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Substrate Nanotopography and Stiffness Modulation of Cell Behaviors for Disease Detection and Modeling

Lin Shi

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Substrate Nanotopography and Stiffness Modulation of Cell Behaviors for Disease Detection and Modeling

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**Dissertation submitted to the
Benjamin M. Statler College of Engineering and Mineral Resources
at West Virginia University**

in partial fulfillment of the requirements for the degree of

**Doctor of Philosophy in
the Chemical and Biomedical Engineering Department**

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2017

Keywords: Stiffness, Nanotopography, Circulating tumor cells, Carbon nanotubes, Lung fibrosis

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ABSTRACT

Substrate Nanotopography and Stiffness Modulation of Cell Behaviors for Disease Detection and Modeling

Lin Shi

In most living tissue, cells resident in a complex microenvironment where these cells interact with the extracellular matrix (ECM) and the neighboring cells. The interactions between cells and ECM could regulate the cell behavior. Similar to *in vivo*, *in vitro* models have been reported that cells have the ability to sense the nanotopography and stiffness of synthetic substrate and, the variation of substrate nanotopography configuration and stiffness could affect cell phenotype and function.

The first project focused on applying nanotopography to capture circulating tumor cells (CTCs) for early cancer detection. CTCs shed from primary tumors, transport through the bloodstream to distant sites, and cause 90% of cancer deaths. Although different techniques have been developed to isolate CTCs for cancer detection, diagnosis and treatment, the heterogeneity of expression of the target antigen and the significant size variance in CTCs limit clinical applications of antibody- and size-based isolation techniques. Cell adhesion using nanotopography has been suggested as a promising approach to isolate CTCs independent of surface marker expression or size of CTCs. However, the influence of nanotopography configuration (geometry and dimensions) on CTC capture efficiency has not been investigated. This study examined capture performance of several cancer cell lines of different types, surface marker expression and

metastatic status on nanotopographies of various geometry and dimensions without antibody conjugation. Compared with flat surfaces and isotropic, discrete nanopillars, anisotropic nanogratings favored cancer cell adhesion, thus improving the capture efficiency. This study provides useful information to optimize nanotopography to further enhance CTC capture efficiency.

The second project focused on understanding the effects of substrate stiffness on fibrogenic responses of human lung fibroblasts to engineered nanomaterials. Most existing *in vitro* models focused on conducting the experiment using the rigid tissue culture polystyrene (TCPS) surfaces, which were much stiffer than the actual *in vivo* cell microenvironment. Thus, the behavior and nanomaterial responsiveness of cells could be largely changed due to the deviation of substrate stiffness when cultured on TCPS. Therefore, it is of the critical need to create physiologically relevant tissue models to mimic the *in vivo* environment by introducing stiffness cue. This study used the synthesized polyacrylamide (PAAm) hydrogel to represent the normal and fibrotic conditions of lung tissues to conduct *in vitro* models. The fibrogenic responses and mechanosensing of fibroblasts to carbon nanotubes (CNTs) at different stiffness conditions have been explored. This study provides understanding of the regulatory pathways and mediators of fibrogenic activities, which will potentially help identify therapeutic targets against fibrosis.

The incorporation of substrate nanotopography and stiffness could be further applied in three-dimensional culture model as the difference in dimensionality could also substantially change the cell behavior and function. Cells under *in vivo* conditions are embedded in multiple ECM components and experiencing different biophysical stimuli compared to those cultured on top of the two-dimensional substrate. Moreover, by introducing flow and shear stress into the *in vivo* system could largely replicate the *in vivo* condition, like the ultimate organ-on-a-chip

microfluidic device. This study provides insight on building physiologically relevant *in vitro* model for disease detection and modeling and could be future applied in drug development and disease treatment.

Dedication

To my parents, Limei Zhang and Shigang Shi

To my husband, Kai Wang and our son Alex M. Wang who is about to be born

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Table of Contents

ABSTRACT.....	ii
Dedication	v
Acknowledgement.....	vi
Table of Contents	ix
List of Figures	xi
List of Tables	xvi
List of Acronyms.....	xviii
References.....	xxii
Chapter 1: Introduction	1
References.....	16
Chapter 2: Incorporating Nanotopography for Disease Detection – Adhesion-based Circulating Tumor Cell Capture.....	28
Abstract	29
Introduction.....	30
Materials and Methods.....	33
Results and Discussion	37
Conclusion	49
References.....	51

Chapter 3: Incorporating Substrate Stiffness for Disease Modeling – *In Vitro* Model of Cell Sensing Nanomaterials.....59

Abstract.....	59
Introduction.....	61
Materials and Methods.....	68
Results and Discussion	79
Conclusion	105
References.....	107

Chapter 4: Path Forward.....120

References.....	130

List of Figures

Chapter 1: Introduction

Figure 1-1: The biophysical cues that exist in typical human tissues.¹

Figure 1-2: hMSCs differentiation directed by pre-culturing them on TCPS for different days.²

Figure 1-3: Nanotopographical features exhibited in various tissues.³

Figure 1-4: Classification of nanotopographies according to the shape.⁴

Chapter 2: Incorporating Nanotopography for Disease Detection – Adhesion-based Circulating Tumor Cell Capture

Figure 2-1: SEM micrographs of the PDMS nanotopographies and the flat substrate.

Figure 2-2: MCF7 cell capture on the nanotopographies.

Figure 2-3: (A) Fluorescent images of captured MCF7 cells on the nanotopographies and flat surfaces. (B) Cell area measurement of captured MCF7 cells on the nanotopographies and flat surfaces.

Figure 2-4: SEM micrographs of MCF7 cells adhered to the nanotopographies and flat surface.

Figure 2-5: Capture efficiency of MCF7, A549, MDA-MB-231 and HeLa cells after 4 h of incubation on the nanotopographies and flat surfaces.

Figure 2-6: SEM micrographs of (A) A549 and (B) HeLa cells on the nanotopographies and flat surfaces.

Chapter 3: Incorporating Substrate Stiffness for Disease Modeling – *In Vitro* Model of Cell Sensing Nanomaterials

Figure 3-1: Cell behaviors regulated by the variation of matrix mechanics.⁵

Figure 3-2: Cell contractility, motility and spreading influenced by the mechanical properties of the substrate.⁶

Figure 3-3: Effect of substrate stiffness on cell elongation and polarization.⁷

Figure 3-4: Schematic of the assembly of sandwich gels.⁸

Figure 3-5: Cell morphologies of NHLFs on different PAAm gel substrates (3 kPa, 100 kPa) and TCPS.

Figure 3-6: Determination of MWCNTs dosage by cell proliferation assay on TCPS.

Figure 3-7: Dose-dependent collagen production of fibroblasts treated with MWCNTs.⁹

Figure 3-8: Immunofluorescent staining of F-actin, α -SMA and nuclei of NHLFs on different substrates: 3 kPa, 100 kPa and TCPS.

Figure 3-9: MWCNTs effects on nuclei.

Figure 3-10: Cell proliferation of NHLFs on different PAAm gel substrates and TCPS control.

Figure 3-11: Collagen I expression of NHLFs treated with or without 0.2 $\mu\text{g}/\text{cm}^2$ MWCNTs.

Figure 3-12: The influence of ROS generation of NHLFs on substrate stiffness in response to MWCNTs.

Figure 3-13: Protein expression of NHLFs treated with or without $0.2 \mu\text{g}/\text{cm}^2$ MWCNTs.

Figure 3-14: Bright field images of cells treated with different dosages of Y27632.

Figure 3-15: Images of NHLFs cell morphologies treated with different dosages of Y27632.

Figure 3-16: Collagen I expression of NHLFs exposed to $0.2 \mu\text{g}/\text{cm}^2$ MWCNTs when treated with different dosages of Y27632 on TCPS.

Figure 3-17: Collagen I expression of NHLFs exposed to $0.2 \mu\text{g}/\text{cm}^2$ MWCNTs and (5 + 5) μM Y27632 on 100 kPa PAAm gel substrates.

Figure 3-18: Collagen I expression of NHLFs exposed to $0.2 \mu\text{g}/\text{cm}^2$ MWCNTs and (5 + 5) μM Y27632 on 100 kPa PDMS gel substrates.

Chapter 4: Path Forward

Figure 4-1: RIE generated nanoroughened surface for cancer cell capture.¹⁰

Figure 4-2: Nanoroughened surfaces incorporated microfluidic device for cancer cell capture.¹¹

Figure 4-3: The difference between 2D culture (collagen-coated glass) and 3D culture (collagen gel) in regard to adhesion, polarity, spreading, matrix distribution and matrix stiffness.¹²

Figure 4-4: “Organ-on-a-chip” microfluidic devices that were fabricated to mimic tissue microenvironment for different disease models.¹³

List of Tables

Chapter 1: Introduction

Table 1-1: Different materials and their stiffness as in Young's modulus.

Chapter 3: Incorporating Substrate Stiffness for Disease Modeling – *In Vitro* Model of Cell Sensing Nanomaterials

Table 3-1: Polyacrylamide Young's modulus table.

Table 3-2: The administrative dosage adding pattern for Y27632 treatment.

List of Acronyms

Abbreviation	Meaning
3-APTMS	(3-Aminopropyl)trimethoxysilan
A549	An adenocarcinomic basal epithelial cell line
AFM	Atomic Force Microscope
ALP	Alkaline Phosphatase
ANOVA	Analysis of Variance
APS	Ammonium Persulfate
ATCC	American Type Culture Collection
BCMn	Block Copolymer Micelle Nanolithography
BSA	Bovine Serum Albumin
CNTs	Carbon Nanotubes
CPPs	Cell Penetrating Peptides
CTCs	Circulating Tumor Cells
DAPI	4,6-diamidino-2-phenylindole
DCFDA	Dichlorodihydrofluorescein Diacetate
DI	Deionized
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide

EBL	Electron Beam Lithography
ECM	Extracellular Matrix
EpCAM	Epithelial Cell Adhesion Molecule
EPCs	Endothelial Progenitor Cells
FAs	Focal Adhesions
FAK	Focal Adhesion Kinase
F-actin	Filamentous Actin
FBM	Fibroblast Basal Medium
FBS	Fetal Bovine Serum
HBSS	Hank's Balanced Salt Solution
HeLa	A cervical cancer cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFFs	Human Foreskin Fibroblasts
HMSCs	Human Mesenchymal Stem Cells
IMCD	Inner Medullary Collecting Duct
MCF7	A luminal non-metastatic breast cancer cell line
MDA-MB-231	A basal aggressive metastatic breast cancer cell line
MEM	Minimum Essential Media
MSCs	Mesenchymal Stem Cells

MWCNTs	Multi-Wall Carbon Nanotubes
NGs	Nanogratings
NHLF	Normal Human Lung Fibroblasts
NHS	N-hydroxysuccinimide
NIH	National Institutes of Health
NPs	Nanopillars
OCN	Osteocalcin
PBS	Phosphate Buffer Saline
PDMS	Polydimethylsiloxane
PEG	Poly(ethylene glycol)
PFA	Paraformaldehyde
pFAK	Phosphorylated Tyrosine-397 FAK
PMMA	Poly(methylmethacrylate)
PPARγ	Peroxisome Proliferator-activated Receptor Gamma
PS	Polystyrene
PVDF	Polyvinylidene Difluoride
RIE	Reactive Ion Etching
RIPA	Radioimmune Precipitation Assay
ROCK	Rho-associated Protein Kinase

ROI	Region of Interest
ROS	Reactive Oxygen Species
S.E.M.	Standard Error of Mean
SDS-PAGE	Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscope
α-SMA	Alpha-Smooth Muscle Actin
Sulfo-SANPAH	Sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate
TBST	1X Tris-Buffered Saline With 0.1% Tween-20
TCPS	Tissue Culture Polystyrene
TEMED	N,N,N',N'-Tetramethylethylenediamine
VICs	Valvular Interstitial Cells
Y27632	Trans-4-[(1R)-1-Aminoethyl]-N-4-pyridinylcyclohexanecarboxamide Dihydrochloride

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Chapter 1: Introduction

The nature of *in vivo* condition

The nature of *in vivo* environment where cells typically resident in is consist of various topographical structures and different degrees of stiffness. In the cell microenvironment, the ECM plays a significant role in regulating the phenotype and function of cells.¹ The role of this dynamic structure is not only to provide solid support surrounding the cells, but also transduce physical signals to cells, thus regulating cell phenotype and function. The composition of ECM was identified years ago,^{1,2,3} and the important role of ECM has been more and more realized recently. The complex network of ECM is formed by a diversity of macromolecules secreted by cells, including different types of collagen, proteoglycans, glycoproteins, extracellular vesicles and many other structures, result in serious diversification in ECM structure.^{1,4,5,6,7,8,9,10} Tissues that come from different organs may contain different ECM structure. Such diversity and variation are a natural selection that would satisfy the different biological related function requirement from different organ systems. For example, the extracellular components in the alveolar region of the lung include both noncellular interstitium and basement membranes.¹¹ Most basement membranes are consist of proteins like type IV collagen, entactin and laminin, as well as proteoglycans.¹² However, the constituents of lung interstitium are fibrillar collagens, elastic fibers, and proteoglycans.^{11,13,14}

Typically, the various macromolecules present in ECM often exhibit three-dimensional nanotopography, as shown in Figure 1-1 (a).¹⁵ Furthermore, the differential composition of ECM structure in different organs results in various stiffness, as shown in Figure 1-1 (b).¹⁵ These biophysical cues, including nanotopographical cue and stiffness cue, are highly organized within

the ECM¹, where the regulation of ECM allows cells to exhibit different phenotypes and functions.¹⁵

Figure 1-1: The biophysical cues that exist in typical human tissues. (a) Illustration of the three-dimensional nanotopographical structures in different human tissues. The enlarged box shows SEM images of the nanostructures in bone, nerve, skin and alveolar interstitium. (b) Different human tissues fall into a wide range of stiffness from 10 Pa to over 1 GPa (Adapted with permission from Ref. [15])

Growing and compelling evidence has shown that other than the chemical cues that can provide modulations to cells, the mechanical properties of these physical cues (topography and rigidity) plays significant roles in directing the cell fate, cell elongation, motility, migration and differentiation, thus have an impact on various ongoing process *in vivo*.^{16, 17, 18, 19, 20} As for topography cue, for example, the nervous system is composed of a complex network of three-dimensional topographical structures, which covers a broad range of size for each component.¹⁶ In

in vivo studies have suggested that some of the topographical features guide cell migration during the development of cerebral cortex²¹ and cerebellum²². However, since there are constant alterations in chemical composition that intrinsically present in the actual *in vivo* condition, it is difficult to interpret the cell behavior by topographical guidance exclusively. An increasing number of *in vitro* models have been established using engineered substrates to understand the topography regulated cell behaviors. Evidence has shown that roughly aligned collagen fibrils can provide contact guidance to axons *in vitro*, suggesting that axons can sense ECM topography.²³ Recently, electron beam lithography has become widely adopted to engineer a diversity of nanostructured substrates to conduct *in vitro* experiments. For example, a controlled nanodisorder surface has been developed that was proved to be able to modulate the cell adhesion and direct stem cell fate, which could induce fast osteogenesis from skeletal stem cells.²⁴ As for stiffness cue, for example, tissue stiffness could substantially change during different stages of the disease. The stiffness of normal breast tissue is 150 Pa, where the cancerous breast tissue could become 10 times stiffer than normal tissue.^{25, 26} The connective tissue of lung is mainly composed of collagens and elastin fibers, where the elastic moduli are around 1200 MPa and 1 MPa, respectively, during normal condition.^{27, 28} The expression of collagen and elastin is largely controlled. However, when experiencing pulmonary fibrosis, the stiffness of the fibrotic lung tissue increases significantly due to the excessive deposition of collagen, altering the mechanical properties of the ECM, thus changing the cellular behavior and function.²⁹ Therefore, the ECM has been identified significantly as in maintaining the steady state of normal tissue while the disruption of homeostasis would potentially cause the development of disease.³⁰

Limitations of current *in vitro* models for disease therapy and diagnosis

From the perspective of the natural *in vivo* condition, current *in vitro* platforms for disease therapy and diagnosis show significant drawback. The majority of the platforms for cell culture are established on TCPS, where it is much stiffer ($E \sim 3 \text{ GPa}$)³¹ than the most human tissues in the actual *in vivo* condition. Moreover, TCPS is lack of three-dimensional topographical structure as it is completely flat. Therefore, most of the cells are randomly spread and largely stretched when cultured on TCPS, which is substantially deviating from their *in vivo* microenvironment. For example, a study has shown that substrate stiffness could direct human mesenchymal stem cells (hMSCs) fate, and the increase of the time that stem cells were cultured on TCPS tended to modulate hMSCs differentiation to osteoblasts.³¹ As shown in Figure 1-2 (b), compared to culturing strictly on soft substrate (So7), increased mechanical dosing on day 10 (DT10/So7) significantly resulted in hMSCs differentiation towards osteogenesis. When hMSCs were precultured on TCPS for 1 to 10 days, most hMSCs tended to lose their multipotency and could only differentiate into osteoblasts.

