA surface which led to successful endothelialization.

4.4.2 Cell adhesion strengthening mechanism

In this study, modification of PLLA with gelatin or chitosan improved the cell adhesion strength under shear stress (Figure 4-7), which is crucial for blood-contacting application. To assess adhesion strength, we analyzed the cellular morphology, FA and fibrillar FN formation on various PLLA substrates to study the underlying cell adhesion strengthening mechanism.

4.4.2.1 Contribution of cell spreading area

The correlation between cell spreading areas on various PLLA substrates with cell adhesion strength was statistically tested. The correlation coefficient was tabulated in Table 4-4, together with other contributing factors to cell adhesion strength. Similar to our finding that greater cell spreading area was correlated with stronger cell adhesion, McClay *et al.* showed that initial adhesion strength within 15 min culture is positively correlated with areas of cell-substrate close contact (Lotz, 1989; Gallant, 2005). Gallant *et al.* used micropatterned FN dots with diameter ranged from 2 μm to 20 μm to control the available adhesive area. Cell adhesion strength of fibroblasts was found to vary linearly with the available adhesive area, up to the threshold value of 78.5 μm^2 (Gallant, 2005). Other researchers also showed that better cell spreading resulted in greater cell adhesion strength (Yan Huang, 2005).

A macroscopic model was developed by Gallant *et al.* to analyze cell adhesion strength under hydrodynamic forces on FN micropatterns (Gallant, 2007). In their model, adherent cell was considered as a hemispherical hat sited on substrates as shown in Figure 4-12. In the force equilibrium, the applied hydrodynamic shear force (F_s) and torque (T_s) are balanced by a tangential force (F_{tan}), tensile (F_T) and compressive (F_C) force at the adhesive interface. Cell detachment from substrate is assumed through “peeling detachment mechanism”. The farthest attachment points on substrate resist most of the

detachment force. Once these bonds are broken, cell could detach from substrate (Gallant, 2007). Along the leading edge of cell-substrate contact area, the tensile force is highly non-uniform with maximum at the periphery and decaying rapidly toward the center of the cell. As the cell spreading area increases, the distance of the farthest cell-substrate contact point from the center of the spreading area increases. Thus, the lever arm of the resultant bond force is effectively increased. That may explain the positive correlation between spreading area and the cell adhesion strength observed in current study. The correlation coefficient of spreading area with respect to the cell retention listed in Table 4-3 is 0.8942 at 15 dyn/cm² and 0.9654 at 25 dyn/cm².

Figure 4-12. Macroscopic model of adherent cells resisting hydrodynamic force.

Table 4-3. Correlation coefficient of shape index, FA size, FA per cell and fibrillar FN size with respect to the cell retention.

	R% at 15 dyn/cm ²	R% at 25 dyn/cm ²
Spreading area	0.8942	0.9654
FA size	0.9654	0.8842
FA-per-cell	0.9841	0.9209
Length of FN Fibrillar	0.8951	0.9713

4.4.2.2 Contribution of focal adhesion

By comparing the FA size and FA-per-cell on different PLLA substrates (Figure 4-9), PLLA-gAA-gelatin presented the most suitable surface to promote FA formation since the most predominant and dense FA were detected, followed by PLLA-gAA-chitosan. This observation fits with the theory proposed by Kato *et al.*, according to which, FA formation process consisted of two consecutive events, nucleation and growth; the initial clustering of integrin-mediated adhesions and their subsequent coarsening. The number and size of FA formation are dependent on integrin-ligand affinity (Kato, 2004). For certain ligand densities, high affinity ligands result in smaller FA with greater density per cell, whilst low affinity ligands result in larger FA with less density. In current study, comparisons of FA formation on different PLLA substrates were analyzed to assess the importance and role of the following surface of the PLLA surfaces.

Surface density of cell adhesion ligands. The surface density of cell adhesion ligands varies on different PLLA substrates with respect to their surface properties. In the case of PLLA-gAA-gelatin, surface immobilized gelatin, contains cell adhesion ligands (such as RGD) and promotes cell adhesion (Ruoslahti, 1987). In contrast, PLLA and PLLA-gAA-chitosan lack cell adhesion ligands on their surface (Refers to Table 3-2). Therefore, the density of integrin-binding ligands was considerably higher on PLLA-gAA-gelatin than that on the other two PLLA substrates. Higher ligand density provides more chance to initiate FA formation (nucleation), which most likely leads to higher FA-per-cell, as demonstrated in HUVEC grown on PLLA-gAA-gelatin, in comparison of that on PLLA or PLLA-gAA-chitosan (Figure 4-9).

Surface chemistry. The available cell adhesion ligands may come from pre-adsorbed proteins as well, such as FN (Dekker, 1991; Tamada, 1993; Steele, 1995; Barrias, 2009). The surface chemistry was found to modulate the pre-adsorbed FN

conformation and to affect the binding affinity to integrins (Keselowsky, 2003). It was reported that the binding of $\alpha_5\beta_1$ integrins to adsorbed FN on surfaces presenting either $-\text{NH}_2$ or $-\text{COOH}$ were more than that on surface presenting $-\text{CH}_3$. In current study, $-\text{NH}_2$ and $-\text{COOH}$ are present on PLLA-gAA-chitosan surface, while $-\text{CH}_3$ is present on unmodified PLLA surface (Table 3-2). This implies that the effective density of integrin-binding ligands on PLLA-gAA-chitosan was more than that on PLLA. Subsequently, this difference in surface chemistry of these two substrates led to the larger number of FA-per-cell observed on PLLA-gAA-chitosan compared with unmodified PLLA.

Surface wettability. Surface wettability influence FA formation in two aspects. First, hydrophobic surfaces decrease the accessibility of specific domains of the adsorbed proteins (Tzoneva, 2007), and effectively prohibit cell adhesion due to competitive adsorption between albumin and adhesive proteins (FN and vitronectin) (Arima, 2007). It further explained the fact that the availability of integrin-binding ligands was relatively rare on PLLA in comparison to the hydrophilic surfaces of PLLA-gAA-gelatin and PLLA-gAA-chitosan. Second, surface wettability influences the size of FA. According to Kato *et al.*, the growth of FA can be described as the subsequent process of individual receptors or smaller and mobile clusters diffusing to the nucleated clusters (Kato, 2004). It implies that the readiness of integrin diffusion influences the growth of FA. It has recently been proved that protein diffusion coefficients are strongly dependent on surface chemistry: protein diffuses four times faster on hydrophilic surfaces than on hydrophobic surfaces (Vieira, 2009). On PLLA whose surface is hydrophobic (Table 3-3), integrin diffusion was most likely hindered as a result of the strong hydrophobic interaction between integrin-ligand complexes and surface, thus FA growth was somewhat restricted. By contrast, immobilization of gelatin and chitosan on PLLA improved the PLLA wettability (Table 3-3). The protein diffusion on gelatin and chitosan modified PLLA surfaces was supposedly faster (Vieira, 2009), thus FA growth was most likely faster.

Hence, the dependence of protein diffusion rate on surface wettability explained the punctured and short FA found on PLLA, and significantly longer FA on PLLA-gAA-chitosan and PLLA-gAA-gelatin (Figure 4-9).

In summary, the variation in size and number-per-cell of FA on different PLLA substrates was found to be associated with substrate surface physiochemical properties. FA assembly were reported to contribute approximately 30% of the cell adhesion strength in vinculin-deficient NIH3T3 cells through siRNA knock down of vinculin expression (Gallant, 2005). The cell adhesion strength was also correlated with the area or length of the FA (Balaban, 2001). In this study, FA was most predominant on PLLA-gAA-gelatin in terms of both larger size and greater number-per-cell in comparison to that on PLLA-gAA-chitosan and PLLA. The strong association of cell adhesion strength with FA size and FA-per-cell were found in present study from their correlation analysis in Table 4-4 where various correlation coefficients were listed. The experimental findings suggest that FA formation is important to the strength of cell adhesion, in that larger and denser FA provide stronger cell anchorage on substrate.

4.4.2.3 Contribution of FN fibril formation

The formation of FN fibrils or FN fibrillogenesis was found to regulate the composition and stability of extracellular matrix (Sottile, 2002). Recent studies have shown that the physiochemical properties of material surface can promote or inhibit the formation of FN matrix fibrils (Faucheux, 2004; Faucheux, 2006; Tzoneva, 2007). Two aspects of surface properties could be discussed to interpret the FN fibrils formation observed in this study (Figure 4-11):

Surface wettability is one of very important surface properties affect FN matrix formation (Tzoneva, 2007). It has been proven that hydrophobic substrates inhibit fibrillar adhesion (FB) formation and FN fibrillogenesis in fibroblasts (Altankov, 1997; Faucheux,

2006). Lack of FN fibrillogenesis in HUVEC grown on hydrophobic surface was reported as well (Tzoneva, 2007). It was believed that strong hydrophobic interaction between adsorbed FN and substrate altered FN conformation and its binding affinity to integrins (Andres J. García, 1999; Keselowsky, 2003; Keselowsky, 2004). The lack of FN fibrillogenesis observed on PLLA in current study may be explained by its hydrophobicity as well (Figure 4-11).

Surface chemistry was reported to have a strong influence on FN fibril formation by promoting or inhibiting segregation of FA and FB which is the prerequisite for FN fibrillogenesis (Faucheux, 2006). It was reported that the presence of $-\text{COOH}$ promoted FB, whereas the presence of $-\text{NH}_2$ inhibits FB in fibroblasts incubated in serum-free medium (Faucheux, 2006). The stronger electrostatic interaction between the negatively charged FN and the positively charged $-\text{NH}_2$ surface was ascribed to this finding (Faucheux, 2006). In this study, surface of PLLA-gAA-chitosan is rich in $-\text{NH}_2$ groups in comparison to PLLA-gAA-gelatin, which is most likely to be the reason of the better FN fibril formation on PLLA-gAA-gelatin.

