Cell adhesion behavior on molecularly engineered surfaces

Cai, Ning

2010

Cai, N. (2010). Cell adhesion behavior on molecularly engineered surfaces. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/42229>

<https://doi.org/10.32657/10356/42229>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

CELL ADHESION BEHAVIOR ON
MOLECULARLY ENGINEERED SURFACES

**CELL ADHESION BEHAVIOR
ON MOLECULARLY ENGINEERED SURFACES**

CAI NING

**CAI NING
SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING
2010**

2010

CELL ADHESION BEHAVIOR ON MOLECULARLY ENGINEERED SURFACES

CAI NING

School of Chemical and Biomedical Engineering

A thesis submitted to the Nanyang Technological University
in fulfillment of the requirement for the degree of
Doctor of Philosophy

2010

Acknowledgements

First and foremost, I would like to express my greatest appreciation to my advisor, Prof. Liao Kin, for his insight, patience, motivation, and invaluable assistance, without which this thesis would not have been possible. His guidance helped me in all the time of research and writing of this thesis.

Besides my advisor, I would like to thank Prof. Chan Vincent. He imparted his knowledge and experience to me and gave me the privilege to use the equipment in his lab.

I am especially grateful to all the colleagues in Prof. Liao's group for their kindly help. Special thank goes to Dr. Ma Gang, Dr. Fang Ning, Dr. Gong Ying Xue, Wong Chee Chung, Tan Samuel, Yu Ting, Yang Tianyi, and Chen Bei Yi for their sincere encouragement and assistance in my experiments during this period.

Last but not the least, I would like to thank my parents, for their love and continuous support throughout my life.

Table of Contents

Acknowledgements	I
Table of Contents	II
List of Figures	VI
List of Tables	XII
Abbreviations	XIII
Summary	XV
Chapter 1 Introduction	1
1.1 Background	1
1.2 Objectives and Scope	4
1.3 Organization.....	5
Chapter 2 Literature Review	7
2.1 Surface Modification Methods for Poly(lactic acid)	7
2.1.1 Physical Absorption	8
2.1.2 Entrapment	9
2.1.3 Chemical Treatment	10
2.1.4 Physical Treatment	11
2.2 Surface Modification Methods for Carbon Nanotubes	11
2.2.1 Noncovalent Attachment of Proteins to CNTs	12
2.2.2 Covalent Linkage of Proteins to CNTs	13
2.3 Extracellular Matrix-Cell Interactions	14
2.3.1 ECM Protein Structure and Function	15
2.3.2 Mutual Recognition between Integrin Receptors and Ligands	17
2.3.3 Cytoskeleton Reorganization and Focal Adhesion Formation.....	20
2.3.4 Cell Adhesion Strengthening.....	21

2.4 Experimental Techniques for Investigating Cell Adhesion.....	23
2.4.1 Confocal Reflection Interference Contrast Microscopy	24
2.4.2 Micropipette Aspiration	26
2.4.3 Total Internal Reflection Fluorescence Microscopy	29
2.5 Summary	30
Chapter 3 Materials and Experimental Methods	32
3.1 Materials	32
3.2 Substrate Preparation	32
3.2.1 Coverslip Cleaning.....	33
3.2.2 Spin Coating.....	33
3.2.3 ECM Protein Immobilization.....	33
3.3 Bicinchoninic Acid Assay	35
3.4 Atomic Force Microscopy.....	35
3.5 Contact Angle Measurement.....	36
3.6 Culture of Porcine Esophageal Fibroblasts.....	36
3.7 Integrin Blocking	37
3.8 Phase Contrast Microscopy and Confocal Reflection Interference Contrast Microscopy	37
3.9 Immunostaining	40
3.10 Total Internal Reflection Fluorescence Microscopy	41
3.11 Scanning Electron Microscopy	42
3.12 Cell Adhesion Force and Strength	43
3.13 Methylthiazoletetrazolium Assay.....	46
3.14 Cell Attachment Assay	46
3.15 Statistical Analysis	47
Chapter 4 Adhesion Dynamics of Porcine Esophageal Fibroblasts on ECM Protein-immobilized Poly(lactic acid)	49
4.1 Introduction.....	49

4.2 Results and Discussion	50
4.2.1 Surface Modification of PLA.....	50
4.2.2 Spreading and Adhesion Dynamics of PEFs on PLA, PLA-CHO, PLA-COL, and PLA-FN Surfaces	51
4.2.3 Evolvement of Distribution of F-actin in Cell Adhesion	63
4.2.4 Cell Attachment Efficiency on PLA, PLA-CHO, PLA-COL, and PLA-FN Surfaces	66
4.3 Summary	67
Chapter 5 Adhesion Dynamics of Porcine Esophageal Fibroblasts on ECM Protein-immobilized CNT Monolayer	69
5.1 Introduction.....	69
5.2 Results and Discussion	70
5.2.1 Surface Characterization	70
5.2.2 Adhesion Dynamics of PEFs on CNT-ML, CNT-COL, and CNT-FN Surfaces.....	73
5.2.3 Metabolic Activity and Attachment Ratio of PEFs on CNT-ML, CNT-COL, and CNT-FN Surfaces	80
5.2.4 Cell Morphology of PEFs on CNT-ML, CNT-COL, and CNT-FN Surfaces.....	82
5.2.5 Actin Filament Distribution of PEFs on CNT-ML, CNT-COL, and CNT-FN Surfaces	85
5.3 Summary	87
Chapter 6 Temporal Effect of Functional Blocking of β_1 Integrin on Cell Adhesion Strength under Serum Depletion	88
6.1 Introduction.....	88
6.2 Results.....	90
6.2.1 Surface Characterization	90
6.2.2 Time-dependent Cell Spreading and Morphological Change	91

6.2.3 Time-dependent Evolvement of F-actin Distribution and Vinculin Pattern	96
6.2.4 Time-dependent Change of Adhesion Force and Strength.....	98
6.2.5 Time-dependent Change of Number of Attached Cells	99
6.3 Discussion	100
6.4 Summary	106
Chapter 7 Weibull Statistical Analysis of Adhesion Strength	107
7.1 Introduction.....	107
7.2 Results and Discussion	108
7.3 Summary	119
Chapter 8 Conclusions and Future Work.....	120
8.1 Conclusions.....	120
8.2 Future work.....	123
References.....	125
Publications	147

List of Figures

Figure 2.1 Covalent binding of proteins to CNTs through three-steps process (adapted from reference [56]). Reprinted in part from [Jiang KY, et al. Journal of Materials Chemistry 2004;14:37-39 Copyright (2004)] with permission from Royal Society of Chemistry.....	14
Figure 2.2 The schematic of collagen fiber formation.....	16
Figure 2.3 A schematic of micropipette aspiration system (adapted from reference [99]). Reprinted in part from [Missirlis YF, et al. Biomolecular Engineering 2002;19:287-294 Copyright (2002)] with permission from Elsevier.....	27
Figure 3.1 Schematic procedures of immobilization of ECM proteins on PLA. PLA was first aminolyzed by 1,6-hexanediamine to introduce amino groups on PLA surface. Then glutaraldehyde was adopted to covalently bind to PLA surface with free carboxyl. Finally, ECM proteins were grafted to the PLA surface with the aid of GA as the cross-linker between aminolyzed PLA and ECM proteins.....	34
Figure 3.2 Schematic diagram of a cell adhering to a flat substrate. Mid-plane radius, contact zone radius, and contact angle are represented by R , a and θ , respectively.	39
Figure 3.3 A series of phase contrast images of a cell on PLA surface at different time following cell seeding.....	40
Figure 3.4 Schematic diagram of spread area and perimeter of a cell in a SEM image. ...	43
Figure 3.5 Illustration of shapes with different Sfc.	43
Figure 3.6 Schematic diagram of micropipette aspiration system. The syringe was used to produce negative pressure in the pipelines to aspirate cell, or positive pressure to push cell out of micropipette. Based on the readings of multimeter (not shown here) connected to the pressure transducer, the pressure in the pipelines can be measured and controlled.....	45
Figure 3.7 Experimental procedure of measuring adhesion force. (a) The suction pressure	

is applied on the cell. (b) Micropipette is moved horizontally, and cell does not move at low levels of suction pressures. (c) Micropipette is moved back, and suction pressure is increased step by step and applied on cell. (d) Cell is moved away at certain level of aspiration pressure. Scale bar = 50 μm45

Figure 3.8 A cell under suction by micropipette.46

Figure 4.1 AFM topographic image of a typical region ($1\mu\text{m}\times 1\mu\text{m}$) on (A) PLA, (B) PLA-CHO, (C) PLA-COL, and (D) PLA-FN surfaces. The height scale is shown in the (E).51

Figure 4.2 Phase contrast images of PEFs on PLA, PLA-CHO, PLA-COL, and PLA-FN surfaces 5 min and 24 h after cell seeding. Scale bar = 30 μm52

Figure 4.3 The 2D spread area of individual PEFs (A) and average normalized 2D spread area of PEFs (B) again time on PLA, PLA-CHO, PLA-COL, and PLA-FN surfaces within 24 h after cell seeding. In (B), the variation in the first 60 min is shown in the inset with enlarged scale.54

Figure 4.4 A series of C-RICM images of a typical PEF in a cell population adhering on PLA surface from 5 min to 24 h after cell seeding.55

Figure 4.5 A series of C-RICM images of a typical PEF in a cell population adhering on PLA-CHO surface from 5 min to 24 h after cell seeding.56

Figure 4.6 A series of C-RICM images of a typical PEF in a cell population adhering on PLA-COL surface from 5 min to 24 h after cell seeding.57

Figure 4.7 A series of C-RICM images of a typical PEF in a cell population adhering on PLA-FN surface from 5 min to 24 h after cell seeding.58

Figure 4.8 Average degree of deformation versus time for PEFs on PLA, PLA-CHO, PLA-COL, and PLA-FN surfaces within 24 h after cell seeding (Logarithmic time scale). The variation from 10 min to 90 min is shown in the inset with linear time axis.59

Figure 4.9 Average adhesion energy of adherent PEFs versus time within 24 h after cell

seeding (Logarithmic time scale). The variation from 40 min to 80 min is shown in the inset with linear time axis.....	61
Figure 4.10 Normalized radius of the contact zone versus time for PEFs on PLA, PLA-CHO, PLA-COL, and PLA-FN surfaces within 24 h after cell seeding.....	63
Figure 4.11 Actin filament distribution in PEFs adhering to PLA, PLA-CHO, PLA-COL, and PLA-FN surfaces 20 min, 60 min, 4 h, and 24 h after cell seeding. The arrows in (B), (C) and (N) indicate the filopodia, stress fibers, and the actin filament bundles in cellular periphery, respectively. Scale bar = 30µm.....	66
Figure 4.12 Attachment ratio of PEFs on PLA, PLA-CHO, PLA-COL, and PLA-FN surfaces 2 h after cell seeding. There is significant difference in attachment ratio among PLA, PLA-COL, and PLA-FN surfaces (Student's t-test: $P<0.05$).....	67
Figure 5.1 SEM image of CNT monolayer on a silica coverslip.....	71
Figure 5.2 AFM images of CNT-ML, CNT-COL, and CNT-FN surfaces.....	72
Figure 5.3 A series of phase contrast and C-RICM images of a typical PEF in a cell population adhering to the CNT-ML surface from 5 min to 120 min after cell seeding. The white arrow indicates the adhesion contact forming at 20 min. Scale bar = 20 µm.	74
Figure 5.4 A series of phase contrast and C-RICM images of a typical PEF in a cell population adhering to the CNT-COL surface from 5 min to 120 min after cell seeding. The white arrow indicates the adhesion contact forming at 20 min. Scale bar = 20 µm. ..	76
Figure 5.5 A series of phase contrast and C-RICM images of a typical PEF in a cell population adhering to the CNT-FN surface from 5 min to 120 min after cell seeding. The white arrow indicates the adhesion contact forming at 5 min. Scale bar = 20 µm.	77
Figure 5.6 Average degree of deformation versus time for PEFs on CNT-ML, CNT-COL, and CNT-FN surfaces within 2 h after cell seeding.....	78
Figure 5.7 Average adhesion energy versus time for PEFs on CNT-ML, CNT-COL, and CNT-FN surfaces within 2 h after cell seeding.....	80

Figure 5.8 MTT assay for PEFs cultured on CNT-ML, CNT-COL, and CNT-FN surfaces for 1 or 3 days. Cell metabolic activity is represented as the relative MTT value of a surface to that of the control surface (CNT-ML). * denotes significant difference compared with CNT-ML surface after 1 day (Student's t-test: $P < 0.05$); ** denotes significant difference compared with CNT-ML surface after 3 days (Student's t-test: $P < 0.01$); Δ denotes significant difference compared with CNT-COL surface after 3 days (Student's t-test: $P < 0.05$).....81

Figure 5.9 Attachment ratio of PEFs on CNT-ML, CNT-COL, and CNT-FN surfaces after 2 h of culture.....82

Figure 5.10 SEM images of PEFs adhering to CNT-ML, CNT-COL, and CNT-FN surfaces after 24 h of culture. The arrows in (A), (E) and (H) indicate the filopodia of a typical PEF on CNT-ML, CNT-COL, and CNT-FN surfaces, respectively. The corresponding details of the filopodia in (A), (E) and (H) are shown in (C), (F) and (I), respectively.84

Figure 5.11 Actin filament distribution of PEFs on CNT-ML, CNT-COL, and CNT-FN surfaces after 24 h of culture. The arrows in (A) indicate less spreaded cells with concentrated actin filaments near cell cortex. The arrow in (B) indicates the stress fibers. Scale bar = 50 μm86

Figure 6.1 AFM images of uncoated glass coverslip and fibronectin-coated glass coverslip.91

Figure 6.2 Time course study of cell morphology, F-actin distribution, and vinculin pattern of non-blocked PEFs. (A)-(F), phase contrast images; (G)-(L), fluorescence images of F-actin; (M)-(R), fluorescence images of vinculin; S-X, merged images of F-actin and vinculin. Vin=vinculin. Scale bar, 20 μm93

Figure 6.3 Time course study of cell morphology, F-actin distribution, and vinculin pattern of β_1 -blocked PEFs. (A)-(F), phase contrast images; (G)-(L), fluorescence images of F-actin; (M)-(R), fluorescence images of vinculin; S-X, merged images of F-actin and

vinculin. Vin=vinculin. Scale bar, 20 μ m.94

Figure 6.4 The spread area against time for non-blocked and β_1 -blocked PEFs on fibronectin-coated surface within 4 h after cell seeding. n=80-100 for each data point. *Significant difference from the corresponding value in β_1 -blocked PEFs at the same time point (Student's t-test: $P<0.05$).95

Figure 6.5 The focal adhesion area (A) and focal adhesion density (B) against time for non-blocked and β_1 -blocked PEFs on fibronectin-coated surface within 4 h after cell seeding. n=80-100 for each data point. *Significant difference from the corresponding value in β_1 -blocked PEFs at the same time point (Student's t-test: $P<0.05$).98

Figure 6.6 The adhesion force (A) and adhesion strength (B) against time for non-blocked and β_1 -blocked PEFs on fibronectin-coated surface within 4 h after cell seeding. n=80-100 for each data points. *Significant difference from the corresponding value in β_1 -blocked PEFs at the same time point (Student's t-test: $P<0.05$).99

Figure 6.7 Number of attached cells against time for non-blocked and β_1 -blocked PEFs on fibronectin-coated surface within 4 h after cell seeding. *Significant difference from corresponding value in β_1 -blocked PEFs at the same time point (Student's t-test: $P<0.05$).100

Figure 6.8 The relationship between adhesion strength and focal adhesion density. (A) Non-blocked PEFs on fibronectin-coated surface; coefficient of determination =0.98 for the linear fit. (B) β_1 -blocked PEFs on fibronectin-coated surface; coefficient of determination=0.97 for the linear fit.102

Figure 7.1 Regression lines for the adhesion strength data obtained through micropipette aspiration technique using linearized Weibull cumulative probability function. (A), non-blocked PEFs on fibronectin coated surface; (B) β_1 -blocked PEFs on fibronectin coated surface. The unit of shear stress, τ , is Pa.110

Figure 7.2 The average and characteristic adhesion strength against time for non-blocked (A) and β_1 -blocked (B) PEFs on fibronectin-coated surface within 4 h after cell seeding.

.....	111
Figure 7.3 The relationship between characteristic adhesion strength and focal adhesion density. $R^2=0.96$ (non-blocked PEFs) or 0.97 (β_1 -blocked PEFs) for the linear fit.	112
Figure 7.4 Weibull cumulative probability and probability density graphs of non-blocked PEFs on fibronectin-coated surface from 30 min to 240 min after cell seeding. Graphs and experimental data points are shown together in (B).	117
Figure 7.5 Weibull cumulative probability and probability density graphs of β_1 -blocked PEFs on fibronectin-coated surface from 30 min to 240 min after cell seeding. Graphs and experimental data points are shown together in (B).	118

List of Tables

Table 2.1 Selective amino acid sequences of extracellular matrix proteins involving in cell adhesion (adapted from references [71]). Reprinted in part from [Shin H, et al. Biomaterials 2003;24:4353-4364 Copyright (2003)] with permission from Elsevier.	18
Table 2.2 The integrin family of adhesion receptora (adapted from reference [72]). Reprinted in part from [Anselme K. Biomaterials 2000;21:667-681 Copyright (2000)] with permission from Elsevier.	19
Table 5.1 Spread area and Sfc of PEFs on CNT-ML, CNT-COL, and CNT-FN Surfaces.	85
Table 6.1 Focal adhesion strength (τ_{FA}) and non-focal adhesion strength (τ_{NFA}) of non-blocked and β_1 -blocked PEFs on fibronectin-coated surface.	103
Table 7.1 Weibull moduli for non-blocked and β_1 -blocked PEFs on fibronectin-coated surface from 30 min to 240 min after cell seeding.	111
Table 7.2 Average and characteristic non-focal adhesion strength (τ_{NFA}) and focal adhesion strength (τ_{FA}) of non-blocked and β_1 -blocked PEFs.	112

Abbreviations

AFM	atomic force microscopy
BCA	bicinchoninic acid
BSA	bovine serum albumin
CNT	carbon nanotube
CNT-COL	collagen-coated CNT monolayer
CNT-FN	fibronectin-coated CNT monolayer
CNT-ML	CNT monolayer
COL	collagen type I
C-RICM	confocal reflection interference contrast microscopy
CS-PLGA	azide-chitosan-immobilized PLGA
DAPI	4',6-diamidino-2-phenylindole
ECM	extracellular matrix
EDC	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide
FAK	focal adhesion kinase
FN	Fibronectin
GA	Glutaraldehyde
Gel-CS-PLGA	gelatin-immobilized CS-PLGA
GFP	green fluorescent protein
hIgG	human immunoglobulin G
MD	molecular dynamics
MLV	multilamellar vesicle
MTT	Methylthiazoletetrazolium
MWCNT	multi-walled carbon nanotube
NHS	N-hydroxysulfosuccinimide
PCL	Polycaprolactone
PDL	poly-D-lysine

PEF	porcine esophageal fibroblast
PEG	poly(ethylene glycol)
PLA	poly(lactic acid)
PLA-CHO	glutaraldehyde-modified PLA
PLA-COL	collagen-immobilized PLA
PLA-FN	fibronectin-immobilized PLA
PLGA	poly(lactide-co-glycolide)
PLL	poly(L-lysine)
SDS	sodium dodecyl sulfate
SF	silk fibroin
Sfc	shape factor coefficient
SWCNT	single wall carbon nanotube
TIRFM	total internal reflection fluorescence microscopy
ULV	unilamellar vesicle
UV	Ultraviolet

Summary

Effective attachment of cells on biomaterials is one important requirement in designing engineered tissue substitute in application of tissue engineering. Engineering biomaterials surface with biomolecules including ECM proteins is a widely-used method to enhance cell adhesion. The advancement in tissue engineering requires the design of optimized biomaterial surface to facilitate tissue regeneration, which relies on a better understanding of cell adhesion behavior on engineered biomaterial surfaces. In this work, poly(lactic acid) (PLA) and carbon nanotubes (CNT), chosen as the representative of conventional biomaterials and novel nano-biomaterials, respectively, were surface-modified with ECM proteins, collagen type I (COL) and fibronectin (FN), to improve their compatibility. The accomplishment of immobilization of ECM proteins on PLA and CNT monolayer were confirmed by atomic force microscopy and BCA assay. Adhesion dynamics of porcine esophageal fibroblasts (PEFs) on ECM protein-immobilized PLA and CNT monolayer was examined by confocal reflection interference contrast microscopy (C-RICM) in conjunction with phase contrast microscopy. It was found that biophysical responses of PEFs on unmodified and ECM protein-modified surfaces are different. ECM protein immobilization accelerated adhesion of PEFs to biomaterials surfaces. PEFs on FN-immobilized biomaterial surface required shorter time to reach its maximum adhesion energy than on unmodified and COL-immobilized surfaces. Attachment ratio of PEFs on unmodified and ECM protein-immobilized biomaterials surfaces was in the order of FN-immobilized > COL-immobilized > unmodified. Furthermore, PEFs on FN-immobilized surface displayed more elongated bipolar morphology which resembled the morphology of fibroblast *in vivo* more than unmodified and COL-immobilized surfaces. Engineering biomaterial surface with ECM proteins possesses potential for application in esophageal tissue engineering.

Because of the conspicuous effect of fibronectin immobilization to promote cell adhesion, adhesion strengthening process for PEFs on FN-coated coverslip was examined and the importance of β_1 integrin to cell adhesion strength was evaluated. During 4 h of culture, spread area, adhesion force, and adhesion strength of PEFs on FN-coated coverslip increased gradually. Adhesion strength was tightly correlated to focal adhesion density. Peeling and fracture models were used to explain the variation of adhesion strength with focal adhesion density. Particularly, linear regression was performed to deduce averaged focal adhesion strength and non-focal adhesion strength. Adhesion strength β_1 -blocked PEFs was smaller than that of non-blocked cells, which was the collective effect of lower local adhesion strength (focal adhesion strength and non-focal adhesion strength) and focal adhesion density due to the functional blocking of β_1 integrin.

Weibull statistical analysis of adhesion strength data were carried out to study cell adhesion process. The distribution of adhesion strength in cell population can be described satisfactorily by Weibull distribution. Characteristic adhesion strength was proved to be a reliable parameter to characterize cell-substrate interactions. The development of cell adhesion was signified by the shift of Weibull cumulative probability and probability density graphs.

This work not only provides useful information about the design of optimized biomaterial surfaces for tissue engineering application, but also offers fundamental insights of cell-substrate interactions which enrich the understanding of cell adhesion development.

Chapter 1 Introduction

1.1 Background

Tissue loss or organ failure, as a result of physical injuries or diseases, is one of the major threats to human health [1]. In medical practice, tissue or organ transplantation is often employed to repair or restore the normal function of tissue or organ in these patients. However, this “standard” therapy has faced great challenge including immune rejection and limited donor source [2]. Although there are other alternative therapies for these patients such as surgical reconstruction and synthetic prosthesis, these methods of treatment have their own shortcoming. For instance, synthetic prosthesis cannot effectively restore the normal function of damaged organ or tissue [3]. The efforts in resolving these problems have prompt researchers to explore other ways to restore normal function of damaged tissue.

Tissue engineering integrates the principles of engineering with the fundamentals of biological sciences towards the development of biological substitutes to restore, maintain, or improve tissue function [4, 5]. Comparing with autograft and allograft procedure, tissue engineering techniques employing biomaterials are more effective because it can avoid problems of donor scarcity, supply limitation, pathogen transfer, as well as immune rejection [6].

In application of tissue engineering, biomaterial scaffolds provide the temporary site to guide new tissue growth and even supply biological signals to ensure the tissue-specific gene expression [7]. A successful tissue scaffold which can induce the regeneration of host tissue, often has several essential features of an ideal scaffold, such as high porosity, proper mechanical properties and biocompatibility [1, 8]. For most types of cells, adhesion to ECM/scaffold is indispensable for survival [9-11]. Without anchorage of cells to biomaterial scaffolds, tissue regeneration would not take place [7, 12]. Furthermore, the

interactions between biomaterials and adherent cells affect the downstream physiological processes of cells including cell proliferation, differentiation and tissue regeneration [13]. Therefore, effective supporting cell adhesion to biomaterial implant has become one of key factors to ensure the success of application of engineered tissue scaffold [2].

To fulfill the diverse needs in tissue engineering, various types of materials have been exploited as scaffold candidates for tissue regeneration [14]. Although many biomaterials exhibit some excellent properties required by tissue engineering, these biomaterials scaffolds cannot provide favorable microenvironment for regenerative cells to support their attachment, proliferation, differentiation, and neo tissue genesis [2].

In native tissues *in vivo*, cellular activities are mediated through the interactions between integrin receptors of cells and ECM adhesion proteins, such as collagen, fibronectin, laminin, and vitronectin [15]. Therefore, to promote cell adhesion and growth, ECM proteins can be introduced onto biomaterials surface to provide specific signal for cells to facilitate tissue formation or regeneration [16]. Immobilized extracellular matrix proteins including fibronectin and collagen entail the ligands for interaction with integrin receptors of cells [17]. Thus, ECM protein-engineered scaffold surfaces imitate certain advantageous feature of natural ECM and act as temporary ECM for regenerative cells [18].

Cell adhesion to biomaterials surface/ECM is a dynamic process, which is mainly mediated by ligand-integrin receptor interactions [19]. During cell adhesion, cells initially need to settle down to a biomaterial surface and ligand-integrin receptor binding occurs which allows the cells to withstand gentle shear force. Then adherent cells spread out to assume a flat morphology. At last, stress fibers appear and focal adhesions form. In addition, a series of signal transduction cascades such as focal adhesion kinase (FAK) phosphorylation, and gene expression, are triggered.

Biophysical responses of adherent cells on engineered biomaterial surface are

believed to be correlated to many critical cellular activities [18]. Better understanding of biophysical responses of adherent cells is of great importance in optimizing biomaterial surface for tissue engineering applications [13]. For example, robust mechanical interaction between adherent cells and biomaterials is requisite in the design of tissue-engineered scaffold [20]. In vascular tissue engineering, rapid and robust adhesion of endothelial to vascular graft surface would be beneficial for adherent cells to withstand the shear forces applied by the blood flow [21]. In connection, information about adhesion energy or adhesion strength acquired through investigating biophysical responses in cell adhesion, allows researchers to choose proper engineered biomaterial surface for tissue engineering application.

Previously, researchers have put great efforts to synthesize novel biomaterials, and have sought new methods to modify biomaterial surfaces to improve their biocompatibility. Only a few studies are focused on monitoring biophysical responses of cells to biomaterials surface during cell adhesion process [22, 23]. Particularly, there is lack of understanding of the change in mechanical property of cell-biomaterial surface interactions in the development of cell adhesion [19].

With the advancement of experimental apparatus and technique, in deep study of biophysical responses including adhesion energy and strength becomes possible now. Confocal reflection interference contrast microscopy (C-RICM) has emerged as an ideal real-time imaging technique to probe the cell-substrate interface [24]. Using C-RICM in conjunction with phase contrast microscopy, valuable information about the exact geometry of adherent cells is obtained under physiological conditions. Total internal reflection fluorescence microscopy (TIRFM), which is adept in examine molecular events within cell-substrate interface [25], can precisely record the focal adhesion plaques through obtained images with very low-level noise. In addition, adhesion strength of adherent cells can be acquired conveniently through micropipette aspiration technique combined with phase contrast microscopy. The integration of molecular biology techniques

with the aforementioned biophysical approaches, make it possible to examine biophysical responses of adherent cells on molecularly engineered biomaterial surface comprehensively and explore the possible relationship between cell-biomaterial surface interactions and intracellular responses. In this work, cell adhesion behavior including adhesion dynamics, deformation, morphology, cytoskeleton reorganization, focal adhesion formation, adhesion energy, and adhesion strength, was investigated systematically.

1.2 Objectives and Scope

The aim of this work is to elucidate the effect of ECM protein immobilization on cell adhesion behavior on biomaterial surface, and explore the inherent relationship between ligand-integrin receptor interactions and intracellular responses during cell adhesion process.

Nanomaterials is often defined as those materials with morphological features smaller than 100 nm in at least one dimension [26, 27]. The fact that the dimensions of nanomaterials are analogous to those of nanostructured ECM enables nanomaterials to be promising candidates for improving traditional tissue engineering materials [27]. Moreover, nanomaterials have exhibited superior cytocompatible, mechanical, electrical, optical, and magnetic properties compared to conventional microstructured materials [28], implying their great potential for numerous tissue engineering applications. To date, researchers have made big progress in using nanomaterials for tissue regeneration of bone, cartilage, bladder, and so on [27].

In this study, poly(lactic acid) (PLA) and carbon nanotube (CNT), as the representative of conventional biomaterials and newly-developed nano-biomaterials, respectively, went through surface modification with ECM proteins (collagen type I and fibronectin). Adhesion behavior of porcine esophageal fibroblasts (PEFs) on ECM protein-immobilized PLA or CNT surface,

as a model system, was studied to elucidate the underlying correlation between immobilized ligands and biophysical responses of adherent cells in the development of cell adhesion. Specifically, the following topics were studied in detail.

- To investigate adhesion dynamics of porcine esophageal fibroblasts on ECM-protein immobilized PLA using C-RICM. Cytoskeleton remodeling was visualized via immunostaining technique to correlate with adhesion dynamics.
- To study the effect of ECM protein coating on adhesion dynamics, cell attachment ratio, cell proliferation, and actin filament distribution of PEFs on CNT monolayer.
- To explore the relationship among adhesion force, adhesion strength, cell spreading and focal adhesion formation during cell adhesion process for PEFs on a model ECM (fibronectin-coated coverslip). Furthermore, the importance of β_1 integrin to cell adhesion strengthening process was evaluated after functional blocking of β_1 integrin by monoclonal antibodies.
- To apply Weibull statistics to analyze adhesion strength of PEFs on fibronectin-coated coverslip.

The results obtained in the aforementioned works will help to gain a better understanding of cellular response to molecularly engineered biomaterials surface in cell adhesion process, which is expected to provide useful instructions in designing optimized engineered tissue surface for potential application in esophageal tissue engineering.

