Solid-supported lipid bilayer as a platform to study cell-extracellular matrix interactions

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NANYANG
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**SOLID-SUPPORTED LIPID BILAYER AS A PLATFORM TO
STUDY CELL-EXTRACELLULAR MATRIX INTERACTIONS**

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2017

SOLID-SUPPORTED LIPID BILAYER AS A PLATFORM TO STUDY CELL-EXTRACELLULAR MATRIX INTERACTIONS

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

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

2017

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

10 Jan 2017

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Date

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Abstract

Development of functionalized surfaces that can interface with cells is essential for many biotechnological applications ranging from routine cell culture to advanced applications such as cell-based diagnostics, tissue engineering, medical implants and biosensor development. Supported lipid bilayer (SLB), is a versatile synthetic platform which mimics the fundamental properties of native biological membranes and enables studying membrane-associated cellular processes in sufficiently simplified and more controlled conditions. SLBs composed of zwitterionic lipids are resistant to nonspecific adsorption of cells and proteins. However, they can be functionalized with specific cell recognition motifs by introducing lipids with chemically selective headgroups into the bilayer for direct covalent or non-covalent coupling of biomolecules.

The primary aim of this thesis work has been to explore and advance the application of SLBs as cell-surface mimetic substrates for cell interaction studies, with the special focus on functionalization of SLBs with the extracellular matrix (ECM) components. The ECM is a tissue-specific network of extracellular bio-active molecules that provides support and anchorage for cells and plays the key role in numerous cellular processes including cell growth and differentiation as well as intercellular communications.

The behaviour of cells in a tissue is regulated by both chemical as well as mechanical signals arising from their microenvironment. First, we developed an experimental platform using SLB functionalized with ECM proteins for mimicking a range of substrate rigidities by tuning the bilayer viscosity. We used a recently developed method of SLB formation called solvent-assisted lipid bilayer (SALB) formation to fabricate bilayers containing various fractions of a functional lipid with a reactive headgroup to attach two major ECM components; collagen type I (Col I) and fibronectin (FN) covalently to the bilayer surface. Using, quartz crystal microbalance with dissipation (QCM-D), we demonstrated that the density of the proteins can be controlled by the fraction of reactive lipids included in the lipid precursor solution used in the SALB

method. The ECM-SLB platform, supported the cell attachment, growth, and function. Importantly, fluorescent recovery after photobleaching (FRAP) analysis showed that bilayer retained its fluidity after protein conjugation and cell adhesion. The fluid nature of the bilayer led to the lateral displacement of anchored Col and FN and their accumulation underneath the cell in response to the traction forces exerted by the cells during the cell spreading process. We could control the displacement by increasing the viscosity of the membrane via incorporation of a high fraction of cholesterol (Chol) (up to 40%), a possibility offered by the SALB method.

Second, we investigate the biological activity of Col I and FN which are covalently attached to the SLB and compared with those of nonspecifically adsorbed to SiO₂ surface. The characterization of protein adsorption by QCM-D revealed that Col I and FN that attached to SLB have higher structural flexibility than those adsorbed onto SiO₂, and their activity was higher with respect to Col I-FN interaction and cell adhesion efficiency.

Finally, in attempt to develop a substrate which more closely mimics the native cell-surface environment, we functionalized the SLB with decellularized extracellular matrix (dECM) components. The dECM was obtained through a combination of chemical and enzymatic treatments of mouse adipose tissue and was solubilized under acidic conditions. We demonstrated that dECM functionalized bilayers sustain cell growth and maintain their function.

The strategy and results presented in this thesis work stress the potential of ECM-SLB platform for studying cell-matrix interactions as well as studying cellular processes that occur at the membrane interface.

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Table of Contents

Abstract.....	i
Acknowledgements	iii
Table of Contents	v
Table Caption	xi
Figure Captions.....	xiii
Abbreviations	xxv
Chapter 1 Introduction.....	1
1.1. Problem Statement	2
1.2. Objectives and Scope	5
1.3. Dissertation Organization	8
1.4. Main Contributions of the Thesis	9
References:.....	11
Chapter 2 Literature Review	15
2.1. The Cell Membrane	16
2.1.1. The Chemical Constituent of the Cell Membrane.....	17
2.1.2. Membrane Fluidity	20
2.2. The Extracellular Matrix.....	22
2.2.1. Collagen	23
2.2.2. Proteoglycans	26
2.2.3. Fibronectin	27
2.2.4. Laminin	28
2.2.5. Liver Extracellular Matrix.....	29

2.2.6. Interactions between Cells and the ECM	30
2.2.7. Biomolecule Engineering at Interface	32
2.3. Biomimetic Models of the Cell Membrane	35
2.3.1. Supported Lipid Bilayers	36
2.4. PhD in Context of Literature	49
References:	52
Chapter 3 Experimental Methodologies	63
3.1. Rationale for the Selection of Materials and Experimental Techniques	64
3.2. Materials	66
3.2.1. Lipid Reagent	66
3.2.2. Modification Reagent	67
3.2.3. Protein Reagents	67
3.2.4. Buffer Reagents	67
3.2.5. Chemical Reagents	67
3.2.6. Cell Culture Reagents	67
3.2.7. Cell Lines	68
3.2.8. Substrates	68
3.3. Experimental Technique	68
3.3.1. Solvent-Assisted Lipid Bilayer Formation Method	68
3.3.2. Silane Chemistry	69
3.3.3. Amine-Reactive Crosslinker Chemistry	70
3.3.4. Quartz Crystal Microbalance with Dissipation Monitoring	71
3.3.5. Fluorescence Microscope	73
3.3.6. Fluorescence Microscopy after Photobleaching	76
3.3.7. Immunofluorescence	78
3.3.8. Cell Culture	79
3.3.9. Cell Counting Kit-8	80
3.3.10. The Live/Dead Viability/Cytotoxicity Assay for Mammalian Cells	82
3.3.11. Enzyme-Linked Immunosorbent Assay	82

3.3.12. Decellularization	84
3.3.13. Scanning Electron Microscope.....	85
3.3.14. The Bicinchoninic Acid Assay.....	87
3.3.15. Hydroxyproline Assay Kit	88
3.3.16. Pico Green Assay	90
3.3.17. Glycosaminoglycan Assay	90
3.4. Statistical Analysis.....	92
References:.....	93
 Chapter 4 Dynamic Cellular Interactions with Extracellular Matrix Triggered by Biomechanical Tuning of Supported Lipid Membranes*	95
4.1. Introduction.....	96
4.2. Materials and Methods.....	98
4.2.1. Lipid Sample Preparation.....	98
4.2.2. Substrate Preparation.....	98
4.2.3. Silanization of Glass Substrates	99
4.2.4. Lipid Bilayer Formation.....	99
4.2.5. Activation of Carboxylic Group of DP-NGPE Lipid Included-SLB by EDC/NHS Treatment	99
4.2.6. Protein Sample Preparation and Adsorption on SLB and Glass/SiO ₂	99
4.2.7. Quartz Crystal Microbalance with Dissipation Monitoring	100
4.2.8. Fluorescence Recovery after Photobleaching	100
4.2.9. Interference Reflection Microscopy	100
4.2.10. Estimation of Membrane Viscosity.....	101
4.2.11. Cell Culture	101
4.2.12. Immunofluorescent Stain for ECM Proteins	102
4.3. Results and Discussion	103
4.3.1. The SLB Formation and Functionalization with ECM Proteins	103
4.3.2. Interaction of Huh 7.5 Cells with ECM Functionalized SLB and Glass.....	111

4.3.3. Interaction of Huh 7.5 Cells with ECM Functionalized SLBs of Different Viscosities	115
4.4. Conclusion	118
References:.....	120
 Chapter 5 Optimizing the Performance of Supported Lipid Bilayers as Cell Culture Platforms Based on Extracellular Matrix Functionalization*	
5.1. Introduction.....	126
5.2. Materials and methods	127
5.2.1. Lipid Bilayer Formation.....	127
5.2.2. Activating Carboxyl Groups of DP-NGPE Lipid Using EDC/NHS Chemistry	128
5.2.3. Functionalization of SLB and Glass/SiO ₂ with ECM Proteins	128
5.2.4. Quartz Crystal Microbalance with Dissipation (QCM-D)	129
5.2.5. Fluorescence Recovery after Photobleaching (FRAP).....	129
5.2.6. Cell Culture	130
5.2.7. Evaluation of Huh 7.5 Cell Morphology.....	130
5.2.8. Quantification of Huh 7.5 Cell Surface Area.....	131
5.2.9. Determination of Cell Viability by LIVE/DEAD Kit	131
5.2.10. Determination of Cell Number by Cell Counting Kit-8 (CCK-8)	131
5.2.11. Determination of Human Albumin in Cell Culture Supernatants	132
5.2.12. Immunofluorescent Stain for ECM Proteins	132
5.3. Results and Discussion	132
5.3.1. The SLB Formation and Functionalization with ECM Proteins	132
5.3.2. Comparison of the Adsorption of Col I and FN on SLB and SiO ₂	135
5.3.3. Comparison of the Biological Activity of Col I and FN Adsorbed on SLB and SiO ₂	138
5.3.4. Comparison of Huh 7.5 Cells Activity on Col I and FN-Coated SLB and Glass... ..	141
5.4. Conclusions.....	147

References:.....	149
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Chapter 6 Hybrid Biomimetic Interfaces Integrating Supported Lipid Bilayers with Decellularized Extracellular Matrix Components* 153

6.1. Introduction.....	154
6.2. Materials and Methods.....	156
6.2.1. Decellularization of White Adipose Tissue	156
6.2.2. Solubilization of Decellularized Adipose Tissue	156
6.2.3. DNA Content Quantification	156
6.2.4. Scanning Electron Microscopy	157
6.2.5. Total Protein Content Quantification	157
6.2.6. Collagen Content Quantification.....	158
6.2.7. GAG Content Quantification	158
6.2.8. Lipid Bilayer Formation.....	159
6.2.9. The Activation of Carboxyl Groups of DP-NGPE Lipid Embedded in the SLB....	159
6.2.10. Quartz Crystal Microbalance with Dissipation (QCM-D)	160
6.2.11. Fluorescence Recovery after Photobleaching (FRAP).....	160
6.2.12. Cell Culture and Cell Seeding on SLB Platform.....	161
6.2.13. Quantification of Huh 7.5 Cell Surface Area.....	161
6.2.14. Determination of Cell Viability by LIVE/DEAD Kit	161
6.2.15. Determination of Huh 7.5 Cell Number by Cell Counting Kit-8.....	162
6.2.16. Determination of Human Albumin in Cell Culture Supernatants	162
6.2.17. Immunofluorescent Stain for ECM Proteins	162
6.2.18. Statistical Analysis	163
6.3. Results and Discussion	163
6.3.1. Decellularization and Characterization of Mouse White Adipose Tissue ...	163
.....	
6.3.2. The SLB Formation and Functionalization.....	165
6.3.3. The Behavior of Huh 7.5 on dECM-Functionalized SLB.....	169

6.4. Conclusion	173
References:.....	174
Chapter 7 Conclusion and Future Outlook	179
7.1. Conclusion	180
7.2. Future outlook.....	183
7.2.1. <i>To Map Out the Influences of Full Length ECM Proteins and Peptide Fragments on Cell Adhesion and Related Activities Such as Spreading, Growth, and Function</i>	183
7.2.2. <i>To Control Stem Cell Differentiation by Physical Interaction with Extracellular Space.</i>	184
7.2.3. <i>Studying Cell-Receptor Interactions in the Cell–ECM-SLB Interface...</i>	185
References:.....	187
Appendix.....	189
List of Publications	191

Table Caption

Table 2.1 Amino acid composition of Col I. Adapted from [15].

Figure Captions

Figure 2.1 An illustration of the natural cell membrane. This complex structure composed of a lipid bilayer consists of a large variety of lipids such as phospholipids, glycolipids, and cholesterol (Chol). Different membrane proteins are embedded or anchored in the membrane including integral membrane proteins, peripheral proteins, and lipid-anchored proteins. Membrane carbohydrates are associated with lipids (glycolipids) or proteins (glycoproteins). The figure is taken from <http://www.ck12.org>.

Figure 2.2 A chemical structure of three main lipid molecules in the cell membrane including phospholipids, glycolipids and cholesterol (Chol). Adapted from <https://en.wikipedia.org>.

Figure 2.3 Schematic of translocation of lipid molecules in the planar bilayer structure. Lipid molecules can move in different manners including rotation, lateral diffusion, flip-flop, and exchange.

Figure 2.4 Illustration depicting the two main part of the ECM including basement membrane and interstitial matrix and their interaction with specific cells surface receptor (integrin) in human skin. Reprinted from <https://www.studyblue.com>.

Figure 2.5 The schematic illustration of (a) a polypeptide chain (α chain) and (b) Col triple helix structure. Col molecules are composed of three identical or different α chains which are twisted around each other, forming a stiff triple helical structure.

Figure 2.6 The diagram represents the self-assembly of Col I into fibrils and fiber structures. The assembly of Col into fibril structure is called fibrillogenesis and it is the result of intermolecular covalent aldol cross-links between two lysine or hydroxylysine amino acids at the C and N-terminus of adjacent Col molecules. Col molecules are staggered with respect to one another approximately every 67 nm. Several fibrils are packed in parallel bundles forming Col fibers. Col fibrils and fibers provide high tensile and mechanical strength for the tissues that need to tolerate high forces such as tendons. Adapted with permission from [16]. Copyright © 2009, Royal Society of Chemistry.

Figure 2.7 The structure of proteoglycans. These molecules consist of a core protein anchored covalently to the GAGs. Proteoglycans in the cartilage matrix can be bound individually to hyaluronic acids, a non-sulfated GAG through their core proteins and create a gigantic complex structure as large as a bacteria cell.

Figure 2.8 The structure of fibronectin molecule and its binding sites. The RGD is a typical sequence in cellular recognition.

Figure 2.9 The structure of LM molecule and its binding site. They exist as a trimer constituted of three distinct polypeptide chains namely α , β , and γ . Adapted from <https://answersingenesis.org>.

Figure 2.10 Schematic illustration of ECM localization in the liver tissue. Adapted with permission from [31]. Copyright © 2007 Blackwell Publishing Ltd.

Figure 2.11 Decellularized tissues/organs and their properties. The ECM-derived tissues/organs have the same physical and biochemical properties of their origin. Adapted with permission from [52]. Copyright © 2016 Copyright Clearance Center, Inc.

Figure 2.12 A schematic illustration of solid supported bilayer. The gap between solid and lipid bilayer is filled by a thin layer of aqueous solution (~1nm).

Figure 2.13 A schematic representation of a hybrid lipid bilayer membrane (h-LBM).

Figure 2.14 Illustration of vesicle fusion on a hydrophilic solid support and formation of SLBs. Adapted with permission from [100]. Copyright © 2008 Elsevier B.V.

Figure 2.15 The principal of solvent assisted lipid bilayer method. Lipids in a mixture of an organic solvent (e.g. isopropanol) and water can be deposited on a hydrophilic substrate. After lipid deposition, if the solvent's water fraction is then gradually increased up to 80-90%, a phase transition occurs to form a planar bilayer. Adapted from [104].

Figure 2.16 Selected strategies for bilayer modification. a) Use of functionalized lipids with various head groups such as maleimide, NHS, biotin, Ni-NTA. b) Use of amphiphiles molecules using different lipid anchors such as cholesterol. Adapted with

permission from [134]. Copyright 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Figure 3.1 Chemical structures of the lipid molecules utilized in this thesis.

Figure 3.2 A schematic illustration of a SAM structure.

Figure 3.3 Schematic illustration of QCM-D working principle. (a) Representative QCM-D quartz crystal that is sandwiched between metal electrodes. (b) An alternating voltage is applied to the electrodes covered with rigid and soft film that result in oscillation of the crystal at different resonance frequency. (c) The voltage is switched off which allows the crystal covered with a rigid/soft film to oscillate freely, respectively. Adapted from <http://www.biolinscientific.com>.

Figure 3.4 A typical Jablonski diagram representing three steps of the energy state of a molecule.

Figure 3.5 A Schematic representation of an epi-fluorescence microscope. Adapted from [12].

Figure 3.6 Schematic illustration of the working principle of FRAP technique. (a) A surface labeled with fluorescent molecules before photobleaching. (b) A defined area of the surface is photobleached using a powerful laser. (c) A mixture of the bleached and fluorescent molecules immediately after photobleaching. (d) The fluorescence recovery of the bleached area. (e) FRAP snapshots of R-DHPE molecules in an egg PC bilayer at four different times showing almost complete recovery. Adapted from [12].

Figure 3.7 Schematic representation of direct and indirect immunofluorescence method. Adapted from <http://www.abcam.com>.

Figure 3.8 (a) The mechanism of cell viability detection by CCK-8. (b) The three simple steps of CCK-8 assay: 1) addition of the reagent to the samples containing cells and cell culture media, 2) Incubation of the sample, and 3) Color absorbance measurement at 450nm using a microplate reader.

Figure 3.9 Overview of the sandwich ELISA assay. Adapted from <http://www.abcam.com>.

Figure 3.10 Schematic represents the layout of main components of a SEM instrument. Adapted from <https://www.purdue.edu>.

Figure 3.11 Schematic diagrams showing (a) the reduction of Cu^{+2} to Cu^{+} by protein in the presence of alkaline solution, and (b) The chelation of Cu^{+} by BCA and production of the BCA- Cu^{+} complex. Adapted with permission from [20]. Copyright 2012, Royal Society of Chemistry.

Figure 3.12 Schematic representation of different steps involved in hydroxyproline assay.

Figure 4.1 Characterization of ECM-functionalized SLB. (a) Schematic illustration of Col I bound to SLB via amine coupling. (b) Structures of the lipids used in this study, together with their chemical names. (c) The QCM-D ΔF and ΔD change after Col I binding to DOPC bilayers prepared by SALB method using varying mole fractions of functional lipid bearing a free carboxylic acid for amine coupling (DP-NGPE) in the lipid precursor mixture (between 0 and 20 mol %). After bilayer fabrication, the frequency shift was then normalized with $\Delta F = 0$ Hz corresponding to the planar lipid bilayer in each experiment. Changes in resonance frequency indicate the adsorption of Col I onto the bilayers. (d) The absolute frequency shift ($|\Delta F|$) upon Col I binding onto bilayer versus mole fraction of DP-NGPE included in the precursor mixture.

Figure 4.2 (a) Typical QCM-D frequency shift (ΔF , top panel) and dissipation (ΔD , bottom panel) for the third overtone were measured as a function of time during the formation of planar bilayer and subsequent protein addition. SLB was formed on SiO_2 via solvent assisted lipid bilayer (SALB) formation method using an isopropanol solution of a DOPC:DP-NGPE (1:4). After establishing the baseline in a buffer solution, pure isopropanol was injected (arrow i) followed by injection of 0.5 mg/mL DOPC/DP-NGPE (arrow ii) which caused a small change in resonance frequency and no change in the energy dissipation. Subsequently, the buffer was injected (arrow iii) leading to a final ΔF

and ΔD of $\sim -26 \pm 2$ Hz and $0.5 \times 10^{-6} \pm 0.3$, respectively which corresponds to a planar bilayer. In order to functionalize SLBs with ECM proteins, EDC/NHS was introduced into the chamber (bracket) to activate free carboxylic acid of DP-NGPE. In the last step, a solution of Col I (0.2 mg/mL) was injected to the bilayer. (b) QCM-D measurements recorded the addition of Col I to the DOPC:DP-NGPE (1:4) bilayer with no EDC/NHS activation. (c) Frequency shift corresponding to Col I adsorption to DOPC:DP-NGPE (1:4) bilayer with and without EDC/NHS activation. The frequency shifts associated with Col I adsorption onto bilayer with and without EDC/NHS activation step are -126 ± 8 and -11.3 ± 2.1 Hz, respectively.

Figure 4.3 Fluorescence immunostaining micrographs of Col I fibers on SLB prepared using varying fraction of DP-NGPE in precursor mixture. Noticeably, the Col I fibers were almost homogeneously distributed on the bilayers prepared with $> 10\%$ mole fraction of the functional lipid. Scale bars are 20 μm .

Figure 4.4 FRAP snap shots of bilayer (20% DP-NGPE) decorated with Col I. The Left and right panels show fluorescence micrographs of the bilayer at $t = 0$ and 60 s after photobleaching, respectively, revealing a near-complete recovery of the fluorescence at the photobleached spot. The fluorescent Rh-PE lipid was included in the bilayer for this. Analysis of the fluorescence recovery data revealed a lipid diffusion coefficient of $2.0 \pm 0.1 \mu\text{m}^2/\text{s}$, which is slightly lower than the diffusion coefficient of the non-functionalized bilayers with identical lipid composition ($2.4 \pm 0.1 \mu\text{m}^2/\text{s}$).

Figure 4.5 (a) Schematic illustration of FN decorated supported bilayer. (b) QCM-D monitoring of FN adsorption onto SLBs prepared by including different fraction of DP-NGPE. QCM-D monitoring confirmed the adsorption of FN to bilayers containing functional lipids. QCM-D results also showed that a small fraction of FN was removed after buffer wash, indicating that FN displayed some degree of nonspecific binding to SLB surface. Similar to Col I-functionalized SLBs, the final frequency change after FN conjugation increased with the fraction of the DP-NGPE lipid. (c) The absolute ΔF upon FN binding as a function of DP-NGPE fraction.

Figure 4.6 Fluorescence immunostaining micrographs of FN on SLB prepared using varying fraction of DP-NGPE in precursor mixture. Scale bars are 20 μm .

Figure 4.7 FRAP snap shots of bilayer (20% DP-NGPE) decorated with FN. The Left and right panels show fluorescence micrographs of the bilayer at $t = 0$ and 90 s after photobleaching, respectively, revealing a near-complete recovery of the fluorescence at the photobleached spot. The fluorescent Rh-PE lipid was included in the bilayer for this. Analysis of the fluorescence recovery data revealed a lipid diffusion coefficient of $1.4 \pm 0.1 \mu\text{m}^2/\text{s}$, which is lower than the diffusion coefficient of the non-functionalized bilayers with identical lipid composition ($2.4 \pm 0.1 \mu\text{m}^2/\text{s}$).

Figure 4.8 Cell behavior on ECM-coated bilayer and glass surfaces. (a) Representative fluorescence image of stained Col I fibers on SLB before (left) and 3 h after plating (right) of Huh 7.5 cells. Col I fibers around the cell were depleted by the cell following adhesion and spreading. (b) Fluorescence micrograph of an adherent cell 3 h after cell attachment on Col I coated glass showed no Col I depletion. (c) Representative fluorescence image of FN-coated bilayer, before and 3 h after cell adhesion demonstrating FN depletion. FN depletion by cell was not observed on (d) FN-coated glass. (e & f) FRAP snapshots of supported bilayer underneath the cell plated on (e) Col I and (f) FN-coated bilayer after three h after cell adhesion. Fluorescence was recovered nearly completely suggesting bilayer retains its mobility underneath the cell. (g) RISM images of cells adhering Col I and FN-functionalized glass and bilayer substrates. Note the greater spread of the cells on the glass substrate compared to the bilayer substrate. (h & i) Graph showing adhesion area (contact) (h) and circularity (i) of cells on different substrates. Note that lower cell adhesion area and higher circularity of cells on bilayer substrates. Error bar is s.e.m. of measurements made from ~ 10 cells in each group. * indicates p -value less than 0.05 while *** indicates p -value less than 0.001.

Figure 4.9 The QCM-D frequency (ΔF) and dissipation (ΔD) change after (a) Col I and (b) FN binding to DOPC:DP-NGPE (4:1) bilayers prepared by SALB method using varying mole fractions of Chol in the lipid precursor mixture (10, 20 and 40 mol %). After bilayer fabrication, the frequency shift was then normalized with $\Delta F = 0$ Hz

corresponding to the planar lipid bilayer in each experiment. Changes in resonance frequency indicate the adsorption of Col I and FN onto the bilayers.

Figure 4.10 Effect of membrane viscosity on cell behavior plated on ECM-functionalized bilayer platforms. (a) Membrane diffusivity and (b) the corresponding viscosity of bare and ECM-functionalized SLBs containing varying fraction of Chol. Fluorescence micrograph of Col I (c) and FN (e) on supported bilayer with varying fraction of Chol (0, 20 and 40%) incubated with Huh 7.5 for 3 h. With increasing the percentage of Chol, the protein depletion decreased. Percentage of cells showing complete protein depletion on Col I (d) and FN (f) functionalized bilayers with varying fraction of Chol. Fluorescence micrographs of (g) Col I and (h) FN on hybrid bilayers before and after incubation with Huh 7.5 for 3 h. No depletion was observed on hybrid bilayers.

Figure 4.11 Typical quantification of the area of a cell and its depletion zone (the area that were cleared of fluorescently-labeled protein). The areas (marked in red) were measured using ImageJ software.

Figure 5.1 ECM-functionalized SLB platform. (a) Schematic illustration of model membranes functionalized with ECM molecules via covalent bonding to lipids with reactive headgroups. (b) Structures of the lipids used in this study, together with their chemical names. (c) QCM-D frequency shift (ΔF , top panel) and dissipation (ΔD , bottom panel) for the third overtone were measured as a function of time during the formation of the planar bilayer on SiO₂. SLBs were formed on SiO₂ via the SALB formation method using an isopropanol solution of a zwitterionic lipid (DOPC) and a lipid bearing a free carboxylic acid for amine coupling (DP-NGPE). Arrows indicate the injection of (i) Tris buffer (10 mM Tris, 150 mM NaCl, pH 7.5), (ii) isopropanol, (iii) lipid mixture [0.5 mg/mL of DOPC: DP-NGPE (4:1) in isopropanol], and (iv) Tris buffer solution leading to a final ΔF and ΔD of -25 ± 1 Hz and $0.4 \pm 0.2 \times 10^{-6}$, respectively, which correspond to a planar bilayer. (d) FRAP snapshots of DP-NGPE doped bilayer containing 0.5 wt % Rho-PE lipid, immediately (top) and 1 min (below) after photobleaching, showing almost complete recovery, indicating the fluidity of the SLB.

Figure 5.2 QCM-D analysis of ECM adsorption to SLB and SiO₂ platforms. QCM-D monitoring of (a) Col I and (d) FN attachment to bilayer via covalent bonding. The SLB on SiO₂ was initially formed by the SALB method, and then the measurement time was normalized. Frequency shift (ΔF , blue) and energy dissipation shift (ΔD , red) for the third overtone ($n = 3$) were measured as a function of time. Protein solution (0.2 mg/mL) was introduced to the surface after treatment of SLBs with EDC/NHS. The insets show the fluorescence immunostaining micrograph of SLB functionalized with Col I fibers (a) and FN (d). Proteins were stained using fluorescently-labeled anti-Col I or anti-FN antibodies. Dissipation-frequency curves (ΔD vs. ΔF) upon (c) Col I and (f) FN adsorption to the SLB and SiO₂ surface. The dashed line shows that at any given frequency, the corresponding dissipation was bigger for protein layer on the SLB. Scale bars are 20 μm .

Figure 5.3 Binding of FN to Col I fibers on SLB and SiO₂ platforms. (a) QCM-D curves of ΔF and ΔD for the adsorption of FN to Col I-functionalized SLB. (b) Fluorescence micrograph of Col I-coated SLB after incubation with FN, stained by fluorescently-labeled anti-FN antibodies. (c) FRAP snapshots of Col I-FN-coated bilayer showing almost complete recovery, indicating the fluidity of the SLB. (d) QCM-D measurement of FN adsorption to Col I-coated SiO₂. The initial baseline values in (a) and (d) are the normalized frequency and dissipation shifts for the bilayer (defined as $\Delta F_{\text{SLB}} = 0$). (e) Fluorescence micrograph of Col I-coated glass, after incubation with FN, stained by fluorescently labeled anti-FN antibodies. Immunostaining of FN revealed its co-localization with Col I fibers. All scale bars are 20 μm .

Figure 5.4 QCM-D curves of ΔF and ΔD upon addition of BSA (0.1 mg/mL) to Col I-functionalized SLB.

Figure 5.5 Quantification of cell spreading on ECM-functionalized SLBs and glass. Representative confocal images of one day plated Huh 7.5 cells on (a) Col I and (b) FN-functionalized SLB and (d) Col I and (e) FN-functionalized glass. Representative confocal images of one day plated Huh 7.5 cells on ECM-free (c) SLB and (f) glass. F-actin was stained by Alexa Fluor 555-labeled phalloidin (red), and nuclei were stained by DAPI (blue). (Scale bar is 40 μm). (g) The average projected area of Huh 7.5 cells plated

on different platforms after 24 h. The images were taken by optical microscope and the observation field was 0.5 mm^2 . (h) The number of cells per absolute values of the maximum QCM-D frequency shifts due to the amount of adsorbed proteins. ($n = 3$, mean $\pm$ SD; $**P \leq 0.01$, $***P \leq 0.001$). All the images were analyzed with imageJ.

Figure 5.6 Cell viability and metabolic functions on ECM coated platforms. (a) Representative fluorescence micrograph images of detecting live (green) and dead (red) Huh 7.5 cells cultured on SLB and glass coated with Col I and FN and Col I-FN at 1 and 3 days post seeding. Scale bar is $200\mu\text{m}$. (b) The proliferation of Huh 7.5 cells was measured over a 3day period. The values were normalized to the absolute ΔF due to the amount of adsorbed proteins. (c) Albumin production by 10^6 Huh 7.5 cells plated on different platforms measured after 24 h of cell growth. The value was normalized to the total number of cells ($n = 3$, mean $\pm$ SD; $* P \leq 0.05$, $**P \leq 0.01$, $***: P \leq 0.001$, and $****P \leq 0.0001$).

Figure 6.1 Schematic illustration of various steps involved in the preparation of supported bilayer functionalized with adipose tissue dECM components for cell study applications. Adipose tissue was decellularized using a combination of chemical and enzymatic treatment. The dECM was then solubilized in acidic condition and was covalently attached via amine coupling to the surface of a supported bilayer containing lipids with head group bearing free carboxylic acid. Subsequently cells were seeded and their attachment, spreading, growth and function were studied.

Figure 6.2 Characterization of decellularized mouse-derived adipose tissue. (a) Representative SEM images of native adipose tissue. (b) Low magnification SEM image for decellularized adipose tissue indicates a three-dimensional scaffold composed of fibrous ECM material that is devoid of cells and lipids. (c) High magnification SEM image of decellularized tissue demonstrates a three-dimensional architecture of the Col fiber. Biochemical evaluation of the content of (d) DNA, (e) Col and (f) GAGs present in the native and decellularized adipose tissue. ($n = 3$, mean $\pm$ SD; $**P \leq 0.01$ and $****P \leq 0.0001$).

Figure 6.3 Modification of SLBs using adipose dECM. (a) The QCM-D signal exhibited different steps of bilayer fabrication and functionalization. ΔF (blue) and ΔD (red) for the third overtone ($n = 3$) were measured as a function of time during the formation of 20% DP-NGPE SLB on SiO₂ using SALB method. After establishing the baseline in a buffer solution, pure isopropanol was injected (arrow i) followed by injection of 0.5 mg/mL DOPC / DP-NGPE (20%) (arrow ii) which caused a small change in ΔF and no change in the ΔD . Subsequently, the buffer was injected (arrow iii) leading to a final ΔF and ΔD of $\sim -25 \pm 2$ Hz and $0.5 \times 10^{-6} \pm 0.3$, respectively which corresponds to a planar bilayer. To functionalize the SLB with ECM proteins, EDC/NHS was introduced into the chamber (arrow iv) to activate free carboxylic acid of DP-NGPE. In the last step, the dECM was injected (arrow v). The significant change in ΔF and ΔD indicates the adsorption of dECM to the SLB. (b) QCM-D characterization of the dECM binding to bilayers containing no (triangles) or 20 % DP-NGPE (rectangles). Fluorescence micrographs of dECM functionalized bilayer incubated with antibodies against (c) Col I and (d) FN. (e) Fluorescence recovery after photobleaching analysis of dECM functionalized bilayer. A ~ 20 μm -wide circular spot was photobleached, followed by time-lapse recording. Fluorescence was recovered nearly completely after 60 s suggesting bilayer retains its mobility.

Figure 6.4 Huh 7.5 Cells behavior on the bare and dECM-functionalized SLB (dECM-SLB). The typical bright field image of Huh 7.5 grown on the (a) dECM- SLB (b) bare after 24 h. Cells did not spread on bare SLB and remained rounded. (c) Quantification of a number of cells attached to the bare and dECM-SLB. (d) The measurement of Huh 7.5 cell spreading size on the bare and dECM-SLB after 24 h. The images were taken by an optical microscope and analyzing with ImageJ. The observation field was 0.5 mm^2 . (e) FRAP analysis of dECM-SLBs after cell adhesion. A $20 \mu\text{m}$ -wide circular spot was photobleached under the cell, followed by time-lapse recording. The fluorescence was recovered nearly completely after 80 s suggesting bilayer retains its mobility under ECM materials and cells.

Figure 6.5 Evaluation of Huh 7.5 cells growth and functions on dECM-functionalized SLB (dECM-SLB). (a) Fluorescence image of calcein AM-stained Huh 7.5 cells plated

on dECM-SLB after 24 and 72 h. The green-stained cells are alive and the red-stained cells are dead. The viability of the cells was over than 90% within three days (b) Quantification of cell viability, cell proliferation and albumin production of Huh 7.5 on dECM-SLB. Cell viability was analyzed using ImageJ. Cell proliferation amount was calculated by normalization of cell number at day 3 to the cell number at day 1. The amount of albumin was obtained by normalization of albumin secretion at day 1 to 10^6 cells.