Figure 1-2: hMSCs differentiation directed by pre-culturing them on TCPS for different days. (a) hMSCs were first cultured on TCPS for 1 day, 5 days and 10 days and then transferred to soft hydrogel substrate, followed by 7 days of culturing. hMSCs cultured strictly on the soft substrate from day 0 was used as a control (So7) (b) Immunostaining of PPAR γ (green) and OCN (red) of hMSCs of the aforementioned samples after 7 days culturing on soft hydrogels. (PPAR γ is mainly present in adipose tissue, colon and macrophages, which is often used as adipogenesis marker. OCN is produced by osteoblasts, which is often used as a biochemical marker for bone formation.) (c) Staining for ALP (osteogenic markers) of hMSCs of the aforementioned samples before transferred to soft hydrogels. The scale bars are 20 μ m. (Adapted with permission from Ref. [31])

The ECM also play a significant role in cell spreading. When cultured on TCPS, cells tend to increase proliferation and the formation of actin fibers and focal adhesions.^{32, 33, 34, 35, 36} For example, one study has shown that increased cell number has been observed due to the promotion of cell proliferation when cultured on TCPS compared with polyacrylamide (PAAm) hydrogels (~ 0.3 to 55 kPa)³⁷ Another study compared the proliferation of cells cultured on TCPS and the fabricated highly aligned microfibrinous tubular scaffold, where the results showed that cell proliferation was significantly higher on TCPS than the engineered scaffold, which had similar mechanical properties compared to the native esophagus.³⁸

Therefore, taking the limitations of current *in vitro* model into consideration, instead of using flat, rigid TCPS surfaces, novel physiologically relevant platforms that incorporate both topographical cue and stiffness cue should be established to closely mimic the *in vivo* conditions.

Biophysical cues modulation of cell phenotype and function

Stiffness cue. A wide range of natural as well as engineered materials with a stiffness varying from several pascal to hundreds gigapascal that have been applied in different biological related studies (see Table 1), including hard materials like silicon (Young's modulus ranging from 50.9 to 180 GPa³⁹), glass (50-90 GPa⁴⁰), TCPS (~ 3 GPa³¹). However, since the ECM exhibits various degree of stiffness in the actual *in vivo* condition, and there are lots of tissues which cover the range below the magnitude of gigapascal (see Figure 1-1b), engineered soft materials have been more and more commonly used to conduct *in vitro* models to mimic the stiffness in different tissues for disease detection and modeling.

For example, elastic polymer polydimethylsiloxane (PDMS) has been widely adopted as substrates/ matrices for biological related study, not only because its great biocompatibility, but

also for the fact that it is a versatile polymeric material which stiffness could be tuned by varying the base material and curing agent, as well as the curing time/ temperature. PDMS typically has an elastic modulus around 1 MPa and holds the great potential of tunable stiffness. One study has shown the ability to tune the stiffness of PDMS from 800 kPa to 10 MPa by varying the base material and crosslinker reagent from 1:2 to 1:19 under different curing temperature and time.⁴¹ A much soft stiffness in the lower limit range has been reported that by varying the base material and crosslinker reagent ratio from 100:1 to 10:1, the PDMS substrates could achieve a range from 0.1 kPa to 2.3 MPa.⁴² Poly(ethylene glycol) (PEG) based hydrogel is another commonly used soft material, which has been realized as a suitable, biocompatible soft material that is widely applied in a diversity of cellular and biomedical related applications, including 3D *in vitro* model for cell study,⁴³ cell encapsulation,⁴⁴ tissue scaffold for regenerative medicine.⁴⁵ The stiffness of PEG is highly tunable via the cross-linking polymerization process by varying the ratio between precursors and cross-linkers. The stiffness of PEG is typically lower than ~1 kPa.⁴⁶ An elastic modulus range from 4 kPa to 70 kPa has been reported by using a radical-free, Michael addition to prepare PEG hydrogel,^{47,48} in which range has covered many soft tissues in human body.⁴⁹ PEG-based hydrogel can also cover a much more rigid elasticity range from 10 kPa to 1 MPa by changing the cross-linking density and mechanism. Specifically, the protein accumulation effects by the crosslinking process have been studied and investigated.⁵⁰ Polyacrylamide (PAAm) gel has been more and more routinely used as an engineered soft substrate for studying the mechanosensing in various *in vitro* models.⁵¹ Typically, mostly used PAAm gels are in a very soft range and the stiffness is ~ 1 kPa but they are also highly tunable by varying the ratio of the two components for polymerization, acrylamide and bis-acrylamide. A wide range of 1.3-166 kPa has been reported (Young's modulus was calculated from shear modulus in the original reference, ν is

assumed to be 0.5).⁵² A wider range has also been reported up to 740 kPa in the upper limit of the stiffness.⁴²

Table 1-1: Different materials and their stiffness as in Young's modulus.

Material	Young's Modulus	Reference
Silicon	50.9-180 GPa	[39]
Glass	50-90 GPa	[40]
TCPS	~ 3 GPa	[31]
PDMS	0.8-10 MPa	[41]
	0.1 kPa – 2.3 MPa	[42]
PEG	4-70 kPa	[47], [48]
	10 kPa – 1 MPa	[50]
PAAm	1.3-166 kPa	[52]
	2 Pa – 55 kPa	[53]
	0.5-740 kPa	[42]

The effect of cellular response to different stiffness has been extensively studied. The variation of stiffness could affect cell adhesion,⁵⁴ cell spreading area, actin organization and cell elongation,^{53, 54, 55, 56} cell migration,^{54, 55, 56} cell proliferation, growth and apoptosis,⁵⁷ cellular contractility,^{54, 55, 58} and stem cell differentiation.^{31, 59}

Among the studies conducted to investigate the cellular response to substrate stiffness, fibroblasts have been widely adopted as a cell model, besides, epithelial cells, endothelial cells and neuron cells were also used. Extensive reports have shown that cell spreading increased with the increase of substrate stiffness increase, along with a noticeable formation and organization of actin stress fiber as for fibroblasts.^{53, 54, 55, 56} For example, one group reported that fibroblasts on soft substrate (2.68 ± 0.99 kPa) have poor cell adhesion, which results in a poorly spread morphology

and has the tendency to aggregate with neighboring cells to form spheroids that are tightly attached and tissue-like. Moreover, the focal adhesions observed on the soft substrate were short, limited quantity and dot-like. On the contrary, cells cultured on the stiff substrate (7.69 ± 2.85 kPa) exhibit similar behavior as that on TCPS surface, with a stronger cell adhesion, elongation, and large cell spreading area, as well as a great number of large, elongated focal adhesions.⁵⁴

Cell migration, which is involved in a number of physiological process *in vivo*, can also be affected and guided by substrate stiffness *in vitro*. For example, PAAm hydrogel sheets that incorporated with different gradient of stiffness have been engineered to test the cell migration of NIH 3T3 fibroblast cells, with the results showing that preferentially, NIH 3T3 cells tended to migrate toward the stiff side of the PAAm sheets with a decreased migration speed.⁵⁵ Another important aspect is that matrix stiffness can also have an impact on activating the signaling pathways of cell contractility.⁵⁸ For example, tumors generally have stiffer elastic moduli than normal tissues where Rho proteins are found to be overexpressed in stiff tumors.⁶⁰ Therefore, it has been found that increased in matrix stiffness resulted in elevated cell contractility because Rho activity has been identified to be closely associated with generating cell contractility.^{58, 60, 61}

Substrate stiffness also affects cell proliferation, growth and apoptosis. NIH 3T3 cells and PAAm gels were used in one study to test the cell proliferation affected by different matrix stiffness, where the cell proliferation showed a 2-fold increase on stiff PAAm gels (0.06% bis-acrylamide) compared to that on soft PAAm gels (0.012% bis-acrylamide) after 24 h incubation and the difference has been increased up to 4-fold after 48 h of incubation.⁵⁷

Substrate rigidity could also substantially affect stem cell differentiation. For example, one study investigated the stem cell differentiation directed by matrix stiffness using mesenchymal

stem cells (MSCs). Results showed that MSCs on soft substrates (0.1-1 kPa) were largely differentiated into neurogenic cell types; on intermediate stiff substrates (11 kPa), the differentiation was directed to myogenic cell types while on the stiffest substrates (34 kPa), osteogenic differentiation occurred at the most.⁵⁹ It has been suggested that the direction of MSCs differentiation tended to develop towards the direction of mimicking the actual *in vivo* environment, where these three stiffness represented brain, muscle and bone tissues.⁶²

Given that the mechanical properties of substrates have a wide range of impact on cell behaviors, substrate stiffness regulation can be well adopted in clinical studies for disease detection and modeling. One of the applications is the impacts of substrate stiffness on cellular uptake of nanoparticles for cancer diagnosis and clinical therapy. One systematical study showed that higher membrane tension that existed in cells on stiffer substrate (3.81 ± 0.12 kPa for intermediate gels and 5.71 ± 0.51 kPa for stiff gels) caused barrier that prevented the nanoparticles from entering the cells, whereas cells on soft gels (1.61 ± 0.11 kPa) had lower membrane tension that promoted the cell uptake of nanoparticles.⁶³ Moreover, this study shed light on the potential application in cancer cell targeting and detection using nanoparticles to inhibit tumor growth by taking the advantages of the stiffness different between tumors and normal tissues.⁶³

Nanotopographical cue. Nanotopographical cue has been more and more realized as an important mechanical property that could modulate cell behaviors for disease detection and modeling. One of the reasons is that nanoscale topographies existed in numerous human tissues that are closely related the function of the specific organ (see Figure 1-3). For example, three-dimensional hierarchical structures and organizations can be seen in many tissues like bone, ligaments and tendon, blood vessels.⁶⁴ Specifically, as shown in Figure 1-3, the bone tissue contains dense-packed, organized cylindrical structures. Such structures possibly existed because

of the supportive and protective function of the bone, which could be able to sustain the possible external force. Particularly, as previously mentioned, the ECM is composed of a diversity of well-defined, three-dimensional nanostructures such as collagens fibers, elastins, laminins, all of which have a feature size in the nanometers range, where cells are highly sensitive to.⁶⁵

Figure 1-3: Nanotopographical features exhibited in various tissues. According to the living environment and the functions of the tissue, there are four major categories: protective tissues, mechano-sensitive tissues, electro-active tissues and shear stress-sensitive tissues. (Adapted with permission from Ref. [64])

Taking the advantage of the rapid evolution of modern nanotechnologies, it is now possible and convenient to generate the designed nanostructures with various shapes and dimensions. For

example, a high resolution with a lateral dimension of 30 nm feature size could be obtained with two-photon lithography, which could be potentially used in fabricating nanoscale structures with a large scale like photonic crystals.⁶⁶ Self-assembly techniques that can fabricate various complex micro- and nano-sized features, also gain many of the interests in the field of nanofabrication,⁶⁷ including block copolymer micelle nanolithography (BCMNL)^{68, 69, 70} and colloidal lithography⁷ were also used to form a large area of nanopatterns at a relatively low cost. Moreover, electron beam lithography (EBL) and focused ion beam lithography were largely developed to generate well-defined patterns with a high resolution of ~ 5 nm in recent decades.^{71, 72} Besides of the high resolution, EBL technique has other advantages such as fabricating customized patterns, performing pattern fabrication without a mask and eliminating the diffraction problem. The EBL fabricated nanotopographies could be further served as the molds to prepare the PDMS replicas.

According to the different shape of the repeating unit of the nanofeature, the designed nanotopographies that have been used in tissue engineering can be divided into two types, the isotropic nanotopography (nanopillars, nanopits, nanorod arrays) and the anisotropic nanotopography (nanogratings, aligned fibers). Besides, there is also nanotopography gradient, where the isotropic or the anisotropic nanotopography can be presented at a density gradient (see Figure 1-4).⁷³ Different design, shapes, as well as the variation of dimension of the nanotopographies, may have different impact on cell behaviors, including cell adhesion, cell spreading (elongation, alignment), cell migration, and stem cell differentiation.