1.3 Organization

This thesis is organized into eight chapters. The background, objective and scope of present research are introduced in Chapter 1. In Chapter 2, we present a literature review on surface modification methods for PLA and CNT,

integrin-mediated cell adhesion, and experimental techniques for investigating cell adhesion. Materials and experimental methods are covered in Chapter 3. The adhesion dynamics of ECM protein-immobilized PLA and CNT monolayer are described in Chapters 4 and 5, respectively. In Chapter 6, the temporal effect of functional blocking of β_1 integrin on adhesion strength is discussed. In Chapter 7, results of adhesion strength of PEFs on fibronectin-coated coverslip are processed using Weibull statistical method. Finally, conclusions and future work are given in Chapter 8.

Chapter 2 Literature Review

This chapter reviews the background information about surface modification methods for poly(lactic acid) and carbon nanotube, extracellular matrix-cell interactions, and experimental techniques for investigating cell adhesion. Various approaches to immobilize proteins onto poly(lactic acid) and carbon nanotube, are overviewed in section 2.1 and 2.2, respectively. As cell adhesion on biomaterial surfaces involves extracellular matrix-cell interactions, background knowledge of ECM protein structure and function, mutual recognition between integrin receptors and ligands, cytoskeleton reorganization and focal adhesion formation, as well as cell adhesion strengthening are introduced in section 2.3. Some newly-developed experimental techniques, such as confocal reflection interference contrast microscopy and total internal reflection fluorescence microscopy were used in our study. Their major advantages and recent applications in the study of cell adhesion are reviewed in section 2.4.

2.1 Surface Modification Methods for Poly(lactic acid)

Poly(lactic acid) (PLA) is a biodegradable and mechanically strong polymer [29]. The adoption of synthetic PLA in clinical practices can be traced back 30 years ago when they were used as biomaterials for suture and bone fixation devices [30]. As one of the biomaterials approved by the US Food and Drug Administration, PLA has been used widely as biodegradable scaffolds in tissue engineering since then. However, PLA cannot effectively support cell attachment due to the lack of bioactive ligands on its surface. Surface modification of PLA by introducing specific ligands, such as ECM proteins or the peptides containing cell recognition motif of ECM proteins, can improve the biocompatibility and promote cell adhesion [17].

Protein (or peptide) immobilization on PLA is generally accomplished by

formation of covalent or non-covalent bonds. ECM proteins can directly be immobilized to the polymer surface through physical absorption or entrapment. In this case, the binding of proteins to the polymer surface is often through non-covalent bonds. Comparatively, proteins can be covalently attached to the polymer surface in more controllable way after surface treatment of PLA. Since PLA is chemically inert to proteins, functional groups should be produced by chemical or physical treatment for the following covalent immobilization of proteins.

2.1.1 Physical Absorption

It is convenient to immobilize proteins on polymer surface by physical absorption. ECM Protein coated on PLA surface is recognized by integrin receptors of seeded cells and acts as adhesive layer for attachment of seeded cells.

To improve the performance of PLA films as skin culture support, Garric et al. modified PLA surface by coating collagen type I [31]. It is shown from the experimental results that collagen coating enhances skin cell (human keratinocyte and dermal fibroblast) adhesion and proliferation. Aframian et al. examined the effect of surface coating of PLA with matrix proteins (fibronectin, laminin, collagen type I, collagen type IV, and gelatin), on growth and morphology of a salivary epithelial cell line (HSG) [32]. It is found that HSG cells do not adhere to the polymer disks without pre-adsorbed proteins. On fibronectin-coated PLA disks, a uniform monolayer of HSG cells forms through culture. Coating of disks with laminin, collagen type I and IV, and gelatin also is beneficial to the formation of cell monolayer. The study demonstrates the potential capability of ECM protein-coated PLA towards developing an artificial salivary gland.

Protein/peptide immobilization can also be realized via the efficient physical absorption of carrier molecule of protein/peptide on PLA surface. Quirk et al. immobilized GRGDS peptide on PLA with poly(L-lysine)-GRGDS as a biomimetic surface modifier [33]. In their study, poly(L-lysine)-GRGDS

conjugate, was synthesized through straightforward carbodiimide attachment of GRGDS peptide to poly(L-lysine) (PLL) first. Then the poly(L-lysine)-GRGDS was attached to the poly(lactic acid) by physical adsorption due to the existence of high affinity between PLL and PLA. It is found that bovine aortic endothelial cells seeded spreads more extensively on the modified PLA than on unmodified surface, demonstrating improvement of the cell-biomaterial interactions on GRGDS-contained PLA surface.

2.1.2 Entrapment

Entrapment is a simple method to incorporate natural polymers with synthetic polymer, originally proposed by Desai and Hubbell for the modification of polymeric materials such as poly(ethylene terephthalate) [34, 35]. The process of entrapment of proteins to polymer mainly includes immersion of polymer into protein solution and the final drying.

Cai et al. modified PLA surface through entrapment of silk fibroin (SF) [36]. To SF-entrapped surfaces, PLA films were placed into acetone/water solution (v/v = 3:1) for 1 h and then soaked in the SF solution for 24 h. Following washing thoroughly and drying, silk fibroin were entrapped into PLA surfaces successfully. Through entrapment of SF, cell attachment, proliferation, viability and differentiation function were greatly enhanced on PLA. The immobilization of gelatin on PLA through entrapment was also realized by other researchers [37].

Zhu et al. entrapped the alginate-amino acid derivatives into poly(DL-lactic acid) with the reversible swelling property [38]. In their study, covalent attachment of amino acid (L-lysine, L-arginine, L-aspartic acid, and L-phenylalanine) to sodium alginate was performed using water-soluble carbodiimide (1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide. Then, sodium alginate and its amino acid derivatives were dissolved in a mixed solution of water and acetone (v/v = 70/30). After immersing the poly(DL-lactic acid) membrane into the solution and through the subsequent process, a thin layer of alginate on

poly(DL-lactic acid) formed which can promote chondrogenesis.

2.1.3 Chemical Treatment

Production of functional groups on polymer surface by means of chemical treatment is also an efficient method for surface modification. Major chemical treatment methods used widely at present include hydrolysis, aminolysis, and oxidation.

Polymer hydrolysis involves the scission of susceptible molecular group by reaction with H_2O . After hydrolysis of the polymer, some functional groups will form for further surface modification. Cai et al. adopted hydrolysis to process poly(DL-lactic acid) [36]. After poly(DL-lactic acid) films were treated in NaOH solution for at $50^{\circ}C$ for 1 h, the carboxylic acid groups were produced on the modified films. The 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC), as a peptide coupling agent, was used to activate carboxylic acid group on the films. Finally, silk fibroin was grafted to the film by reacting the activated carboxylic acid group with the nucleophilic N-terminus of the peptide. In addition, entrapment method to immobilize the silk fibroin was also employed for comparison. Interestingly, in the methylthiazoletetrazolium (MTT) assay, the viability of osteoblasts on entrapment-treated poly(DL-lactic acid) film was significantly higher than that found on EDC-treated poly(DL-lactic acid) films. A similar trend was also observed from alkaline phosphatase activity and cell attachment. It is obvious that the entrapment method is better than EDC treatment for surface modification of poly(DL-lactic acid) in their study.

Zhu et al. devised a simple method to create amino groups on PLA surface through aminolysis [39]. After the treatment of 1,6-hexanediamine, amino groups were introduced onto PLA surface through the reaction between 1,6-hexanediamine and the ester groups of PLA. Gelatin, chitosan, or collagen can be further immobilized onto the aminolyzed PLA membrane via a cross-linker. Immobilization of the biomacromolecules through the above method has a

positive influence on endothelial regeneration *in vitro*, confirming the effectiveness of the method for grafting proteins.

Ma et al. made hydroperoxide groups on PLA film through photooxidization [40]. PLA was treated with peroxide solution under ultraviolet (UV) irradiation. After the photooxidization step, the PLA film with hydroperoxide groups underwent polymerization with methacrylic acid by induction of UV. The resultant product is PLA film with grafted poly(methacrylic acid) on the surface. Then it reacted with EDC to activate the carboxyl groups. Through the aforementioned steps, the protein can be grafted to the PLA surface via covalent bond.

2.1.4 Physical Treatment

Physical treatment is another effective way to modify biomaterial surface. Poly(lactic acid) and polycaprolactone (PCL) can be amino-functionalized through allylamine pulsed plasma deposition [41]. Carlisle et al. coated a thin layer of amine on PCL and PLA surface by pulsed plasma deposition [41]. Adhesion peptides (RGD or YIGSR) coupled to poly(ethylene glycol) (PEG) were linked to the aminated PCL and PLA surface. Further adhesion assays were carried out on the peptide-grafted polymer surfaces. Hepatocyte adhesion to PCL and PLLA surfaces was enhanced by this novel adhesion peptide grafting technique using pulsed plasma deposition and PEG cross-linking.

2.2 Surface Modification Methods for Carbon Nanotubes

Carbon nanotubes (CNTs), as a novel materials, discovered in 1991 by Iijima [42]. This class of nanomaterial possesses various excellent properties such as high surface area, and high mechanical strength with ultra-light weight. Because of their unique physical properties, carbon nanotubes are potentially useful in a wide variety of applications, such as electronics, optics, and advanced materials

[43, 44]. Recently, the advent of the field of CNT has stimulated an interest in its potential application in biomedical devices [45]. For example, the unique properties of CNTs, such as a large surface area to volume ratio and high mechanical strength, make them an excellent candidate for providing the needed structural reinforcement for tissue engineering scaffolds [46-52]. However, carbon nanotubes do not effectively support cell adhesion due to lack of bioactive domain on its surface, which may hinder its application in tissue engineering.

To improve the biocompatibility of CNTs for cell regeneration, CNTs can be functionalized with various biomolecules including ECM proteins with or without chemical coupling. In a simple way, proteins are immobilized onto the wall of CNTs via noncovalent bonding through physical adsorption. Nevertheless, CNTs can be chemically modified and then covalently bound to proteins.

2.2.1 Noncovalent Attachment of Proteins to CNTs

The simplest scheme to immobilize proteins to CNTs is physical absorption. The large aromatic (π -electrons) surface of CNTs makes it easy for biomolecules to bind through hydrophobic forces [53]. In this process of physical absorption, proteins are nonspecifically bound to CNT and form noncovalent protein-nanotube conjugates. Various lines of evidence indicate that proteins can be adsorbed spontaneously on the sidewalls of oxidized CNTs [54, 55]. Despite the advantages of physical absorption including simple operation, the relative size of some proteins to single wall carbon nanotube (SWCNT) may hinder its application for immobilization of particular large size proteins. In a study by Kam et al., protein-SWCNT conjugates are poor and nearly nonexistent for a large protein including human immunoglobulin G (hIgG, 150 kDa in molecular weight) [54]. As the diameter of hIgG is significantly bigger than that of SWCNTs (~7-8 nm in diameter for hIgG; 1-2 nm in diameter for SWCNTs), scarcity of hIgG-SWCNT may possibly be attributed to size mismatch between large protein and SWCNT [54]. However, it is not a hurdle for large protein to be absorbed

onto multi-walled carbon nanotube (MWCNT) as the diameter of MWCNT is bigger enough.

2.2.2 Covalent Linkage of Proteins to CNTs

Covalent absorption of proteins onto the CNTs bears some advantages including more controllability and tighter binding in CNT-protein conjugate. There are a number of ways to achieve this goal. One widely-used way to do this is to covalently cross-link proteins to CNTs with carboxyl functional groups. Carboxylic functional group can be produced on either sidewall or the ends of the CNTs by acid oxidization. After activation of carboxyl groups, protein can be covalently bound to CNT. Jiang et al. immobilize ferritin and bovine serum albumin (BSA) on CNT surface through a three-step process using this method [56]. The overview of covalent attachment process is shown in Figure 2.1. First, MWCNTs are acid-oxidized to form carboxyl groups on the surface of the carbon nanotubes. Then, these groups can be further activated using EDC (also called EDAC) as a coupling agent. As acylurea derivative produced by EDC treatment is highly reactive, it forms a more stable active ester in the presence of N-hydroxysulfosuccinimide (NHS). Finally, through nucleophilic substitution reaction between the active ester and the amine group of the protein, a protein can cross-linked to CNT via amide bond. Due to avoidance of intermolecular conjugation of proteins, the attachment of proteins to MWCNTs through this process is uniform. Therefore, this approach provides a universal and efficient method for protein (or peptide) immobilization on carbon nanotubes.

Carbon nanotubes can also be functionalized with amino groups for the subsequent grafting of protein (or peptide). This method of functionalization is based on the 1,3-dipolar cycloaddition reaction of the azomethine group to CNT sidewalls [57]. Pantarotto et al. successfully used amino functionalized CNT to bind peptides [58]. In their study, foot-and-mouth disease virus (FMDV) peptides were covalently coupled to CNT surface. Antigenicity of the FMDV peptide

conjugated to CNT was further proved by a surface plasmon resonance analysis and an enzyme-linked immunosorbent assay, suggesting that the peptide bound to the CNT took up correct secondary conformation recognized by specific antibodies.

Figure 2.1 Covalent binding of proteins to CNTs through three-steps process (adapted from reference [56]). Reprinted in part from [Jiang KY, et al. Journal of Materials Chemistry 2004;14:37-39 Copyright (2004)] with permission from Royal Society of Chemistry.

2.3 Extracellular Matrix-Cell Interactions

The interactions between cell and ECM are important physiological activities for most mammalian. Bidirectional signals between the extracellular matrix and intracellular compartments are transduced through the cell-ECM interactions [59]. Only after attaching to the ECM and interact with it, the anchorage-dependent cells can survive, grow, and differentiate [60]. Although the subcellular machinery

that mediates cell–ECM adhesion and signaling is complicated, it consists of the binding of ligands to transmembrane integrin receptors and multiple intracellular proteins interactions triggered by receptors [61, 62].

2.3.1 ECM Protein Structure and Function

Extracellular matrix is an insoluble macromolecular network. It is composed of a great variety of biomolecules, including the collagen family, elastic fibers, glycosaminoglycans, proteoglycans, and adhesive glycoproteins [7]. These biomacromolecules form a network for cells to organize into tissue and interact with their environment [63].

Many proteins in the extracellular matrix, such as collagen, fibronectin, laminin, vitronectin, tenascin and nidogen, are found to interact with cells [63]. These huge ECM proteins usually not only have several cell-binding domains but also contain the domains to connect themselves to other ECM proteins [63]. The first well-characterization protein from ECM is collagen, which is the structural component of the matrix [64]. Collagens are the most abundant proteins found in mammals. Collagens are predominantly synthesized by fibroblasts but epithelial cells can also synthesize these proteins. There are more than 15 types of collagen but most of them are type I, II, III. The collagen has the triple-helical structure, with two α_1 chains and one α_2 chain for collagen type I. The collagen molecules with the triple-helical structure can associate side by side to form collagen fibrils, which are further assembled into collagen fibers. The schematic of collagen fiber formation is shown in Figure 2.2. Collagen fibers ensure the necessary strength and rigidity of extracellular matrix for cell to adhere and migrate.

Figure 2.2 The schematic of collagen fiber formation.

Fibronectin (FN) is one kind of adhesive glycoproteins in ECM. Many different cell types synthesize and secrete FN. Fibronectin consists of two nearly identical polypeptide chains, each with 220 kD in molecular weight, linked together by disulfide bonds at the C terminus [65]. It mainly contains three types of molecular repeats, FN type I, II, and III. In cell adhesion, the fibronectin dimmers bind to integrins. Then interaction of fibronectin with cytoplasmic proteins such as actin via integrin leads to the formation of focal adhesion. Through oligomerization of fibronectin at FN I₁₋₅ and FN III₁ domains [66, 67], the fibronectin fibrils are elongated. The disulfide cross-linking and integrin clustering in focal adhesion lead to the transformation of fibrils into insoluble structure. This process for the formation of insoluble structure of fibronectin is called fibrillogenesis, which is critical for some cellular activities. For example, Yi et al. reported that FN III fragments inhibits tumor growth, angiogenesis, and metastasis when administered in mice [68].

2.3.2 Mutual Recognition between Integrin Receptors and Ligands

The interactions between cells and extracellular matrix are mediated by cell adhesion receptors [15]. Among them, integrins are a major class of transmembrane receptors, which play a central role in the transduction of cell-matrix adhesion signals [61, 62]. Integrin signaling contributes not only to cell adhesion and migration, but also regulates cell proliferation, survival, and differentiation [61]. Integrins are a family of heterodimers. Each integrin comprises subunit α and β . To date, at least 18 α subunits, 8 β subunits, and 24 different integrins have been reported [69]. Each subunit consists of three domains, namely, the extracellular domain, the transmembrane domain, and the cytoplasmic domain. The integrins bind to short amino acid sequences present in ECM proteins. Selective amino acid sequences in ECM proteins mediating cell adhesion are listed in Table 2.1. Among these sequences, the Arg-Gly-Asp (RGD) sequence is widely known because it is found in many types of ECM proteins, such as collagen, fibronectin and vitronectin, and binds to integrin receptors in various types of cells [70]. Proteins or peptides containing RGD sequence are used widely to modify biomaterials surface to enhance cell adhesion and activate integrin signaling pathways in tissue engineering [71].

For ECM protein-integrin interaction, there is no simple one-to-one correspondence in the ligand-integrin pair. One integrin can interact with several ligands. At the same time, one specific ligand can bind to different types of integrins (Table 2.2). For example, $\alpha_3\beta_1$ binds to fibronectin, collagen, laminin and so on while fibronectin can interact with over several types of integrins including $\alpha_3\beta_1$, $\alpha_5\beta_1$ and $\alpha_v\beta_3$ [72]. Despite the existence of multiple integrin dimers interacting with collagen, $\alpha_2\beta_1$ is the major type I collagen receptor [73]. For fibronectin, cell adhesion to fibronectin is mediated primarily by the $\alpha_5\beta_1$ integrin.

Table 2.1 Selective amino acid sequences of extracellular matrix proteins involving in cell adhesion (adapted from references [71]). Reprinted in part from [Shin H, et al. Biomaterials 2003;24:4353-4364 Copyright (2003)] with permission from Elsevier.

Sequences	Origin	Function
RGD	Fibronectin, Vitronectin, Collagen	Cell adhesion
KQAGDV		Smooth muscle cell adhesion
YIGSR	Laminin B1	Cell adhesion
REDV	Fibronectin	Endothelial cell adhesion
KHIFSDDSSSE	Neural cell adhesion molecules	Astrocyte adhesion
DEGA	Collagen	Cell adhesion
KRSR	Heparin binding domain	Osteoblast adhesion

Table 2.2 The integrin family of adhesion receptora (adapted from reference [72]).

Reprinted in part from [Anselme K. Biomaterials 2000;21:667-681 Copyright (2000)] with permission from Elsevier.

Sub-units		Other names	Ligands
β_1	α_1	VLA-1	Collagen-I, collagen-IV and laminin
	α_2	VLA-2, P1a-IIa, ECMRII	Collagens-I to IV, laminin and possibly Fibronectin
	α_3	VLA-3, VCA-2, ECMRI, Gapb-3	Fibronectin, Collagen-I, Epiligrin, Laminin and Nidogen/Entactin
	α_4	VLA-4, LPAM-2	Fibronectin, VCAM-1
	α_5	VLA-5, FNR, GPIc-IIa, ECMRVI	Fibronectin
	α_6	VLA-6, GPIc-IIa	Laminin-1, -2, -4 and -5 (only <i>in vitro</i>)
	α_7	VLA-7	Laminin-1
	α_8		Fibronectin
	α_9		Tenascin-C, osteopontin
	α_V		Fibronectin, vitronectin
β_2	α_D		?
	α_L	LFA-1, CD11a-CD18	ICAM-1 to -3
	α_M	Mac-1, CR-3, CD11b-CD18	C3bi, coagulation factor X, fibrinogen and ICAM-1
β_3	α_X	p150, CR-4, CD11c-CD18	Fibrinogen
	α_V	VNR, CD51	Fibrinogen, fibronectin, Von Willebrand's factor, vitronectin, thrombospondin, osteopontin and bone sialoprotein 1
	α_{IIb}	GPIIb-IIIa, CD41	Fibrinogen, fibronectin, Von Willebrand's factor and vitronectin
β_4	α_6		Laminin-1
β_5	α_V		Vitronectin
β_6	α_V		Fibronectin
β_7	α_4	LPAM-1	MadCAM, fibronectin and VCAM-1 ?
	α_{IELb}	M290 IEL, α_H	?
β_8	α_V		?

^aVLA: very late activation antigen, VCA: very common antigen, ECMR: extra cellular matrix receptor, GP: glycoprotein, Gap: galactoprotein, LFA: leukocyte function associated antigen, M290 IEL: mouse intraepithelial lymphocyte antigen recognized by monoclonal antibody M290, FNR: fibronectin receptor, ICAM: inter cellular adhesion molecule, LPAM: lymphocyte Peyer's patch HEV adhesion molecule (mouse), VCAM: vascular cellular adhesion molecule, CR: C3bi receptor, VNR: vitronectin receptor, MAC: macrophage receptor.

2.3.3 Cytoskeleton Reorganization and Focal Adhesion Formation

During cell adhesion process, signal transduction cascades are triggered [15]. The actin cytoskeleton is one of the most important targets of integrin-mediated signal transduction [74]. After ligand binds to the cellular receptors, integrin begins to cluster and signal transduction cascade is initiated. The early adhesion initiated signal transduction, include activation of Rac and Cdc42, which stimulates the formation of lamellipodia and filopodia extension, respectively [75]. The Rho GTPases play an important role in cytoskeletal rearrangements [76]. They act as molecular switches, which are active when they are bound to GTP and inactive when bound to GDP. Integrins can activate these small GTPases including Rac and Cdc42. RhoA is another important member of Rho GTPases. Different from Cdc42 and Rac, RhoA serves as upstream regulator for integrin activation [77]. Furthermore, it plays a central role in the formation of both stress fibers and focal adhesions in spread cells [76]. Two other small GTPases, R-ras and Rap1 are also found to act upstream of integrin activation [78, 79].

Focal adhesions are multi-component structure situated within the region of close contact between cell membrane and the underlying extracellular matrix. In focal adhesion, integrins, signaling and cytoskeletal molecules are colocalized. Focal adhesion is often shown in the form of flat, elongated plaque with several square microns in area. Many types of proteins including vinculin, talin and paxillin, participate in the formation of focal adhesion. It is observed that the recruitment of focal adhesion components is a sequential process during the aggregation of integrins following binding of integrins to ligands [74, 80]. Furthermore, there are differences in the constituents and the state of activation of the focal adhesion components. Tensin and FAK are recruited in the first place following integrin clustering. Clustered integrin-ligand binding induces recruitment of focal adhesion components such as vinculin, α -actinin, and talin. In addition, phosphorylation events at focal adhesions are triggered by members of

the Src protein kinase family, including c-Src, Csk, and Fyn. Subsequently, downstream signaling molecules including Rho-like GTPases are activated and the ERK and JNK signal transduction cascades are triggered [81, 82]. Finally, this signaling transduction cascade initiated by integrin-ligand binding leads to cytoskeleton reorganization, anchorage-dependent entry into S-phase of cell cycle, and integrin-mediated gene transcription [83].

2.3.4 Cell Adhesion Strengthening

Integrin-mediated adhesion is a dynamic process involving strengthening of mechanical property of cell adhesion between adherent cells and substrate. Many mechanical models have been proposed to analyzing the enhancement of adhesion force/adhesion strength (adhesion force per unit spread area) during cell adhesion process [84-88].

One of the classic models to describe the development of cell adhesion force proposed by Lotz et al. was traced back to 1989 [85]. In their study, adhesion force was measured by a centrifugal force-based adhesion assay and close contact between cell and substrate was visualized by interference reflection microscopy. It is shown in their results that the values of initial cell adhesion force at 4°C maintains on the order of 10^{-5} dynes/cell and there is no involvement of cytoskeleton in this adhesion process. Through 15 min of culture at 37°C, cell adhesion force to fibronectin coated surface experiences an order of magnitude increase which requires cytoskeletal involvement. In contrast to the evident adhesion strengthening on fibronectin, there is not enhancement of adhesion for cells on tenascin during 15 min of culture at 37°C. Therefore, the variation of adhesion is dependent on the type of protein and cell. In addition, there is tight correlation between the area of close contact with separation distance of less than 15 nm and adhesion force. Further experimental results of inhibitory activity of cytochalasin B imply that the adhesion strengthening after initial binding requires the recruitment of additional biomolecules into the adhesive site and actin

polymerization. Therefore, integrin-mediated adhesion can be divided into two major stages: (1) the initial ligand-integrin receptor binding and (2) the following adhesion strengthening process which involves a series of post-binding events, including integrin clustering, focal adhesion formation, and cytoskeleton remodeling.

Adhesion strength is influenced by many factors, such as bond number, integrin clustering, and focal adhesion assembly. Mathematical models provide useful tools to theoretically analyze the contribution of different factors to adhesion strengthening process. Bell did pioneering work to propose a theoretical framework for the analysis of cell adhesion force [89]. Based on Bell's theory, Ward and Hammer did work to model the effects of focal contact formation on adhesion strength [86]. In their study, a one-dimensional tape peeling model was adopted to deduce the critical tension necessary for peeling the membrane to obtain adhesive strength with or without focal contacts. Through mathematical analysis, formulas for calculating the critical tension to initiate peeling of cell without focal contacts were derived. Focal contacts are modeled as congregation sites of cytoplasmic adhesion components, whose structural integrity is determined by cytoskeletal connections. The strengthening effect of focal contact formation on adhesion strength depends on the elastic rigidity of the cytoskeletal connections. In the condition of rigid cytoskeletal connections, greater attachment strength is deduced. Finally, their predicted results were compared with the adhesion strengthening responses of glioma cells on fibronectin-coated surface. Comparison of the model with experimental data at 37°C suggests that cells may experience a switch of detachment mechanism with the maturation of focal adhesion contact. Before the maturation of focal adhesion, a loose cytoskeleton network with little mechanical stiffness leads to negligible adhesion strengthening. Under this condition, detachment of cells proceeds through peeling. As the process of focal contact maturation involves a combination of receptor clustering and focal contact solidification, the entire adherent cell membrane shares the applied load. Under this

condition, cell detachment means simultaneous failure of the bonds between cell membrane and surface, which is called fracture mode. As cell detachment moves from peeling towards fracture, adhesion strength increases.

Although Ward's model can be employed to analyze adhesion strengthening process comprehensively, it relies on many biophysical parameters that are difficult to derive experimentally, which hinder its application. Gallant et al. used a force balance, macroscopic-to-microscopic model with only a few of parameters to analyze adhesion strengthening process [87, 90].

A principal advantage of Gallant's approach is that the main parameters of the analysis can be easily obtained in the customized experimental system, allowing direct comparisons between theoretical and experimental results. In their experiment, micropatterned substrates with engineered adhesive area and a hydrodynamic assay are integrated to investigate fibroblast adhesion strengthening on fibronectin. It is shown that integrin binding and focal adhesion assembly induce seven-fold increase of adhesion strength rapidly until reaching steady state level. Through simulation by a force balance, macroscopic-to-microscopic model, adhesion strength demonstrates exponential increases with the number of bound integrins, as well as recruited vinculin and talin, consistent with experimental findings. Furthermore, the contribution of focal adhesion assembly to adhesion strength is determined to about 30%.

2.4 Experimental Techniques for Investigating Cell Adhesion

With of the advances in technology, novel experimental techniques, such as confocal reflection interference contrast microscopy (C-RICM), micropipette aspiration, and total internal reflection fluorescence microscopy (TIRFM) are developed to study cell adhesion process. These newly-developed methods make it possible for us to study the biophysical responses of cells on molecular

engineered surfaces comprehensively.

2.4.1 Confocal Reflection Interference Contrast Microscopy

The formation of cell adhesion contact is related to the biophysical responses of cell upon adhesion. The contour of membrane-surface separation in the cohesive zone of adherent cell contains much meaningful information about cell adhesion such as the adhesion strength and membrane bending modulus. Characterization of cell adhesion contact is very important for the investigation of cell-surface interactions. Several techniques based on confocal microscope including side-view phase contrast microscope, have been adopted in studying cell adhesion. However, they only provide information about cell contour and cannot probe the cell-surface separation, whose distance is less than 1 μm typically [91].

Base on the interference of light reflected from the cell and the underlying substrate, confocal reflection interference contrast microscopy has been developed to examine the adhesion contact [91]. By integrating the virtue of C-RICM and the advantage of laser source, it can achieve a dynamics range between 0.01 μm and 1 μm and improve the spatial resolution in cell adhesion contact [91].

Fang et al. reported the theory of C-RICM and its application for probing the deformation and adhesion of phospholipids vesicles induced by colloidal forces on solid surface [91]. The effect of wall thickness, pH, and osmotic stress on the non-specific adhesion of giant unilamellar vesicle (ULV) and multilamellar vesicle (MLV) on fused silica was investigated by the C-RICM in conjunction with cross-polarization light microscope. To analyzing the data obtained from the experiments, the adhering vesicle was simplified as a truncated sphere and the adhesion energy is calculated base on a mechanical model proposed by Wan et al [92].

Yin et al. began to extend the application of C-RICM to cell-surface interactions [93]. In their study, C-RICM in conjunction with phase contrast microscopy was utilized to investigate the adhesion contact and two-dimensional

spreading kinetics of HepG2 cells on galactose immobilized and collagen gel coated substrates. It is shown from the experimental results that HepG2 cells keep nearly round shape in presence of galactose while they spread very much on collagen-coated substrate. Although there is difference in morphology for cells on the galactose-immobilized and collagen-coated substrates, HepG2 cells forms strong adhesion contact on the two types of substrates. Moreover, the initial rate of adhesion contact formation and stabilized adhesion energy of HepG2 cells on galactose-immobilized substrate are higher than those collagen-coated surfaces. That result provides meaningful information of cell adhesion to elucidate the cell adhesion mechanism. By a similar method, the adhesion contact dynamics of fibroblasts on other biomacromolecule-immobilized surfaces was studied through C-RICM [94-96].