Figure 6.6 Comparison of Huh 7.5 cells growth and functions on dECM and Col I-FN functionalized SLB. Cell proliferation amount was calculated by normalization of cell number at day 3 to the cell number at day 1. The amount of albumin was obtained by normalization of albumin secretion at day 1 to 10^6 cells.

Abbreviations

2D	Two Dimensional
3D	Three Dimensional
AFM	Atomic Force Microscopy
ASCs	Adipose-Derived Mesenchymal Stem Cells
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
CCK-8	Cell Counting Kit-8
Chol	Cholesterol
Col	Collagen
Col I	Collagen Type I
Col-SLB	Collagen-Functionalized Supported Lipid Bilayer
Col IV	Collagen Type IV
Col VI	Collagen Type VI
D	Diffusion Coefficient
DAPI	4',6-Diamidino-2-Phenylindole
dECM	Decellularized Extracellular Matrix
DMEM	Dulbecco's Modified Eagle's Medium
DOPC	1,2-Dioleoyl-sn-Glycero-3-Phosphocholine
DP-NGPE	1,2-Dipalmitoyl-sn-Glycero-3-Phosphoethanolamine-N-(Glutaryl).
ECM	Extracellular Matrix
ECM-SLB	Extracellular Matrix-Functionalized Supported Lipid Bilayer
EDC	1-Ethyl-3-(3-Dimethylaminopropyl) Carbodiimide
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
FN	Fibronectin
FN-SLB	Fibronectin-Functionalized Supported Lipid Bilayer
FRAP	Fluorescence Recovery after Photobleaching
GAGs	Glycosaminoglycans
GF	Growth Factor

GFOGER	Glycine-Phenylalanine-Hydroxyproline-Glycine-Glutamate-Arginine
Gly	Glycine
GPa	Gigapascal
GPI	Glycosylphosphatidylinositol
HCl	Hydrochloric Acid
HSPGs	Heparin Sulfate Proteoglycans
Huh 7.5	Human Hepatocarcinoma Cell Line
IgG	Immunoglobulin G
IKVAV	Isoleucine-Lysine-Valine-Alanine- Valine
IMPs	Integral Membrane Proteins
kPa	Kilopascal
LM	Laminin
Lys	Lysine
N ₂	Nitrogen Gas
NHS ester	N-Hydroxysuccinimide ester
NTA	Nitrilotriacetic Acid
OVA	Ovalbumin
PAA	Poly (Acrylic Acid)
PBS	Phosphate Buffered Saline
PC	Phosphatidylcholine
PDMS	Polydimethylsiloxane
PE	Phosphatidylethanolamine
PEG	Polyethylene Glycol
PHSRN	Proline-Histidine-Serine-Arginine-Asparagine
PS	Phosphatidylserine
PUA	Poly (Urethane Acrylate)
QCM-D	Quartz Crystal Microbalance with Dissipation
RGD	Arginylglycylaspartic Acid
RIPA	RadioImmunoprecipitation Assay Buffer
RTKs	Receptor Tyrosine Kinases
SALB	Solvent- Assisted Lipid Bilayer

SAM	Self-Assembled Monolayer
SDS	Sodium Dodecyl Sulfate
SiO ₂	Silicon Dioxide
SLB	Supported Lipid Bilayer
SPR	Surface Plasmon Resonance
TIRF	Total Internal Fluorescence
WAT	White Adipose Tissue
YIGSR	Tyrosine- Isoleucine-Glycine-Serine-Arginine

Chapter 1

Introduction

This chapter presents a brief introduction to the structure and function of the cell membrane and extracellular matrix (ECM) as well as an overview of the biomimetic membrane platforms. Briefly, the research is aimed at developing a platform which mimics the natural cell membrane environment by functionalization of the supported lipid bilayer (SLB) as a simplified model of the cell membrane with ECM signaling molecules. At the end of this chapter, the objectives and scope of the research, as well as an overview of each chapter of the thesis are provided. The novelty of the research also has been discussed.

1.1. Problem Statement

The functional and structural units of the human body are the cells. All human cells are enclosed by a selectively permeable barrier called the cell membrane. The cell membrane defines the structure of the cells and isolates the interior of the cells from the extracellular space. In addition, all internal cellular organelles such as the cell nucleus and the mitochondria are compartmentalized by a membrane. The structure and organization of the cell membrane are very complex. It is made up of a self-assembled lipid bilayer composed of many different lipids decorated with various types of proteins and carbohydrates, forming a 5-9 nm thick membrane [1]. The components of the cell membrane are held together through non-covalent bonds and are distributed asymmetrically in the two leaflets of the lipid bilayer, forming domains of different compositions, thickness, and mobility [2]. The cell membrane provides a scaffold within which biochemical interactions can occur more efficiently. This way, it participates extensively in many vital cellular functions such as signal and energy transduction as well as cell-cell recognition and adherence [1].

Although cells are enclosed and defined by the membrane, many cells are surrounded by the ECM, which are secreted by cells into their immediate environment. The ECM contains a wide variety of bio-active macromolecules which have both structural and growth regulating functions. There are two major classes of ECM macromolecules including proteoglycans with glycosaminoglycan (GAGs) polysaccharide side chains and fibrous proteins categorized into structural (e.g., collagen (Col), and elastin) and adhesive (e.g., fibronectin (FN), and laminin (LM)) types [3]. These molecules contain specific cell binding motifs that are recognized by receptors on the surface of their target cells. Ligand binding to transmembrane adhesion-receptor proteins such as integrin [4] initiates the transmission of specific intra-and extracellular reactions that result for instance in changes in gene expression, leading to different cellular behaviors such as growth, migration, proliferation, and differentiation [5].

Any abnormal changes in chemistry or function of cell membranes or its ECM or failure in membrane-associated processes, for instance, due to the malfunction of cell surface receptors, would result in severe pathological conditions such as cancer, atherosclerosis and neurodegenerative diseases [6]. In fact, more than 60% of current drug targets are membrane proteins [7]. For instance, G-protein-coupled receptors (GPCRs) make up 44% of human drug targets [8]. Therefore, studying the detailed function of cell membranes and their constituents as well as membrane-associated processes are essential for understanding the mechanism of disease and for drug discovery. However, given the complexity of biological membranes, this has remained a challenge. To this end, different simplified model systems for in vitro studies of cellular behavior have been developed. Generally, these platforms consist of a solid substrate such as glass or polystyrene which is coated with different signaling molecules to mimic the cellular environment. However, the cell membrane and its environment are highly dynamic; its lipids and proteins organization changes during various cellular processes. For instance, receptor reorganization and clustering occurs during many receptor-mediated signal transduction processes. Thus, due to the static nature of these platforms, such dynamic properties of the cell membrane cannot be effectively modeled.

The dynamic nature of the cell membrane is mainly facilitated by the fluidity of its lipid bilayer which allows lateral diffusion of lipids and membrane-associated biomolecules. Many of the most basic cellular processes, including cell division, formation of intercellular junctions, cell growth, and endocytosis, depend on the movement of membrane components [1]. Therefore, to better mimic the cell membrane structure, various lipid based model systems with the physical and chemical characteristics comparable to natural membranes have been developed [9]. The lipid-based platforms, such as small [10], and giant lipid vesicles [11], bilayer nanodiscs [12], and SLB [13] when functionalized with biological ligands, allow studying membrane-associated cellular processes as well as visualizing their dynamic and organizations in sufficiently simplified and more controlled conditions [9]. Among them, the SLB which is comprised of a single planar lipid bilayer associated with a solid substrate is particularly effective thanks to its excellent mechanical stability and preservation of the

fundamental characteristics of natural membranes such as two-dimensional fluidity and electrical sealing properties [14]. In addition, the association of the lipid bilayer to a solid support makes it possible to characterize the SLB with a wide variety of surface sensitive analytical techniques such as atomic force microscopy (AFM) [15], quartz crystal microbalance with dissipation (QCM-D) [16], total internal fluorescence (TIRF) microscopy [17], and electrochemical measurements [18].

The chemical composition of SLBs can be easily tuned to manipulate their biophysical properties such as surface charge [19], lipid diffusivity [20], their membrane organization such as phase separation [21] and formation of lipid rafts [22]. Importantly, SLBs can be functionalized with biological receptors and signaling ligands by introducing synthetic head-group modified lipids into the bilayer for direct covalent or non-covalent coupling of biomolecules [23]. Functionalized SLBs have been used to examine range essential biological processes that occur at the membrane interfaces including viral fusion [24], ligand-receptor interactions [25], enzymatic activities [26], cellular signaling and cell-cell recognition [27]. Owing to their inherent antifouling properties; SLBs are resistant to nonspecific adsorption of cells [28] and proteins [29]; a feature which makes them a suitable interface for detection of specific biomolecular interactions. In particular, they are effective tools for studying receptor dynamics during cell-cell interactions as well as triggering various intercellular reactions when interfaced with live cells [23,27,30].

While simplified models have been proven to be useful for many biological applications, development of models with greater compositional complexities would be the next step toward platforms that more closely mimics the cell surface environment. In this regard, we propose that the functionalization of SLBs with ECM components is the next logical step toward this goal. Therefore, the purpose of this thesis work is to provide a natural cell environment condition by functionalization of SLB as a simplified model of cell membrane with native individual or collection of ECM signaling molecules, to study the interaction of mammalian cells with such an environment.

1.2. Objectives and Scope

Development of platforms that mimic biological membranes is a critical step for studying membrane related biological processes. An ideal platform should offer the ability to be functionalized with a precise density of signaling molecules, reduced non-specific interactions and should preserve the bioactivity of attached biomolecules. The main goal of this thesis is to design such a platform that not only mimic the biological membranes but also mimic the cell membrane environment for studying cell-cell and cell-ECM interactions.

The objectives and scope of this thesis are as follows:

1. To Construct an ECM-Functionalized SLB with Controlled ECM Protein Density

In this work, the SLB was selected as a platform to be functionalized with ECM proteins in order to mimic the cellular environment. Zwitterionic lipid bilayer coatings doped with lipids with chemically selective headgroups, provide a robust platform for immobilization of biomolecules in an antifouling, protein resistant background. In this work SLBs were fabricated using a recently developed technique namely solvent-assisted lipid bilayer (SALB) formation method. The SALB is based on using lipids dissolved in alcohol (e.g., isopropanol) to form bilayer on solid supports by the slow replacement of the organic solvent in the lipid-solvent mixture with aqueous buffer. A unique feature of the SALB is that it enables preparation of SLBs that cannot be obtained using conventional techniques (i.e., vesicle fusion), e.g., bilayers containing high fraction of cholesterol (Chol).

The common strategy to functionalize lipid bilayers is to incorporate lipid-like molecules with chemically-reactive headgroups into the bilayer structure. The functionalized lipids act as an anchor to immobilize specifically the biomolecules containing the corresponding binding moiety to the membrane surface. Importantly, the

degree of biomolecule conjugation to the bilayer can be controlled by the fraction of functional lipids included in the bilayer.

We used SALB method to form bilayers composed of a neutral, zwitterionic lipid (DOPC) which provides resistance to protein adhesion and gives the bilayer its antifouling capabilities and a lipid-like molecule (DP-NGPE) with a chemically-reactive headgroup (free carboxylic acid) which serves as a linker to attach ECM proteins to the SLB surface through covalent bonding via amine coupling chemistry. After bilayer formation, SLBs were functionalized with collagen type I (Col I) and fibronectin (FN) through amine coupling. The Col I and FN are two major ECM proteins that widely used to mimic cellular environment. In attempt to control the level of protein conjugation (i.e., surface density of proteins), bilayers containing varying fraction (up to 20%) of the reactive lipid (DP-NGPE) were tested. To check the behavior of cell at Col I and FN-bilayer interface, human hepatocellular carcinoma cell line (Huh 7.5) were plated onto the Col I and FN functionalized bilayers. Cell adhesion and its interaction with ECM proteins were monitored by fluorescence microscopy which enables to visualize changes in the integrity and distribution of fluorescently labeled ECM proteins during their interaction with individual cells.

2. To Modulate the Viscosity of ECM-Functionalized SLB by Controlling the Content of Chol Incorporated into the SLBs

The physical environment of mammalian cells is important for regulating the different cellular process. For instance, the mechanical properties of ECM affect the cell adhesion [31], differentiation [32-34] and migration [31,35]. We altered the physical property of the SLBs by changing the viscosity of the underlying membrane. Controlling the viscosity of plasma membrane is utilized by different cells to regulate the dynamics of membrane proteins [36] and cell migration [37].

The SLB viscosity can be modulated by incorporation of Chol in the membrane. Incorporation of Chol results in a decrease in the lipid mobility and hence an increase in

the membrane viscosity. The formation of the SLBs with a high concentration of Chol (> 20%) is only possible with the SALB method. We used QCM-D combined with fluorescence recovery after photobleaching (FRAP) to characterize and measure the bilayer formation with the different fraction of Chol. We studied the behavior of cells attached to Col I and FN-coated membrane with varying fraction of Chol (0, 20 and 40%).

3. To Compare the Biological Activity of the ECM Components that Covalently Attached to Lipid Bilayer and Nonspecifically Adsorbed to the SiO₂ Surface

Strategies to coat surfaces with biofunctionalized layers are essential for many biotechnological applications such as cell-based diagnostics, tissue engineering, medical implants and biosensor development [38]. Proteins usually physically adsorb to the commonly used surfaces such as metal dioxide, glass, polystyrene, and gold. However, depending on the interaction strength between proteins and the surface, they may undergo structural rearrangements or even denature upon adsorption [39]. The biological activity and function of proteins such as the presentation of ligands for specific receptors are usually excreted by functional domains in their three dimensional (3D) structures which specifically interact with other biomolecules. Consequently, any surface-induced structural deformation may alter the protein function.

We examined the biological activity of two important components of ECM; FN and Col I which are covalently attached to a SLB and compare with those of nonspecifically adsorbed to SiO₂ surface. Cell adhesion, proliferation, and function, as well as FN-Col I interaction, were studied on ECM-functionalized SLB and ECM-coated SiO₂.

4. To Functionalize SLB with Native Decellularized Extracellular matrix (dECM) Components

Towards the development of biomimetic platforms that more closely mimics the cell surface environment, we functionalized solid supported phospholipid bilayer with dECM components. So far, SLBs functionalized with individual ECM components such as collagen [40], and FN [41] or peptides derived from these proteins (e.g., IKVAV [42]) have been reported. For the first time, we demonstrate functionalization of SLBs with solubilized adipose tissue dECM and characterize their formation with QCM-D and immunofluorescence microscopy. The dECM was obtained through a combination of chemical and enzymatic treatments of mouse adipose tissue and was solubilized under acidic conditions. The dECM was characterized using different biological assays to measure protein concentration, DNA concentration, and GAGs concentrations. We studied cell attachment, growth, survival and function on the dECM functionalized bilayers platform.

1.3. Dissertation Organization

This dissertation is accordingly divided into the following chapters:

Chapter 1: “Introduction” describes the background of the research field and outlines the objectives and scope of the Ph.D. research.

Chapter 2: “Literature Review” reviews the literature concerning cell membrane, ECM, biomimetic model systems, and SLB and its role in mimicking cell environment.

Chapter 3: “Experimental Methodologies” presents the materials and experimental techniques used in this thesis work.

Chapter 4: “Dynamic Cellular Interactions with Extracellular Matrix Triggered by Biomechanical Tuning of Supported Lipid Membranes” introduces (i) SLB formation

using SALB method, (ii) functionalization of SLBs with controlled density of Col I and FN proteins, (iii) studying the interaction between cell and ECM-functionalized SLBs, and (iv) modulating the fluidity of the substrate by controlling the concentration of Chol embedded into SLBs.

Chapter 5: “Optimizing the Performance of Supported Lipid Bilayers as Cell Culture Platforms based on Extracellular Matrix Functionalization” discusses the biological activity of Col I and FN, (i) adsorbed nonspecifically to the glass surface, (ii) attached to the surface of SLB via covalent bonding. Cell adhesion, proliferation, and function, as well as FN-Col I interaction, were studied on these platforms. The structural properties of protein coatings on both platforms were characterized by QCM-D.

Chapter 6: “Hybrid Biomimetic Interfaces Integrating Supported Lipid Bilayers with Decellularized Extracellular Matrix Components” presents the decellularization of adipose tissue and functionalization of SLBs with the resulting decellularized ECM proteins. The characterization of the dECM, QCM-D monitoring of the bilayer formation and subsequent dECM adsorption, as well as cell growth and function on the dECM-SLB platform, are presented.

Chapter 7: “Conclusion and Future Outlook” summarizes concluding remarks of the above chapters and makes recommendations for the future work based on the findings in this dissertation.

1.4. Main Contributions of the Thesis

This dissertation led to several novel outcomes by:

1. Fabrication and characterization of SLBs functionalized with controlled density of individual ECM proteins.

2. Formation of SLBs with high fractions (up to 40%) of Chol using SALB formation method to study the effect of membrane viscosity on cell behavior as well as cell-ECM interactions.
3. Characterization of the biological activity and viscoelastic properties of ECM components attached to SLBs and glass surface.
4. Fabrication and characterization of SLBs functionalized with natural tissue-derived ECM components toward developing models with greater compositional complexities that more closely mimics the cell surface environment.

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Chapter 2

Literature Review

This chapter provides an overview of previous research and studies related to the work presented in the dissertation. Overall, it describes the background and current knowledge about: (i) the biological membrane structure, compositions and functions, (ii) the extracellular matrix (ECM) and its roles in regulating mammalian cell behaviors; and (iii) the biomimetic model membrane systems with the specific focus on the supported lipid bilayer (SLB) and its applications in cell studies. In the end, the objective of the Ph.D. work is discussed within the context of the field.

Conventional plastic cell culture plates present an extremely stiff substrate for mammalian cell culture applications. In addition, while gel-based substrates have been widely used for mimicking a range of substrate rigidities, they still have difficulty accessing the softest and most fluid interfaces that cells may experience in a physiological environment. Thus, there is a need for the development of soft substrates for mimicking the physical properties of soft tissues. In this thesis, we filled this gap by developing a SLB-based substrate for cell adhesion studies.

2.1. The Cell Membrane

The human body is composed of various types of cells with different shapes and functions. Despite these diversities, all the human cells are surrounded by a semi-permeable barrier called the cell membrane. The cell membrane defines the structure of cells and acts as an interface between the interior of the cells and their extracellular space. It also separates all internal cellular organelle such as the cell nucleus and the Golgi apparatus to provide a proper condition for their activities without external interference. Moreover, the cell membrane plays critical roles in signal transduction, intercellular interaction, and energy transduction [1].

The cell membrane is a very complex system with a thickness around 5-10 nm [1] consists of a lipid bilayer in which different proteins and carbohydrates are anchored or incorporated (see **Figure 2.1**). This structure was described by a famous membrane model called the fluid mosaic model. The fluid mosaic model was introduced by SJ Singer and GL Nicolson in 1972 [2]. Based on this model, the lipid bilayer is a two-dimensional liquid in which proteins and lipid molecules are distributed homogeneously in each leaflet and also can diffuse freely. In 1997 the fluid mosaic model was updated regarding membrane organization [3,4]. It was discovered that the natural cell membrane is not a homogenous mixture of lipids and proteins as was assumed in the fluid mosaic model. Remarkably, it contains small (10 - 30 nm diameters) domains of distinctive lipid compositions called lipid rafts. Lipid rafts are concentrated with specific protein receptors which participate in critical cellular processes such as signaling and cell-cell interactions [1]. The discovery of membrane composition and organization was a very crucial step for understanding the mechanism of membrane related cellular processes.

The membrane compositions and functions will be discussed in more detail in this section.

Figure 2.1 An illustration of the natural cell membrane. This complex structure composed of a lipid bilayer consists of a large variety of lipids such as phospholipids, glycolipids, and cholesterol (Chol). Different membrane proteins are embedded or anchored in the membrane including integral membrane proteins, peripheral proteins, and lipid-anchored proteins. Membrane carbohydrates are associated with lipids (glycolipids) or proteins (glycoproteins). The figure is taken from <http://www.ck12.org>.

2.1.1. The Chemical Constituent of the Cell Membrane

Lipid Membrane

The cell membrane contains three major types of lipids molecules including: phospholipids, glycolipids, and cholesterol (Chol) as shown in **Figure 2.2**. All these lipid molecules have amphipathic character consisting of both hydrophilic and hydrophobic regions. In aqueous solutions, phospholipids spontaneously align themselves into a bilayer structure in which all hydrophilic head groups face water, and all hydrophobic tails face inward to the core of the bilayer to avoid contact with water molecules.

Phospholipids: The major lipids of the cell membrane are phospholipids. A common phospholipid has two hydrophobic fatty acid chains via a glycerol backbone that is linked to a hydrophilic phosphate-based polar head group. Due to the existence of glycerol in most membrane phospholipids, they are called glycerophospholipids.

Glycerophospholipids carry additional group (lipid head groups) attached to the phosphate such as phosphatidylserine (PS), phosphatidylcholine (PC), and phosphatidylethanolamine (PE). PS is negatively charged while PE and PC have a net zero charges at pH 7. Phospholipids are variously dispersed in two different leaflets of the bilayer forming more than half of the total membrane lipid molecules (up to 60%).

Glycolipids: Glycolipid molecules carry carbohydrate chains instead of a phosphate-containing headgroup. In animal membranes, usually, the backbone of glycolipids is sphingosine. Glycolipids are in the outer leaflet of the cell bilayer and play important roles in different cellular functions such as cell growth, cell-cell interactions, and communications.

Cholesterol (Chol): Chol is one of the major and abundant components of animal cell membrane and belongs to the steroid family [5], consisting of four steroid rings that are linked to a short hydrocarbon tail and a hydroxyl group. Although Chol molecules have amphipathic character, they are not able to form bilayer by themselves. The Chol are the main components of lipid rafts.

Figure 2.2 A chemical structure of three main lipid molecules in the cell membrane including phospholipids, glycolipids and cholesterol (Chol). Adapted from <https://en.wikipedia.org>.

Membrane Proteins

There are three classes of membrane proteins, which are defined by their specific interaction with the lipid bilayer including integral membrane proteins, peripheral proteins and lipid-anchored proteins [1].

Integral Membrane Proteins (IMPs): Integral membrane proteins are mainly transmembrane proteins which pass through the lipid bilayer and thus, have two domains exposed to the extracellular space and the cytosol of the cell. Integral proteins have an amphipathic nature, having both hydrophobic and hydrophilic regions. The part of the proteins that is located within a lipid bilayer has hydrophobic character and adopts an alpha-helical structure. Those regions of the integral proteins that are exposed to both sides of the membrane (non-embedded domains) have hydrophilic characters and interact with water-soluble components. Some integral proteins span the membrane once, whereas the others do it several times. Integral membrane proteins play some vital roles in the cell membrane. They can act as a receptor such as integrin to mediate different cellular behaviors or function as ion channels or transporters for diffusion of ions and other molecules throughout the cell membrane [6] .

Peripheral Proteins: Peripheral Proteins are placed on the surface of the cell membrane and attach to the membrane and integral proteins by non-covalent interaction. They can work as a membrane enzymes, mechanical support, and signal transmitters.

Lipid-Anchored Proteins: Like the peripheral proteins, lipid-anchored proteins are placed on the surface of the cell membrane, but they are anchored covalently to the fatty acid domains of the lipid molecules in the core of the bilayer. This type of membrane proteins are involved in cell signaling.

Membrane Carbohydrate

Membrane carbohydrates constitute 2-10% of the cell membrane [7]. These molecules are linked covalently to the lipids (glycolipids) and proteins (glycoproteins). More than 90% of the membrane carbohydrates are attached to the membrane proteins and are short branched oligosaccharides. They perform several key roles in cell-cell recognition, communication, and intracellular adhesion.

2.1.2. Membrane Fluidity

The cell membrane has a two-dimensional fluid structure in which lipids and protein molecules are laterally mobile. This unique physical property of the cell membrane facilitates different interactions within the planar membrane. For instance, the membrane fluidity allows reorganization and clustering of membrane proteins necessary for the different cellular processes such as intercellular signaling [8]. In addition, many of cellular processes such as cell growth, cell migration, cell division, endocytosis, and intercellular junction depend on the movement and reorganization of membrane compositions.

Figure 2.3 Schematic of translocation of lipid molecules in the planar bilayer structure. Lipid molecules can move in different manners including rotation, lateral diffusion, flip-flop, and exchange.

The position and orientation of the phospholipid molecules within the membrane are constantly changing over time. There are several types of lipid motions such as rotation, lateral diffusion, transverse diffusion or flip-flop, and molecular exchange (**Figure 2.3**).

The diffusion coefficient (D) of lipid molecules in the bilayer plane is usually used as a measure of the membrane fluidity. Membrane fluidity refers to the viscosity of the lipid bilayer arising from the friction of the lipid molecules as they rearrange to accommodate shape changes. In this context, the fluidity of membrane is a result of lateral motion of phospholipids in the plane of membrane. Thus, the diffusivity of lipid molecules in the membrane plane can be used a measure of membrane viscosity and hence membrane fluidity. Lateral motion of molecules within the membrane and their diffusion coefficients can be measured by a number of fluorescence techniques including fluorescence recovery after photobleaching (FRAP) [9]. Accordingly, several models have been developed which correlates diffusion coefficient of lipid molecules to the membrane viscosity [10].

The fluidity of lipid bilayers is controlled by temperature and lipid composition. The temperature at which the lipids undergo a phase transition from a fluid to a gel phase called transition temperature. Phase transition temperature of lipids depends on the lengths of the fatty acid chains as well as the number of double bonds along these chains. Lipids with short chains have a weaker interaction with each other in comparison to the lipids with longer. The existence of double bonds in the hydrocarbon tails forms a kink structure that perturbs packing of fatty acid chains.

The Chol content also influences the fluidity of membranes. Above the transition temperature, the steroid rings of Chol molecules interfere with the fatty acid chains of phospholipid molecules in the bilayer and hinder their movement that results in the reduction of membrane fluidity. Below the transition temperature, Chol molecules maintain the fluidity of the bilayer by preventing the fatty acid chains of phospholipids to

be packed together and be crystallized. Therefore, Chol content of the membrane can regulate the membrane fluidity.

2.2. The Extracellular Matrix

The multicellular structures in vertebrates are supported mechanically and biochemically by an organized network of macromolecules forming the ECM [6]. The ECM is secreted, synthesized and remodeled by tissue-specific cells and its composition alters from tissue to tissue. This highly complex network provides biochemical and mechanical stimuli to affect cellular behavior.

The basement membrane and the interstitial matrix are the two main types of the ECM (**Figure 2.4**). The basement membrane with a sheet-like structure acts as a barrier between epithelium and stroma. In adult tissues, the major components of basement membrane are collagen type IV (Col IV), laminin (LM), and heparin sulfate proteoglycans (HSPGs). The interstitial matrix fills the intercellular space and is mainly composed of fibrillar collagen (Col), elastin, fibronectin (FN), and proteoglycans.

The ECM components continuously interact with the mammalian cells through specific cell surface receptors such as integrin and play critical roles in regulating different cellular behaviors such as cell attachment, migration, proliferation, differentiation and cell-cell communications [1,11]. Any abnormal change in the ECM structure and composition causes failure in the signaling pathway that results in different disease and disorders [1].

In this section, some of the key components of the ECM and their roles are described in detail.

Figure 2.4 Illustration depicting the two main part of the ECM including basement membrane and interstitial matrix and their interaction with specific cells surface receptor (integrin) in human skin. Reprinted from <https://www.studyblue.com>.

2.2.1. Collagen

Collagen (Col) is a fibrous protein in the ECM which constitutes up to 25% of all proteins (the most abundant protein) in the mammals and is mainly produced by fibroblasts [1]. They provide strength and flexibility for different tissues such as tendon, cartilage, bone and skin and also are important for a wide variety of functions such as cell attachment, cell migration, tissue morphogenesis, and repair [12]. These molecules are made of three polypeptide chains called α chain (**Figure 2.5a**) that can be identical or a mixture of different chains with different chemical composition [1]. These chains are linked by hydrogen bonds and at least in some parts of their length, are twisted around each other forming a stiff triple helical structure (**Figure 2.5b**). Each α chain in Col structure composed of repeats of the amino acid sequence glycine (Gly)-X-Y, where X and Y can be any amino acids but are frequently occupied with proline and hydroxyproline [13]. Gly as a smallest amino acid plays important roles in Col triple helical formation and is placed in the center of triple helical strictures. The side chains of the X and Y amino acids are placed outward and can have intermolecular interactions with the other molecules.

Figure 2.5 The schematic illustration of (a) a polypeptide chain (α chain) and (b) Col triple helix structure. Col molecules are composed of three identical or different α chains which are twisted around each other, forming a stiff triple helical structure.

To date, at least 28 different types of human Col have been determined with the different combination of α -chains [1]. Depending on the type of α chains, the Col molecules also can self-assemble into fibrils [13]. For instance, collagen types I (Col I), II, and III fibrils structure are found in fibrous connective tissues such as skin, tendon, bone cartilage, etc.

Among fibrillar Col, type I is the most abundant type in the mammals (more than 70%) [14]. Col I is composed of two identical $\alpha 1$ (I) chains and one $\alpha 2$ (I) which each chain contains similar amino acid composition (**Table 2.1**). The molecular length and width of Col I are about 300 and 1.5 nm, respectively [13]. The type I fibrils have a diameter of 50 to 200 nm with a length of hundreds of micrometers [13] (**Figure 2.6**). Col fibrils can also self-assemble into parallel bundles forming Col fibers (**Figure 2.6**).

Table 2.1 Amino acid composition of Col I. Adapted from [15].

Amino acid	$\alpha 1(I)$	Percentage(%)	$\alpha 2(I)$	Percentage(%)
3-Hydroxyproline	1	0.1	1	0.1
4-Hydroxyproline	108	10.8	93	9.3
Aspartic acid	42	4.2	44	4.4
Threonine	16	1.6	19	1.9
Serine	34	3.4	30	3
Glutamic acid	73	7.3	68	6.8
Proline	124	12.4	113	11.3
Glycine	333	33.3	338	33.8
Alanine	115	11.5	102	10.2
Half-cystine	0	0	0	0
Valine	21	2.1	35	3.5
Methionine	7	0.7	5	0.5
Isoleucine	6	0.6	14	1.4
Leucine	19	1.9	30	3
Tyrosine	1	0.1	4	0.4
Phenylalanine	12	1.2	12	1.2
Hydroxylysine	9	0.9	12	1.2

Figure 2.6 The diagram represents the self-assembly of Col I into fibrils and fiber structures. The assembly of Col into fibril structure is called fibrillogenesis and it is the result of intermolecular covalent aldol cross-links between two lysine or hydroxylysine amino acids at the C and N-terminus of adjacent Col molecules. Col molecules are staggered with respect to one

another approximately every 67 nm. Several fibrils are packed in parallel bundles forming Col fibers. Col fibrils and fibers provide high tensile and mechanical strength for the tissues that need to tolerate high forces such as tendons. Adapted with permission from [16]. Copyright © 2009, Royal Society of Chemistry.

2.2.2. Proteoglycans

Proteoglycans are another important component of the ECM that exists in large amounts in the basement membrane. Proteoglycans are composed of a core protein that is covalently attached to glycosaminoglycans (GAGs) chains (**Figure 2.7**). The GAGs are polysaccharides made of repeating disaccharide units. The four main classes of the GAGs molecules exist in proteoglycans structures are: hyaluronan, chondroitin sulfate /dermatan sulfate, heparan sulfate, and keratan sulfate [6].

Due to the presence of sulfate and carboxyl subunits in their sugars structures, GAGs are highly acidic and negatively charged. Therefore, these molecules are highly hydrophilic and adsorbed a huge number of water molecules that results in the formation of a porous, hydrated gel-like structure that fills the extracellular environment. This property of glycoproteins protects the cells and tissues from compression forces. Glycoproteins along with Col that resist pulling forces give strength to the ECM and support them against deformation [1]. Additionally, proteoglycans involve intercellular and cell-matrix interactions and also have critical roles in cell migration, cell differentiation and cell proliferation [17,18].

Figure 2.7 The structure of proteoglycans. These molecules consist of a core protein anchored covalently to the GAGs. Proteoglycans in the cartilage matrix can be bound individually to hyaluronic acids, a non-sulfated GAG through their core proteins and create a gigantic complex structure as large as a bacteria cell.

2.2.3. Fibronectin

Fibronectin (FN) is an essential ECM protein with a molecular weight of ~440 kDa and a molecular length of 60-70 nm. These macromolecules are produced by a wide variety of cells such as fibroblasts, and hepatocytes and can be found in both soluble and insoluble forms (fibrils). Soluble FN exists in blood plasma, while the fibrils are the main component of the ECM. The FN molecules consist of two linear polypeptides linked covalently by a pair of disulfide bonds near the C-termini [1]. Each polypeptide is made of three repeated modular forms: FN type I, FN type II, and FN type III. The FN modules are organized into multiple functional domains (**Figure 2.8**). Each domain has one or more specific binding sites for:

- Different components of the ECM including Col, proteoglycans, and fibrin molecules. These binding domains help to connect the ECM molecules together and stabilize the matrix.
- Cell surface receptors such as integrin. FN molecules contain a specific cell binding domain composed of arginine, glycine and aspartic acid (RGD). Integrin can bind to the RGD sequence and facilitates the attachment of cells to the ECM.