4.4.2.4 The overall mechanism of cell adhesion strengthening

This study compared cell adhesion strength on three types of PLLA with different physiochemical surface properties. Cell morphology, FA and FN fibril formation were studied as contribution to cell adhesion strengthening. The overall mechanism of cell adhesion strengthening on substrate is illustrated in Figure 4-13. When shear stress is applied to adherent cell, the peeling effect of shear stress is countered by the anchoring to the ECM. The anchoring force or traction tension is generated from cytoskeleton stress fibers and distributed to fibrillar extracellular matrix (predominantly FN fibrils) through transmembrane connections, *i. e.* FA and FB. In addition, larger spreading area

contributes to stronger cell adhesion by increasing the lever arm of farthest adhesion points where the strongest of detaching force is exposed.

Figure 4-13. Schematic illustration on cell adhesion strengthening mechanisms from contribution of: (I) spreading area, (II) focal and fibrillar adhesion formation and (III) fibrillar FN formation.

4.5 Summary

The experimental results on cell affinity study of surface-modified PLLA substrates were reported according to three aspects: (1) Cell morphology, the expression of cell phenotype with regards to different modified surface; (2) Cell adhesion and proliferation, the ability of HUVEC to adhere and proliferate onto biomaterials; (3) Cell adhesion strength, the retention of HUVEC under shear flow. It was found that gelatin and chitosan modified PLLA significantly improved the EC affinity of PLLA substrate by enhancing cell adhesion and proliferation. Furthermore, complete endothelialization was observed within 7 days culture on both the modified PLLA substrates with seeding density of 10^4 cells/cm², but not on the unmodified PLLA. Cell spreading area and FA formation on both modified PLLA were significantly improved, which indicates better cell affinity and spreading compared to unmodified PLLA. Cell adhesion strength was assessed using parallel flow chamber system under three different levels of shear stress

representing three typical physiological shear forces experienced by blood vessel at varying locations. Gelatin modified PLLA exhibited the highest cell adhesion strength, followed by chitosan modified PLLA. In contrast, PLLA had comparably less cell adhesion.

The cell adhesion strengthening mechanism on gelatin and chitosan modified PLLA surface was further studied. It was found that cell morphology, FA and fibrillar FN formation contributed to cell adhesion strength. Cells grown on PLLA-gAA-gelatin showed the best cell spreading, FA and FN fibrils formation. All the three features were correlated to the strongest cell adhesion observed on PLLA-gAA-gelatin. Though cells on PLLA-gAA-chitosan failed to exhibit the typical cobblestone morphology, the FA and FN fibril formation were moderately improved. This contributed to the greater cell adhesion strength found on PLLA-gAA-chitosan in comparison to PLLA.

According to the second objective of the work (Chapter 1), the experimental findings described in this chapter indicated substantial modulation of cell-biomaterial interaction by surface modification of PLLA. Gelatin and chitosan modified PLLA improved cell adhesion, proliferation and adhesion strength. Focal adhesion and extracellular matrix remodelling (in particular of FN fibrillogenesis) are more predominant on these two modified PLLA substrates which contributed to more stable cell adhesion in physiological flow condition.

Chapter 5 Seeding Density Matters

5.1 Introduction

Work on *in vitro* studies for EC-biomaterial interactions have previously employed a wide range of cell seeding densities from 4×10^3 to 2×10^5 cells/cm² (Lu, 2001; Gumpenberger, 2003; Yang, 2003; Miller, 2004; Crombez, 2005; Boura, 2006; Bérard, 2009; Z. G. Chen, 2010). To date, no standard protocol has been established to define the experimental seeding density required. This adds a complexity in comparing the observations between different studies carried out on the same substrate. It may be logical to assume that the biocompatibility of certain materials may be evaluated using high seeding densities, such as 2×10^5 cells/cm², as the exposure of cells in excess will emphasize the ability of a material to promote cell adhesion. However, the use of an exaggerated cell density may produce artifactual observations. For instance, a biomaterial that is required to sustain cell-material interactions in an environment in which supply of recruitable cells is limited. In the case of *in situ* endothelialization of blood-contacting devices, regrowth of the EC layer may be derived from the migration of EC from adjacent tissue (“trans-mural endothelialization”) and/or attachment and proliferation or the circulating EPC (Rotmans, 2005; Brewster, 2007; Avci-Adali, 2008). Available cell density in both situations is considered to be low (Hill, 2003; Q. Lin, 2010).

Cell-matrix adhesion is predominantly mediated by integrins, involving integrin-based FA complexes tightly associated with the F-actin cytoskeleton (Geiger, 2009). Cadherins is a key cell-cell adhesion molecule localized at adherens junctions (Nelson, 2008). Cell-cell adhesions are sites of physical connection and signaling transduction to regulate cell behaviors (Dejana, 2004). Integrins and cadherins are two distinct families of transmembrane cell adhesion receptor, yet cadherins has a function similar to that of integrins within FA complexes. The distinguishing differences between

the two are that integrins bind to the ECM; cadherins bind homotypically to the cadherins on the neighboring cells. Arthur *et al.* (Arthur, 2002) showed that the signaling cascades of both cell-matrix and cell-cell adhesion, transmitted through integrins and cadherins respectively, involve Rho proteins, which mediate in the reorganization of the actin cytoskeleton. Other studies have demonstrated cross-talk between cell-matrix and cell-cell junctions and that both types of junctions cooperatively regulate cell movement, proliferation, adhesion and polarization (Geiger, 2001; Schwartz, 2002; Sakamoto, 2006).

The strength of stimuli from neighboring cells via interaction of cell-surface receptors and secreted growth factors/cytokines are strongly dependent on the cell density. When the cell density is low, direct cell-cell contacts are limited. Therefore cell-biomaterial interactions are expected to be pre-dominantly influenced by cell-substrate contact. As cell density increases, cell-cell interaction becomes more extensive and is expected to profoundly influence cellular responses to biomaterials (Nagahara, 1996).

Due to the conditions described above, there is a distinct possibility that, *in vivo*, the EC will interact with the biomaterial surface with minimal influence from intercellular contact with other EC. Hence, there is a need to study the effects of initial seeding density on assessing the EC-biomaterial interaction. In this study, a low seeding density of 5000 cells/cm² was compared with a high seeding density of 50,000 cells/cm². Cell-biomaterial interaction was studied in terms of cell adhesion, morphology and proliferation of HUVEC seeded on various PLLA substrates. It will reveal the sensitivity of the biomaterial to cell number, thus allowing an assessment of possible artifacts generated at “non-representative” cell densities. Three type PLLA substrates, PLLA, PLLA-gAA-chitosan and PLLA-gAA-gelatin, were prepared following the surface modification method described in chapter 3.

5.2 Materials and Methods

5.2.1 Cell adhesion and proliferation

The materials and methods used in this chapter are as same as that described in chapter 4, unless is specified.

The low seeding density of 5000 cells/cm² represented the confluent level whereby HUVEC were sparsely distributed on the substrate. The high seeding density of 50,000 cells/cm² represented the 100% confluent level whereby HUVEC were saturated on substrate.

5.2.2 Mechanistic study on improved cell attachment in high-density seeding

Two mechanisms are possible for the growth of cells under high seeding density conditions. First, the secreted growth factors and cytokines in high-density seeding are more concentrated than that in low-density seeding. In order to study the influence from secreted growth factors and cytokines on cell attachment, seeding of 5000 cells/cm² HUVEC on unmodified PLLA substrate was carried out in either conditioned or fresh medium. Conditioned medium was prepared by incubating fresh EGM with confluent HUVEC in a tissue culture flask for 24 h. Then the conditioned medium was pipetted out for centrifugation (3600rpm, 10 min) to remove detached cells and debris. HUVEC constituted in either conditioned or fresh culture media were seeded at a density of 5000 cells/cm² on PLLA films placed within 24-well tissue culture plate. The cells were allowed to attach for 2 h. Cell attachment study was performed following the method the WST-8 assay described in Chapter 4.

Second, frequent cell-cell interaction between neighboring cells at higher density can promote initial cell attachment. In order to study the effects of extensive cell-cell interactions at high-density seeding, seeding of 5000 cells/cm² HUVEC was carried out on both pristine and pre-seeded PLLA. Pre-seeded PLLA was prepared by seeding

HUVEC at a density of 25,000 cells/cm² on PLLA films placed within 24-well tissue culture plate for 24 h. HUVEC at density of 5,000cell/cm² were seeded on either bare PLLA or pre-seeded PLLA. Cells were allowed to attach for 2 h, and the number of attached cells was quantified by WST-8. The cell attachment percentage on bare PLLA was calculated as $N_{2h}/5000 \times 100\%$. The cell attachment percentage on pre-seeded PLLA was calculated as $(N_{2h}-N_{pre})/5000 \times 100\%$, where N_{2h} and N_{pre} were the cell count at 2 h and the initial pre-seeding respectively.

5.3 Results and discussion

5.3.1 High seeding density masks the surface dependence of cell adhesion

Cell adhesion percentage values were compared between low and high seeding density experimental groups, with the results illustrated in Figure 5-1A. In the case of 5000 cells/cm² seeding density, the cell adhesion percentage appeared to be surface dependent (ANOVA, * $p < 0.05$). After 2 h incubation, about 30% of seeded cells had adhered on unmodified PLLA. The presence of immobilized biomolecules on the PLLA surface enhanced cell adhesion: 90% adhesion was observed on PLLA-gAA-gelatin and 50% on PLLA-gAA-chitosan. These observations agreed well with our previous results (Figure 4-2). In contrast, the surface dependence of cell adhesion was not observed at high seeding density of 50,000 cells/cm². Instead, all substrates appeared to be equivalent: about 70% of seeded cells were able to attach on all three PLLA substrates regardless of their differences in surface properties. High seeding density significantly improved cell adhesion on PLLA and PLLA-gAA-chitosan ([#] $p < 0.05$). Interestingly, cell adhesion percentage on PLLA-gAA-gelatin decreased from 90% to 70%. However, the total cell count on PLLA-gAA-gelatin in fact increased from 4500 to 35,000. It is conceivable that high seeding density enhanced cell adhesion during the early phase of cell-biomaterial interaction and masked any

differences in the substrate surface. Surface dependence of cell adhesion was only observed at low seeding density by eliminating intensive cell-cell interaction.