Cell adhesion on the surface-modified polymer can also studied using C-RICM [22, 23]. Zhu et al. implemented C-RICM to investigate cell adhesion on surface modified poly(lactide-co-glycolide) (PLGA) [23]. They immobilized azide-chitosan (Az-CS) onto PLGA first. Then gelatin (Gel) was grafted to the polymer surface with the aid of glutaraldehyde as the crossing-linking agent. The adhesion dynamics of 3T3 fibroblasts on three PLGA surfaces (Gel-CS-PLGA, CS-PLGA and unmodified PLGA surface) was examined by C-RICM and phase contrast microscopy. The cell adhesion was greatly enhanced on the Gel-CS-PLGA surface because the maximum adhesion energy at steady state was achieved on this surface. The remarkable effect of gelatin and chitosan immobilization on cell adhesion, difficult to obtain by other experimental techniques, was demonstrated from experimental results.

It is interesting that Feng et al. compared the adhesion dynamics of intact HepG2 cells and GFP-actin transfected HepG2 cells on a model extracellular matrix by C-RICM [97]. The initial rate of cell deformation of intact HepG2 cells on the model ECM was higher than that of transfected HepG2 cells. That result was possibly related to the fact that the internal structure of HepG2 cells

was altered during GFP-actin transfection process.

Recently, He et al. integrated C-RICM with AFM to evaluate attachment of both bacteria and mammalian cells towards a functionalized biomedical device surface [98]. It is shown that immobilized RGD sequence only promotes fibroblast adhesion but has no apparent influence on *E. coli* and *S. aureus* adhesion. In contrast, fibroblast and *S. aureus* adhesions were enhanced together through collagen immobilization. Their findings bring new insight into the design of biomedical device surface which requires promoting cell adhesion and inhibiting bacterial adhesion simultaneously.

From the above cases about the application of C-RICM, it is found that the development of adhesion contact can be easily monitored by C-RICM. Moreover, adhesion energy can also be obtained conveniently from C-RICM and phase contrast images with the aid of mechanical model. Therefore, C-RICM provides an easy way to study cell adhesion behavior on engineered biomaterial surfaces.

2.4.2 Micropipette Aspiration

The micropipette aspiration technique is a versatile tool in capturing and manipulating cells [99]. It has many applications in the study of cells such as determining the mechanical property of single cell and measuring the adhesion strength between cell and ECM [100]. The basic principle of this technique is simple. A glass micropipette is connected to a water container. By change the vertical position of water container, the pressure in the micropipette tip can be controlled precisely for sucking a cell. A schematic of micropipette aspiration system is shown in Figure 2.3.

Figure 2.3 A schematic of micropipette aspiration system (adapted from reference [99]). Reprinted in part from [Missirlis YF, et al. *Biomolecular Engineering* 2002;19:287-294 Copyright (2002)] with permission from Elsevier.

Micropipette aspiration is useful technique to determine the cell-cell adhesion force [101]. Shao et al. presented a novel method to measure piconewton-scale adhesion forces [102]. By using this technique, the minimum force required to form a membrane tether from neutrophils was determined. At the same time, the length of the microvilli on the surface of neutrophils was deduced through the experimental result. They continued to use the micropipette aspiration technique to measure the strength of anchoring of transmembrane receptors to cell membrane [103]. In their experiment, several beads were coated with antibodies to CD62L (L-selectin), CD18 (β_2 integrins), or CD45. With micropipette suction, the bead was manipulated to adhere to the neutrophil and then detach from neutrophil surfaces. The force ranges to extract L-selectin, β_2 integrin, and CD45 from neutrophil surfaces were determined. The obtained results supported the conclusion that the bond between L-selectin and its ligand is broken during neutrophil rolling on endothelium.

Besides cell-cell adhesion, micropipette aspiration is used to widely measure cell adhesion to engineered biomaterials surface.

Sung and colleagues carried out a series of works in investigating adhesion of ligament fibroblasts on various substrate through micropipette aspiration

technique [104-107]. In their study, adhesion force and strength of fibroblast from the anterior cruciate ligament and the medial collateral ligament are compared on laminin, fibronectin, collagen types I and III coated surfaces. In addition, the effect of tropomodulin, talin, vinculin, and alpha-actinin on ligament fibroblast adhesion was also determined in terms of differential adhesion forces. These results are expected to provide some theoretical basis of healing ligament damage in clinical practice.

Qin et al. measured of adhesion strength of human embryonic tenocytes (HETCs) and transformed human embryonic tenocytes (THETCs) on fibronectin(FN) and collagen type I (COL) immobilized PLGA surface through micropipette aspiration technique [108]. FN and COL were immobilized on the Poly-D-lysine-coated PLGA surface. HETCs and THETCs were cultured on the surface-modified PLGA for different time and then subjected to suction and manipulation by micropipette aspiration. It is indicated from the experimental results that the adhesion strength of cells increases with the culture time. Anti-fibronectin antibody and anti-collagen type I antibody coated on protein-modified surface compromise the function of FN or COL in promoting cell adhesion. That result proves that FN or COL-modified PLGA is beneficial for the THETC and HETC adhesion and has potential application in tissue engineering as tendon scaffold.

Both micropipette aspiration and C-RICM can be used to characterize mechanical property of cell adhesion between adherent cells and substrate as adhesion force and adhesion energy can be acquired by micropipette aspiration and C-RICM, respectively. However, there is difference in their application. For micropipette aspiration, adhesion force is directly measured through experiments. Mechanical model is not required to analyzing experimental data to obtain adhesion force. In contrast, adhesion energy can only be acquired after analyzing C-RICM images with mechanical model. Therefore, C-RICM is applied to the characterization of mechanical property of cell adhesion in an indirect way. As

some simplifications are made in the mechanical model, there must be deviation between deduced adhesion energy and the actual value.

2.4.3 Total Internal Reflection Fluorescence Microscopy

Total internal reflection fluorescence microscopy (TIRFM) is a novel technique to excite and visualize fluorophores in an aqueous environment very near a solid surface (within ≤ 100 nm) [109, 110]. Because only fluorescent molecules at the cell-substrate interface are selectively illuminated by evanescent light in TIRFM, fluorescence from fluorophores in the bulk solution or inside the cells is markedly low. Therefore, TIRFM technique provides better fluorescence images with higher contrast and lower background than traditional epifluorescence microscopy [109, 110].

Generally, cells can be fixed and immunostained for the subsequent observation using TIRFM. Guignandon et al. adopted TIRFM as the major tool in studying the effect of microgravity exposure on cell adhesion and its related cell cycle [111]. In their study, vinculin was stained by FITC-conjugated antibody and observed by TIRFM. The acquired TIRFM images were analyzed to obtain parameters describing morphologic parameters of adhesion plaques (vinculin), such as area and number of adhesion plaques. Quantification of focal adhesion parameters demonstrated that microgravity acted on rat osteosarcoma cells by disorganizing cytoskeletal actin and vinculin. Using a similar method, Senju et al. visualized vinculin-containing focal adhesions in fibroblasts [112]. It is demonstrated in their experimental results myosin-II inhibitors (Y-27632) effectively affect adhesion dynamics of Swiss 3T3 cells, which is testified by disappearance of stress fibers and smaller vinculin plaques after 4 h of culture in the presence of 10 mM Y-27632.

To monitor molecular behavior of live cells at the cell-substrate interface by TIRFM, fluorescence protein-tagged targeted protein can be introduced into cells by transfection technique. Iino et al. imaged single green fluorescent protein

(GFP) molecule successfully for the first time [113]. In their experiment, GFP were linked to the cytoplasmic carboxyl terminus of E-cadherin (E-cad-GFP) and the oligomerization of E-cad-GFP was examined through TIRFM. It is found that many E-cad-GFP oligomers greater than dimmers, appear on the free surface, suggesting the function of greater oligomers as the basic components of adherent junctions.

Slack-Davis et al. applied TIRFM to the analysis of focal adhesion turnover [114]. In their experiment, NIH 3T3 cells were stably transfected with green fluorescent protein-paxillin first. Then transfected 3T3 cells in confluence were treated with a novel focal adhesion kinase inhibitor (PF-573,228). Finally, focal adhesions in cells at the edge of the wound were analyzed by TIRFM. The lifetime of single adhesion plaque was determined by monitoring its fluorescence intensity over time. It is shown that untreated cells possess shorter adhesion lifetime and more active adhesion assembly and disassembly than pretreated cells with PF-573,228, demonstrating the effectiveness of PF-573,228, as a focal adhesion kinase inhibitor in depressing cell migration.

2.5 Summary

Immobilization of ECM proteins on biomaterial surfaces can be used to promote cell adhesion. The advancement in tissue engineering requires the design of optimized biomaterial surface to facilitate tissue regeneration, which relies on a better understanding of biophysical responses on engineered biomaterial surfaces.

Cell adhesion is strengthening process, which is often indexed by the augment of adhesion energy or strength. With the aid of C-RICM, adhesion energy can be easily obtained. Therefore, poly(lactic acid) (conventional biomaterials) and carbon nanotubes (nano-biomaterials) was chosen to go through surface modification to improve their biocompatibility, and adhesion strengthening process of cells of ECM protein engineered PLA or CNT can be studied by

C-RICM.

Cell adhesion involves integrin-ECM interactions. β_1 integrin subunit plays an important role in integrin-ECM adhesion. It is necessary to elucidate the importance of β_1 integrin subunit in adhesion strengthening process. Furthermore, the contribution of focal adhesion to adhesion strengthening process has not been understood thoroughly. Through micropipette aspiration technique and TIRFM, the relationship between adhesion strength (by micropipette aspiration) and focal adhesion (by TIRFM) is determined. In addition, the effect of functional blocking of β_1 integrin on cell adhesion is evaluated. Systematic investigation of cell adhesion behavior on molecularly engineered biomaterials using newly-developed apparatuses will not only enrich the understanding of cell adhesion on biomaterial surface, but also provide beneficial instructions in modifying biomaterial surface for tissue engineering applications.

Chapter 3 Materials and Experimental Methods ^{*†}

In this Chapter, the source of materials used in this study is introduced. In addition, the details of experimental methods such as substrate preparation, BCA assay, atomic force microscopy, and contact angle measurement, are presented.

3.1 Materials

Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), Trypsin-EDTA, Alexa Fluor® 635 phalloidin, penicillin-streptomycin solution, and kanamycin were obtained from Invitrogen (USA). Phosphate buffer saline (PBS), collagen type I, fibronectin, sodium dodecyl sulfate (SDS), bovine serum albumin (BSA), FITC-labeled phalloidin, QuantiPro bicinchoninic acid (BCA) assay kit, methylthiazolotetrazolium (MTT) solution, glutaraldehyde and paraformaldehyde, Triton X-100, 4',6-diamidino-2-phenylindole (DAPI), and PLA were purchased from Sigma-Aldrich (USA). The CNT monolayers supported on glass substrate were obtained from Nanolab biosystem Inc. (USA). Mouse anti-vinculin monoclonal antibody and FITC conjugated Goat anti-Mouse IgG were provided by Chemicon (USA). Anti-integrin β_1 antibodies (4B4 clone) were bought from Beckman Coulter (USA).

3.2 Substrate Preparation

^{*} Reprinted in part from [Cai N, et al. ACS Applied Materials & Interfaces. 2010; 2:1038-1047 Copyright (2010) and Cai N, et al. Langmuir. 2009; 25:10939-10947 Copyright (2009)] with permission from American Chemical Society.

[†] Reprinted in part from [Cai N, et al. Biomedical Materials. 2008; 3:015014 Copyright (2008)] with permission from Institute of Physics.

3.2.1 Coverslip Cleaning

Silica coverslips were cleaned in Piranha solution (a 3:7 mixture of 30% hydrogen peroxide and sulfuric acid) for 2 h at 80°C. They were subsequently thoroughly rinsed with 18.2 MΩ distilled water and dried in a vacuum oven at 120 °C for 1 h.

3.2.2 Spin Coating

To prepare a PLA-coated glass coverslip, approximately 0.4 ml of PLA solution in dichloromethane (5 g/L) was dispensed and allowed to spread thoroughly on a cleaned coverslip fixed on a spin coater. The spin coating was conducted at 3000 rpm for 90 seconds. A layer of PLA film was formed on the coverslip through spin coating. The PLA-coated coverslips were placed in a vacuum oven overnight to remove the remnant of dichloromethane on the coverslips.

3.2.3 ECM Protein Immobilization

Immobilization of ECM proteins on PLA was carried out by the following procedures [115]. The procedure is schematically shown in Figure 3.1. First, a small volume of 1,6-hexanediamine/propanol solution (6% w/v) was added onto the PLA coated coverslips. After being placed for 2 min at room temperature (22°C), the coverslips were washed with large quantity of deionized water to remove the free 1,6-hexanediamine. Through this step, the amino groups are produced on the PLA surface through the reaction between amine of the 1,6-hexanediamine and ester groups of PLA. Second, glutaraldehyde (GA) was introduced as the cross-linker between aminolyzed PLA and ECM proteins. In brief, the PLA coated coverslip was immersed in GA solution (1% w/w) for 2 h at room temperature, followed by rinsing with a great amount of deionized water to remove free GA. Then, the coverslips were air-dried and sterilized under UV light

for 30 min. The last step was the covalent binding of ECM proteins to GA-modified PLA surface. Collagen or fibronectin solution (0.5 mg/ml) was placed on the GA-modified PLA surface and was allowed to spread out evenly. After 24 h of absorption at 4°C, the coverslip was rinsed three times with 1× PBS (pH=7.4) to remove the unbound collagen or fibronectin. For simplicity, GA-modified PLA, collagen or fibronectin-immobilized PLA surfaces are abbreviated to PLA-CHO, PLA-COL, and PLA-FN, respectively.

Figure 3.1 Schematic procedures of immobilization of ECM proteins on PLA. PLA was first aminolyzed by 1,6-hexanediamine to introduce amino groups on PLA surface. Then glutaraldehyde was adopted to covalently bind to PLA surface with free carboxyl. Finally, ECM proteins were grafted to the PLA surface with the aid of GA as the cross-linker between aminolyzed PLA and ECM proteins.

The CNT monolayer used in our study was a layer of multi-walled carbon nanotubes covalently bound to a silica coverslip [116]. Before usage, each coverslip with CNT monolayer was thoroughly washed by deionized water. Subsequently, the coverslip was dry by air and exposed to UV light for 30 min. Collagen type I solution or fibronectin solution (0.6 mg/ml in 1× PBS) was dispensed onto the CNT monolayer followed by incubation for 24 h at 4°C. Finally, the coverslip was washed three times with 1× PBS to remove unbound

collagen or fibronectin. For convenience, CNT monolayer, collagen-coated CNT monolayer, and fibronectin-coated CNT monolayer are abbreviated as CNT-ML, CNT-COL, and CNT-FN, respectively, hereafter.

To prepare fibronectin-coated coverslips, the cleaned coverslips were sterilized through 2 h of UV exposure before protein coating. Then fibronectin solution (0.6 mg/ml in 1× PBS) was placed onto the surface of each coverslip. After incubation overnight at 4°C, the coverslips were washed three times with 1× PBS.

3.3 Bicinchoninic Acid Assay

The density of immobilized collagen or fibronectin was determined by QuantiPro BCA assay kit. In brief, the protein-coated substrate were immersed in 1% SDS and shaken for 30 min to desorb the immobilized proteins. Then the desorbed protein solution was transferred into a centrifuge tube and QuantiPro BCA working reagent was added to the tube with protein solution, followed by gentle mixing. After incubation for 2 h at 37°C, the sample was cooled down to room temperature and its absorbance was measured at 562 nm with UV-visible spectrophotometer (Shimadzu UV-2450, Shimadzu Corp., Japan). Each measurement was repeated at least three times and the results are presented as average $\pm$ standard deviation.

3.4 Atomic Force Microscopy

AFM was used to examine topographical feature of all surfaces. All measurements were performed with a NanoMan AFM system (Veeco Inc., USA). A cantilever with a spring constant of 0.03 N/m and a scan rate of 1 Hz were used in all the tests. Each measurement was performed on at least six regions (1 μm ×1 μm in dimension for each region) for each of the four samples. Each AFM topographic image was a typical one for each sample. The root-mean-square

(RMS) roughness of the each image was determined by AFM associated software, Nanoscope Software v5.31. (Veeco Inc., USA).

3.5 Contact Angle Measurement

The contact angle of distilled water (18.2 MΩ) on the different surfaces was measured by the sessile drop technique using a contact angle measurement equipment (FTA 1000, First Ten Angstroms Inc., USA) at room temperature. Water droplets of 5 µl formed by an automatic micro-syringe were dropped onto different points of each surface, and the images of attached droplets were recorded after 30 seconds. Water contact angle was obtained by analyzing the image of the droplet. The average value of contact angle was calculated based on over eight data points collected from three sample surfaces.

3.6 Culture of Porcine Esophageal Fibroblasts

Porcine esophageal fibroblasts were prepared from pig esophagus according to a published protocol [115]. In brief, porcine esophagus was cut open longitudinally and rinsed thoroughly in 1× PBS. Mucosa-submucosa layer was peeled off from the esophagus and cut into small pieces. Then these small parts of tissues were washed three times for 5 min each time with 1× PBS containing 1000 U/ml penicillin, 1000 U/ml streptomycin, and 1 mg/ml kanamycin, and collected by centrifugation for 5 min at 1000 rpm. The obtained tissue pellets were responded in DMEM supplemented with 10% FBS, 500 U/ml penicillin, 500 U/ml streptomycin, and 0.5 mg/ml kanamycin and seeded onto a fibronectin-coated culture flask. After two or three days of culture, fibroblasts extended from tissue pellets and adhered to the flask due to their strong attachment capability. These primary porcine esophageal fibroblasts were collected and transferred into tissue culture polystyrene dish for culture in DMEM, supplemented with 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin at 37°C in 5% CO₂ atmosphere.

When PEFs reached 80-90% confluence, the cells were harvested with Trypsin-EDTA solution. The cell suspension was centrifuged at 1000 rpm for 5 min. The supernatant was discarded and the cell pellet was resuspended for sub-culturing or experiments. The cells used in the experiments were between passage 6 and passage 10.

3.7 Integrin Blocking

Anti-integrin β_1 antibodies were applied to block binding of integrin β_1 to fibronectin. To prepare non-blocked PEFs (the control group) and integrin β_1 -blocked PEFs (the experimental group), cells were harvested and suspended in blocking buffer (0.5% BSA in DMEM) to a final density of 1×10^6 cells/ml. Then cells in blocking buffer were maintained at 4°C for 30 min.

For the control group, cells were kept in this condition for another 30 min. For the experiment group, monoclonal anti-integrin β_1 antibodies were added into cell solution at a volume ratio of 1:100 and the cells were incubated in blocking buffer with antibodies at 4°C for 30 min. Before usage, non-blocked and β_1 -blocked PEFs were collected by centrifugation followed by the dispersion in DMEM.

3.8 Phase Contrast Microscopy and Confocal Reflection Interference Contrast Microscopy

Confocal reflection interference contrast microscopy (C-RICM) in conjunction with phase contrast microscopy used in our experiments is based on Pascal 5 confocal microscope system (Carl Zeiss, Germany) with a 40 $\times$ oil-immersion objective (Neofluar, N.A.:1.25) [24]. Its illumination source was an Argon-ion laser with a maximum power of 1 mW and excitation wavelength of 488 nm. Phase contrast microscopy and C-RICM were used to visualize the contour of adherent cells and their adhesion contact, respectively. Strong adhesion contact of an adherent cell appears as a dark or grey region on the C-RICM image.

Throughout the experiment, the cells were kept at 37°C and 5% CO₂ through a customized chamber (Carl Zeiss, Germany). Following the seeding of the cells with 1 ml of culture medium on the substrates, a series of phase contrast and C-RICM images were taken at regular intervals. Using an image analysis software (ZSM5, Carl Zeiss, Germany), the mid-plane diameters of adhering cells (R) and contact zone radius (a) were obtained.

A contact mechanics model of adherent cells has been proposed previously [92]. In the model, an adherent cell is modeled as a truncated capsule with mid-plane radius, R , contact zone radius, a , and contact angle, θ . Schematic diagram of a cell adhering to a flat substrate is shown in Figure 3.2. The degree of deformation a/R is related to other parameters in the model through the equation, $\alpha = a/R = \sin\theta$. The cell wall is assumed to be under a uniform equi-biaxial stretch; the biaxial stress, σ , equates to $C\varepsilon$ where C is equivalent to $Eh/(1-\nu)$ in a linear system under small strain with the elastic modulus, E , and Poisson's ratio, ν . The average biaxial strain, ε , is given by

$$\varepsilon = \frac{1}{2} \left[\frac{2 + 2(1 - \alpha^2)^{1/2}}{4/R^2 - \alpha^2} - 1 \right] \quad (3.1)$$

After cell seeding, the cell spontaneously adjusts its distance of approach toward the substrate until the equilibrium is achieved. The adhesion energy, W , can be shown as [92]

$$W = (1 - \cos\theta)C\varepsilon + C\varepsilon^2 \quad (3.2)$$

Based on the experimental measurements of the mid-plane diameter, R (from phase contrast microscopy), and the radius of contact zone, a (from C-RICM), W can be determined by Equations 3.1 and 3.2. Elastic modulus, E , of porcine esophageal fibroblast is taken as 12 kN/m² according to the measurement of 3T3 fibroblasts by AFM indentation [117].

It is worthy to be pointed out that this model was proposed based on the assumption of negligible bending in cell membrane. The bending will play its role

when cell shape is not perfect spherical. Therefore, the adhesion energy deduced by this model is lower than its actual value.

Figure 3.2 Schematic diagram of a cell adhering to a flat substrate. Mid-plane radius, contact zone radius, and contact angle are represented by R , a and θ , respectively.

In addition, two parameters are defined herein to obviate the influence of widely distributed cell size to analyze the two-dimensional (2D) spreading and adhesion contact kinetics. Normalized 2D spread area, A_N , is defined as the ratio of 2D spread area, A , to the initial 2D spread area (or called mid-plane area), A_0 , as given in Equate 3.3. Similarly, normalized radius of contact zone, a_N , is defined as the radius of contact zone, a , divided by the initial mid-plane radius, R_0 , as given in Equation 3.4. The initial 2D spread area and mid-plane radius are obtained by analyzing the phase contrast image during the initial 2-3 min after cell seeding.

$$A_N = \frac{A}{A_0} \quad (3.3)$$

$$a_N = \frac{a}{R_0} \quad (3.4)$$

Figure 3.3 is composed of a series of phases contrast images of a cell on PLA surface at different time following cell seeding. The spread area of this cell is

measured to be $653 \mu\text{m}^2$ (3 min), $669 \mu\text{m}^2$ (20 min), and $2155 \mu\text{m}^2$ (8 h), respectively. According the above definition, $A_0 = 653 \mu\text{m}^2$. Therefore, A_N (20 min) = $669/653 = 1.02$ and A_N (8 h) = $2155/653 = 3.30$.

Figure 3.3 A series of phase contrast images of a cell on PLA surface at different time following cell seeding.

3.9 Immunostaining

Cells at a density of 2×10^4 cells/cm² were seeded on PLA, PLA-CHO, PLA-COL, and PLA-FN surfaces. After being cultured at 37°C and 5% CO₂ for a predetermined period, the cells were washed with 1× PBS and fixed with 3.7% formaldehyde for 20 min. The sample was further rinsed three times with 1× PBS and permeabilized with 0.2% Triton X-100. The cells were subsequently incubated with FITC-labeled phalloidin in 1× PBS containing 0.5% BSA for 1 h. Confocal fluorescence microscopy was carried out on the cells with a laser scanning microscope (Pascal 5, Carl Zeiss, Germany).

To visualize actin filaments, PEFs were cultured on CNT-ML, CNT-COL, and CNT-FN surfaces for 24 h. The cells was then washed in 1× PBS, fixed in 4% paraformaldehyde solution for 30 min, washed again in 1× PBS and stained with FITC-labeled phalloidin (5 μg/ml) for 40 min. Finally, the samples were washed

in 1× PBS to remove residual stain and viewed using confocal laser scanning microscope (Pascal 5, Carl Zeiss, Germany).

To examine F-actin and vinculin, Non-blocked and β_1 -blocked PEFs were seeded onto fibronectin-coated coverslips. After cultured for different time periods, cells were fixed with 4% paraformaldehyde in 1× PBS for 30 min followed by washing twice with wash buffer (1× PBS containing 0.05% Tween-20) at room temperature. The cells were then permeabilized with 0.1% Triton X-100 in 1× PBS for 10 min. Following two washes with wash buffer, the sample was saturated with blocking solution (1% BSA in 1× PBS) for 40 min. After an overnight incubation in dark at 4°C with mouse anti-vinculin monoclonal antibodies (1:300 dilution in blocking solution), the sample was washed three times with wash buffer again. Finally, FITC conjugated Goat anti-Mouse IgG at 1:300 dilution in 1× PBS was applied together with Alexa Fluor® 635 phalloidin (3 Unit/ml) to the sample for 1 h at room temperature. The stained sample was covered with antifade mounting solution (Vector laboratories, USA) for microscopy observation.

3.10 Total Internal Reflection Fluorescence Microscopy

The TIRFM system is based on Zeiss Axiovert inverted microscope system (Carl Zeiss, Germany) equipped with a 100× 1.45NA objective lens (Carl Zeiss, Germany), a 488 nm argon laser, and X-Cite 120 fluorescence illumination system. FITC-labelled vinculin (Green) was detected in TIRFM mode with excitation at 488 nm. Alexa Fluor® 635-labelled actin (Red) was visualized using an X-cite 120 illumination system. In addition, a bright field image was also taken for the same field of vision to examine cell morphology.

All the obtained bright field and TIRFM images were analyzed using ImageJ (National Institute of Health, USA) to measure cell spread area and focal adhesion area, respectively. Focal adhesion area was acquired by following a published

procedure [118]. First, TIRFM image of vinculin was converted into a grey level image. Second, the binary image was generated by thresholding the grey level image. The threshold value was calculated by the formula, $\text{Threshold} = \text{Mean} - \text{SD}$, where *Mean* and *SD* refer to the average value and standard deviation of the grey level distribution of all pixels in the grey level image, respectively. Pixels with a grey level above the threshold value are set to 1 (black in a binary image), while the rest are set to 0 (white in a binary image). Finally, focal adhesion area was obtained from the binary image by counting the black pixels. In addition, cell spread area was acquired after analyzing bright field image by ImageJ software. The obtained results of focal adhesion area and focal adhesion density (defined to be focal adhesion area per unit spread area) are shown in the form of mean $\pm$ standard deviation.

3.11 Scanning Electron Microscopy

The detailed morphology of the cells was examined by scanning electron microscopy (SEM). Firstly, PEFs seeded on different substrate (CNT-ML, CNT-COL, and CNT-FN) were cultured for 24 h. Then the substrates with adherent cells were rinsed with $1\times$ PBS followed by fixation with 2.5% glutaraldehyde (SEM grade) for 2 h. After three rinses with $1\times$ PBS, the specimens were immersed in 1% osmium tetroxide solution for 1 h. The fixed specimen were dehydrate with a graded ethanol series (30%, 50%, 70%, 90%, 95%, $2\times 100\%$) for 10 min at each concentration, soaked in hexamethyldisilazane for 10 min and dried in air. Finally, the specimens were sputtered with a layer of platinum and visualized by a field emission SEM (JSM-6700, JOEL Ltd., Japan).

The SEM images were analyzed using AxioVision, an image analysis software (Carl Zeiss, Germany). After measuring spread area and perimeter of cells (Figure 3.4), cell shape factor coefficient (Sfc) [119], defined as $4\pi \times (\text{spread}$

area)/(perimeter²), was calculated. Sfc ranges from 0 to 1, where 0 implies a straight line, and 1 denotes circular shape (Figure 3.5).

Figure 3.4 Schematic diagram of spread area and perimeter of a cell in a SEM image.

Figure 3.5 Illustration of shapes with different Sfc.

3.12 Cell Adhesion Force and Strength

Cell adhesion force was measured with a micropipette aspiration system. Its complete setup was shown in Figure 3.6. Before measurement, equilibrium

voltage, V_{eq} , corresponding to equilibrium liquid pressure at which no water or media went in or out of the micropipette, was measured. Then, a coverslip with adherent cells cultured on fibronectin-coated surface for specified time was mounted onto the stage of an inverted microscope (Carl Zeiss, Germany). One adherent cell was chosen randomly for measurement and a phase contrast image was taken to capture its shape. Figure 3.7 showed the basic experimental procedure. During experiment, a small aspiration pressure was applied in micropipette, by using the syringe, to catch an adhering cell. Then micropipette was moved by micromanipulator horizontally away from the cell. Initially, a cell cannot move together with micropipette as the aspiration pressure is not big enough. The aspiration and retraction of cell was repeated with the stepwise increase in suction pressure until the entire cell body moved together with micropipette. The voltage corresponding to the detachment of the cell, V_d , was recorded. Based on calibration result, the liquid pressure–voltage scale is 554.89 Pa/V. Therefore, the aspiration pressure (P_a) needed to detach the cell is expressed as following equation.

$$P_a = 554.89 \times (V_d - V_{eq}) \quad (3.5)$$

The inner diameter of the micropipette tip (d_m) was measured using SEM. The adhesion force (F_s) was calculated by

$$F_s = P_a A_{micropipette} = P_a \times \left(\frac{\pi}{4} d_m^2 \right) \quad (3.6)$$

The schematic diagram of d_m and P_a was shown in Figure 3.8. Cell spread area was acquired from phase contrast images of adherent cells. Adhesion strength was determined by dividing adhesion force by cell spread area, where. At least 80 cells cultured for a specified time period were used for micropipette aspiration test. The adhesion strength results are presented in form of mean $\pm$ standard error.

Figure 3.6 Schematic diagram of micropipette aspiration system. The syringe was used to produce negative pressure in the pipelines to aspirate cell, or positive pressure to push cell out of micropipette. Based on the readings of multimeter (not shown here) connected to the pressure transducer, the pressure in the pipelines can be measured and controlled.

Figure 3.7 Experimental procedure of measuring adhesion force. (a) The suction pressure is applied on the cell. (b) Micropipette is moved horizontally, and cell does not move at low levels of suction pressures. (c) Micropipette is moved back, and suction pressure is increased step by step and applied on cell. (d) Cell is moved away at certain level of aspiration pressure. Scale bar = 50 μm .