These characteristics of FN molecules make them one of the vital extracellular components that mediate different cellular interactions with the ECM and have significant roles in cell attachment, growth, differentiation, and migration [19-21].

Figure 2.8 The structure of fibronectin molecule and its binding sites. The RGD is a typical sequence in cellular recognition.

2.2.4. Laminin

Laminins (LM) are the main extracellular proteins in the basement membrane [22]. They exist as a trimer constituted of three distinct polypeptide chains namely α , β , and γ which are joined by the disulfide bond as shown in **Figure 2.9**. These three polypeptide chains are organized into a crossed shape with three short arms and one long arm. To date, 15 different types of LM have been characterized. LMs, like the other extracellular components such as Col and FN, can bind to cell surface receptors such as integrin to perform critical roles in cell migration, cell growth, and cell differentiation [1]. In

addition to binding with integrin, LM can bind to proteoglycans and different ECM components that are in the basement membrane.

Figure 2.9 The structure of LM molecule and its binding site. They exist as a trimer constituted of three distinct polypeptide chains namely α , β , and γ . Adapted from <https://answersingenesis.org>.

2.2.5. Liver Extracellular Matrix

In this thesis work, hepatocarcinoma cell line (Huh 7.5) was employed as model cells. The Huh 7.5 cells are a subclone of Huh 7 derived from hepatocyte cells in the liver tissue which shows liver specific functions such as albumin production [23]. This thesis work was part of a bigger project dealing with liver tissue engineering. Therefore, the model cell for liver tissue was used.

This section explains a brief overview of liver ECM.

While most of the ECM components are similar, each tissue or organ in the body has particular ECM compositions that determine their functions [24-26].

Figure 2.10 shows the distribution of ECMs in the liver. Col and FN are the main components in the liver ECM that support liver tissue physically and biochemically [27,28]. These molecules participate in a wide variety of functions such as liver development, regeneration, and modulating hepatocyte differentiation state [28,29]. Since the proper functions of the ECM molecules are vital for liver tissue, any modification, and change in the ECM structure and composition can affect liver functions and cause disease such as liver fibrosis [30].

Figure 2.10 Schematic illustration of ECM localization in the liver tissue. Adapted with permission from [31]. Copyright © 2007 Blackwell Publishing Ltd.

2.2.6. Interactions between Cells and the ECM

As described previously, the human cells are embedded within a complex network of the ECM macromolecules that are influenced by their chemical and mechanical properties. One of the major roles of the ECM is providing chemical signals for mammalian cells. Chemical signals are transmitted within the cells through the interaction of cell surface receptors with specific cell binding domains present in the ECM protein structure. These signals are essential for regulating different cellular processes such as cell adhesion, cell migration, and cell differentiation. It has been shown that the activation of different signaling cascades such as cell proliferation,

differentiation, and apoptosis depend on the interaction between the RGD sequence and integrin [32,33]. In addition to RGD, other cell binding domains also found within ECM proteins such as (IKVAV) [34] and YIGSR [35] from laminin, GFOGER from Col I [36], PHSRN from FN [37] that mediate different cellular behaviors. Furthermore, the ECM is a reservoir for growth factors [38]. Growth factors such as vitamin or hormones can bind to cell surface receptors and regulate different cellular process in the body.

While historically, most of the study has been done on the chemical effect of the ECM, the role of matrix mechanics is less well known. The mechanical environment of mammalian cells is important for regulating different cellular functions such as adhesion, differentiation, and migration. Mammalian cells reside in tissue structures and frequently are influenced by different mechanical effectors such as external forces (e.g. shear stress) and the ECM stiffness. The ECM stiffness not only protects cells from mechanical stress but also play a significant role in the cellular fate and behavior [39-41].

Stiffness is the ability of an object to resist deformation during applying forces. Depending on the ECM composition and organization, the stiffness of human tissues differs from soft tissues to hard tissues. Additionally, the stiffness of mammalian tissue is not fixed and can be varied in different physiological and pathological conditions such as wound healing [42,43], and cancer [43,44].

A wide variety of substrates were designed to investigate the effect of the ECM stiffness on cellular behaviors such as poly (dimethylsiloxane) (PDMS) [45], and hydrogels formed from polyacrylamide (PAA) [46]. Example of using these materials is culturing epithelial and fibroblast cells on collagen-coated polyacrylamide to study the effect of matrix flexibility on cell adhesion and spreading [47]. It has been shown that cell adhesion and spreading were reduced on flexible substrate compare to the rigid substrate. Similarly, fibroblast cultured on the soft and rigid substrate shown different cellular morphology. These cells were rounded on the soft substrate while shown an increase in cell spread area on the stiff substrate [48]. Endothelial cells also showed higher spreading area on the substrate with greater rigidity [49]. Additionally, it has been

demonstrated that the stiffness of the substrate can regulate stem cell lineage specification [50]. However, changes in matrix stiffness in vitro can simultaneously affect other properties of the substrates such as surface chemistry or topography that provide some limitations to study independently the effect of ECM stiffness on cellular functions [51].

2.2.7. Biomolecule Engineering at Interface

In the last section (**Section 2.2.6**) the effects of the ECM on cellular behavior were discussed. Now, we are going to talk about natural and synthetic ECM components, the substrate and also different surface engineering techniques that were used to mimic extracellular environment for different applications from routine cell culture to tissue engineering.

Natural and Synthetic ECM Components for Cell Studies

ECM components in the form of full-length proteins or short peptides (**Section 2.2.6**) are usually used to coat the substrate to mimic extracellular environment for cell studies. Full-length proteins such as Col, FN and LM are commonly used for investigation of cellular behaviors. These molecules can be extracted individually or collectively from different mammal's tissues and organs via decellularization processes. Decellularization is a process by which cells are removed from the tissues, leaving a native three-dimensional ECM scaffold with the same physical and biochemical properties of its origin [24] (see **Figure 2.11**).

Decellularized tissues /organs have been widely used in tissue engineering and regenerative medicine applications [52]. The ECM scaffold provides the necessary chemical and mechanical cues required for cell growth, differentiation, and tissue development. Moreover, due to the absence of the host cells in the decellularized tissues, they can be re-seeded with recipient-derived cells to be used as a biocompatible transplantation that eliminates the adverse immune response [53].

Many methods have been introduced for decellularization of tissues or organs which can be varied based on the tissue or organ cellularity, density, lipid concentration and thickness [54]. In general, a proper decellularization protocol minimizes the adverse effects on ECM components and structures. Decellularization methods can be categorized to physical, chemical and enzymatic methods [24,52]. The physical methods involve freezing, agitation, sonication, mechanical massage, and direct pressure to lysis the cell membrane and release cell materials. The chemical methods use acids, alkaline, organic solvents, detergents, hypotonic, and hypertonic solutions, to solubilize the cytoplasmic component of the cells and also to remove nucleic acids. The enzymatic method applies different enzymes such as protease, nucleases and lipase to remove and separate the cellular material from the ECM. All these methods have advantages and disadvantages and usually the combination of them makes the most robust and high efficient decellularization protocols.

There is a broad range of the ECM scaffolds derived from different tissues and organs using decellularization process such as liver [55,56], and adipose tissue [57,58]. In particular, adipose tissue has been extensively used as a source of the ECM for cell studies. In mammals, there are two types of adipose tissues namely white adipose tissue and brown adipose tissue. White adipose tissue mainly serves as an energy reservoir while brown adipose tissue serves to dissipate energy. The ECM components of adipose tissue are comprehensively reviewed by Edwin C. M. Mariman and coworkers [59]. In general, adipose tissue is enriched in Col proteins. Other ECM components also can be found in adipose tissue such as LM, and FN.

White adipose tissue has been used as a bioactive coating material for studying mammalian cells behaviors. For instance, decellularized white adipose tissue conjugated to polycaprolactone (PCL) substrate [60] was used as an scaffold for plating mesenchymal stem cells (ASCs). The ASCs cells were plated on this platform and cell adhesion, proliferation, and viability were studied. It has been shown that the ECM-modified PCL promotes cell attachment, proliferation, and viability. Additionally, human

adipose tissue-derived ECM was used as a bioink for 3D printing of adipose tissue constructs in order to study soft tissue regeneration [61].

Figure 2.11 Decellularized tissues/organs and their properties. The ECM-derived tissues/organs have the same physical and biochemical properties of their origin. Adapted with permission from [52]. Copyright © 2016 Copyright Clearance Center, Inc.

Surface Engineering for Cell Studies

The model substrate used for cell studies should have several essential characteristics: First of all, it should provide a condition that bioactive molecules distribute homogeneously and their density is controllable. Additionally, the substrate should be resistant to nonspecific ligand or protein adsorption. Moreover, this model system should be compatible with adherent cell culture condition as well as different characterization methods for cell studies [62].

To date, a large variety of surfaces and chemistries have been developed in order to immobilize bioactive molecules on different substrates such as glass [63,64], polystyrene, metals oxide [65], quartz [66], polymers [67], self-assembled monolayer (SAM) [62] and the SLBs [68,69]. The simplest way to immobilize the bioactive molecules on the surfaces such as glass or polystyrene is the incubation of these surfaces with the bioactive molecules in the physiological conditions. The interaction between the solid surfaces and the bioactive molecules is affected by several parameters including i) environmental condition such as pH, salinity, and temperature, ii) protein properties such as size, structural stability, and composition as well as iii) surface properties such as

polarity, charge, and roughness [70]. The disadvantages of this type of system are as follows: this interaction is nonspecific and mediated by physical forces; therefore, there is no control on the amount, orientation and spatial distribution of the adsorbed molecules. Additionally, this type of interaction can cause protein structural rearrangements or even protein denaturation that may alter the protein function [70-72]. Furthermore, the part of the protein molecule which interacts with the solid surface is not available for other interactions.

To overcome these drawbacks, the bioactive molecules were attached on different surfaces using specific coupling methods. There are several chemical approaches to modified the solid substrate for covalent attachment of ligands including silanized silica [73,74], thiolated gold [75,76], N-hydroxysuccinimide ester (NHS ester)–amine coupling [66,68]. Bioactive molecules also can be conjugated specifically to the substrate via histidine/nitrilotriacetic acid interaction [77] or an interaction between streptavidin-labeled proteins/peptide with biotin molecules attached to the surface [78,79]. These methods allow modulating the densities of the molecules and also enabling us to more easily control the spatial distribution and orientation of the bioactive molecules to the surface. In many cases, the solid surfaces are passivated using poly (ethylene glycol) (PEG) [75,76] or proteins [80] such as albumin to minimize nonspecific adsorption of cells and proteins in cell growth media.

2.3. Biomimetic Models of the Cell Membrane

Natural cell membranes are highly complex systems. Therefore, for studying the structures and functions of the biological membranes various lipid-based model systems have been developed which their physical and chemical characteristics such as fluidity and ability to host membrane proteins are comparable to those of natural membranes [81]. To date, a wide variety of biomimetic models such as small [82], and giant lipid vesicles [83], bilayer nanodiscs [84], and SLBs [85] have been developed. These platforms when functionalized with biological ligands, allow studying membrane-associated cellular processes in a sufficiently simplified and more controlled conditions.

2.3.1. Supported Lipid Bilayers

SLB is an effective membrane model system that was first introduced by Tamm and McConnell in early 1980s [86]. This platform consists of a single lipid bilayer associated with a planar solid substrate. An important feature of the SLB is that the bilayer is not in direct contact with the substrate but separated from the solid surface by a thin (10-20 Å) layer of water (**Figure 2.12**). Despite the presence of the water layer, the interaction of bilayer with the surface is strong enough to hold it attached to the surface. This water-filled space between bilayer and the substrate is important as it allows the lipids in the bilayer to retain their lateral mobility despite its association to the surface. As mentioned before, membrane fluidity is a critical property of biological membranes which facilitates many vital cell membrane functions. The advantages of the surface-association of SLBs are two folds: 1) the solid support imparts mechanical stability to the SLB and makes it a robust platform, 2) a wide range of ultrasensitive surface-based analytical techniques can be used to study the SLB.

Figure 2.12 A schematic illustration of solid supported bilayer. The gap between solid and lipid bilayer is filled by a thin layer of aqueous solution (~1nm).

Several types of SLBs have been fabricated based on different applications such as hybrid bilayer, tethered bilayer, suspended lipid bilayer, and supported vesicle layer [87].

Hybrid Lipid Bilayer Membrane

Hybrid lipid bilayers consist of a solid support (e.g., silicon or gold), a hydrophobic self-assembled monolayer (SAM) and a lipid monolayer. Alkylsilane and alkanethiol are frequently used to form hydrophobic SAM on glass and gold, respectively. A lipid monolayer can be deposited on the hydrophobic SAM via vesicle fusion or other techniques forming a hybrid bilayer as shown in **Figure 2.13** [88,89]. Due to the strong interaction between the hydrophobic SAM layer and the substrate, this platform is highly stable. However, due to the lack of water-filled space between the lipids and the support, the mobility of lipids in this platform is less than the mobility of lipids in the supported bilayers.

Figure 2.13 A schematic representation of a hybrid lipid bilayer membrane (h-LBM).

Fabrication Methods of Supported Lipid Bilayers

SLBs are commonly formed using the vesicle fusion method [90]. Different steps involved in the formation of SLB using vesicle fusion method are depicted in **Figure 2.14**. In this method nano-sized ($< 100\text{nm}$) lipid vesicles are used. Lipid vesicles are closed bilayer structures surrounding an aqueous cavity. Upon addition of vesicles to hydrophilic surfaces, they adsorb to the surface and deform due to the interaction between membrane and the surface. If the adhesion energy between the membrane and the surface is strong enough, the vesicles eventually rupture. The ruptured vesicles then fuse together and form a continuous bilayer on the solid surface. Therefore, vesicle rupture is the critical step in bilayer formation using vesicles fusion, and thus the success

of the bilayer formation using this method strongly depends on the vesicle-surface interactions.

The vesicle-surface interaction is a function of both surface and vesicle physical properties such as surface hydrophilicity, roughness, and polarity of the solid surface as well as rigidity, surface charge and size of the vesicles. With respect to substrates, under physiological conditions, vesicle fusion is limited to a subset of surface and lipid compositions. It mainly occurs on silica-based material such as mica and silicon dioxide [91], while on the other surfaces such as gold [92], titanium oxide [93] and aluminum oxide [94]; vesicles adsorb but do not rupture and remain intact. With respect to lipid composition, incorporation of a high amount of Chol or negatively charged lipids into the vesicle hinders vesicle rupture [95]. The reason is the high adhesion energy required for the vesicle to rupture [96]. In some cases, the experimental conditions such as solution pH [97], ionic strength [98], and temperature [99] can be optimized to overcome these limitations.

Figure 2.14 Illustration of vesicle fusion on a hydrophilic solid support and formation of SLBs. Adapted with permission from [100]. Copyright © 2008 Elsevier B.V.

In response to these limitations, other methods of bilayer formation that do not require vesicles have been developed including spin coating [101], air bubble collapse [102], and solvent-assisted lipid bilayer (SALB) formation method [103]. In this thesis, SALB formation method was used to fabricate bilayers, thus it will be explained in more detail below.

Solvent-Assisted Lipid Bilayer Formation Method

The SALB formation method is a simple and robust technique that recently has been developed in our group [103]. The method was mainly developed to overcome the limitations of vesicle fusion method. In this technique, first, lipids dissolved in a water miscible alcohol (isopropanol or ethanol) are incubated with the substrate. In this step, lipid molecules adsorb to the surface until equilibrium is reached. Next, the lipid-alcohol mixture is gradually replaced by water. The introduction of water to the system leads to several bulk phase transitions in the tertiary mixture of lipid-alcohol-water. When the water fraction in the mixture exceeds a threshold (about 90%), lipids in the bulk form bilayer lamellar structures and a supported bilayer form on the surface (see **Figure 2.15**).

Figure 2.15 The principal of solvent assisted lipid bilayer method. Lipids in a mixture of an organic solvent (e.g. isopropanol) and water can be deposited on a hydrophilic substrate. After lipid deposition, if the solvent's water fraction is then gradually increased up to 80-90%, a phase transition occurs to form a planar bilayer. Adapted from [104].

While the exact mechanism of the bilayer formation is still not clear, the quality of the formed bilayer depends on several operating parameters including lipid concentration, solvent exchange rate, and chamber dimensions as well as the solvent type [103,105]. It has been demonstrated that the formation of a high quality lipid bilayer on silicon dioxide (SiO_2) required the introduction of high lipid concentration (0.5mg/mL) with low flow rate (50 $\mu\text{l/min}$) [105]. However, for other surfaces, the optimum condition might be a bit different, thus one might need to fine tune the operating condition in particular lipid concentration to find the optimum condition.

The SALB has been successfully used to form bilayer on substrates which are intractable to vesicle fusion such as TiO_2 [105], Al_2O_3 [106], Cr and Au [103]. Another unique feature of this technique is the ability to form lipid bilayers composed of complex lipid compositions. Importantly, the method allows the formation of bilayer with a high fraction of Chol which is not possible using the vesicle fusion method. The vesicle fusion method only permits to form SLBs containing up to 10 mol % Chol [95]. In contrast, SALB method allows the formation of SLBs containing up to ~ 63 mol % Chol [107]. The formation of Chol-enriched SLBs is necessary to study the plasma membrane of some cells such as erythrocytes that contain 45-50 mol% Chol within its membrane. Additionally, Chol content can be varied in the variety of diseases states and affecting normal cellular behaviors. Moreover, Chol is an important component for regulating the mobility of the bilayer as mentioned in **Section 2.1.2**. In addition to Chol, SALB method was utilized to fabricate different fractions of anionic (DOPS) and cationic (DOEPC) bilayer on SiO_2 and Al_2O_3 [106].

In this thesis work, we used the SALB method to form bilayer with different fractions of Chol to control the viscosity of the membrane.

Analytical Methods to Study Supported Lipid Bilayer Platform

Attachment of SLB to a solid surface increases its stability and allows characterizing this platform with a wide variety of surface-based analytical tools such as

surface plasmon resonance spectroscopy (SPR) [108], quartz crystal microbalance with dissipation (QCM-D) [109], fluorescent microscopy [110], and atomic force microscopy (AFM) [111]. These methods provide the information about the structural properties of SLBs as well as the associated biological process. Below we discuss the application of these techniques for studying SLBs.

QCM-D is a surface sensitive technique which measuring the free oscillation of the quartz crystal, recording the changes in frequency (ΔF) and dissipation (ΔD). The ΔF is related to the mass adsorbed on the crystal, while ΔD represents viscoelastic properties of adsorbing layers such as rigidity and softness. This technique has been used extensively to study the kinetics of lipid adsorption onto solid support and bilayer formation. In particular, QCM-D provides a signature for adsorption and rupture of lipid vesicle on solid supports resulting in the formation of planar bilayers [112]. Also, this technique enables us to monitor and characterize adsorption [113] and structural properties of biomolecules on the SLB.

Fluorescence microscopy is a fundamental technique used in different fields of science especially in biology. This technique is applied to various specimens that are fluorescent in its natural form or conjugated with fluorescent chemicals. Fluorescence microscopy commonly used to investigate physical properties of SLBs such as homogeneity, the formation of membrane domains, and phase separation. Additionally, this technique permits to visualize the interaction of biomolecules and cells with SLBs. Lipid molecules do not fluorescence. Therefore, there is an essential need to incorporate fluorescently labeled lipids in the bilayer structure to be studied by fluorescence microscopy. Depending on the nature of the lipid and the fluorescent label, the labeled lipid may exhibit specific properties. For instance, rhodamine-PE lipids are known as a fluorescence labeled lipids that preferentially accumulates in liquid phase domain. This lipid has been used to visualize phase separation on SLBs by fluorescence microscopy [107].

Lipid lateral mobility as a fundamental property of SLBs is measured using a fluorescence-based technique called fluorescence recovery after photobleaching (FRAP) [9]. In this technique, a defined area of fluorescent-labeled SLB bilayer is photobleached by a powerful laser and the fluorescent recovery caused by lipid diffusion is monitored by fluorescence microscopy. FRAP allows measuring the percentage and the speed of the fluorescence recovery. This information indicates the mobile fraction of the bilayer and also diffusion coefficient (D) of the lipids within the SLBs.

AFM is a type of scanning probe microscopy with a high resolution which is used to measure the interaction force between the tip and the sample. AFM is a suitable method for imaging soft biological materials such as SLBs at nanometer resolution [114]. This technique widely used to investigate the thickness of SLBs as well as the presence of lipid domains [115]. AFM has been used to distinguish between the liquid phase and solid phase in Chol-enriched SLBs [95]. Additionally, this technique can be applied to monitor time-dependent process such as the interaction of SLBs with biomolecules [116].

SPR has been thoroughly established and recognized as a versatile, label-free monitoring for the interaction of biomolecular interactions in fluids. SPR has been used to characterize the adsorption of range of biomolecules such as proteins onto SLBs [117].

In this thesis work, QCM-D, fluorescent microscopy, and FRAP were used as main methods for studying SLBs and associated biomolecules. These methods will be described in more detail in **Chapter 3**.

Antifouling Character of Supported Lipid Bilayers

Proteins can be easily immobilized on different surfaces such as glass and polystyrene through nonspecific interactions [118]. Some proteins also can bind to charged SLBs [119], while zwitterionic SLBs is highly resistant to protein adsorption. For instance, QCM-D study showed a negligible adsorption of proteins such as human fibrinogen, human serum albumin, and bovine hemoglobin on SLBs composed of

zwitterionic PC [113]. The PCs are one of the head groups of phospholipid family that are neutrally charged in the solutions with a wide pH range, $3 < \text{pH} < 10$ [120]. Therefore, proteins cannot interact with SLBs through electrostatic interactions. Additionally, the surface of PC SLBs is surrounded by water layer that prevents the interaction of proteins with SLBs [120]. Furthermore, lipid molecules contain PCs have high lateral lipid mobility that is considered as one the reason for protein repletion [113]. Gel-phase SLBs also can retain nonfouling characteristics to some extent. For example, QCM measurements showed a negligible absorbance of serum proteins on MPPC-based gel-phase SLBs [121].

SLBs are also resistant to cell adhesion [122,123]. Cell adhesion usually occurs through the interaction of cell surface proteins with the substrate. Since no protein can adsorb on SLBs, therefore, cells cannot adhere to the bilayer. The cell repellent properties of SLBs were early shown by culturing two different types of epithelial cells on PC SLBs [122]. No cells were attached and proliferated on this platform whereas epithelial cells could adhere and proliferate on the control surfaces including SiO_2 , glass, and TiO_2 . QCM-D measurement confirmed that the absence of serum proteins on SLB is the main reason for cell resistivity.

The antifouling nature of SLB prevents nonspecific protein interaction with SLBs that is critical for different applications such as cells studies. Cell-SLBs interactions will be discussed in more detail later in the section.

Functionalization of Supported Lipid Bilayer

The SLBs are passive but functionalizable surfaces. Functionalization of SLBs is a process in which the functionality is introduced to the surface of the lipid bilayer with the aim of its specific interaction with proteins and cells. Membrane functionalization is commonly used to study biological processes such as cell-cell [124,125] and cell-ECM interactions [68,69,126].

Several strategies have been used to functionalize SLBs as shown in **Figure 2.16**. Based on these strategies, specific molecules such as amphiphilic or functionalized lipids doped into SLBs and act as specific receptors for protein adsorption and cell attachment.

Functionalized lipids are a type of lipid molecules with reactive head groups that are capable of interacting with specific target molecules. Among these head groups, one can name nitrilotriacetic acid (NTA), maleimide, biotin, and free carboxylic acid group.

Histidine-tagged proteins can bind especially to NTA-functionalized SLBs loaded with Ni^{+2} metal ions using chelation chemistry [77]. This interaction is highly selective, non-covalent, and reversible. Proteins with multiple histidine residues preserve proper orientation and stable attachment over SLBs. Conjugation of proteins with NTA-modified SLB has been used in different biological studies such as T cell immunological synapse [77].

Alternately, maleimide-modified lipids that are embedded into SLBs bond covalently to a thiol group of proteins and peptides [69,127]. Due to the covalent nature of this interaction, it is stable and irreversible.

Biotinylated lipids are the other functionalized lipids that can be incorporated into SLBs. These lipid molecules bound to biotinylated target through a stable linking bridge including streptavidin, neutravidin, and avidin [128-130]. This interaction is stable, irreversible and non-covalent and commonly used for different biological applications. For instance, using fluorescently labeled neutravidin allows to detect the lateral diffusivity of ligands and also provide the information about the amount of molecules per surface area [131].

Another chemical functional head group that was used in this thesis is a free carboxylic acid group in 1,2-dipalmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine-N-(glutaryl) or DP-NGPE. This functionalized lipid can be doped in SLBs and react with primary amines ($-\text{NH}_2$) placed at the N-terminus of polypeptide chains and side chains of

lysine amino acid residues through a so-called amine coupling. This coupling chemistry has been used to conjugate full-length proteins such as Col I and FN to the SLB [68,126]. A Free carboxylic acid group of DP-NGPE needs to be activated before interacting with proteins. This activation is done using N-hydroxysuccinimide (NHS) or 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). These molecules act as a cross-linker and are highly efficient when used together in pH (4.5-6). In the activation process, EDC binds NHS to a carboxyl group of DP-NGPE and forms an NHS ester. NHS ester is ready to interact with the primary amine group of lysine residues in proteins. Lysine constitutes ~3% of Col I amino acids. The primary amines of these molecules are nucleophilic and positively charge at pH 7 that usually placed on the outside of protein surfaces. Therefore, these molecules have high accessibility for activated carboxylic acid group of DP-NGPE.

In addition to chemical functionalization, SLBs can be modified by amphiphilic molecules. These molecules can be inserted into SLBs and serve as an anchor between SLBs and ligand of interest. For example, glycosylphosphatidylinositol (GPI) linkage can bind with specific proteins and embedded into SLBs through the insertion of their tails to the bilayer [132]. Chol also can be modified with specific biomolecules and act as an anchor for bilayer functionalization [133].

Figure 2.16 Selected strategies for bilayer modification. a) Use of functionalized lipids with various head groups such as maleimide, NHS, biotin, Ni-NTA. b) Use of amphiphiles molecules

using different lipid anchors such as cholesterol. Adapted with permission from [134]. Copyright 2015 WILEY - VCH Verlag GmbH & Co. KGaA, Weinheim.

Supported Lipid Bilayer in Cell Studies

The SLB is an enabling platform for cell studies thanks to its biocompatibility and the capacity to host range of signaling and adhering molecules. Due to its two-dimensional fluidity, lipids and anchored molecules retain their mobility and diffuse freely within the membrane [135]. This feature of SLBs facilitates reorganization and cluster formation of membrane-anchored proteins that is essential for effective ligand-receptor interactions and further signal activation [136,137]. Furthermore, it is possible to tune the physical properties of SLB such as surface charge [138,139], fluidity, as well as the density of signaling molecules [77,140] by controlling the bilayer composition. Additionally, functionalization of SLBs and its interaction with cells can be characterized with different surface sensitive techniques [108].

To study cellular process, SLB can be used as a platform for growth of mammalian cells or it can be used as a cell membrane mimics for cell-cell interaction and signaling process.

Supported Lipid Bilayer in Cell-Cell Interaction studies

Cell-cell interactions occur via extensive formation of membrane protein complexes at the cell interface. The process is dynamics and involves large scale rearrangements of protein complexes over lengths scales of tens of micrometers [141]. Intercellular engagement play crucial roles in physiological and pathological processes such as differentiation [142], adhesion [5] and cancer [143].

SLBs have been extensively employed to study cell-cell interaction in immunosystem [144]. For instance, the function of FC receptor (typical immune cell receptor) was investigated using SLBs. In this study, SLBs was modified with specific

antibodies contain FC region and exposed to the live cells with FC receptors. This interaction regulates different functions such as cell adhesion.

The next step was using SLBs to investigate the effect of ligand lateral mobility on cell adhesion. In this strategy, the action of membrane-anchored CD58 or lymphocyte function-associated antigen 3 (LFA-3) was compared with its transmembrane form. The anchored form of CD58 is mobile while the transmembrane form is immobile in SLBs. It has been shown that the mobile form CD58 was 50-times more potent and also higher stabilized adherence compare to the immobile form. Due to the mobility of the anchored CD58 they can accumulate under the cells that result in higher cell surface receptor-ligand interactions [137].

Also, SLBs was used to study the function of epidermal growth factor (EGF). EGF was tethered with SLBs and exposed to target cells. It was found that the fluidity of the SLBs enables the formation of EGF clusters underneath of the cells that reflect the distribution of EGF receptors (EGFR) on the cell surface [124].

Supported Lipid Bilayer in Cell-ECM Interactions

The importance of the interaction between cells and their extracellular environment in health and disease was described in **Section 2.2**. The SLBs functionalized with short peptides, and full-length proteins have been used as a simplified model of ECM for cell studies.

In the case of short peptides, these molecules were tethered to SLBs to promote several cellular functions such as cell adhesion, cell spreading, and cell differentiation. For example, SLBs was functionalized with IKVAV to investigate their influence on neural cell adhesion and neurite outgrowth [145]. It was demonstrated that SLBs functionalized with cysteine-terminated IKVAV peptides can promote PC12 cells adhesion [69]. IKVAV functionalized-SLB also was used as a substrate to study the influence of ligand density and mobility on AHPs cells adhesion, growth, and

differentiation. AHPs cells attached on peptide-functionalized SLBs and formed cluster morphology but remained undifferentiated [127].

SLBs decorated with RGD-containing peptides have also been used to mimic cell-ECM interactions[146]. For instance, SLBs modified with different densities of a multivalent cyclic RGD ligand was used to investigate the influence of ligand density and distance on HEK293 cells attachment and spreading. The result demonstrated that HEK293 cells could attach to SLB with 1 mol % of the ligand (10 nm spacing) and were able to spread and form filopodial extensions while at lower densities cell could not spread over the surface and remain round [147].

In addition to peptide molecules, SLBs have also been functionalized with native ECM proteins such as Col I and FN. These full-length proteins incorporated in SLBs can better mimic *in vivo* extracellular space. Col I was the first native ECM proteins that was conjugated with SLBs to serve as cell culture platform [68]. In this study, A10 cells were cultured on both Col I-functionalized SLBs and bare SLB to evaluate cell adhesion and growth on these two platforms. Cell attachment and spreading was 2.5-fold and 6.5-fold higher on Col I-functionalized SLBs in compare to bare SLBs.

In addition to Col I, FN and bovine serum albumin also were conjugated with SLBs using the same chemistry [126]. These proteins were used to study NIH3T3 cell growth and function.

Polysaccharides such as hyaluronic acid also can be used to promote cell adhesion onto SLBs. These molecules are recognized by specific cell surface receptors such as CD44. Hyaluronic acid was conjugated with SLBs through recombinant His-tagged p32 hyaluronic acid binding protein [148]. More recently, SLBs was functionalized with hyaluronic using biotin-streptavidin chemistry [149].

Cell Adhesion on Charged Supported Lipid Bilayers

Phospholipid bilayers found in cell membranes are usually negatively charged. Therefore, while they do not adhere to zwitterionic SLBs, they adhere to positively charged bilayers. Therefore, positively charged bilayer can be used as cell culture platform without using cell adhesion macromolecules. For instance, positively charged SLBs were used as substrates for neural cell culture [150]. This was achieved by introducing DOTAP; a positively charged lipid, into SLBs. Neuron cells successfully attached and grew on positively charged SLBs. The membrane of neuron cells is negatively charged and can interact with DOTAP through electrostatic interaction. In this study, neuron cells could sustain growth up to 10 days on positively charged bilayer.

2.4. PhD in Context of Literature

In response to the high complexity of native biological membranes and their microenvironments, various lipid based model systems have been developed and their physical and chemical characteristics are comparable to those of natural membranes [81]. The lipid based platforms, such as small [82], and giant lipid vesicles [83], bilayer nanodiscs [84], and SLB [85], when functionalized with biological ligands, allow studying membrane-associated cellular processes in sufficiently simplified and more controlled conditions.

Among them, the SLB comprised of a single planar lipid bilayer association with a solid substrate is particularly effective thanks to its excellent stability while preserving the fundamental characteristics of natural membranes such as two-dimensional fluidity and electrical sealing properties [151]. SLB is more “stable” and “durable” in compare to other membrane model system. For instance they better survive external mechanical stresses such as shear force exerted by flow in microfluidic devices. Also, SLBs chemical composition can be easily tuned to manipulate their biophysical properties such as surface charge [152], and lipid diffusivity [95], and their membrane organization such as phase separation [107] and formation of lipid rafts [153].

Importantly, SLBs can be functionalized with biological receptors and signaling ligands by introducing synthetic head-group modified lipids into the bilayer for direct covalent or non-covalent coupling of biomolecules [154]. Functionalized SLBs, have been used to examine range essential biological processes that occur at the membrane interfaces including viral fusion [155], ligand-receptor interactions [156], enzymatic activities [119], cellular signaling and cell-cell recognition [125]. Owing to their inherent antifouling properties; SLBs are resistant to nonspecific adsorption of cells [122] and proteins [113]; a feature which makes them a suitable interface for detection of specific biomolecular interactions. In particular, they are effective tools for studying receptor dynamics during cell-cell interactions as well as triggering various intercellular reactions when interfaced with live cells [125,154,157].