The enhancement of HUVEC adhesion at high seeding density can possibly arise from two mechanisms: (a) the increased concentrations of secreted growth factors and cytokines at higher seeding densities can stimulate cell adhesion signaling pathways; (b) intensive cell-cell interactions between neighboring cells at higher density can activate cell attachment signaling pathways through crosstalk between cell-cell and cell-substrate adhesion.

To investigate the first possible mechanism, cell adhesion on PLLA at 5000 cells/cm² in conditioned medium was compared with that in fresh medium. As illustrated in Figure 5-1B, cell adhesion percentage values were about 37% in both conditions. This implies that increased secretion of growth factors and cytokines at high seeding densities had negligible effect on cell adhesion during 2 h incubation.

To investigate the second possible mechanism, cell adhesion on PLLA at 5000 cells/cm² was compared with that on pre-seeded PLLA. As shown in Figure 5-1B, a significant increase of cell adhesion percentage from 37% to 72% was observed by pre-seeding PLLA. This suggested that the presence of pre-seeded HUVEC promoted the adhesion of incoming cells. This test demonstrated a possible scenario occurring at high seeding density, whereby adherent cells hastened the adhesion of cells from free suspension, through cell-cell interaction followed by triggered cell-substrate adhesion. Another possible scenario is that high seeding density favors the formation of cell-cell adhesion in suspension even prior to cell-substrate adhesion. Clumps of associated cells sediment and attach on the substrate simultaneously, such that cell adhesion was significantly improved even on low cell affinity substrates. At present, we cannot distinguish between these two possible mechanisms.

Zhu *et al.* (Zhu, Gao, Liu, He, , 2004; Zhu, Gao, Liu, & Shen, 2004)

demonstrated that cell adhesion on gelatin and chitosan modified PLLA (modified through aminolysis) was comparable at a seeding density of 12×10^4 cell/cm², which agreed with our results at high seeding density. However, in current study, it was discovered the superiority of cell adhesion on PLLA-gAA-gelatin compared to that on PLLA-gAA-chitosan at a relatively low seeding density of 5000 cell/cm². The data showed that the differences among substrates were more pronounced at low seeding density, but was masked at high seeding density due to cell saturation. This should be taken into consideration whenever biomaterials are to be evaluated for use in applications involving low cell densities.

Figure 5-1. (A) High seeding density masks the surface dependence of cell adhesion. Surface-dependent cell adhesion was analyzed with ANOVA, $*p < 0.05$. Density-dependent cell adhesion was analyzed with paired Student's t-test, $\#p < 0.05$. (B) Growth factors and cytokines secretion showed no significant effect on cell adhesion by comparing seeding in fresh medium to that in conditioned medium. Pre-seeded cells on PLLA greatly promoted cell adhesion ($**p < 0.05$) by comparing seeding HUVEC on unmodified PLLA to that on 25000 cell/cm² pre-seeded PLLA.

5.3.2 The influence of surface chemistry on cell morphology, independent of cell density

After 2 h incubation, the cell morphology was observed by immunostaining of F-actin, vinculin and nuclei. As shown in Figure 5-2, the cellular morphology did not exhibit distinctive differences upon comparing cells grown on the same substrate at low (5000 cells/cm²) and high (50,000 cells/cm²) seeding densities. On PLLA-gAA-gelatin (Figure 5-2 C and D), cells observed at both seeding densities exhibited the characteristic morphology of spreading HUVEC. Bundles of F-actin were found in cell lamellipodia, which are the broad, flat protrusions at the leading edge of a motile cell. Filopodia, the thin finger-like structures filled with tight parallel bundles of F-actin, were readily detected, protruding from the lamellipodia. Both of these are well-known structures involved in cell spreading and migration (Mattila, 2008). On PLLA-gAA-chitosan (Figure 5-2 E and F) seeded with either 5000 or 50,000 cells/cm², the cells were observed to have significantly reduced spreading area in high-density seeding. This was further confirmed in the subsequent morphometric parameter analysis. Nevertheless, the typical spreading cell morphology was observed under both seeding density conditions, *i.e.* defined lamellipodia and filopodia with F-actin bundles localized mostly near the cell periphery. On PLLA (Figure 5-2 A and B), no obvious difference in cell morphology between low and high density seeding was found, other than the reduced cell spreading area. Compared to the cell morphology on gelatin and chitosan modified PLLA, cells on unmodified PLLA developed much thinner F-actin bundles beneath the cell membrane and hardly any vinculin expression, thus indicating poor EC affinity for unmodified PLLA as demonstrated in our previous study.

Figure 5-2. Surface chemistry, rather than seeding density, influences cell morphogenesis. HUVEC were seeded on PLLA (A and B), PLLA-gAA-gelatin (C and D), and PLLA-gAA-chitosan (E and F) at low seeding density of 5000/cm² (A, C and E) or high seeding density of 50,000/cm² (B, D and F). After 2 h culture, unattached cells were removed by PBS rinsing. The remaining cells were fluorescently stained to label nuclei (blue), vinculin (green) and the F-actin (red). The images were captured by Leica TCS SP5 CLSM. Scale bar = 20μm.

Cell morphology was evaluated using two morphometric parameters, cell spreading area and aspect ratio. Cell spreading area measures the extent of cell spreading on a substrate as projection area. As shown in Figure 5-3, the cell spreading area observed from a confluent EC monolayer grown on tissue culture plate (TCPS) was 1400 μm^2 on average. After 2 h incubation, cell spreading under any of the experimental conditions was not able to reach that value. It is also noted that cell spreading areas from the high seeding density group were significantly smaller than those observed in the low seeding density group. Spatial restriction is believed to be the main reason for the decrease in spreading area in the high seeding density group.

Normal EC displays a typical ‘cobble-stone’ morphology at confluence with an epithelioid phenotype, whereas sparsely distributed cells shows a fibroblastoid/mesenchymal morphology (Dejana, 2004). Cell aspect ratio is defined as the ratio of the major axis to the minor axis of an equivalent ellipse. In Figure 5-3, the cell aspect ratio of confluent EC monolayer grown on TCPS displaying cobble-stone morphology was about 2.38 on average.

Amongst the low-density seeding experimental group, the mean aspect ratio on PLLA-gAA-gelatin was about 2.80, suggesting a mesenchymal phenotype. By contrast, aspect ratios on PLLA and PLLA-gAA-chitosan were about 1.5, which were significantly less than that of cobble-stone morphology. This implied that although cells adhered on both surfaces, their cell morphology was somehow compromised. The absence of cell adhesion ligands on PLLA and PLLA-gAA-chitosan is believed to be the main reason for the compromised cell morphology observed on both substrates. Unlike PLLA-gAA-gelatin, PLLA and PLLA-gAA-chitosan contained no cell adhesion molecules/ligands on their surfaces. Cell adhesion in serum-free medium relied largely on hydrophobic or electrostatic interaction between substrate and adhesion ligands on the cell membrane. Amongst the high-density seeding experimental groups, cell aspect

ratio on PLLA-gAA-gelatin was reduced to 1.90, mostly due to cell-cell contact inhibition. The cell aspect ratio on PLLA and PLLA-gAA-chitosan was similar to that at low density seeding. It suggests that the dominant factor for aspect ratio is surface chemistry.

The data presented in Figure 5-3 suggest that cell morphogenesis during the early stage of cell-biomaterial interaction was strongly dependent on substrate chemistry rather than seeding density. However, seeding density did influence some cell morphometric parameters. Reduced cell spreading area was observed on all three PLLA substrates and a less elongated cell phenotype was seen on PLLA-gAA-gelatin at high seeding density.

Figure 5-3. Seeding density influenced (a) cell spreading area and (b) aspect ratio. Cell morphometric indicators observed on (A) TCPS, (B and C) PLLA, (D and E) PLLA-gAA-gelatin, (F and G) PLLA-gAA-chitosan. Cells on TCPS were confluent and incubated for 4 days. Low seeding density of 5000 cells/cm² was used in B, D and F. High seeding density of 50,000 cells/cm² was used in C, E, and G. High resolution immunofluorescent images of individual cells were taken by Leica TCS SP5 CLSM, and then analyzed by Image Pro Plus software. At least 50 cells were measured for each condition.

5.3.3 High seeding density masks surface dependence of cell proliferation

When EC are sparsely-seeded in the sub-confluent state, they are actively proliferating and are sensitive to growth-factor stimulation. Once confluence has been achieved, the cell proliferation is inhibited and cell apoptosis is protected (Liebner, 2006).