Figure 1-4: Classification of nanotopographies according to the shape, including anisotropic topography (nanogroove, aligned fibers), isotropic nanotopography (nanopillar, nanopost and nanopit arrays) and topography gradients. (Adapted with permission from Ref. [73])

Isotropic nanotopographies are uniform, homogeneous repeating units, which provides discrete contact guidance in every direction. Cell behavior could be altered by isotropic nanotopographies from numerous aspects including increased⁷⁴ or reduced cell adhesion,⁷⁵ limited cell spreading,^{76, 77} cell migration⁷⁸ and directed stem cell differentiation.^{24, 76} For example, one group discovered that compared to bare polyethylene glycol (PEG) surfaces, fabricated PEG nanopillars could enhance the cell adhesion of cultured cardiomyocytes for the reason that those pillars could not only serve as anchor posts for cell to attach on by extruding filopodia and

lamellipodia, but also favor the actin formation.⁷⁴ The influence of nanotopography on cell adhesion is highly dependent on the shape of the nanotopography. Another study reported a reduced adhesion complex formation and a limited cell spreading on highly-ordered nanopit arrays compared to the planar control.⁷⁵ It seemed that the nanopit topography could disrupt or inhibit the adhesion formation. A similar study has been done by fabricating square (sq) and hexagonal (hex) ordered nanopits that proved to reduce cell spreading and the adhesion formation.⁷⁷ Cell migration mostly occurred on anisotropic nanogratings, where there have been limited studies conducted on isotropic nanotopographies. One study that systematically investigated the mechanical properties modulated cell migration by combining the substrate stiffness and topographical cues, which showed that the cell migration was highly dependent on the substrate anisotropy, and the migration orientation was appeared to be along the spatial direction of pillar arrangement.⁷⁸ Another important aspect is the influence on stem cell differentiation. Results suggested that the MSCs differentiation could be altered by varying the order of isotropic nanotopographies. It was noted on DSQ50 nanopits, which is a disordered square array with dots displaced randomly by up to 50 nm on both axes from their position in a true square, exhibited bone nodule formation whereas the highly ordered hexagon and square array decreased the cell adhesion and low amount of osteoblastic differentiation marker was detected.²⁴

In contrast to the isotropic nanotopographies, anisotropic topographies provide continuous contact guidance in a single direction, which results in different impact on cell behaviors, including enhanced cell elongation and alignment,^{79, 80, 81} increased⁸² or reduced cell adhesion,⁸¹ cell proliferation,^{79, 80, 81, 82} directional migration^{79, 83} and stem cell differentiation.^{80, 84} For example, endothelial progenitor cells (EPCs) cultured on nanogratings that featured 1200 nm in width and 600 nm in height showed a highly elongated morphology aligned with the groove direction,

reduced cell proliferation and an enhanced directional migration along with an overall high speed.⁷⁹ It has also been realized that the influence on cell proliferation may closely related to the dimension of the nanotopographies. One study showed that compared to flat PDMS, cell proliferation all showed a reduction on nanogratings with 350 nm, 1 μm and 10 μm in width but it decreased more significantly as the width getting smaller.⁸⁰ Other than the lateral dimension of the nanogratings, height also serves as an important factor in affecting the cell spreading and proliferation. One study reported that the enhancement or reduction of cell proliferation and cell spreading followed a height dependent manner. In comparison to control, cell spreading area was increased on nanogratings with 13 nm in height and decreased on higher islands with 95 nm in height.⁸⁵

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Chapter 2: Incorporating Nanotopography for Disease Detection – Adhesion-based Circulating Tumor Cell Capture

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Abstract

CTCs shed from primary tumors, transport through the bloodstream to distant sites, and cause 90% of cancer deaths.¹ Although different techniques have been developed to isolate CTCs for cancer detection, diagnosis and treatment, the heterogeneity of expression of the target antigen and the significant size variance in CTCs limit clinical applications of antibody- and size-based isolation techniques. Cell adhesion using nanotopography has been suggested as a promising approach to isolate CTCs independent of surface marker expression or size of CTCs. However, the influence of nanotopography configuration (geometry and dimensions) on CTC capture efficiency has not been investigated. This study examined capture performance of several cancer cell lines of different types, surface marker expression and metastatic status on nanotopographies of various geometry and dimensions without antibody conjugation. The cancer cells exhibited differential capture performance on the nanotopographies with an efficiency up to 52%. Compared with flat surfaces and isotropic, discrete nanopillars, anisotropic nanogratings favored cancer cell adhesion, thus improving the capture efficiency. The influence of nanotopography height studied, on the other hand, was less significant. This study provides useful information to optimize nanotopography to further improve CTC capture efficiency.

Introduction

Cancer metastasis, initiated by CTCs migration from primary tumors through the bloodstream to distant sites of the body, causes 90% of cancer deaths.¹ Although the cellular and molecular characterization of CTCs holds great promise for cancer detection, diagnosis and treatment, the realization of this potential remains limited by current challenges associated with CTC isolation techniques.² The major hurdles in advancing CTC isolation techniques include rarity and heterogeneity of CTCs. CTCs are extremely rare, as few as one CTC per 10^9 normal blood cells in the blood of patients with metastatic cancer.³ CTCs also display heterogeneity in the expression of target antigens and variation in cell size. Nevertheless, different techniques have been developed to capture CTCs.

The most widely used CTC capture techniques rely on positive selection based on surface biomarkers such as epithelial cell adhesion molecule (EpCAM) expression on tumor cells.⁴ For instance, CellSearch assay, the most standardized technology, uses ferrofluids loaded with an EpCAM antibody (anti-EpCAM) to capture CTCs through a magnetic field. The advent of microfluidic technology advances CTC capture techniques. CTC capture efficiency has been improved by enhancing CTCs-antibody interactions via optimizing the microchannel dimensions,⁵ introducing microscale pillars,⁶ and generating microvortices.⁷ Although EpCAM is expressed in the cells of epithelial origin, the EpCAM expression on tumor cells varies with tumor type,⁸ some cells even express no EpCAM.⁹ Additionally, invasive tumor cells tend to lose their epithelial antigens via the epithelial-to-mesenchymal transition process.¹⁰ The antibody-based capture techniques are thereby limited to the tumor cells expressing the specific antigen.^{11, 12, 13} Because of relatively low sensitivity on some tumor cells expressing low or no EpCAM, the CellSearch assay only achieved a median yield of approximately one CTC per milliliter and typically low

purity.^{14, 15} To overcome the limitation of heterogeneous surface marker expression, several physical properties distinguishing CTCs from most normal blood cells have been utilized to capture CTCs. The properties include the larger size of most epithelial cells and differences in density, charge, migratory properties, and some properties of specific cell types (e.g., melanocytic granules in melanoma cells).^{16, 17, 18} For instance, the size-based microsieve and microfiltration device have been developed to isolate breast, gastric, and colon tumor cells lines including EpCAM-negative tumor cells.^{17, 19, 20, 21} However, the fact that CTCs are not always larger than leukocytes and monocytes have a similar size (15-25 μm) to CTCs²² impedes the clinical applications of size-based capture techniques.²³

Emerging compelling evidence continues to show that substrate nanotopography has a profound influence on cell adhesion, suggesting cell adhesion as a promising approach to CTCs capture. Inspired by nanostructured surface (e.g., microvilli, microridges and cilia) of cancer cells²⁴ and enhanced cancer cells-nanotopography interactions,²⁵ silicon nanowires,^{26, 27} polystyrene (PS) nanotubes²⁸ and hierarchical nanowire arrays²⁹ have been fabricated and conjugated with anti-EpCAM to significantly improve sensitivity and efficiency of CTC capture. Strikingly, by taking advantage of the differential adhesion preference of cancer cells to nanotopography compared with normal blood cells, Chen *et al.* demonstrated high selectivity and high efficiency capture of CTCs by using nanorough glass surfaces prepared by using reactive ion etching (RIE) regardless of the surface marker expression or physical size of the CTCs.³⁰ To successfully translate adhesion-based CTC capture technique using nanotopography to clinical settings, it is highly desirable to understand how nanotopography configuration parameters such as geometry and dimensions affect tumor cell adhesion.

Herein, we investigated capture performance of four human cancer cell lines on representative nanotopographies. The nanotopographies of various geometry (nanoscale gratings and pillars) and dimensions (in particular height) were engineered on elastomeric PDMS substrates. The cancer cell lines were MCF7 (a luminal non-metastatic breast cancer cell line), MDA-MB-231 (a basal aggressive metastatic breast cancer cell line), A549 (an adenocarcinomic basal epithelial cell line), and HeLa (a cervical cancer cell line). Thereby, we were able to examine the influence of geometry and dimensions of nanotopography on cell adhesion and capture performance of the cancer cells of different cell types, surface marker expression (EpCAM positive MCF7 and A549 cells vs EpCAM negative MDA-MB-231 and HeLa cells), and metastatic status (non-metastatic MCF7 vs metastatic MDA-MB-231). This study helped our understanding of nanotopography enhanced CTC capture.

Materials and Methods

Cell culture

All cancer cell lines were purchased from American Type Culture Collection (ATCC; Manassas, VA). MCF7 were cultured in Eagle's Minimum Essential Medium (EMEM; ATCC) supplemented with 10% (v/v) fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA), 1% (v/v) penicillin/streptomycin (Life Technologies, Carlsbad, CA) and 0.01 mg/ml human recombinant insulin (Sigma-Aldrich, St Louis, MO). MDA-MB-231 were cultured in Minimum Essential Media (MEM; Thermo Fisher Scientific) supplemented with 10% (v/v) FBS, 1% (v/v) L-glutamine (Life Technologies) and 1% (v/v) penicillin/streptomycin. HeLa and A549 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich) supplemented with 10% (v/v) FBS, 1% (v/v) L-glutamine 1% (v/v) penicillin/streptomycin. The cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. The culture media was replaced every 3 days.

Preparation of nanotopographies

Nanotopography was generated by using EBL and replicated into PDMS substrate as previously described.^{31, 32} Briefly, the nanopattern was designed and written onto a poly(methylmethacrylate) (PMMA) thin film on a silicon substrate by using EBL and then etched in the silicon substrate by applying RIE process. The generated nanotopography was cast with a mixture of PDMS resin and curing agent (Sylgard 184 silicone elastomer kit; Corning, Corning, NY) at a 10:1.05 w/w ratio, followed by curing at 70 °C for 4 h. The PDMS nanotopography was expanded to a large area by applying a stitch technique.³³ The stitched mold was then imprinted

into PS substrates to make a PS master mold, from which working PDMS nanotopographies were produced.

The working PDMS nanotopographies were punched to discs fitting in the wells of a 48-well plate. The discs were oxygen plasma treated at 300 mT, 50 W for 30 s in a PX-250 Plasma Asher (Nordson MARCH, Concord, CA) to render a hydrophilic surface. The PDMS nanotopographies and flat surfaces as control were sterilized in 70% ethanol followed by UV exposure, each for 30 min, and then incubated with 5 $\mu\text{g}/\text{cm}^2$ collagen I (Corning) for 1 h prior to cell seeding.

Cancer cell capture

The cancer cells were first labeled with either CellTracker Green or CellTracker Red (Life Technologies) according to the manufacturer's instruction. Briefly, the cells were incubated in 5 mM CellTracker in DMEM solution (serum free) at 37°C for 30 min. The cells were then trypsinized using 0.25% trypsin-EDTA (Sigma-Aldrich) after phosphate buffer saline (PBS; Sigma-Aldrich) rinse, resuspended in 1 mL fresh media, and diluted to the desired seeding density of 500 cells/well. The cell suspension was added onto the PDMS discs in 48-well plates and incubated for a predefined capture time (1 h, 2 h or 4 h).

To achieve accurate cell counting, the whole PDMS surface with the cells was first scanned by using a Nikon Swept Field microscope with a 10x objective (Nikon, Melville, NY) at the predefined time point. Multiple images from the scanned field of 1 cm x 1.03 cm rectangle were stitched to a single image using Nikon NIS-Element software, covering the whole well of 48-well plates. Subsequently, the culture media was carefully aspirated and the sample was gently rinsed

with PBS to remove any non-adhered cells. The PDMS disc with captured cells was scanned again to count the captured cells by repeating the aforementioned scanning process.

ImageJ (<http://imagej.nih.gov/ij/>) was used to quantify the numbers of seeded cells and captured cells using the “analyze particle” function. The cells were highlighted by adjusting the threshold and the image was converted to a binary image prior to analyzing the particles.

Cell area measurement

MCF7 cells were pre-labeled with CellTracker Red and seeded at a density of 1000 cells/cm² on the PDMS substrates including nanotopographies and flat surfaces. After 4 h cultivation, the cells were fixed using 4% paraformaldehyde (PFA; Electron Microscopy Sciences, Hatfield, PA) in PBS for 30 min, and mounted on a coverglass using ProLong Gold Antifade Reagent with 4,6-diamidino-2-phenylindole (DAPI; Life Technologies) overnight at room temperature. At least 50 cells were imaged using a Nikon Swept Field microscope with 40x oil objective. Cell area was quantified using ImageJ software. Firstly, the cells were highlighted by adjusting the threshold and the image was converted to a binary image. After the scale has been properly calibrated, the areas of the cells were measured using ROI (Region of Interest) manager.

SEM observations

The substrates with cells grown were rinsed with PBS and fixed in a mixture of 4% PFA and 2% glutaraldehyde (Thermo Fisher Scientific) in PBS at ambient for 4 h. The samples were then rinsed with deionized (DI) water and progressively dehydrated using a Baltec CO₂ critical point dryer (Bal-Tec, Los Angeles, CA). The substrates with(out) cells grown were sputter-coated with a gold layer of ~10 nm thick by using a Denton Vacuum Desk V sputter unit (Denton Vacuum,

LLC, Moorestown, NJ, USA), and imaged in a field emission SEM (JEOL JSM-7600F, Peabody, MA).

Statistical analysis

Capture efficiency and cell areas were expressed as mean $\pm$ standard error of mean (S.E.M). The differences between groups were analyzed by one-way ANOVA using student T-test for multiple comparisons at a confidence interval level of 95%.

Results and Discussion

Design of nanotopographies

Recent studies indicated that nanotopography modulated cell phenotype and function were dictated by the geometry (e.g., gratings, pillars and wells), dimensions and arrangement of nanofeatures, which is cell type-specific.^{34, 35, 36, 37, 38, 39} Our studies also demonstrated that cell adhesion, nuclear volume and cell function such as proliferation, transfection and ECM protein production of human lung fibroblasts could be modulated by the geometry (nanoscale anisotropic gratings and pillars) and dimensions, in particular the height of nanotopography.³¹ In light of our previous results, we chose representative nanogratings (NGs) and nanopillars (NPs) of various heights in this study (see Figure 2-1). The nanotopographies had the same feature size (500 nm in line width of NGs and diameter of NPs), similar spacing (500 and 450 nm for the edge-to-edge spacing of NGs and NPs, respectively) and two heights (150 and 560 nm). Additionally, equally spaced nanogratings of 1000 nm in line width were fabricated, which had 15% and 56% surface area increase for 150 and 560 nm heights, respectively. For the convenience of discussion, the nanotopography was labeled by using a combination of feature size, edge-to-edge spacing and height. For example, 500-0.9×-150 referred to the nanogratings of 500 nm in diameter, 0.9 times edge-to-edge distance (450 nm) in spacing and 150 nm in height. The lateral dimensions of nanotopographies were defined by EBL and confirmed by using SEM, and the height was controlled by RIE processing time and measure by using atomic force microscope (AFM).³¹ Such design of nanotopographies allowed systematic investigation of capture performance of different cancer cells on nanotopography of various configuration parameters (geometry and height).

Figure 2-1: SEM micrographs of the PDMS nanotopographies and the flat substrate. The scale bars are 1 μm .

MCF7 cell capture on nanotopographies

We first examined the capture efficiency of MCF7 cells on the nanotopographies as a function of incubation time. Capture efficiency was defined as the ratio of the number of cells adhered to the substrate surface to the number of cells initially seeded. To avoid possible errors in initial cell counting from the cell suspension preparation step, the seeded cells were precisely counted by scanning the whole PDMS surface after the predefined time, but before rinsing off the non-adherent cells. Because early cell-nanotopography interactions were critical to mechanosensing⁴⁰ and the reported CTC capture experiment was usually performed within 4 h,^{26, 30, 41} the incubation time was determined at 1, 2 and 4 h. At all the time points, it was observed that some cells were captured on the PDMS substrates including the nanotopographies and the flat surfaces, as exemplified in Figure 2-2A. The measurement showed that on the same PDMS substrate, the capture efficiency increased as a function of the incubation time (see Figure 2-2B).

The capture efficiency on the flat surfaces and the nanopillars slightly increased over time. For instance, the efficiency on the flat surfaces increased from $3.1 \pm 1.5\%$ at 1 h to $4.3 \pm 2.0\%$ and $10.4 \pm 2.3\%$ at 2 and 4 h, respectively. By contrast, the nanogratings displayed a remarkable enhancement on MCF7 capture, and the efficiency reached up to $41.0 \pm 5.0\%$ on 500-1 $\times$ -150 at 4 h. For the same nanotopography geometry, the decrease in height did not result in significantly different capture efficiency on the nanopillars (except the capture at 2 h), yet improved MCF7 capture on the nanogratings, $20.7 \pm 1.3\%$ on 500-1 $\times$ -560 vs $29.1 \pm 0.3\%$ on 500-1 $\times$ -150 at 2 h, and $27.9 \pm 3.9\%$ on 500-1 $\times$ -560 vs $41.0 \pm 5.0\%$ on 500-1 $\times$ -150 at 4 h. The results suggested that while the nanotopographies could improve CTC capture, the nanogratings were more effective on MCF7 capture than the nanopillars, and increase in the height of nanogratings undermined MCF7 cell capture.