Moreover, a wide range of ultrasensitive surface-based analytical techniques such as atomic force microscopy (AFM) [158], quartz crystal microbalance with dissipation (QCM-D) [159], total internal fluorescence (TIRF) microscopy [160], and electrochemical measurements [161], can be used to characterize SLBs structure and the biochemical interactions occurs at their interface.

In **Chapter 4** of this thesis we utilized a recently developed method of bilayer formation; SALB formation method, to fabricate SLBs functionalized with different ECM components. The ECM is a tissue-specific network of extracellular bio-active molecules [162,163] that provides support and anchorage for cells and play a key role in numerous cellular processes including cell growth and differentiation as well as intercellular communications [164]. The ECM is mainly composed of fibrous proteins such as Col [165], FN [20], and LM [166], as well as proteoglycans with GAGs side-chains [167]. We investigated strategies for conjugation of various ECM proteins with controlled density onto the SLB and explored the cell-ECM interactions on such platform. In addition, we studied the effect of membrane viscosity the cell behavior and its interaction with the ECM components.

The biological activity and function of proteins such as enzymatic activity or presentation of ligands for specific receptors are usually exerted by functional domains in their 3D structures which specifically interact with other biomolecules. Consequently, any surface-induced structural deformation [70-72] may alter the protein function. Moreover, the part of the protein molecule which interacts with the surface is not available for other interactions. Thus strategies to coat surfaces with proteins while preserving their biological activities are of importance for the successful development of tailored biomaterial surfaces. In this regard, SLBs offer unique possibilities for controlled functionalization of solid surfaces. In **Chapter 5**, we provide a comparative assessment of the biological activity of FN and Col I that are (i) attached to SLB via covalent bonding and (ii) are adsorbed nonspecifically to SiO₂ surface.

While simplified models have been proven to be useful for many biological applications, development of models with greater compositional complexities would be the next step toward platforms that more closely mimic the cell surface environment. In this regard, decoration of SLBs with the ECM components holds great promise. Numerous protocols have been developed to obtain natural ECM through decellularization of tissue via a series of physical, chemical and enzymatic treatments [54,168,169]. Decellularized ECM (dECM) has shown to retain its natural 3D-ultrastructure and support the attachment and survival of cells while maintaining their phenotypic and functional characteristics. Thus, these biomaterials have attracted much attention as natural 3D-scaffolds for tissue engineering and regenerative medicine applications [170-178].

So far, SLBs functionalized with individual ECM components such as collagen [68], and fibronectin [179], or peptides derived from these proteins (e.g., RGD, IKVAV)[69,146,180-183] have been reported. In **Chapter 6**, for the first time, we demonstrate functionalization of SLBs with solubilized adipose tissue dECM and characterize their formation with QCM-D and immunofluorescence microscopy. We demonstrate that dECM functionalized bilayers sustain cell growth and maintain their function.

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Chapter 3

Experimental Methodologies

This chapter provides detailed information regarding the experimental approaches including fluorescence microscopy, scanning electron microscopy (SEM), quartz crystal microbalance with dissipation (QCM-D), fluorescence recovery after photobleaching (FRAP) and various biochemical assays such as ELISA, live/dead assay, and cell counting assay (CCK-8) as well as protein, DNA, and hydroxyproline quantification assays, used for (i) SLBs fabrication, functionalization, and characterization, (ii) cell culture and characterizations; and (iii) tissue isolation and characterization. Additionally, data analysis was included in each experimental protocol.

3.1. Rationale for the Selection of Materials and Experimental Techniques

Materials and experimental techniques used in this dissertation have been selected based on five different experimental plans. The first plan was the fabrication of a functionalizable and tunable SLB. Two different lipid types including DOPC and DP-NGPE were used for bilayer formation on a hydrophilic solid supports (SiO_2 and glass). DOPC lipid is a zwitterionic phospholipid which is the most common lipids in the animal cell membrane. This type of lipid is resistant to nonspecific proteins and cell adsorption. In contrast, DP-NGPE is a type of functionalized lipids containing a free carboxylic acid group allowing conjugation of proteins with the bilayer through amine coupling. These two lipids were assembled in bilayer structure using solvent-assisted lipid bilayer (SALB) formation method. This method is very simple and robust allowing the formation of a lipid bilayer that fully coats the solid support and also is independent of substrates material and lipid compositions.

After formation of the bilayer, it was decorated with two extracellular matrixes (ECM) proteins collagen type I (Col I) and fibronectin (FN) (second plan). The reason for this selection was that in our cell studies, we used human hepatocellular carcinoma cell line (Huh-7.5) which is derived from hepatocyte in the liver tissue. Therefore, Col I and FN which are two main ECM proteins in the liver tissue were used to mimic liver cell ECM.

The bilayer formation and functionalization were characterized using quartz crystal microbalance with dissipation (QCM-D), fluorescence microscopy, and fluorescence recovery after photobleaching (FRAP). QCM-D is a real-time surface sensitive technique commonly used to monitor the kinetics of thin film formation and characterization of its thickness and viscoelastic properties. It is widely used in soft matter research and interfacial science applications [1,2], particularly for studying lipid bilayer formation and its *in situ* modification [3]. The method also has been used for studying cell attachment and spreading on solid supports [4,5]. The method records the frequency change (ΔF) of an oscillating quartz crystal which depends on the total effective mass of the adlayer

which consists of the mass of the coating material plus entrapped and coupled water. A negative change in ΔF indicates an increase in the mass of the adlayer. In addition, QCM-D measures energy dissipation (ΔD) which reflects the softness/rigidity of the adlayer. A perfectly rigid film such as SLB has a near to zero energy dissipation, while softer adlayers such as proteins exhibit greater energy dissipation.

Fluorescence microscopy was used to visualize the homogeneity of the fluorescently-labeled lipid bilayer as well as the adsorption of the fluorescently-labeled proteins onto bilayer surface. In addition, FRAP was applied to check the fluidity of the membrane and to measure the diffusion coefficient of lipid molecules in the bilayer.

After establishing a strategy to fabricate ECM-functionalized supported bilayers with controlled density of proteins (Plan one and two), we used this platform as a substrate to study cell-ECM interactions (Plan three). Huh 7.5 cells were seeded on this platform and cells viability, proliferation, and albumin production were measured using standard techniques; Live/Dead assay, cell counting kit-8 (CCK-8) and enzyme-linked immunosorbent assay (ELISA), respectively. All the listed assays are very simple, accurate, stable, and sensitive.

In the next plan, we studied the effect of mechanical properties of the ECM-SLB on cellular behavior. We used cholesterol (Chol) to manipulate the viscosity underlying bilayers. The Chol increase the viscosity of the bilayer and reduces lipids mobility.

At the final plan, we functionalized SLB with tissue-derived ECM to closely mimic the extracellular environment. We used adipose tissue for decellularization, as the extraction of this tissue is easy compared to other tissues and it is enriched in ECM components. Adipose tissue was decellularized using a combination of chemical and enzymatic treatments. The chemical and enzymatic treatment using organic solvents removes the cells and dissolves the lipid molecules. Proteins and other cell components such as DNA and RNA can be degraded using specific enzymes.

After decellularization, the composition the decellularized-ECM (dECM) components were characterized using different bioanalytical assays including the bicinchoninic acid assay, picogreen assay, hydroxyproline assay, glycosaminoglycans (GAGs) assay. The morphology of the dECM was visualized by scanning electron microscope (SEM). All these assays and techniques were necessary to confirm that the isolated ECM is empty of DNA and fat and at the same time is full of proteins and sugars. Picogreen assay is used for measuring the amount of DNA in the tissue. Hydroxyproline assay was used to calculate the amount of Col that is the major proteins in adipose tissue. GAGs assay was applied to measure the amount of the glycosaminoglycan. The bicinchoninic acid assay was applied to measure the amount of total proteins inside the decellularized tissue. All the listed assays are very simple, stable, and sensitive

3.2. Materials

3.2.1. Lipid Reagent

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(glutaryl)(DP-NGPE), cholesterol (Chol), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Ammonium salt) (Liss Rhod-PE fluorophore) were obtained as a powder from Avanti Polar Lipids (Alabaster, AL, USA). The chemical structures of the lipid molecules used in this dissertation are presented in **Figure 3.1**.

Figure 3.1 Chemical structures of the lipid molecules utilized in this thesis.

3.2.2. Modification Reagent

N-Hydroxysulfosuccinimide sodium (sNHS) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) were received from Alfa Aesar, USA.

3.2.3. Protein Reagents

The Col I and was purchased from Sigma-Aldrich, USA, FN was obtained from Calbiochem, UK. Mouse monoclonal anti-Col I and mouse monoclonal IgG1 anti-FN were received from Abcam (USA), and Santa Cruz (USA), respectively. Alexa Fluor 488 Goat Anti-mouse IgG (H+L) and Alexa Fluor 555 Rabbit Anti-Mouse IgG (H+L) were purchased from LifeTechnology, USA.

3.2.4. Buffer Reagents

Radioimmunoprecipitation assay (RIPA) buffer and HEPES buffer were obtained from Sigma-Aldrich, USA. Phosphate-buffer saline (PBS) was received from Thermo Fisher Scientific, USA.

3.2.5. Chemical Reagents

All chemicals mentioned in this thesis were purchased from Sigma-Aldrich, USA, unless otherwise specified.

3.2.6. Cell Culture Reagents

Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum were received from Hyclone, USA. Penicillin, streptomycin, antibiotic-antimycotic and 0.25 % trypsin were purchased from Gibco, USA.

3.2.7. Cell Lines

Human hepatocellular carcinoma cell line (Huh-7.5) was obtained from Apath (NY, USA).

3.2.8. Substrates

Glass substrates and sticky-slid VI^{0.4} were obtained from ibidi, Germany. SiO₂-coated AT-cut piezoelectric quartz crystals (model no. QSX303) were purchased from Q-sense AB (Gothenburg, Sweden). Tissue culture dishes were purchased from Thermo Fisher Scientific, US.

3.3. Experimental Technique

3.3.1. Solvent-Assisted Lipid Bilayer Formation Method

Solvent assisted-lipid bilayer (SALB) formation method is a recently developed technique for bilayer fabrication [6]. The SALB method is based on the reverse-phase evaporation phenomenon and involves lipid deposition onto a hydrophilic solid surface in an alcohol, e.g., isopropanol, followed by the slow exchange of the alcohol with water, resulting in the formation of a lipid bilayer on the surface.

This technique enables us to overcome the limitations of the commonly used method of bilayer formation, namely vesicle fusion method. In fact SALB allows bilayer formation on surfaces such as Au [6], and Al₂O₃ [7], which are intractable to vesicles fusion. In addition, SALB can be used to form bilayers with compositions that cannot be formed with vesicles fusion. While using vesicle fusion, bilayer containing up to 10% Chol can be formed; SALB allows the formation of bilayers containing very high fraction of Chol (up to 60%). Since in this thesis we used Chol to modulate the bilayer viscosity, we needed a technique which allows formation of bilayers with wide range of Chol content. Therefore, we used SALB to form supported bilayers.

Experimental Protocol: Right before SALB experiment, DOPC, and Chol powders were dissolved in isopropanol at a nominal concentration of 10 mg/mL, and 25 mg/mL, respectively. Due to the insolubility of DP-NGPE in isopropanol, the powder was dissolved in ethanol (99.99%) at 5 mg/mL lipid concentration and was heated to 50°C and gently vortexed. The stock solutions were diluted in isopropanol to the desired concentration. Finally, isopropanol mixture of DOPC with various fractions of DP-NGPE (0%, 2.5%, 5%, 7.5%, 10%, and 20%) was prepared. For Chol experiment, different fraction of Chol (0%, 10%, 20%, 30%, and 40%) was mixed with isopropanol mixture of DOPC and 20% DP-NGPE. The final lipid concentration for all experiments was 0.5 mg/mL.

The substrates including glass and SiO₂ were immersed in 1% sodium dodecyl sulfate (SDS) and rinsed with Milli-Q water (Millipore) extensively. After drying the substrates with nitrogen gas (N₂), SiO₂ and glass were treated with oxygen plasma (March Plasmod Plasma Etcher, March Instruments, California) at about 180W for 1 minute. Bilayers were formed either in QCM-D chamber or commercially available microfluidic flow cells (stick-Slide). The lipid solution was introduced into the chamber for 10 min. An aqueous buffer solution (10mM Tris, 100mM NaCl, pH 7.2) was then carefully added and replaced the lipid-isopropanol solution completely. The flow rate was 50 µL/min and experiments were done at room temperature

3.3.2. Silane Chemistry

Coating of the substrate with a silane monolayer is called silanization which is the result of the adsorption, self-assembly, and covalent binding of silane molecules onto silica-based such as glass, silica, and mica. Silane-self-assembled monolayer (SAM) is a versatile tool using to control the physical and chemical properties of surfaces such as hydrophilicity and conductivity [8].

The self-assembling molecules usually composed of three regions: the reactive head group, the alkyl spacer, and the end group as shown in **Figure 3.2**. The head group

can be trichloro, trimethoxy, or triethoxy silane that binds to silica-based surfaces via covalent bonding. The alkyl spacer is responsible for the stability of the monolayer, and the end groups provide chemical functionality for the monolayer that is critical for overall surface properties. These modified surfaces are widely used many technologies such as micro-contact printing, preparation of bioactive surfaces, and semiconductor sensors.

Figure 3.2 A schematic illustration of a SAM structure.

Experimental Protocol: In this thesis, silane SAM film was used to form hybrid lipid bilayer. Glass were silanized in a 1 vol% solution of Octa-decyl-tri-chloro-silane (Sigma-Aldrich, US) in dry toluene (p.a., Merck) at room temperature for 1 h. Silanized substrates were finally rinsed with toluene, methanol (p.a., Merck), and Milli-Q water (Millipore).

3.3.3. Amine-Reactive Crosslinker Chemistry

Amine coupling is a standard approach for protein/peptide conjugation, chemical labeling, crosslinking and solid-phase immobilization applications. In this method, the primary amines (-NH_2) of proteins covalently interact with molecules presenting free carboxylic acid. These primary amines usually are located at the N-terminus of each polypeptide chain as well as in the side-chain of lysine residues. To form covalent bond, the carboxylic acid should be first activated. Usually, a mixture of NHS (N-

hydroxysuccinimide), and EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide) is used to convert carboxylic groups to amine-reactive NHS ester.

Experimental Protocol: Immediately before the experiment, EDC and NHS powders were dissolved in 10 mM HEPES buffer + 100mM NaCl (pH 5.5) at a final concentration of 10 mg/mL. The solution was incubated with SLBs containing lipids presenting free carboxylic acid (DP-NGPE), for 1 h at room temperature. After carboxylic activation, the SLBs were washed three times with the HEPES buffer.

3.3.4. Quartz Crystal Microbalance with Dissipation Monitoring

The QCM is a well-established technique that is designed to monitor and measure the adoption of thin films onto a surface in vacuum, gas, and liquid condition. This technique works based on the inherent piezoelectric character of an AT cut-quartz crystal. Piezoelectric materials can produce the electric charge when they are subjected to mechanical stresses or be mechanically deformed by applying an electrical field.

In QCM-D method, quartz crystal is commonly sandwiched by two thin layers (100-nm) of gold electrodes. When the alternating current (AC) is applied to the Au crystals, a shear deformation is applied onto the crystal by the voltage, causing the crystal to oscillate at a specific resonance frequency that is on the order of MHz. At the resonance frequency, the crystal oscillates harmonically with the largest amplitude and lowest energy loss. The fundamental resonance frequency of quartz crystals is not invariant and affected by the thickness of the crystals. In other words, the thinner crystals have higher resonance frequency and are more sensitive compared to the thicker ones. A typical QCM-D quartz crystal is approximately 300 μm thick and resonates at 5 MHz. Mass adsorption to the crystal surface leads to a shift in the resonance frequency (ΔF) of the crystal. The frequency shift can be used to calculate the mass adsorbed to the surface. If the deposited film is rigid, one can apply the Sauerbrey equation to find the mass of adlayer as follows [9]:

$$\Delta m = \frac{C}{n} \times \Delta f \quad \text{Equation 3.1}$$

Where Δm is the deposited mass, Δf is the frequency shift, n corresponds to the odd-numbered overtones, and C is the sensitivity constant that is $17.7 \text{ ng Hz}^{-1} \text{ cm}^{-2}$ for a 5 MHz quartz crystal [10].

In addition to measuring the mass of adsorbed layers, having the information about their viscoelastic properties is also crucial to realize their conformation upon the adsorption on the surface and over time. A newly developed QCM technique that called quartz crystal microbalance with dissipation (QCM-D), enable us to measure the mass and the viscoelastic properties of the adsorbed layer simultaneously (**Figure 3.3**). In this system, the energy dissipation (ΔD) of adsorbed layer is quantified by removing the driving voltage during periodic intervals of quartz crystals that allow the crystal to oscillate freely. The resonance decays for soft adsorbed layer more quickly compare to the rigid films, indicating higher energy dissipation.

Figure 3.3 Schematic illustration of QCM-D working principle. (a) Representative QCM-D quartz crystal that is sandwiched between metal electrodes. (b) An alternating voltage is applied to the electrodes covered with rigid and soft film that result in oscillation of the crystal at different resonance frequency. (c) The voltage is switched off which allows the crystal covered with a rigid/soft film to oscillate freely, respectively. Adapted from <http://www.biolinscientific.com>.

Experimental Protocol: A Q-Sense E4 instrument (Q-sense AB, Gothenburg, Sweden) was used in this thesis work to measure the mass and viscoelastic properties of the SLBs and associated ECM proteins. The QCM-D measurements were taken at the 3, 5, 7 and 11 overtones, and the presented data was measured and normalized at the third overtone (15 MHz ($\Delta f_{n=3}/3$)).

Substrate Preparation: Immediately before the experiments the crystals were cleaned with 1% sodium dodecyl sulfate (SDS) and rinsed with Milli-Q water (Millipore). The crystals were dried with N₂ and treated with an oxygen plasma (model no; PDC-002-Harrick Plasma, Ithaca, NY) at 180W for 1 minute to remove any residual organic molecules bound to the surface while making the substrate more hydrophilic.

Measurement Conditions: The QCM-D measurements were done under continues flow condition. A peristaltic pump (Ismatec, Glattbrugg, Switzerland) was used to introduce the sample into the measurement chamber, with the flow rate fixed at 50 μ L/min for all measurements. The working temperature in the flow cell was maintained at 24.00 $\pm$ 0.5°C.

3.3.5. Fluorescence Microscope

Fluorescence is a physical phenomenon of certain organic and inorganic specimens that can adsorb light at an excitation wavelength and emit light at red-shifted emission wavelength. This process is governed through an electronic transition within a fluorescent molecule (also called a fluorophore) and is accomplished in three different steps as shown in **Figure 3.4**. Firstly, the adsorption of light or photon by fluorescent molecule causes the transition of a single electron from the ground state to a higher energy state or excited state. Next, the excited molecule loses some of its energy through an internal conversion or vibrational relaxation. Finally, the electron transfers back to the ground state by emitting the energy as radiation. This energy is lower than the excitation energy and the difference between them called Stokes shift.

Figure 3.4 A typical Jablonski diagram representing three steps of the energy state of a molecule.

A fluorescence microscope is a developed form of conventional light microscope that can visualize fluorescence or phosphorescence in addition to the magnifying properties. This technique is very sensitive which applied in many fields of science, and in particular in life science [11]. A typical fluorescence microscope is composed of objectives, a dichroic mirror, excitation filter, emission filter and detector as shown in **Figure 3.5**. To observe a sample, light is generated by a mercury lamp and passes through an excitation filter. The excitation filter only allows the specific wavelength that is needed for excitation of the fluorescence molecules pass through, while other wavelengths are filtered. The light of the excitation wavelength is reflected by a dichroic mirror and focused on the sample by the objective lens. Light emitted from the sample can be classified into two types including elastically scattered light and fluorescent light. The source of elastically scattered light can be from different parts of the sample and has the same wavelength as the excitation light. In contrast, the source of fluorescent light is from the fluorescent molecules and has a longer wavelength compare to the excitation

light. The emission filter blocks the elastically scattered light, and only fluorescent light can pass through this filter and reaches to the detector.

Figure 3.5 A Schematic representation of an epi-fluorescence microscope. Adapted from [12].

Experimental Protocols: In this thesis work, an inverted epifluorescence Eclipse TE 2000 microscope (Nikon) equipped with a 60× oil immersion and a 10× objective combined with an Andor iXon + EMCCD camera (Andor Technology, Belfast, Northern Ireland) were used to investigate:

- Homogeneity of SLBs labeled 0.5 wt % with rhodamine (fluorescent dye).
- The interaction of fluorescently labeled proteins and cells with each other and with the SLBs.
- The viability of Huh 7.5 cells.

All the samples were prepared inside a custom-made microfluidic flow cell (stick-slide) that was attached to a glass substrate with dimensions of 25 mm x 75 mm x 170 μm (Coverslips for sticky slides, ibidi GmbH, Germany). The collected images consisted of 512×512 pixels with a pixel size of $0.267 \mu\text{m} \times 0.267 \mu\text{m}$ and were analyzed using ImageJ software.

3.3.6. Fluorescence Microscopy after Photobleaching

Fluorescence recovery after photobleaching (FRAP) is a fluorescence-based method that was introduced in the mid-1970s to study the fluidity of membrane and mobility of membrane-associated molecules [13,14]. The working principle of FRAP is based on the phenomenon called photobleaching. Photobleaching is a photochemical reaction that causes damage to the structure of fluorescence molecules that result in losing their fluorescence property permanently. In FRAP technique, a high-intensity light source such as a laser is used to bleach the fluorescent molecules within a defined area (small spot). Since the molecules in the samples are mobile, the bleached molecules will be mixed with non-bleached fluorescent molecules resulting in a gradual disappearance of the bleached area (**Figure 3.6**). This process is called fluorescence recovery.

Figure 3.6 Schematic illustration of the working principle of FRAP technique. (a) A surface labeled with fluorescent molecules before photobleaching. (b) A defined area of the surface is photobleached using a powerful laser. (c) A mixture of the bleached and fluorescent molecules immediately after photobleaching. (d) The fluorescence recovery of the bleached area. (e) FRAP snapshots of R-DHPE molecules in an egg PC bilayer at four different times showing almost complete recovery. Adapted from [12].

After obtaining the recovery curve, a proper curve fitting is applied to obtain the FRAP kinetics including the rate of fluorescence recovery. There are two important parameters that can be extracted from FRAP analysis: 1) the percentage of fluorescence recovery (how much) and, 2) the speed of fluorescence recovery (how fast). Fundamentally, the rate of fluorescence recovery is determined by the diffusion coefficient of lipids in the plane of the membrane. Several mathematical models as well as empirical formula have been developed to correlate the time constant of fluorescence recovery with molecular diffusion coefficient. The details of these models and how they were constructed are beyond the scope of this thesis. Numerous commercially available as well as costume-made softwares have been developed which use a set of numerical techniques to solve the models to obtain the diffusion coefficient from FRAP kinetics.

In this thesis we used a MATLAB program called `frap_analysis`, implementing all the steps in the Hankel transform method, which was developed by Jonsson et al. [15]. and is available upon request. A detailed description of the Hankel transform method is beyond the scope of this thesis. The MATLAB program extracts the experimental recovery curve and diffusion coefficient, given a stack of fluorescence images representing a FRAP experiment.

Experimental Protocols: FRAP was used to estimate lateral lipid mobility in planar SLBs with and without the presence of proteins and cells. Same as fluorescence microscopy protocol, the SLBs was tagged with 0.5 wt % rhodamine. A 532 nm, 100 mW laser (Klasech Laser Technologies, Dortmund) was applied to a confined area of the lipid bilayer for 5 seconds resulting in the formation of a circular black spot of $\sim 20\ \mu\text{m}$ diameter. Fluorescence recovery was monitored and measured by time-laps recording. All the images collected at a rate of 1 image per two second intervals. Images were analyzed using ImageJ software and the diffusion coefficient was measured using a custom-made MATLAB program implementing the Hankel transform method provided by Jonsson et al. [15].

3.3.7. Immunofluorescence

Immunofluorescence (IF) is a typical laboratory method that is used to detect specific biological macromolecules in the sample. The working principle of the IF technique is based on the conjugation of fluorescently labeled antibodies directly or indirectly to the antigen which enables to quantify or visualize the antigen through different fluorescent techniques. The immunofluorescent labeling can be direct or indirect (**Figure 3.7**).

Direct or primary IF: in this approach, a specific primary antibody that is conjugated to a fluorescent dye such as fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC) directly interact with the antigen of interest.

Indirect or secondary IF: In this method, first, a specific primary antibody is used to detect the antigen of interest. Then, a fluorescently labeled secondary antibody is utilized against the primary antibody. This method is more common and is inexpensive compared to the direct method.

Experimental Protocol: In this thesis work, immunofluorescence technique was utilized to stain Huh 7.5 cells and proteins. Huh 7.5 was stained using direct IF technique. Firstly, the cells were fixed by incubating in 4% paraformaldehyde (Sigma, Aldrich) for three min. After washing the samples three times with PBS, a 3% BSA blocking buffer was added and incubated for 1 h. Alexa Flour 555-labeled phalloidin (Life Technologies) was used to mark filamentous actin (F-actin) and was incubated with cells for 2 h at room temperature. The nucleus of each cell was labeled with the addition of 10 µg/ mL of 1, 2, 6-diamidino-2-phenylindole, dihydrochloride (DAPI; Life Technologies) for five min. The fluorescence images were captured using a confocal microscope, LSM710 with a ZEN program (Carl Zeiss) and were analyzed using ImageJ software.

Col I and FN was stained using indirect IF technique. In brief, primary antibody for Col I: mouse monoclonal anti-Col I and FN: mouse monoclonal IgG₁ anti-FN was added to the samples with a volumetric dilution of 1:1000 and 1:50 in PBS, respectively. After incubation for 2 h at room temperature, the samples were washed with PBS properly. Secondary antibodies including, Alexa Fluor 488 Goat Anti-mouse IgG (H+L) (Life Technology) and Alexa Fluor 555 Rabbit Anti-Mouse IgG (H+L) (Life Technology) were diluted in PBS (1:3000) and incubated with the Col I and FN for 15 min, respectively. Finally, the samples were washed with PBS and covered with aluminum foil for light protection. The fluorescence images were captured using an inverted epifluorescence Eclipse TE 2000 microscope (Nikon) equipped with a 60× oil immersion combined with an Andor iXon + EMCCD camera (Andor Technology, Belfast, Northern Ireland).

Figure 3.7 Schematic representation of direct and indirect immunofluorescence method. Adapted from <http://www.abcam.com>.

3.3.8. Cell Culture

Cell culture is a promising technique providing an artificial environment for growing and maintaining prokaryotic, eukaryotic or plant cells under controlled condition. This method allows investigating normal physiology and biochemistry behavior of cells independent of whole organism as well as the influence of different drugs and toxic materials on cellular viability and function. Additionally, this technique plays important roles in drug screening and development.

Each cell types need specific culture conditions. However, a cell culture environment invariably composed of:

- A substrate that is needed to support the attachment of anchorage-dependent cells.
- Growth medium or culture medium as source of the essential nutrients such as minerals, carbohydrate, vitamins, amino acids).
- Grows factors and hormones.
- The gasses (O_2 and CO_2)
- Regulated physiochemical environment such as pH, temperature, osmotic pressure.

A typical animal cell culture consists of three main procedures: cell culture thawing procedure, cell culture subculture procedure, and cell culture freezing procedure. The animal cells that are using for cell culture can be divided into two categories: primary cells and cell lines. If the isolated cells from the animals be cultured and proliferated in vitro, they known as primary cells. Primary cell converts to cell line or sub-clone after the first subculture. The cell line has a limited life span and will lose its capability after several subcultures; however, these cells can become immortal by a process called transformation.

Experimental Protocols: Huh-7.5 cells were plated on a tissue culture dish (100 mm) containing a 10 mL of DMEM, 10% FBS and 1% penicillin and streptomycin, for several days to reach a confluency of 80%. Cells were detached from the plate by incubating the culture dish with 0.25 % trypsin for ~ 3-5 min. After the cell suspension was dispersed in the growth medium, the cell count of 2×10^4 / 1×10^3 was determined via a hemocytometer. A flow chamber was used for cell seeding. The cell growth area per channel was ~ 0.6 cm².

3.3.9. Cell Counting Kit-8

Cell counting kit-8 or CCK-8 is a colorimetric assay that is used to quantify the cellular viability in cell proliferation assays and cytotoxicity tests. This assay consists of a highly water-soluble tetrazolium salt (WST-8) and an electron mediator called 1-

Methoxy PMS. The WST-8 salt is reduced by dehydrogenase activity of living cells that result in the production of an orange water-soluble formazan dye with light absorbance at 450 nm (**Figure 3.8**). The amount of formazan dye in the sample is corresponding to the number of viable cells. CCK-8 is more sensitive and stable assay compare to the other tetrazolium salts such as MTT, XTT, MTS or WST-1. Additionally, this assay has a low cytotoxicity that makes it possible to use the same cells for further experiments.

Experimental Protocol: The CCK-8 assay was used to determine the number of cells. This assay was obtained from Dojindo Molecular Technologies, Japan. Based on the manufacturer's protocol, a 10% CCK-8 reagent in complete media was added to the cells. After incubation for 1 h at 37°C, the absorbance was measured at 450 nm using a microplate reader (TECAN, Switzerland). The number of cells was calculated against the standard curve.

Figure 3.8 (a) The mechanism of cell viability detection by CCK-8. (b) The three simple steps of CCK-8 assay: 1) addition of the reagent to the samples containing cells and cell culture media, 2) Incubation of the sample, and 3) Color absorbance measurement at 450nm using a microplate reader.

3.3.10. The Live/Dead Viability/Cytotoxicity Assay for Mammalian Cells

The Live/Dead Cell Viability/ Cytotoxicity assay allows distinguishing live and dead cells simultaneously. This assay contains two dyes including calcein AM and ethidium homodimer (EthD-1) which are used to stain live and dead cells, respectively. Calcein-AM is non-fluorescent, hydrophobic and cell membrane permeable molecules. Due to the presence of intracellular esterase enzyme in living cells, these molecules are converted to fluorescent calcein that led to the production of an intense green fluorescence (λ_{ex} ~495 nm, λ_{em} ~515 nm). By contrast, EthD-1 can only pass through damaged cell membrane. These molecules bind to nucleic acid of dead cells, thereby producing red fluorescence (λ_{ex} ~495 nm, λ_{em} ~635 nm). This assay is simple, stable, fast, and accurate with low fluorescence background. Additionally, this assay is compatible with different instruments including flow cytometry, fluorescence microscopy, and fluorescence microplate readers (TECAN, Switzerland).

Experimental Protocol: The Live/Dead Cell Viability/ Cytotoxicity assay was used in this thesis to visualize cellular viability. This assay was received from Life Technologies, USA. According to manufacturer's protocol, the proper volume of 2 μ M calcein AM and 4 μ M EthD-1 were prepared in cell culture media and added to the sample containing the cells. The reagents were incubated with cells for 20 min at 37°C. The cell viability was determined using a fluorescence microscopy.

3.3.11. Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent (ELISA) is a highly sensitive biochemical technique that was introduced by Engvall and Perlmann, 1971 [16]. This technique also called enzyme immunoassay (EIA), designed for quantitative and qualitative detection of specific analytes such as proteins, antibodies, peptides, and hormones. Due to the simplicity and accuracy of ELISA, it has become one of the popular diagnostic assays in different fields such as medicine and industries. The working principle of this assay is based on the specific interaction between surface-immobilized antigen or antibody with

their substances. In other words, immobilization of an antigen to a solid surface enables us to detect its antibody; correspondingly, immobilized antibody allows catching the antigen. These interactions are visualized using enzyme-conjugated reagents. This enzyme is activated by incubating with a chromogenic substrate that results in the production of a measurable color. The intensity of this color is proportional to the concentration of the substances. A detection enzyme can be conjugated directly to primary antibodies or can be linked to a secondary antibody that detects the primary antibody. Additionally, it can be bound to streptavidin if the primary antibody is biotinylated. There are several types of ELISA assay including direct ELISA, indirect ELISA, competitive ELISA, and sandwich ELISA. The last is more powerful, sensitive and flexible compared to the other types. Double sandwich ELISA was used in this thesis and consists of nine steps (Summarized in **Figure 3.9**). In the first step, a polystyrene microplate is coated with a certain amount of capture antibodies of interest. Second, the unbounded antibodies are removed, and the residual protein binding capacity of the surface is blocked by irrelevant proteins such as bovine serum albumin. This step is done to prevent nonspecific interaction of antigen with the surface that results in false-positive signals. Third, a test sample containing the antigen is introduced in the surfaces and incubated with the immobilized antibody for a defined time. Fourth, the unbounded antibodies are removed from the plate by rinsing. Fifth, a specific biotinylated detection antibody is introduced to the surface and incubated for a certain time (the antigen is sandwiched among two primary antibodies). Sixth, the unbounded antibodies are washed away from the plate. Seventh, enzyme-conjugated streptavidin is added and incubated. Eighth, a substrate solution such as TMB is applied to hydrolyze the enzyme and produce a measurable color. At the final step, the optical density (OD) of this color is measured using a plate reader that the measurement is proportional to the concentration of the antibody.