Figure 3.8 A cell under suction by micropipette.

3.13 Methylthiazoletetrazolium Assay

MTT assay was carried out to determine the metabolic activity of attached cells. Cellular damage will inevitably accompanied by loss of the ability of the cell to provide energy for metabolic function. As tetrazolium salts are reduced to a colored formazan only by metabolically active cells, MTT assay is devised based on this principle. MTT assay is based on the cleavage of tetrazolium ring of MTT by mitochondrial dehydrogenases of viable cells, yielding purple formazan crystals which are insoluble in aqueous solutions. Briefly, PEFs at the seeding density of 1×10^5 cells/cm² were cultured on three different types of substrates for 1 or 3 days. In the last 4 h of each culture period, cells were covered with 1 mg/ml of MTT solution. Following 4 h of incubation, the MTT solution was removed and the specimen was washed with $1 \times$ PBS. The formazan crystals produced were dissolved in dimethyl sulfoxide and a microplate reader (GENios, Tecan Inc., USA) was used to measure the absorbance at a wavelength of 570 nm. Each test was repeated three times for each surface.

3.14 Cell Attachment Assay

In order to study the different response of PEFs on unmodified and modified PLA or CNT monolayer surfaces, cell attachment ratio was determined to quantify the adhesiveness of cells to the different surfaces. The attachment ratio (R_a) is

defined as the percentage of the number of the cells adhering to the substrate for a given time after cell plating:

$$R_a = \frac{N_a}{N_0} \times 100\% \quad (3.7)$$

where N_a and N_0 is the number of cells adhering to the substrate 2 h after cell seeding, and the initial number of cells applied on the substrate, respectively.

In cell attachment test on unmodified and ECM protein-immobilized CNT monolayer, PEFs at a density 1×10^5 cells/cm² were plated and allowed to adhere on the substrates in the incubator for 2 h at 37°C and 5% CO₂. At the end of this time period, the substrates were washed three times with 1× PBS in order to remove non-adherent cell. Then the adherent cells on the substrate were harvested by trypsinization. The number of the harvested cells by trypsinization was determined using a haemocytometer. Each assay was repeated over three times for each type of surface.

The affinity of fibronectin-coated coverslips to non-blocked and integrin β_1 -blocked PEFs was evaluated according to the number of attached cells after the same culture period. Firstly, cells were plated onto fibronectin-coated surface at about 5×10^4 cells/cm². Following each incubation period, the surfaces were rinsed with 1× PBS to remove unbound cells. The adhered cells were fixed using 4% paraformaldehyde in 1× PBS for 20 min followed by permeabilization in 0.2% Triton X-100 for 2 min. After three wash by 1× PBS, cell nuclei were stained by DAPI (50 ng/ml). For counting cells, at least 20 separate zones on each substrate were imaged at 200 times amplification by an Olympus IX71 microscope. Each assay was repeated five times separately for each type of cells.

3.15 Statistical Analysis

All data is shown as mean $\pm$ standard deviation unless otherwise specified. Statistical analysis was performed by the Student's t-test. A value of $P < 0.05$ was

considered statistically significant. Weibull statistics was also used to analyze the distribution of adhesion strength.

Chapter 4 Adhesion Dynamics of Porcine Esophageal Fibroblasts on ECM Protein-immobilized Poly(lactic acid) [‡]

4.1 Introduction

Esophagus cancer is currently ranked as the 7th most common cancer with the diagnosis of over 400 000 new cases annually worldwide [120]. Surgery, chemotherapy, and radiation therapy are commonly used to treat esophagus cancer patients [121]. In combination with other treatments, surgery serves to minimize the patient's suffering and improve his/her quality of life. The treatment of esophageal cancer by surgery often comprises removal of a portion of esophagus and the reconnection of the remaining portion of the esophagus with specific implant [122]. Alternatively, several types of human innate tissues including stomach and colon, have been tried as possible esophagus substitutes but resulted in limited efficacy [123, 124].

Replacement of the cancerous esophagus by engineered tissue equivalent is considered an ideal treatment in the future. However, there is currently a lack of focus on the development of novel materials for enabling esophagus regeneration. Surface-modified biomaterials, particularly those that are widely used in clinical practices, are potential candidates for engineered esophagus. By surface modification, the biocompatibility of biomaterials could be improved effectively [125]. One of the key tasks of esophageal tissue engineering is to develop bio-functional materials which can mimic the biological microenvironment of esophageal cells and eventually induce the tissue regeneration for esophagus substitute.

[‡] Reprinted in part from [Cai N, et al. Biomedical Materials. 2008; 3:015014 Copyright (2008)] with permission from Institute of Physics.

In this study, poly(lactic acid) as a widely-used biomaterials was functionalized with ECM proteins to explore its potential application in esophageal tissue engineering. The main objective of our work is to gain a better understanding of the biophysical responses of primary esophageal fibroblasts on surface-modified PLA surface in cell adhesion. In detail, two typical ECM proteins, collagen type I (COL) and fibronectin (FN), were immobilized on PLA film through chemical cross-linking. Monitoring of the dynamics in cell deformation and adhesion energy of porcine esophageal fibroblasts (PEFs) on PLA, PLA-CHO, PLA-COL, and PLA-FN surfaces was performed by using C-RICM and phase contrast microscopy. F-actin immunostaining was employed to study the transformation in the spatial organization of F-actin in PEFs, in relation to adhesion kinetics and adhesion affinity at the population level.

4.2 Results and Discussion

4.2.1 Surface Modification of PLA

The immobilization of ECM proteins on PLA-coated coverslips can possibly leads to the change of surface morphology on PLA surface. Typical AFM topographic images ($1 \times 1 \mu\text{m}$) of PLA, PLA-CHO, PLA-COL, and PLA-FN surfaces are shown in Figure 4.1. The root-mean-square (RMS) roughness of a pure silica coverslip was determined as $0.18 \pm 0.05 \text{ nm}$ [97]. The AFM measurement shows the emergence of small clusters on PLA. The RMS roughness of PLA surface is $1.15 \pm 0.16 \text{ nm}$ and higher than that of a pure silica coverslip due to the deposition of PLA film on the substrate. The RMS roughness of the PLA-CHO, PLA-COL, and PLA-FN surfaces is $3.56 \pm 0.23 \text{ nm}$, $2.45 \pm 0.28 \text{ nm}$, and $4.12 \pm 0.33 \text{ nm}$, respectively. Moreover, there is a significant change on the nano-topology on both PLA-COL and PLA-FN compared to PLA surface. It is shown that the immobilization of ECM proteins on PLA surface results in the increase of RMS roughness. The difference of RMS roughness was

also reported in collagen and fibronectin-coated coverslips [97]. The densities of immobilized collagen and fibronectin on PLA surface were determined to be $1.02 \pm 0.23 \mu\text{g}/\text{cm}^2$ (PLA-COL) and $1.19 \pm 0.27 \mu\text{g}/\text{cm}^2$ (PLA-FN), respectively. The result demonstrates that PLA-COL and PLA-FN is fully covered with proteins as the required density to forming collagen and fibronectin monolayer is $0.3 \mu\text{g}/\text{cm}^2$ and $0.25 \mu\text{g}/\text{cm}^2$, respectively[22, 126].

Figure 4.1 AFM topographic image of a typical region ($1\mu\text{m}\times 1\mu\text{m}$) on (A) PLA, (B) PLA-CHO, (C) PLA-COL, and (D) PLA-FN surfaces. The height scale is shown in the (E).

4.2.2 Spreading and Adhesion Dynamics of PEFs on PLA, PLA-CHO, PLA-COL, and PLA-FN Surfaces

Cell spreading is one of the typical phenotypes for strong cell adhesion. Figure 4.2 shows the phase contrast images of PEFs on PLA, PLA-CHO, PLA-COL, and PLA-FN surfaces 5 min and 24 h after cell seeding. Due to lack of specific ligands, it is difficult for cells to spread on the PLA film. As shown in the figure, many cells take on a round shape and smooth contour without forming filopodia or lamellipodia on PLA even after 24 h. Similarly, only a small portion of seeded PEFs attach and spread on the control surface (PLA-CHO) at 24 h. This result suggests that PLA surface fails to provide an ideal microenvironment for activating PEFs. Through immobilization of collagen or fibronectin on PLA surface, the activation of PEFs is greatly improved. It is evident in Figure 4.2 that most seeded cells extensively spread on PLA-COL and PLA-FN surfaces after 24 h of seeding. The attached PEFs on these two surfaces exhibit polygonal morphology at 24 h.

Figure 4.2 Phase contrast images of PEFs on PLA, PLA-CHO, PLA-COL, and PLA-FN surfaces 5 min and 24 h after cell seeding. Scale bar = 30 μm .

Cell spreading, which exists almost throughout the cell adhesion process, is related to many physiological activities of the cell [127]. As demonstrated in

Figure 4.3(A), spread area of the individual cell ascends over time. The average normalized 2D spread area of PEFs is plotted against time within 24 h after cell seeding as shown in Figure 4.3(B). Error bar represents the standard deviation of the data for at least 60 cells on eight identical samples. In general, it is seen that the average normalized 2D spread area on four surfaces (PLA, PLA-CHO, PLA-COL, and PLA-FN) increases with time. However, the initial rate of increase is different on the four surfaces. The spread area increases faster on PLA-FN and PLA-COL surfaces than on PLA and PLA-CHO surfaces. Particularly, the 2D spread area of PEFs on PLA-FN surface demonstrates abrupt increase only after seeded for 20 min. The difference in initial rate of increase of 2D spread area demonstrates the effect of immobilized ECM proteins on cell adhesion. Fibronectin contains more than two adhesion motifs including Arg-Gly-Asp (RGD) and Arg-Glu-Asp-Val (REDV) [71], resulting in a higher probability of binding the integrins of PEFs. That may help to explain the most rapid responses of PEFs on PLA-FN surface among four cases. The spread area reaches steady state after 20 h of culture. The normalized spread area, A_N , of PEFs on PLA, PLA-CHO, PLA-COL, and PLA-FN surfaces at 24 h of cell seeding is 3.78, 3.96, 4.79* and 8.20*, respectively (*Student's t-test: $P < 0.01$, compared to PLA and PLA-COL surfaces).

Cell spreading is correlated to the cytoskeleton remodeling triggered by receptor-ligand interactions [62]. The different extent of spreading for PEFs on four surfaces suggests the innate interaction between integrin receptors and immobilized ECM proteins. It is known that fibroblast adhesion to collagen is primarily mediated through interactions with the integrin $\alpha_1\beta_1$ and $\alpha_2\beta_1$ [128]. However, several integrins including $\alpha_5\beta_1$, $\alpha_2\beta_1$, and $\alpha_v\beta_3$ can interact with fibronectin [61, 129]. There are more types of ligand-receptor interactions between fibronectin and integrins. In addition, it has been found that the sequence Pro-His-Ser-Arg-Asn (PHSRN) in fibronectin acts synergistically with RGD to enhance integrin-mediated adhesion and cell signaling via integrin $\alpha_{15}\beta_1$ and

$\alpha_{IIB}\beta_3$ [130, 131]. All these factors may contribute to more intensive responses inside the cell on PLA-FN surface.

Figure 4.3 The 2D spread area of individual PEFs (A) and average normalized 2D spread area of PEFs (B) again time on PLA, PLA-CHO, PLA-COL, and PLA-FN surfaces within 24 h after cell seeding. In (B), the variation in the first 60 min is shown in the inset with enlarged scale.

Langenbach et al. reported that NIH 3T3 fibroblasts on collagen film spread less than those on fibronectin film [132]. Higher expression level of tenascin-C

of 3T3 fibroblasts on fibronectin film was also found, which confirmed the different ligand-receptor interactions on the two kinds of films. Higher spreading on fibronectin-coated surface than on collagen-coated surface was also observed previously by our group in HepG2 cells [97].

Because the change of cell morphology may not necessarily be correlated with the adhesion contact evolution, the adhesion contact cannot be monitored by phase contrast microscopy, which only provides the 2D geometry of the adherent cells. C-RICM provides the capability for probing the kinetics of adhesion contact formation between the cells and the substrate. A series of C-RICM images of a typical PEF on PLA surface within 24 h after cell seeding are shown in Figure 4.4.

Figure 4.4 A series of C-RICM images of a typical PEF in a cell population adhering on PLA surface from 5 min to 24 h after cell seeding.

In the C-RICM images, strong adhesion contact is seen as dark patch. Despite tight bounding on PLA, a cell has a lag time of around 10 min for initiating adhesion contact formation. At 10 min post seeding, it is evident that PEF forms a small cluster of strong adhesion contact (with an area of $23 \mu\text{m}^2$) to PLA surface. The contact zone keeps on expanding until it reaches steady state at 20 h. The adhesion contact area has increased a hundred times, from $23 \mu\text{m}^2$ to $2378 \mu\text{m}^2$ in 20 h after cell seeding. A series of C-RICM images of a typical PEF on PLA-CHO surface within 24 h after cell seeding are shown in Figure 4.5. As demonstrated in the Figure 4.5, the contact zone of PEF on PLA-CHO surfaces extends in 20 h of culture before stabilization. The contact area climbs from $27 \mu\text{m}^2$ at 10 min to $2509 \mu\text{m}^2$ at 20 h.

Figure 4.5 A series of C-RICM images of a typical PEF in a cell population adhering on PLA-CHO surface from 5 min to 24 h after cell seeding.

The immobilization of ECM protein on PLA likely tunes the adhesion contact formation. A series of phase contrast and C-RICM images of a typical PEF on PLA-COL surface within 24 h after cell seeding is shown in Figure 4.6. As shown in these C-RICM images, a small dark patch of $14 \mu\text{m}^2$ appears at 10 min post seeding. The contact area increases with time until reaches $2985 \mu\text{m}^2$ at 24 h.

Figure 4.6 A series of C-RICM images of a typical PEF in a cell population adhering on PLA-COL surface from 5 min to 24 h after cell seeding.

A series of C-RICM images of a typical PEF on PLA-FN surface within 24 h after cell seeding is shown in Figure 4.7. Only after 5 min of cell seeding, adhesion contact zone with an area of $152 \mu\text{m}^2$ forms between the cell and the substrate. The contact zone goes through significant augment within 8 h. The

contact area of the cell on PLA-FN surface shoots up to $5230 \mu\text{m}^2$ at 8 h post seeding. The rate of increase of adhesion contact for PEFs is largest on PLA-FN compared to the other three surfaces. These observations suggest the tight correlation between adhesion contact and biological recognition of cells to immobilized ligands.

Figure 4.7 A series of C-RICM images of a typical PEF in a cell population adhering on PLA-FN surface from 5 min to 24 h after cell seeding.

Cell adhesion is inevitably accompanied by the deformation of cell. Through monitoring cell deformation in the process of cell adhesion, information about biophysical responses induced by the integrin-ligand interactions can be obtained.

By simultaneously analyzing the phase contrast and C-RICM images, we can quantify degree of deformation and adhesion energy in cell adhesion.

The average degree of deformation (a/R) with time for PEFs on four surfaces (PLA, PLA-CHO, PLA-COL, and PLA-FN) during 24 h culture is shown in Figure 4.8. Error bar represents the standard deviation of the data for at least 60 cells on eight identical samples. On all the four surfaces, a/R increases against time and reaches maximum level after cell seeding for given time. The time needed for PEFs on PLA, PLA-CHO, PLA-COL, and PLA-FN surfaces to reach maximum a/R is 90, 90, 80, and 30 min, respectively. For comparison, the average increase rate of a/R is defined as the maximum a/R divided by the time needed for the cells to reach maximum a/R after cell seeding. The average increase rate of a/R on PLA, PLA-CHO, PLA-COL, and PLA-FN surfaces is 0.011, 0.011, 0.012*, and 0.033* min^{-1} , respectively (*Student's t-test: $P < 0.01$, compared to PLA and PLA-CHO surfaces), indicating that the evolvement of cell adhesion contact on PLA-FN surface is faster than that on the other three surfaces.

Figure 4.8 Average degree of deformation versus time for PEFs on PLA, PLA-CHO, PLA-COL, and PLA-FN surfaces within 24 h after cell seeding (Logarithmic time scale). The variation from 10 min to 90 min is shown in the inset with linear time axis.

It is also observed in Figure 4.8 that the average degree of deformation of PEFs on PLA, PLA-CHO, and PLA-COL surfaces maintains at zero within 5 min after cell seeding while a/R of PEFs on PLA-FN surface experiences rapidly increase during the same period. The response of PEFs to PLA-FN surface is 5 min earlier than that to the other three surfaces. These results suggest that bioactive domains on PLA-FN surface is better recognized by PEFs than those on PLA, PLA-CHO, and PLA-COL surfaces, which is possibly attributed to more binding sites in fibronectin than in collagen.

As illustrated in Figure 4.8, a/R of PEFs adhering to the four surfaces is stabilized after reaching the maximum value. The maximum a/R of PEF on PLA, PLA-CHO, PLA-COL, and PLA-FN surfaces is 0.98, 0.98, 0.98, and 0.99, respectively (Student's t -test: $P > 0.05$), indicating that there is no statistically significant difference between these a/R values. All these values are approximate to unity which implies that all the spreading portions of PEFs have bound to the substrates.

It has been reported by previous studies that the extent of cell spreading has no direct correlations with the adhesion contact formation during initial cell seeding [22, 93, 97]. However, PEF spread area is identical to adhesion contact area after cells are being cultured for a given time (90 min). This result suggests that all spread portions of PEFs form strong adhesion to the substrate after being cultured for certain period of time.

Adhesion energy is an important parameter to characterize the interactions between cell and substrate. The average adhesion energy of PEFs within 24 h after cell seeding is shown in Figure 4.9. Error bar represents the standard deviation of the data for at least 60 cells on eight identical samples. The adhesion energy on the four surfaces increases first and becomes steady. The average adhesion energy of PEFs on PLA, PLA-CHO, and PLA-COL surfaces spans five orders of magnitude. In contrast, it only spans two orders of magnitude on PLA-FN surface. PEFs on PLA-FN surface possess the highest average adhesion energy at 5 min

after cell seeding while the average adhesion energy of PEFs on PLA, PLA-CHO, and PLA-COL surfaces is zero at that time (data not shown up on log scale). It is demonstrated by these data that the response of PEFs to PLA-FN surface is very rapid after cell seeding. As indicated in Figure 4.9, the average adhesion energy is higher on PLA-FN surface than on PLA, PLA-CHO, and PLA-COL surfaces initially from 5 to 50 min post seeding. However, the adhesion energy of PEFs on four surfaces converges after 80 min. The time needed for PEFs on PLA, PLA-CHO, PLA-COL, and PLA-FN surfaces to reach maximum adhesion energy is 80, 80, 70, and 20 min, respectively. The average adhesion energy of PEFs at steady state is $9.5 \times 10^{-7} \text{ J/m}^2$ on the four surfaces. The maximum average adhesion energy of PEFs on four surfaces is the same. Other researchers reported the adhesions energy of different cells on protein-coated surfaces [133-135], which ranges from 0.77×10^{-6} to $76 \times 10^{-6} \text{ J/m}^2$. The maximum adhesion energy ($9.5 \times 10^{-7} \text{ J/m}^2$) obtained in the present study agree with those data.

Figure 4.9 Average adhesion energy of adherent PEFs versus time within 24 h after cell seeding (Logarithmic time scale). The variation from 40 min to 80 min is shown in the inset with linear time axis.

A biophysical model has been presented by de Gennes et al. to simulate the growing process of the adhesion contact [136]. In one of these models, the adhesion contact is related to the seeding time. The kinetics of adhesion contact evolution is described by

$$a_N \propto t^n \quad (4.1)$$

where a_N and n is the normalized radius of contact zone and growth coefficient, respectively. The normalized radius of contact zone with time for PEFs on the four surfaces is shown in Figure 4.10. Logarithmic scale is adopted for both time and radius axes in the figure. As shown in Figure 4.10, the data under each condition form a bilinear curve. Each curve represents the best fit of the data with non-linear least square analysis. The growth coefficients, n , can be obtained from the curve fitted by Equation 4.1. In fact, n is the slope of the fitted line. The slope of the right portion of each bilinear curve of four surfaces is 0.060 (PLA), 0.054 (PLA-CHO), 0.125 (PLA-COL), and 0.076 (PLA-FN), respectively. The slope of the left portion of each bilinear curve is the initial growth coefficient for PEFs on each surface. It is indicated that initial growth coefficient for PEFs adhering on PLA, PLA-CHO, PLA-COL, and PLA-FN surfaces is 0.87, 0.88, 0.91, and 0.49, respectively. Initial growth coefficient of PEFs on PLA-FN surface is smaller than that on PLA-COL surface, which suggests higher reaction rate between integrins and fibronectin on PLA-FN surface during the growth of adhesion contact. That result is consistent with the more rapid adhesion of PEFs on PLA-FN surface.

Furthermore, it is observed that the slope of the right portion of each bilinear curve is lower than that of the left portion of the curve. In other words, the growth coefficient, n , decreases after a period of cell adhesion. This result suggests the exhaustion of the reservoir of receptors with the development of cell adhesion [136].

Figure 4.10 Normalized radius of the contact zone versus time for PEFs on PLA, PLA-CHO, PLA-COL, and PLA-FN surfaces within 24 h after cell seeding.

4.2.3 Evolvement of Distribution of F-actin in Cell Adhesion

In cell adhesion, the integrin-ligand binding transmits external stimuli into cell and triggers a cascade of signal transduction such as actin filament reorganization which leads to the change of cell shape and motility [74]. Actin filaments reorganization is a dynamic process involving the polymerization and depolymerization of actin monomer [137]. By immunostaining of F-actin, it is possible to monitor the dynamic process of F-actin distribution in cell adhesion.

F-actin distribution in PEFs adhering to PLA, PLA-CHO, PLA-COL, and PLA-FN surfaces 20 min, 60 min, 4 h and 24 h after cell seeding are shown in Figure 4.11. Actin filaments in PEFs adhering on PLA surface are highly concentrated as a ring-like structure at the periphery of the cell at 20 min although they are present throughout the cytoplasm. The ring-like structure in the cell cortex is formed by cross-linking of actin filaments with actin-binding proteins; it supports the outer layer of the cell and determines the shape of cell. Shortly after seeding, the cells begin to spread. After PEFs are seeded on PLA surface for 60 min, filopodia (also called microspikes indicated by the arrows in Figure 4.11(B))

forms around the cell membrane. These structures look like spikes protruding from the cell membrane. At 4 h, PEFs adhering on PLA surface deforms and show polygonal shape due to the reorganization of cytoskeleton structure. Stress fibers (indicated by the arrow in Figure 4.11(C)) are present inside the cell, which suggests the formation of focal adhesion [13]. Filopodia and lamellipodia are clearly seen because they contain a high concentration of actin filaments visible by immunofluorescence. The cell morphology continues to change over time. After 24 h of cell seeding, PEFs cluster together and take on elongated morphology. The stress fibers demonstrate anisotropic organization and align in parallel to the long-axis of cells.

The distribution of actin filaments in PEFs on PLA-CHO and PLA-COL surfaces evolves in a similar manner. The PEFs adhering on PLA-CHO and PLA-COL surface also go through the process including the formation of lamellipodia, filopodia, and stress fibers. There is no obvious difference in the distribution of actin filaments in PEFs adhering on PLA, PLA-CHO, and PLA-COL surfaces at the same time after cell seeding. The degree of deformation for PEFs on three surfaces goes through rapid increase within 60 min after cell seeding. However, the distribution of actin filament in PEFs on the three surfaces do not changed prominently within 60 min except the emergence of inconspicuous filopodia or lamellipodia, suggesting the binding of integrin receptors to ligands do not lead to the rapid reorganization of cytoskeleton on a large scale. From 60 min to 24 h, the cytoskeleton continues the remodeling including the formation of stress fibers which induce the persistent spreading of cells.

For PEFs adhering on PLA-FN surface, the distribution of actin filament evolves more quickly than the cells on the other three surfaces. Similarly, a ring-like structure rich in actin filaments at cell periphery formed after 20 min cell seeding. Only after 60 min of cell seeding, PEFs become highly polarized as shown by the highly anisotropic orientation of stress fibers through the cell. Specifically, actin filament bundles accumulate near the cell membrane region and

in the lamellipodium and filopodium edges (the arrow in Figure 4.11(N)). It is seen that, at 60 min, the distribution of actin filaments is similar to that in PEFs on PLA, PLA-CHO, and PLA-COL surfaces at 4 h. The trend is directly correlated with the largest rate of increase of a/R and adhesion energy for cells on PLA-FN. The reorganization of actin filaments in PEFs proceeds more rapidly on PLA-FN surface than on PLA, PLA-CHO, and PLA-COL surfaces. PEFs become elongated with time. After 4 h of cell seeding, PEFs are highly polarized on PLA-FN surface. It is shown in Figure 4.11(P) that PEFs with actin filament bundles concentrated in the cell cortex, take on more highly elongated morphology than PEFs on PLA, PLA-CHO, and PLA-COL surfaces. Previous studies have demonstrated that proper shape of cell is critical because many fundamental biological processes, such as growth, differentiation, and apoptosis, are regulated by changes in cell shape and cytoskeletal integrity [138-140]. The morphology of PEFs adhering on PLA-FN surface at 24 h closely resembles its bipolar morphology for fibroblasts *in vivo*, suggesting that PLA-FN surface provides a proper environment for PEFs to grow.

It should be pointed out that all the morphology of PEFs in phase contrast and C-RICM phase images is not as elongated as that in F-actin staining images. In our experiments, the bottom of Petri dish with adherent cells is needed to contact with microscope lens in the outside environment for observation. Therefore, the Petri dish is not fully covered the customized chamber and the temperature of the atmosphere of the chamber is different from that of culture medium. As the chamber is designed to maintain the temperature of the atmosphere of the chamber at 37°C, the actual temperature of culture medium in the Petri dish is lower than 37°C, which leads to less elongated morphology of PEFs. Through calibration, the actual temperature of the medium is determined to be about 34°C.

Figure 4.11 Actin filament distribution in PEFs adhering to PLA, PLA-CHO, PLA-COL, and PLA-FN surfaces 20 min, 60 min, 4 h, and 24 h after cell seeding. The arrows in (B), (C) and (N) indicate the filopodia, stress fibers, and the actin filament bundles in cellular periphery, respectively. Scale bar = 30 μ m.

4.2.4 Cell Attachment Efficiency on PLA, PLA-CHO, PLA-COL, and PLA-FN Surfaces

The attachment ratio of PEFs on PLA, PLA-CHO, PLA-COL, and PLA-FN surfaces 2 h after cell seeding is shown in Figure 4.12. The percentage of seeded

PEFs that attached to PLA, PLA-CHO, PLA-COL, and PLA-FN surfaces is $26\% \pm 9\%$, $36\% \pm 8\%$, $73\% \pm 11\%$, and $88\% \pm 7\%$, respectively. Without immobilization of ECM proteins, PLA and PLA-CHO are relatively poor substrates for adhesion of PEFs. PLA-COL and PLA-FN surfaces exhibit excellent capability to “seize” the cells. Particularly, the PLA-FN surface allows for the greatest PEF adhesion among all four cases after 2 h of cultured. The adhesion ratio result demonstrates strong affinity of PLA-FN surface to PEFs.

Figure 4.12 Attachment ratio of PEFs on PLA, PLA-CHO, PLA-COL, and PLA-FN surfaces 2 h after cell seeding. There is significant difference in attachment ratio among PLA, PLA-COL, and PLA-FN surfaces (Student’s t-test: $P < 0.05$).

4.3 Summary

In this study, collagen and fibronectin were immobilized to PLA surface via the bridging of glutaraldehyde between protein and aminolyzed PLA for novel application in primary esophageal cell. The adhesion dynamics of PEFs on PLA, PLA-CHO, PLA-COL, and PLA-FN surfaces were investigated by using C-RICM

in conjunction with phase contrast microscopy. It is shown from the experimental results that the adhesion dynamics of PEFs on four surfaces are different. PEFs reach steady state more quickly on PLA-FN surface than on PLA, PLA-CHO, and PLA-COL surfaces. At steady state, PEFs spread more extensively on PLA-FN surface than the other three surfaces. The distribution of actin filaments in PEFs alters after PEFs are seeded on PLA, PLA-CHO, PLA-COL, and PLA-FN surfaces. The evolution of the distribution of actin filaments in PEFs is more rapid on PLA-FN surface than on the other three surfaces. Furthermore, PEFs on PLA-FN surface take on elongated and bipolar morphology which is more similar to the morphology of fibroblast *in vivo* than that on PLA, PLA-CHO, and PLA-COL surfaces. In addition, the result of cell attachment ratio assay shows the higher attachment ratio of PEFs on PLA-FN surface than the other three surfaces although PLA-COL surface possesses higher attachment ratio to PEFs than PLA and PLA-CHO surfaces. All the results support the possible application of fibronectin-immobilized PLA for esophageal tissue engineering in the future.

Chapter 5 Adhesion Dynamics of Porcine Esophageal Fibroblasts on ECM Protein-immobilized CNT Monolayer[§]

5.1 Introduction

Carbon nanotubes (CNTs), cylindrical macromolecules of carbon with novel properties, were discovered back in 1991 [42]. Ever since the discovery of carbon nanotubes, researchers have been exploring their potential in tissue engineering applications [141]. Although there is a concern on the cytotoxicity of CNT [142-145], many published studies supported the biocompatibility of CNT and CNT-based biomaterials [146]. Furthermore, the toxicity of CNT can be possibly mitigated through functionalization with biological molecules [46, 147].

In tissue engineering, effective adhesion of cell to biomaterial surface is an indispensable requirement for designing any engineered tissue equivalent [148]. Functionalization of biomaterials with extracellular matrix (ECM) proteins has been proven to be useful for improving the biocompatibility of biomaterials for cell regeneration [18]. Through physical adsorption, extracellular matrix (ECM) proteins can be immobilized onto the wall of CNTs through noncovalent bonding. After introducing the ligands onto CNTs, it is possible to increase the bio-recognition of cells and promote cell adhesion to CNTs. However, the lack of understanding on the effect of ECM protein immobilization on the adhesion mechanism of cells on CNT becomes a hurdle for the development of CNT-based biomaterials.