Figure 2-2: MCF7 cell capture on the nanotopographies. (A) Fluorescent images of MCF7 captured on nanogratings 500-1x-150. Top row: Field scan images of MCF7 cells captured at 1, 2

and 4 h of incubation time. The scale bars are 2 mm. Bottom row: enlarged view of boxed area in the top row. The arrows point to the captured cells in red. The scale bars are 250 μm . (B) Capture efficiency of MCF7 on the nanotopographies as a function of time. *: Significant different ($p < 0.05$) from the flat controls and #: significant difference ($p < 0.05$) between two groups.

Intuitively, enhanced cell adhesion was associated with a larger cell area. We thus measured the cell spreading area and examined its correlation with capture efficiency on the nanotopographies. As shown in Figure 2-3A, MCF7 cells displayed approximately round morphology on the flat surfaces and the nanopillars but a mixture of round and partially elongated morphology on the nanogratings (SEM images in Figure 2-4). Cell area measurement summarized in Figure 2-3B revealed that MCF7 cells on the nanogratings exhibited average areas ($418.8 \pm 21.5 \mu\text{m}^2$ on 500-1 $\times$ -150, and $443.8 \pm 22.0 \mu\text{m}^2$ on 500-1 $\times$ -560) similar to the flat controls ($410.84 \pm 13.96 \mu\text{m}^2$), but significantly larger than those on the nanopillars ($107.77 \pm 5.13 \mu\text{m}^2$ on 500-1.9 $\times$ -150, and $155.63 \pm 10.62 \mu\text{m}^2$ on 500-1.9 $\times$ -560) ($p < 0.001$). It was also noted that cell areas on the deeper nanotopographies were larger than their shallow counterparts. The observation that the nanogratings induced a larger cell area than the nanopillars was consistent with the MCF7 capture results, indicating increased cell spreading area facilitated the cell capture. However, the increase in height of the nanotopographies from 150 to 560 nm, accompanied with an increased substrate surface area, resulted in an enlarged the cell area, contradictory to the influence of height on MCF7 capture efficiency. To further understand nanotopography modulated cell adhesion, we examined cell-nanotopography interactions by conducting SEM observations. As shown in Figure 2-4, MCF7 cells were well spread on the flat surfaces. However, the cells displayed a round and compact morphology on the nanopillars. The cells on the nanogratings, on the other hand, spread along the nanograting direction. Moreover, the protrusion of lamellipodia and filopodia on the

nanotopographies was not obviously affected by the height difference. It was speculated that although the increase in the height enlarged the area of adherent cells, the adhesion of non-invasive MCF7 on the deeper nanotopography might be weakened, leading to decreased capture efficiency.

Figure 2-3: (A) Fluorescent images of captured MCF7 cells on the nanotopographies and flat surfaces. The cell was labeled with CellTracker (red) and the nucleus was stained with DAPI (blue). The scale bars are 10 μm . (B) Cell area measurement of captured MCF7 cells on the nanotopographies and flat surfaces. *: Significant different ($p < 0.05$) from the flat controls and #: significant difference ($p < 0.05$) between two groups.

Figure 2-4: SEM micrographs of MCF7 cells adhered to the nanotopographies and flat surface. Insets provide an enlarged view of cell-substrate interactions from the boxed area. The scale bars are 10 μm .

It has been suggested that isotropic nanopillars tend to decrease the surface wettability, and consequently reduce protein adsorption, leading to an unfavorable cell adhesion.^{42, 43} That provides a possible explanation for differential influence of nanogratings versus nanopillars on MCF7 cell

capture. Our previous study suggests that the anisotropic nanogratings provide continuous contact guidance for the formation and growth of focal adhesions, thus promoting cell adhesion. The highly ordered, discrete nanopillars, on the contrary, tend to disrupt the development of focal adhesions, accompanied with reduced cell spreading area. Evidently, anisotropic nanogratings are favorable for cell adhesion, leading to increased capture efficiency.

Cell type specific capture on nanotopographies

We further explored nanotopography enhanced CTC capture by extending to more cancer cells of different cell types, surface marker expression and metastatic status. Given the increased MCF7 capture efficiency with incubation time and commonly used capture duration, we assessed CTC capture performance only at 4 h and summarized it in Figure 2-5.

Figure 2-5: Capture efficiency of MCF7, A549, MDA-MB-231 and HeLa cells after 4 h of incubation on the nanotopographies and flat surfaces. *: Significant different ($p < 0.05$) from the flat controls and #: significant difference ($p < 0.05$) between two groups.

A549 lung cancer cells were EpCAM positive similar to MCF7 cells, but demonstrated different capture efficiency from MCF7 cells in response to the alteration in geometry and height of the nanotopographies. Deeper nanotopography achieved significantly higher capture efficiency than its shallow counterpart, i.e., $51.7 \pm 4.9\%$ on 500-1 $\times$ -560 vs $38.2 \pm 3.3\%$ on 500-1 $\times$ -150 ($p < 0.0371$), and $47.5 \pm 2.2\%$ on 500-0.9 $\times$ -560 vs $29.7 \pm 4.0\%$ on 500-0.9 $\times$ -150 ($p < 0.0141$). By comparison, MCF7 cells reached the highest efficiency on the shallow nanogratings 500-1 $\times$ -150. The nanogratings also increased the capture efficiency compared with the nanopillars of the same height, but not statistically significant. The observations indicated that A549 cells were sensitive to the height yet not to the geometry of nanotopography. SEM observations showed that A549 cells on all nanotopographies spread more than the flat controls (see Figure 2-6A). Although the cells distributed randomly on the nanopillars and stretched along nanogratings, the nanotopography geometry did not have evident influence on cell area.

Figure 2-6: SEM micrographs of (A) A549 and (B) HeLa cells on the nanotopographies and flat surfaces. The scale bars are 10 μm .

Compared to non-metastatic MCF7 cells, the aggressive metastatic MDA-MB-231 breast cancer cells exhibited similar yet different capture performance on the nanotopographies. MDA-MB-231 cells captured on the nanogratings reached relatively higher efficiency, indicating that the nanogratings could more effectively enhance the cell adhesion than the nanopillars. However, the influence of nanotopography height on the cell capture was not significant. It was speculated that MDA-MB-231 cells, different from the epithelial-like MCF7 cells, were mesenchymal-like cells and invasive, and thus could readily reach the bottom of the nanotopography. In this regard, the difference in cell-nanotopography adhesion caused by the height change imposed insignificant influence on the cell capture.

EpCAM negative HeLa cells displayed similarity and difference in the capture performance on the nanotopographies in comparison with MCF7 and A549 cells. The nanogratings favored HeLa cell capture compared with the nanopillars of the same height, which was similar to MCF7 cells but different from A549 cells. Deeper nanotopographies, on the other hand, improved the capture efficiency, which was similar to A549 cells but different from MCF7 cells. Compared with A549 cells, HeLa cells were more sensitive to geometry but less sensitive to the height, which was supported by the SEM observations (see Figure 2-6B).

Despite the differential capture efficiency that these cancer cells demonstrated on the nanotopographies, general trends were observed regarding the influence of nanotopography on CTC capture. The nanogratings usually favored cancer cell adhesion. For example, the capture efficiency on the nanogratings was significantly higher than that on the nanopillars of the same height for MCF7, MDA-MB-231 and HeLa cells. The capture efficiency of A549 cells on the nanogratings was also slightly higher than that on the nanopillars. In current study, increase in height can help capture of these cancer cells, in particular A549 cells.

Compared to high capture efficiency using anti-EpCAM modified nanopatterns, the capture efficiency demonstrated in this study was relatively low (up to 52%). In this study, the height of the nanopatterns was up to 560 nm, far shorter than 2.6 μm , the reported characteristic length of long brushes of cancer cells.²⁴ In addition to the antibody enhancement, the previous studies used nanopillars of a height up to 12-15 μm ²⁶, which significantly improve cell-nanopattern interactions. The RIE generated glass surfaces, which had a roughness of 150 nm but no anti-EpCAM conjugation, could achieve an efficiency of up to 80%.³⁰ The improved CTC capture could be attributed to the higher surface free energy of RIE-etched glass surface compared to RIE-treated PDMS substrates. In light of the current study, the CTC capture efficiency is expected to be improved by optimizing the configuration of nanopattern and surface modification.

Conclusion

While more and more studies have been done to realize the significant role that nanotopography played in regulating cell behavior, there have been increased applications in the clinical settings as well. This study fabricated two types of nanotopographies: anisotropic nanogratings and isotropic nanopillars by using EBL and incorporated nanotopographical cue into disease detection for CTCs capture, which systematically evaluated the influence of nanotopography configuration, i.e., geometry and dimension on capture performance of human cancer cell lines of different cell types, surface marker expression and metastatic status. The results showed that compared to flat substrates, nanotopography could enhance cell adhesion and thus improved capture efficiency. Generally speaking, the nanogratings improved cell adhesion and facilitated cancer cell capture although the capture efficiency was also cell type specific. The cancer cells were less sensitive to the height range studied.

For MCF7 cells, the capture efficiency could reach up to 41% on 500-1 $\times$ -150 nm nanogratings after 4 hours of incubation, suggesting that although nanotopography could enhance cell adhesion, nanogratings were more favorable for cell capture compared to nanopillars. The cell spreading area measurement revealed that cells on nanogratings exhibited significantly larger area than that on nanopillars. SEM observations also showed enhanced cell-substrate interactions on nanogratings for all four types of cancer cell lines compared to nanopillars and flat surfaces.

The significance of this study is to systematically investigate the effects of shapes and dimensions of nanotopographies on tumor cell capturing. The novelty of our study is to identify the enhanced cell adhesion by nanogratings and apply the nanotopography into adhesion-based CTCs capture while most studies have been focusing on fabricating nanopillar arrays to perform

CTCs capture by enhanced cell-substrate interaction. Our study shows that the nanogratings can provide continuous contact guidance for focal adhesion formation while discrete nanopillars disrupt the growth of focal adhesions. Meanwhile, the research focus on nanogratings affected cell behavior mostly landed on cell alignment, while we explored the enhanced cell adhesion that caused by the continuous contact guidance provided by nanogratings, which was also a novelty for this study. By optimizing the nanotopography configuration and performing appropriate surface modification for different types of cancer cell lines, the CTCs capture efficiency was expected to be improved. This study is expected to shed light on optimization of nanotopography to improve adhesion-based CTC capture technique for disease detection.

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Chapter 3: Incorporating Substrate Stiffness for Disease Modeling – In Vitro Model of Cell Sensing Nanomaterials

Abstract

As the rapid development of modern nanotechnology, engineered nanomaterials have been used in a wide range of fields because of their unique properties, including physics, chemistry, biochemistry, biomedical engineering, and so many other disciplines. In the same time, an arising issue has been more and more realized by the researchers that nanomaterials toxicity may cause potential risk to the human body.

This study focused on understanding the effects of substrate stiffness on fibrogenic responses of human lung fibroblasts to multi-wall carbon nanotubes (MWCNTs). In most living tissue, cells resident in a complex microenvironment where these cells interact with the ECM and the neighboring cells. The interactions between cells and ECM could regulate the cell behavior. Similar to *in vivo*, *in vitro* models have been reported that cells have the ability to sense the stiffness of synthetic substrate and, the variation of substrate stiffness could affect cell phenotype and function. Most existing *in vitro* models focused on conducting the experiment using the rigid TCPS surfaces, which were much stiffer than the actual *in vivo* cell microenvironment. Thus, the behavior and nanomaterial responsiveness of cells could be largely changed due to the deviation of substrate stiffness when cultured on TCPS. Therefore, it is of the critical need to create physiologically relevant tissue models to mimic the *in vivo* environment by introducing stiffness cue.

This study used the synthesized polyacrylamide (PAAm) hydrogel to represent the normal and fibrotic conditions of lung tissues to conduct *in vitro* models. The fibrogenic responses and mechanosensing of fibroblasts to MWCNTs at different stiffness conditions have been explored. This study provides an understanding of the regulatory pathways and mediators of fibrogenic activities, which will potentially help identify therapeutic targets against fibrosis.

Introduction

Cells live in a dynamic microenvironment that is regulated by the ECM. ECM is a network composed of a wide variety of macromolecules secreted by cells, including the fibers such as collagen, which is the most abundant protein in ECM, even in human body.^{1, 2} A majority of the composition of ECM have been known to have a nanoscaled structure, like the collagen I fibrils, which are 300 nm-long, staggered large-diameter banded fibrils typically found in lung, bone and skin tissue.³ The intrinsic diverse composition of ECM contributes to the fact that it could serve many functions, including supporting and interacting with the surrounding cells,⁴ providing various growth factors, exchanging signals with cells to control the local environment.⁵ Depending on ECM composition and interstitial fluid, the ECM exhibits various degree of stiffness, ranging from <1 kPa to >1 GPa for human tissues.^{6, 7} A growing body of evidence has shown that the mechanical cues of ECM critically influence numerous developmental, physiological and pathological processes *in vivo*,^{8, 9} and have a profound influence on cell phenotype and function *in vitro*.¹⁰

Inspired by the composition of ECM and cell microenvironment, a number of researches have been conducted to study the influence of surface topography and substrate stiffness on cell behavior. It has been found that cell could be highly sensitive when responding to a synthetic surface with different topographical cues as well as stiffness cues. This study focused on how substrate stiffness regulated cell phenotype and function. Figure 3-1 shows a schematic illustration of changes in cell behavior when changing the matrix stiffness from soft to stiff.^{11, 12}

Figure 3-1: Cell behaviors regulated by the variation of matrix mechanics. This schematic illustration shows several behavioral changes (cell growth, proliferation, apoptosis, cell migration, cell adhesion, cell spreading and fibrogenesis response) when changing the matrix stiffness from soft to stiff. (Adapted with permission from Ref. [11])

Generally speaking, cells on soft substrate tends to be rounded and less adhesive and proliferative while on the contrary, cells on the stiff substrate are more proliferative and fibrogenic. Focal adhesions (FAs), as the first and essential step of cell responding to the substrate, have been seen on the stiff matrix and the cells have a spread phenotype. The dynamic changes of focal adhesion induced by substrate stiffness could largely have an impact on cell phenotype and eventually cell function. The signally pathway of cell sensing substrate stiffness as well as surface topography are shown in Figure 3-2.¹³

Figure 3-2: Cell contractility, motility and spreading influenced by the mechanical properties of the substrate. (Adapted with permission from Ref. [13]) (A) Influence of substrate stiffness and topography on cell contractility as described by physical and biochemical signaling pathways.¹⁴ (B) Cell motility speed and traction force change with the increase of gel stiffness. Cells tend to have higher motility speed with less tension on soft substrate,¹⁵ which results in migration from soft towards stiff on a gradient gel.¹⁶ (C) Nonlinear response obtained from a thermodynamic model of cell spreading area versus collagen density and gel elasticity.¹⁷

In our study, we conducted an *in vitro* tissue model based on using the stiffness cue to regulate cell phenotype and function through cell-substrate interaction to examine the cellular response to MWCNTs.