Experimental Protocol: An ELISA assay was used to quantify the amount of secreted albumin by Huh 7.5 cells. Albumin human ELISA kit was purchased from Abcam. The cell culture media containing albumin was collected after 24 h and was centrifuged at 10000 g for 10 min. The supernatant was transferred to a sterile Eppendorf tube, and 50ul

of the solution was added to the 96-well plate containing immobilized antibody. The manufacturer's instructions were subsequently followed. The absorbance of the color was measured by a microplate reader (TECAN, Switzerland) at a wavelength of 450nm immediately. Measured absorbance was converted to the albumin amount using a standard curve. The data were normalized against the number of cells after day 1.

Figure 3.9 Overview of the sandwich ELISA assay. Adapted from <http://www.abcam.com>.

3.3.12. Decellularization

Tissues and organs decellularization is a method for isolating the ECM components by removing the cells and cytoplasmic inclusion. This method was introduced by Stephen F. Badylak [17] and is applicable in tissue engineering and regenerative medicine. Decellularized tissue and organs contain main biological components that can play the role as a natural scaffold for regulating different cellular functions as discussed in **Chapter 2**. Depending on the type tissue/organ and their applications, a wide variety of decellularization protocols are available [18]. These treatments can be physical, chemical, enzymatic or a combination of these three methods. The design of these methods is based on the sufficient tissue/organ decellularization with the minimize damage to the structural and chemical integrity of the tissue.

Physical methods: this method utilized with the aim of removing, lysing, and killing the cells from the tissue and organs through freezing, force and pressure, and electrical disruption.

Chemical method: In this method different chemical components are used for decellularization. The selection of these chemicals depending on the thickness, ECM chemical composition and application of the tissue can be varied. These chemical components that are used for removing and killing the cells include: alkaline, acid, ionic detergents, non-ionic detergents, zwitterionic detergents, tri (n-butyl) phosphate, hypotonic and hypertonic treatment, and chelating agent.

Enzymatic method: This method is applied to break the interactions between nucleic acids, cell-cell interaction, and other cellular components. These enzymes can be categorized to protease digestion, calcium chelating agent, and nucleases.

Experimental Protocols: The white adipose tissue samples were extracted from cadavers of mouse (C5/BL/6) and were decellularized with an in-house method using chloroform/methanol treatment. Briefly, the dissected tissue was cut into small pieces and washed several times with PBS (1X) to remove all the blood and hairs. During the first three days, the samples were treated with chloroform/methanol mixture at room temperature using different volumetric ratios: 2/1 (day 1), 1 /1 (day 2) and 1/2 (day 3). Subsequently, the tissue samples were washed with PBS (1X) several times and treated with trypsin (0.25%) for 24 h on a shaker at 37°C. Finally, the samples were washed with PBS (1X) and then treated with isopropanol for 48 h on a shaker at room temperature. The samples were stored in PBS (1X) and supplemented with 1% antibiotic-antimitotic (Gibco) at 4°C until further use.

3.3.13. Scanning Electron Microscope

Scanning electron microscope (SEM) is a magnification method that utilizes electron beam rather than light to produce images. The interaction between electron beam and atoms in the sample creates a variety of signals providing invaluable information about the surface topography, morphology, chemical composition, and crystallographic structure of the sample. In comparison to light microscopes, SEM has a higher magnification (>100,000×) with a larger depth of field (~ 100×). SEM instrument

composed of different compartments that work with each other to create three-dimensional images including vacuum chamber, electron gun, anode, magnetic focusing lens, scanning coils, detectors sample chamber, and detectors (**Figure 3.10**). A typical SEM works in a high vacuum.

The basic principle is that the electrons are generated by electron guns at the top of the microscope and accelerated down using a high voltage (e.g., 20 kV). The accelerated electrons pass through electromagnetic fields and lenses that result in the creation of a focused beam of electrons. Then, the electron beam travels through a pair of scanning coils which deflect the beam in X and Y axes. Upon interaction of electron beam with a defined area of the sample, electrons, and x-ray are emitted from that and collected by detectors. The detectors convert them to various signals and sends to the screen to produce the final image. The SEM samples must be prepared in dry condition (no water), and all non-metals samples need to be coated with a thin layer of conductive metals such as gold or palladium before imaging.

Figure 3.10 Schematic represents the layout of main components of a SEM instrument. Adapted from <https://www.purdue.edu>.

Experimental Protocols: In this thesis work, SEM was used to study the morphology of adipose tissue samples before and after decellularization. The samples were prepared for SEM by fixing with 2.5% glutaraldehyde (Sigma-Aldrich) overnight at 4°C. Fixed samples were subsequently dehydrated using ethanol series (20%, 40%, 50%, 70%, 90%, and 100%) for 5 min per grade. Further, the samples were immersed in 100% ethanol and then Hexamethyldisilazane (Sigma-Aldrich) for 1 h each. The samples were loaded on a sample holder covered with carbon and copper tape, followed by coating with gold using a sputter coater (SPI Supplies Inc, USA). The SEM (JSM-7600F-JEOL, Japan) was used to image the sample with an acceleration voltage of 5 kV at various magnifications.

3.3.14. The Bicinchoninic Acid Assay

Bicinchoninic acid (BCA) assay is one of the most common colorimetric assays in biochemical research for determination of protein concentration (mg/mL) or the total amount of protein in unknown protein samples (mg) [19]. The working principle of the assay is based on two reactions as shown in **Figure 3.11**. Cu^{2+} ions present in BCA solution is reduced to Cu^{+} by the proteins in alkaline condition. Thus, the concentration of produced Cu^{+} is proportional to the concentration of proteins in the sample. The Cu^{+} ions react with BCA resulting in the formation of a purple color complex with a strong light absorbance at 562 nm. The intensity of this color is proportional to the protein concentration.

The advantages of this assay are that it is sensitive, stable, moderately fast, detergent compatible and applicable over a broad working range of protein concentrations (20-2000 $\mu\text{g/mL}$).

Experimental Protocol: The total protein amount for the tissue samples before and after decellularization was determined by the bicinchoninic acid (BCA) assay using the Pierce™ BCA protein assay kit (Thermo Scientific, USA) according to the manufacturer's manual. Briefly, lyophilized tissue was immersed in RIPA buffer supplemented with protease and phosphatase inhibitor at 4°C for 30 min. The samples

then were homogenized using a probe-sonicator (Qsonica, USA). After homogenization, the samples were centrifuged at 10,000 g for 20 min at 4°C and the supernatant was transferred to a new Eppendorf for measuring the protein content. 25 µl of the protein samples were added in a 96-well microplate and mixed with 200 µl of BCA working reagent. The plate was shaken for 30 s and subsequently incubated at 37°C for 30 min. After cooling the plate, the light absorbance of the samples were measured at 562 nm using a plate reader (TECAN, Switzerland) and the protein concentration was calculated against the standard curve. The amount of proteins was normalized to dry-weight tissue mass.

Figure 3.11 Schematic diagrams showing (a) the reduction of Cu^{+2} to Cu^+ by protein in the presence of alkaline solution, and (b) The chelation of Cu^+ by BCA and production of the BCA- Cu^+ complex. Adapted with permission from [20]. Copyright 2012, Royal Society of Chemistry.

3.3.15. Hydroxyproline Assay Kit

Hydroxyproline assay is a colorimetric assay for measuring the concentration of hydroxyproline in cell and tissue culture supernatant, serum and urine. Since hydroxyproline is a major amino acid in collagen (13.5%) and highly restricted to this protein structure [21], therefore the quantification of hydroxyproline content can be used as an indicator to determine the level of collagen/gelatin in the sample. In this assay, the concentration of hydroxyproline is measured by the interaction between oxidized

hydroxyproline and 4-(Dimethylamino) benzaldehyde (DMAB) that results in the formation of a chromophore product. The light absorbance of this colorimetric product is 560 nm that is corresponding to the hydroxyproline amount (**Figure 3.12**). This assay is simple and sensitive that can measure over the range of 0.1-2 μg .

Experimental Protocol: Hydroxyproline assay (Sigma- Aldrich, USA) was used this thesis to measure the amount of Col in white adipose tissue sample before and after decellularization. After homogenization of tissue with 100 μl water, hydrochloric acid (12 M, 100 μl) was added to the samples and incubated at 120°C for 3 h. The samples were dried under vacuum and mixed with equal volume of chloramine T/Oxidation buffer and incubated for 5 min. Finally, dimethylaminobenzaldehyde (p-DMBA) colorimetric solution was added to each sample and incubated for 90 min at 60°C. The absorbance of the colorimetric product was measured at 560 nm using a microplate reader (TECAN, Switzerland). The concentration of hydroxyproline was calculated based on the standard curve and converted to Col content based on the assumption that hydroxyproline constitutes 13.5% of collagen dry mass [21] .

Figure 3.12 Schematic representation of different steps involved in hydroxyproline assay.

3.3.16. Pico Green Assay

Pico Green Assay is a fluorescent-based method that is designed for determination of double-stranded DNA (dsDNA) in solution. This assay works based on an ultra-sensitive fluorescent nucleic acid stain, Quant-iT™ PicoGreen® dsDNA reagent. This stain is able to selectively bind with dsDNA. There are several key benefits of this assay that are very important for detection of DNA. First of all, this assay has low affinity to interact with non-double stranded nucleic acid; therefore, the risk of interference from non-genomic nucleic acid is minimized. Second, is sensitivity and applicability of this assay that allows detection of a broad working range of DNA concentrations (25pg-100ng/mL). Third, this is a very simple, stable, and accurate assay that can be used with most fluorescence-based microplate readers and fluorometers.

Experimental Protocol: Quant-iT™ PicoGreen ® dsDNA Reagen and Kits was obtained from Life Technologies, USA and was used to quantify the amount of DNA in white adipose tissue. Briefly, lyophilized adipose tissues were treated with 1 mL TE buffer (1X) (from the kit) and 0.1% Triton X-100. The samples were freeze-thawed three times (-80°C to 37°C). 100 µl of the aqueous working solution of the Quant-iT™ PicoGreen® reagent was mixed with 100 µl of the sample and was incubated for 5 min at room temperature. The fluorescence of the sample was measured at 480 and 520 nm using a plate reader (TECAN), and the DNA concentration was obtained from the standard curve.

3.3.17. Glycosaminoglycan Assay

Glycosaminoglycan Assay is a type of dye-binding assay that is used for analyzing sulfated proteoglycans and glycosaminoglycans, (sGAG). In other words, this assay is suitable to measure the O-and N-sulfated glycosaminoglycan ratio as well as the total sulfated glycan (sGAG) content. This assay has been used to measure a wide variety of mammalian proteoglycans that can be in the form of free chains or attached to the protein core such as: chondroitin sulfates (4- and 6-sulfated), keratan sulfates (alkali sensitive

and resistant forms), dermatan sulfate (containing iduronic & glucuronic acid), and heparan sulfates (including heparins). This assay can determine these proteoglycans in different sample materials such as mammalian tissue and extracellular matrix secreted by cells.

Two main components of the assay for measuring sGAG are a dye reagent of 1, 9-dimethyl-methylene blue and the dissociation reagent. The dye reagent is an inorganic buffer that is attached to sulfated proteoglycans (GAGs) specifically at acid pH that results in the production of the dye-GAG complex. The dissociation reagent comprises a chaotropic salt dissolved in an aqueous propan-1-ol solution that has been designed for dissociating the GAG-dye complex and also increasing the spectrophotometric absorption profile of the free dye. The Blycan dye in the dissociation reagent has a maximum light absorbance at 656 nm. The amount of sGAG is proportional to the absorbance and calculated against the standard curve that is provided using a sGAG standard composed of chondroitin 4-sulfate. The absorbance less than 0.1 and more than 1.5 is not detectable using this method.

The Blycan assay is more sensitive and accurate compared to earlier methods and is capable of measuring a wide range of sGAG concentration from 2.5 µg/mL to 500 µg/mL when using 100 to 10 µL sample aliquots.

Experimental Protocol: The Blyscan™ GAG assay (Biocolor, UK) was used to measure the amount of total sGAG in adipose fat tissue samples before and after treatment according to the manufacturer's protocol. Briefly, the lyophilized tissue sample was incubated with 1 mL papain extraction reagent (see **Appendix**) at 65°C overnight. The sample was centrifuged at 10,000 g for 10 min and the supernatant was separated. 100 µL of the sample was mixed with 1 mL of Blyscan dye reagent and mixed thoroughly on a plate shaker for 30 min at room temperature and subsequently was centrifuged at 10,000 g for 10 min. The colored pellet was separated from the supernatant and was dried completely. The pellet was dissociated using 0.5 mL of dissociation reagent and was vortexed. The solution was centrifuged at 10,000 g for 5 min. 200 µL of the solution was transferred inside a 96-well microplate and light absorbance was measured at 656 nm

using a plate reader (TECAN, Switzerland). The amount of GAG was calculated based on the standard curve.

3.4. Statistical Analysis

All experiments were carried out in triplicates unless otherwise stated. The significance of differences between the groups was studied using the one-way ANOVA analysis and PRISM software. The groups were considered statistically significant where $p \leq 0.05$. All data are represented as means $\pm$ SD. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$, and **** $p \leq 0.0001$.

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Chapter 4

Dynamic Cellular Interactions with Extracellular Matrix Triggered by Biomechanical Tuning of Supported Lipid Membranes*

The behavior of cells in a tissue is regulated by both chemical as well as physical signals arising from their microenvironment. Physical signals arising from the microenvironment including the rigidity of the substratum has been shown to decide the fate of pluripotent stem cells. While gel-based substrates have been widely used for mimicking range substrate rigidities, there is a need for the development of soft substrates for mimicking the physical properties of soft tissues. Here we report the development of a supported lipid bilayer (SLB)-based substrate for cell adhesion studies. SLBs are functionalized with either collagen I (Col I) or fibronectin (FN) via covalent, amine coupling to a carboxyl group-modified lipid molecule. While the lipid molecules in the bilayer show long-range lateral mobility, the covalently functionalized extracellular matrix (ECM) proteins are immobile on the bilayer surface. Specific adhesion of cells results in an enrichment of the protein on the bilayer, and the appearance of a zone of depletion around the cells. Further, the lateral reorganization of the ECM proteins is controlled by altering the fluidity of lipid molecules in the substrate. Thus, the experimental platform developed here could be utilized for both addressing basic questions related to cell adhesion on soft substrates as well as biomedical applications requiring adhesion of cells to soft substrates.

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4.1. Introduction

Cells in a tissue are constantly engaged with their microenvironment, and alter their physiology based on the signaling inputs received from their microenvironment. These signals are generally received by the cell through the activation of specific receptors that are localized to the cell membrane. In addition to chemical signals, cells also respond to mechanical [1-3] and geometrical [4-6] aspects of the tissue microenvironment. This natural sensitivity to physical microenvironmental signals presents a difficulty for *in vitro* cellular studies, in which cells are cultured on synthetic substrates. Conventional plastic cell culture plates present an extremely stiff substrate, at the far end of the spectrum of mechanical stiffness experienced by cells in physiological settings [7,8]. Polydimethylsiloxane (PDMS) and other polymeric substrates have been used to access softer regimes, yielding many important insights into the role of mechanical properties of the microenvironment in cellular behavior [7-12]. However, these polymer gel substrates still have difficulty accessing the softest and most fluid interfaces that cells may experience in a physiological environment.

Supported lipid membranes, consisting of continuous phospholipid bilayers assembled on a solid substrate, have proven useful in a wide variety of cellular and biomolecular studies. The supported membrane presents a naturally fluid surface, with lipids and membrane-associated proteins free to diffuse within the membrane. Typical two-dimensional lateral diffusion coefficients in low-defect supported membranes are in the range of a few $\mu\text{m}^2/\text{s}$. As such, supported membranes present a relatively viscous, but not elastic, environment that resembles the natural cell membrane. They have been used extensively to reconstitute and study juxtacrine signaling systems in which receptor and ligand interact across a cell-cell interface. These include immunological T and B cell receptors in the context of adhesion and co-stimulatory molecules [5,13-15], Eph receptor tyrosine kinases (RTKs) in epithelial cells interacting with ephrin ligands in the supported membrane [6,16], and cadherin-cadherin interactions [17,18].

Supported membranes have also been effectively used to study integrin-ECM interactions that would not naturally involve a membrane. In one salient example, Yu et al. [19] used supported membranes to display RGD ligand in a fluid format, which prevented the living cells from being able to apply enough force to trigger focal adhesion formation. In certain cases, this extremely low force substrate led to podosome formation instead [20]. Further, a combination of fluid and rigid display of RGD ligand provided insights into the differential recruitment of an endocytosis-related adaptor protein to integrin clusters [21]. The naturally fluid nature of supported membranes is thus useful to access the extremely low stiffness end of the mechanical spectrum.

Here we develop a general strategy to allow cell adhesion to SLB-based substrates functionalized with either Col I or FN proteins, which are abundant in the ECM. The SLB substrates are covalently functionalized with ECM proteins through amine coupling to the carboxylic group-modified DP-NGPE lipid incorporated in the bilayers that were assembled through the recently developed solvent assisted lipid bilayer (SALB) formation method [22,23]. The degree of ECM protein functionalization strongly depends on the fraction of the functional, DP-NGPE lipid molecules incorporated in the bilayers. Adhesion of the human hepatocellular carcinoma, Huh 7.5, cells to these substrates results in a local enrichment of the ECM proteins at the cell-substrate interface, and a depletion of the proteins in the adjacent areas on the bilayer. This points to the specific interaction of the cells with the ECM proteins, and the application of mechanical forces by the cells on the ECM proteins functionalized on the bilayer. The redistribution of ECM proteins under the adhering cells is dependent on the lateral fluidity of the underlying bilayer. Importantly, the redistribution of the ECM proteins can be abrogated by increasing the viscosity of the bilayer, either by including cholesterol (Chol) in SLBs, or by generating a hybrid substrate in which bottom monolayer of lipid is replaced with a monolayer of silane while the top monolayer is formed of lipid molecules.

4.2. Materials and Methods

4.2.1. Lipid Sample Preparation

To prepare lipid samples, 1,2-dioleoyl-sn-glycero-3-Phosphatidylcholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(glutaryl) (DP-NGPE), Cholesterol (Chol), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)(ammonium salt) (Rh-PE) were purchased from Avanti Polar Lipid (Alabaster, AL). Right before SALB experiment, DOPC, Chol, and Rh-PE powders were dissolved in isopropanol at a nominal concentration of 10 mg/mL, 25 mg/mL and 1 mg/mL, respectively. Due to the insolubility of DP-NGPE in isopropanol, the powder was dissolved in ethanol (99.99%) at 5mg/mL lipid concentration and was heated to 50°C and gently vortexed. The stock solutions were diluted in isopropanol to the desired concentration DOPC:DP-NGPE:Chol molar ratios, and then diluted to 0.5 mg/mL lipid concentration. In fluorescence microscopy experiments, 0.5 wt% Rh-PE was mixed with the lipid composition.

4.2.2. Substrate Preparation

Two different substrates including Silicon Oxide (SiO₂) -coated AT-cut piezoelectric quartz crystals (model no. QSX303) (Q-sense AB) and glass (Coverslips for sticky-Slides: # 1.5H (170 µm +/- 5 µm) D 263 M Schott glass, 25 mm x 75 mm; ibidi) were submerged in 1% sodium dodecyl sulfate (SDS) and rinsed with Milli-Q water (Millipore) extensively. After drying the substrates with nitrogen gas (N₂), SiO₂ and glass were treated with oxygen plasma (March Plasmod Plasma Etcher, March Instruments) at about 180W for 1 min and 5 min, respectively.

4.2.3. Silanization of Glass Substrates

Glass was silanized in a 1 vol% solution of Octa-decyl-tri-chloro-silane (Sigma-Aldrich) in dry toluene (p.a., Merck) at room temperature for 1 h. Silanized substrates were finally rinsed with toluene, methanol (p.a., Merck), and Milli-Q water.

4.2.4. Lipid Bilayer Formation

The SALB method was utilized to form lipid bilayer. In brief, lipids in a mixture of isopropanol were incubated with the substrate for 10 min. After lipid deposition, the organic solvent was gradually exchanged by aqueous buffer (10mM Tris+100mM NaCl, pH 7.2). In case of bilayer formation on the glass-based surface, commercially available microfluidic flow cells (sticky-slide VI^{0.4}, ibidi) were utilized. The rate of injection flow was 50 μ L/min.

4.2.5. Activation of Carboxylic Group of DP-NGPE Lipid Included-SLB by EDC/NHS Treatment

For conjugating protein with SLB, DP-NGPE as a functionalized lipid was activated by a solution of 10 mg/mL N-Hydroxysulfosuccinimide sodium (sNHS) / 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (Alfa Aesar) in pH 5.5 HEPES (10mM HEPES +100mMNaCl) immediately before used. The solution was incubated with SLB for 1 h to convert carboxylic groups to amine-reactive NHS ester.

4.2.6. Protein Sample Preparation and Adsorption on SLB and Glass/SiO₂

Protein samples were prepared in pH 7.5 PBS as following concentrations: 0.2 mg/mL Col I (Sigma-Aldrich) and 0.2 mg/mL FN (Calbiochem). 10 mg/mL Protein solutions were introduced on SLB using a peristaltic pump (Ismatec Reglo Digital) with a flow rate of 50 μ L/min to form Col I-SLB or FN-SLB. Finally, the substrates were

washed with PBS to remove unbounded proteins. As a control experiment, a similar process was performed on glass/SiO₂ substrates.

4.2.7. Quartz Crystal Microbalance with Dissipation Monitoring

A Q-Sense E4 instrument (Q-sense AB) was used to monitor the kinetics of lipid bilayer formation and subsequent protein adsorption on SiO₂ coated 5 MHz, AT-cut piezoelectric quartz crystal. Temporal changes in frequency and dissipation were recorded as previously described [24]. All the QCM-D measurements were recorded at 3, 5, 7 and 11 overtones at $24.00 \pm 0.5^\circ\text{C}$. A peristaltic pump (Ismatec Reglo Digital) was used to introduce sample into the measurement chamber at 50 $\mu\text{L}/\text{min}$.

4.2.8. Fluorescence Recovery after Photobleaching

To do FRAP experiment, a 532 nm, a 100mW laser beam was applied to photobleach fluorescent lipid (Rh-PE) embedded in the bilayer in a specific area (small spot). The duration of photobleaching was around 5 second, and the wideness of the black spot was 20 μm . All the fluorescence image were captured using an inverted epifluorescence Eclipse TE 2000 microscope (Nikon) equipped with a 60 $\times$ oil immersion objective (NA 1.49), and an Andor iXon+ EMCCD camera (Andor Technology). The acquired images had dimensions of 512×512 pixels with a pixel size of 0.267×0.267 μm . The recovery was followed at 2 s intervals, and diffusion coefficients were computed using the Hankel transform method [25]. All image processing was done using ImageJ software.

4.2.9. Interference Reflection Microscopy

Before imaging, Huh 7.5 cells cultured on proteins-functionalized SLBs and glass were fixed by incubating in 4% paraformaldehyde (Sigma, Aldrich). The samples were washed three times with PBS. An interference reflection microscopy (RICM) imaging was performed using an Eclipse Ti inverted microscope (Nikon) equipped with an Evolve

electron multiplying charge-coupled device (EMCCD) camera (Photo-metrics) while images were collected using Metamorph (Molecular Devices). All image processing was done using ImageJ software.

4.2.10. Estimation of Membrane Viscosity

The viscosity of the supported bilayer was calculated using the Evans – Sackmann model for the diffusivity D of a cylindrical particle with radius a_C , embedded within a solid supported, fluidic membrane with viscosity η_M [26]:

$$D = \frac{k_B T}{4\pi\eta_M} \left(\frac{1}{2} \varepsilon^2 + \frac{\varepsilon K_1(\varepsilon)}{K_0(\varepsilon)} \right)^{-1}, \text{ where } \varepsilon = a_C \sqrt{\frac{b}{\eta_M}}. \quad \text{Equation 4.1}$$

In Eq. (4.1) $k_B T = 4.1 \times 10^{-21} \text{ kg m}^2 \text{ s}^{-2}$ is the Boltzmann energy, K_0 and K_1 are the zeroth and first order modified Bessel functions of the second kind and b is a phenomenological constant to account for the friction between the membrane and its support. In order to use Eq. (1) to find the viscosity of the bilayer, based on the measured diffusivity of the lipid molecules within, we make the following assumptions. 1) A lipid molecule is treated as a cylinder with a radius $a_C = 0.45 \text{ nm}$ [27]. 2) The viscosity experienced by this molecule equals the monolayer viscosity η_M , and the bilayer viscosity η_B is twice the monolayer viscosity. 3) The friction experienced by the molecule from the opposing leaflet is accounted for by parameter b , which is referred to as the *inter leaflet friction coefficient*. We use the value, that has been measured in Ref [28]: $b = 10^7 \text{ kg s}^{-1} \text{ m}^{-2}$. Inserting the known values for D , $k_B T$, a_C and b into Eq. (1) leaves a non-linear equation for η_M , which is solved using Newton Raphson iteration. The bilayer viscosity is then found as: $\eta_B = 2 \times \eta_M$.

4.2.11. Cell Culture

Human hepatocellular carcinoma cell line (Huh-7.5, Apath.) was used as a hepatocyte model in this study. The cells were grown on 100 mm tissue culture plates in

supplemented growth media containing Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone), 10% fetal bovine serum (FBS) (Hyclone) and 1% penicillin and streptomycin (Gibco). The media was changed every 3 days. When the confluence of the cells reached about 70%, 0.25 % trypsin (Gibco) was used for 3 min to separate Huh 7.5 cells from the tissue culture plates. Cell number was counted using hemocytometer and 1×10^3 Huh 7.5 cells were seeded on with and without proteins-functionalized SLB for 3 h. As a control experiment, bare glass and protein-functionalized glass were used to compare with SLB-based surfaces.

4.2.12. Immunofluorescent Stain for ECM Proteins

To study ECM protein conformation and their interactions with Huh 7.5, Col I and FN was stained using primary and fluorescent-labeled secondary antibody. In brief, before cell seeding, conjugated Col I and FN were washed with PBS three times to remove all unbounded proteins. Next, primary antibody for Col I: mouse monoclonal anti-Col I (Abcam) and FN: mouse monoclonal IgG₁ anti-FN (Santa Cruz) was added to the samples with a volumetric dilution of 1:1000 and 1:50 in PBS, respectively. After incubation for 2 h at room temperature, the samples were washed with PBS properly. Secondary antibodies including, Alexa Fluor 488 Goat Anti-mouse IgG (H+L) (LifeTechnology) and Alexa Fluor 555 Rabbit Anti-Mouse IgG (H+L) (Life Technology) were diluted in PBS (1:3000) and incubated with the Col I and FN for 15 min, respectively. Finally, the samples were washed with PBS and covered with aluminum foil for light protection. All the images were captured using an inverted epifluorescence Eclipse TE 2000 microscope (Nikon) equipped with a 60× oil immersion combined with an Andor iXon + EMCCD camera (Andor Technology). Image analysis was done using ImageJ software.

4.3. Results and Discussion

4.3.1. The SLB Formation and Functionalization with ECM Proteins

To generate a soft substrate for integrin-mediated cell adhesion, we functionalized ECM proteins on fluid SLBs. **Figure 4.1a** provides a schematic illustration of an SLB platform decorated with Col I. Briefly, SLBs composed of a zwitterionic lipid (DOPC) and varying fraction (up to 20%) of a lipid with a free carboxylic acid (DP-NGPE), (**Figure 4.1b**) were deposited on glass coverslips using the recently developed SALB formation method that is highly flexible with the nature of the constituent lipids [22,23]. This method is based on using lipids dissolved in an alcohol (e.g., isopropanol) to form bilayer on solid supports by the slow replacement of the organic solvent in the lipid-solvent mixture with aqueous buffer. The increase in the water fraction mediates a series of phase transitions in the lipid-alcohol-water mixture, resulting in the formation of bilayers at the contacting solid surfaces upon complete solvent replacement. A unique feature of the SALB method is that it enables preparation of SLBs with high fractions of Chol [29] that cannot be obtained using conventional techniques such as the commonly used vesicle fusion method [6,17-21,30].

The QCM-D was used to monitor both the formation of lipid bilayers as well as the subsequent covalent coupling of ECM proteins to the bilayer. The QCM-D measurements have been successfully utilized in the real-time monitoring of adsorption processes involving proteins and lipid membrane interfaces [31,32].

ECM proteins were functionalized covalently to lipid molecules present on bilayers via amine coupling, similar to a previously described strategy [33]. While a number of other protein functionalization strategies are available including biotin-streptavidin, they may not prove sufficiently stable for long-term interaction of the functionalized proteins with cells that are known to apply forces on the proteins [34]. Bilayers composed of a zwitterionic lipid (DOPC) and varying fraction (up to 20%) of a lipid with a free carboxylic acid (DP-NGPE) were formed on SiO₂ substrates using

SALB formation method. Post assembly of lipid bilayers, the carboxylic acid group on DP-NGPE was activated via the EDC/NHS chemistry. Following the activation of the carboxylic group, ECM proteins were added to allow covalent coupling of the proteins to the bilayer.

Figure 4.1c presents the changes in the QCM-D frequency (ΔF) and energy dissipation (ΔD) upon addition of Col I to bilayers prepared by including different fractions of DP-NGPE in their precursor lipid mixture. The ΔF and ΔD are related to the mass and the viscoelastic properties (rigidity and softness) of the adlayer, respectively. A negative shift in ΔF indicates the increase in the mass due to protein adsorption. Addition of Col I to pure DOPC bilayer resulted in almost no change in QCM-D ΔF and ΔD . This is expected as zwitterionic lipid bilayers resist protein adsorption in general, and has been utilized as antifouling surfaces. However, a negative ΔF was observed for bilayers containing the functional lipid (DP-NGPE) suggesting specific adsorption of Col I to the bilayer. The high- ΔD observed here indicates the softness of the protein-lipid adlayer, suggesting that the Col I fibers attached to the SLB are not rigid. About 3% of the total amino acids in Col I are lysine [35] and thus, a limited number of sites of covalent coupling on the fibers allow them to retain their flexibility while attached to the bilayer. Importantly, only a negligible fraction of the Col I fibers detached from the bilayer upon extensive rinsing with a protein-free buffer solution. This confirms that the Col I fibers are covalently coupled to the bilayer, and should be suitable for long-term interaction with cells.

A key consideration in using the present method for bilayer formation is that not all lipid molecules are equally favored for incorporation into the bilayer. It is likely that anionic lipids such as the one used here i.e. DP-NGPE will be less favorably incorporated into the bilayer that are assembled on a negatively charged SiO_2 substrate [36]. Therefore, a range of mole fractions of the functional lipid was used to form SLBs to assess the functionalization of ECM proteins. **Figure 4.1d** shows that the QCM-D ΔF upon Col I binding to functionalized SLBs depends linearly on the fraction of functional lipids included in the original lipid mixture. This is despite the fact that the adlayer is not

completely rigid, as assessed from the high ΔD , which may suggest that the relationship between ΔF and the actual adsorbed mass may not be linear. Nevertheless, the linear dependence suggests that the amount of Col I covalently coupled can be modulated by the fraction of functional lipids included in the precursor lipid mixture.

Figure 4.1 Characterization of ECM-functionalized SLB. (a) Schematic illustration of Col I bound to SLB via amine coupling. (b) Structures of the lipids used in this study, together with their chemical names. (c) The QCM-D ΔF and ΔD change after Col I binding to DOPC bilayers prepared by SALB method using varying mole fractions of functional lipid bearing a free carboxylic acid for amine coupling (DP-NGPE) in the lipid precursor mixture (between 0 and 20 mol %). After bilayer fabrication, the frequency shift was then normalized with $\Delta F = 0$ Hz corresponding to the planar lipid bilayer in each experiment. Changes in resonance frequency indicate the adsorption of Col I onto the bilayers. (d) The absolute frequency shift ($|\Delta F|$) upon Col I binding onto bilayer versus mole fraction of DP-NGPE included in the precursor mixture.

To check the specific attachment of proteins to NHS-activated free carboxylic acid group of DP-NGPE, Col I was added to the bilayer prepared using 20 mol % DP-NGPE with and without NHS activation step resulted in a negative frequency shifts of -126 ± 8

Hz and 11.3 ± 2.1 Hz, respectively suggesting that majority of proteins specifically attached to the activated free carboxylic acid group of the functional lipid (DP-NGPE) (see **Figure 4.2**).

Figure 4.2 (a) Typical QCM-D frequency shift (ΔF , top panel) and dissipation (ΔD , bottom panel) for the third overtone were measured as a function of time during the formation of planar bilayer and subsequent protein addition. SLB was formed on SiO_2 via solvent assisted lipid bilayer (SALB) formation method using an isopropanol solution of a DOPC:DP-NGPE (1:4). After establishing the baseline in a buffer solution, pure isopropanol was injected (arrow i) followed by injection of 0.5 mg/mL DOPC/DP-NGPE (arrow ii) which caused a small change in

resonance frequency and no change in the energy dissipation. Subsequently, the buffer was injected (arrow iii) leading to a final ΔF and ΔD of $\sim -26 \pm 2$ Hz and $0.5 \times 10^{-6} \pm 0.3$, respectively which corresponds to a planar bilayer. In order to functionalize SLBs with ECM proteins, EDC/NHS was introduced into the chamber (bracket) to activate free carboxylic acid of DP-NGPE. In the last step, a solution of Col I (0.2 mg/mL) was injected to the bilayer. (b) QCM-D measurements recorded the addition of Col I to the DOPC:DP-NGPE (1:4) bilayer with no EDC/NHS activation. (c) Frequency shift corresponding to Col I adsorption to DOPC:DP-NGPE (1:4) bilayer with and without EDC/NHS activation. The frequency shifts associated with Col I adsorption onto bilayer with and without EDC/NHS activation step are -126 ± 8 and -11.3 ± 2.1 Hz, respectively.