Figure 5-4 A shows the cell proliferation profiles on the various PLLA substrates as a function of culture duration, when HUVEC were sparsely seeded at a low density of 5000 cell/cm². Cells grown on TCPS and PLLA-gAA-gelatin proliferated with a doubling time of about 15-16 hours. In contrast, cell proliferation showed stagnation on PLLA-gAA-chitosan and even negative on PLLA during the first 72 hours. Cell growth took place only after 72 h. Figure 5-4B illustrates the cell proliferation profiles of various PLLA substrates, at a high seeding density of 50,000 cells/cm². Cells plated on TCPS, PLLA-gAA-gelatin and PLLA-gAA-chitosan exhibited similar proliferation behavior. Cell proliferation started a few hours after seeding with a doubling time of about 7-9 hours during the first 24 h. During 24-72 h, cell proliferation rate slowed down and eventually stopped. The number of HUVEC on TCPS and PLLA-gAA-gelatin did not increase significantly after 72 h, suggesting that confluence was achieved and that cell-cell contact inhibited proliferation. Cells grown on PLLA displayed an exponential proliferation pattern, which was unique in comparison to the other three substrates.

Comparing Figure 5-4 A to Figure 5-4 B, the profound impact of initial seeding density on cell proliferation is clearly evident. First, low density seeding revealed the superiority of PLLA-gAA-gelatin in supporting cell proliferation compared to PLLA-gAA-chitosan, while extensive intercellular contact at high density seeding masked this difference in EC behavior. Second, by plating a confluent monolayer at high seeding density, the proliferation stagnation on PLLA-gAA-chitosan and PLLA was overcome. In applications involving the *in situ* endothelialization of cardiovascular implants, such as coronary stents (Yingying Huang, 2009) and patent foramen ovale (PFO) occluders (M. Tanaka, 2006), the number of circulating EC or EPC is relatively low. At high seeding density, PLLA-gAA-gelatin and PLLA-gAA-chitosan showed equivalent performance in cell adhesion and proliferation. However, at low seeding density, PLLA-gAA-chitosan showed only marginal improvement in cell adhesion and proliferation over PLLA.

Clearly, PLLA-gAA-gelatin is a better candidate for such applications, a conclusion that could be reached only through studies at low seeding density.

Figure 5-4. Extensive intercellular contact masks surface dependence of cell proliferation. Cell proliferation was monitored over 7 days of culture using (A) low seeding density of 5000cells/cm², and (B) high seeding density of 50,000cells/cm².

After confluence was reached, cell morphology was observed through cytoskeletal and nuclei immunostaining (Figure 5-5). It was found that cell morphology was indistinguishable on the various PLLA surfaces at either low (A, C and E) or high (B, D and F) seeding density. The characteristic cobblestone morphology was observed with bundles of stress fibers extending from the nucleus to lamellipodia across the entire cytoplasm and terminating at FA and cell-cell junctions (Figure 5-5 G and H).

During cell spreading and proliferation, cells produce their own extracellular matrix. The self-synthesized matrix may in turn mask the intrinsic effects of the original substrate on cell proliferation. The results of this study corroborate with earlier reports - that differences in cell behavior rising from differences in seeding conditions (substrate and/or density) are more apparent during the first days of cell-biomaterial interaction (Reilly, 2010).

Figure 5-5. Substrate dependence of cell morphology disappeared at the point of confluence. Fluorescent micrographs of HUVEC were taken on PLLA (A and B), PLLA-gAA-gelatin (C and D), and PLLA-gAA-chitosan (E and F). Low seeding density of 5000cells/cm² was used in A, C and E, and high seeding density of 50,000cells/cm² was used in B, D and F. (G) Matured FA clusters were observed in HUVEC. (H) F-actin bundles terminated at FA clusters by colocalization of F-actin and vinculin. The cells were fluorescently stained to label nuclei (blue), vinculin (green) and F-actin (red). The images were captured by Olympus IX71 inverted microscopy. Scale bar = 50μm.

5.4 Summary

In accordance to the third objective of the work (Chapter 1), this study has demonstrated the important role of seeding density in cell-biomaterial interaction. During the early stage of cell-biomaterial interaction whereby cell adhesion is the pre-dominant cellular activity, low seeding density enabled us to observe surface chemistry-dependent cell adhesion behavior. High seeding density masked differences amongst substrates, and enhanced cell adhesion on all substrates examined. During the later stage of cell-biomaterial interaction, cell proliferation profile was found to be surface-dependent at low seeding density, whereas surface dependence was masked at high seeding density. For practical applications, the *in situ* endothelialization of blood-contacting surfaces will probably proceed from very low initial cell densities, and hence the results of this study are relevant to the clinical situation.

Chapter 6 Immobilization of recombinant vaults on solid substrates

6.1 Introduction

Native vaults are nanoscale particles found abundantly in the cytoplasm of most eukaryotic cells. They have a capsule-like structure with a thin shell surrounding a “hollow” interior compartment which is capable of accommodating hundreds of proteins. Recombinant vault particles were found to self-assemble following expression of the major vault protein (MVP) in a baculovirus expression system, and these particles were virtually identical to native vaults. Such particles have been recently studied as potential delivery vehicles. However, all of the previously reported work to date, which explored the possibility of vaults as a drug/protein delivery system, has used vaults in aqueous solutions. With the view of cardiovascular applications, our group would like to see the feasibility of incorporating vaults particles into solid matrices or onto substrates (such as PLLA). By doing this, the release of encapsulated bioactive agents, such as vascular endothelial cell growth factors (VEGF), could be better controlled and sustained. In addition, short cell adhesion ligands, such as RGD and REDV, could be fused on to C-terminus of recombinant vaults. These cell adhesion ligands expressed on immobilized particles may be able to provide beneficial cell-substrate interaction.

In this study, we focus on immobilization of vault particles on glass and PLLA, as a first step to study cell-substrate interaction on these vaults immobilized substrates. To this end, we first engineered the recombinant vaults by fusing two different tags to the C-terminus of MVP, a 3 amino acid RGD peptide and a 12 amino acid RGD-strep-tag peptide. We have demonstrated two strategies for immobilizing vaults on solid substrates. The barrel-and-cap structure of vault particles was observed for the first time, by atomic force microscopy (AFM), in a dry condition. Cell adhesion, proliferation and morphology of HUVEC were studied to assess the cell affinity of these vaults immobilized substrates.

6.2 Materials and Methods

6.2.1 Materials

(3-Aminopropyl)triethoxysilane (APTES) (99%, Aldrich), 1-ethyl-3-(3-dimethylamioethyl) carbodiimide hydrochloride (EDAC, Pierce), and 2-Morpholinoethanesulfonic acid (MES, Sigma), N-hydroxysuccinimide (NHS), 1% Triton X-100, 1 mM dithiothreitol, 0.5mM phenylmethylsulfonyl fluoride (PMSF), protease inhibitor cocktail, Tris HCl, glycerol, sodium dodecyl sulphate (SDS) and β mercaptoethanol bought from Sigma were used as received. All solvents were either HPLC grades or analytical grades and were purchased from Sigma and Aldrich.

6.2.2 Construction of C-terminus modified recombinant plasmids

The 3 amino acid sequence of the RGD tag is Arg-Gly-Asp. The RGD peptide is cloned in to the C-terminus or 3' end of the rat MVP cDNA using a unique internal *XhoI* site in the 3' end of the rat MVP cDNA (GenBank accession no.U09870). Two primers were designed. The forward primer is 5'-CAGCCCGGATCATTCGAATGGCTGTTTTTGGC-3', which is about 80 bases upstream (5') of the *XhoI* site in the rat MVP cDNA. The reverse primer is 5'-GCGCGGTACCTCAGTCTCCCCGCTTCTGTGCTGGCGGCTG-3', which is complimentary to the 3' end of MVP cDNA and encodes RGD. The 3' end of MVP was PCR amplified using forward and reverse primers with the CP-MVP pFastBac plasmid as the template. The PCR product (containing the 3' end of the MVP cDNA fused in frame to the RGD DNA) was purified on a Qiagen column, digested with *XhoI* and *KpnI*, and ligated to *XhoI/KpnI* digested CP-MVP pFastBac DNA to form CP-MVP-RGD pFastBac. All constructs were confirmed by DNA sequence analysis.

Likewise, CP-MVP-RGD-STP is made by fusing 3 amino acid RGD peptide and 9 amino acid strep-tag to C-terminus of CP-MVP. The 12 amino acid sequence of RGD-

STP tag is Arg-Gly-Asp-Ala-Trp-Arg-His-Pro-Glu-Phe-Gly-Gly. The forward Primer was designed as: 5'-CAGCCCGGATCATTCGAATGGCTGTTTTTGGCTTTGAGATGTCTGAAGACACAGGTCCTGATGGCACA-3'. The reverse was 5'-GCGCGGTACCTCAGCCGCCAAATTCCGGATGTCTCCAGGCGTCTCCCCCGCTTCTGTGCTGGCGGCTG-3', which is complimentary to the last 20 bases of the rat MVP cDNA, and encodes RGD-STP. The 3' end of MVP was PCR amplified using RGD-STP forward and reverse primers with the CP-MVP pFastBac plasmid as the template. The PCR product (containing the 3' end of the MVP cDNA fused in frame to the RGD-STP DNA) was purified on a Qiagen column, digested with XhoI and KpnI, and ligated to XhoI/KpnI digested CP-MVP pFastBac DNA to form CP-MVP-RGD-STP pFastBac. All constructs were confirmed by DNA sequence analysis. The vault targeting construct, mCherry-mINT pFastBac, was a generous gift of Professor Leonard Rome (Kickhoefer, 2009).

6.2.3 Expression, purification and characterization of recombinant vaults

Recombinant baculoviruses were generated according to the Bac-to-Bac protocol (Invitrogen). For vault purification, baculovirus-infected Sf9 insect cells were subjected to a standard protocol (Stephen, 2001). The protein concentration of purified vault proteins was determined by using the BCA assay (Pierce), and their purity was analyzed by fractionating on SDS-PAGE gel followed by staining with coomassie blue. All vault samples were routinely analyzed by staining with uranyl acetate and viewed on an electron microscope (Kedersha, 1986b). Western blot analyses on purified recombinant vaults were performed using anti-MVP rabbit polyclonal antibodies as primary antibody, and peroxidase-conjugated goat anti-rabbit IgG (BioRad) as secondary antibody (Kickhoefer, 2009).