While the quick development of synthesizing advanced nanomaterials, the toxicity of the nanomaterials to human body has more and more been realized by the researchers. For example, CNTs, a novel material that has been used in a variety of biomedical applications as intracellular transporters due to their intrinsic ability to cross cell membranes.¹⁸ Like many other synthetic nanomaterials, CNTs have been rapidly developed for the past decade, which brought an increasing concern of its toxicity to the human body. CNTs could cause potential damage to human lung tissue by inhalation and enter into the respiratory tract.¹⁹ CNTs interacts with lung fibroblasts could cause damage to the tissue and has been reported to be responsible for lung fibrosis.²⁰ Other injuries to the lung could occur like inflammation and tissue damage. The invasion of CNTs to fibroblasts could result in many biological changes including cell proliferation, differentiation and collagen production.¹⁹ The activation and proliferation fibroblast could cause the disturbances of collagen production, which leads to fibrosis.²¹ The increase of collagen production could be a sign of lung fibrosis.²²

Thus, it is of great significance to evaluate the potential toxic effects of CNTs. Both *in vivo*^{23, 24, 25} and *in vitro*^{26, 27, 28} studies have been conducted to assess their toxicity and influence on causing cellular dysfunction. To evaluate the toxicity of nanomaterials, animal studies are necessary but expensive, time-consuming and facility limited; while the most important limitation of current *in vitro* models using flat, stiff plastic surfaces under static conditions is that they do not recapitulate characteristics of the ECM with which cells interact *in vivo* and thus cell behaviors on such surfaces significantly deviate from their *in vivo* counterparts.¹⁰ Cell could sense the environment and respond to the physical signals that pass through the substrate. Therefore, cell could behave completely different when culturing on stiff substrate compared to the actual *in vivo* situation. Therefore, it is of great significance to develop *in vitro* biomimetic models which can

provide reliable, rapid and inexpensive methods for toxicity testing and risk assessment of nanomaterials. By taking the stiffness cues into consideration to evaluate the cell sensing nanomaterials, we could physiologically mimic the cell microenvironment, thus providing more convincing results.

A number of researches have been done using fibroblasts as a cell model to study the cell response to substrate elasticity from a variety of perspective.^{29, 30, 31, 32} Figure 3-3 here shows the effect of substrate stiffness on stress fibers of fibroblast. Stress fibers are more prominent in cells that are grown on rigid than on soft substrates.³³

Figure 3-3: Effect of substrate stiffness on cell elongation and polarization. Human foreskin fibroblasts (HFFs) were fixed with 3% PFA in PBS containing 0.25% Triton X-100 6 h after cell seeding and immunofluorescent stained with TRITC-phalloidin. The images show HFFs spreading on glass coated with fibronectin or engineered substrates PDMS (a) or PAAm (b) with various stiffness. The scale bar is 40 μm . (Reproduced with permission from Ref. [34])

So far, few reports have been shown to integrate the stiffness cue into the toxicity study for cellular response to CNTs. In this study, our hypothesis is that substrate stiffness can regulate fibroblast sensing CNTs through cell-substrate interaction. Therefore, the objective of this project is to develop an *in vitro* cell-based model integrated with stiffness cues of physiological relevance to assess the toxicity of nanomaterials. In this view, we systematically investigated the stiffness effects on the fibrogenic response of normal human lung fibroblasts (NHLFs) to MWCNTs.

Toxicity study

Currently, both *in vivo* and *in vitro* have been established for toxicity study. Animal models have been used to test the toxicity effect by injecting the CNTs to animals.³⁵ Pulmonary inflammation and fibrotic reactions have been widely observed during the *in vivo* study.^{23, 24, 25, 36,}
³⁷ Though *in vivo* study could provide a reliable result, it has the intrinsic limitation of complex process, expensive and humanity. *In vitro* study, however, is more controllable and easy. Traditionally, *in vitro* studies for toxicity are mostly based on rigid TCPS plate. Few reports have emerged of toxicity study of fibroblasts on substrate tunable stiffness. In fact, the elastic moduli of TCPS are much more rigid than lung tissue,³⁸ leading to an unavoidable drawback of the most *in vitro* study – it cannot physiologically mimic the *in vivo* microenvironment, as cell phenotypes

and function could be largely regulated by its environment.¹² Thus, it is critical to develop a physiologically relevant *in vitro* model for toxicity study.

Cellular responses to substrate stiffness cue

Cells live in a complex microenvironment, which is composed of the ECM and the neighboring cells. ECM is secreted by cells and, conversely, giving support to the surrounding cells.⁴ The elasticity of ECM from different tissue can vary from soft to stiff, based on the function of the different organ. The stiffness of ECM has recently been shown to have a great impact on cell phenotype and regulating cell functions, including cell contraction¹³, cell proliferation³⁹ and cell migration⁴⁰. Therefore, by engineering a substrate with tunable stiffness, we could try to mimic the *in vivo* tissue softness instead of using the TCPS rigid culture plate doing *in vitro* study. Besides, cellular responses to different substrate stiffness could also be studied, which could guide us to develop a biomimetic cell culture platform to closely mimic the *in vivo* microenvironment. Furthermore, by optimizing the substrate, we can systematically assess the toxicity testing in a more reliable way.

Materials and Methods

Preparation of PAAm hydrogel substrate

The protocol of synthesizing PAAm hydrogel has been widely reported.^{41, 42, 43, 44} We chose two different stiffness: 3 kPa and 100 kPa to represent the range from soft to stiff. Different from conventional stiff TCPS (reported Young's modulus 3.6 GPa⁴⁵), 3 kPa could physiologically mimic the stiffness of lung tissue as reported normal lung tissue has Young's modulus varying from 1-5 kPa.^{38, 46} However, studies have shown that fibrotic lung tissue is much stiffer, where the stiffness could range from 20-100 kPa.^{38, 47} The process of preparing PAAm gel for cell study based on the "sandwich-gel" approach is shown in Figure 3-4.⁴⁴ Briefly, there are 5 major steps:

- 1) Activate the surface of coverglass.** This process is critical because it will allow PAAm gel to adhere to the glass surface. Failing of the activation process will result in gel peeling from the coverglass.

Firstly, 25 mm coverslips were cleaned by immersing in 70% ethanol and sonicating for approximately 1-2 min. The coverslips were air dried or dried by Kimwipes. After the coverglass was completely cleaned and dried, they were placed in glass dishes which are 10 mm in diameter (approximate 6-8 coverslips per dish for 25 mm coverslips) and then immersed with 0.1 M NaOH. Carefully do not let coverslips overlap each other, placing them as far from each other as possible and then incubate for 3 min. While waiting for the incubation of NaOH, 0.5% (3-aminopropyl)trimethoxysilane (3-APTMS) were prepared (approximate 25 mL for one dish) in the chemical hood by diluting 97% stock solution in DI water. After 3 min incubation was completed, NaOH was recycled to the same bottle.

Secondly, working inside the chemical hood, all coverslips were then immersed with prepared 0.5% 3-APTMS, and then incubated for 30 min. While waiting for the incubation of 3-APTMS, 0.5% glutaraldehyde (approximate 25 mL for one dish) were prepared in the chemical hood by diluting 50% stock solution in DI water. (Note: 0.5% glutaraldehyde must be freshly prepared) After 30 min incubation of 3-APTMS, the chemical was disposed into its own waste container. The coverslips were rinsed with DI water in the same dish three times on rocker (little less or equal to the maximum speed), for 10 min each wash.

Finally, after three times wash was completed, all coverslips were immersed in prepared 0.5% glutaraldehyde solution and incubated for another 30 min. After 30 min, glutaraldehyde was disposed into its own waste container. Rinse the coverslips again three times as described previously. After three times wash was completed, the coverslips were dried completely using Kimwipe. The coverslips activation was completed and it could be good for several weeks in a dry area.

2) PAAm gel polymerization. There are four components involved in the polymerization process: acrylamide, N,N'-Methylenebis(acrylamide), Ammonium persulfate (APS) and N,N,N',N'-Tetramethylethylenediamine (TEMED).

Firstly, prepare every component for gel polymerization: 40% acrylamide (A) stocking solution, 2% bis-acrylamide (B) stocking solution, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, 10% APS and TEMED. 40% acrylamide stocking solution was prepared by dissolving acrylamide powder into DI water by wt% to achieve the desired concentration. The preparation of 2% bis-acrylamide stocking solution was similar as described above. 50 mM HEPES

buffer was first prepared by first dissolving HEPES powder in DI water to achieve a concentration of 1M. Dilute 1 M HEPES solution with DI water to achieve the final concentration of 50 mM. Using NaOH or HCl and a pH meter to adjust the pH to 8.2. The 50 mM HEPES solution was autoclaved prior to use. 10% APS was prepared by dissolving ammonium persulfate powder into DI water by wt% to achieve the desired concentration. Then the stocking solution was aliquoted into small tubes with 100 μ L per tube and stored in -20°C freezer.

Secondly, PAAm gel was prepared by mixing A stocking solution, B stocking solution first, then HEPES buffer was added to reach the desired acrylamide percentage. To be able to tune the stiffness, the concentration of A and B was varied during the mixing process according to Table 1. Then working in the chemical hood, for 500 μ L polyacrylamide solution, 1.5 μ L TEMED were added to the solution, after mixing a little bit by vortex, 5 μ L 10% APS was added to the mixture. The mixture was then vortexed again.

Finally, 115-120 μ L (for each 25 mm coverslip) drop of the solution was pipetted on a big square glass plate (glass plate were pre-treated both sides with rain-x solution to make both surfaces hydrophobic). The activated coverslips were quickly placed on top of each drop before it began to polymerize. The "sandwich" gels were incubated at room temperature for approximately 20 min or until the gels are fully polymerized. After polymerization, the coverslips were carefully peeled off from the glass plate, PAAm gels stayed on coverslips. Immerse PAAm gels in 50 mM HEPES buffer overnight to achieve equilibrium.

Table 3-1: Polyacrylamide Young's modulus table. The formula shows the amount needed for acrylamide (A), bis-acrylamide (B), HEPES, TEMED and 10% APS to make a 500 μ L mixture for the desired Young's modulus.

PAAm stiffness	Final concentration A + B	40% Acrylamide (μL)	2% Bis-acrylamide (μL)	50 mM HEPES (μL)	TEMED (μL)	10% APS (μL)	Young's modulus (kPa)
Soft	5% + 0.1%	62.5	25	412.5	1.5	5	3
Rigid	12% + 0.5%	150	125	225	1.5	5	100

3) Activate PAAm gel surface. This process is critical for cell study because it will allow ECM conjugation on the PAAm surface. Failing of activation PAAm gel will result in cell detaching from the gel surface.

Firstly, a sheet of parafilm was placed on the bottom of a big petri dish. A vial of 24 μ L aliquoted sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate (sulfo-SANPAH) was thawed at room temperature, then 600 μ L 50 mM HEPES buffer was added to the vial to make a 1 mg/mL work solution (sulfo-SANPAH was 25 mg/mL in DMSO).

Secondly, the gels were taken out of the HEPES buffer and the excess buffer was wicked off with a Kimwipe. The coverslips were placed on the parafilm sheet supported by a 150-mm petri dish with gel side up. 200 μ L working solution was added to each gel (for a 25 mm coverslip). The petri dish was placed under UV lamp and the distance between the top of the gel and the UV lamp was 5 cm. The gels were under long wave exposure for 6 min and then the chemical was carefully sucked out using Kimwipe. The PAAm gels were placed back into UV chamber in the same position and were under UV exposure for another 6 min.

Finally, the PAAm gels were taken out of the UV chamber and immersed into HEPES buffer solution. The gels were washed with HEPES buffer three times for 10 min each, until the red color fade away.

4) Coat PAAm gel with ECM protein. Fibronectin coating on the activated PAAm gel surface to allow the cell to attach.

Firstly, fibronectin working solution was prepared by diluting the stock solution into 50 mg/mL.

Then, a wet chamber was prepared using a 15 mm dish with parafilm placing on the bottom and wet paper towels on the side. 200 μ L fibronectin drops were added on the parafilm sheet.

Finally, the PAAm gels were face down on the fibronectin solution drops and incubated overnight at 4 °C or 5 hr at room temperature.

5) Seed cell on coated PAAm gel surface. After surface conjugation of fibronectin, the hydrogels were rinsed three times with sterile PBS under bio-hood and followed by UV exposure for 30 min.

Figure 3-4: Schematic illustration of the sandwich gel assembly process. (i) Activation of glass surfaces with NaOH, 3-APTMS and glutaraldehyde. (ii) Polyacrylamide gel polymerization on the top of the activated glass. (iii) Conjugation of sulfo-SANPAH on the surface of the polyacrylamide gel by exposing gel surfaces to UV light, allowing the photoactivatable nitrophenyl azide group to bond with the polyacrylamide. (iv) Finally, the NHS ester (N-hydroxysuccinimide) group in sulfo-SANPAH is able to bond with the future ECM proteins like fibronectins or collagens, which is essential for cells (green) to be able to adhere to the polyacrylamide surface (v). (Reproduced with permission from Ref. [44], step vi will be excluded from our study)

Preparation, dispersion, and characterization of MWCNTs

MWCNTs were used for toxicity study. The MWCNTs solution was prepared as previously reported.⁴⁸ Briefly, 1 mg of MWCNTs was dispersed in 1 mL of phosphate buffered saline (PBS; Life Technologies, Carlsbad, CA, USA) containing 5 mg of bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) by slightly sonicating in a biological safety hood. The MWCNTs stock solution was stored at 4 °C for long term use. When added to the cells, the MWCNTs was further diluted in the culture medium to obtain the desired concentrations (0.06–0.6 $\mu\text{g}/\text{cm}^2$) before use. The *in vitro* CNT exposure doses were determined based on *in vivo* CNT exposure data normalized to the alveolar surface area in mice. Previously, the *in vitro* surface area dose of CNTs used was of 0.08–0.16 $\mu\text{g}/\text{cm}^2$, relevant to *in vivo* exposure doses of 40–80 $\mu\text{g}/\text{mouse}$.⁴⁹ In this study, the doses of 0.06–0.6 $\mu\text{g}/\text{cm}^2$ were chosen to cover the aforementioned range.

The length (L) and width (W) of individual dry CNT were $8.19 \pm 1.7 \mu\text{m}$ (L) and $81 \pm 5 \text{ nm}$ (W). The size of MWCNTs was measured by using AFM. AFM measurement was performed in tapping mode using a Veeco NanoScope MultiMode scanning probe microscope under ambient

conditions. Topography images were recorded at the fundamental resonance frequency of the cantilever, with a typical scan rate of 1 Hz and a resolution of 512 samples per line.

Production of collagen I was assessed by incubating the cells with MWCNTs at different doses for 72 h on TCPS surfaces as well as PAAm gel surfaces followed by western blot analysis. Previous studies showed that MWCNTs at high doses ($\geq 0.6 \mu\text{g}/\text{cm}^2$) caused severe cytotoxicity.⁴⁹ Because of the dose-dependent collagen I production, the dose of $0.2 \mu\text{g}/\text{cm}^2$ was chosen for assessment of the substrate stiffness mediated fibrogenic response of the fibroblasts to MWCNTs.⁵⁰

Primary cell culture

NHLFs were used as our model for cell study due to the fact that lung fibroblasts are a representative cell type that presents in the lung interstitium, which is considered to be associated with collagen production during fibrogenesis.^{20, 32, 51} NHLFs were cultured in fibroblast basal medium (FBM) supplemented with 2% FBS, 0.1% recombinant human fibroblast growth factor basic (rhFGF-B), 0.1% insulin, 0.1% gentamicin/amphotericin-B (Lonza, Walkersville, MD, USA), 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. Cells were used between passages 3-6.