[§] Reprinted in part from [Cai N, et al. ACS Applied Materials & Interfaces. 2010; 2:1038-1047 Copyright (2010)] with permission from American Chemical Society.

To date, little is known on the interactions between cells and CNT-based surfaces. In the area of esophagus regeneration, there is lack of report on the adhesion behavior of primary esophageal cells on CNT-based materials. In this study, the adhesion dynamics of primary porcine esophageal fibroblasts (PEFs) on CNT monolayer was elucidated with our C-RICM based biophysical approaches. First, collagen type I (COL) and fibronectin (FN), two key ECM proteins, were immobilized on CNT monolayer through physical absorption. Then the initial rate of cell deformation, steady-state degree of deformation and adhesion energy of PEFs on unmodified CNT and ECM protein-immobilized CNT monolayer during initial cell seeding were determined. The result showed that the biophysical parameters of cell adhesion were significantly tuned by ECM immobilization. Moreover, cell proliferation activity and attachment ratio were correlated to the adhesion dynamics on various CNT-based materials. In addition, fluorescence microscopy and scanning electron microscopy (SEM) were used to examine actin filament distribution and degenerated structures of PEFs, respectively.

5.2 Results and Discussion

5.2.1 Surface Characterization

A typical SEM image of unmodified CNT monolayer supported on glass substrate is shown in Figure 5.1. The result shows that long wry multi-walled carbon nanotubes (MWCNTs) with length of $1.7 \pm 0.8 \mu\text{m}$ are randomly distributed on the substrate upon CNT deposition. The diameters of MCNTs on CNT monolayer are in the range of ~ 20 to 40 nm , with average value of 27.4 nm . Their aspect ratio is determined to be 67 ± 28 . In addition, it is observed that these MWCNTs protrude from the bottom of the coverslip and their frontal parts may overlap. Thus the thickness of the monolayer slightly varies from region to region on the substrate.

Figure 5.1 SEM image of CNT monolayer on a silica coverslip.

Proteins can be immobilized onto CNT by spontaneous absorption or chemical reactions [149]. Although protein immobilization onto CNT via chemical reaction may be carried out in a more controllable way, protein coating through physical absorption remains to be common practice due to its simplicity [149]. Because of the hydrophobic property of CNT surface, collagen or fibronectin can non-specifically bound to the wall of CNT through noncovalent bonding. AFM height and phase images of CNT-ML, CNT-COL, and CNT-FN surfaces are shown in Figure 5.2. The peak of a surface is represented as bright region in an AFM phase image. It is shown in Figure 5.2(A) and (B) that CNTs with different length protrude from the coverslip. Compared to Figure 5.2(A), the CNT-COL surface shown in Figure 5.2(C) is relatively flat. However, some very bright regions are observed in Figure 5.2(D), suggesting that some CNTs are exposed outside the protein layer. After collagen immobilization, the ridges between tangled CNTs are filled with collagen, leading to a more homogeneous morphology as shown in the phase image of the CNT-COL surface than that of the CNT monolayer. As depicted in Figure 5.2(E) and (F), similar topographic features are also found on the CNT-FN surface. However, the fibronectin layer

on the CNT-FN surface is not as smooth as the collagen layer on the CNT-COL surface, possibly attributed to the higher molecular weight of fibronectin (approx. 450 kD) compared to collagen (approx. 300 kD). By analyzing the topographic images of the three surfaces, the root-mean-square (RMS) roughness of CNT-ML, CNT-COL, and CNT-FN surfaces are 9.45 ± 1.32 nm, 4.55 ± 0.31 nm and 6.23 ± 0.42 nm, respectively (Student's t-test: $P < 0.05$). ECM immobilization leads to a decrease in RMS roughness compared to neat CNT monolayer, which confirms successful ECM immobilization onto the CNT monolayer.

Figure 5.2 AFM images of CNT-ML, CNT-COL, and CNT-FN surfaces.

Functionalization of CNT monolayer may induce change of its hydrophobic property due to the introduction of ECM proteins. Contact angles of CNT-ML, CNT-COL, and CNT-FN surfaces are $120.6 \pm 3.0^\circ$, $53.3 \pm 2.2^\circ$ and $93.1 \pm 1.5^\circ$, respectively (Student's t-test: $P < 0.01$). The contact angle of CNT monolayer is found to be significantly higher than those of CNT-COL and CNT-FN surfaces, exhibiting the innate hydrophobic property of carbon nanotubes. Because of the immobilization of collagen, the CNT-COL surface becomes more hydrophilic. In contrast, the CNT-FN surface is still somewhat hydrophobic. It was previously reported by Harnett et al. that fibronectin-coated surface retained hydrophobic property and was independent of the underlying substrates [150]. Our result is consistent with their finding. By QuantiPro BCA measurement, the densities of absorbed collagen on CNT-COL surface and fibronectin on CNT-FN surface is determined to be $2.14 \pm 0.26 \mu\text{g}/\text{cm}^2$ and $2.38 \pm 0.31 \mu\text{g}/\text{cm}^2$, respectively. The result shows that both proteins form multiple molecular layer on CNT since the required density for forming collagen and fibronectin monolayer is $0.3 \mu\text{g}/\text{cm}^2$ and $0.25 \mu\text{g}/\text{cm}^2$, respectively [22, 126].

5.2.2 Adhesion Dynamics of PEFs on CNT-ML, CNT-COL, and CNT-FN Surfaces

Cell attachment and spreading are phenotypic responses of all anchorage-dependent cells [151]. A series of phase contrast and C-RICM images of a typical PEF on the CNT-ML surface during 2 h of culture is shown in Figure 5.3. The result shows that the PEF maintains round shape within 40 min after cell seeding on CNT-ML. After 2 h of culture, the PEF begins to spread with filopodia protruding from cellular membrane. Cell spreading was shown to be decoupled from the adhesion contact development in the initial phase of cell adhesion [22, 93, 97]. Thus C-RICM provides additional information about cell adhesion mechanism. As demonstrated in the C-RICM images of Figure 5.3, the

PEF forms strong adhesion contact at 20 min after cell seeding as represented by a grey patch with an area of $31 \mu\text{m}^2$ in the C-RICM image (indicated by the white arrow in Figure 5.3). The grey patch significantly increases in area over time. After 120 min of culture, the adhesion contact area reaches $296 \mu\text{m}^2$. By comparing the phase contrast images with C-RICM images within 40 min, it is found that the evolution of adhesion contact is different from that of cell spreading. The contact area of PEF at 40 min is $204 \mu\text{m}^2$, 560% higher than that of PEF at 20 min. In contrast, the projected area of PEF remains almost constant at about $310 \mu\text{m}^2$ during the initial 40 min of culture.

Figure 5.3 A series of phase contrast and C-RICM images of a typical PEF in a cell population adhering to the CNT-ML surface from 5 min to 120 min after cell seeding. The white arrow indicates the adhesion contact forming at 20 min. Scale bar = $20 \mu\text{m}$.

Despite the lack of adhesive ligands, CNT-ML is effective to induce adhesion contact formation. The result observed herein is likely caused by rapid adsorption of serum proteins on CNT-ML by hydrophobic interaction. In fact, the first stage of cell adhesion (cell attachment) is mainly driven by binding of integrin receptors to the ligands on biomaterials [151], while the development of cell

spreading is controlled by cytoskeleton remodeling [127]. Although the ligand-receptor interactions trigger signal transduction cascades, the downstream biophysical responses, such as cytoskeleton reorganization occurs later than the adhesion contact evolution. This evidence supports the independent trend of adhesion contact evolution and cell spreading during the initial cell adhesion process.

Immobilization of ECM protein on biomaterials provides necessary ligands for cell adhesion, which is critical to the anchorage of seeded cells. A series of phase contrast and C-RICM images of a typical PEF on the CNT-COL surface are shown in Figure 5.4. From the phase contrast images, it is observed that a PEF displays round shape during the initial 40 min of cell seeding. At 40 min, the PEF spreads a little with filopodia formations near cell periphery, which is not found in PEF on CNT monolayer at the same time of observation. Through 2 h of culture, this PEF spreads extensively. Lamellipodia and filopodia are seen to protrude from the cell membrane. As indicated in the C-RICM images of Figure 5.4, the PEF initiates its adhesion contact formation (indicated by the white arrow in Figure 5.4) 20 min post seeding. Thereafter, the adhesion contact area climbs up from $58 \mu\text{m}^2$ at 20 min to $785 \mu\text{m}^2$ at 120 min. The result implies that the interaction between the integrin receptors of PEF and immobilized collagen accelerates adhesion contact formation compared to that for PEF on CNT-ML.

Switching one ECM protein to another alters ligand-receptor interactions, which may induce a shift of downstream signal transductions inside the adherent cells. A series of phase contrast and C-RICM images of a typical PEF on the CNT-FN surface are shown in Figure 5.5. The cell goes through the significant filopodia formation and extensive spreading in 2 h of culture, which is similar to the PEF on the CNT-ML or CNT-COL surface. However, there is a notable distinction between the evolution of adhesion contact of PEFs on the CNT-FN surface compared to that of PEFs on the CNT-ML or CNT-COL surface. Just 5 min after cell seeding, a dark patch (as indicated by the white arrow in Figure 5.5),

with an area of $91 \mu\text{m}^2$ emerges on the CNT-FN surface. This faster adhesion contact formation of PEFs to the CNT-FN surface than that on the CNT-ML and CNT-COL demonstrates better bio-recognition of fibronectin by PEFs. Moreover, the density of adsorbed fibronectin is close to that of collagen. It is because fibronectin contains abundant types of binding domains including Arg-Gly-Asp (RGD) and Arg-Glu-Asp-Val (REDV) [71], which may favor more rapid identification and binding of transmembrane integrin receptors of PEFs to the immobilized fibronectin. From 5 min to 120 min, the contact zone grows quickly. After 2 h of culture, the contact area reaches $1288 \mu\text{m}^2$, which is the biggest for PEFs at 120 min among all of the three surfaces. In our adhesion dynamics results of PEFs on PLA, PLA-COL, and PLA-FN surfaces (refer to Chapter 4), PEFs on the PLA-FN surface also exhibit the fastest adhesion contact formation among three cases. These results support the notion that adhesion contact development is modulated by the interactions between immobilized ECM protein and cellular receptors in the CNT system.

Figure 5.4 A series of phase contrast and C-RICM images of a typical PEF in a cell population adhering to the CNT-COL surface from 5 min to 120 min after cell seeding. The white arrow indicates the adhesion contact forming at 20 min. Scale bar = $20 \mu\text{m}$.

Figure 5.5 A series of phase contrast and C-RICM images of a typical PEF in a cell population adhering to the CNT-FN surface from 5 min to 120 min after cell seeding. The white arrow indicates the adhesion contact forming at 5 min. Scale bar = 20 μm .

Cell adhesion always induces cell deformation through the geometric transformation. The average degree of deformation (a/R) against seeding time for PEFs on three surfaces (CNT-ML, CNT-COL, and CNT-FN) during 2 h culture is shown in Figure 5.6. Each data point and error bar represents the mean value of more than 50 cells and corresponding standard deviation. It is demonstrated that a/R of PEFs on CNT-ML surface retains zero within the initial 10 min of cell seeding. Moreover, the average increase rate of a/R is defined as deformation rate which is a/R divided by the time needed for the cells during initial stage of adhesion contact formation. At 20 min, a/R obtains a value of 0.21 (with deformation rate of 0.021 min^{-1}). The rate of increase of average a/R against time is gradually slackened from 30 min onwards. At 80 min, a/R acquires its maximum value on CNT-ML. Similarly, a/R of PEFs on CNT-COL surface remains to be zero within 10 min followed by the rapid rise from 20 min (with deformation rate of 0.042 min^{-1}).

Figure 5.6 Average degree of deformation versus time for PEFs on CNT-ML, CNT-COL, and CNT-FN surfaces within 2 h after cell seeding.

However, the trend of a/R against time for PEFs on the CNT-FN surface is largely different from those of the other two surfaces. From 0 to 5 min, a/R of PEFs on CNT-FN surface jumped from 0 to 0.52 (with deformation rate of 0.1 min^{-1}). The lag time for PEFs to initiate adhesion to CNT-FN surface is 15 min shorter than that of CNT-ML and CNT-COL surfaces, which may be attributed to the quicker recognition of fibronectin by integrin receptors of PEFs based on the indifference in the density of adsorbed collagen and fibronectin. After 40 min, a/R of PEFs on CNT-FN surface reaches the maximum value. The result strongly indicates that the evolution of cell adhesion contact on CNT-FN surface is the most rapid and has the highest deformation rate among the three surfaces reported herein (Student's t -test: $P < 0.01$).

Through 2 h of culture, PEFs on three surfaces reach a steady state and gain their maximum value of a/R . The maximum a/R of PEFs on CNT-ML, CNT-COL, and CNT-FN surfaces is 0.76, 0.93 and 0.98, respectively (Student's t -test: $P < 0.05$). For spreading cells, a/R is translated into the ratio of the radius of adhesion contact zone to the radius of spreading portion. The maximum a/R value

for PEFs on CNT-COL and CNT-FN surfaces is almost 1, suggesting major spreading membrane of cells is tightly bound to the substrates due to ECM immobilization. The result also shows that CNT-ML alone is not optimized for cell activation.

Adhesion energy is a useful biophysical parameter describing the binding affinity between adherent cells and biomaterials surfaces [152]. The average adhesion energy of PEFs against time of cell seeding on CNT-ML, CNT-COL, and CNT-FN surfaces during 2 h culture is shown in Figure 5.7. Each data point and error bar represents the mean value of more than 50 cells and corresponding standard deviation. For all three surfaces, the adhesion energy of PEFs goes up over time till reaching their steady state. Because the zero value would not appear in log scale, the average adhesion energy of cells on CNT-ML and CNT-COL surfaces before 20 min is not shown on Figure 5.7, implying no formation of strong adhesion contact for PEFs on CNT-ML and CNT-COL surfaces before 20 min. The average adhesion energy of PEFs on CNT-ML surface climbs up from $1.66 \times 10^{-10} \text{ J/m}^2$ at 20 min to $4.86 \times 10^{-7} \text{ J/m}^2$ at 70 min, spanning three orders of magnitude. During the same culture period, it only spans about two orders of magnitude on CNT-COL surface, from $1.16 \times 10^{-8} \text{ J/m}^2$ to $9.54 \times 10^{-7} \text{ J/m}^2$. For PEFs on CNT-FN surface, high adhesion energy with $4.45 \times 10^{-8} \text{ J/m}^2$ shows up only at 5 min after cell seeding. Then it keeps on rising and reaches a steady-state of $9.54 \times 10^{-7} \text{ J/m}^2$. The time needed for PEFs on CNT-ML, CNT-COL, and CNT-FN surfaces to reach maximum adhesion energy is 70, 60 and 30 min, respectively. The adhesion energy of PEFs on CNT-FN surface spend the least time to arrive at the steady state. In our aforementioned study, PEFs on ECM protein-functionalized PLA surfaces follow a similar trend to gain the maximum adhesion energy over time. These results suggest immobilized ECM proteins may act as major mediator of cell adhesion [153].

Figure 5.7 Average adhesion energy versus time for PEFs on CNT-ML, CNT-COL, and CNT-FN surfaces within 2 h after cell seeding.

5.2.3 Metabolic Activity and Attachment Ratio of PEFs on CNT-ML, CNT-COL, and CNT-FN Surfaces

The metabolic activity of PEFs in terms of cell proliferation on the three surfaces was determined by MTT assay, to evaluate their biocompatibility. Data shown in Figure 5.8 are in the form of relative values to the control surface (CNT-ML, 1 day). As demonstrated in Figure 5.8, metabolic activity of PEFs on CNT-COL and CNT-FN surfaces was significantly higher than that on CNT-ML surface after either 1 day or 3 days of culture. After 3 days of culture, PEFs on CNT-FN surface exhibit the highest cell proliferation among the three cases, suggesting CNT-FN surface is more suitable to support the growth of PEFs than the other two surfaces. These results agree well with published studies of primary hippocampal cells cultured on unmodified or bioactive molecule-functionalized carbon nanotube films [147].

Figure 5.8 MTT assay for PEFs cultured on CNT-ML, CNT-COL, and CNT-FN surfaces for 1 or 3 days. Cell metabolic activity is represented as the relative MTT value of a surface to that of the control surface (CNT-ML). * denotes significant difference compared with CNT-ML surface after 1 day (Student's t-test: $P < 0.05$); ** denotes significant difference compared with CNT-ML surface after 3 days (Student's t-test: $P < 0.01$); Δ denotes significant difference compared with CNT-COL surface after 3 days (Student's t-test: $P < 0.05$).

Cell attachment ratio assay was carried out to characterize the global affinity of biomaterial surfaces to PEFs. It is indicated in Figure 5.9 that attachment ratio of PEFs on CNT-ML, CNT-COL, and CNT-FN surfaces after 2 h of culture is $32 \pm 11\%$, $68 \pm 6\%$ and $83 \pm 7\%$, respectively (Student's t-test: $P < 0.05$). Through the introduction of ECM protein as ligands onto CNT monolayer, the attachment ratio is elevated by at least 36%. Particularly, PEFs on CNT-FN surface possess the highest attachment ratio among the three cases, demonstrating that fibronectin immobilization is most effectively enhance cell adhesion among the three cases. Similar effect of ECM immobilization on attachment ratio was found on PLA-COL and PLA-FN surfaces in our aforementioned studies (refer to Chapter 4).

Figure 5.9 Attachment ratio of PEFs on CNT-ML, CNT-COL, and CNT-FN surfaces after 2 h of culture.

5.2.4 Cell Morphology of PEFs on CNT-ML, CNT-COL, and CNT-FN Surfaces

Cell morphology are intimately related to many physiological processes, including cell growth, differentiation and apoptosis [154]. In our experiments, PEFs cultured on different surfaces for 24 h were fixed, dehydrated, and then observed under SEM to examine their morphology.

SEM images of PEFs adhering to CNT-ML, CNT-COL, and CNT-FN surfaces are shown in Figure 5.10. On the CNT-ML surface, it is found that the morphology of cells is not uniform: Some cells are almost circular while others are polygonal. As shown in Figure 5.10(A), the cell spreads slightly and exhibits a relatively round shape. In contrast, the cell as shown in Figure 5.10(B) significantly spreads and display elongated morphology. It is known that anchorage-dependent cells rely on spreading to enter G1 and G2 of the cell cycle [9]. It was found that cells which only bound to ECM and lack spreading did not elevate cyclin D1 levels and downregulate the cell-cycle inhibitor p27 [155],

suggesting PEFs with nearly round morphology on the CNT-ML surface may not proliferate normally. Comparatively, the morphology of PEFs on CNT-COL or CNT-FN surface is uniform. In Figures 5.10(D), (E), (G) and (H), all cells display highly elongated morphology.

The details of filopodia of a typical PEF on CNT-ML, CNT-COL, and CNT-FN surfaces are shown in Figures 5.10(C), (F) and (I), respectively. It is depicted in these figures that filopodia sprawl on the CNT sidewalls. On multi-walled CNT construct, other researchers noticed that filopodia of human osteoblastic cells grasped and deformed the surrounding compliant CNTs [156]. No similar phenomena were found in our study. A possible explanation is that the length of CNTs on our substrate ($1.7 \pm 0.8 \mu\text{m}$) is smaller than that of CNTs on multi-walled CNT construct (over $10 \mu\text{m}$) used by other researchers. Under the same load, the deflection of shorter CNTs is so small that it is unnoticeable under SEM.

To quantitatively compare the different morphology of cells on three surfaces, spread area and shape factor coefficient (Sfc) of cells are determined by analyzing the SEM images. Spread area and Sfc of PEFs on the three surfaces after 24 h of culture are listed in Table 5.1. These two parameters are used to evaluate the degree of cell spreading and cell elongation, respectively. The lower the Sfc value for a cell, the more elongated its morphology. It is indicated in Table 5.1 that spread area of cells on CNT-COL and CNT-FN surfaces is bigger than that on CNT-ML surface. In contrast, Sfc of cells on CNT-COL and CNT-FN surfaces is lower than that on CNT-ML surface. The functionalization of CNT-ML with ECM proteins modulates the morphology of adherent cells. For instance, cells adhering on functionalized CNT monolayer possess larger spread area and more elongated morphology than those on unmodified CNT monolayer. Particularly, cells on CNT-FN surface display most expanded and elongated morphology among the three surfaces, very similar to the bipolar morphology of PEFs *in vivo*. The

difference in cell morphology on three surfaces reflects the variation of ligand-receptor interactions, which tune cell morphology.

Figure 5.10 SEM images of PEFs adhering to CNT-ML, CNT-COL, and CNT-FN surfaces after 24 h of culture. The arrows in (A), (E) and (H) indicate the filopodia of a typical PEF on CNT-ML, CNT-COL, and CNT-FN surfaces, respectively. The corresponding details of the filopodia in (A), (E) and (H) are shown in (C), (F) and (I), respectively.

It is worth emphasizing that inconsistency of cell morphology was not found on bio-functionalized CNT monolayer. A possible explanation was related to the improved uniformity of the surface chemistry through ECM protein immobilization. It was reported that spread area of human osteoblastic cells cultured on CNT construct was negatively correlated with nanotube diameter

[156]. The diameters of CNTs on CNT-ML surface used herein ranged from 20 to 40 nm. The dissimilar cellular shape and spread area for cells on the CNT-ML surface may just act as the mirror of underlying CNTs with different size. On the CNT-COL and CNT-FN surfaces, the ECM protein layer encapsulating the CNT sidewalls possible insulated the effect of size of CNT on cell morphology. In our aforementioned studies, PEFs also exhibited more spread area and elongated morphology on PLA-FN surface than PLA and PLA-COL surfaces (refer to Chapter 4). These results supports that immobilized ligands, not the underlying supporting materials mainly dictates the resulted phenotypes of adherent cells.

Table 5.1 Spread area and Sfc of PEFs on CNT-ML, CNT-COL, and CNT-FN Surfaces.

Surfaces	Spread area*(μm^2)	Sfc*
CNT-ML	1326 ± 594	0.81 ± 0.10
CNT-COL	2456 ± 467	0.67 ± 0.07
CNT-FN	2886 ± 536	0.62 ± 0.05

* Student's t-test: $P < 0.05$; $n > 50$.

5.2.5 Actin Filament Distribution of PEFs on CNT-ML, CNT-COL, and CNT-FN Surfaces

Cell adhesion to biomaterials triggers signal transduction cascades, inducing cytoskeleton reorganization. The change of biomaterial surface may lead to alteration of signal transduction pathways and cytoskeleton organization of adherent cells. Representative images of stained PEFs on three surfaces after 24 h of culture are shown in Figure 5.11. It is found in Figure 5.11(A) that the F-actin distribution of PEFs on CNT-ML surface is not consistent. In general, there are two types of actin organization on CNT-ML surface: one is in the less spreaded

cells (indicated by the white arrows in Figure 5.11(A)), with concentrated actin filaments near cell cortex; the other is in well-spread cells, with actin filament bundles all over the cytosol. In the former case, actin micro-filament bundles are not observed. Only the randomly oriented actin filaments near cell cortex form a roughly ring-like structure correlating with its nearly round shape. In contrast, no circular actin structure is found in the latter case, but the cells exhibit clearly visible stress fibers.

Figure 5.11 Actin filament distribution of PEFs on CNT-ML, CNT-COL, and CNT-FN surfaces after 24 h of culture. The arrows in (A) indicate less spreaded cells with concentrated actin filaments near cell cortex. The arrow in (B) indicates the stress fibers. Scale bar = 50 μm .

Functionalization of CNT monolayer affects the actin organization of adherent cells. In contrary to the CNT-ML, the distribution of F-actin inside various PEFs on the CNT-COL surface is more consistent in comparison with that on CNT-ML. As demonstrated in Figure 5.11(B), PEFs spread extensively on CNT-COL surface. Stress fibers (indicated by the white arrow in Figure 5.11(B)) align parallel to the long axis of the cells, indicating the formation of focal adhesion. The result shows that the cells are highly polarized on CNT-COL. It is shown in Figure 5.11(C) that F-actin distribution of PEFs on CNT-FN surface is very similar to that on the CNT-COL surface. Highly anisotropic orientation of stress

fibers are seen inside the highly polarized PEFs. Comparing the F-actin distribution of PEFs on the three surfaces, it is obvious that ECM protein immobilization on CNT monolayer has greatly transformed the diverse actin organizations of PEFs into consistent actin organization with parallel alignment of stress fibers, which demonstrates the critical role of immobilized ligands in modulating cell adhesion. The results of actin organization of PEFs on three surfaces coincide with the SEM observations.

5.3 Summary

In summary, the adhesion dynamics of PEFs on CNT-ML surface and functionalized CNT-ML surfaces (CNT-COL and CNT-FN) was investigated. First, the effect of ECM immobilization on the interfacial properties of CNT-ML has been thoroughly characterized. It is shown that adhesion dynamics of PEFs on three surfaces is different. In general, a/R and adhesion energy for PEFs reach steady state more quickly on functionalized CNT-ML surfaces than on CNT-ML surface. Particularly, PEFs on the CNT-FN surface initiate adhesion contact formation and reach steady state earlier than that on CNT-COL surface, reflecting the different innate bioactive domains inside ECM proteins. Attachment ratio and metabolic activity of PEFs on three surfaces are in the order of CNT-FN > CNT-COL > CNT-ML. The most prominent result in this study is that the irregular cell morphology and actin organization on CNT-ML surface become uniform on functionalized CNT-ML surface. PEFs on CNT-FN surface possess the highest elongated morphology and largest spread area among three cases after 24 hr culture. ECM protein functionalization effectively promotes PEF adhesion to CNT monolayer. Therefore, ECM protein-functionalized CNT, particularly fibronectin-functionalized CNT, demonstrates its promising potentials for application in esophageal tissue engineering.

Chapter 6 Temporal Effect of Functional Blocking of β_1 Integrin on Cell Adhesion Strength under Serum Depletion **

6.1 Introduction

Cell adhesion to extracellular matrix (ECM) is critical to many physiological processes such as cell migration, tissue organization, and cell differentiation [12]. Integrin-mediated adhesion is a dynamic process comprised of two major stages: the initial ligand-integrin receptor binding and the following adhesion strengthening process which involves a series of post-binding events, including integrin clustering, recruitment of structure proteins to form focal adhesion, and cytoskeleton remodeling [87, 157]. Focal adhesions, specific sites on the cell membrane where integrins link ECM to cytoskeleton, contains multiple structural proteins such as vinculin, talin, paxillin, as well as signaling proteins including focal adhesion kinase (FAK) [61, 158]. Focal adhesions not only mechanically combine with integrins and cytoskeleton, but also act as a signaling hub to relay biochemical and mechanical stimuli from the extracellular environment to cell nucleus [159, 160].

Integrin-mediated adhesion have been extensively studied with the intent to decipher its mechanism [61, 62]. However, most of these efforts are focused on seeking the proteins involved in cell adhesion and the downstream signal transduction pathway [62], and less attention has been paid to the mechanical aspect of cell adhesion [90]. For example, although the positive influence of focal adhesion density on overall adhesion strength has been observed previously by

** Reprinted in part from [Cai N, et al. Langmuir. 2009; 25:10939-10947 Copyright (2009)] with permission from American Chemical Society.

researchers [90, 161], there is still lack of experimental results to quantitatively correlate adhesion strength with focal adhesion density.

Fibronectin is one of key components in ECM which can be recognized by various integrin receptors such as $\alpha_5\beta_1$, $\alpha_{II}\beta_3$, $\alpha_4\beta_1$, and $\alpha_V\beta_3$ [162]. Among fibronectin-binding receptors, integrin $\alpha_5\beta_1$ is the primary receptor in mediating integrin-fibronectin interactions [162]. β_1 integrin subunit plays a key role in $\alpha_5\beta_1$ integrin-mediated adhesion [163]. The cytoplasmic domains of β_1 integrin subunits is indispensable in the establishment of these connections between focal adhesion protein and actin filament [164]. It has been found that altered expression of the β_1 integrin affects both physiological and pathological processes including epidermal growth, neural differentiation development, wound healing, and tumor angiogenesis [165-168]. In contrast to the clear understanding of the function of β_1 in other physiological process, the influence of functional blocking of integrin β_1 on adhesion strength and focal adhesion formation is poorly known.

Currently, little is known of temporal effect of functional blocking of β_1 integrin on cell adhesion strength. The motivation of this study is to explore the quantitative relation among adhesion strength, focal adhesion density, and cell spreading, as well as the contribution of β_1 integrin subunit to adhesion strengthening process. In the present work, fibronectin coated surface was used as a model ECM. Adhesion-blocking antibody was employed to inhibit the function of integrin β_1 subunit in porcine esophageal fibroblasts (PEFs). The evolution of cell morphology, F-actin distribution, and vinculin pattern in non-blocked and β_1 -blocked PEFs during 4 h of culture was examined by phase contrast microscopy, fluorescence microscopy, and TIRFM, respectively. Time-dependent change of adhesion force of non-blocked and β_1 -blocked PEFs on fibronectin-coated surface was measured by micropipette aspiration technique. As serum contained various types of unknown proteins which may enhance cell adhesion, all of our adhesion experiments were carried out in serum free condition, where only coated fibronectin is involved in integrin-mediated

adhesion. Based on data acquired in our experiments, the contribution of cell spreading and variation of focal adhesion density to adhesion strengthening process were studied and interpreted, and the effect of functional blocking of integrin β_1 on adhesion strength were discussed.

6.2 Results

6.2.1 Surface Characterization

The topographic morphology of uncoated coverslip and fibronectin-coated coverslip was examined by AFM. The AFM images of the uncoated coverslip and fibronectin-coated coverslip in phase and height mode are shown in Figure 6.1. It is displayed in Figure 6.1(A) and (B) that uncoated glass surface is rather smooth. Only a few of spherical bulges and dents, possibly brought by fabrication process are found in some parts of the region examined. In contrast, fibronectin lies on smooth coverslip surface in globular form as shown in Figure 6.1(C) and (D). Furthermore, the root-mean-square (RMS) roughness of uncoated coverslip and fibronectin-coated coverslip are 0.31 ± 0.03 nm and 1.13 ± 0.15 nm (Student's t-test: $P < 0.05$), respectively. The immobilization of fibronectin on clean coverslip brings the increase of roughness. Similar variation in morphology after fibronectin coating on glass was also seen by other researchers [169].