To establish whether the C-terminal tags were functionally exposed, a bead binding assay was carried out. Lysates of sf9 cells infected with the appropriate baculovirus were prepared by lysing cells in 50 mM Tris-Cl, pH7.4, 75 mM NaCl, 0.5 mM MgCl₂ containing 1% Triton X-100, protease inhibitor cocktail (Sigma), and 1 mM PMSF, followed by centrifugation at 20000g for 10 min. The supernatants (S20) were used for the binding assays. Streptavidin beads (Streptavidin UltraLink[®] Resin, Pierce) were equilibrated in lysis buffer. S20 lysates from CP-MVP-RGD and CP-MVP-RGD-STP infected cells were mixed with beads for 1 hr at 4 °C. The beads were then washed with phosphate buffered saline (PBS) and boiled in SDS-PAGE samples buffer. The eluted proteins were loaded onto to 6% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with polyclonal anti-MVP antibodies followed by peroxidase-conjugated goat anti-rabbit IgG.

6.2.4 Immobilization of vaults on solid substrates

Glass substrate. As shown in Figure 6-1, glass coverslips (DI=15mm) were sonicated in a mixture of acetone and water (v/v=1:1) for 15 min, and then immersed in piranha solution (H₂SO₄: H₂O₂ = 7:3 v/v) for 60 min at 120°C. They were rinsed with copious amount of MilliQ water, and dried under a stream of nitrogen. The glass treated in this way is rich in hydroxyls at the oxide surface and suitable for silanization (H. Li, 2009).

PLLA substrate. In order to get ultra-smooth PLLA surface, spin coating technique was used to coat a thin layer of PLLA solution in dichloromethane solution (3.0 wt. %) at 3000 rpm on cleaned glass slide. After drying, the resultant PLLA film obtained is ~650nm. Then, the smooth PLLA film was treated by oxygen plasma cleaner (PDC-32G-2, Harrick Plasma, Ithaca, NY) with a power of 10.8 W for 1 min at a pressure of 120 mTorr. The PLLA surface treated in this way is rich in hydroxyls and suitable for silanization.

These hydroxyl-rich substrate then were immersed in 1 vol% APTES in Milli-Q water for 15 min. Then the substrates were rinsed with water to remove any excess of silanes, and subsequently dried under a stream of nitrogen. APTES-modified glass was heated in oven at 120°C for 1 hr, APTES-modified PLLA surface was heated at 55°C for 1 h.

In order to demonstrate the feasibility of immobilizing vault particles on a substrate for studies of cellular interaction and/or localized drug/gene delivery. Two strategies were developed as shown in Figure 6-1.

(1) *With electrostatic adsorption.* The first method is based on the electrostatic adsorption. Vault particles are reported to be negatively charged overall. We found that the vault particles could be easily adsorbed on a positively charged APTES-modified glass surface. 4 µg vaults, dissolved in 50 µl Milli-Q water, was deposited on APTES-modified glass and kept for overnight in humid box.

(2) *By covalent bonding.* The second method is based on the covalent bonding between the amine groups in the APTES-modified surface and the carboxylic groups in the exposed C-termini of vault particles *in* water soluble carbodiimide (WSC) solution. There are between 39 and 48 C-termini localized at each vault half at the caps, and exposed outside of the vault particle. EDAC and NHS were added into 25 mM MES solution, pH 6.5, to form the water soluble carbodiimide (WSC). APTES-modified glass was immersed into this WSC solution with 4µg of protein (MVP vaults or MVP-RGD vaults) loading. The reaction was kept for 10 h at room temperature.

Figure 6-1. The surface treatment protocol to immobilize vaults on solid substrates.

6.2.5 Surface characterization of vaults immobilized substrates

Surface density of immobilized vaults. After the immobilization reaction, glass substrates were washed with Milli-Q water to remove loosely adsorbed vault particles or free mCherry-mINT released from the broken vault particles. The amount of vault particles immobilized on APTES-modified glass was measured by fluorescent microplate reader (Tecan infinite M200) at excitation wavelength of 540 nm and emission wavelength of 640 nm. The amount of vaults immobilized on the APTES-modified glass coverslips were quantified using a standard curve constructed with mCherry fluorescent intensity against vaults particle mass.

Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR). IR spectra were measured using a Perkin-Elmer Spectrum™ GX system equipped with a Graseby Specac ATR detector. For one spectrum, 256 scans were coadded at a resolution of 4 cm⁻¹. The bare glass was measured for background spectra.

XPS. The surface composition of protein-coated APTES-modified glass was analyzed by XPS (Kratos AXIS Ultra) with monochromatic Al K α (1486.71 eV) X-ray

radiation (15 kV and 10 mA). 160 eV pass energy was used for survey scan whereas 40 eV was used for the high-resolution scan.

AFM. The protein-coated APTES-modified glass was washed by Milli-Q water and dried with nitrogen gas. A commercial AFM instrument (Dimension 3100 with Nanoscope IIIa controller, Veeco Instruments Inc., CA) equipped with a scanner (90 μm $\times$ 90 μm) was employed. The tapping mode in air was performed to observe the MVP particles on APTES-modified glass. Super sharp silicon cantilevers with the normal resonance frequency of 330 kHz and spring constants of 42 N m⁻¹ (SSS-NCH, Nanosensors) were used. All images were captured with scan rate at 1~2 Hz and 512 $\times$ 512 pixel resolution. At least 7 images were taken for each experimental sample.

6.2.6 Cell affinity of recombinant vaults immobilized substrates

Cell affinity of those recombinant vaults immobilized substrates, involving cell adhesion and proliferation, was assessed using the WST-8 assay described in Section 4.2.2. The results are presented as means $\pm$ SD (n=6). The cell morphology was imaged using Olympus IX71 light microscope. At least 7 images were taken for each experimental sample.

6.3 Results and Discussion

6.3.1 Structural characterization of C-terminally tagged vaults.

The C-terminus of MVP is localized in the “caps of the barrel”, and peptide additions are accessible. In this study, two types of C-terminally tagged recombinant vault particles, CP-MVP-RGD and CP-MVP-RGD-STP, were expressed. The expression of RGD at the C-termini of vault particles would be expected to lead to an increase of cell adhesion on the vault-immobilized substrate. Strep-tag is widely used in protein purification and detection because of its strong affinity to streptavidin. The expression of

the additional strep-tag at the C-terminus of CP-MVP-RGD is solely for detection of C-terminal tag expression, i.e., confirming the incorporation of RGD sequence in MVP.

Fractionation on a discontinuous sucrose gradient is the most important step during vault purification. Generally, vaults fractionate in the 40% and 45% layer depending on whether or not the particles are empty or filled with mINT fusion proteins. Empty particles are found in higher concentrations in the 40% layer, and filled particles fractionate in the 45% layer. Recombinant CP-MVP (Figure 6-2A, lane 1) as positive control, CP-MVP-RGD (Figure 6-2A, lane 2) and CP-MVP-RGD-STP (Figure 6- 2A, lane 4), purified from the 40% sucrose gradient fractions, were analyzed by immunoblotting with anti-MVP antibody. All three fractions contained MVP, indicating that proper vaults were formed for the three different constructs. S20 extract of Sf9 cells without infection of any baculovirus was analyzed as negative control (Figure 6-2A, lane 3), and showed no MVP production.

To establish whether the C-terminal tags were functionally exposed, a pull-down experiment with streptavidin beads was carried out. S20 extracts of CP-MVP-RGD-STP and CP-MVP-RGD were used for binding experiments, followed by subsequent immunoblotting analysis with anti-MVP antibody. S20 extracts of CP-MVP-RGD-STP and CP-MVP-RGD contained MVP as detected by Western blot (Figure 6-2B, lane 1 and 3) with only CP-MVP-RGD-STP being successfully purified by streptavidin beads, compared to CP-MVP-RGD (compare Figure 6-2B, lane 2 and 4). This confirms that the RGD-STP and by analogy the RGD tag on its own were successfully cloned in frame with the MVP, and that the RGD tag was functionally exposed to the outside of the vault particles. Importantly, the use of streptavidin beads to purify vaults from S20 extracts may in future be a valuable technique for large-scale purification of vaults, which is very difficult to achieve using the current sucrose gradient approach.

In this study, a fluorochrome (mCherry) was also incorporated into the vault by attaching to the previously defined vault-targeting domain, mINT. Purified recombinant vaults (CP-MVP-RGD/mCherry-mINT and CP-MVP-RGD-STP/mCherry-mINT) were studied by coomassie staining (Figure 6-2 C), showing that mCherry-mINT was successfully co-purified with the vaults and was enriched in the 45% sucrose gradient layer. This strongly suggests that both the CP-MVP-RGD and the CP-MVP-RGD-STP constructs produce the fully assembled vaults.

For the final confirmation of the correct assembly of the vault with the C-terminal tagged MVP, the morphology of assembled CP-MVP, CP-MVP-RGD and CP-MVP-RGD-STP vault particles was further analyzed by transmission electron microscopy (TEM), demonstrating the abundant, barrel-shaped recombinant particles (Figure 6- 3). These particles varied from 32-37 nm in width and 59-65 nm in length, and displayed the distinctive bifold symmetric central barrel of the vault with dual caps.