Characterization of cellular response

Cell proliferation, immunofluorescent staining, and western blotting were used to conduct various experiments to examine the cellular response of NHLFs to substrate stiffness.

Cell viability. Cell viability on PAAm gel with two different stiffness, 3 kPa and 100 kPa, as well as on TCPS was evaluated by using CellTiter 96® AQueous One Solution Cell Proliferation Assay. The data on TCPS was used as a control. NHLFs were seeded on PAAm gels (3 kPa and 100 kPa) that were made on 8 mm coverglass, which could fit into 48-well plate. Cells seeded

directly on TCPS in 48-well plate were used as a control. For each group, three parallel samples were prepared. Cells were allowed overnight to fully attach to the substrate. MWCNTs with a concentration of $0.2 \mu\text{g}/\text{cm}^2$ were added on the following day and incubated with the cells. After 72 h of incubation, cell viability assay was performed by using CellTiter 96® AQueous One Solution Cell Proliferation kit according to the manufacturer protocol. Briefly, the PAAm gels with cells were transferred to a new 48-well plate and the cell proliferation kit reagent, CellTiter 96® AQueous One Solution Reagent, was diluted in fibroblasts growth media by a concentration of 1:5 and added to the new plate. For TCPS samples, the original media was replaced by the same amount of cell proliferation reagent. The 48-well plates were then incubated for an additional 4 h at 37 °C. After 4 hours of incubation, 200 μL solution was sucked out from each well and added to a 96-well plate. The absorbance of each well at a wavelength of 490 nm was measured by means of an FLUOstar OPTIMA Microplate Reader (BMG LABTECH, Cary, NC, USA). The culture medium containing the reagent was set as background.

Immunofluorescent staining. NHLFs on 3 kPa, 100 kPa PAAm gel as well as on TCPS substrates were fixed with 4% PFA for 30 min at room temperature, permeabilized with PBS containing 0.2% Triton-X 100 for 20 min at room temperature, and blocked using a PBS solution containing 0.03 g/mL BSA, 1% goat serum (Sigma-Aldrich), and 0.2% Triton-X 100 for 1 h. Primary antibody was conjugated in PBS solution with 0.2% Triton-X 100 for 2 h in ambient condition. The samples were then washed with PBS solution containing 0.2% Triton-X 100 for three times, 5 min for each wash. After the completion of washing, the secondary antibody was conjugated in the same solution for 1h in dark at room temperature. F-actin was stained with Alexa Fluor 488 phalloidin (Life Technologies), alpha-smooth muscle actin (α -SMA) was stained with anti- α -SMA mouse polyclonal antibody (Abcam) and the nuclei were counterstained and mounted

using ProLong Gold Antifade Reagent with DAPI. The fluorescent images were taken by using a Nikon Ti Eclipse Fluorescence Microscope. The confocal images were taken by using Zeiss Violet Confocal Microscope. 3D deconvolution was processed by using Imaris (Bitplane USA, Concord, MA).

Reactive oxygen species (ROS) staining. NHLFs were seeded on the 3 kPa PAAm gel substrates at a density of 15000 cells per cm^2 and 100 kPa PAAm gel substrates at a density of 10000 cells per cm^2 . Cells cultured on TCPS at a density of 7500 cells per cm^2 were set as the control. Cells were cultured and allowed fully attached overnight and treated with $0.2 \mu\text{g}/\text{cm}^2$ MWCNTs on the following day. Cells were then incubated with MWCNTs for another 16 h. The culture medium was then replaced with 1 mL Hank's balanced salt solution (HBSS) containing $10 \mu\text{M}$ dichlorodihydrofluorescein diacetate (DCFDA) and incubated for an additional 30 min. The cells were finally fixed using 4% PFA solution and the nuclei were visualized using ProLong Gold Antifade Reagent with DAPI. At least 10 images for each group were taken under the Nikon Ti Eclipse fluorescence microscope with the same setting. The fluorescence intensities of images were analyzed by using ImageJ and the ROS production per cell was calculated by subtracting the background fluorescence intensity from Image fluorescence intensity, then divided by cell number.

Western blot. The western blotting assay was performed as previously reported.⁵² Passage 3 NHLFs were seeded on the 3 kPa PAAm gel substrates at a density of 15000 cells per cm^2 and 100 kPa PAAm gel substrates at a density of 10000 cells per cm^2 . Cells cultured on TCPS at a density of 7500 cells per cm^2 were set as the control. Cells were cultured and allowed fully attached overnight and treated with $0.2 \mu\text{g}/\text{cm}^2$ MWCNTs on the following day. After incubation with MWCNTs for 72 h, the whole cell protein was extracted by lysing cells with radioimmune precipitation assay (RIPA) buffer (Santa Cruz Biotechnology, CA, USA) containing protease and

phosphatase inhibitors for 30 min on ice. The cell protein was then separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Darmstadt, Germany). The PVDF membranes were then blocked with 5% nonfat milk in 1× Tris-buffered saline with 0.1% Tween-20 (TBST), followed by blotting with primary antibodies at 4 °C overnight with shaking, and incubating with a horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich) for 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescence detection reagents (EMD Millipore) and acquired by using a C DiGit Blot Scanner (LI-COR Biosciences, Lincoln, NE, USA). The immunoblotting acquired was then analyzed by using ImageJ software (<https://imagej.nih.gov/ij/>).

Rho-associated Protein Kinase (ROCK) inhibitor Y27632 treatment

Y27632, a specific drug to inhibit ROCK pathway was used to inhibit Rho kinase without changing other kinase pathways.^{53, 54} After cells were seeded and attached overnight, Y27632 was added on the following day 2 hours before MWCNTs treatment. The desired concentration was achieved by diluting the stock 10 mM Y27632 solution with fibroblast complete growth media. See Table 2 for the predefined concentration used in the experiment. Observation of the cells using bright field microscope to see if there are any morphology changes after Y27632 has been added. See supporting information for the bright field images taken to record the daily changes of the cell morphologies.

Table 3-2: The administrative dosage adding pattern for Y27632 treatment.

Total dosage	10 μ M			16 μ M
Day 1	3 μ M	5 μ M	10 μ M	8 μ M
Day 2	3 μ M	5 μ M		8 μ M
Day 3	4 μ M			

Statistical analysis

The data will be presented as mean $\pm$ standard error of mean (S.E.M.). Student's *t*-test will be used to determine the statistical difference. The proliferation ($n = 3$), collagen I expression ($n = 3$) and ROS generation ($n > 10$) of NHLFs were measured from three independent replicates for each condition. Statistically significant differences will be considered at a level of $p < 0.05$.

Results and Discussion

Cell morphology

Cell morphologies on 3 kPa, 100 kPa PAAm gel as well as on TCPS were examined first under bright field. The phase contrast images were shown on the top row in Figure 3-5. Fibroblasts displayed different morphologies as the substrate stiffness changed. The majority of the cells on soft substrate (3 kPa) remained round shape without spreading out, indicating a weak cell-substrate interaction. Cells on the stiff substrate (100 kPa) were similar to that on TCPS, which were elongated and stretched out mostly.

Immunofluorescent staining of F-actin was also performed to visualize the actin fibers and the cell morphology difference between 3 kPa, 100 kPa PAAm gels and TCPS. The fluorescent images were shown on the bottom row in Figure 3-5. Similar to what was observed in the bright field, cells on soft substrate (3 kPa) remained round shape without spreading out and there was no obvious actin fiber that has been seen; cells on 100 kPa PAAm gel as well on TCPS were largely stretched out and elongated, with more visible actin fibers.

Figure 3-5: Cell morphologies of NHLFs on different PAAm gel substrates (3 kPa, 100 kPa) and TCPS. Top row: phase contrast images of NHLFs cell morphologies on different PAAm gel substrates. The scale bars are 400 μm . Bottom row: fluorescent images of fibroblasts cell morphologies on different PAAm gel substrates. The F-actin (green) was immunostained with Alexa Fluor 488® phalloidin and the nuclei (blue) were stained with DAPI. The scale bars are 50 μm .

CNT dosages determination

To further determine the dosage of MWCNTs, cell proliferation assay on TCPS with various MWCNTs dosages was performed, with different incubation length from 1 day to 4 days. The results of cell proliferation assay on TCPS with various MWCNTs dosages are shown in Figure 3-6. Cells cultured without adding MWCNTs were used as a control. The reason of this experiment was to give an idea on the choice of different MWCNTs dosage as well as the culture length. We found that on day 3, the CNT group with $0.06 \mu\text{g}/\text{cm}^2$ and $0.2 \mu\text{g}/\text{cm}^2$ both showed a

significant increase in cell proliferation, while for a higher dosage of $0.6 \mu\text{g}/\text{cm}^2$, the proliferation started to decline at day 3, and further decreased on day 4 culture. This result was consistent with the previous report that MWCNTs showed toxicity to the cells at a higher dosage doses ($\geq 0.6 \mu\text{g}/\text{cm}^2$).⁴⁹

Figure 3-6: Determination of MWCNTs dosage by cell proliferation assay on TCPS. The cells were treated with MWCNTs at a dosage of $0.06 \mu\text{g}/\text{cm}^2$, $0.2 \mu\text{g}/\text{cm}^2$ and $0.6 \mu\text{g}/\text{cm}^2$. Cells treated without MWCNTs were used as a control. Cells treated with different dosages of MWCNTs were incubated for 4 days. Proliferation data was obtained every 24 h. *: significant difference ($p < 0.05$) from the control.

According to previous study,⁵² a dose of $0.2 \mu\text{g}/\text{cm}^2$ was hence chosen for establishing the *in vitro* model of cell sensing nanomaterials. As shown in Figure 3-7, compared to $0.06 \mu\text{g}/\text{cm}^2$, adding $0.2 \mu\text{g}/\text{cm}^2$ MWCNTs could induce more collagen production although these two dosages showed similar proliferation after 72 h of incubation. Meanwhile, since MWCNTs showed toxicity

to the cells at a higher dosage doses ($\geq 0.6 \mu\text{g}/\text{cm}^2$), $0.2 \mu\text{g}/\text{cm}^2$ was finally chosen for assessment of substrate stiffness regulated the fibrogenic response of NHLFs to MWCNTs.

Figure 3-7: Dose-dependent collagen production of fibroblasts treated with MWCNTs. (A) Immunoblotting of collagen I. β -actin was used as the loading control. Fibroblasts were treated with MWCNTs at different dosages of 0.06, 0.2 and $0.6 \mu\text{g}/\text{cm}^2$ for 48 h. (B) Densitometric analysis of collagen I production based on the results of the immunoblotting in (A). (Adapted with permission from Ref. [52])

CNT effects on cytoskeleton structure and nuclei

We further examined the cell cytoskeleton structure by performing immunofluorescent staining of F-actin and α -SMA. F-actin was stained to demonstrate the presence of actin stress fibers in NHLFs in order to visualize the cytoskeleton structure. α -SMA is a commonly used molecular marker that has been found expressed by myofibroblasts, which is highly associated with the lung fibrosis.^{55, 56} An important hallmark during fibrogenesis is the differentiation of fibroblasts to myofibroblasts.⁵⁷

F-actin was immunostained with Alexa Fluor 488® phalloidin, which is a high-affinity F-actin probe. α -SMA was stained with anti- α -SMA mouse polyclonal antibody. The confocal images were shown in Figure 3-8. Corresponding to the previous cell morphology results, fibroblasts exhibited different morphologies as the substrate stiffness changed. Cells on 3 kPa remained compact morphologies with no visible stress fiber. On the contrary, cells on 100 kPa substrate were elongated and stretched out with stress fiber formation. The stress fiber formation became more obvious on TCPS substrates, suggesting that the expression of F-actin increased when increasing the substrate stiffness. The stiffer the substrate was, the more expression of F-actin.

Figure 3-8: Immunofluorescent staining of F-actin, α -SMA and nuclei of NHLFs on different substrates: 3 kPa, 100 kPa and TCPS. The last column on the right highlights the nuclei of the cells cultured on TCPS. Top row: cells treated without MWCNTs were used as controls. Second row: cells treated with $0.2 \mu\text{g}/\text{cm}^2$ MWCNTs. Third row: bright field images of the second row. The arrows point to the MWCNTs localization inside the cells. The F-actin (green) was immunostained with Alexa Fluor 488® phalloidin, the α -SMA (red) was stained with anti- α -SMA mouse polyclonal antibody and the nuclei (blue) were stained with DAPI. The scale bars are $20 \mu\text{m}$.

The influence of MWCNTs to cytoskeleton structure was revealed in Figure 3-8 with a combination of the second and the third row. The CNTs structures could be seen clearly in the bright field images and it was observed that when MWCNTs were present, the actin fiber was distorted or disrupted, as well as the nuclei structure shown in the last column. Without MWCNTs, the actin fiber was continuous and intact and the nuclei structure was in oval shape and smooth. The detailed explanation for this phenomenon is still unclear and is open to investigation. However, several studies have been done which revealed that CNTs or functionalized CNTs possessed the ability to penetrate cell membranes and reach the nucleus of cancer cells.^{58, 59, 60, 61} It still remains to be elucidated that how CNTs translocate across the cell membrane. One of the studies proposed that the translocation mechanism of CNTs was comparable to cell penetrating peptides (CPPs), where they were able to reach nuclei due to their positive charge and the nuclear localization signal conveyed by the amino acid sequences.⁶⁰ It seemed from our results that substrate stiffness could also be responsible for the CNTs uptake mechanism inside the cells (see Figure 3-9). Compared to TCPS substrate, the nuclei of cells cultured on soft PAAm gel substrate were less disrupted. However, solid evidence and more investigation are needed to explain this observation.

Figure 3-9: MWCNTs effects on nuclei. The first column shows the immunofluorescent staining of F-actin (green), α -SMA (red) and nuclei (blue) of NHLFs on different substrates: 3 kPa, 100 kPa and TCPS. The second column shows the single channel of nuclei. The insets are the 3D deconvolution of nuclei. The last column on the right shows the overlay images of the nuclei and the bright field. The scale bars are 20 μ m.

Fibrogenesis response

Cell Viability. Previous study has shown that increased cell proliferation is one of the hallmarks of lung fibrosis,²⁰ which was first investigated in the study to evaluate the fibrogenesis response of NHLFs to MWCNTs on substrates with different stiffness. The results of cell viability assay on 3 kPa and 100 kPa PAAm gel substrates as well as on TCPS with an MWCNTs dosage of 0.2 μ g/cm² were shown in Figure 3-10.

Figure 3-10: Cell viability of NHLFs on different PAAm gel substrates and TCPS control. The cells were treated with MWCNTs for 72 h (labeled as MWCNTs in the plot). Cells treated without MWCNTs were used as controls (labeled as CTRL in the plot). *: significant difference ($p < 0.05$) from the TCPS controls; +: significant difference ($p < 0.05$) between groups.