To determine the hydrophobic/hydrophilic nature of the two surfaces, contact angle measurement was implemented. The contact angle of uncoated coverslip and fibronectin-coated coverslip is $39 \pm 2^\circ$ and $94 \pm 3^\circ$ (Student's t-test: $P < 0.01$), respectively, indicating the hydrophilic nature of uncoated coverslip surface is turned significantly more hydrophobic following fibronectin coating. In addition, the density of fibronectin on fibronectin-coated coverslip is quantified to be 0.45 ± 0.06 $\mu\text{g}/\text{cm}^2$ by BCA assay, which is nearly the double of fibronectin monolayer density (0.25 $\mu\text{g}/\text{cm}^2$) [126], revealing that more than one layer of fibronectin was deposited onto the coverslip. Moreover, the minimum density of adsorbed

fibronectin on culture plastics for inducing cell adhesion is approximately 10–15 ng/cm² [170]. Thus the amount of immobilized ECM proteins on the coverslip is believed to be sufficient for supporting cell adhesion.

Figure 6.1 AFM images of uncoated glass coverslip and fibronectin-coated glass coverslip.

6.2.2 Time-dependent Cell Spreading and Morphological Change

Cell adhesion is often accompanied by the change in cell morphology [75]. Phase contrast and immunofluorescence images of F-actin and vinculin for a typical non-blocked PEF on fibronectin-coated surface within 4 h after cell seeding are shown Figure 6.2. As demonstrated in Figure 6.2(A)-(F), the non-blocked cell significantly spreads during 4 h of culture. At the same time, the cell is transformed into polygonal morphology from round shape. β_1 integrin

submit is a major component of fibronectin receptor prototype, $\alpha_5\beta_1$ integrin [162], and blocking β_1 subunit affects ligand-receptor interaction, possibly altering the time course of the morphological change. Phase contrast and immunofluorescence images of F-actin and vinculin for a typical β_1 -blocked PEF on fibronectin-coated surface within 4 h after cell seeding are shown Figure 6.3. It is noticed in Figure 6.3(A)-(F) that the β_1 -blocked cell on fibronectin-coated surface also goes through cell spreading and morphological evolution during 4 h of culture, similar to the non-blocked cell. However, the initiation of change in cell shape for the β_1 -blocked PEF lags behind the non-blocked cell. For the non-blocked cell, it displays polygonal morphology after only 60 min of culture (Figure 6.2(B)). In contrast, the β_1 -blocked cell maintains nearly round shape within 90 min (Figure 6.3(A)-(C)). This difference reflects β_1 integrin subunit exerts important influence on cell adhesion process.

Figure 6.2 Time course study of cell morphology, F-actin distribution, and vinculin pattern of non-blocked PEFs. (A)-(F), phase contrast images; (G)-(L), fluorescence images of F-actin; (M)-(R), fluorescence images of vinculin; S-X, merged images of F-actin and vinculin. Vin=vinculin. Scale bar, 20 μ m.

Figure 6.3 Time course study of cell morphology, F-actin distribution, and vinculin pattern of β_1 -blocked PEFs. (A)-(F), phase contrast images; (G)-(L), fluorescence images of F-actin; (M)-(R), fluorescence images of vinculin; S-X, merged images of F-actin and vinculin. Vin=vinculin. Scale bar, 20 μ m.

To quantitatively compare the spreading process of non-blocked and β_1 -blocked cells, phase contrast images were analyzed to obtain spread area. Figure 6.4 shows the plot of spread area against culture time for non-blocked and β_1 -blocked PEFs on fibronectin-coated surface. The result indicates that either non-blocked or β_1 -blocked cells keep spreading after cell seeding. During the initial 90 min of culture, non-blocked cells possess higher spread area than β_1 -blocked cells. However, after 240 min of culture, the spread area of the two types of cells is almost comparable. At 240 min, spread area of non-blocked and β_1 -blocked cells are $2855 \mu\text{m}^2$ and $2712 \mu\text{m}^2$ (Student's t-test: $P>0.05$), respectively, indicating there is no significant difference in extent of spread for the two type of cells. The independence of final spreading extent on β_1 integrin blocking also occurs in osteoblasts, chondrocytes and mesenchymal stem cells [171, 172].

Figure 6.4 The spread area against time for non-blocked and β_1 -blocked PEFs on fibronectin-coated surface within 4 h after cell seeding. $n=80-100$ for each data point. *Significant difference from the corresponding value in β_1 -blocked PEFs at the same time point (Student's t-test: $P<0.05$).

6.2.3 Time-dependent Evolvment of F-actin Distribution and Vinculin Pattern

Cell adhesion process always involves cytoskeleton remodeling and focal adhesion formation [62]. The non-blocked PEF undergoes a process of actin filament reorganization after being seeded onto fibronectin-coated surface during 4 h of culture (Figure 6.2(G)-(L)). At 30 min, actin filaments are distributed throughout the nearly round cell body. The appearance of actin filament bundles (stress fibers) suggests the formation of focal adhesion in cell cortex (Figure 6.2(G)). After another 30 min of culture, sparser and thicker actin filament bundles are localized in the edge of the cell in polygonal shape (Figure 6.2(H)). However, actin filaments are still found in the cytoplasm. From 90 min onwards, actin filament bundles become more prominent in the cellular periphery (Figure 6.2(I)-(L)). After 240 min of incubation, stress fibers are aligned to the edges of the cell in a highly-order manner (Figure 6.2(L)).

The appearance of stress fibers, as a result of cytoskeleton remodeling, is coupled with the formation of focal adhesion [158]. As shown in Figure 6.2(M), focal adhesion lay at cell periphery at 30 min where stress fibers exist. The corresponding merged image of actin and vinculin confirmed the colocalization of actin and vinculin near cellular cortex (Figure 6.2(S)). After 60 min of culture, disparate and big focal adhesion plaques were found near cell cortex. There are few focal adhesion plaques in the central portion of the cell at both 30 min and 60 min. This phenomenon is consistent with the devoid of stress fiber in central portion of cell body. From 90 min onwards, vinculin pattern becomes different. In Figure 6.2(O), it is found that vinculin is scattered all over the cell body although focal adhesion plaques in cell periphery appears larger than those in other regions. Similar focal adhesion pattern to that at 90 min is displayed after 120 min of culture (Figure 6.2(P)-(R)). At 240 min, focal adhesion is clearly spotted at the

end of stress fibers (Figure 6.2(X)), confirming the colocalization of stress fiber and vinculin.

Blocking β_1 may affect actin filament remodeling and focal adhesion formation besides morphological evolution. At 30 min after cell seeding, actin filaments are present throughout the cytoplasm of the β_1 -blocked PEF with round contour (Figure 6.3(G)). At the same time, vinculin is not observed in TIRFM image as green background of the TIRFM image means no captured signals (Figure 6.3(M)). After another 30 min of culture, actin filaments still exist all over the cell body and vinculin becomes visible in cell periphery and particular regions of cell body (Figure 6.3(H) and (N)). The actin-rich lamellipodia in cell membrane forms at 90 min after cell plating (Figure 6.3(O)). Vinculin is mainly colocalized with actin filament bundles at the cell edge (Figure 6.3(U)). After cultured for more than 120 min, stress fibers are aligned parallel to the edge of the cell (Figure 6.3(J)-(L)) and vinculin were dispersed throughout the cell body (Figure 6.3(P)-(R)).

Comparing the evolution of F-actin distribution and focal adhesion pattern in the non-blocked and β_1 -blocked cells, the biggest difference is the lag time for initiating focal adhesion. For non-blocked cells, a lag time of around 30 min is needed for initiating focal adhesion formation. However, blocking β_1 integrin delays the focal adhesion formation, leading to an increase of lag time to 60 min, reflecting the dependence of activation of focal adhesion on β_1 integrin.

Focal adhesion area, the area occupied by vinculin for each cell, was acquired from TIRFM images. Correspondingly, focal adhesion density, defined as focal adhesion area per spread area, was derived from analyzing the TIRFM and phase contrast images. The effect of culture time on focal adhesion area and focal adhesion density of non-blocked and β_1 -blocked PEFs on fibronectin-coated surface is shown in Figure 6.5. A time-dependent increase in focal adhesion area of non-blocked and β_1 -blocked cells is observed (Figure 6.5(A)). During the ascending process, focal adhesion area of non-blocked cells always exceeded that

of β_1 -blocked cells. At 240 min post seeding, focal adhesion area of non-blocked cells is $667 \mu\text{m}^2$, 59% higher than that of β_1 -blocked cells. Time-dependent change in focal adhesion density is shown in Figure 6.5(B). No single downward/upward trends in focal adhesion density against time are found in whether non-blocked or β_1 -blocked cells. For non-blocked cells, their focal adhesion density increases initially but then declines after 60 min post seeding. From 90 min, it starts to ascend again till reaching maximum value at 240 min. A similar trend is also observed in β_1 -blocked cells. During the late culture period (120 min-240 min), focal adhesion density of non-blocked cells is persistently higher than that of β_1 -blocked cells.

Figure 6.5 The focal adhesion area (A) and focal adhesion density (B) against time for non-blocked and β_1 -blocked PEFs on fibronectin-coated surface within 4 h after cell seeding. $n=80-100$ for each data point. *Significant difference from the corresponding value in β_1 -blocked PEFs at the same time point (Student's t-test: $P<0.05$).

6.2.4 Time-dependent Change of Adhesion Force and Strength

Typically, cell adhesion is a strengthening process [87]. The effect of culture time on adhesion force is presented in Figure 6.6(A). Adhesion force of non-blocked cells keeps on rising against time until stabilizes after 180 min.

Similarly, adhesion force of β_1 -blocked cells goes up with time. However, the adhesion force-time curve of non-blocked cells lies above that of β_1 -blocked cells, indicating higher force is needed for the former to detach from the substrate following the same period of culture. Adhesion strength, defined as adhesion force per unit spread area, represents the maximum shear stress to detach cells. The effect of culture time on adhesion strength is shown in Figure 6.6(B). As illustrated in Figure 6.6(B), adhesion strength maintains upwards trend over time during 240 min of culture period. The maximum adhesion strength of non-blocked and β_1 -blocked cells is 52.8 Pa and 34.4 Pa, respectively. Similar tendency for adhesion force to ascend with time was also noticed on chondrocytes by other researchers [173, 174].

Figure 6.6 The adhesion force (A) and adhesion strength (B) against time for non-blocked and β_1 -blocked PEFs on fibronectin-coated surface within 4 h after cell seeding. $n=80-100$ for each data points. *Significant difference from the corresponding value in β_1 -blocked PEFs at the same time point (Student's t-test: $P < 0.05$).

6.2.5 Time-dependent Change of Number of Attached Cells

To evaluate the affinity between cells and substrates, cell attachment assay was carried out. On fibronectin-coated surface, both number of attached

non-blocked PEFs and number of attached β_1 -blocked PEFs exhibit increase trend with culture time and reach a plateau (Figure 6.7). Comparatively, it is quicker for number of attached non-blocked PEFs to reach its plateau. After 4 h of culture, number of attached β_1 -blocked cells is 43% less than that of non-blocked cells, implying functional blocking of integrin β_1 significantly deteriorates the affinity of integrin receptors for fibronectin ligands.

Figure 6.7 Number of attached cells against time for non-blocked and β_1 -blocked PEFs on fibronectin-coated surface within 4 h after cell seeding. *Significant difference from corresponding value in β_1 -blocked PEFs at the same time point (Student's t-test: $P < 0.05$).

6.3 Discussion

Integrin-mediated cell adhesion is often considered to be an adhesion strengthening process [87]. The initial integrin-ligand binding triggers cell spreading, integrin clustering, and focal adhesion, all of which contribute to the enhancement of adhesion force between cell and substrate [90]. Previous experimental data from other researchers unanimously demonstrated time-dependent increase in adhesion force for various types of cells on different

substrates [107, 108, 173-177]. However, no result was reported on temporal fluctuation of adhesion force for β_1 -blocked cells. It is demonstrated in our results that blocking β_1 integrin subunit does not alter the ascending trend of adhesion force over time.

Cell spreading and focal adhesion formation contribute significantly to enhancement of cell adhesion [90]. There is a consensus on the fact that cells keep on spreading in the initial cell adhesion process [75, 173, 175]. To separate the effect of cell spreading and other factors, adhesion strength is defined to exclude the contribution of cell spreading to adhesion strengthening process. As depicted in most published works, there was a trend of increase in adhesion strength over culture time [107, 173, 175]. In our results, the time-dependent increase of spread area and adhesion strength confirms cell spreading alone cannot account for the augment of adhesion force during cell adhesion.

To explain the strengthening process during cell adhesion, many mechanical models have been proposed [86, 87, 178]. In these theoretical analyses, focal adhesion formation has been positively correlated with the enhancement of adhesion strength. At focal adhesion sites, cell membrane stiffens and binding strength intensifies due to the recruitment of focal adhesion protein and cytoskeleton remodeling [86, 179, 180]. For example, Ward et al. predicted adhesion strength in focal contact zone was 60% ~ 650% higher than that in non-focal contact zone [86]. In contrast, focal adhesion formation only resulted in 50% ~ 200% of increase in adhesion strength through Gallant's simulation [87].

Focal adhesion force (adhesion force at focal adhesion sites) is believed to be tightly connected with focal adhesion area. In Ward's model, focal adhesion force is nearly proportional to the focal adhesion density when the focal adhesion density is below a critical value [86]. Until now, there has been lack of experimental results about quantitative relation between focal adhesion force and focal adhesion area due to the difficulty in the measurement of local adhesion force. However, the study of the association of traction force with focal adhesion

provides some analogic results [181, 182]. It is demonstrated in these works that traction force is almost linearly dependent on focal adhesion area, suggesting possible linear relationship between focal adhesion force and focal adhesion area. If adhesion force within non-focal adhesion zone is also proportional to the non-focal adhesion area, the linear relationship between adhesion strength and focal adhesion density may exist under particular conditions.

To check this hypothesis, adhesion strength-focal adhesion density curves for non-blocked and β_1 -blocked PEFs were plotted in Figure 6.8. Beyond our initial expectation, six data points did not display a linear trend in Figure 6.8(A) and (B).

Figure 6.8 The relationship between adhesion strength and focal adhesion density. (A) Non-blocked PEFs on fibronectin-coated surface; coefficient of determination = 0.98 for the linear fit. (B) β_1 -blocked PEFs on fibronectin-coated surface; coefficient of determination = 0.97 for the linear fit.

Nevertheless, the four data points at 90, 120, 180, and 240 min are linearly correlated in Figure 6.8(A). Similarly, the three data points at 120, 180, and 240 min appear to lie roughly on a straight line in Figure 6.8(B). Regression of the four data points in Figure 6.8(A) and the three data points in Figure 6.8(B) was carried out using the following function

$$\tau_A = \tau_{FA} \cdot P_{FA} + \tau_{NFA} (1 - P_{FA}) \quad (6.1)$$

where τ_A , τ_{FA} , τ_{NFA} , and P_{FA} are adhesion strength (or overall adhesion strength), focal adhesion strength, non-focal adhesion strength, and focal adhesion density, respectively. The slope and intercept of the fitted line correspond to $\tau_{FA}-\tau_{NFA}$ and τ_{NFA} , respectively. The focal adhesion and non-focal adhesion strength of non-blocked and β_1 -blocked PEFs obtained by linear fitting are listed in Table 6.1.

Table 6.1 Focal adhesion strength (τ_{FA}) and non-focal adhesion strength (τ_{NFA}) of non-blocked and β_1 -blocked PEFs on fibronectin-coated surface.

Cell type	τ_{NFA} (Pa)	τ_{FA} (Pa)
Non-blocked PEFs	24.7	145.3
β_1 -blocked PEFs	19.2	104.5

The variation of adhesion strength of the two types of cells with respect to focal adhesion density during cell adhesion process follows a similar trend. For instance, as shown in Figure 6.8(A), adhesion strength of non-blocked cells increases with focal adhesion density from 30 min to 60 min. After 60 min, the trend is reversed. The augment of focal adhesion density does not elevate the measured adhesion strength from 60 min to 90 min. However, adhesion strength returns back to the escalating trend from 90 min onwards. During 240 min of culture, increase of focal adhesion density does not always induce the ascent of adhesion strength. To explain this phenomenon, the peeling model and fracture model of cell detachment proposed by Ward et al are tentatively used for reference [86]. According the distribution of focal adhesion within cell boundary, adherent cells examined in our experiments are classified into two groups. The first group is cells whose focal adhesions are extremely concentrated on cell periphery. The second group is cells with relatively uniform distribution of focal adhesion. After examining vinculin patterns in Figures 6.2 and 6.3, non-blocked PEFs at 30 min and 60 min, and β_1 -blocked PEFs at 60 min and 90 min were classified the first group according to this criterion. Other cells except β_1 -blocked

PEFs at 30 min without the formation of focal adhesion are included in the second group. For the first group of cells, the detachment of adherent cells is believed to start from a progressive breaking of bonds at the central part of the cells where binding strength is weaker and cell membrane is more flexible than cell periphery. Afterwards, the simultaneous failure of the bonds at cell periphery with dense focal adhesion structure leads to separation of the whole cell body from the substrate. Therefore, the dominant factor to determine adhesion strength of the cells in the first group is focal adhesion density. In other words, the cellular portion with non-focal adhesion makes little contribution to the overall adhesion strength. For the second group of cells, the distribution of focal adhesion is relatively uniform in cell adhesion patch. Because of stiffening effect of uniformly distributed focal adhesion on cell membrane, the whole contact portion of the cells can be considered as a totally rigid entity approximately. Thus, adherent part of cell membrane with/without focal adhesion acts collectively to withstand the shear force applied by micropipette. Under this condition, adherent portion of cell membrane without focal adhesions also made a definite contribution to the overall adhesion strength.

During adhesion process of non-blocked cells, focal adhesion sites are mainly localized to cell periphery at 30 min and 60 min. Cell detachment goes in the peeling mode. Adhesion strength ascends with an increase in focal adhesion density during this culture period. At or after 90 min, focal adhesions become uniformly dispersed in the cytoplasm of the cells. Cell detachment proceeds in fracture mode. Non-focal adhesion portion of membrane starts to make its contribution to the overall adhesion strength. That may explain the rise of adhesion strength under the circumstance that focal adhesion density declines from 60 min and 90 min. After 90 min, focal adhesion is still dispersed throughout the spread portion of cell membrane. As a result, adhesion strength increases in an approximate linear relationship over focal adhesion density. The variation of

adhesion strength with focal adhesion density in β_1 -blocked PEFs during 240 min of culture can be interpreted in a similar way.

The linear fit of adhesion strength with respect to focal adhesion density produces some useful information to comprehend cell adhesion. As shown in Table 6.1, focal adhesion strength is significantly higher than non-focal adhesion strength regardless to the type of cells used herein. The ratio of focal adhesion strength to non-focal adhesion strength in non-blocked and β_1 -blocked PEFs is 5.9 and 5.4, respectively. These values just fall within the range predicted by Ward et al. [86]. As focal adhesion strength, non-focal adhesion strength, and focal adhesion density are known, the contribution rate of focal adhesion to the overall adhesion strength can be derived. At 240 min, the contribution of focal adhesion to the overall adhesion strength is 64% in non-blocked cells and 55% in β_1 -blocked cells. Gallant et al. employed siRNA technique to knockdown vinculin expression in order to investigate the importance of focal adhesion to adhesion strength [90]. In their results, focal adhesion makes up around 30% of the overall adhesion strength of NIH 3T3 fibroblasts. As shown in our results, slight variation of focal adhesion density may induce big change in adhesion strength because of high ratio of focal adhesion strength to non-focal adhesion strength. In Gallant's report, very few vinculin plaques are found in NIH 3T3 fibroblasts compared to the higher focal adhesion density in PEFs. Therefore more contribution of focal adhesion to the overall adhesion strength is found in our results herein.

Functional blocking of β_1 integrin subunit was believed to impair the binding between integrin receptor and immobilized fibronectin and to adhesion strength [183, 184]. It is demonstrated in our results that non-blocked PEFs always possesses higher adhesion strength than β_1 -blocked PEFs during 4 h of culture. Diminishment of adhesion strength by functional blocking of β_1 integrin was also confirmed indirectly on the adhesion of chondrocytes to cartilage tissue through flow cytometry by Kurtis et al. [185, 186]. In addition, it is found in our results

that functional blocking of β_1 integrin reduces focal adhesion density, suggesting downregulation of vinculin expression which may be attributed to the impairment of fibronectin-integrin interaction. It was reported that targeted ablation or functional blocking of β_1 integrin results in a decrease of FAK expression level [187, 188]. Thus it is not surprising that functional blocking of β_1 integrin leads to a decrease of focal adhesion density. Furthermore, there is smaller focal adhesion strength and non-focal adhesion strength in β_1 -blocked PEFs than in non-blocked PEFs as listed in Table 6.1. Therefore, it is the collective effect of smaller local adhesion strength (focal adhesion strength and non-focal adhesion strength) and lower focal adhesion density to account for the weaker adhesion strength in β_1 -blocked PEFs on fibronectin-coated surface.

6.4 Summary

In this study, monoclonal antibodies were applied to block β_1 integrin subunit in PEFs. The temporal effect of functional blocking of β_1 integrin on mechanotransduction of cell was investigated with TIRFM and micropipette aspiration technique. It is shown in our results that spread area, adhesion force, and adhesion strength of non-blocked and β_1 -blocked PEFs on fibronectin-coated surface tend to increase over time during 4 h of cell seeding. Adhesion force and adhesion strength of β_1 -blocked cells are always smaller than those of non-blocked cells, respectively. Interestingly, there is tight relationship between adhesion strength and focal adhesion density in PEFs that can be explained by the peeling and fracture models. During the late culture period, adhesion strength increases linearly with focal adhesion density. Based on the fracture model, averaged focal adhesion strength and non-focal adhesion strength are deduced. Smaller adhesion strength is possessed by β_1 -blocked PEFs than non-blocked cells, which is attributed to lower focal and non-focal adhesion strength as well as decreased focal adhesion density because of the blocking of β_1 integrin.

Chapter 7 Weibull Statistical Analysis of Adhesion Strength

7.1 Introduction

Weibull distribution is a statistical method named after Waloddi Weibull [189]. As a Swedish physicist, Waloddi Weibull used it in 1939 to describe the breaking strength of materials [190]. Since then, its application has been extended to many other fields. Currently, it can be used to model the lifetime of products, estimate the correlation between the risk factors and disease mortality, make weather forecast, and so on [191-193].

Flow chamber assay is a widely used method to determine cell adhesion strength [194]. In flow chamber test, shear stress applied to adherent cells is controlled through varying volumetric flow rate. Due to the heterogeneity in the adhesion strength of cell, the number of attached cells decreases gradually with the increase of flow rate. After quantification of the number of cells bound to the substrate at different shear stress, the relationship between shear stress and fraction of attached cells is set up to determine adhesion strength.

To analyze detachment data obtained from flow chamber experiments, sigmoidal, normal, lognormal, and Weibull distributions are often employed to represent the variation of adhesion strength among cell population in order to determine overall adhesion strength of adherent cells [195-203]. Among the aforementioned four types of distributions, Weibull distribution is widely used in the analysis of the data of flow chamber test. In particular, Rezanian et al. used sigmoidal, lognormal, and Weibull distributions to fit adhesion strength data of bone cells on chemically modified surfaces [200]. It is found that Weibull distribution is more representative of the observed cell-substrate adhesion strength compared to the sigmoidal and log-normal distributions.

Cell adhesion to substrate is adhesion strengthening processes characterized by augment of average adhesion strength against culture time. In Chapter 6, micropipette aspiration technique was employed to measure adhesion strength of individual PEF on fibronectin coated coverslip within 4 h of culture. It is shown that average adhesion strength goes up during the development of cell adhesion. However, it is highly possible that there is shift of distribution of adhesion strength in the adhesion strengthening process besides the change of average adhesion strength. The information about the evolution of adhesion strength distribution may be beneficial to better understanding of cell adhesion process.

To date, there are no reported studies on analysis of distribution of adhesion strength quantified by micropipette aspiration technique. In this study, adhesion strength data for non-blocked and integrin β_1 -blocked PEFs on fibronectin-coated substrate following different culture period were fitted with Weibull distribution function. Characteristic adhesion strength and Weibull modulus were deduced and compared for PEFs in cell adhesion process. In addition, Weibull cumulative probability and probability density graphs for non-blocked and β_1 -blocked PEFs were plotted for analysis.

7.2 Results and Discussion

A two-parameter Weibull distribution was used to characterize the strength of cell adhesion to the fibronectin coated surfaces. The Weibull cumulative probability function is given as:

$$P = 1 - e^{-(\tau/\tau_0)^m} \quad (7.1)$$

where P is the Weibull cumulative probability of cell detachment or failure, τ_0 is the characteristic adhesion strength, τ is shear stress applied by micropipette aspiration (or adhesion strength), and m is the Weibull modulus. To determine τ_0 and m , it is a common practice to linearize Equation 7.1 by taking natural logarithms twice into the following form:

$$\ln[-\ln(1-P)] = m(\ln \tau - \ln \tau_0) \quad (7.2)$$

The cumulative probability of cell detachment at a given shear stress, P , was obtained through the median rank formula,

$$P = (i - 0.3)/(n + 0.4) \quad (7.3)$$

where i is the rank of ascending adhesion strength data, and n is the total number of cell failures. Using Equations 7.2 and 7.3, regression of original adhesion strength data is implemented and shown in Figure 7.1. As illustrated in Figure 7.1, Weibull distribution fits the experimental data well. Coefficients of determination (R^2) of these regressions range from 0.967 to 0.986. Relatively large value of R^2 validates the reliability of Weibull distribution to model adhesion strength variation among the adherent cell population.

The characteristic adhesion strength and Weibull modulus, the two parameters in Weibull probability function, are obtained through the regression of experimental data. To compare characteristic adhesion strength and average adhesion strength, the two types of adhesion strength-time curves were plotted in Figure 7.2. It is found that characteristic strength-time curve is always above the average strength-time curve for both non-blocked and β_1 -blocked PEFs, indicating characteristic strength is higher than average strength for the same cell population. Characteristic adhesion strength, as an important parameter in Weibull analysis, represents the shear stress at which the probability of detachment/failure is equal to 0.632. In theory, whether characteristic adhesion strength is higher or lower than average adhesion strength is dependent on the value of Weibull modulus, m . If m is bigger than 1, characteristic adhesion strength will surpass average adhesion strength [204].

The Weibull moduli for non-blocked and β_1 -blocked PEFs are shown in Table 7.1. As indicated in Table 7.1, all the Weibull modulus are bigger than 1. That explains why characteristic strength curve is located above average strength curve in Figure 7.2. It is also shown in Figure 7.2 that characteristic and average

adhesion strength goes up with time. The tendencies of the two types of adhesion strength variation in 4 h of culture are very similar, demonstrating the feasibility of adoption of characteristic adhesion strength to describe cell-substrate interactions besides average adhesion strength.

Figure 7.1 Regression lines for the adhesion strength data obtained through micropipette aspiration technique using linearized Weibull cumulative probability function. (A), non-blocked PEFs on fibronectin coated surface; (B) β_1 -blocked PEFs on fibronectin coated surface. The unit of shear stress, τ , is Pa.

Figure 7.2 The average and characteristic adhesion strength against time for non-blocked (A) and β_1 -blocked (B) PEFs on fibronectin-coated surface within 4 h after cell seeding.

Table 7.1 Weibull moduli for non-blocked and β_1 -blocked PEFs on fibronectin-coated surface from 30 min to 240 min after cell seeding.

Cell type	Weibull modulus (m)					
	30 min	60 min	90 min	120 min	180 min	240 min
Non-blocked	3.3	3.8	4.6	4.8	5.5	4.4
β_1 -blocked	2.1	2.9	3.2	4.1	3.8	4.2

To further verify reliability of the usage of characteristic adhesion strength, the variation of characteristic adhesion strength with focal adhesion density is shown in Figure 7.3. In Chapter 6, the relationship between average adhesion strength and focal adhesion strength has been systematically studied. As depicted in Figure 6.8, average adhesion strength initially ascends, then diminishes at a critical time point, and finally resumes increasing linearly against the increase of focal adhesion density. The change of average adhesion strength with time is explained by the peeling and fracture model. The variation of characteristic adhesion strength with time follows a similar trend as that of average adhesion strength

(Figure 7.3). With the aid of Equation 6.1, linear regressions of the four data points of non-blocked PEFs and the three data points of β_1 -blocked PEFs were employed to deduce characteristic focal and non-focal adhesion strength. Average and characteristic non-focal/focal adhesion strength were listed in Table 7.2 for comparison. It is found that characteristic non-focal or focal adhesion strength is higher than the corresponding average adhesion strength, which is consistent the results in Figure 7.2.

Figure 7.3 The relationship between characteristic adhesion strength and focal adhesion density. $R^2=0.96$ (non-blocked PEFs) or 0.97 (β_1 -blocked PEFs) for the linear fit.

Table 7.2 Average and characteristic non-focal adhesion strength (τ_{NFA}) and focal adhesion strength (τ_{FA}) of non-blocked and β_1 -blocked PEFs.

Cell type	τ_{NFA} (Pa)		τ_{FA} (Pa)	
	Average	Characteristic	Average	Characteristic
Non-blocked PEFs	24.7	28.1	145.3	158.2
β_1 -blocked PEFs	19.2	21.2	104.5	117.0

Moreover, characteristic focal adhesion strength is several-fold higher than non-focal characteristic strength regardless to the type of cells. Similar result is shown in the comparison of average τ_{NFA} and τ_{FA} . The above results prove that characteristic adhesion strength is as reliable as average adhesion strength in characterize cell-substrate interactions.