Figure 6-2. The C-terminal peptide tag is located on the exterior of the caps of recombinant vaults. (A) Western blot analysis of purified CP-MVP-RGD (lane 2) and CP-MVP-RGD-STP (lane 4), compared with CP-MVP as positive control (lane 1), and S20 extract Sf9 cells without any baculovirus infection as negative control (lane 3). (B) Western blot analysis of streptavidin beads affinity purified proteins from S20 extracts. Lane 1 and lane 3 are from S20 extracts of CP-MVP-RGD-STP and CP-MVP-RGD, respectively. Lane 2 and lane 4 are from bead affinity test of CP-MVP-RGD-STP and CP-MVP-RGD, respectively. (C) Coomassie stain of purified CP-MVP-RGD-STP (lane 1, 40% fraction and lane 2, 45% fraction) and CP-MVP-RGD (lane 3, 40% fraction and lane 4, 45% fraction) recombinant vaults containing mCherry-mINT fractionated on 4-15% SDS-PAGE. The major protein bands are the ~100 kDa CP-MVP-RGD/CP-MVP-RGD-STP and the ~45 kDa mCherry-mINT (note: the 45 kDa mCherry-mINT is a triplet that is likely caused by leaky translation due to the presence of three in-frame methionines in the first 17 amino acids of mCherry) (Kickhoefer, 2009).

Figure 6-3. TEM micrograph of the negative-stained recombinant (A) CP-MVP, (B) CP-MVP-RGD and (C) CP-MVP-RGD-STP vault particles.

6.3.2 Immobilization of recombinant vaults on APTES-modified glass

6.3.2.1 *The surface concentration of immobilized vaults*

As mentioned above, CP-MVP and CP-MVP-RGD vault particles used in this study contain the fluorescent proteins, mCherry-mINT. The mINT domain on mCherry-mINT is able to interact with MVP, and to direct mCherry-mINT into the lumen of the recombinant vaults. Nonspecific bound vaults with mCherry-mINT can be washed away; hence any detected fluorescence indicates the presence of half- or fully-assembled vaults. It means that the amount of structurally assembled vaults immobilized on substrate can be quantified by the measuring the mCherry fluorescent intensity. As mINT domain binds the inside of vaults at at two locations, above and the below the waist of the vault particles, detection of mCherry fluorescent intensity confirms the presence of full or half-assembled vaults, and cannot unequivocally confirm the fully-assembled structure.

The protein concentration of purified vaults containing mCherry-mINT (CP-MVP/mCherry-mINT) was measured using the standard bicinchoninic acid assay (BCA). The fluorescent intensity of CP-MVP/mCherry-mINT solution corresponding to different total protein concentrations was measured by fluorescent microplate reader. Thus, the vaults quantification calibration curve could be established by plotting mCherry

fluorescent intensity against vault concentration (Figure 6- 4). The above calibration was used to quantify the amount of (half- and fully)-assembled vaults on the substrates by measuring the fluorescence intensity, and the results are tabulated below (Table 6-1).

Figure 6-4. The calibration curve for vaults, with a fluorescent “tag” CP-MVP/mCherry-mINT. The fluorescent intensity was measured at *excitation* wavelength of 540 nm and emission wavelength of 640 nm.

Table 6-1. The loading efficiency of vault immobilization on APTES-modified glass

Unit: µg	Covalent bonding		Electrostatic adsorption	
	CP-MVP	CP-MVP-RGD	CP-MVP	CP-MVP-RGD
Immobilized amount	1.98	1.79	2.13	1.32
Loading amount	4	4	4	4
Loading efficiency	49.5%	44.8%	53.2%	33.1%

In general, the mCherry-mINT data showed that the vaults can be immobilized on APTES-modified glass surface, and further that a substantial portion of the original vault loading is immobilized in the half-vault or full-vault configuration. Both types of vaults, CP-MVP and CP-MVP-RGD could be immobilized by electrostatic adsorption or covalent bonding, although the relative efficiency was somewhat different. The

preference for either method will depend on the nature of the application. Electrostatic immobilization is a simpler process and imposes less damage to the vaults structure. On the other hand, covalent immobilization is relatively stable and less sensitive to change of physiological environments. For instance, in the application where the pH value fluctuation is negligible, electrostatic immobilization is feasible; otherwise, covalent immobilization process is recommended.

6.3.2.2 Surface characterization of vaults immobilized glass substrates

The mCherry-mINT fluorescent intensity measurement was carried out in an aqueous environment for the vaults, and it would be instructive to check whether this configuration is maintained in a ‘dry’ environment, as it will be critical for some applications. We report on efforts to do this in this section.

Firstly, we characterized the surface (in a dry state) for the presence of vaults as well as the success of the surface functionalization. In order to do this, after the substrate was coated with vault particles, it was dried in vacuum oven at 37 °C for 12 h. The chemical and structural stability of the immobilized vaults was studied by various surface characterization techniques. In order to confirm the success of silanization on glass, and to characterize surface chemical composition of the vaults-coated substrate, XPS was carried out on bare glass, APTES- modified glass, CP-MVP coated APTES- modified glass and CP-MVP-RGD coated APTES- modified glass (Figure 6- 5). The atomic percentage was tabulated in Table 6-2, together with the measured nitrogen to carbon ratio (N/C ratio), compared with the theoretical N/C ratio. On a bare glass, no nitrogen peak was detected. The presence of the N1s peak at ca. 400 eV on the APTES- modified glass was a strong evidence of existence of the APTES layer. The N/C ratio of APTES- modified glass was expected to be from 0.14 to 0.33 depending on the degree of hydrolysis of ethoxy groups into silanol groups (Beari, 2001). The measured N/C ratio of

0.16 was reasonably within its theoretical range. CP-MVP and CP-MVP-RGD coated APTES-modified glass showed N1s peaks as well. As MVP is a large protein with molecular weight around 100 kDa, its theoretical N/C ratio was calculated by taking the average N/C ratio of the most common 20 amino acids. The N/C ratios measured on vaults coated substrates were about 0.21-0.23, which were slightly higher than that measured on APTES- modified glass. However, this N1s peak might arise from vaults as well as from the exposed APTES layer on glass. The protein nature of CP-MVP and CP-MVP-RGD coating on APTES- modified glass was therefore further characterized by attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR).

Figure 6-5. XPS of (A) bare glass, (B) APTES- modified glass, (C) CP-MVP coated APTES-modified glass, and (D) CP-MVP-RGD coated APTES- modified glass.

Table 6-2. Atomic percentage and N/C ratio of (A) bare glass, (B) ATPES- modified glass, (C) CP-MVP coated ATPES- modified glass, and (D) CP-MVP-RGD coated ATPES- modified glass.

Sample	Measured N/C ratio	Theoretical N/C ratio	Atomic percentage %		
			O 1s (532eV)	N 1s (400eV)	C 1s (285eV)
A	0	0	75.69		24.31
B	0.16	0.14-0.33*	72.15	3.83	24.02
C	0.21	0.33	26.81	12.61	60.58
D	0.23	0.33	27.81	13.56	58.63

*Due to incomplete hydrolysis of ethoxy groups, theoretical N/C ratio of APTES modified surface ranges from 0.14 with hydrolysis of one ethoxy group, to 0.33 with hydrolysis of three ethoxy groups (Beari, 2001).

The ATR-FTIR measurement of the vault layer was performed using a bare glass as the background. In Figure 6- 6 A, APTES-modified glass, coated with CP-MVP based on electrostatic adsorption, showed the characteristic peaks of amide I bond around 1650 cm^{-1} , which was assigned to the carboxyl stretching (Kong, 2007). A similar spectrum was observed on APTES-modified glass coated with CP-MVP-RGD (Figure 6-6 B). Both main chains of immobilized vaults and APTES-vault covalent coupling are able to contribute to amide I signal. However, main chains of immobilized vaults on substrate should be the main signal source. That means the observation of amide I peak only proved the presence of proteins on the substrate, i.e. the immobilized recombinant vault proteins. The self-assembled vault structure has to be confirmed by other topological techniques, such as atomic force microscopy (AFM). The characteristic N-H stretching peak around $3200\text{ to }3400\text{ cm}^{-1}$ could originate either from APTES coating due to the incomplete surface coverage of vaults (Herlem, 2008), or from –NH side chain of major vaults proteins, or from both of them.

Figure 6-6. ATR-FTIR spectra of (A) CP-MVP immobilized on APTES-modified glass, and (B) CP-MVP-RGD immobilized on APTES-modified glass. The amide I bond is assigned to C=O stretching shown in 1600-1690 cm^{-1} .

XPS and ATR-FTIR techniques yield no information about whether the recombinant vaults assumed the characteristic barrel-and-cap structure after they were immobilized on the surface. It is not possible to image the vaults on a glass substrate by TEM, therefore the CP-MVP and CP-MVP-RGD vault particles-coated, APTES-modified glass substrates were imaged by AFM (these substrates were dried after the coatings were applied, hence are in a non-aqueous environment). It was found that the vaults adsorbed on these substrates (Figure 6-7 A-E), and their characteristic barrel-and-cap structure was retained. Some of the smaller particles appeared in AFM imaging as well. This might be

due to the adsorbed salts coming from the solution and other contaminations, as well as due to some disassembled vaults.

In Figure 6- 7 F, TEM images of CP-MVP vault particles are compared to the AFM images. Most vault particles in AFM images are barrel-shaped and similar to those observed in TEM images. Caps at the two ends of vault particles can also be seen quite clearly. The dimensions of vault particles in AFM images are compared with those in TEM images as well. From the AFM images, the observed width and length of CP-MVP vault particles are 63.2 ± 2.8 and 90.2 ± 2.1 nm, respectively. Due to the tip-sample convolution (Wong, 2007), the lateral dimensions of vault particles in AFM images are larger than those in TEM images (32-37 nm in width and 59-65 nm in length). The height of CP-MVP vault particles measured by AFM is about 8 nm, which is lower than 41 nm measured by cryoEM (Mikyas, 2004). Two possible reasons are given for this smaller vertical dimension of vaults imaged by AFM. Firstly, at ambient conditions, the AFM tip exerts a relatively large force on the soft vault particles, sufficient to compress the vaults somewhat (Lyubchenko, 1993). It was also reported that the measured height of dsDNA by tapping mode AFM in air was about 0.7 nm, though the diameter of dsDNA of 2 nm was well accepted and proven by many experiments (X.-J. Li, 2003). Secondly, the smaller height could also be caused by the interaction between the vaults and APTES-modified glass (Maeda, 1999; Zhou, 2008). Importantly, this is the first time that the barrel-and-cap structure of recombinant vault particles has been observed in a dry condition. The successful immobilization of intact vault nanoparticles on a solid substrate may open up future applications for selective cellular interactions as well as for localized delivery of bioactive agents.