Immunofluorescence imaging of the Col I functionalized bilayer showed randomly oriented fibers on the membrane surface (**Figure 4.3**). Consistent with the QCM-D results, the density of Col I fibers adsorbed to the bilayer increased with the fraction of functional lipids in the original lipid mixture. Noticeably, the Col I fibers were almost homogeneously distributed on the bilayers prepared with $> 10\%$ mole fraction of the functional lipid.

Figure 4.3 Fluorescence immunostaining micrographs of Col I fibers on SLB prepared using varying fraction of DP-NGPE in precursor mixture. Noticeably, the Col I fibers were almost homogeneously distributed on the bilayers prepared with $> 10\%$ mole fraction of the functional lipid. Scale bars are 20 μm .

A requirement for full-filling the criterion of a soft substrate is that the lipid molecules are diffusive after functionalization of the ECM proteins. In order to determine

if the bilayers functionalized with Col I exhibit long-range lateral diffusion, a microscope-based FRAP experiment was performed on the bilayers. The fluorescent Rh-PE lipid was included in the bilayer for this. In **Figure 4.4**, left and right panels show fluorescence micrographs of the bilayer at $t = 0$ and 60 s after photobleaching, respectively, revealing a near-complete recovery of the fluorescence at the photobleached spot. Analysis of the fluorescence recovery data revealed a lipid diffusion coefficient of $2 \pm 0.1 \mu\text{m}^2/\text{s}$, which is slightly lower than the diffusion coefficient of the non-functionalized bilayers with identical lipid composition ($2.4 \pm 0.1 \mu\text{m}^2/\text{s}$). While the lipid diffusion coefficient on ECM protein functionalized bilayer was found to be slightly lower than the non-functionalized bilayer, a bilayer with a diffusion coefficient of $2.0 \pm 0.1 \mu\text{m}^2/\text{s}$ is sufficiently fluid to be used as extremely soft substrates.

Figure 4.4 FRAP snap shots of bilayer (20% DP-NGPE) decorated with Col I. The Left and right panels show fluorescence micrographs of the bilayer at $t = 0$ and 60 s after photobleaching, respectively, revealing a near-complete recovery of the fluorescence at the photobleached spot. The fluorescent Rh-PE lipid was included in the bilayer for this. Analysis of the fluorescence recovery data revealed a lipid diffusion coefficient of $2.0 \pm 0.1 \mu\text{m}^2/\text{s}$, which is slightly lower than the diffusion coefficient of the non-functionalized bilayers with identical lipid composition ($2.4 \pm 0.1 \mu\text{m}^2/\text{s}$).

We followed essentially the same approach for preparing SLB substrates functionalized with FN (**Figure 4.5a** and **Figure 4.5b**). QCM-D monitoring confirmed the adsorption of FN to bilayers containing functional lipids (**Figure 4.5b**). QCM-D results also showed that a small fraction of FN was removed after buffer wash, indicating

that FN displayed some degree of nonspecific binding to SLB surface (**Figure 4.5b**). Similar to Col I-functionalized SLBs, the final ΔF after FN conjugation increased with the fraction of the DP-NGPE lipid (**Figure 4.5c**). The final ΔF at each condition was however smaller than that of Col I, as would be expected from a smaller molecular weight of FN in comparison to Col I at similar molar surface densities.

Figure 4.5 (a) Schematic illustration of FN decorated supported bilayer. (b) QCM-D monitoring of FN adsorption onto SLBs prepared by including different fraction of DP-NGPE. QCM-D monitoring confirmed the adsorption of FN to bilayers containing functional lipids. QCM-D results also showed that a small fraction of FN was removed after buffer wash, indicating that FN displayed some degree of nonspecific binding to SLB surface. Similar to Col I-functionalized SLBs, the final frequency change after FN conjugation increased with the fraction of the DP-NGPE lipid. (c) The absolute ΔF upon FN binding as a function of DP-NGPE fraction.

In agreement with the QCM-D data, immunofluorescence microscopy of the FN functionalized SLBs showed increased protein densities with increasing fraction of functional lipids (**Figure 4.6**). The fluorescence micrographs also show the presence of some unevenly distributed FN, which appeared as particularly brighter spots. This is in agreement with a previous atomic force microscopy-based study of the interaction of FN with supported bilayer, which revealed the formation of FN aggregate at the membrane interface [37].

Figure 4.6 Fluorescence immunostaining micrographs of FN on SLB prepared using varying fraction of DP-NGPE in precursor mixture. Scale bars are 20 μm .

FRAP experiment was also performed on FN-functionalized lipid bilayer. In **Figure 4.7**, left and right panels show fluorescence micrographs of the bilayer at $t = 0$ and 90 s after photobleaching, respectively, revealing a near-complete recovery of the fluorescence at the photobleached spot. FRAP analysis of FN functionalized bilayer showed that the membrane retains its long-range lateral mobility, albeit with a reduced diffusivity ($D = 1.4 \pm 0.1 \mu\text{m}^2/\text{s}$).

Figure 4.7 FRAP snap shots of bilayer (20% DP-NGPE) decorated with FN. The Left and right panels show fluorescence micrographs of the bilayer at $t = 0$ and 90 s after photobleaching, respectively, revealing a near-complete recovery of the fluorescence at the photobleached spot. The fluorescent Rh-PE lipid was included in the bilayer for this. Analysis of the fluorescence recovery data revealed a lipid diffusion coefficient of $1.4 \pm 0.1 \mu\text{m}^2/\text{s}$, which is lower than the diffusion coefficient of the non-functionalized bilayers with identical lipid composition ($2.4 \pm 0.1 \mu\text{m}^2/\text{s}$).

Having observed the long-range, lateral diffusion of lipid molecules in the bilayer, we attempted to determine the diffusion behavior of the covalently bound proteins on the bilayer. Protein diffusion on a membrane is thought to be primarily governed by the diffusive properties of the lipid molecules [38]. However, fluorescence imaging showed that FN and Col I fibers practically were immobile in the time scale of experiments (3 h). This perhaps reflects the fact that individual ECM proteins are covalently bound to multiple lipid molecules, and hence, displays a reduced diffusion [39,40]. Such an idea is supported by the observed reduction in the diffusion coefficient of lipid molecules on ECM protein functionalized bilayers (2.0 ± 0.1 and $1.4 \pm 0.1 \mu\text{m}^2/\text{s}$ for Col I and FN functionalized bilayers, respectively, versus $2.4 \pm 0.1 \mu\text{m}^2/\text{s}$ for non-functionalized bilayers). However, unlike the display of highly diffusive proteins on bilayers [5,6,19-21], this display of immobile proteins on fluid bilayers is better suited for cell adhesion studies that require a reduction in the diffusion of the protein [17,41]. More specifically, this immobile display of ECM proteins on fluid bilayer appears to be a better mimic of soft matrices that also display immobilized proteins [42-44].

4.3.2. Interaction of Huh 7.5 Cells with ECM Functionalized SLB and Glass

Having established an ECM protein functionalized bilayer substrates, we went ahead with characterizing their interaction with cells. We chose Huh 7.5 cell, which has been previously utilized for studies related to the effect of ECM proteins on hepatocyte cell behavior and differentiation [45,46]. Cells were plated on both the Col I and FN-functionalized bilayers, and their interaction with ECM proteins were monitored by fluorescence as well as RISM [47] microscopy. Fluorescence microscopy enables visualization of changes in the integrity and distribution of fluorescently-labeled ECM proteins during their interaction with individual cells. Cell adhesion and spreading depend strongly on the interaction between cell and ECM molecules. Huh 7.5 cells readily attached to Col I-functionalized bilayer (**Figure 4.8a**). In contrast, almost no attachment was observed for cells plated onto non-functionalized bilayers, consistent with previous observations [48-50], suggesting that the functionalization of the bilayer with Col I resulted in the specific adhesion of the cells, probably through the engagement

of the integrin family of cell adhesion receptors [51]. Importantly, we found that the interaction of the cells with the bilayer resulted in a local enrichment of the fluorescently-labeled Col I underneath cell while a zone of depletion was observed around the cell after 3 h (**Figure 4.8a**). This observation was specific to Col I-functionalized bilayer since neither enrichment or depletion of the fluorescently-labeled Col I was observed on glass surfaces, even though the cells showed robust adhesion on such surfaces (**Figure 4.8b**). Importantly, RICM imaging of the adhering cells showed a close association of the adhering cells with both types of surfaces (**Figure 4.8g**). Additionally, adhering cells showed reduced contact area and polarization (as assessed by the circularity of the cell adhesion zone) [8] on Col I-functionalized bilayers in comparison to Col I-functionalized glass surfaces (**Figure 4.8h** and **4.8i**). Similar results were obtained in experiments with FN (**Figure 4.8c, 4.8d, 4.8g, 4.8h, and 4.8i**). These results suggest that application of forces by the adhering cells on the bilayer-bound Col I molecules causes a lateral movement of the molecule that eventually results in an enrichment of the protein underneath the cell. However, such forces are probably not sufficient to cause dislocation of the protein that are adsorb on glass surfaces.

Figure 4.8 Cell behavior on ECM-coated bilayer and glass surfaces. (a) Representative fluorescence image of stained Col I fibers on SLB before (left) and 3 h after plating (right) of Huh 7.5 cells. Col I fibers around the cell were depleted by the cell following adhesion and spreading. (b) Fluorescence micrograph of an adherent cell 3 h after cell attachment on Col I coated glass showed no Col I depletion. (c) Representative fluorescence image of FN-coated bilayer, before and 3 h after cell adhesion demonstrating FN depletion. FN depletion by cell was not observed on (d) FN-coated glass. (e & f) FRAP snapshots of supported bilayer underneath the cell plated on (e) Col I and (f) FN-coated bilayer after three h after cell adhesion. Fluorescence was recovered nearly completely suggesting bilayer retains its mobility underneath the cell. (g) RICM images of cells adhering Col I and FN-functionalized glass and bilayer substrates. Note the greater spread of the cells on the glass substrate compared to the bilayer substrate. (h & i) Graph showing adhesion area (contact) (h) and circularity (i) of cells on different substrates. Note that lower cell adhesion area and higher circularity of cells on bilayer substrates. Error bar is s.e.m. of measurements

made from ~10 cells in each group. * indicates p -value less than 0.05 while *** indicates p -value less than 0.001.

As we have mentioned in the introduction part, SLBs are viscous but not elastic, and thus application of cellular forces on ECM proteins will lead to their dislocation in the lateral dimension. However, given the viscous nature of SLBs, the lateral translocation of the ECM proteins will result in the development of a viscous drag force. Such a force has been estimated using the Einstein-Smoluchowski relation on SLBs functionalized with cadherin adhesion protein and was found to be in the range of femtoNewtons (fN) [17], which are much lower than the few to tens of picon reported for integrin-ECM interaction on stiff substrates [52,53].

The local depletion of the ECM proteins observed here appears to be similar to previous results obtained with nanoparticle coated solid surfaces [54-56]. While these local depletion of the ECM proteins appear to be similar to the engulfment of nanocrystals, these two systems are fundamentally different in that the ECM proteins are covalently linked to the supported lipid bilayer while the nanocrystals adhere to the Col I coated glass surface in a nonspecific manner.

A lateral translocation of the Col I molecules underneath adhering cells will require that the bilayer retains its long-range lateral mobility after the cell adhesion. Therefore, we performed FRAP experiments at the zone of cell adhesion to assess the mobility of lipid molecules (**Figure 4.8e** and **4.8f**). FRAP experiments revealed a diffusion coefficient of 1.99 ± 0.06 and $1.4 \pm 0.05 \mu\text{m}^2/\text{s}$ for Col I and FN-functionalized bilayers, respectively, confirming that the bilayers still retain their fluidity. The slight reduction in the diffusion coefficient observed under adhering cells is likely due to the greater accumulation of the lipid molecules that are covalently bound to the ECM proteins. This confirms that the enrichment of Col I and FN observed on bilayers is enabled by the lateral mobility of the lipid molecules.

4.3.3. Interaction of Huh 7.5 Cells with ECM Functionalized SLBs of Different Viscosities

After establishing the SLB-based substrate for cell adhesion, we manipulated the bilayer viscosity to determine its role in the cell adhesion and enrichment of ECM proteins. Membrane viscosity directly controls the tension developed upon the application of cellular forces on the covalently bound ECM proteins [17]. We altered the viscosity of the bilayers by adding Chol in the bilayers. Controlling Chol content in the plasma membrane of cells have been previously utilized to regulate their membrane viscosity [57-59], which in turn regulate the dynamics of membrane proteins [60] and the rate of cell migration [61,62]. We chose the SALB method of bilayer formation for this as it has been already shown to allow assembly of bilayers with high Chol content [29]. Bilayers were prepared with a range of Chol fraction (up to 40%) and functionalized with either Col I or FN as described in the previous section. The protein adsorption level to Chol-containing bilayers was almost the same as that of bilayers with no Chol (see **Figure 4.9**). It is expected as Chol only affects the viscosity of the membrane while the protein adsorption is mainly determined by the fraction of activated functional lipids present in the membrane.

Figure 4.9 The QCM-D frequency (ΔF) and dissipation (ΔD) change after (a) Col I and (b) FN binding to DOPC:DP-NGPE (4:1) bilayers prepared by SALB method using varying mole fractions of Chol in the lipid precursor mixture (10, 20 and 40 mol %). After bilayer fabrication, the frequency shift was then normalized with $\Delta F = 0$ Hz corresponding to the planar lipid bilayer

in each experiment. Changes in resonance frequency indicate the adsorption of Col I and FN onto the bilayers.

Diffusion coefficients of lipids were determined by FRAP, and membrane viscosity was estimated from the diffusion coefficients by applying the Evans–Sackman model [26]. **Figure 4.10a** and **4.10b** show the diffusivity and membrane viscosity of non-functionalized and ECM protein-functionalized bilayers with varying fraction of Chol, respectively. Generally, the diffusivity of lipids decreased with increased fraction of Chol. For instance, the lipid diffusivity of non-functionalized membranes was reduced almost four times from 2.3 ± 0.3 to $0.6 \pm 0.2 \mu\text{m}^2/\text{s}$, when 40% Chol was included in the bilayer (**Figure 4.10a**). Accordingly, the membrane viscosity was increased almost six times from $6.7 \pm 1.1 \times 10^{-10}$ to $37.4 \pm 4.5 \times 10^{-10} \text{ kg/s}$ (**Figure 4.10b**). Importantly, while the lipid diffusion coefficients at low Chol fractions ($\leq 10\%$) were different between the non-functionalized and ECM protein functionalized bilayers (**Figure 4.10a**), they were similar at higher Chol fractions indicating a dominating effect of Chol on bilayer diffusivity in comparison to the effect of covalent coupling of the ECM proteins to the bilayer.

Increasing the fraction of Chol in the bilayer resulted in a reduction in the amount of Col I depletion by adhering cells. The majority ($68 \pm 10\%$) of cells on bilayers with no Chol showed complete Col I depletion (**Figure 4.10d**, inset), however, only 39 ± 7 and $28 \pm 5\%$ of cells on bilayer containing 20 and 40% Chol, respectively, showed complete depletion. The rest of the cells in these samples showed partial or no depletion (**Figure 4.10c** and **4.10d**). A similar trend was observed for cells on FN-functionalized bilayers (**Figure 4.10e** and **4.10f**). These results suggest that increasing the viscosity of the bilayer results in a decrease in the lateral translocation of the ECM proteins on the bilayer. The reduction in the lateral translocation of the ECM proteins on 40% Chol-containing bilayers was interesting. Although Chol leads to a reduction in the fluidity of the bilayer, it is not clear if the effects seen with cells were entirely due to the increase in the viscosity, and is not due to any chemical effects of Chol.

Figure 4.10 Effect of membrane viscosity on cell behavior plated on ECM-functionalized bilayer platforms. (a) Membrane diffusivity and (b) the corresponding viscosity of bare and ECM-functionalized SLBs containing varying fraction of Chol. Fluorescence micrograph of Col I (c) and FN (e) on supported bilayer with varying fraction of Chol (0, 20 and 40%) incubated with Huh 7.5 for 3 h. With increasing the percentage of Chol, the protein depletion decreased. Percentage of cells showing complete protein depletion on Col I (d) and FN (f) functionalized bilayers with varying fraction of Chol. Fluorescence micrographs of (g) Col I and (h) FN on hybrid bilayers before and after incubation with Huh 7.5 for 3 h. No depletion was observed on hybrid bilayers.

To confirm that the effects seen with cells are purely due to the change in the physical properties of the substrate, we prepared hybrid substrates composed of a phospholipid monolayer on top of a hydrophobic alkane-silane self-assembled monolayer on glass using the SALB method [22,23]. The lipid mobility in these substrates was very slow, and a FRAP analysis revealed a diffusivity of $0.2 \pm 0.04 \mu\text{m}^2/\text{s}$, which is in the

range previously reported values [63], and corresponds to a viscosity of $123 \pm 30 \times 10^{-10}$ kg/s. Col I and FN were readily conjugated to the hybrid bilayers containing 20% functional lipids, as judged from fluorescence imaging (**Figure 4.10g** and **4.10h**). Similar to the results obtained with high Chol-containing bilayers, less than 10% of cells showed a depletion of either Col I or FN on these substrates. This confirms that the reduction in the fraction of cells showing the lateral movement of ECM proteins is controlled by the viscosity of the underlying bilayer.

It needs to be mentioned that the areas of depletion zones around cells that were cleared of fluorescently-labeled protein and the areas of the corresponding cells were determined using ImageJ software. We assigned cases which the area of the depletion zone was greater than the area of the corresponding cell to the complete depletion group. A typical analysis was shown in **Figure 4.11**.

Figure 4.11 Typical quantification of the area of a cell and its depletion zone (the area that were cleared of fluorescently-labeled protein). The areas (marked in red) were measured using ImageJ software.

4.4. Conclusion

In conclusion, we have developed a bilayer-based substrate displaying ECM proteins through covalent coupling that allows cell adhesion. Cells adhering to these substrates show lateral translocation of the ECM protein resulting in the formation of a

zone of enrichment of ECM proteins underneath them, and a zone of depletion of ECM proteins around them. This lateral translocation is enabled by the long-range diffusivity of the underlying SLB, and can be controlled by altering the diffusivity of the bilayer. We envisage that the substrate developed here will be useful in enabling both the short-term (within hours) adhesion, and long-term (within days) gene expression changes and differentiation of stem cells. In addition, these substrates will be useful in biomedical applications requiring cell adhesion to extremely soft surfaces such as in the case of neuronal tissues.

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Chapter 5

Optimizing the Performance of Supported Lipid Bilayers as Cell Culture Platforms Based on Extracellular Matrix Functionalization*

Strategies to fabricate biofunctionalized surfaces are essential for many biotechnological applications. Zwitterionic lipid bilayer coatings doped with lipids with chemically selective headgroups provide a robust platform for immobilization of biomolecules in an antifouling, protein resistant background. Herein, we assess the biological activity of two important components of the extracellular matrix (ECM); collagen type I (Col I) and fibronectin (FN) which are covalently attached to a supported lipid bilayer (SLB) and compare their activity with those of nonspecifically adsorbed to SiO₂ surface. The characterization of protein coatings by quartz crystal microbalance with dissipation (QCM-D) revealed that Col I and FN attached to SLB are less dense and has higher structural flexibility than when adsorbed onto SiO₂. Cell adhesion, proliferation, and function, as well as Col I-FN interactions, were more efficient on ECM-functionalized SLB making it a promising platform for cell-based diagnostics, tissue engineering, medical implants, and biosensor development.

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5.1. Introduction

Controlled functionalization of solid surfaces with proteins is a critical step in the development of biointeractive surfaces employed in advanced technologies such as cell-based diagnostics and therapies, tissue engineering, biomolecular therapies, and biosensors [1-6]. Proteins usually physically adsorb to commonly used surfaces such as metal dioxide, glass, polystyrene, and gold. The protein-surface interaction is a result of a complex interplay between many factors including (i) environmental conditions such as temperature [7], pH, and salinity [8], (ii) protein properties [9] such as size, structural stability, and composition, as well as, (iii) surface properties such as polarity, charge, and roughness [10]. However, depending on the interaction strength between proteins and the surface, proteins may undergo structural rearrangements or even denature upon adsorption [10-12]. The biological activity and function of proteins, such as enzymatic activity or presentation of ligands for specific receptors, usually originated from the functional domains in their 3D structures that specifically interact with other biomolecules. Consequently, any surface-induced structural deformation may alter the protein function. Moreover, the part of the protein molecule which interacts with the surface is not available for other interactions. Thus, strategies to coat surfaces with proteins whilst preserving their biological activities are of importance for the successful development of tailored biomaterial surfaces. In this regard, SLBs [13] offer unique possibilities for controlled functionalization of solid surfaces [14,15].

SLBs are biomembrane-mimetic coating materials that are biocompatible. Importantly, lipid membrane coatings composed of zwitterionic lipids that have both a positively and a negatively charged moiety within the same headgroup (such as phosphorylcholine (PC)), are nonfouling and resistant to nonspecific adsorption of proteins [16] and cells [17]. However, specific lipids containing reactive headgroups such as amine or free carboxylic acid [18,19], or specific chemical functionalities, such as biotin [20,21] or DNA oligonucleotides [22], can be introduced into the membrane to act as linkers to specifically attach proteins or any biomolecule of interest to the membrane surface. The protein resistance but functionalizable characteristics of lipid membranes

have led to many applications in biomedical research and biotechnology. The applications include the design of various innovative biointerfaces for optically or electrically active substrates in biosensors, passivation of micro and nanofluidics [23], and functionalization of nanoparticles for targeted drug delivery applications [24]. In addition, SLBs functionalized with cell-binding peptides [25-27] have been developed for studying cell behavior. In this regard, it is important to evaluate the biological activity of proteins that covalently attach to SLBs.

Col [28] and FN [29] belong to a collection of molecules called the ECM [30] which are secreted by cells and provide structural and biochemical support to the surrounding cells. They are of immense biomedical importance and have central roles in cell adhesion. This feature has been exploited to mediate adhesion of cells to synthetic surfaces coated with FN and Col for biomaterial and tissue engineering applications [31-35]. In addition, SLBs functionalized with Col I and FN have also been demonstrated to be an efficient platform for mammalian cell culture, and it has been shown that, for instance, smooth muscle cells retained normal growth behavior on such platforms [36,37].

Herein, we provide a comparative assessment of the biological activity of Col I and FN when they are (i) attached to the SLB via covalent bonding and (ii) adsorbed nonspecifically to a SiO₂ surface. To this end, protein adsorption to each surface was quantified by QCM-D. The amount of adsorbed protein to each platform was used as a framework for comparison of the protein biological activities including the cell attachment, proliferation, and function, as well as protein-protein interactions.

5.2. Materials and methods

5.2.1. Lipid Bilayer Formation

The lipid reagents 1,2-dioleoyl-sn-glycero-3-Phosphatidylcholine (DOPC) and 1,2-dipalmitoyl-sn-glycero-3 phosphoethanolamine-N-(glutaryl) (DP-NGPE) were purchased

in a lyophilized powder form from Avanti Polar Lipid (Alabaster, AL). Isopropanol and ethanol were used to dissolve DOPC and DP-NGPE to a concentration of 10 and 5 mg/mL, respectively. The DP-NGPE solution was heated to 50°C and gently vortexed, to enhance the solubility. All of the stock solutions were diluted and mixed in isopropanol to prepare a lipid solution containing 80% DOPC and 20% DP-NGPE to a final concentration of 0.5 mg/mL. Lipid bilayers were formed using solvent-assisted lipid bilayer (SALB) formation method, whereby an isopropanol mixture of DOPC and DP-NGPE was introduced to a hydrophilic SiO₂/glass substrate and was incubated for 10 min. A buffer solution (10mM Tris, 100mM NaCl, pH 7.2) was carefully added and replaced with the isopropanol mixture of lipids. In case of bilayer formation on the glass-based surface, commercially available microfluidic flow cells (sticky-slide VI^{0.4}, ibidi) were utilized.

5.2.2. Activating Carboxyl Groups of DP-NGPE Lipid Using EDC/NHS Chemistry

N-Hydroxysulfosuccinimide sodium (sNHS) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) were received from Alfa Aesar. Immediately before the experiment, EDC/NHS powders were dissolved in 10 mM HEPES buffer and 100mM NaCl (pH 5.5) at a final concentration of 10 mg/mL. The solution was incubated with SLBs for 1 h at room temperature. After carboxylic activation, SLBs were washed three times with a HEPES solution.

5.2.3. Functionalization of SLB and Glass/SiO₂ with ECM Proteins

The Proteins reagents were prepared in pH 7.5 PBS (10mM Phosphate buffer, 2.7mM KCL, and 150mM NaCl) as a following concentrations: 0.2 mg/mL Col I (Sigma-Aldrich), 0.2 mg/mL FN (Calbiochem) (for direct protein conjugation), 0.05 mg/mL FN and 0.1 mg/mL bovine serum albumin (BSA) (Sigma-Aldrich) (for multiple protein conjugation). For direct protein conjugation, 10 mL 0.2 mg/mL Col I or 10 mL 0.2 mg/mL FN solution was introduced on SLB and SiO₂/glass using a peristaltic pump (Ismatec Reglo Digital) with a flow rate of 50 µL/min. The surface was then rinsed

thoroughly with PBS to remove unbound proteins. For multiple protein systems, 10 mL 0.05mg/mL FN solution/ 0.1mg/mL BSA was injected on Col I-SLB (20% DP-NGPE) to form FN/Col I-SLB/ BSA/Col I-SLB. Finally, the substrates were washed with PBS to remove unbound proteins.

5.2.4. Quartz Crystal Microbalance with Dissipation (QCM-D)

The formation of a lipid bilayer and binding of proteins to the lipid interface was monitored and characterized by a Q-Sense E4 instrument (Q-sense AB). SiO₂-coated AT-cut piezoelectric quartz crystals (model no. QSX303) (Q-sense AB) were cleaned with 1% sodium dodecyl sulfate (SDS) and rinsed with Milli-Q water (Millipore). The crystals were dried with nitrogen gas (N₂) and treated with oxygen plasma (March Plasmod Plasma Etcher, March Instruments) at 180W for 30 s to remove any residual organic molecules bound to the surface while making the substrate more hydrophilic. QCM-D measurements were taken at the 3, 5, 7, and 11 overtones, and the presented data were measured and normalized at the third overtone (15 MHz). The samples were injected into the QCM chamber using a peristaltic pump (Ismatec Reglo Digital) with a flow rate of 50 μ L/min at room temperature.

5.2.5. Fluorescence Recovery after Photobleaching (FRAP)

Lateral lipid mobility was estimated using FRAP technique. 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (Rh-PE) (Avanti Polar Lipid), (0.5 wt %) was included in the lipid precursor solution to fluorescently label the lipid bilayers. A commercially available flow chamber (sticky-slide VI^{0.4}, ibidi) was used for bilayer formation using the SALB method. The SLBs were photobleached using a 532 nm, 100mW laser beam that resulted in the formation of a $\sim$ 20 μ m bleached spot. The bleaching period was $\sim$ 5s, and the fluorescence recovery of the bilayer was measured using an inverted epifluorescence Eclipse TE 2000 microscope (Nikon) equipped with a 60 $\times$ oil immersion objective (NA 1.49) combined with an Andor iXon+ EMCCD camera (Andor Technology). The images were recorded at 2 s intervals

with dimensions of 512×512 pixels with a pixel area of $0.267 \times 0.267 \mu\text{m}$ and analyzed using ImageJ software. The lipid diffusivity was determined by the Hankel transform method [38]. For all fluorescence imaging experiments, glass coverslips (coverslips for sticky-slides: # 1.5H ($170 \mu\text{m} \pm 5 \mu\text{m}$) D 263 M Schott glass, 25 mm x 75 mm; ibidi) were used.

5.2.6. Cell Culture

The human hepatocellular carcinoma cell line (Huh-7.5) was obtained from Apath. Cells were plated on a tissue culture dish (100 mm) containing a 10 mL of Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone), 10% fetal bovine serum (Hyclone) and 1% penicillin, and streptomycin (Gibco) for several days to reach a confluency of ~80%. Cells were detached from the plate by incubating the cells with 0.25% trypsin (Gibco) for ~ 3-5 min. After the cell suspension was dispersed in the growth medium, the cell count of 2×10^4 was determined via a hemocytometer. A flow chamber (sticky-slide VI, ^{0.4} ibidi) was used for cell seeding. The cell growth area per channel was $\sim 0.6 \text{ cm}^2$.

5.2.7. Evaluation of Huh 7.5 Cell Morphology

Cell morphology was visualized over a period of 24 h, wherein Huh 7.5 cells were fixed using 4% paraformaldehyde (Sigma-Aldrich) for 3min. After washing the samples with PBS three times, a 3% BSA blocking buffer was added and incubated for 1 h. Alexa Flour 555-labeled phalloidin (Life Technologies) was used to label filamentous actin (F-actin) and was incubated with cells for 2 h at room temperature. The nucleus of each cell was labeled with the addition of $10 \mu\text{g}/\text{mL}$ of 1, 2, 6-diamidino-2-phenylindole, dihydrochloride (DAPI; Life Technologies) for 5 min. The fluorescence images were captured using a confocal microscope, LSM710 with a ZEN program (Carl Zeiss), and were analyzed using ImageJ software.

5.2.8. Quantification of Huh 7.5 Cell Surface Area

The optical images of Huh 7.5 cells were captured after 24 h using an inverted microscope (Nikon) with a 10× objective. The cell surface area was measured and analyzed using ImageJ software.

5.2.9. Determination of Cell Viability by LIVE/DEAD Kit

The qualitative investigation of Huh 7.5 cell viability was done using LIVE/DEAD Cell Viability Assay (Life Technologies), following the manufacturer's protocol. The proper volume of 2 μ M calcein AM and 4 μ M ethidium homodimer-1 (EthD-1) was prepared in DMEM media. The solution was added to each sample and incubated for 20 min at 37°C. After incubation, the trapped calcein inside the living cells yielded a strong green fluorescence and was imaged using a 494-517 nm emission filter. For dead cells, EthD-1 was bonded with the nucleic acids inside the cell and produced a red fluorescence that was imaged using a 517-617 nm emission filter. An inverted epifluorescence Eclipse TE 2000 microscope (Nikon) equipped with a 10× objectives (NA 1.49) combined with an Andor iXon+ EMCCD camera (Andor Technology) was used for capturing fluorescence images. The images were analyzed using ImageJ software.

5.2.10. Determination of Cell Number by Cell Counting Kit-8 (CCK-8)

Cell proliferation for days 1 and 3 was measured using a colorimetric assay CCK-8 (Dojindo Molecular Technologies). Based on the manufacturer's protocol, a 10 % CCK-8 reagent in DMEM was added to the cells. After incubation for 1 h at 37°C, the absorbance was measured at 450 nm using a microplate reader. The measured absorbance was converted to cell number according to a CCK-8 standard curve for Huh 7.5.

5.2.11. Determination of Human Albumin in Cell Culture Supernatants

Albumin secretions from the Huh 7.5 cells were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Abcam). The cell culture media was collected after 24 h and was centrifuged at 10000 g for 10 min. The supernatant was transferred to a sterile Eppendorf tube and stored at -20°C. Before the experiment, the samples were kept at room temperature and the provided protocols were subsequently followed.

5.2.12. Immunofluorescent Stain for ECM Proteins

The primary antibodies, mouse monoclonal anti-Col I (Abcam) and mouse monoclonal IgG1 anti-FN (Santa Cruz) were incubated with conjugated Col I and FN, respectively, for 1 h at room temperature. The samples were then washed with PBS to remove unbound antibodies. After washing, the secondary antibodies Alexa Fluor 488 Goat Anti-mouse IgG (H+L) (green-fluorescent dye, Life Technology) and Alexa Fluor 555 Rabbit Anti-Mouse IgG (H+L) (red-fluorescent dye, Life Technology) were added and incubated for 15 min to fluorescently label Col I and FN, respectively. Fluorescence images were captured with an inverted epifluorescence Eclipse TE 2000 microscope (Nikon) equipped with a 60 × oil immersion objective (NA 1.49) combined with an Andor iXon+ EMCCD camera (Andor Technology). The data were analyzed using ImageJ software.