The development of cell adhesion is possibly accompanied by the variation of adhesion strength distribution. The Weibull cumulative probability and probability density graphs for non-blocked PEFs on fibronectin-coated surface are shown in Figure 7.4. It is conspicuous in Figure 7.4(A) that the probabilities of detachment for adherent cells after cultured for given time increases with applied shear stress. In details, the speed of increase in probabilities of detachment experience an acceleration phase followed by a slacken phase until probabilities reach the maximum value, 1. Another finding is that the probability curves of different time in Figure 7.4(A) are orderly arranged except the two curves of 180 min and 240 min. The probability curve of longer time is situated at the right side of that of shorter time, indicating that shear stress should be elevated with culture time in order to maintain determined probability of cell detachment. However, the probability curves of 180 min and 240 min intersect at adhesion strength ≈ 61 Pa and probability ≈ 0.7 , which can be translated as follows: if PEFs at 180 min were cultured for another 60 min, lower stress strength is required to detach less than 70% of adherent cells. It is interesting that culture for longer time will not inevitably enhance adhesion of all adherent cells.

It is worthy to be pointed out that the probability graphs were generated through regression of experimental data. Therefore, there is deviation between predicted value and actual experimental value. In fact, the deduced crossing point (61 Pa, 0.7) is only accurate on the basis that Weibull distribution can describe actual distribution of adhesion strength accurately. The coefficients of determination range from 0.967 to 0.987, indicating these regressions are not

100% believable. As demonstrated in Figure 7.4(B), cumulative probability graphs do not fit experimental data points perfectly. Therefore, other experiments including flow chamber assay may be needed to verify this above result obtained from cumulative probability graphs.

Weibull distribution can also be displayed in the form of probability density graph. In Figure 7.4(C), the shape of six probability density curves is dissimilar. For instance, the morphology of probability density curve of 240 min looks like more swollen than that of the one of 180 min. The range of probability density curve of 240 min span more widely than that of 180 min, indicating that the minimum value of adhesion strength at 240 min is lower than that at 180 min (Figure 7.4(C)). Meredith et al. reported that S-phase cells show smaller focal adhesion density than non-S-phase cells [205]. As adhesion strength is positively correlated to focal adhesion density, the decrease in the minimum value of adhesion strength from 180 min to 240 min may suggest part of adherent cells enter the S-phase of their cycle from G1.

Weibull modulus, as an important parameter in Weibull distribution function, determines shape of the distribution. It is noticed in Table 7.1 that the Weibull modulus for non-blocked PEFs of 240 min is smaller than that for the one of 180 min. Weibull modulus, m , indicates the scatter of adhesion strength values, and a higher m value implicates narrower and less skewed distribution [206]. The difference in m is consistent with the fact that the morphology of probability density curve of 240 min is more swollen than that of the one of 180 min. Moreover, characteristic adhesion strength or average adhesion strength for non-blocked PEFs at 180 min is almost equal to that of 240 min (Figure 7.2). Therefore, stability in either characteristic adhesion strength or average adhesion strength can be interpreted to be more a dynamic steady state than a static status. Interestingly, a general trend of increase in Weibull modulus of non-blocked PEFs is found in Table 7.1, implying that the cell-substrate interactions in cell population become more consistent after cultured for longer time.

Functional blocking of β_1 integrin possibly affects the distribution of adhesion strength during cell adhesion process. The Weibull cumulative probability and probability density graphs for β_1 -blocked PEFs are shown in Figure 7.5. These probability curves are aligned side by side from left to right with the increase of time, as presented in Figure 7.5(A). Hence, higher shear stress is needed to apply to maintain any given probability of cell detachment with extension of culture period for β_1 -blocked PEFs. It is demonstrated in Figure 7.5(C) that the probability density curves of different time are not uniform in shape, which is confirmed by the distinct value of m listed in Table 7.1.

Among these curves in Figure 7.5(C), the probability density curve of 30 min is conspicuous as its value ascends rapidly in the vicinity of origin of coordinate while other probability density curves are almost superposed with adhesion strength axis in the same range. That means a small portion of adherent cells cannot withstand very small shear force applied by micropipette aspiration. This singular phenomenon appearing in β_1 -blocked PEFs at 30 min is believed to result from the devoid of focal adhesions. As depicted in Figure 6.3(M), vinculin was not observed in β_1 -blocked PEFs after 30 min of culture. Due to the soft adherent membrane without the formation of focal adhesions, the detachment of adherent cells still proceeds in peeling mode. In this condition, very low shear stress may separate a tiny part of membrane in a weakly attached PEF from the substrate. Following that, the detached portion of cell membrane extends steadily till the whole cell body is dissociated from the fibronectin-coated substrate. In addition, a general upward trend of Weibull modulus also exists in β_1 -blocked PEFs (Table 7.1), suggesting cell-substrate adhesion activities becomes more uniform with increased culture time.

As shown in the above results, adhesion strength of PEFs obeys Weibull distribution. Now, we will attempt to explain why probability density graphs of adhesion strength display the characteristic shape of Weibull distribution. As the change in the shape of probability density graphs for β_1 -blocked cells during 240

min of culture is more evident than that for non-blocked cells, the Weibull distribution of β_1 -blocked cells was analyzed. In Figure 7.5(C), the probability density graph of 30 min is skewed to right, showing a relatively long right-hand tail. It is found that there was no appearance of focal adhesion formation at 30 min for the major portion of PEFs (Figure 6.3). However, it is very possible that a small portion of “active” cells adhere to the surface more quickly than others and focal adhesions form. According to the results in Table 6.1, focal adhesion strength is over 4 times higher than non-focal adhesion strength for β_1 -blocked cells. Thus, these “active” cells will possess significantly higher adhesion strength than other cells, leading to the appearance of long right-hand tail in the probability density graph.

The development of cell adhesion proceeds with time. After cultured for 120 min, Weibull modulus (m) has increased to 4.1 from 2.1 at 30 min. With the increase of m , probability density graphs become more symmetrical. Despite that, the shape of probability density graph will change from positively skewed (skewed to the right or steep on the left-hand side) to negatively skewed (skewed to the left or steep on the right-hand side) [207]. The probability density graphs of 120 min, 180 min and 240 min, are skewed to the left, showing a left-hand tail. Possible explanation of the appearance of this type of tail is that some cells progress from G1 to S-phase. S-phase cells have smaller focal adhesion density than non-S-phase cells [205]. The decrease of focal adhesion density may possibly lead to the appearance of highly uneven distribution of focal adhesion. Under this condition, cell detachment mode is changed from “fracture” to “peeling” and non-focal adhesions do not make substantial contribution to adhesion strength. Consequently, adhesion strength for this portion of cells decreased notably. Thus, the three probability density graphs for β_1 -blocked cells at 120 min, 180 min and 240 min are shown to be skewed to the left.

Figure 7.4 Weibull cumulative probability and probability density graphs of non-blocked PEFs on fibronectin-coated surface from 30 min to 240 min after cell seeding. Graphs and experimental data points are shown together in (B).

Figure 7.5 Weibull cumulative probability and probability density graphs of β_1 -blocked PEFs on fibronectin-coated surface from 30 min to 240 min after cell seeding. Graphs and experimental data points are shown together in (B).

7.3 Summary

Weibull statistics was adopted to analyze adhesion strength data obtained through micropipette aspiration technique. It is demonstrated that Weibull distribution can describe the distribution of adhesion strength for PEFs on fibronectin-coated surfaces satisfactorily. Similar to average adhesion strength, characteristic adhesion strength acquired through Weibull statistical analysis is a reliable parameter to characterize cell-substrate interactions. Furthermore, the shift of Weibull cumulative probability and probability density graphs for PEFs within 4 h of culture reflects the development of cell adhesion, providing information for understanding cell adhesion process.

Chapter 8 Conclusions and Future Work

8.1 Conclusions

In this work, biophysical experiments were conducted to elucidate adhesion behavior of PEFs on molecular engineered surfaces. First, the effect of ECM protein immobilization on adhesion behavior of PEFs was investigated on PLA and CNT monolayer.

PLA was subjected to surface modification by coupling ECM proteins on its surface to promote cell adhesion. Two typical ECM proteins, collagen type I (COL) and fibronectin (FN) were immobilized on the PLA surface with the aid of glutaraldehyde as the cross-linker between aminolyzed PLA and ECM proteins. By using C-RICM integrated with phase contrast microscopy, adhesion dynamics of porcine esophageal fibroblasts (PEFs) on four types of surfaces (unmodified PLA, PLA-CHO, PLA-COL, and PLA-FN) was investigated during 24 h of culture. It is demonstrated by C-RICM results that PEFs form strong adhesion contact on all four types of surfaces at different stages of cell seeding. Among four surfaces, PEFs on PLA-FN surface climb up to its maximum adhesion energy ($9.5 \times 10^{-7} \text{ J/m}^2$) in the shortest time (20 min) during the initial stage of cell seeding. After adhesion energy reaches maximum, PEFs maintain their highly deformed geometries until they reached steady state after 20 h of culture. Results from F-actin immunostaining show that the evolvement of spatial organization of F-actin is tightly correlated to the formation of adhesion contact and cell spreading. Furthermore, cell attachment ratio of PEFs on PLA in 2 h is only 26% comparing with 88% on PLA-FN, 73% on PLA-COL, and 36% on PLA-CHO.

CNT monolayer (CNT-ML) was also functionalized with two types of ECM proteins including collagen type I and fibronectin in order to promote its biocompatibility. Adhesion dynamics of PEFs on functionalized-CNT monolayer were elucidated with C-RICM integrated with phase contrast microscopy. It is

found that the topographical features of CNT-ML are dependent on the types of immobilized ECM protein, as shown by atomic force microscopy. There is a significant time lag in adhesion contact evolution of around 10 min for PEFs on both CNT-ML and CNT-COL compared to the negligible time lag on CNT-FN. Adhesion energy of PEFs on the CNT-COL and CNT-FN surfaces reach steady state at 60 min and 30 min after cell seeding compared to 70 min on CNT-ML surface. At steady state, the adhesion energy of PEFs on the CNT-COL and CNT-FN surfaces is almost twice that of the CNT-ML surface. Moreover, immobilization of collagen or fibronectin on CNT monolayer leads to an increase of proliferations and attachment ratio of PEFs. Scanning electron microscopy and immunostaining together demonstrated that PEFs display an elongated morphology and highly polarized actin network on both CNT-COL and CNT-FN surfaces, whereas PEFs display non-uniform cell morphology and actin organization on the CNT-ML surface.

All of the aforementioned results of adhesion dynamics of PEFs on ECM protein-immobilized PLA and CNT monolayer surfaces demonstrate the effect of surface modification on the biophysical responses of PEFs in cell adhesion. Functionalization of PLA or CNT through extracellular matrix protein immobilization effectively promotes cell adhesion. Fibronectin-immobilized PLA and CNT demonstrate promising potentials for tissue engineering application.

Second, adhesion strengthening process of PEFs was investigated on fibronectin-coated coverslip due to excellent performance of fibronectin immobilization in enhancing cell adhesion. In this study, we attempt to bridge the quantitative connection between focal adhesion density and cell-substrate interactions and evaluate the influence on functional blocking of β_1 integrin on adhesion strength. Total internal reflection fluorescence microscopy, fluorescence microscopy, and phase contrast microscopy were employed to study the time-dependent evolvement of vinculin pattern, distribution of actin filament, and morphological change, respectively, during 4 h of culture for PEFs on

fibronectin-coated surface. Micropipette aspiration technique was used to study the change of mechanotransduction through the determination of adhesion force and strength. It is shown in our experimental results that spread area, adhesion force, and adhesion strength increase over time on the two types of cells. Throughout the culture period, the two key mechanotransduction parameters of non-blocked cells is higher those of β_1 -blocked cells. Interestingly, adhesion strength increases initially, then begins to diminish after a critical time point, and finally resumes increasing linearly against the increase of focal adhesion density. This variation as mentioned above can be explained by peeling and fracture models based on the dissimilar vinculin pattern of cells after cultured for different time period. Moreover, the averaged focal adhesion strength and non-focal adhesion strength of β_1 -blocked cells are significantly less than those of non-blocked of cells. The weaker adhesion strength on β_1 -blocked cells is directly caused by lower focal and non-focal adhesion strength as well as smaller focal adhesion density.

Finally, Weibull statistics was performed for the first time to analyze adhesion strength data obtained by micropipette aspiration technique. Characteristic adhesion strength is proved to be as reliable as average adhesion strength to describe cell-substrate interactions during cell adhesion process. The augment of Weibull modulus with culture time reveals that the distribution of adhesion strength of cell population becomes more uniform with the development of cell adhesion. Furthermore, the shift of Weibull cumulative probability and probability density graphs for PEFs within 4 h of culture reflects the development of cell adhesion.

In brief, immobilization of ECM proteins on biomaterials alters biophysical responses of cells and promotes cell adhesion. There is positive linear correlation between focal adhesion density and adhesion strength, demonstrating the key role of focal adhesion in cell adhesion strengthening process. β_1 integrin subunit is important in cell-fibronectin interaction and functional blocking of β_1 integrin

deteriorates adhesion strength between cell and fibronectin-coated surface. Moreover, Weibull statistics provides an effective way to analyze adhesion strength data obtained by micropipette aspiration.

8.2 Future work

Based on the experimental results mentioned above, future study can be directed to the following aspects for deeper understanding of cell adhesion behavior and the underlying adhesion mechanism of cells on molecularly engineered surface.

1) Real-time monitoring focal adhesion dynamics

Gene transfection technique is a useful technique to investigate intracellular molecular events. When combined with TIRFM, real-time study of focal adhesion dynamics with/without external stimuli becomes practical. In this study, cells can be transfected with green fluorescent protein (GFP)–vinculin. Using GFP-tagged vinculin as the focal adhesion site marker, the focal adhesion dynamics can be monitored in real time after cell seeding. If external stimuli, such as mechanical stimuli to compress cells and chemical stimuli by infusion of growth factors, are exerted to adherent transfected cells, the change of individual vinculin plaque can be captured for analysis. Furthermore, results of RT-PCR and Western blotting can be integrated with the change of focal adhesion dynamics for analyzing cell adhesion mechanisms.

2) The function of adhesion-related proteins in regulating cell adhesion dynamics

Small interfering RNA (siRNA) technique, as a newly-developed method, can be employed to knock down gene expression. To investigate the importance of adhesion-related protein including Cdc42, Rac, and RhoA to adhesion behavior, cells will be treated by siRNA to silence the expression of the corresponding gene.

Using C-RICM based technique, adhesion dynamics of experimental cells (siRNA treated) and the control cells (untreated) on molecularly engineered surfaces will be studied and compared to delineate the function of these proteins.

3) Molecular dynamics simulation of ligand-receptor interactions

Molecular dynamics (MD) simulation is an effective and powerful tool to study fine details of ligand-receptor interactions at the molecular level. The interactions between $\alpha_5\beta_1$ integrin (non-blocked and β_1 -blocked) and fibronectin can be studied by MD simulation. Adhesion energy and adhesion strength will be deduced. As adhesion strength of cells to fibronectin-coated surface can be acquired by micropipette aspiration technique, the theoretical results obtained by MD simulation can be compared with the experimental data.

References

- [1] Chen, GP, Ushida, T, Tateishi, T. Scaffold design for tissue engineering. *Macromolecular Bioscience* 2002;2:67-77.
- [2] Vasita, R, Shanmugam, K, Katti, DS. Improved biomaterials for tissue engineering applications: Surface modification of polymers. *Current Topics in Medicinal Chemistry* 2008;8:341-353.
- [3] Kim, BS, Mooney, DJ. Development of biocompatible synthetic extracellular matrices for tissue engineering. *Trends in Biotechnology* 1998;16:224-230.
- [4] Langer, R, Vacanti, JP. Tissue engineering. *Science* 1993;260:920-926.
- [5] Griffith, LG, Naughton, G. Tissue engineering - Current challenges and expanding opportunities. *Science* 2002;295:1009-1014.
- [6] Liu, XH, Ma, PX. Polymeric scaffolds for bone tissue engineering. *Annals of Biomedical Engineering* 2004;32:477-486.
- [7] Rosso, F, Giordano, A, Barbarisi, M, Barbarisi, A. From cell-ECM interactions to tissue engineering. *Journal of Cellular Physiology* 2004;199:174-180.
- [8] Ma, PX. Biomimetic materials for tissue engineering. *Advanced Drug Delivery Reviews* 2008;60:184-198.
- [9] Folkman, J, Moscona, A. Role of cell shape in growth control. *Nature* 1978;273:345-349.
- [10] Benzeev, A, Farmer, SR, Penman, S. Protein synthesis requires cell-surface contact while nuclear events respond to cell shape in anchorage-dependent fibroblasts. *Cell* 1980;21:365-372.
- [11] Ruoslahti, E, Reed, JC. Anchorage dependence, integrins, and apoptosis. *Cell* 1994;77:477-478.
- [12] Gumbiner, BM. *Cell Adhesion: The Molecular Basis of Tissue*

- Architecture and Morphogenesis. *Cell* 1996;84:345-357.
- [13] Lutolf, MP, Hubbell, JA. Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. *Nature Biotechnology* 2005;23:47-55.
- [14] Nair, LS, Laurencin, CT. Biodegradable polymers as biomaterials. *Progress in Polymer Science* 2007;32:762-798.
- [15] Berrier, AL, Yamada, KM. Cell-matrix adhesion. *Journal of Cellular Physiology* 2007;213:565-573.
- [16] Ma, ZW, Mao, ZW, Gao, CY. Surface modification and property analysis of biomedical polymers used for tissue engineering. *Colloids and Surfaces B-Biointerfaces* 2007;60:137-157.
- [17] Jiao, YP, Cui, FZ. Surface modification of polyester biomaterials for tissue engineering. *Biomedical Materials* 2007;2:R24-R37.
- [18] Wilson, CJ, Clegg, RE, Leavesley, DI, Percy, MJ. Mediation of biomaterial-cell interactions by adsorbed proteins: A review. *Tissue Engineering* 2005;11:1-18.
- [19] Garcia, AJ, Gallant, ND. Stick and grip - Measurement systems and quantitative analyses of integrin-mediated cell adhesion strength. *Cell Biochemistry and Biophysics* 2003;39:61-73.
- [20] Eisenbarth, E, Velten, D, Breme, J. Biomimetic implant coatings. *Biomolecular Engineering* 2007;24:27-32.
- [21] Chan, BP, Bhat, VD, Yegnasubramanian, S, Reichert, WM, Truskey, GA. An equilibrium model of endothelial cell adhesion via integrin-dependent and integrin-independent ligands. *Biomaterials* 1999;20:2395-2403.
- [22] Tan, WJ, Teo, GP, Liao, K, Leong, KW, Mao, HQ, Chan, V. Adhesion contact dynamics of primary hepatocytes on poly(ethylene terephthalate) surface. *Biomaterials* 2005;26:891-898.
- [23] Zhu, AP, Fang, N, Chan-Park, MB, Chan, V. Adhesion contact dynamics of 3T3 fibroblasts on poly (lactide-co-glycolide acid) surface modified by

- photochemical immobilization of biomacromolecules. *Biomaterials* 2006;27:2566-2576.
- [24] Chan, V, Wan, KT. Thermal-induced modification of the contact mechanics of adhering liposomes. *Langmuir* 2002;18:3134-3141.
- [25] Schneckenburger, H. Total internal reflection fluorescence microscopy: technical innovations and novel applications. *Current Opinion in Biotechnology* 2005;16:13-18.
- [26] Rao, CNR, Cheetham, AK. Science and technology of nanomaterials: current status and future prospects. *Journal of Materials Chemistry* 2001;11:2887-2894.
- [27] Zhang, LJ, Webster, TJ. Nanotechnology and nanomaterials: Promises for improved tissue regeneration. *Nano Today* 2009;4:66-80.
- [28] Balasundaram, G, Webster, TJ. An overview of nano-polymers for orthopedic applications. *Macromolecular Bioscience* 2007;7:635-642.
- [29] Wang, SG, Cui, WJ, Bei, JZ. Bulk and surface modifications of polylactide. *Analytical and Bioanalytical Chemistry* 2005;381:547-556.
- [30] Kulkarni, RK, Moore, EG, Hegyeli, AF, Leonard, F. Biodegradable poly(lactic acid) polymers. *Journal of Biomedical Materials Research* 1971;5:169-181.
- [31] Garric, X, Moles, JP, Garreau, H, Guilhou, JJ, Vert, M. Human skin cell cultures onto PLA(50) (PDLA) bioresorbable polymers: Influence of chemical and morphological surface modifications. *Journal of Biomedical Materials Research Part A* 2005;72A:180-189.
- [32] Aframian, DJ, Cukierman, E, Nikolovski, J, Mooney, DJ, Yamada, KM, Baum, BJ. The growth and morphological behavior of salivary epithelial cells on matrix protein-coated biodegradable substrata. *Tissue Engineering* 2000;6:209-216.
- [33] Quirk, RA, Chan, WC, Davies, MC, Tendler, SJB, Shakesheff, KM. Poly(L-lysine)-GRGDS as a biomimetic surface modifier for poly(lactic

- acid). *Biomaterials* 2001;22:865-872.
- [34] Desai, NP, Hubbell, JA. Solution technique to incorporate polyethylene oxide and other water-soluble polymers into surfaces of polymeric biomaterials. *Biomaterials* 1991;12:144-153.
- [35] Desai, NP, Hubbell, JA. Surface physical interpenetrating networks of poly(ethylene terephthalate) and poly(ethylene oxide) with biomedical applications. *Macromolecules* 1992;25:226-232.
- [36] Cai, KY, Yao, KD, Cui, YL, Yang, ZM, Li, XQ, Xie, HQ, Qing, TW, Gao, LB. Influence of different surface modification treatments on poly(D,L-lactic acid) with silk fibroin and their effects on the culture of osteoblast in vitro. *Biomaterials* 2002;23:1603-1611.
- [37] Cui, YL, Hou, X, Qi, AD, Wang, XH, Wang, H, Cai, KY, Yin, YJ, Yao, KD. Biomimetic surface modification of poly (L-lactic acid) with gelatin and its effects on articular chondrocytes in vitro. *Journal of Biomedical Materials Research Part A* 2003;66A:770-778.
- [38] Zhu, HG, Ji, J, Lin, RY, Gao, CY, Feng, LX, Shen, JC. Surface engineering of poly(DL-lactic acid) by entrapment of alginate-amino acid derivatives for promotion of chondrogenesis. *Biomaterials* 2002;23:3141-3148.
- [39] Zhu, YB, Gao, CY, Liu, XY, He, T, Shen, JC. Immobilization of biomacromolecules onto aminolyzed poly(L-lactic acid) toward acceleration of endothelium regeneration. *Tissue Engineering* 2004;10:53-61.
- [40] Ma, ZW, Gao, CY, Ji, JA, Shen, JC. Protein immobilization on the surface of poly-L-lactic acid films for improvement of cellular interactions. *European Polymer Journal* 2002;38:2279-2284.
- [41] Carlisle, ES, Mariappan, MR, Nelson, KD, Thomes, BE, Timmons, RB, Constantinescu, A, Eberhart, RC, Bankey, PE. Enhancing hepatocyte adhesion by pulsed plasma deposition and polyethylene glycol coupling. *Tissue Engineering* 2000;6:45-52.

- [42] Iijima, S. Helical Microtubules of Graphitic Carbon. *Nature* 1991;354:56-58.
- [43] Baughman, RH, Zakhidov, AA, de Heer, WA. Carbon nanotubes - the route toward applications. *Science* 2002;297:787-792.
- [44] Arnold, MS, Green, AA, Hulvat, JF, Stupp, SI, Hersam, MC. Sorting carbon nanotubes by electronic structure using density differentiation. *Nature Nanotechnology* 2006;1:60-65.
- [45] Yang, WR, Thordarson, P, Gooding, JJ, Ringer, SP, Braet, F. Carbon nanotubes for biological and biomedical applications. *Nanotechnology* 2007;18:412001.
- [46] Harrison, BS, Atala, A. Carbon nanotube applications for tissue engineering. *Biomaterials* 2007;28:344-353.
- [47] MacDonald, RA, Laurenzi, BF, Viswanathan, G, Ajayan, PM, Stegemann, JP. Collagen-carbon nanotube composite materials as scaffolds in tissue engineering. *Journal of Biomedical Materials Research Part A* 2005;74A:489-496.
- [48] Abarrategi, A, Gutierrez, MC, Moreno-Vicente, C, Hortiguela, MJ, Ramos, V, Lopez-Lacomba, JL, Ferrer, ML, del Monte, F. Multiwall carbon nanotube scaffolds for tissue engineering purposes. *Biomaterials* 2008;29:94-102.
- [49] Shi, X, Sitharaman, B, Pham, QP, Liang, F, Wu, K, Edward Billups, W, Wilson, LJ, Mikos, AG. Fabrication of porous ultra-short single-walled carbon nanotube nanocomposite scaffolds for bone tissue engineering. *Biomaterials* 2007;28:4078-4090.
- [50] Edwards, SL, Church, JS, Werkmeister, JA, Ramshaw, JAM. Tubular micro-scale multiwalled carbon nanotube-based scaffolds for tissue engineering. *Biomaterials* 2009;30:1725-1731.
- [51] Yildirim, ED, Yin, X, Nair, K, Sun, W. Fabrication, Characterization, and Biocompatibility of Single-Walled Carbon Nanotube-Reinforced Alginate

- Composite Scaffolds Manufactured Using Freeform Fabrication Technique. Journal of Biomedical Materials Research Part B-Applied Biomaterials 2008;87B:406-414.
- [52] Lu, YL, Cheng, CM, Leduc, PR, Ho, MS. Controlling the mechanics and nanotopography of biocompatible scaffolds through dielectrophoresis with carbon nanotubes. Electrophoresis 2008;29:3123-3127.
- [53] Katz, E, Willner, I. Biomolecule-functionalized carbon nanotubes: Applications in nanobioelectronics. Chemphyschem 2004;5:1085-1104.
- [54] Kam, NWS, Dai, HJ. Carbon nanotubes as intracellular protein transporters: Generality and biological functionality. Journal of the American Chemical Society 2005;127:6021-6026.
- [55] Guo, ZJ, Sadler, PJ, Tsang, SC. Immobilization and visualization of DNA and proteins on carbon nanotubes. Advanced Materials 1998;10:701-703.
- [56] Jiang, KY, Schadler, LS, Siegel, RW, Zhang, XJ, Zhang, HF, Terrones, M. Protein immobilization on carbon nanotubes via a two-step process of diimide-activated amidation. Journal of Materials Chemistry 2004;14:37-39.
- [57] Georgakilas, V, Kordatos, K, Prato, M, Guldi, DM, Holzinger, M, Hirsch, A. Organic functionalization of carbon nanotubes. Journal of the American Chemical Society 2002;124:760-761.
- [58] Pantarotto, D, Partidos, CD, Graff, R, Hoebeke, J, Briand, JP, Prato, M, Bianco, A. Synthesis, structural characterization, and immunological properties of carbon nanotubes functionalized with peptides. Journal of the American Chemical Society 2003;125:6160-6164.
- [59] Geiger, B, Bershadsky, A, Pankov, R, Yamada, KM. Transmembrane extracellular matrix-cytoskeleton crosstalk. Nature Reviews Molecular Cell Biology 2001;2:793-805.
- [60] Clark, EA, Brugge, JS. Integrins and signal transduction pathways: the road taken. Science 1995;268:233-239.

- [61] Giancotti, FG, Ruoslahti, E. Integrin signaling. Science 1999;285:1028-1032.
- [62] Hynes, RO. Integrins: Bidirectional, allosteric signaling machines. Cell 2002;110:673-687.
- [63] Hay, ED, Cell biology of extracellular matrix (2nd Edition). 1991, New York: Plenum Press.
- [64] Eyre, DR. Collagen: Molecular diversity in the body's protein scaffold. Science 1980;207:1315-1322.
- [65] Wang, RX, Clark, RAF, Mosher, DF, Ren, XD. Fibronectin's central cell-binding domain supports focal adhesion formation and rho signal transduction. Journal of Biological Chemistry 2005;280:28803-28810.
- [66] Morla, A, Ruoslahti, E. A fibronectin self-assembly site involved in fibronectin matrix assembly: Reconstruction in a synthetic peptide. Journal of Cell Biology 1992;118:421-429.
- [67] Morla, A, Zhang, ZH, Ruoslahti, E. Superfibronectin is a functionally distinct form of fibronectin. Nature 1994;367:193-196.
- [68] Yi, M, Ruoslahti, E. A fibronectin fragment inhibits tumor growth, angiogenesis, and metastasis. Proceedings of the National Academy of Sciences of the United States of America 2001;98:620-624.
- [69] Luo, BH, Carman, CV, Springer, TA. Structural basis of integrin regulation and signaling. Annual Review of Immunology 2007;25:619-647.
- [70] Hwang, DS, Sim, SB, Cha, HJ. Cell adhesion biomaterial based on mussel adhesive protein fused with RGD peptide. Biomaterials 2007;28:4039-4046.
- [71] Shin, H, Jo, S, Mikos, AG. Biomimetic materials for tissue engineering. Biomaterials 2003;24:4353-4364.
- [72] Anselme, K. Osteoblast adhesion on biomaterials. Biomaterials 2000;21:667-681.
- [73] Xu, Y, Gurusiddappa, S, Rich, RL, Owens, RT, Keene, DR, Mayne, R,

- Hook, A, Hook, M. Multiple binding sites in collagen type I for the integrins $\alpha(1)\beta(1)$ and $\alpha(2)\beta(1)$. *Journal of Biological Chemistry* 2000;275:38981-38989.
- [74] Yamada, KM, Miyamoto, S. Integrin transmembrane signaling and cytoskeletal control. *Current Opinion in Cell Biology* 1995;7:681-689.
- [75] Price, LS, Leng, J, Schwartz, MA, Bokoch, GM. Activation of Rac and Cdc42 by integrins mediates cell spreading. *Molecular Biology of the Cell* 1998;9:1863-1871.
- [76] Hall, A. Rho GTPases and the actin cytoskeleton. *Science* 1998;279:509-514.
- [77] Burridge, K, Chrzanowska-Wodnicka, M, Zhong, CL. Focal adhesion assembly. *Trends in Cell Biology* 1997;7:342-347.
- [78] Zhang, ZH, Vuori, K, Wang, HG, Reed, JC, Ruoslahti, E. Integrin activation by R-ras. *Cell* 1996;85:61-69.
- [79] Reedquist, KA, Ross, E, Koop, EA, Wolthuis, RMF, Zwartkruis, FJT, van Kooyk, Y, Salmon, M, Buckley, CD, Bos, JL. The small GTPase, Rap1, mediates CD31-induced integrin adhesion. *Journal of Cell Biology* 2000;148:1151-1158.
- [80] Miyamoto, S, Teramoto, H, Coso, OA, Gutkind, JS, Burbelo, PD, Akiyama, SK, Yamada, KM. Integrin function: Molecular hierarchies of cytoskeletal and signaling molecules. *Journal of Cell Biology* 1995;131:791-805.
- [81] Schlaepfer, DD, Hunter, T. Integrin signalling and tyrosine phosphorylation: just the FAKs? *Trends in Cell Biology* 1998;8:151-157.
- [82] Aplin, AE, Howe, AK, Juliano, RL. Cell adhesion molecules, signal transduction and cell growth. *Current Opinion in Cell Biology* 1999;11:737-744.
- [83] Boudreau, N, Bissell, MJ. Extracellular matrix signaling: integration of form and function in normal and malignant cells. *Current Opinion in Cell Biology* 1998;10:640-646.