Figure 6-7. AFM topographic images of (A, C) CP-MVP and (D) CP-MVP-RGD adsorbed on APTES-modified glass respectively. AFM phase images of (B) CP-MVP and (E) CP-MVP-RGD adsorbed on APTES-modified glass respectively. (F) Comparison between AFM topographic images (top row) and TEM images (bottom row). The Z scale for AFM topographic images in A, C and D is 20 nm. The Z scale for AFM phase image in B and E is 20° and 60°, respectively.

6.3.2.3 Cell affinity of recombinant vaults immobilized glass

Cell affinity of recombinant vaults immobilized glass was studied in three aspects: cell adhesion, proliferation and morphology .

Cell adhesion. HUVEC of 10^4 cell/cm² were allowed to incubate with substrates for 2 h. The unattached cells were rinsed off; the attached cells were quantified using WST-8 assay. Cell adhesion on vaults coated glass substrates was studied in comparison to that on APTES-modified and RGD-coated glass substrates. At 2 h, as shown in Figure 6-8, the highest absorbance at 450 nm which linearly correlate with the number of adherent cell was observed on APTES modified glass. This may be attributed to strong

electrostatic interaction between negatively charged cell surface with the positively charged APTES-modified glass (J. H. Lee, 1997). The cell adhesion on RGD coated glass was significantly greater than that on CP-MVP-RGD vaults-immobilized substrates as shown in Figure 6-8. It implies that the accessibility of RGD fused at C-terminus of vaults may be not as good as RGD short peptides coated on glass. When comparing MVP to MVP-RGD vaults coating on glass substrates, no statistical significance in cell adhesion was found. It suggests the addition of RGD sequence at vaults C-terminus did not gain beneficial advantage in cell-substrate interaction. It could be a further evident of poor accessibility of RGD fused at C-terminus of vaults when considering cell adhesion.

Figure 6-8. Cell adhesion at 2 h on various substrates was represented by the absorbance of WST-8 assay measured on corresponding substrates. Cell seeding density is 10^4 cell/cm².

Cell proliferation. The adherent cells were allowed to grow over 5 days of incubation. Cell proliferation was assessed using WST-8 assay, whereby the absorbance at 450 nm represented the cell number at the measurement time-point. Cell proliferation profile was plotted in Figure 6-9. Despite the difference found in cell adhesion on various glass substrates at 2 h, HUVEC shared the similar proliferation profile on each substrate. Cell proliferation rate (the slope of proliferation curve) was relatively slow before 72 h

and then picked up onwards. It suggests that cell proliferation on different glass substrates was comparable.

Figure 6-9. Cell proliferation profile on various glass substrates was represented by the absorbance of WST-8 assay measured on the corresponding substrates plotted against incubation duration over 5 days. Cell seeding density is 10^4 cell/cm².

Cell morphology. The cell affinity was also evaluated by the expression of cell phenotype with regards to different coating. As presented in Figure 6-10, HUVEC on each substrate were able to express the classic cobblestone morphology and to form the monolayer of endothelium, which indicates the good cell affinity of the substrate.

Figure 6-10. HUVEC grown on (A) CP-MVP vaults coated gs-APTES, (B) CP-MVP-RGD vaults coated gs-APTES (C) RGD coated gs-APTES. HUVEC were allowed to

proliferate over 5 days with initial seeding density of 10^4 cell/cm². Then HUVEC were fixed and stained using crystal violet. The scale bar = 100 μ m.

In summary, the preliminary results of cell affinity study demonstrated the cytocompatibility of these recombinant vaults coated glass substrates. Though cell adhesion on vaults immobilized substrates was not as good as RGD coated substrates, the success of vaults immobilization leads possibility to use vaults as located and sustained drug carrier with acceptable cytocompatibility.

6.3.3 Immobilization of recombinant vaults on APTES-modified PLLA

6.3.3.1 Surface characterization of vaults immobilized PLLA substrates

As illustrated in Figure 6-1, PLLA film was fabricated on glass by spin-coating and then treated by oxygen plasma which introduced hydroxyl groups on PLLA surface. By immersing plasma-treated PLLA films into APTES solution, self-assembled monolayers formed on PLLA surface presenting a positive charged surface in rich of –NH₂ function groups which were utilized to immobilize vaults on PLLA surface.

PLLA films before and after surface chemical modifications were characterized using AFM. The roughness of various PLLA films was compared in Table 6-3. As Figure 6-11A shows, a smooth film was fabricated on glass by spin-coating, the surface roughness root-mean-square (RMS) of this as-spun PLLA film was 0.268 nm in $5 \times 5 \mu\text{m}^2$. After oxygen plasma treatment, the RMS increased to 0.77 nm (Figure 6-11 B). This indicates that oxygen plasma treated PLLA film was rougher than that of as-spun PLLA surface (Figure 6-11A and B). The surface of APTES modified PLLA (PLLA-APTES) was further roughened, as the RMS increased to 0.882 nm (Figure 6-11C), which is about 10% of the height of vaults immobilized on glass (Figure 6-7). Hence, the morphology of immobilized vaults (indicated by a red circle) on PLLA-APTES substrates was difficult to observe, as shown in Figure 6-11D.

Table 6-3. Surface roughness measurements of various substrates ($5 \times 5 \mu\text{m}^2$) by AFM

Sample	Surface roughness (RMS) (nm)
As spun PLLA	0.268
Oxygen plasma treated PLLA	0.774
PLLA-APTES	0.882

Figure 6-11. AFM images of (A) as-spun PLLA, (B) oxygen plasma treated PLLA, (C) PLLA-APTES, and (D) CP-MVP vaults coated PLLA-APTES. The Z scale is 20 nm for (A), (B), and (C), 80 nm for (D) respectively.

6.3.3.2 Cell affinity of recombinant vaults immobilized PLLA

Cell affinity of recombinant vaults immobilized PLLA was studied in three aspects: cell adhesion, proliferation and morphology using the same protocol described previously.

Cell adhesion. HUVEC of 10^4 cell/cm² were allowed to incubate with substrates for 2 h. The unattached cells were rinsed off; the attached cells were quantified using WST-8 assay. Cell adhesion on vaults coated PLLA-APTES, namely PLLA-MVP, was studied in comparison to that on as-spun PLLA and PLLA-APTES. Cell adhesion on TCPS was considered as the reference. At 2 h (Figure 6-12), cell adhesion on as-spun PLLA was comparable to that on TCPS; whereas cell adhesion on PLLA-MVP was significantly lower than that on PLLA-APTES and as-spun PLLA (* $p=0.01771$ and * $p=6.5E-06$ respectively). This significant decrease in cell adhesion on PLLA-MVP may be due to the unfavorable electrostatic interaction between the negatively charged MVP vaults particles coated on PLLA and the negatively charged cell surface. In contrast, APTES self-assembled layer was positively charged due to the rich present of $-NH_2$ groups, which may contribute to favorable cell adhesion on PLLA-APTES in comparison to PLLA-MVP.

Figure 6-12. Cell adhesion at 2 h on various PLLA substrates was represented by the absorbance of WST-8 assay measured on corresponding substrates. Cell seeding density is 10^4 cell/cm².

Cell proliferation. The adherent cells were allowed to grow over 5 days of incubation. Cell proliferation was assessed using WST-8 assay, whereby the absorbance at 450 nm represented the cell number at the measurement time-point. Cell proliferation profile was plotted in Figure 6-13. Cell proliferation on TCPS was considered as a reference. Cell grown on various PLLA substrates shared the similar proliferation profile. Cell proliferation rate (the slope of proliferation curve) was relatively slow before 72 h and then picked up onwards.

Figure 6-13. Cell proliferation profile on various substrates was represented by the absorbance of WST-8 assay measured on corresponding substrates plotted against incubation duration over 5 days. Cell seeding density is 10^4 cell/cm².

Cell morphology. The cell affinity was evaluated by the expression of cell morphology with regards to different coating after 2 h attachment. As presented in Figure 6-14, HUVEC on each substrate were able to adhere and spread after 2 h post seeding. The expressed phenotype on different PLLA substrates showed the classic cobblestone morphology, which indicates the good cell affinity of these substrates.

Figure 6-14. HUVEC grown on (A) as-spun PLLA, (B) PLLA-APTES and (C) PLLA-MVP. HUVEC were allowed to incubate for 2 h with initial seeding density of 10^4 cell/cm². Then the images were taken by IX71 Olympus microscope. The scale bar = 100 μ m.

In summary, the preliminary study of cell-substrate interaction, including the study of cell adhesion, proliferation and morphology, showed that immobilization of vaults did not have significant impact on cell affinity on PLLA substrate. Despite the lowest cell adhesion observed on PLLA-MVP, cell proliferation and morphology were not influenced by the presence of vaults on PLLA.