As shown in Figure 3-10, cell number increased when stiffness increased, indicating that on stiffer substrate, cell proliferation was more active than that on soft substrate. To compare the MWCNTs effects within group, there was no significant increase on soft substrate (3 kPa) while on 100 kPa gel substrate and TCPS, cell number was significantly increased, suggesting that MWCNTs could enhance proliferation in a stiffness-dependent manner. Moreover, according to the p -value from T-test, although on 100 kPa and TCPS, cell proliferation both showed a significant increase, it increased much more on 100 kPa ($p = 0.003$) compared to that on TCPS ($p = 0.020$). This phenomenon was agreed with a previous finding suggesting that soft substrate has a much lower membrane tension compared to the stiff substrate, where the high membrane tension of the cells on TCPS makes it hard for nanoparticles to enter, resulting in a higher cellular uptake of nanoparticles on the soft substrate.⁶² This could be a possible explanation for the difference in enhancement of CNTs on 100 kPa and TCPS. However, our results showed that cell proliferation on the softest substrate (3 kPa) did not result in a noticeable increase, which may seem contradictory to the previous theory. This could possibly result from the limited spreading area that cells remain on the soft substrate. Previous results showed a rounded morphology of fibroblasts that cultured on 3 kPa PAAm gel, whereas cells cultured on 100 kPa PAAm gel and TCPS substrates were more spread and elongated. It has been also studied that when the cell area was substantially decreased compared to the larger spreading area on two stiffer substrates, cellular uptake of nanoparticles resulted in a much lower total amount.⁶²

Collagen I Expression. Western blotting was used to qualitatively and quantitatively measure the collagen I production with or without adding MWCNTs. Figure 3-11 here showed the western blot result of collagen I protein band and quantitative analysis.

Figure 3-11: Collagen I expression of NHLFs treated with or without $0.2 \mu\text{g}/\text{cm}^2$ MWCNTs. (A) Immunoblotting of collagen I on 3 kPa, 100 kPa PAAm gel substrates as well as on TCPS. Tubulin was used as the loading control. Fibroblasts were treated with MWCNTs for 72 h in positive group (labeled as MWCNTs in the plot). Cells treated without MWCNTs were used as controls (labeled as CTRL in the plot). (B) Densitometric analysis of collagen I expression based on the results of immunoblotting in (A). *: significant difference ($p < 0.05$) compared to the TCPS controls.

The collagen I production of fibroblasts on 3 kPa and 100 kPa was much lower than that on TCPS, indicating that the expression of collagen depended on the stiffness of substrates. The expression on 3 kPa substrates was significantly lower than TCPS with or without CNT treatment, while on 100 kPa substrates it was less significant.

Other than cell proliferation, excessive deposition of collagen matrix was also considered as a hallmark in lung fibrosis.⁶³ Previous study has shown that collagen I production could be significantly increased by dispersed SWCNT and MWCNT both *in vivo* and *in vitro*.⁶⁴ From our results, collagen I production was enhanced on all substrates with the presence of MWCNTs but not significantly. It is speculated that this insignificant influence of MWCNTs was due to the competing effects between CNTs induced collagen I production and the disruption of cytoskeleton structure by CNTs. Previous studies have shown that CNT could induce lung fibroblasts differentiation to myfibroblasts both *in vivo* and *in vitro*.^{22, 48, 65} The presence of myfibroblast would result in synthesizing and secreting the ECM, which will increase the collagen I production.^{57, 66} From another perspective, our previous observation of immunofluorescent staining in Figure 3-8 showed that the cytoskeleton structure seemed to be disrupted when treated with CNTs, along with the decreasing of α -SMA expression (red), which was a competitive effect compared to CNTs induced myfibroblast differentiation because α -SMA was a commonly used molecular marker indicating the formation of myfibroblast. The collagen I production under this circumstance was expected to decrease. In the view of these two contradictory effects, the significance of CNTs induced collagen production was compromised. However, this was not a conclusive result where more solid evidence regarding the cytoskeleton organization was needed to consolidate our finding.

ROS Generation. Previous studies have shown that oxidative stress was associated with the toxicity of the nanoparticles, hence played an important role in nanomaterial-induced fibrogenesis.^{48, 67, 68} Lung tissue is generally exposed to more oxygen, which potentially leads it to a more vulnerable condition of experiencing oxidative stress.⁶⁹ MWCNTs could enter lung interstitial via inhalation, where the deposition of nanoparticles could have the potential to generate oxidative stress, thus triggering fibrogenic response. Therefore, the influence of substrate stiffness on ROS generation in response to MWCNTs has been examined.

Figure 3-12: The influence of ROS generation of NHLFs on substrate stiffness in response to MWCNTs. The NHLFs were treated with MWCNT for 16 h (labeled as MWCNTs in the plot). Cells treated without MWCNTs were used as controls (labeled as CTRL in the plot). ROS was stained with DCF-DA and nuclei were stained with DAPI. Quantitative ROS image analysis was obtained from at least ten separate fields. *: significant difference ($p < 0.05$) compared to the TCPS controls. +: significant difference ($p < 0.05$) between groups.

As shown in Figure 3-12, the quantitative analysis of ROS generation suggested that as the stiffness increased, the ROS generation increased with or without CNTs exposure, suggesting that substrate stiffness could regulate ROS generation of NHLFs. The effects of MWCNTs treatment resulted in enhanced ROS generation on all substrates. The level of increase was different between these three substrates, which was correlated with the previous cell proliferation results. The increase was not obvious and no significance on the soft substrate (3 kPa) whereas significant increases were both observed on 100 kPa PAAm substrate and TCPS.

Although extensive studies have been done that suggested the CNTs induced substantial ROS production, less effort has been made to explore the stiffness mediated ROS generation when exposed to CNTs. One studies used MSCs as a model cell to investigated the stiffness affected ROS generation by using PAAm gel substrate in kilopascal range compared to the fibronectin-coated glass surface in gigapascal range, which revealed that ROS generation on the soft substrate was increased by a 2-4 fold when remained noncytotoxic.⁷⁰ The result was not agreed with our findings possibly due to the difference in cell model and the different *in vivo* condition. Our result indicating that fibroblasts endured more oxidative stress when cultured on stiffer substrates (100 kPa), which represented the fibrotic lung tissue. On the contrary, on soft substrate (3 kPa), normal lung tissue was under lower oxidative stress. This result highlighted the importance of incorporating stiffness cue to build a physiologically relevant model to investigate cell behavior for disease model.

Mechanosensing

Influence of stiffness on focal adhesion kinase (FAK) and phosphorylated tyrosine-397 FAK (pFAK). FAs are dynamic protein molecules that regulate signals reflecting how cells

respond to ECM, serving as a link between cytoskeleton and ECM.^{71, 72, 73} FAK is a key signaling kinase in FAs which could respond to substrate stiffness, thus mediate mechanotransduction.⁷⁴ Western blotting was performed to examine the total and activated FAK levels by using antibodies specific for FAK and pFAK.

Figure 3-13: Protein expression of NHLFs treated with or without 0.2 μg/cm² MWCNTs. (A) Immunoblotting of F-actin, α-SMA, FAK and pFAK on 3 kPa, 100 kPa PAAm gel substrates as well as on TCPS. Tubulin was used as the loading control. Fibroblasts were treated with MWCNTs

for 72 h in positive group. (labeled as MWCNTs in the plot). Cells treated without MWCNTs were used as controls (labeled as CTRL in the plot). (B) Densitometric analysis of the protein level of F-actin, α -SMA, FAK and pFAK based on the immunoblotting results of (A). *: significant difference ($p < 0.05$) compared to the TCPS controls.

Generally speaking, the stiffness effects of all four proteins were significant on 3kPa PAAm gel while the CNTs effects on the protein expression didn't show significant influence. From the immunoblotting in Figure 3-13A, both FAK and pFAK showed a similar trend of increased level when increasing the substrate stiffness. Quantitative analysis of the protein bands in Figure 3-13B showed that compared to TCPS controls, both the protein level of FAK and pFAK showed significantly decreased expression on soft substrate (3 kPa) regardless of whether under CNTs treatment. The effects of CNTs on the expression of FAK was highly stiffness-dependent. On soft substrate (3 kPa), the CNTs significantly increased the FAK expression, whereas on 100 kPa gel substrate and TCPS, FAK expression showed slightly increase and decrease, respectively. For pFAK, CNTs induced expression on all three substrates while none of which was significant.

Influence of stiffness on F-actin and α -SMA. One of the important characters of lung fibrosis is the differentiation of lung fibroblasts into myofibroblasts which synthesize and secrete the ECM.^{57, 66} Fibroblasts in normal tissue exhibit little ECM production whereas in damaged tissue, they actively migrated towards the injured tissue and began to produce ECM.^{56, 75} We thus investigated the F-actin expression and also α -SMA expression, a molecular expressed by myofibroblasts to identify the influence of substrate stiffness affected cell sensing CNTs.

As shown in Figure 3-13A, cells on soft substrate (3 kPa) generally expressed little F-actin as well as α -SMA. The soft substrate is comparable to normal lung tissue stiffness *in vivo*. When

the stiffness increase to 100 kPa and as well as on TCPS, the expression of F-actin and α -SMA were augmented. It has been suggested that fibroblasts could form contractile stress fibers when activated by stiffer substrate because matrix mechanical change caused constant change of ECM remodeling. from, whereas little to no stress fibers were formed on soft substrate.^{56, 65, 76} From the quantitative analysis shown in Figure 3-13B, the CNTs effects on F-actin formation was not clearly shown on softer substrate (3 kPa and 100 kPa). The increase on TCPS induced by CNTs was not significant, either. Moreover, it was observed that when treated with CNTs, α -SMA expression was significantly decreased on softer substrate (3 kPa and 100 kPa) compared to the TCPS control.

Effects of intracellular tension

Effects of intracellular tension on TCPS. To further understand how cells respond to substrate stiffness in molecular level, we examined the effects of intracellular tension on fibroblasts by inhibition of the Rho-ROCK signally pathway. RhoA is one of the most studied that belongs to Rho family of GTPase, which is a family of small G proteins that involves in intracellular signal transduction. Extensive studies have shown that RhoA could regulate the remodeling of actin cytoskeleton, inducing stress fiber formation and upregulating the focal adhesion formation.⁷⁷ It has been reported that higher intracellular tension was observed on stiff substrate, thus cells are more spreading and elongated, generating more stress fibers and where they adversely exerted augment traction force on the substrate.^{77, 78, 79, 80} By inhibiting ROCK signally pathway, which is activated by RhoA, we could further investigated the mechanism of substrate stiffness modulated cellular response. Y27632, a pharmacological inhibitor of ROCK, was used inhibit the ROCK signally pathway.

To determine the proper dosage for Y27632, we first conducted the experiment on TCPS by using several dosages of Y27632 and different administrative adding pattern (see Table 2). Cell morphologies were taken under bright field as a record (see Figure 3-14).

Figure 3-14: Bright field images of cells treated with different dosages of Y27632. NHLFs were seeded on day 0. Y27632 was added from day 1 according to the predetermined dosages described in Table 3-2.

Since the most commonly used dosage used for Y27632 is $10\text{ }\mu\text{M}$ ^{54, 81}, we chose $10\text{ }\mu\text{M}$ as the primary targeting dosage. We overserved that when adding a total dosage of $10\text{ }\mu\text{M}$, cells experienced a distinct morphology change as they were being highly attenuated and elongated (see Figure 3-15). While when adding $5\text{ }\mu\text{M}$ for each day for two days in a row, cells largely remained as their original morphologies. In this view, $(5 + 5)\text{ }\mu\text{M}$ was chosen for the following stiffness study. Moreover, fluorescent images showed that stress fibers were largely decreased when treated with Y27632, while there were clear stress fibers present in the control group (no Y27632 treatment). This result agreed with the previous theory that Rho-ROCK signally pathway is associated with stress fiber formation.

Figure 3-15: Images of NHLFs cell morphologies treated with different dosages of Y27632. Cells were cultured on TCPS. Fibroblasts were treated with MWCNTs for 72 h in positive group (labeled as MWCNT in the figure). Cells treated without MWCNTs were used as controls (labeled as CTRL). The F-actin (green) was immunostained with Alexa Fluor 488® phalloidin and the nuclei (blue) were stained with DAPI. The bright field images represent MWCNTs localized in the cells. The scale bars are 50 μm .

To further evaluate the effects of ROCK signaling in regard to different Y27632 dosages treatment, western blotting was performed to examine the collagen I expression under various Y27632 treatment. Figure 3-16 showed the immunoblotting and the quantitative analysis of

collagen I expression, which indicated a downregulated mechanism of Y27632 to collagen I expression regardless of the dosage. However, statistical analysis did not reveal a significant decrease for either of the three dosages. Moreover, the treatment of MWCNTs didn't result in a significant increase in collagen expression. This observation was possibly related to the cell morphology change when adding Y27632 and decreased cell spreading area. It was observed that when treated with Y27632, cells were stretched and changed their morphologies in a dose-dependent manner. When increased the dosage from 10 μM total to a (8 + 8) μM total, the elongation and morphology change was increased, resulting in a decreased cell spreading area. For a total of 10 μM , adding in one day caused more morphology change than adding 5 μM each day. The reduced cell area compared to control could result in a decrease in cellular uptake of the nanoparticle. However, inhibiting ROCK pathway by Y27632 would initially lower the intracellular tension, and previous reports showed a upregulated uptake of nanoparticle with lower membrane tension.⁶² According to Figure 3-16B, the collagen I production showed no significant change after adding CNTs, it was speculated that it was because of a neutralization of the two competing mechanisms.

Figure 3-16: Collagen I expression of NHLFs exposed to $0.2 \mu\text{g}/\text{cm}^2$ MWCNTs when treated with different dosages of Y27632 on TCPS. (A) Immunoblotting of collagen I treated with different dosages of Y27632 TCPS. Tubulin was used as the loading control. Fibroblasts were treated with MWCNTs for 72 h in positive group (labeled as MWCNTs in the plot). Cells treated without MWCNTs were used as controls (labeled as CTRL in the plot). (B) Densitometric analysis of collagen I expression based on the results of immunoblotting in (A).

Effects of intracellular tension on 100 kPa PAAm gel. Based on the immunofluorescent results and western blotting performed on TCPS, we chose $(5 + 5) \mu\text{M}$ for the following study of intracellular tension effects on 100 kPa PAAm gel. Western blotting also performed to show the collagen I expression when inhibiting ROCK on 100 kPa PAAm gel (see Figure 3-17). In contrary

to the results obtained from TCPS, Y27632 exhibited an upregulated mechanism when cultured on 100 kPa PAAm gel. Although the detailed explanation of this phenomena remained unclear and needed to be further explored, we speculated that it might because culturing on soft gel (100 kPa compared to TCPS) lowered the membrane tension, which favored the cellular uptake of nanoparticles,⁶² thus increasing the collagen I production when treated with both Y27632 and MWCNTs. The preliminary condition of this explanation based on the similar spreading area that we observed before and after adding Y27632 on 100 kPa PAAm gel (see Figure 3-17B).

Figure 3-17: Collagen I expression of NHLFs exposed to $0.2 \mu\text{g}/\text{cm}^2$ MWCNTs and (5 + 5) μM Y27632 on 100 kPa PAAm gel substrates. (A) Immunoblotting of collagen I treated with (5 + 5)

μM Y27632. Tubulin was used as the loading control. Fibroblasts were treated with MWCNTs for 72 h in positive group (labeled as MWCNTs in the plot). Cells treated without MWCNTs were used as controls (labeled as CTRL in the plot). (B) Comparison of cell morphologies before and after adding Y27632. The scale bars are 200 μm .