5.3. Results and Discussion

5.3.1. The SLB Formation and Functionalization with ECM Proteins

First, we demonstrate the preparation and characterization of ECM-functionalized bilayer platforms; then, we compare the biological activity of ECM proteins on SLB platforms with those prepared on glass. As mentioned before, the common strategy to functionalize lipid bilayers is to incorporate lipid-like molecules with chemically reactive headgroups into the bilayer structure [39]. The functionalized lipids act as an anchor to

immobilize specific biomolecules containing the corresponding binding moiety to the membrane surface. Importantly, the degree of biomolecule conjugation to the bilayer can be controlled by the fraction of functional lipids included in the bilayer. **Figure 5.1a** illustrates the schematics of a solid surface coated with a lipid bilayer that is functionalized with ECM proteins. In the current work, we prepared bilayers composed of DOPC doped with DP-NGPE. DOPC (**Figure 5.1b**) is a neutral, zwitterionic lipid that provides resistance to protein adhesion and gives the bilayer its antifouling capabilities. DP-NGPE serves as a functionalized lipid whose headgroup bears a free carboxylic acid (**Figure 5.1b**). After activation, DP-NGPE can react covalently with amine-containing biomolecules (e.g., proteins).

Figure 5.1 ECM-functionalized SLB platform. (a) Schematic illustration of model membranes functionalized with ECM molecules via covalent bonding to lipids with reactive headgroups. (b) Structures of the lipids used in this study, together with their chemical names. (c) QCM-D frequency shift (ΔF , top panel) and dissipation (ΔD , bottom panel) for the third overtone were measured as a function of time during the formation of the planar bilayer on SiO_2 . SLBs were formed on SiO_2 via the SALB formation method using an isopropanol solution of a zwitterionic lipid (DOPC) and a lipid bearing a free carboxylic acid for amine coupling (DP-NGPE). Arrows indicate the injection of (i) Tris buffer (10 mM Tris, 150 mM NaCl, pH 7.5), (ii) isopropanol, (iii) lipid mixture [0.5 mg/mL of DOPC: DP-NGPE (4:1) in isopropanol], and (iv) Tris buffer solution leading to a final ΔF and ΔD of -25 ± 1 Hz and $0.4 \pm 0.2 \times 10^{-6}$, respectively, which correspond to a planar bilayer. (d) FRAP snapshots of DP-NGPE doped bilayer containing 0.5 wt % Rho-PE lipid, immediately (top) and 1 min (below) after photobleaching, showing almost complete recovery, indicating the fluidity of the SLB.

Previously, Huang et al. [36,37], used the vesicle fusion method to form a SLB containing various fraction of DP-NGPE. Vesicle fusion, which involves the spontaneous adsorption and rupture of phospholipid vesicles, is the most commonly used method to form SLBs. However, under physiological conditions, vesicle fusion is limited to only a subset of lipid compositions and solid supports. In this work, the surface was coated with phospholipid bilayer using the so-called SALB formation method [40]. The SALB method is based on the reverse-phase evaporation phenomenon and involves lipid deposition onto a hydrophilic solid surface in an alcohol, for example, isopropanol, followed by the slow exchange of the alcohol with water, resulting in the formation of a lipid bilayer on the surface [41]. The SALB method has been applied successfully to a wide variety of surfaces including SiO_2 , and those which are intractable to the conventional vesicle fusion method, such as Au [40], Al_2O_3 [42], and graphene [43]. In addition, the SALB method enables formation of bilayers composed of lipids that cannot be formed by vesicle fusion method for example, those containing large fractions of cholesterol (Chol) [44,45].

The bilayer formation on a solid surface and subsequent protein conjugation were monitored by the QCM-D measurement technique [36,37]. QCM-D allows real-time monitoring of adsorption processes involving lipid membranes and proteins [46]. The method records ΔF which depends on the total effective mass of the adlayer, which consists of the mass of the coating material plus entrapped and coupled water. A negative change in ΔF indicates an increase in the mass of the adlayer. In addition, QCM-D measures ΔD which reflects the softness/rigidity of the adlayer. A perfectly rigid film, such as SLB has a near zero energy dissipation, whereas softer adlayers, such as proteins, exhibit greater energy dissipation.

Figure 5.1c represents the time course of ΔF (top panel) and ΔD (bottom panels) shift during SALB formation on SiO_2 . A typical SALB formation experiment involved baseline recording in the aqueous buffer solution (i), the addition of isopropanol (ii), the addition of the lipid mixture [0.5 m/mL, DOPC: DP-NGPE (4:1)] in isopropanol (iii), and solvent exchange back into the initial buffer solution (iv). The final ΔF and ΔD values

after complete solvent replacement were -25 ± 1 Hz and 0.4 ± 0.2 , respectively, corresponding to formation of a planar bilayer.

The formation of the bilayer was further confirmed by FRAP analysis showing lateral mobility of lipids in the two-dimensional plane of the membrane with a diffusion coefficient of $2 \pm 0.3 \mu\text{m}^2/\text{s}$ (**Figure 5.1d**) which is comparable to previously reported values [40].

5.3.2. Comparison of the Adsorption of Col I and FN on SLB and SiO_2

Next, we studied the adsorption of Col I and FN to the SLB and compared the results with their adsorption to SiO_2 . All steps were monitored by QCM-D. To this end, after the SLB formed, it was incubated with EDC/NHS to activate the carboxylic groups of DP-NGPE, followed by injection of the protein solution. **Figure 5.2a** shows the changes in ΔF and ΔD , during Col I conjugation. In this and other QCM-D graphs, the bilayer formation and EDC/NHS incubation were not shown and the frequency shift was normalized with $\Delta F = 0$ Hz corresponding to the planar lipid bilayer in each experiment. During the adsorption of Col I to the SLB, ΔF decreased and saturated at $\Delta F_{\text{Col I}} = -122 \pm 5$ Hz. At the same time, $\Delta D_{\text{Col I}}$ increased and stabilized at $\Delta D = 32.7 \pm 0.6 \times 10^{-6}$. After the protein adsorption approached the saturation level, the pure buffer was injected, which resulted in almost no change in ΔF and ΔD , indicating that Col I had irreversibly adsorbed to the surface. The high energy dissipation indicates the softness and viscoelastic nature of the Col I film, suggesting that Col I fibers did not rigidly attach to the underlying bilayer. In addition, the high dissipation indicates the presence of a high amount of water molecules coupled to the Col I fibers resulting in a hydrated layer with high mass and dissipation.

The immunofluorescence microscopy of the Col I coated SLBs, prepared using the same protocol in a microfluidic chamber for microscopy, also showed attachment of Col I fibers randomly to the SLB surface (**Figure 5.2a**, inset).

Figure 5.2 QCM-D analysis of ECM adsorption to SLB and SiO₂ platforms. QCM-D monitoring of (a) Col I and (d) FN attachment to bilayer via covalent bonding. The SLB on SiO₂ was initially formed by the SALB method, and then the measurement time was normalized. Frequency shift (ΔF , blue) and energy dissipation shift (ΔD , red) for the third overtone ($n = 3$) were measured as a function of time. Protein solution (0.2 mg/mL) was introduced to the surface after treatment of SLBs with EDC/NHS. The insets show the fluorescence immunostaining micrograph of SLB functionalized with Col I fibers (a) and FN (d). Proteins were stained using fluorescently labeled anti-Col I or anti-FN antibodies. Dissipation-frequency curves (ΔD vs. ΔF) upon (c) Col I and (f) FN adsorption to the SLB and SiO₂ surface. The dashed line shows that at any given frequency, the corresponding dissipation was bigger for protein layer on the SLB. Scale bars are 20 μm .

For comparison, Col I adsorption on the SiO₂ substrate with no SLB coating was examined by QCM-D, as shown in **Figure 5.2b**. Adsorption of Col I to the surface was revealed by a negative frequency shift and a simultaneous increase in the dissipation reaching a saturation value of $\Delta F_{\text{Col I}} = -186 \pm 60$ Hz and $\Delta D_{\text{Col I}} = 42 \pm 19 \times 10^{-6}$. A buffer wash step resulted in a small change in ΔF (1.75 ± 1 Hz) and ΔD (1.25 ± 0.5), indicating the majority of Col I irreversibly adsorbed to the surface. Comparing the $\Delta F_{\text{Col I}}$ for SLB and SiO₂ suggests that more Col I adsorbed to SiO₂ than to SLB. The ΔF for Col I adsorption to SiO₂ was almost 1.5 times greater than that of SLB. The higher ΔF

indicates that more Col I adsorption to the bare SiO₂ surface. In agreement with QCM-D results, the fluorescent images also revealed that a greater amount of Col I fibers adsorbed to the bare glass (**Figure 5.2b**, inset).

The proteins adsorbed nonspecifically to the glass surface. In contrast, zwitterionic lipid bilayer has previously been shown to be quite efficient in preventing protein adsorption at interfaces. The exact mechanism for the nonfouling behavior of zwitterionic bilayers is not known; however, the cooperative contribution of the following factors has been proposed to be important [16]: (i) charged neutrality of zwitterionic lipids in a wide range of pH weakens the electrostatic interaction between bilayer and proteins, (ii) the presence of a hydration layer which strongly associates with lipid headgroups acts as a barrier for protein adsorption, and (iii) the high lateral mobility of lipids in the membrane plane. Because of the nonfouling feature of the bilayer, Col I fibers attach to the bilayer surface exclusively via the anchoring lipids (DP-NGPE). The activated carboxylic acid of DP-NGPE covalently binds to lysine residues which contain the primary amine side chains. Therefore, the Col I fibers attached to the bilayer surface via a limited number of lysine residues that are dispersed along the fibers. As a result, fewer fibers attach to the membrane surface in comparison to those on the SiO₂ substrate.

To gain more information about the structure of the Col I film on SLB and SiO₂, the correlation between ΔD and ΔF was studied. **Figure 5.2c** shows ΔD versus ΔF during Col I adsorption on SLB and SiO₂. At any given ΔF , the corresponding ΔD is higher for Col I on the bilayer. In other word, the Col I film on SLB has a higher structural flexibility than that on SiO₂. This is expected as, due to the limited number of anchoring points along the fiber, the Col I film on SLB is less dense than that on SiO₂.

The same series of experiments were performed to prepare and characterize the FN-coated bilayer and SiO₂, as shown in **Figure 5.2d-5.2f**. The QCM-D results show a ΔF and ΔD of -21 ± 2.1 Hz and $1.4 \pm 0.4 \times 10^{-6}$ for the FN-coated bilayer, respectively (**Figure 5.2d**). The fluorescence micrograph of FN on SLB shows the presence of some unevenly distributed FN aggregates which appear as particularly brighter spots (**Figure**

5.2d, inset). The interaction of FN with the supported bilayer has been previously studied and the formation of FN aggregates at the membrane interface has been shown by atomic force microscopy (AFM) [47]. ΔF and ΔD for FN adsorption to SiO₂ were -101.3 ± 6 Hz and $6.9 \pm 1.3 \times 10^{-6}$, respectively (**Figure 5.2e**) which are similar to the Col I samples, and are higher than that those of FN adsorption to SLB. In contrast to FN on SLB, the fluorescence micrograph of FN on SiO₂ revealed a uniform, highly packed layer (**Figure 5.2e**, inset). FN adsorbed to the bilayer only through the covalent binding to DP-NGPE, but nonspecifically, entirely covering the SiO₂ surface. Similar to the trend observed for the Col I samples (**Figure 5.2c**), the FN film on SLB was softer than that on SiO₂ (**Figure 5.2f**). In addition, the ΔF and ΔD corresponding to the adsorption of FN on SLB and SiO₂ were lower than those of Col I, due to its lower molecular weight and compact globular structure [48].

5.3.3. Comparison of the Biological Activity of Col I and FN Adsorbed on SLB and SiO₂

Next, we examined the biological activity of Col I and FN adsorbed sequentially to SLB and SiO₂. We began by studying the Col I-FN interaction on the SLB and SiO₂ platforms. The interaction of Col I and FN is essential for cell adhesion and migration. FN molecules can bind to a specific domain sequence within the Col I fiber [49]. To study the Col-FN interaction, FN was added to the Col I-coated platforms, and its binding was monitored by QCM-D and visualized by fluorescence microscopy. **Figure 5.3a** shows the QCM-D results for the adsorption of FN to Col I-coated SLB. After bilayer formation and subsequent Col I conjugation, FN was injected. Due to the organization of Col I in a fiber structure, there are many FN binding sites available on Col I fibers. The significant changes of resonance frequency ($\Delta F_{\text{Col I-FN}} = -420.7 \pm 70.5$ Hz) and energy dissipation ($\Delta D_{\text{Col I-FN}} = 84.7 \pm 19.2 \times 10^{-6}$) indicate the tendency of FN to bind to Col I on SLB.

Figure 5.3b and **5.3e** show the fluorescence micrograph of Col I coated SLB and glass after addition of FN. In these figures, only FN was stained using a specific

antibody. Fluorescence staining of FN in these samples revealed the co-localization with Col I fibers due to Col I-FN binding. Also, we checked if the underlying bilayer retains its fluidity after conjugation with Col I and FN (**Figure 5.3c**). FRAP analysis indicated that the bilayer maintains its mobility after functionalization with multiple protein systems (Col I-FN) with a long-range lateral mobility of $0.7 \pm 0.2 \mu\text{m}^2\text{s}^{-1}$. The high lateral mobility of the underlying membrane suggests that protein conjugation was mainly mediated via the anchoring lipids, and the rest of the lipids were free to diffuse. The mobility of the bilayer may assist the lipid anchors in displacing laterally in the membrane plane. Thus, the attached fibers are not locked in their places. As a result, fiber segments motions are not completely restricted and the fiber may have more freedom to change conformation.

Next, for comparison, we examined the sequential adsorption of Col I and FN to the SiO_2 or glass surfaces. **Figure 5.3d** shows the time course of ΔF and ΔD upon subsequent addition of Col I and FN to the SiO_2 substrate. The adsorption of FN to the Col I-coated SiO_2 surface resulted in frequency and dissipation shifts of $\Delta F_{\text{Col-FN}} = -465 \pm 144.2 \text{ Hz}$ and $\Delta D_{\text{Col-FN}} = 72.5 \pm 37.5 \times 10^{-6}$, respectively. These results suggest that in both platforms, FN could specifically bind to the Col I fibers. However, the ratio of ΔF_{FN} to $\Delta F_{\text{Col I}}$ was higher for SLB (2.4 ± 0.4) than that for SiO_2 (1.5 ± 0.09). In other word, the amount of FN molecules per Col I fiber was greater ($p \leq 0.01$) for SLB than that for SiO_2 . The data suggest that the FN binding sites on Col I fibers that adsorbed to SLB were more accessible than the ones on SiO_2 . This is consistent with QCM-D results showing that Col I fibers on SLB have a higher structural flexibility.

Figure 5.3 Binding of FN to Col I fibers on SLB and SiO_2 platforms. (a) QCM-D curves of ΔF and ΔD for the adsorption of FN to Col I-functionalized SLB. (b) Fluorescence micrograph of Col I-coated SLB after incubation with FN, stained by fluorescently-labeled anti-FN antibodies. (c) FRAP snapshots of Col I-FN-coated bilayer showing almost complete recovery, indicating the fluidity of the SLB. (d) QCM-D measurement of FN adsorption to Col I-coated SiO_2 . The initial baseline values in (a) and (d) are the normalized frequency and dissipation shifts for the bilayer (defined as $\Delta F_{\text{SLB}} = 0$). (e) Fluorescence micrograph of Col I-coated glass, after incubation with FN, stained by fluorescently labeled anti-FN antibodies. Immunostaining of FN revealed its co-localization with Col I fibers. All scale bars are 20 μm .

As a control, BSA was added to Col I-functionalized bilayer (see **Figure 5.4**). No change in ΔF and ΔD was observed after BSA injection, indicating that BSA did not adsorb to the Col I-decorated SLB. This observation suggests that FN adsorption to this platform was the result of specific interaction between Col and FN.

Figure 5.4 QCM-D curves of ΔF and ΔD upon addition of BSA (0.1 mg/mL) to Col I-functionalized SLB.

5.3.4. Comparison of Huh 7.5 Cells Activity on Col I and FN-Coated SLB and Glass

Next, we studied the cell-adhering activity of FN and Col I adsorbed to SLB and glass. The various ECM components including FN [50] and Col [51,52] interact specifically with different cells through integrins as specialized cell surface receptors. Integrins bind to specific domains within the ECM proteins and mediate adhesion of cells to the matrix proteins. In addition, integrin receptor interaction with ECM proteins also initiates an intracellular signaling cascade that directs cellular processes such as cell survival [53], proliferation [54], differentiation [55], and migration [56].

We plated the same number of cells on the Col I or FN-coated SLB and glass surfaces and measured the number and area of adhered cells. The aim was to determine

the effect of protein coverage on the extent of cell adhesion and spreading. As a model cell, we used Huh-7.5 cells, which are a human hepatocellular carcinoma cell line. The hepatic ECM consists mostly of FN and some Col I, which makes Huh-7.5 cells a suitable model for our study [57].

To assess qualitatively cell attachment and spreading, cells were stained and analyzed with an epifluorescence microscope. **Figure 5.5a** and **5.5b** present the fluorescence images for actin-stained Huh 7.5 cells attached on Col I and FN-coated SLB after 24 h and show cells attached and spread well on these platforms. However, in agreement with previous reports [17,25,36,37], when cells were seeded on ECM-free SLB, only a limited number of cells attached and remained rounded and did not appreciably spread over 24 h (see **Figure 5.5c**). As cells attach to surfaces via their surface proteins, the limited cell attachment to the ECM-free bilayer can be related to the SLB's resistance to the adsorption of cell adhesive proteins.

On glass, cells attached and spread on the ECM-coated (**Figure 5.5d** and **5.5e**) as well as bare, surface (**Figure 5.5f**). A quantitative analysis of the bright images indicated that the projected area of cells adhered to glass platforms was larger than that of their SLB counterparts (**Figure 5.5g**). This is because Col I and FN on SLBs are less resistant to displacement when experiencing force from the cell. The reason is that the functional lipids, which anchor the proteins to the membrane, are free to move in the plane of the fluid underlying the membrane [As reported in **Chapter 4**]. In addition, for both SLB and glass, the same trend for cell area was observed; the cell area was larger on the FN-coated surfaces as compared to that on the Col I coated and uncoated surfaces [37].

To determine how the cell-adhering activity of FN and Col I was influenced by adsorption to SLB and glass, the number of attached cells on each platform was measured using CCK-8 assay and divided by protein surface coverage. The proteins coverage can be estimated from the QCM-D ΔF . Although the Sauerbrey model [58] can be used to convert ΔF to mass for rigid films, mass determination for soft layers is not trivial. However, as the exact value for protein mass is not required for our analysis, we used ΔF

as an indicator for the adsorbed mass and applied them for comparative analysis. **Figure 5.5h** shows the number of cells normalized as $\text{cells}/\Delta F_{\text{Col I or FN}}$, for glass and SLB. For both proteins, the normalized cell number was higher for SLB than that for glass. In particular, the cell adhesion on Col I and FN-coated SLB were significantly greater than that of glass. This result suggests that even though the protein coverage on the glass surface was greater than that on SLB, the biological activity of FN, and Col I was greater on SLB. The cell attachment is mediated by integrin interaction with specific domains in FN and Col I. RGD is the principal integrin-binding domain present within ECM proteins such as FN and Col I [59]. As Col I and FN interact strongly with the glass surface, they form a relatively dense layer upon surface adsorption and possibly undergo surface-induced conformational changes. In addition, there is a possibility of denaturing when the interaction between a passively adsorbed protein and the surface is too strong [60,61]. As a result, the cell-binding sites (RGD) of a substantial population of adsorbed protein molecules may not be accessible to the cells in the solution phase. However, on SLB, the proteins retain their structural flexibility. Also, the local chemical environment of the phospholipid bilayer mimics that of the natural cell membrane and helps the immobilized protein molecules to maintain their activity and accessibility.

Figure 5.5 Quantification of cell spreading on ECM-functionalized SLBs and glass. Representative confocal images of one day plated Huh 7.5 cells on (a) Col I and (b) FN-functionalized SLB and (d) Col I and (e) FN-functionalized glass. Representative confocal images of one day plated Huh 7.5 cells on ECM-free (c) SLB and (f) glass. F-actin was stained by Alexa Fluor 555-labeled phalloidin (red), and nuclei were stained by DAPI (blue). (Scale bar is 40 μm). (g) The average projected area of Huh 7.5 cells plated on different platforms after 24 h. The images were taken by optical microscope and the observation field was 0.5 mm². (h) The number of cells per absolute values of the maximum QCM-D frequency shifts due to the amount of adsorbed proteins. ($n = 3$, mean $\pm$ SD; ** $P \leq 0.01$, *** $P \leq 0.001$). All the images were analyzed with imageJ.

To further investigate the biological activity of FN and Col I on SLB and glass, we compared the viability and function of the cells plated on the Col I, FN and Col I-FN platforms (see **Figure 5.6**). The physical contact between cell and ECM through integrin [62] and nonintegrin cell surface proteins can trigger chemical pathways leading to cell

growth and division. Therefore, we measured Huh7.5 proliferation on ECM-coated platforms as a function of protein coverage.

The viability of cells on the ECM-coated SLB and glass surfaces were quantified using a live/dead assay combined with a fluorescence microscope at day 1 and 3. As shown in **Figure 5.6a** over 90% of Huh 7.5 cells were viable on all platforms within three days. Next, cell proliferation was measured using the CCK-8 assay [63,64], by dividing the number of cells at day 3 by those at day 1. The obtained proliferation value for each surface was then normalized to its corresponding QCM-D ΔF upon protein adsorption (**Figure 5.6b**). For all cases, except for Col I-FN platforms, the normalized proliferation was significantly higher for the SLB platforms. FN-Col I platforms were prepared by sequential addition of Col I and FN to SLB and SiO₂. Therefore, in both platforms, the majority of FN bound to the Col I layer and not to the underlying surfaces. Thus, in both cases the interaction of FN with underlying substrates is minimal and hence the conformation of FN molecules is perhaps similar. In addition, the frequency change corresponding to FN binding to Col I functionalized SLB and SiO₂ were not significantly different; 298 ± 65.5 and 279 ± 93.3 Hz, respectively, indicating that the amount of FN were similar in both platforms. Moreover, since most of Col I fibers are coated with FN, the interaction of cells with substrate are most probably mediated through FN molecules. Taken together, the conformation and amount of FN which interact with cells are not significantly different in Col I-FN platforms. This similarity might explain the insignificant difference in cell proliferation on Col I-FN platforms, as cell proliferation is mainly modulated by cell-ECM interactions.”

The interaction of cells with ECM can also alter the cell functions by stimulating the transcription of a subset of genes [65]. In hepatocytes, it has been shown that the interaction with ECM can promote albumin production [66,67]. Therefore, we selected albumin secretion as one of the major functions of Huh 7.5 and measured its production on cells plated on the SLB and glass platforms. **Figure 5.6c** shows the average amount of albumin produced by 10^6 cells after 24 h. On average, cells grown on SLB platforms produced more albumin than those grown on glass-based surfaces. This result suggests

that the biological activity of the proteins on the SLB was better reserved than on glass surfaces.

Figure 5.6 Cell viability and metabolic functions on ECM coated platforms. (a) Representative fluorescence micrograph images of detecting live (green) and dead (red) Huh 7.5 cells cultured on SLB and glass coated with Col I and FN and Col I-FN at 1 and 3 days post seeding. Scale bar is 200 μ m. (b) The proliferation of Huh 7.5 cells was measured over a 3day period. The values were normalized to the absolute ΔF due to the amount of adsorbed proteins. (c) Albumin production by 10^6 Huh 7.5 cells plated on different platforms measured after 24 h of

cell growth. The value was normalized to the total number of cells ($n = 3$, mean $\pm$ SD; * $P \leq 0.05$, ** $P \leq 0.01$, ***: $P \leq 0.001$, and **** $P \leq 0.0001$).

However, it is widely known that in hepatocyte the albumin secretion is strongly dependent on the adhesion area [68]. Considering the fact that the cell adhesion area is smaller on SLB substrate, as compared with glass substrate (**Figure 5.5g**), our results may suggest that other factors such as conformation of ECM affect the cell behavior as well as albumin production. For instance our QCM-D results indicate that while the attachment of proteins to the underlying bilayer is firm, the protein molecules retain their flexibility. In another word, the ECM film on SLB has a higher structural flexibility than that on SiO₂. In addition, the mobility of the bilayer assists the lipid anchors and hence the ECM protein in displacing laterally in the membrane plane which might promote cell functions such as albumin production via receptor-ligand binding clustering. In this regard, it has been previously shown that restriction of receptor movement alters cellular response [69].

5.4. Conclusions

The adsorption behavior and biological activity of FN and Col I attached to SLB via covalent bonding were compared with those of FN and Col I adsorbed nonspecifically to SiO₂/glass surface. The characterization of protein adsorption by QCM-D revealed that Col I and FN that attached to SLB have a higher structural flexibility than those adsorbed onto SiO₂. The QCM-D study also showed that the interaction of FN with Col I is more efficient on SLB than on SiO₂, suggesting that the Col I-FN interaction on the SiO₂ surface may be inhibited by limited accessible binding sites on the surface-adsorbed Col I. In addition, the ratio of the number of cells attached to protein-coated SLB and glass, to the amount of adsorbed proteins, revealed that the interaction of cells with the anchorage sites for cell adhesion on FN and Col I is more efficient on SLB than on glass. The same trend was observed for cell proliferation and albumin production by cells attached to the functionalized SLB and glass. Taken together, our results show that FN and Col I attached to SLB via covalent bonding retain their biological activities better than when

attached on SiO₂/glass. Thus, SLB functionalization can serve as an effective strategy to interface biological molecules to inorganic surfaces. The benefit of this platform is expected to be more pronounced when smaller protein/peptide motifs are needed to be used for functionalization.

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Chapter 6

Hybrid Biomimetic Interfaces Integrating Supported Lipid Bilayers with Decellularized Extracellular Matrix Components*

This study describes the functionalization of solid supported phospholipid bilayer with decellularized extracellular matrix (dECM) components, towards the development of biomimetic platforms that more closely mimics the cell surface environment. The dECM was obtained through a combination of chemical and enzymatic treatments of mouse adipose tissue that contains collagen (Col), Fibronectin (FN), and glycosaminoglycans (GAGs) Using amine coupling chemistry, the dECM components were attached covalently to the surface of a supported lipid bilayer (SLB) containing phospholipids with reactive carboxylic acid headgroups. The bilayer formation and the kinetics of subsequent dECM conjugation were monitored by quartz crystal microbalance with dissipation (QCM-D). Fluorescence recovery after photobleaching (FRAP) confirmed the fluidity of the membrane after functionalization with dECM. The resulting hybrid biomimetic interface supported the attachment and survival of the human hepatocellular carcinoma cell line, Huh 7.5 and maintained the representative hepatocellular function. Importantly, the platform is suitable for monitoring the lateral organization and clustering of cell-binding ligands and growth factor receptors in the presence of the rich biochemical profile of tissue-derived ECM components.

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6.1. Introduction

In response to the high complexity of native biological membranes and their microenvironments, various lipid-based model systems have been developed. The physical and chemical characteristics of these model systems are comparable to those of natural membranes [1]. The lipid-based platforms, such as small [2], and giant lipid vesicles [3], bilayer nanodiscs [4], and SLB [5], when functionalized with biological ligands, allows the studying of membrane-associated cellular processes in simplified and controlled conditions.

Amongst these lipid-based platforms, the SLB comprising of a single planar lipid bilayer associated with a solid substrate is particularly effective owing to its excellent mechanical stability while preserving the fundamental characteristics of natural membranes such as two-dimensional fluidity and electrical sealing properties [6]. In addition, The chemical composition of SLBs can be easily tuned to manipulate their biophysical properties such as surface charge [7], and lipid diffusivity [8], and their membrane organization such as phase separation [9] and formation of lipid rafts [10].

Importantly, SLBs can be functionalized with biological receptors and signaling ligands by introducing synthetic head-group modified lipids into the bilayer for direct covalent or non-covalent coupling of biomolecules [11]. Functionalized SLBs, have been used to examine range essential biological processes that occur at the membrane interfaces including viral fusion [12], ligand-receptor interactions [13], enzymatic activities [14], cellular signaling and cell-cell recognition [15]. Owing to their inherent antifouling properties; SLBs are resistant to nonspecific adsorption of cells [16] and proteins [17]; this feature makes them a suitable interface for detection of specific biomolecular interactions. In particular, they are effective tools for studying receptor dynamics during cell-cell interactions as well as triggering various intercellular reactions when interfaced with live cells [11,15,18]. Moreover, a wide range of ultrasensitive surface-based analytical techniques such as atomic force microscopy (AFM) [19], QCM-D [20], total internal fluorescence (TIRF) microscopy [21], and electrochemical

measurements [22], can be used to characterize SLBs in terms of their structure and the biochemical interactions that occur at their interface.

While simplified models have been proven to be useful for many biological applications, development of models with greater compositional complexities would be the next step toward platforms that more closely mimics the cell surface environment. In this regard, decoration of SLBs with the ECM components holds great promise.

The ECM is a tissue-specific network of extracellular bio-active molecules [23,24] that provides support and anchorage for cells and plays a key role in numerous cellular processes including cell growth and differentiation as well as intercellular communications [25]. The ECM is mainly composed of fibrous proteins such as Col [26], FN [27], and laminin [28], as well as proteoglycans with GAGs side-chains [29].

Numerous protocols have been developed to obtain natural ECM through decellularization of tissue via a series of physical, chemical and enzymatic treatments [30-32]. The dECM has been shown to retain its natural 3D-ultrastructure and support the attachment and survival of cells while maintaining their phenotypic and functional characteristics [33,34]. Thus, these biomaterials have attracted much attention as natural 3D-scaffolds for tissue engineering and regenerative medicine applications [35-43].

Previously, SLBs functionalized with individual ECM components such as Col [44], and FN [45], or peptides derived from these proteins (e.g., RGD, IKVAV) [46-51] have been reported. Herein, for the first time, we demonstrate functionalization of SLBs with solubilized adipose tissue dECM and characterize their formation with QCM-D and immunofluorescence microscopy. We demonstrate that dECM functionalized bilayers sustain cell growth and maintain cell function.

6.2. Materials and Methods

6.2.1. Decellularization of White Adipose Tissue

The white adipose tissue samples were extracted from cadavers of mouse (C5/BL/6) and were decellularized with an in-house method using chloroform/methanol treatment. Briefly, the dissected tissue was cut into small pieces and washed several times with PBS (1X) to remove all the blood and hairs. During the first three days, the samples were treated with chloroform/methanol mixture at room temperature using different volumetric ratios: 2/1 (day 1), 1 /1 (day 2) and 1/2 (day 3). Subsequently, the tissue samples were washed with PBS (1X) several times and treated with trypsin (0.25%) for 24 h on a shaker at 37°C. Finally, the samples were washed with PBS (1X) and then treated with isopropanol for 48 h on a shaker at room temperature. The samples were stored in PBS (1X) and supplemented with 1% antibiotic-antimitotic (Gibco) at 4°C until further use.

6.2.2. Solubilization of Decellularized Adipose Tissue

Lyophilized dECM was crushed into powder form using a mortar and pestle. The powder was dissolved in 0.1 M HCl and incubated with 1 mg of pepsin (Sigma, Aldrich) for 48 h on a stirrer at 4°C. After that, the solubilized sample was centrifuged and the resulting sample neutralized using 1M NaOH solution.

6.2.3. DNA Content Quantification

The amount of DNA before and after decellularization was quantified using a Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies) according to the manufacturer's protocol. Briefly, lyophilized adipose tissues were treated with 1 mL TE buffer (1X) (from the kit) and 0.1% Triton X-100. The samples were freeze-thawed three times (-80°C to 37°C). 100 µl of the aqueous working solution of the Quant-iT™ PicoGreen® reagent was mixed with 100 µl of the sample and was incubated for 5 min at

room temperature. The fluorescence of the sample was measured at 480 and 520 nm using a plate reader (TECAN), and the DNA concentration was obtained from the standard curve.

6.2.4. Scanning Electron Microscopy

Scanning electron microscopy (SEM) was utilized to study the morphology of adipose tissue sample before and after decellularization. Samples were prepared for SEM by fixing with 2.5% glutaraldehyde (Sigma-Aldrich) overnight at 4°C. Fixed samples were subsequently dehydrated using ethanol series (20%, 40%, 50%, 70%, 90%, and 100%) for 5 min per grade. Further, the samples were immersed in 100% ethanol and then Hexamethyldisilazane (Sigma-Aldrich) for 1 h each. The samples were loaded on a sample holder covered with carbon and copper tape, followed by coating with gold using a sputter coater (SPI Supplies Inc). The SEM (JSM-7600F-JEOL) was used to image the sample with an acceleration voltage of 5 kV at various magnifications.

6.2.5. Total Protein Content Quantification

The total protein amount for the lyophilized adipose tissues samples before and after decellularization was determined by the Bicinchoninic Acid (BCA) using the Pierce™ BCA protein assay kit (Thermo Scientific) according to the manufacturer's protocol. Briefly, lyophilized tissue was immersed in radioimmunoprecipitation assay (RIPA) buffer supplemented with protease and phosphatase inhibitor (Sigma, Aldrich) at 4°C for 30 min. The samples then were homogenized using a probe-sonication. After homogenization, the samples were centrifuged at 10,000 g for 20 min at 4°C and the supernatant was transferred to a new Eppendorf for measuring the protein content. 25 µl of the protein samples were added in a 96-well microplate and mixed with 200 µl of BCA working reagent. The plate was shaken for 30 s and subsequently incubated at 37°C for 30 min. After cooling the plate, the light absorbance of the samples was measured at 562 nm using a plate reader (TECAN) and the protein concentration was calculated against the standard curve. The amount of proteins was normalized to dry-weight tissue mass.