- [84] Hammer, DA, Lauffenburger, DA. A dynamic model for receptor-mediated cell adhesion to surfaces *Biophysical Journal* 1987;52:475-487.
- [85] Lotz, MM, Burdsal, CA, Erickson, HP, McClay, DR. Cell adhesion to fibronectin and tenascin: Quantitative measurements of initial binding and subsequent strengthening response. *Journal of Cell Biology* 1989;109:1795-1805.
- [86] Ward, MD, Hammer, DA. A theoretical analysis for the effect of focal contact formation on cell-substrate attachment strength. *Biophysical Journal* 1993;64:936-959.
- [87] Gallant, ND, Garcia, AJ. Model of integrin-mediated cell adhesion strengthening. *Journal of Biomechanics* 2007;40:1301-1309.
- [88] Kong, D, Ji, BH, Dai, LH. Nonlinear mechanical modeling of cell adhesion. *Journal of Theoretical Biology* 2008;250:75-84.
- [89] Bell, GI. Models for specific adhesion of cells to cells. *Science* 1978;200:618-627.
- [90] Gallant, ND, Michael, KE, Garcia, AJ. Cell adhesion strengthening: Contributions of adhesive area, integrin binding, and focal adhesion assembly. *Molecular Biology of the Cell* 2005;16:4329-4340.
- [91] Fang, N, Chan, V, Wan, KT, Mao, HQ, Leong, KW. Colloidal adhesion of phospholipid vesicles: high-resolution reflection interference contrast microscopy and theory. *Colloids and Surfaces B-Biointerfaces* 2002;25:347-362.
- [92] Wan, KT, Liu, KK. Contact mechanics of a thin-walled capsule adhered onto a rigid planar substrate. *Medical & Biological Engineering & Computing* 2001;39:605-608.
- [93] Yin, C, Liao, K, Mao, HQ, Leong, KW, Zhuo, RX, Chan, V. Adhesion contact dynamics of HepG2 cells on galactose-immobilized substrates. *Biomaterials* 2003;24:837-850.
- [94] Zhu, AP, Fang, N. Adhesion dynamics, morphology, and organization of

- 3T3 fibroblast on chitosan and its derivative: The effect of O-carboxymethylation. *Biomacromolecules* 2005;6:2607-2614.
- [95] Fang, N, Zhu, AP, Chan-Park, MB, Chan, V. Adhesion contact dynamics of fibroblasts on biomacromolecular surfaces. *Macromolecular Bioscience* 2005;5:1022-1031.
- [96] Feng, Z, Chian, KS, Ong, WF, Mhaisalka, PS, Chan, V, Ratner, BD. Dual requirements of extracellular matrix protein and chitosan for inducing adhesion contact evolution of esophageal epithelia. *Journal of Biomedical Materials Research Part A* 2007;82A:788-801.
- [97] Feng, ZQ, Chen, WN, Lee, PVS, Liao, K, Chan, V. The influence of GFP-actin expression on the adhesion dynamics of HepG2 cells on a model extracellular matrix. *Biomaterials* 2005;26:5348-5358.
- [98] He, T, Shi, ZL, Fang, N, Neoh, KG, Kang, ET, Chan, V. The effect of adhesive ligands on bacterial and fibroblast adhesions to surfaces. *Biomaterials* 2009;30:317-326.
- [99] Missirlis, YF, Spiliotis, AD. Assessment of techniques used in calculating cell-material interactions. *Biomolecular Engineering* 2002;19:287-294.
- [100] Hochmuth, RM. Micropipette aspiration of living cells. *Journal of Biomechanics* 2000;33:15-22.
- [101] Lim, CT, Zhou, EH, Li, A, Vedula, SRK, Fu, HX. Experimental techniques for single cell and single molecule biomechanics. *Materials Science and Engineering: C* 2006;26:1278-1288.
- [102] Shao, JY, Hochmuth, RM. Micropipette suction for measuring piconewton forces of adhesion and tether formation from neutrophil membranes. *Biophysical Journal* 1996;71:2892-2901.
- [103] Shao, JY, Hochmuth, RM. Mechanical anchoring strength of L-selectin, beta(2) integrins, and CD45 to neutrophil cytoskeleton and membrane. *Biophysical Journal* 1999;77:587-596.
- [104] Sung, KLP, Kwan, MK, Maldonado, F, Akeson, WH. Adhesion strength of

- human ligament fibroblasts. Journal of Biomechanical Engineering-Transactions of the ASME 1994;116:237-242.
- [105] Sung, KLP, Steele, LL, Whittermore, D, Hagan, J, Akeson, WH. Adhesiveness of human ligament fibroblasts to laminin. Journal of Orthopaedic Research 1995;13:166-173.
- [106] Sung, KLP, Yang, L, Whittermore, DE, Shi, Y, Jin, G, Hsieh, AH, Akeson, WH, Sung, LA. The differential adhesion forces of anterior cruciate and medial collateral ligament fibroblasts: Effects of tropomodulin, talin, vinculin, and alpha-actinin. Proceedings of the National Academy of Sciences of the United States of America 1996;93:9182-9187.
- [107] Yang, L, Tsai, CMH, Hsieh, AH, Lin, VS, Akeson, WH, Sung, KLP. Adhesion strength differential of human ligament fibroblasts to collagen types I and III. Journal of Orthopaedic Research 1999;17:755-762.
- [108] Qin, TW, Yang, ZM, Wu, ZZ, Xie, HQ, Qin, H, Cai, SX. Adhesion strength of human tenocytes to extracellular matrix component-modified poly(DL-lactide-co-glycolide) substrates. Biomaterials 2005;26:6635-6642.
- [109] Axelrod, D, Total internal reflection fluorescence microscopy. 2007: Springer Berlin / Heidelberg. p. 195-236.
- [110] Axelrod, D. Cell-substrate contacts illuminated by total internal reflection fluorescence. Journal of Cell Biology 1981;89:141-145.
- [111] Guignandon, A, Lafage-Proust, MH, Usson, Y, Laroche, N, Caillot-Augusseau, A, Alexandre, C, Vico, L. Cell cycling determines integrin-mediated adhesion in osteoblastic ROS 17/2.8 cells exposed to space-related conditions. FASEB Journal 2001;15:2036-2038.
- [112] Senju, Y, Miyata, H. The Role of Actomyosin Contractility in the Formation and Dynamics of Actin Bundles During Fibroblast Spreading. Journal of Biochemistry 2009;145:137-150.
- [113] Iino, R, Koyama, I, Kusumi, A. Single molecule imaging of green

- fluorescent proteins in living cells: E-cadherin forms oligomers on the free cell surface. *Biophysical Journal* 2001;80:2667-2677.
- [114] Slack-Davis, JK, Martin, KH, Tilghman, RW, Iwanicki, M, Ung, EJ, Autry, C, Luzzio, MJ, Cooper, B, Kath, JC, Roberts, WG, Parsons, JT. Cellular characterization of a novel focal adhesion kinase inhibitor. *Journal of Biological Chemistry* 2007;282:14845-14852.
- [115] Zhu, YB, Chian, KS, Chan-Park, MB, Mhaisalkar, PS, Ratner, BD. Protein bonding on biodegradable poly(L-lactide-co-caprolactone) membrane for esophageal tissue engineering. *Biomaterials* 2006;27:68-78.
- [116] Kumar, S, Kumar, R, Jindal, VK, Bharadwaj, LM. Immobilization of single walled carbon nanotubes on glass surface. *Materials Letters* 2008;62:731-734.
- [117] Rotsch, C, Jacobson, K, Radmacher, M. Dimensional and mechanical dynamics of active and stable edges in motile fibroblasts investigated by using atomic force microscopy. *Proceedings of the National Academy of Sciences of the United States of America* 1999;96:921-926.
- [118] Kim, YJ, Park, S, Lee, YJ, Shin, JW, Kim, DH, Heo, SJ, Park, KD. Effects of intermittent hydrostatic pressure on cell adhesive forces and other related parameters under various resting periods. *Journal of Biomedical Materials Research Part B-Applied Biomaterials* 2008;85B:353-360.
- [119] Allen, LT, Tosetto, M, Miller, IS, O'Connor, DP, Penney, SC, Lynch, I, Keenan, AK, Pennington, SR, Dawson, KA, Gallagher, WM. Surface-induced changes in protein adsorption and implications for cellular phenotypic responses to surface interaction. *Biomaterials* 2006;27:3096-3108.
- [120] Parkin, DM, Bray, F, Ferlay, J, Pisani, P. Estimating the world cancer burden: GLOBOCAN 2000. *International Journal of Cancer* 2001;94:153-156.
- [121] Griffin, SM, Lamb, P. Oesophageal cancer. *Surgery* (Oxford)

2006;24:97-100.

- [122] Wu, PC, Posner, MC. The role of surgery in the management of oesophageal cancer. *Lancet Oncology* 2003;4:481-488.
- [123] Beckstead, BL, Pan, S, Bhrany, AD, Bratt-Leal, AM, Ratner, BD, Giachelli, CM. Esophageal epithelial cell interaction with synthetic and natural scaffolds for tissue engineering. *Biomaterials* 2005;26:6217-6228.
- [124] Fuchs, JR, Nasser, BA, Vacanti, JP. Tissue engineering: A 21st century solution to surgical reconstruction. *Annals of Thoracic Surgery* 2001;72:577-591.
- [125] Ratner, BD, Bryant, SJ. Biomaterials: Where we have been and where we are going. *Annual Review of Biomedical Engineering* 2004;6:41-75.
- [126] Goodman, SL, Cooper, SL, Albrecht, RM. Integrin receptors and platelet adhesion to synthetic surfaces. *Journal of Biomedical Materials Research* 1993;27:683-695.
- [127] Schoenwaelder, SM, Burridge, K. Bidirectional signaling between the cytoskeleton and integrins. *Current Opinion in Cell Biology* 1999;11:274-286.
- [128] Heino, J. The collagen receptor integrins have distinct ligand recognition and signaling functions. *Matrix Biology* 2000;19:319-323.
- [129] Danen, EHJ, Yamada, KM. Fibronectin, integrins, and growth control. *Journal of Cellular Physiology* 2001;189:1-13.
- [130] Aota, S, Nomizu, M, Yamada, KM. The short amino acid sequence Pro-His-Ser-Arg-Asn in human fibronectin enhances cell-adhesive function. *Journal of Biological Chemistry* 1994;269:24756-24761.
- [131] Bowditch, RD, Hariharan, M, Tominna, EF, Smith, JW, Yamada, KM, Getzoff, ED, Ginsberg, MH. Identification of a novel integrin binding site in fibronectin - Differential utilization by beta-3 integrins. *Journal of Biological Chemistry* 1994;269:10856-10863.
- [132] Langenbach, KJ, T Elliott, J, Tona, A, McDaniel, D, Plant, AL. Thin films

- p of Type I collagen for cell by cell analysis of morphology and tenascin-C promoter activity.
- BMC Biotechnology*
- 2006;6:14.
- [133] Ivanov, IB, Hadjiiski, A, Denkov, ND, Gurkov, TD, Kralchevsky, PA, Koyasu, S. Energy of adhesion of human T cells to adsorption layers of monoclonal antibodies measured by a film trapping technique. *Biophysical Journal* 1998;75:545-556.
 - [134] Werner, C. Cell adhesion to polymer substrates characterized by the micropipette aspiration technique. *Journal of Adhesion* 2004;80:895-911.
 - [135] Pierrat, S, Brochard-Wyart, F, Nassoy, P. Enforced detachment of red blood cells adhering to surfaces: Statics and dynamics. *Biophysical Journal* 2004;87:2855-2869.
 - [136] de Gennes, PG, Puech, PH, Brochard-Wyart, F. Adhesion induced by mobile stickers: A list of scenarios. *Langmuir* 2003;19:7112-7119.
 - [137] Welch, MD, Mallavarapu, A, Rosenblatt, J, Mitchison, TJ. Actin dynamics in vivo. *Current Opinion in Cell Biology* 1997;9:54-61.
 - [138] Chen, CS, Alonso, JL, Ostuni, E, Whitesides, GM, Ingber, DE. Cell shape provides global control of focal adhesion assembly. *Biochemical and Biophysical Research Communications* 2003;307:355-361.
 - [139] Chen, CS, Mrksich, M, Huang, S, Whitesides, GM, Ingber, DE. Geometric control of cell life and death. *Science* 1997;276:1425-1428.
 - [140] McBeath, R, Pirone, DM, Nelson, CM, Bhadriraju, K, Chen, CS. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. *Developmental Cell* 2004;6:483-495.
 - [141] Veetil, JV, Ye, KM. Tailored carbon nanotubes for tissue engineering applications. *Biotechnology Progress* 2009;25:709-721.
 - [142] Jia, G, Wang, HF, Yan, L, Wang, X, Pei, RJ, Yan, T, Zhao, YL, Guo, XB. Cytotoxicity of carbon nanomaterials: Single-wall nanotube, multi-wall nanotube, and fullerene. *Environmental Science & Technology* 2005;39:1378-1383.

- [143] Lam, CW, James, JT, McCluskey, R, Arepalli, S, Hunter, RL. A review of carbon nanotube toxicity and assessment of potential occupational and environmental health risks. *Critical Reviews in Toxicology* 2006;36:189-217.
- [144] Cui, DX, Tian, FR, Ozkan, CS, Wang, M, Gao, HJ. Effect of single wall carbon nanotubes on human HEK293 cells. *Toxicology Letters* 2005;155:73-85.
- [145] Soto, K, Garza, KM, Murr, LE. Cytotoxic effects of aggregated nanomaterials. *Acta Biomaterialia* 2007;3:351-358.
- [146] Smart, SK, Cassady, AI, Lu, GQ, Martin, DJ. The biocompatibility of carbon nanotubes. *Carbon* 2006;44:1034-1047.
- [147] Mattson, MP, Haddon, RC, Rao, AM. Molecular functionalization of carbon nanotubes and use as substrates for neuronal growth. *Journal of Molecular Neuroscience* 2000;14:175-182.
- [148] Liu, CZ, Czernuszka, JT. Development of biodegradable scaffolds for tissue engineering: a perspective on emerging technology. *Materials Science and Technology* 2007;23:379-391.
- [149] Lin, Y, Taylor, S, Li, HP, Fernando, KAS, Qu, LW, Wang, W, Gu, LR, Zhou, B, Sun, YP. Advances toward bioapplications of carbon nanotubes. *Journal of Materials Chemistry* 2004;14:527-541.
- [150] Harnett, EM, Alderman, J, Wood, T. The surface energy of various biomaterials coated with adhesion molecules used in cell culture. *Colloids and Surfaces B-Biointerfaces* 2007;55:90-97.
- [151] Murphy-Ullrich, JE. The de-adhesive activity of matricellular proteins: is intermediate cell adhesion an adaptive state? *Journal of Clinical Investigation* 2001;107:785-790.
- [152] Moy, VT, Jiao, YK, Hillmann, T, Lehmann, H, Sano, T. Adhesion energy of receptor-mediated interaction measured by elastic deformation. *Biophysical Journal* 1999;76:1632-1638.

- [153] Lebaron, RG, Athanasiou, KA. Extracellular matrix cell adhesion peptides: Functional applications in orthopedic materials. *Tissue Engineering* 2000;6:85-103.
- [154] Huang, S, Ingber, DE. Shape-dependent control of cell growth, differentiation, and apoptosis: Switching between attractors in cell regulatory networks. *Experimental Cell Research* 2000;261:91-103.
- [155] Huang, S, Ingber, DE. The structural and mechanical complexity of cell-growth control. *Nature Cell Biology* 1999;1:E131-E138.
- [156] Mwenifumbo, S, Shaffer, MS, Stevens, MM. Exploring cellular behaviour with multi-walled carbon nanotube constructs. *Journal of Materials Chemistry* 2007;17:1894-1902.
- [157] Lotz, MM, Burdsal, CA, Erickson, HP, McClay, DR. Cell adhesion to fibronectin and tenascin: Quantitative measurements of initial binding and subsequent strengthening response. *Journal of Cell Biology* 1989;109:1795-1805.
- [158] Carragher, NO, Frame, MC. Focal adhesion and actin dynamics: A place where kinases and proteases meet to promote invasion. *Trends in Cell Biology* 2004;14:241-249.
- [159] Geiger, B, Bershadsky, A. Exploring the neighborhood: Adhesion-coupled cell mechanosensors. *Cell* 2002;110:139-142.
- [160] Geiger, B, Spatz, JP, Bershadsky, AD. Environmental sensing through focal adhesions. *Nature Reviews Molecular Cell Biology* 2009;10:21-33.
- [161] Griffin, MA, Engler, AJ, Barber, TA, Healy, KE, Sweeney, HL, Discher, DE. Patterning, prestress, and peeling dynamics of myocytes. *Biophysical Journal* 2004;86:1209-1222.
- [162] Johansson, S, Svineng, G, Wennerberg, K, Armulik, A, Lohikangas, L. Fibronectin-integrin interactions. *Frontiers in Bioscience* 1997;2:d126-146.
- [163] Al-Jamal, R, Harrison, DJ. Beta1 integrin in tissue remodelling and repair:

- From phenomena to concepts. *Pharmacology & Therapeutics* 2008;120:81-101.
- [164] Bershadsky, AD, Balaban, NQ, Geiger, B. Adhesion-dependent cell mechanosensitivity. *Annual Review of Cell and Developmental Biology* 2003;19:677-695.
- [165] Andressen, C, Arnhold, S, Puschmann, M, Bloch, W, Hescheler, J, Fassler, R, Addicks, K. Beta 1 integrin deficiency impairs migration and differentiation of mouse embryonic stem cell derived neurons. *Neuroscience Letters* 1998;251:165-168.
- [166] Grose, R, Hutter, C, Bloch, W, Thorey, I, Watt, FM, Fassler, R, Brakebusch, C, Werner, S. A crucial role of beta 1 integrins for keratinocyte migration in vitro and during cutaneous wound repair. *Development* 2002;129:2303-2315.
- [167] Watt, FM. Role of integrins in regulating epidermal adhesion, growth and differentiation. *EMBO Journal* 2002;21:3919-3926.
- [168] Mettouchi, A, Meneguzzi, G. Distinct roles of beta 1 integrins during angiogenesis. *European Journal of Cell Biology* 2005;85:243-247.
- [169] Vallieres, K, Chevallier, P, Sarra-Bournett, C, Turgeon, S, Laroche, G. AFM Imaging of immobilized fibronectin: Does the surface conjugation scheme affect the protein orientation/conformation? *Langmuir* 2007;23:9745-9751.
- [170] Grainger, DW, Pavon-Djavid, G, Migonney, V, Josefowicz, M. Assessment of fibronectin conformation adsorbed to polytetrafluoroethylene surfaces from serum protein mixtures and correlation to support of cell attachment in culture. *Journal of Biomaterials Science-Polymer Edition* 2003;14:973-988.
- [171] Lee, JW, Kim, YH, Park, KD, Jee, KS, Shin, JW, Hahn, SB. Importance of integrin beta 1-mediated cell adhesion on biodegradable polymers under serum depletion in mesenchymal stem cells and chondrocytes.

Biomaterials 2004;25:1901-1909.

- [172] Siebers, MC, Walboomers, XF, van den Dolder, J, Leeuwenburgh, SCG, Wolke, JGC, Jansen, JA. The behavior of osteoblast-like cells on various substrates with functional blocking of integrin-beta 1 and integrin-beta 3. Journal of Materials Science-Materials in Medicine 2008;19:861-868.
- [173] Yamamoto, K, Tomita, N, Fukuda, Y, Suzuki, S, Igarashi, N, Suguro, T, Tamada, Y. Time-dependent changes in adhesive force between chondrocytes and silk fibroin substrate. Biomaterials 2007;28:1838-1846.
- [174] Lee, MC, Sung, KLP, Kurtis, MS, Akeson, WH, Sah, RL. Adhesive force of chondrocytes to cartilage - Effects of chondroitinase ABC. Clinical Orthopaedics and Related Research 2000:286-294.
- [175] Huang, W, Anvari, B, Torres, JH, LeBaron, RG, Athanasiou, KA. Temporal effects of cell adhesion on mechanical characteristics of the single chondrocyte. Journal of Orthopaedic Research 2003;21:88-95.
- [176] Schinagl, RM, Kurtis, MS, Ellis, KD, Chien, S, Sah, RL. Effect of seeding duration on the strength of chondrocyte adhesion to articular cartilage. Journal of Orthopaedic Research 1999;17:121-129.
- [177] Rezaia, A, Healy, KE. Integrin subunits responsible for adhesion of human osteoblast-like cells to biomimetic peptide surfaces. Journal of Orthopaedic Research 1999;17:615-623.
- [178] Ra, HJ, Picart, C, Feng, HS, Sweeney, HL, Discher, DE. Muscle cell peeling from micropatterned collagen: direct probing of focal and molecular properties of matrix adhesion. Journal of Cell Science 1999;112:1425-1436.
- [179] Glogauer, M, Arora, P, Yao, G, Sokholov, I, Ferrier, J, McCulloch, CAG. Calcium ions and tyrosine phosphorylation interact coordinately with actin to regulate cytoprotective responses to stretching. Journal of Cell Science 1997;110:11-21.
- [180] Martinez, EJP, Lanir, Y, Einav, S. Effects of contact-induced membrane

- stiffening on platelet adhesion. *Biomechanics and Modeling in Mechanobiology* 2004;2:157-167.
- [181] Balaban, NQ, Schwarz, US, Rivelino, D, Goichberg, P, Tzur, G, Sabanay, I, Mahalu, D, Safran, S, Bershadsky, A, Addadi, L, Geiger, B. Force and focal adhesion assembly: a close relationship studied using elastic micropatterned substrates. *Nature Cell Biology* 2001;3:466-472.
- [182] Tan, JL, Tien, J, Pirone, DM, Gray, DS, Bhadriraju, K, Chen, CS. Cells lying on a bed of microneedles: An approach to isolate mechanical force. *Proceedings of the National Academy of Sciences of the United States of America* 2003;100:1484-1489.
- [183] Kuo, SC, Lauffenburger, DA. Relationship between receptor/ligand binding affinity and adhesion strength. *Biophysical Journal* 1993;65:2191-2200.
- [184] Xiao, Y, Truskey, GA. Effect of receptor-ligand affinity on the strength of endothelial cell adhesion. *Biophysical Journal* 1996;71:2869-2884.
- [185] Kurtis, MS, Schmidt, TA, Bugbee, WD, Loeser, RF, Sah, RL. Integrin-mediated adhesion of human articular chondrocytes to cartilage. *Arthritis and Rheumatism* 2003;48:110-118.
- [186] Kurtis, MS, Tu, BP, Gaya, OA, Mollenhauer, J, Knudson, W, Loeser, RF, Knudson, CB, Sah, RL. Mechanisms of chondrocyte adhesion to cartilage: role of beta 1-integrins, CD44, and annexin V. *Journal of Orthopaedic Research* 2001;19:1122-1130.
- [187] Bernard-Trifilo, JA, Kramar, EA, Torp, R, Lin, CY, Pineda, EA, Lynch, G, Gall, CM. Integrin signaling cascades are operational in adult hippocampal synapses and modulate NMDA receptor physiology. *Journal of Neurochemistry* 2005;93:834-849.
- [188] Li, N, Zhang, Y, Naylor, MJ, Schatzmann, F, Maurer, F, Wintermantel, T, Schuetz, G, Mueller, U, Streuli, CH, Hynes, NE. beta 1 integrins regulate mammary gland proliferation and maintain the integrity of mammary

- alveoli. EMBO Journal 2005;24:1942-1953.
- [189] Weibull, W. A statistical distribution function of wide applicability. Journal of Applied Mechanics-Transactions of the ASME 1951;18:293-297.
- [190] Weibull, W. A statistical theory of the strength of materials. Proceedings of Royal Swedish Institute for Engineering Research 1939;151:1-45.
- [191] Menotti, A, Puddu, PE, Lanti, M, Kromhout, D, Blackburn, H, Nissinen, A. Twenty-five-year coronary mortality trends in the seven countries study using the accelerated failure time model. European Journal of Epidemiology 2003;18:113-122.
- [192] Castillo, E, Fernandez-Canteli, A. A general regression model for lifetime evaluation and prediction. International Journal of Fracture 2001;107:117-137.
- [193] Kelly, KS, Krzysztofowicz, R. Precipitation uncertainty processor for probabilistic river stage forecasting. Water Resources Research 2000;36:2643-2653.
- [194] Pierres, A, Benoliel, AM, Bongrand, P. Studying receptor-mediated cell adhesion at the single molecule level. Cell Adhesion and Communication 1998;5:375-395.
- [195] Bearinger, JP, Castner, DG, Golledge, SL, Rezanian, A, Hubchak, S, Healy, KE. P(AAm-co-EG) interpenetrating polymer networks grafted to oxide surfaces: Surface characterization, protein adsorption, and cell detachment studies. Langmuir 1997;13:5175-5183.
- [196] Bhat, VD, Truskey, GA, Reichert, WM. Using avidin-mediated binding to enhance initial endothelial cell attachment and spreading. Journal of Biomedical Materials Research 1998;40:57-65.
- [197] Deligianni, DD, Katsala, ND, Koutsoukos, PG, Missirlis, YF. Effect of surface roughness of hydroxyapatite on human bone marrow cell adhesion, proliferation, differentiation and detachment strength. Biomaterials 2001;22:87-96.

- [198] Goldstein, AS, DiMilla, PA. Effect of adsorbed fibronectin concentration on cell adhesion and deformation under shear on hydrophobic surfaces. *Journal of Biomedical Materials Research* 2002;59:665-675.
- [199] Rezanian, A, Healy, KE. Biomimetic peptide surfaces that regulate adhesion, spreading, cytoskeletal organization, and mineralization of the matrix deposited by osteoblast-like cells. *Biotechnology Progress* 1999;15:19-32.
- [200] Rezanian, A, Thomas, CH, Healy, KE. A probabilistic approach to measure the strength of bone cell adhesion to chemically modified surfaces. *Annals of Biomedical Engineering* 1997;25:190-203.
- [201] Takamizawa, K, Shoda, K, Matsuda, T. Pull-out mechanical measurement of tissue-substrate adhesive strength: Endothelial cell monolayer sheet formed on a thermoresponsive gelatin layer. *Journal of Biomaterials Science-Polymer Edition* 2002;13:81-94.
- [202] Bhat, VD, Truskey, GA, Reichert, WM. Fibronectin and avidin-biotin as a heterogeneous ligand system for enhanced endothelial cell adhesion. *Journal of Biomedical Materials Research* 1998;41:377-385.
- [203] Rezanian, A, Thomas, CH, Branger, AB, Waters, CM, Healy, KE. The detachment strength and morphology of bone cells contacting materials modified with a peptide sequence found within bone sialoprotein. *Journal of Biomedical Materials Research* 1997;37:9-19.
- [204] Lin, CK, Berndt, CC. Measurement and analysis of adhesion strength for thermally sprayed coatings. *Journal of Thermal Spray Technology* 1994;3:75-104.
- [205] Meredith, DO, Owen, GR, ap Gwynn, I, Richards, RG. Variation in cell-substratum adhesion in relation to cell cycle phases. *Experimental Cell Research* 2004;293:58-67.
- [206] Lima, RS, Marple, BR. High Weibull modulus HVOF Titania coatings. *Journal of Thermal Spray Technology* 2003;12:240-249.
- [207] Rinne, H, The Weibull distribution: a handbook. 2009, Boca Raton: CRC

Press.

Publications

1. **Cai N**, Wong CC, Gong YX, Tan SCW, Chan V, Liao K. Modulating cell adhesion dynamics on carbon nanotube monolayer engineered with extracellular matrix proteins. *ACS Applied Materials & Interfaces*. 2010; 2:1038-1047.
2. **Cai N**, Wong CC, Tan SCW, Chan V, Liao K. Temporal effect of functional blocking of $\beta 1$ integrin on cell adhesion strength under serum depletion. *Langmuir*. 2009;25:10939-10947.
3. **Cai N**, Gong YX, Chian KS, Chan V, Liao K. Adhesion dynamics of porcine esophageal fibroblasts on extracellular matrix protein-functionalized poly(lactic acid). *Biomedical Materials* 2008;3:015014. (Featured in IOP Select and Highlights of 2008 for *Biomedical Materials*)
4. Tan SCW, Pan WX, Ma G, **Cai N**, Leong KW, Liao K. Viscoelastic behaviour of human mesenchymal stem cells. *BMC Cell Biology* 2008; 9:40.
5. Feng Z, **Cai N**, Chan V, Chian KS, Ratner BD and Liao K. Novel biophysical techniques for investigating long-term cell adhesion dynamics on biomaterial surfaces. *Advances in Experimental Medicine and Biology* 2006;585:151-165.
6. **Cai N**, Ma G, Mhaisalkar PS, Chian KS, Chan V, Liao K. Adhesion contact dynamics of porcine esophageal fibroblasts on ECM protein-Immobilized poly (lactic acid). *Proceedings of the XVth international conference on mechanics in medicine and biology*. pp.500-503, Dec 6th-8th, Singapore, 2006.
7. Pan WX, Petersen E, **Cai N**, Ma G, Lee JR, Feng ZQ, Liao K, Leong KW. Viscoelastic properties of human mesenchymal stem cells. *Journal of Biotechnology* 2005; 118:S67.