6.4 Summary

In this chapter, we have described the production of two successful C-terminal modifications of recombinant vaults, CP-MVP-RGD, and CP-MVP-RGD-STP. Surface modification methods to immobilize vaults on both glass and PLLA substrates were successful through either electrostatic adsorption or covalent bonding. The immobilized recombinant vaults on glass substrate were found to retain their barrel-and-cap structure even when the substrate was dried, as observed for the first time by AFM. Cell affinity of vaults immobilized substrate was proved through cell adhesion, proliferation and morphology studies; however, neither the immobilized CP-MVP nor the CP-MVP-RGD recombinant vaults on substrates enhanced endothelialization over APTES-modified

substrates. Most likely this is due to the negative vault charges and relative inaccessibility of the RGD motif on the vaults.

The success in immobilizing recombinant vault nanoparticles contributed in two aspects. Firstly, this work proved the feasibility of immobilizing those nanoparticles on a solid surface. By doing this, the release of encapsulated bioactive agents could be better controlled and sustained. VEGF could be one of examples of these bioactive agents that are related to promote endothelial cell proliferation in cardiovascular applications. Secondly, in order to improve cell affinity of vaults coated substrate, we designed and expressed RGD tagged recombinant vault particles for the first time, although cell-biomaterial interaction study did not show significant advantage by using these cell adhesion ligands expressed at C-terminus of vaults. REDV is another adhesion receptor found on the human endothelial cells. REDV has been reported to selectively promote the adhesion of endothelial cell rather than smooth muscle cells (Hubbell, 1991). Since the RGD tagged recombinant vaults particles have been successfully expressed, the expression of REDV tagged particles could be promising. Nevertheless, our work in immobilization of intact vault nanoparticles on a solid substrate may open up applications for improved endothelial cellular affinity as well as for localized delivery of bioactive agents in application of cardiovascular prostheses.

Chapter 7 Conclusions and Perspectives

7.1 Conclusions

The primary achievement of this work is the development and evaluation of the three-step surface modification method, with a view of its application in cardiovascular therapeutic devices. This is a concise and easily-controlled surface modification method to immobilize biomolecules (gelatin and chitosan) onto biodegradable polymer (PLLA). Furthermore the procedure employs relatively low hazard chemicals and procedures. This three-step surface modification method, as described in Chapter 3, involved (1) argon plasma treatment, (2) acrylic acid grafting polymerization and (3) water soluble carbodiimide coupling reaction. We proved the feasibility and stability of this method through a series of surface characterization analysis, including XPS, contact angle measurement and colorimetric methods for surface density of functional groups.

This surface modification method has considerable usefulness as it can be potentially applied to other biodegradable polyester polymers and copolymers. Moreover, the final step is not restricted to gelatin and chitosan. A wide range of bioactive molecules functioning as cell-biomaterial interaction cues could be immobilized on polymeric surfaces. For instance, collagen IV and laminin could be immobilized on PLLA to mimic the basal membrane of endothelium, and vascular endothelial growth factor (VEGF) to stimulate faster endothelialization. This flexibility in application will allow the surface modification method to be an important tool in biomaterial construction.

In Chapter 4, improved endothelial cell affinity on gelatin and chitosan modified PLLA was demonstrated by assessing cell adhesion, proliferation and retention under shear stress. We were able to observe the complete endothelialization on both the modified PLLA substrates but not on the unmodified PLLA under *in vitro* experimental conditions. These findings supported the feasibility of the three-step surface modification

method in producing surfaces that promote endothelialization. In the study of cell retention, we found that cell retention was positively correlated with cell morphometric parameters such as cell spreading area, FA size, FA density, and FN fibril length. It implied that surface physiochemical properties modulated the cell adhesion strength via influencing cell morphology, focal adhesion and FN fibril formation.

Further scientific findings of note were reported in this thesis, such as the important role of initial seeding density on evaluating cell-biomaterial interaction. In the course of studying cell-biomaterial interactions, we observed that initial seeding density affected the cellular behaviour. Additionally, following an extensive literature search, it became apparent that a wide range of initial seeding density had been used in previous studies across several groups, to evaluate cell-biomaterial interaction. Hence, we characterised the effect of seeding density on assessing cell-biomaterial interactions as reported in Chapter 5 to determine the correct density for accurate analysis of cell biomaterial interaction. We were able to demonstrate that high seeding density (50,000 cell/cm²) promoted cell adhesion and proliferation, which masked the differences in cellular response on different substrates. We assume cell-cell interactions were able to mediate a greater effect at high cell density and the influences of surfaces features were lost. In contrast, low seeding density (5,000 cell/cm²) enabled us to clearly observe surface chemistry-dependent cell adhesion and proliferation behaviors. This novel study gave the justification for us to make the recommendation to use low seeding density range in evaluating cell-biomaterials interaction to eliminate the influence of cell-to-cell interactions.

The study in Chapter 6 describes the preliminary investigation to immobilize recombinant vault nanoparticles with the aim of improving endothelial cell affinity on solid substrate. To this end, we first designed and expressed the RGD tagged recombinant

vaults by fusing RGD sequence to the C-terminus of MVP. Our pioneer work demonstrated that recombinant vaults particles (CP-MVP, CP-MVP-RGD) could be immobilized on a solid substrate (glass and PLLA) by either electrostatic adsorption or covalent bonding. And such immobilized particles were found to retain their barrel-and-cap structure on solid substrates in dry conditions. However, the coating of RGD tagged vaults particles on substrates did not show improved endothelial cell affinity of substrates. Nevertheless, the success of immobilizing and characterizing the vaults particles provided a basis for further study in the application of immobilized vaults both as a carrier for bioactive agents and as a biochemical cue for cellular interactions.

In summary, the work presented in this thesis demonstrates the successful optimization and characterization of the three-step modification method with the use of selected biodegradable polymers and immobilized biomolecules to promote endothelialization. It further described the interactions cells with the synthesized surfaces. Finally the examination of recombinant vaults as a potential surface cell recruitment factor was performed, establishing an interesting line of study.

7.2 Perspectives

Proteins are omnipresent in both body fluids and culture medium, and their adsorption on implants always takes place before cellular adhesion. Biomaterials surface properties determine protein adsorption which later on modulates the subsequent cell behavior. Hence, studying the relation between biomaterials surface properties and protein adsorption and the relation between protein adsorption and subsequent cell responses could lead a better understanding of cell-biomaterial interaction.

The function of the monolayer of endothelial cells formed on modified PLLA substrate could be tested in two aspects: the anti-thrombogenicity to inhibit thrombosis and the anti-proliferation of smooth muscle cells to inhibit restenosis.

Study on cell detachment mechanism could enable better understanding of cellular responses to shear stress. Cell detachment from fibronectin-coated surface is mediated by breaking integrin-FN bonds (Andrés J. García, 1998). By contrast, cell detachment from non-endogenous derived fibrillar matrix occurred through a novel mechanism of fibril matrix breakage (Engler, 2009). During the process of endothelialization on substrate, cells assemble and remodel endogenous extracellular matrix. It is likely the mechanism of cell detachment might be different from both reported mechanisms.

The successful immobilization of intact vault nanoparticles on a solid substrate may open up future applications for selective cellular interactions as well as for localized delivery of bioactive agents, which are worth to pursuit.

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Publications

- 1) **Xia Y**, Ramgopal Y, Li H, Shang L, Srinivas P, Kickhoefer VA, Rome LH, Preiser PR, Boey F, Zhang H, Venkatraman SS. *Immobilization of recombinant vault nanoparticles on solid substrates*. **ACS Nano** 2010, 23, 1417-1424. DOI:10.1021/nn901167s.
- 2) **Xia Y**, Boey F, Venkatraman SS. *Surface modification of poly(L-lactic acid) with biomolecules to promote endothelialization*. **Biointerphases** 2010, 5, FA32-FA40. DOI:10.1116/1.3467508.
- 3) **Xia Y**, Prawirasatya M, Heng BC, Boey FYC, Venkatraman SS. *Seeding Density matters: extensive interacellular contact masks the surface dependence of endothelial cell-biomaterial interactions*. **Journal of materials science: materials in medicine**, 2011, 22, 389-396. DOI: 10.1007/s10856-010-4211-5.
- 4) Heng BC, **Xia Y**, Shang XB, Preiser PR, Law SK, Boey FYC, Venkatraman SS. *Comparison of the adhesion and proliferation characteristics of HUVEC and two endothelial cell lines (CRL 2922 and CRL 2873) on various substrata*. **Biotechnology and Bioprocess Engineering**, 2011, 16, 127-135. DOI: 10.1007/s12257-010-0141-9
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- 7) **Xia Y**, Prawirasatya M, Ma LL, Ng KW, Irvine SA, Yang Y, Boey FYC, Venkatraman SS. *Surface chemistry modulates cell retention under shear stress on biomolecules modified poly(L-lactic acid)*. **Journal of Tissue engineering and Regenerative medicine**, 2011, under revision.
- 8) Li H, **Xia Y**, He Q, Zhou X, Lu G, Shang, L, Boey FYC, Venkatraman, SS, Zhang H, *Surface modification of smooth poly(L-lactic acid) film by silanization and oxygen plasma to immobilize gelatin*. **Langmuir**, 2011, under revision.

Appendix

Figure A-1. Carboxylic group concentration standard curve measured by rhodamine-carboxyl interaction method.

Figure A-2. Gelatin concentration standard curve by Bradford method.

Figure A-3. Cell number standard curve measured by WST-8 assay.

Table A-1. The fusion enthalpy (ΔH_m) of PLLA films subjected to different plasma treatment condition.

ΔH_m (J/g)	60(s)	90(s)	120(s)	150(s)	180(s)
80W	35.6±0.5	34.0±2.2	36.9±1.4	32.2±0.9	33.7±0.1
120W	38.6±1.0	38.1±0.7	34.9±1.0	35.4±0.3	37.0±0.4
160W	34.9±1.2	37.9±0.4	38.8±0.7	35.7±0.6	36.0±0.4
Fusion enthalpy of unmodified PLLA (IV=8.4) is 28.1 J/g (M. Tanaka, 2006)					