We further conducted the same experiment by changing the substrate material to PDMS (see Figure 3-18) while remaining the same substrate stiffness. PDMS with a 100 kPa stiffness was obtained by varying the ratio of the base material and curing agent (30:1) as well as controlling the curing condition.^{82, 83, 84}

Figure 3-18: Collagen I expression of NHLFs exposed to $0.2 \mu\text{g}/\text{cm}^2$ MWCNTs and $(5 + 5) \mu\text{M}$ Y27632 on 100 kPa PDMS gel substrates. (A) Immunoblotting of collagen I treated with $(5 + 5) \mu\text{M}$ Y27632. Tubulin was used as the loading control. Fibroblasts were treated with MWCNTs for 72 h in positive group (labeled as MWCNTs in the plot). Cells treated without MWCNTs were used as controls (labeled as CTRL in the plot). (B) Densitometric analysis of collagen I expression based on the results of immunoblotting in (A).

From the immunoblotting, we could see that the collagen I expression under different condition showed no obvious change either treated with Y27632 or MWCNTs. The different result obtained from PDMS compared to previous PAAm result was possibly due to the structural difference between PDMS and PAAm, although they were designed for the same stiffness. One study has shown that cell responses to the substrate stiffness were not from the bulk stiffness but the coated ECM anchor point.⁸⁵ The structure of PAAm is porous, where the pore size is associated with stiffness. Even under the same bulk stiffness, PDMS didn't have the porous structure, which might result in difference in cell behavior.

Conclusion

There has been evolving and compelling evidence showed that substrate stiffness could have a profound influence on cell behavior including cell adhesion, cell spreading, cell migration, cell proliferation and cell migration. More and more studies have been done to realize the important roles that substrate stiffness played in regulating cell behavior. This study systematically investigated cellular responses to CNTs by incorporating stiffness cue to replicate the physiological condition of the human body. PAAm gel substrates were fabricated to represent normal human lung tissue and fibrotic lung tissue, respectively. NHLFs were cultured on PAAm substrates with different stiffness as well as on TCPS. Our findings indicated that substrate stiffness played a significant role in regulating cell phenotype and function, including cell adhesion, cell spreading, cell elongation and cell proliferation.

NHLFs on soft (3 kPa) substrates were rounded without actin fibers, on the contrary, cells on stiffer (100 kPa) substrates and TCPS were largely elongated with visible stress fibers. The fibrogenesis response of the cell to MWCNTs could also be mediated by substrate stiffness, including the increased collagen I expression, cell proliferation and ROS generation. Moreover, we found out that CNTs did induce the production of collagen I but not significant. The CNTs effects on cell proliferation and ROS generation were highly dependent on the substrate stiffness, where they didn't induce significant increase on soft substrates, but the increases in cell proliferation and ROS generation were both significant on 100 kPa substrates and TCPS. Furthermore, results of the mechanosensing showed similar trends on stiffness modulated the protein level expression of F-actin, α -SMA, FAK expression. Finally, by inhibiting the Rho-ROCK signally pathway, we were trying to understand the substrate stiffness affected cellular responses to CNTs down to molecular level. We found out that ROCK pathway was associated with the

collagen I synthesis and the CNTs induced collagen I effect was compromised by inhibiting ROCK. However, there are still needs to explore the mechanism of substrate stiffness effect.

This study provides a basic understanding of stiffness modulated cell behaviors and could potentially serve as an *in vitro* platform for disease modeling. While nanotechnology has been widely used in a diversity of different applications, the increasing concern of the toxicity of nanomaterials has been brought to attention. Efforts have been made to address the issues regarding the toxicity study of nanomaterials, while most of the established *in vitro* models were based on using stiff TCPS where the stiffness is far from most of the tissues existing in the human body. The novelty of our study was to build a physiologically relevant *in vitro* model by introducing stiffness cue to mimic the microenvironment of normal and fibrotic lung tissue for disease toxicology study of cellular response to MWCNTs, where there have been few reports on the idea of studying the toxicity by varying the substrate stiffness. We systematically investigated the substrate stiffness affected cell behaviors, as well as the fibrogenesis response of cellular responses to CNTs. Also, we targeted the cellular responses to CNTs through mechanosensing and intracellular tension, which helped further understanding the mechanism of substrate stiffness modulation of cell behaviors.

The stiffness cue could also be further applied to three-dimensional culture model to largely replicate the actual *in vivo* condition. Ultimately, the *in vitro* platform for disease modeling could be further optimized by introducing flow and shear stress to mimic the dynamic *in vivo* microenvironment into microfluidic devices. For example, simulating the breathing condition of the lung in the “lung-on-a-chip” could be potentially used as a reliable disease model for understanding and studying lung fibrosis.

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Chapter 4: Path Forward

From the perspective of understanding the mechanism of the human disease and achieving ultimate treatment, it is of great significance to build *in vitro* disease model that could largely represent the actual *in vivo* condition. Although numerous experiments and researches have been done using animal models, there are still gaps existing between the results obtained from animal and human, causing the barrier to disease detection and drug development. In this dissertation, our work emphasized incorporating two biophysical cues, nanotopography cue and stiffness cue, into the process of disease detection and *in vitro* disease modeling, which had the potential to mimic the *in vivo* microenvironment. Both studies were basic research to understand the two biophysical cues, which eventually should be optimized and integrated into a platform for disease detection, diagnosis and treatment.

Incorporating nanotopography into CTCs capture

For nanotopography cue, we used adhesion preference on nanotopographies with various shapes and dimensions to capture tumor cells instead of culturing on flat substrate, which was inspired by the tremendous nanoscale structures inside human tissue. Our study provides useful information to optimize nanotopography to further improve tumor cells capture efficiency, which plays a major role in early cancer detection and diagnosis. Based on what we obtained from our results and the current research studies, there are multiple future aspects: optimization of shape and dimensions of nanotopographies, surface chemistry modification, surface biomarker conjugation, the use of artificial blood sample or patient blood sample and ultimately integration of nanotopography into a microfluidic device.

Optimization of shape and dimensions. Motivated by the present work, the first path forward is going to be testing the different geometry and dimension of the nanotopography surface. In our study, we fabricated the representative nanopillars and nanogratings with specific dimensions to capture tumor cells. Results showed that the geometry did put an impact on the capture efficiency, which made the next step further be optimizing the nanotopography to further improve the cell adhesion, thus favored cell capture. Although nanogratings and nanopillars are representative ones, they are not the optimized ones. The optimization could focus on increasing cell-nanotopography interactions and facilitating focal adhesion formation. The previous reported RIE generated nanoroughened surface could achieve a capture efficiency up to 95% within 1 hour (see Figure 4-1).¹ One of the reasons that such a high capture efficiency could be reached was the nanoroughened, chaotic structure that has been generated by RIE that largely enhanced the cell-topographical interactions. Another reason might need to be taken into consideration was that the material they used was glass instead of PDMS in our study. PDMS is hydrophobic and typically inert to cell adhesion, whereas glass is more hydrophilic. The material that is used to perform the CTCs capture could also be carefully selected and modified.

Figure 4-1: RIE generated nanoroughened surface for cancer cell capture. (A) The process of generating nanotopography on glass surface by RIE. (B) Schematic of nanotopography fabricated by RIE on glass surface compared to smooth surface and the cell capture performance on each substrate, respectively. Enlarged views show cell captured on the illustrated nanotopography (left) and SEM (right) micrographs of cancer cells captured on nanoroughened glass surfaces. (Reproduced with permission from Ref. [1])

Surface chemistry. As previously mentioned RIE generated nanoroughened surfaces, while it created a nanotopography surface to enhance the cell-topographical interaction, the RIE treatment could also chemically activate the surface, thus favoring cell adhesion (see Figure 4-1).

Surface biomarker conjugation. The next future direction could focus on shortening the capture time by conjugating surface biomarkers on the nanotopography surfaces to capture specific tumor cells. As shown in our results, the capture time used was 4 h, which was not fast enough to capture or detect CTCs. However, the importance of early diagnosis has a substantial influence on the disease treatment and patient survival. For example, statistics showed that the number of survived women who diagnosed with breast cancer at the earliest stage is 6-fold compared to that who diagnosed at the advanced stage.² Based on our findings that 500-1x-150 nanogratings could enhance the adhesion of tumor cells, thus enhanced the capture efficiency, it is possible to shorten the capture time by chemically modifying the surface of the optimized nanogratings. Because, as previously mentioned in Chapter 2, the most widely used CTC capture techniques rely on positive selection based on surface biomarkers such as EpCAM expression on tumor cells.³ Besides EpCAM, there are various surface markers that expressed by different types of CTCs, which has been widely used for CTC isolation for cancer diagnosis.⁴ By identifying the specific biomarker that expressed on CTC to justify the proper antibody that could be conjugated to the surface, the capture efficiency is expected to be improved. By combining the topographical modulation and the chemical modification, the capture time could expect to be shortened and the capture efficiency is going to be further improved.

The use of artificial blood sample or patient blood sample and ultimately integration of nanotopography into a microfluidic device. The next step would be testing our system by using artificial blood sample, and ultimately, the optimized capture platform could be incorporated into the microfluidic system to largely mimic the dynamic blood flow. In real *in vivo* condition, CTCs are always circulating inside the bloodstream instead of static growing. The capture efficiency that obtained from static culturing was still substantially deviating the actual condition.

The flow rate, shear stress in the dynamic culture condition would potentially change the outcome of the cancer cell capture and disease detection. Recently, a group reported a microfluidic CTC capture chip by using nanoroughened glass substrate and achieve a significantly high capture yield of CTCs from blood samples (see Figure 4-2).⁵

Figure 4-2: Nanoroughened surfaces incorporated microfluidic device for cancer cell capture. (a) Image for the cancer cell capture microfluidic device. The enlarged views show the SEM micrographs of the nanotopography surface (upper) and the cell captured on the nanotopography (lower). (b) Quantitative measurement of capture efficiency of cell captured on nanotopography compared with smooth substrates. The artificial blood sample was spiked with 1,000 cells for each

cell type. **: significant difference ($p < 0.01$) between groups. (Adapted with permission from Ref. [5])

Incorporating stiffness into disease modeling

For stiffness cue, we used both soft and stiff PAAm gel to represent normal and fibrotic lung tissue, respectively, to understand how the matrix stiffness affects cell sensing nanomaterials. Our findings suggested that substrate stiffness played an important role in modulating cell behaviors and function, including cell elongation, proliferation, fibrogenic response and mechanosensing, which established a basic understanding of how substrate stiffness affected cell behavior, leading the next step to land on incorporating stiffness cue into three-dimensional culture model, which advanced the current *in vitro* two-dimensional model and more closely mimic the *in vivo* condition. As in *in vivo* microenvironment, cells are embedded in multiple ECM components whereas in our model, cells were cultured on top of the substrate with various stiffness, which was essentially a two-dimensional model. Technically speaking, the identification of whether cells are cultured under two-dimensional or three-dimensional condition is based on how adhesion proteins are formed, where under two-dimensional they are formed on the top of the cells and around the cells from all 360 degrees under three-dimensional condition (see Figure 4-3).⁶

Figure 4-3: The difference between 2D culture (collagen-coated glass) and 3D culture (collagen gel) in regard to adhesion, polarity, spreading, matrix distribution and matrix stiffness. This illustration shows that the parameters cell are faced with under these two culture conditions are substantially different because of the dimensionality. (Adapted with permission from Ref. [6])

Currently, there have been more and more *in vitro* study that shifted their focus from traditional two-dimensional culture to three-dimensional culture to better understand how cell responses to the surrounding environment and the mechanical properties of the matrix.⁷ As cell behavior could possibly be altered by the variation of cultural environment. For example, as our

study suggested that when cultured on PAAm gel, the increase in substrate stiffness could enhance cell elongation and spreading, where the results seemed counterintuitive while incorporating the system into three-dimensional culture. A studied has shown that valvular interstitial cells (VICs) encapsulated in soft gels expressed more actin fibers while in stiffened gels, there was no obvious exhibition of α -SMA or F-actin fibers.⁷ This results confirmed what previously proposed that cell behaviors could be affected and changed by the alteration of culture dimensionality.

Moreover, the future model establishment may combine both nanotopographical cue and stiffness cue as they both serve important functions in the composition of the ECM. For example, one study that incorporated both nanotopography cue and stiffness cue by embedded Calu-3 and MDCK-II cells in Matrigel that was composed of important proteins existed in the ECM like laminins, collagen IV, and entactins.⁸ The cells were cultured on fabricated nanoglass substrates. Results showed that this model could reflect the cell microenvironment as the cells formed tissue-like structures that were similar to their morphologies *in vivo*.⁹

Finally, the ultimate direction for our work would be the establishment of “organ-on-a-chip”, which incorporates everything into microfluidics to conduct three-dimensional dynamic culturing. Although the current three-dimensional *in vitro* model has advanced applications in closely replicating the *in vivo* microenvironment for disease study, it still has the intrinsic limitation of being a static model. The ECM in the human body is a dynamic environment with numerous substance and signal exchanges that happen all the time. Cells that are embedded into three-dimensional culture model without external flow are normally lack of the mechanical cues including the shear stress, physical force, tension,^{10,11} which are crucial factors to the development of tissue and organ when analyzing the disease model.¹² In the perspective of introducing fluid and to overcome the limitations of the current three-dimensional model, the recent development of

microfluidic-based organ-on-a-chip attracts substantial interests across the biomedical field including disease detection and modeling as well as drug development,^{13, 14} including lung on a chip,^{15, 16} kidney on a chip,^{17, 18} liver on a chip,^{19, 20} and body on a chip.²¹ Figure 4-4 here listed several microfluidic chips that mimic the different organs in human body.²²

Figure 4-4: “Organ-on-a-chip” microfluidic devices that were fabricated to mimic tissue microenvironment for different disease models. (Reproduced with permission from Ref. [22]) (a) Liver Chip: The liver-on-a-chip microfluidics device was designed to mimic the hepatic cords structure in liver tissue. The cell culture area and the medium flow channel was divided by an endothelial-like barrier. This specific design made it possible that the rat primary hepatocytes formed a two line alignment which the formation of bile canaliculi along the hepatic cords-like structure could be largely facilitated.¹⁹ (b) Kidney Chip: The kidney-on-a-chip microfluidics device was designed to mimic the tubular microenvironment *in vivo* for primary rat inner medullary collecting duct (IMCD) cells. This particular multi-layer microfluidics design incorporated PDMS channel and a porous membrane which was able to introduce fluid flow for 5 hours.¹⁷ (c) Lung Chip: The lung-on-a-chip microfluidics device was designed to create a

physiologically relevant model to mimic the alveolar system of human lung organ. The alveolar-capillary barrier was formed between two PDMS compartments on a porous membrane. This specific bioinspired microdevice also had the capability of mimicking the breathing movement, which had the potential application in nanotoxicity studies to test the lung inflammatory responses to silica nanoparticles.¹⁵ (d) Body Chip: The body-on-a-chip microfluidics device was designed to regenerate the *in vivo* microenvironment that reproduced organ-organ interactions. To achieve the desired purpose, different types of cell lines representing the liver, tumor, and marrow were embedded in the three-dimensional collagen hydrogel that was linked by the media flow of the microchannel that mimicked the blood flow. The specific type of the design had great potential by replicating organ-organ interactions, establishing a new platform for *in vitro* disease detection and modeling in a more realistic, *in vivo*-like microenvironment.²¹

This research direction of incorporating nanotopographical cue and substrate stiffness cue to build an organ-on-a-chip model should be based on optimizing the previous results for two-dimensional study and three-dimensional static study, choosing the suitable conditions for three-dimensional dynamic microfluidic study, which will ultimately provide great insight and useful reference for disease detection and modeling. The “organ-on-a-chip” microdevices that integrated biophysical cues could have the large potential of expanding the current cell culture *in vitro* models and providing the feasible alternatives to lower the cost of expensive *in vivo* animal models.¹⁵

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