6.2.6. Collagen Content Quantification

The Col content of intact and decellularized samples was quantified by measuring the hydroxyproline content of the samples, using hydroxyproline assay (Sigma-Aldrich), according to the manufacturer's manual. The hydroxylproline contents of the samples were converted to the amount of Col. Briefly, the samples were hydrolyzed in 12M HCl at 120°C for 3 h. The samples were dried under vacuum and mixed with equal volume of chloramine T/Oxidation buffer and incubated for 5 min. Finally, dimethylaminobenzaldehyde (p-DMBA) colorimetric solution was added to each sample and incubated for 90 min at 60 °C. The absorbance of the colorimetric product was measured at 560 nm using a microplate reader (TECAN). The concentration of hydroxyproline was calculated based on the standard curve and converted to Col content based on the assumption that hydroxyproline constitutes 13.5% of collagen dry mass [52].

6.2.7. GAG Content Quantification

The Blyscan™ GAG assay (Biocolor) was used to measure the amount of total sulfated glycosaminoglycans (sGAG) in the lyophilized adipose tissues samples before and after decellularization according to the manufacturer's protocol. Briefly, the lyophilized tissue sample was incubated with 1 mL papain extraction reagent (see **Appendix**) at 65°C overnight. The sample was centrifuged at 10,000 g for 10 min and the supernatant was separated. 100 µl of the sample was mixed with 1 mL of Blyscan dye reagent and mixed thoroughly on a plate shaker for 30 min at room temperature and subsequently was centrifuged at 10,000 g for 10 min. The colored pellet was separated from the supernatant and was dried completely. The pellet was dissociated using 0.5 mL of dissociation reagent and was vortexed. The solution was centrifuged at 10,000 g for 5 min. 200 µl of the solution was transferred inside a 96-well microplate and light absorbance was measured at 656 nm using a plate reader (TECAN). The amount of GAG was calculated based on the standard curve.

6.2.8. Lipid Bilayer Formation

The SLB was prepared using solvent-assisted lipid bilayer (SALB) formation method. Zwitterionic lipid, 1, 2-dioleoyl-sn-glycero-3-Phosphatidylcholine (DOPC) and a head-group modified lipid, 2-dipalmitoyl-sn-glycero-3 phosphoethanolamine-N-(glutaryl) (DP-NGPE) were obtained in powder from Avanti Polar Lipid (Alabaster, AL). Immediately before the experiment, 10 mg DOPC and 5mg DP-NGPE were dissolved in 1 mL isopropanol and ethanol, respectively. To aid dissolution of DP-NGPE in ethanol, the solution was heated to 50°C and vortexed gently. This process was repeated several times to a transparent solution was obtained. The stock solutions were diluted using isopropanol to prepare 0.5 mg/mL lipid mixture containing 80% DOPC and 20% DP-NGPE. 200 µl of the lipid mixture was injected into a microfluidic channel (sticky-slide VI,^{0.4} ibidi) which has a clean hydrophilic glass (glass coverslip, ibidi) (25 mm x 75 mm x 170 µm) at the bottom. The lipid solution was incubated in the channel for 10 min and was gradually replaced by Tris buffer (Sigma-Aldrich) (10mM Tris, 100mM NaCl, pH7.5). After complete replacement of the buffer with isopropanol, a SLB was formed on the glass surface.

6.2.9. The Activation of Carboxyl Groups of DP-NGPE Lipid Embedded in the SLB

The carboxylic group of DP-NGPE incorporated in SLBs was activated using N-Hydroxysulfosuccinimide sodium (sNHS) (Alfa Aesar) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (Alfa Aesar) for amine coupling. A mixture of 60 mg EDC/NHS in 3 mL HEPES buffer (Sigma-Aldrich) (10mM HEPES, 100mM NaCl, pH 5.5) was freshly prepared and introduced into the sample and incubated for 1 h at room temperature followed by washing with HEPES buffer.

6.2.10. Quartz Crystal Microbalance with Dissipation (QCM-D)

QCM-D was used as a surface sensitive technique to measure and monitor the change of resonance frequency (ΔF) and energy dissipation (ΔD) caused by the adsorption of lipid and proteins on silicon oxide (SiO₂)-coated quartz crystals (model no. QSX303) (Q-sense AB). Before the experiment, the quartz crystals were immersed in 1% sodium dodecyl sulfate (SDS) (Sigma-Aldrich) and subsequently washed with Milli-Q water (Millipore). After drying the crystals with nitrogen gas (N₂), they were placed in an oxygen plasma (March Plasmod Plasma Etcher, March Instruments) at 180 W for 1 min. This step was done to clean the surface of the crystal from residual organic molecules and also to make the surface hydrophilic. All measurements were done using a Q-Sense E4 instrument (Q-sense AB) under continuous flow condition with a flow rate of 50 μ L/min. The flow condition was conducted and controlled using a peristaltic pump (Ismatec Reglo Digital). QCM-D measurements were taken at the 3, 5, 7 and 11 overtones, and the presented data was measured and normalized at the third overtone [$15 \text{ MHz } (\Delta F n=3/3)$].

6.2.11. Fluorescence Recovery after Photobleaching (FRAP)

Estimation of the diffusion coefficient of the lipid molecules in the presence and absence of proteins and cells was carried out by the Fluorescence Recovery after Photobleaching (FRAP). Briefly, 0.5 wt % of 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (Rh-PE) (Avanti Polar Lipid) was added to the lipid mixture of 80% DOPC and 20% DP-NGPE. After formation of the bilayer on the glass surface, a 532 nm, 100mW laser beam (Klaser Laser Technologies) was used to bleach a defined area of SLBs for 5 s that result in the formation of a $\sim 20 \mu\text{m}$ bleached spot. The fluorescence recovery was monitored and measured using an epifluorescence Eclipse TE 2000 microscope (Nikon) combined with an Andor iXon+ EMCCD camera (Andor Technology). All images were collected at a rate of 1 image per 2 s intervals. Images were analyzed using ImageJ software and diffusion coefficients were measured using the Hankel transform method [53].

6.2.12. Cell Culture and Cell Seeding on SLB Platform

Human hepatocellular carcinoma cell line (Huh 7.5), purchased from Apath, were cultured in a humidified atmosphere with 5% CO₂ at 37°C and maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone), supplemented with 10% fetal bovine serum (FBS) (Hyclone), 1% penicillin and streptomycin (Gibco). The media was changed every three days. Cells were detached using 0.25% trypsin (Gibco) and suspended in media. The total cell number was counted by a hemocytometer. 20,000 cells were seeded onto each of the supported bilayers and cultured for three days, with media changes every 24 h.

6.2.13. Quantification of Huh 7.5 Cell Surface Area

The cell surface area of Huh7.5 cells was quantified after 24 h. An inverted optical microscope (Nikon) with a 10× objective was utilized to capture the Huh 7.5 cells images on the bare and dECM-functionalized SLBs. All images were analyzed, and the average cell surface area was measured using ImageJ software.

6.2.14. Determination of Cell Viability by LIVE/DEAD Kit

Viability of Huh 7.5 cells viability was observed qualitatively using LIVE/DEAD assay (Life Technologies). Briefly, cells cultured on dECM-functionalized SLBs were washed with PBS (1X) and treated with 2 μM calcein AM and 4 μM EthD-1 in DMEM media at 24 and 72 h timepoints. Cells were incubated with the assay solution for 20 min at 37°C and subsequently washed with PBS (1X) before visualization under an epifluorescence Eclipse TE 2000 microscope (Nikon) (10× objective) combined with an Andor iXon+ EMCCD camera (Andor Technology). The living cells were stained with calcein and emitted a strong green fluorescence that was visualized by a 494-517 nm emission filter. In contrast, the dead cells were stained with EthD-1 and emitted a red fluorescence that was imaged using a 517-617 nm emission filter.

6.2.15. Determination of Huh 7.5 Cell Number by Cell Counting Kit-8

Cell numbers were measured for Huh 7.5 cells cultured on dECM-functionalized SLB for day 1 and day 3 using cell counting kit-8 (CCK-8) (Dojindo Molecular Technologies, Japan). In brief, the cells were incubated with the CCK-8 solution for 1 h at 37°C. Absorbance of a range number of cells was plotted for a standard curve, which was used to calculate the number of viable cells present on the dECM-functionalized SLB on the different timepoints. Cell proliferation was determined by normalizing the cell number at day 3 to the cell number at day 1.

6.2.16. Determination of Human Albumin in Cell Culture Supernatants

The amount of human albumin secreted into the cell culture media by the Huh 7.5 cells cultured on SLB platforms was determined using an enzyme-linked immunosorbent assay (ELISA) kit (Abcam), 24 h after seeding. The amount of albumin produced was normalized to the number of viable cells on the platforms at respective timepoints.

6.2.17. Immunofluorescent Stain for ECM Proteins

In order to qualitatively visualize the presence of collagen type I (Col I) and FN in the dECM-SLB, the sample was stained with mouse monoclonal anti-Col I (Abcam) and mouse monoclonal IgG1 anti-FN (Santa Cruz), following the incubation time (~ 1 h) at room temperature. The samples were washed with PBS (1X) properly and subsequently the secondary antibodies Alexa Fluor 488 Goat Anti-mouse IgG (H+L) (green-fluorescent dye, Life Technology) and Alexa Fluor 555 Rabbit Anti-Mouse IgG (H+L) (red-fluorescent dye, Life Technology) were added to the sample to fluorescently label the Col I and FN antibodies, respectively. Fluorescence images were captured with an inverted epifluorescence Eclipse TE 2000 microscope (Nikon) equipped with a 60 × oil immersion objective (NA 1.49) in combination with an Andor iXon+ EMCCD camera (Andor Technology). Fluorescence images were analyzed using ImageJ software.

6.2.18. Statistical Analysis

All experiments were carried out in triplicates unless otherwise stated. The significance of differences between two groups was studied using PRISM software. All data are represented as means $\pm$ SD. **, $p \leq 0.01$; ****, $p \leq 0.0001$.

6.3. Results and Discussion

6.3.1. Decellularization and Characterization of Mouse White Adipose Tissue

Multiple steps involved in the preparation of the dECM based SLB platform as a cell surface mimetic are summarized in **Figure 6.1**. The first step is to decellularize the Col-rich ECM of adipose tissue [54]. An effective decellularization process allows us to achieve the maximum removal of cellular components with minimum ECM loss and damage. We used a combination of organic solvent extraction and enzymatic digestion processes. Briefly, the whole-tissue biopsy from mouse adipose tissue was subjected to a sequential treatment with organic solvents for fat removal and trypsin to assist cell detachments (Refer to materials and method for detailed protocol).

Figure 6.1 Schematic illustration of various steps involved in the preparation of supported bilayer functionalized with adipose tissue dECM components for cell study applications. Adipose tissue was decellularized using a combination of chemical and enzymatic treatment. The dECM was then solubilized in acidic condition and was covalently attached via amine coupling to the surface of a supported bilayer containing lipids with head group bearing free carboxylic acid.

Subsequently cells were seeded and their attachment, spreading, growth and function were studied.

Next, we examined the effectiveness of the decellularization process by visualizing the ultrastructure of the matrix and determining its chemical composition. The matrix visualization was done by SEM. **Figure 6.2a** and **6.2b** shows the micrographs of adipose tissue before and after the decellularization process, respectively. **Figure 6.2b** shows that no cell or cell fragments were present after decellularization, indicating efficient cell removal. In addition, the discrete organized micro fiber structure of the Col matrix (**Figure 6.2c**), indicates that the fibrillar form of the dECM components was preserved.

The efficiency of the cell removal from the adipose tissue was also evaluated via quantification of the residual DNA. Only 7.7 ± 2.2 ng of DNA was retained in each mg of dry tissue (**Figure 6.2d**) corresponding to about 98% reduction in the DNA level of the sample. Additionally, the dECM components; Col and sulfated GAGs were quantified (**Figure 6.2e** and **6.2f**) as the amount per content of total protein in each sample. The normalized Col content for dECM was higher than that of the native tissue (**Figure 6.2e**), indicating that Col was enriched during the decellularization process. This phenomenon is expected as the removal of cellular and lipid material results in the concentration of the collagenous ECM after decellularization (**Figure 6.2e**). The normalized value for GAGs was almost unaltered indicating that they were preserved during the decellularization process (**Figure 6.2f**).

Figure 6.2 Characterization of decellularized mouse-derived adipose tissue. (a) Representative SEM images of native adipose tissue. (b) Low magnification SEM image for decellularized adipose tissue indicates a three-dimensional scaffold composed of fibrous ECM material that is devoid of cells and lipids. (c) High magnification SEM image of decellularized tissue demonstrates a three-dimensional architecture of the Col fiber. Biochemical evaluation of the content of (d) DNA, (e) Col and (f) GAGs present in the native and decellularized adipose tissue. ($n = 3$, mean $\pm$ SD; ** $P \leq 0.01$ and **** $P \leq 0.0001$).

6.3.2. The SLB Formation and Functionalization

After decellularization, the remaining ECM matrix was solubilized completely by acid treatment [55]. The solubilized dECM then were used for functionalization of the SLB. The dECM proteins were attached to the SLB via covalent bonding between free amine present in the protein components of the dECM matrix and free carboxylic acid present in the head group of a functional lipid (DP-NGPE) incorporated in the bilayer. A similar approach was previously used to functionalize the SLB with purified Col I and FN [44,45]. Application of lipids with reactive head groups such as amine, carboxylic acid, thiol, or maleimide are efficient approaches for ligation of lipid bilayers and have been used successfully for surface functionalization of supported bilayers and lipidic nanoparticles such as liposomes [56].

We used the SALB formation method to fabricate lipid bilayer [57]. The method involves the adsorption of lipid molecules onto a solid surface in the presence of alcohol (e.g., isopropanol) followed by gradual replacement of the lipid-alcohol mixture with an aqueous buffer solution which leads to lipid phase transition in bulk and finally formation of lipid bilayer membrane on solid support surface [58]. The SALB method enables fabrication of SLBs on various hydrophilic solid surfaces, including those which are not suitable for vesicle fusion method [59]. In addition, the method allows the formation of SLBs with lipid compositions which cannot be formed using the vesicle fusion method (e.g., containing a high fraction of cholesterol (Chol) [8].

QCM-D was used to monitor SLB formation and the kinetics of adsorption of solubilized adipose dECM to the bilayer. QCM-D is a real-time surface sensitive technique commonly used to monitor the kinetics of thin film formation and characterization of its thickness and viscoelastic properties. It is widely used in soft matter research and interfacial science applications [60,61], particularly for studying lipid bilayer formation and its *in situ* modification [62]. The method has also been used for studying cell attachment and spreading on solid supports [63,64]. **Figure 6.3a**, shows the QCM-D ΔF ; (related to the mass of the adlayer including mass of the coupled water molecules) and ΔD ; (related to the frictional (viscous) losses in the adlayer) responses corresponding to different steps involved in the formation of a DOPC bilayer containing 20% functional lipid (DP-NGPE) via the SALB method (arrow i-iii), activation of the free carboxylic acid via incubation EDC/NHS (arrow iv) and the subsequent addition of protein to the bilayer surface (arrow v). After complete solvent exchange (arrow iii), a ΔF and ΔD shifts of -25 ± 2 Hz and $0.5 \pm 0.3 \times 10^{-6}$, respectively, were obtained, which correspond to the formation of a planar bilayer [62]. The close to zero energy dissipation indicates very low friction loss because water molecules rest on the bilayer surface.

Figure 6.3b, shows an enlarged view of the QCM-D curve corresponding to the protein adsorption step (arrow v in **Figure 6.3a**). The addition of dECM to the bilayer led to a negative ΔF of ~ -60 Hz. The negative ΔF indicates an increase in the mass of the adlayer due to the protein adsorption. As a control, the adipose-dECM was added to a

bilayer containing no functional lipid (marked with blue triangles), showing almost no change in the ΔF indicating no adsorption. Thus, the adsorption of proteins to the bilayer containing functional lipids was due to the formation of covalent bond between proteins and the lipids with reactive headgroups. The relatively high energy dissipation of the protein adlayer is related to the frictional losses due to a conformation changes in the protein structure [17]. These changes are reversible and are caused by the periodic shear motion of the QCM crystal surface [65]. This observation indicates that the protein layer on supported bilayer is soft and relatively high amount of water molecules are coupled to its structure. The softness and structural flexibility of protein layer are expected as they attached to the underlying bilayer surface through limited number of anchoring points. The anchoring points are limited free amine-bearing lysine residues which are dispersed along the protein structure. Thus, while the attachment of proteins to the underlying bilayer is firm, the protein molecules retain their flexibility. It is noteworthy that the DOPC bilayer is zwitterionic and has an antifouling property and thus is resistant to non-specific protein adsorption [17].

Additional confirmation of the attachment of ECM components to the bilayer was achieved by immunofluorescence microscopy. **Figure 6.3c** and **6.3d**, presents background-corrected fluorescence microscopy images for a dECM coated bilayer incubated with fluorescently labeled anti-Col I and anti-FN antibody, respectively. The results show that both Col I and FN are present in the dECM matrix. In addition to the uniformly distributed fluorescent signal, there are randomly distributed brighter spots indicative of the presence of antibody clusters. The presence of antibody clusters suggests the presence of protein aggregates on the SLB surface. The protein aggregates might be due to the presence of non-solubilized proteins or defects in the underlying bilayer.

The membrane fluidity is a crucial property of biological membranes. Importantly, despite the strong association of the supported bilayer with the solid surface, individual lipid molecules are highly mobile in the plane of the membrane [66]. To check if the underlying lipid bilayer after functionalization with adipose-dECM retained its fluidity, FRAP experiment was carried out. Representative fluorescence micrographs, (**Figure**

6.3e), confirm the retention of the long-range lateral fluidity of lipids with a lipid diffusivity of $2 \pm 0.1 \mu\text{m}^2/\text{s}$ after functionalization with adipose-dECM proteins.

Figure 6.3 Modification of SLBs using adipose dECM. (a) The QCM-D signal exhibited different steps of bilayer fabrication and functionalization. ΔF (blue) and ΔD (red) for the third overtone ($n = 3$) were measured as a function of time during the formation of 20% DP-NGPE SLB on SiO_2 using SALB method. After establishing the baseline in a buffer solution, pure isopropanol was injected (arrow i) followed by injection of 0.5 mg/mL DOPC / DP-NGPE (20%) (arrow ii) which caused a small change in ΔF and no change in the ΔD . Subsequently, the buffer was injected (arrow iii) leading to a final ΔF and ΔD of $\sim -25 \pm 2 \text{ Hz}$ and $0.5 \times 10^{-6} \pm 0.3$, respectively which corresponds to a planar bilayer. To functionalize the SLB with ECM proteins, EDC/NHS was introduced into the chamber (arrow iv) to activate free carboxylic acid of DP-NGPE. In the last step, the dECM was injected (arrow v). The significant change in ΔF and ΔD indicates the adsorption of dECM to the SLB. (b) QCM-D characterization of the dECM binding to bilayers containing no (triangles) or 20 % DP-NGPE (rectangles). Fluorescence micrographs of dECM functionalized bilayer incubated with antibodies against (c) Col I and (d) FN. (e) Fluorescence recovery after photobleaching analysis of dECM functionalized bilayer. A $\sim 20 \mu\text{m}$ -wide circular spot was photobleached, followed by time-lapse recording. Fluorescence was recovered nearly completely after 60 s suggesting bilayer retains its mobility.

6.3.3. The Behavior of Huh 7.5 on dECM-Functionalized SLB

Next, we studied the attachment and growth behavior of cells on the adipose-dECM functionalized bilayer platform. Cell adhesion to the ECM is mediated mainly by integrins present at the cell surface [67]. Different ECM component such as Col and FN possess specific integrin binding domains which facilitate cell attachment and subsequent intracellular signaling responsible for cell survival [68], proliferation [69], differentiation [70], and migration [71]. **Figure 6.4a**, represents typical bright field image of Huh 7.5 cells seeded on adipose-dECM functionalized bilayer after 24 h showing attachment and spreading. In contrast, cells did not spread even after 24 h, on the supported bilayer with no dECM (**Figure 6.4b**). This is expected and consistent with previous reports showing that the antifouling properties of the zwitterionic bilayer prevent the adsorption of proteins [17] as well as cells [16]. Quantification of images (**Figure 6.4c** and **6.4d**), shows that the number of cells attached to the dECM-SLB (~ 140 cell μm^{-2}) as well as their projected area ($1100 \mu\text{m}^2/\text{cell}$) was more than twice of that of bare SLB. Thus, the dECM components support the growth and adhesion of Huh 7.5 cells on the lipid bilayer platform.

We further checked if the bilayer retained its mobility after the cell seeding, by performing FRAP on membrane area underneath the cell. **Figure 6.4e** shows that the bilayer retained its mobility underneath the cell, however the lipid diffusivity ($1.4 \pm 0.05 \mu\text{m}^2/\text{s}$) of the bilayer underneath the cell was lower than that of areas with no cells ($2 \pm 0.1 \mu\text{m}^2/\text{s}$). Such decrease in the lipid diffusivity is expected as the adsorption of macromolecules to lipids hinders the lateral mobility of the interacting lipids in the underlying bilayer [72,73].

Figure 6.4 Huh 7.5 Cells behavior on the bare and dECM-functionalized SLB (dECM-SLB). The typical bright field image of Huh 7.5 grown on the (a) dECM- SLB (b) bare after 24 h. Cells did not spread on bare SLB and remained rounded. (c) Quantification of a number of cells attached to the bare and dECM-SLB. (d) The measurement of Huh 7.5 cell spreading size on the bare and dECM-SLB after 24 h. The images were taken by an optical microscope and analyzing with ImageJ. The observation field was 0.5 mm². (e) FRAP analysis of dECM-SLBs after cell adhesion. A 20 µm-wide circular spot was photobleached under the cell, followed by time-lapse recording. The fluorescence was recovered nearly completely after 80 s suggesting bilayer retains its mobility under ECM materials and cells.

To further investigate the biological activity of adipose-dECM on SLB, we examined the viability, proliferation, and function of the cells plated on this platform. The cell viability was quantified using live/dead assay combined with fluorescence microscopy. Over 90% of Huh 7.5 cells were viable within three days, as shown in **Figure 6.5a**. The cell proliferation measured using CCK-8 assay showed that the number of cells after 72 h was around 1.5 times higher than the number of the cells after 24 h (**Figure 6.5b**).

The interaction between cells and ECM component control many cellular activities including tissue-specific expressions [74]. Albumin production by hepatocyte is one such cellular function which is affected by cell-ECM interaction [75]. To check the activity of Huh 7.5, we measured the amount of albumin secreted by cells grown on dECM coated bilayer. The average amount of albumin produced by the cells after 24 h was $3.1 \pm 0.2 \mu\text{g}$ per 10^6 cells, which is in the of our results obtained on culture dishes (**Figure 6.5b**).

Figure 6.5 Evaluation of Huh 7.5 cells growth and functions on dECM-functionalized SLB (dECM-SLB). (a) Fluorescence image of calcein AM-stained Huh 7.5 cells plated on dECM-SLB

after 24 and 72 h. The green-stained cells are alive and the red-stained cells are dead. The viability of the cells was over than 90% within three days (b) Quantification of cell viability, cell proliferation and albumin production of Huh 7.5 on dECM-SLB. Cell viability was analyzed using ImageJ. Cell proliferation amount was calculated by normalization of cell number at day 3 to the cell number at day 1. The amount of albumin was obtained by normalization of albumin secretion at day 1 to 10^6 cells.

Finally, we compared the cell behavior on supported bilayer functionalized with dECM and Col I-FN. Both cell proliferation and albumin production on Col I-FN were higher than on dECM (**Figure 6.6**). These results may suggest that some of structural motifs responsible for cell proliferation and albumin production were denatured during acid solubilization step.

Figure 6.6 Comparison of Huh 7.5 cells growth and functions on dECM and Col I-FN functionalized SLB. Cell proliferation amount was calculated by normalization of cell number at day 3 to the cell number at day 1. The amount of albumin was obtained by normalization of albumin secretion at day 1 to 10^6 cells.

6.4. Conclusion

In summary, a cell-surface mimetic substrate consisting of a solid-SLB decorated with dECM components was fabricated. The adipose tissue was successfully decellularized using a combination of chemical and enzymatic processes. The remaining ECM components were attached to the surface of a SLB via covalent bonding using amine coupling methodology. The attachment process and the final surface were characterized by QCM-D and immunofluorescence microscopy. While, denaturation of dECM component after re-solubilization in acidic conditions is possible, the ECM components attached to the bilayer retained their biochemical functionality and promoted cell adhesion and proliferation to the otherwise nonfouling cell resistant phospholipid bilayer. The cell binding activity is known to be mainly mediated via RGD which is the principal integrin-binding domain present within ECM proteins such as FN and Col I. Therefore our results suggest that at least RGD ligands were preserved after re-solubilized in acidic conditions. FRAP analysis revealed that the underlying bilayer retained its fluidity after ECM conjugation as well as cell anchorage and spreading. SLBs functionalized with proteins have been shown to be an enabling platform for studying cell-cell interactions, thanks to the fluidity of the underlying supported bilayers. For instance, the membrane fluidity has been exploited to examine the spatial reorganization of immune recognition receptors and cluster formation that occur during the immune signaling processes [76]. In this context, the fact that lipids in the system developed in this work are laterally mobile, enables studying such process in a platform that more closely mimics the *in the vivo* extracellular environment. Moreover, the ECM is an important component of the stem cell niche which regulates stem cell differentiation and function. Thus dECM-SLB can serve as a platform for studying stem cell-matrix interactions. Taken together, this platform could thereby serve as a more relevant option for studying cellular processes that occur at the membrane interface.

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Chapter 7

Conclusion and Future Outlook

In this chapter, a summary of all the findings in this dissertation along with implications is discussed. The three proposals for future outlook indicated by the research are suggested. Our findings set a new precedent for utilizing molecular self-assembly to develop tunable functional materials in order to address basic questions related to cell adhesion on soft substrates as well as biomedical applications requiring adhesion of cells to soft substrates. In addition, owing to the rich biochemical diversity of the natural ECM components and their essential roles in many membrane-related cellular functions, such platform should better mimic the in vivo extracellular environment.

7.1. Conclusion

The major purpose of this dissertation was to develop a biomimetic platform that more closely mimics the cell surface environment. Toward this goal, the specific objective was to fabricate and explore the possibilities offered by supported lipid bilayers (SLBs) which are decorated with extracellular matrix (ECM) proteins. The overall hypothesis was that the ECM-functionalized SLBs (ECM-SLBs) could serve as cell-surface mimetic substrates for cell interaction studies. The rationale behind this hypothesis was (i), SLBs mimics the fundamental properties of the cell membrane such as fluidity and the ability to host membrane proteins, and (ii), ECM provides support and anchorage for cells and play key role in numerous cellular processes including cell growth and differentiation as well as intercellular communications. In this setting, the following studies were conducted as follows:

In **Chapter 4**, a strategy was developed to functionalize SLBs with ECM proteins. Zwitterionic (DOPC) SLBs were fabricated using a recently developed solvent-assisted lipid bilayer (SALB) formation method which does not require vesicles. The method enables the formation of bilayers on a broad range of surfaces, with lipid compositions that cannot be prepared by the conventional method of bilayer formation i.e. vesicle fusion method. To make the bilayers functionalizable, the bilayers were doped with a headgroup modified lipids (DP-NGPE) which contain a reactive free carboxylic acid. The doping was done by including the reactive lipid (DP-NGPE) along with the dominant lipid (DOPC) in the precursor lipid mixture in the organic solvent used in the SALB procedure. After activation, the free carboxylic acid of the reactive lipid incorporated in the bilayer, covalently binds to the free amine groups present in proteins. We tested formation of bilayers containing different fraction of reactive lipid (up to 20%). The bilayer formation and protein attachment to its surface was monitored by quartz crystal microbalance with dissipations (QCM-D). Importantly, we found that adsorption of the tested ECM proteins; collagen type I (Col I) and fibronectin (FN), to the bilayer surface was dependent on the fraction of reactive lipids included in the precursor lipid mixture. A feature which allows controlling the level of protein conjugation.

The model cells (Huh 7.5) were seeded on the ECM-SLB platforms and cells attachment and their interactions with both Col I and FN-functionalized SLBs; Col I-SLB and FN-SLB, respectively was visualized by fluorescence microscopy. Interestingly, after cell attachment to Col I and FN on the SLB, the proteins were depleted from the cell surroundings. Fluorescence microscopy images revealed the accumulation of Col I and FN underneath the cell in response to the traction forces exerted by the cells during the cell spreading process. However, such depletion was not observed when cells were seeded on Col I or FN-coated glass surface. In addition, the depletion was much less when cells were seeded on the functionalized membranes containing a high fraction of cholesterol (Chol). Incorporation of Chol into the membrane reduces the lipid mobility in the membrane due to an increase in the viscous molecular friction between lipids. Our findings indicate that the observed Col I and FN remodelling on the bilayer surface was due to the fluid nature of the underlying bilayer which allows lateral displacement of anchored proteins when experiencing traction forces. Therefore, the lower depletion observed in bilayers rich in Chol was due to the greater resistance of the anchoring lipids against the force exerted by the cell. Importantly, the cell shape was also dependent on the rigidity of the substrates.

In **Chapter 5**, we examined the biological activity of Col I and FN which are covalently attached to the SLB and compared their biological activity with those that are nonspecifically adsorbed to SiO₂ surface. Our hypothesis was that the specific attachment of ECM proteins to SLBs via covalent bonding through amine coupling, would better preserve the structural flexibility and hence the biological activity of proteins. The rationale behind this hypothesis was as follows: zwitterionic SLBs are nonfouling and resistant to nonspecific adsorption of proteins. Thus, the covalent attachment of Col I fibre and FN to the SLB would occur only via a limited number of potential anchor points (free amine group of lysine residues), (ii) attachment of Col I and FN to SiO₂ is non-specific and thus most part of the protein structure interacts with the surface which in turn substantially alter the natural structure of the protein. Indeed, the characterization of protein adsorption by QCM-D revealed that Col I and FN that attached to SLB have higher structural flexibility than those adsorbed onto SiO₂. QCM-D study also showed

that the interaction of FN with Col I is more efficient on SLB than on SiO₂, suggesting that the Col I-FN interaction on SiO₂ surface may be inhibited by limited accessible binding sites on the surface-adsorbed Col I. In addition, the ratio of number of cells attached to the protein-coated SLB and SiO₂, to the amount of adsorbed proteins, revealed that the interaction of cells with the anchorage sites for cell adhesion on FN and Col I are more efficient on SLB than on SiO₂. Thus, SLB functionalization can serve as an effective strategy to interface biological molecules to inorganic surfaces. The benefit of this platform is expected to be more pronounced when smaller protein/peptide motifs are needed to be used for functionalization.

In **Chapter 6**, we conducted the functionalization of solid supported phospholipid bilayer with decellularized adipose tissue ECM (dECM) components. Owing to the rich biochemical diversity of the ECM components and their essential roles in many membrane-related cellular functions, such platform should better mimic the *in vivo* extracellular environment. We demonstrated that the dECM components attached to the bilayer retained their biochemical functionality and promoted cell adhesion and proliferation to the otherwise nonfouling cell resistant phospholipid bilayer. This result indicates that the decellularization process as well as acid-aided solubilization and subsequent covalent attachment of dECM components, did not damage the components significantly. To be more precise, at least the cell binding properties of dECM components were preserved. Thus, dECM-SLB can serve as a platform for studying cell-matrix interactions as well as studying cellular processes that occur at the membrane interface. Importantly, the underlying bilayer retained its fluidity after dECM conjugation as well as cell anchorage and spreading. The fluidity of the underlying supported bilayers opens an opportunity to study receptor reorganization and protein clustering which occur for instance during cell-cell interaction studies. The dECM-SLB platform allows performing such studies in the presence of ECM components.

Taken together, these works presented in this dissertation offers a tunable and functionalizable platform for cell study applications.

7.2. Future outlook

The strategy for developing engineered bioactive interfaces based on SLBs presented in this thesis provide a basis and guidance for future research. In this chapter, a few potential projects utilizing ECM-SLB platform that could be explored in future studies are discussed.

7.2.1. To Map Out the Influences of Full Length ECM Proteins and Peptide Fragments on Cell Adhesion and Related Activities Such as Spreading, Growth, and Function

The interaction between mammalian cells and ECM components is not only critical for tissue morphogenesis, differentiation, and homeostasis but also for any failure in the signalling pathway which can result in diseases and other disorders [1]. There is a critical need to have enough information about the source of the signals and to identify the signals that are promoting specific behaviour of mammalian cells such as growth, differentiation, migration. However, ECM proteins such as collagen, fibronectin, and laminin are large biomolecules with a variety of subunits and functions. These large proteins fold randomly upon adsorption on the solid surfaces; thus, their receptor binding domains might be sterically inaccessible. Accordingly, in recent years, application of short peptides as well as engineered proteins has been increased. These types of molecules not only retain many fundamental properties of ECM proteins, but also their recognition sequences are highly accessible for cell surface receptors.

In this proposed study, the aim will be to use SLB to prepare platforms with a diverse set of pro-adhesive factors. Several types of ECM proteins such as various collagens, fibronectin, laminin, elastin, vitronectin and ECM-derived adhesive peptides such as IKVAV, RGD, and GFOGER, can be immobilized with a controlled density on SLB using the strategy developed in the current work. These molecules mediate the specific attachment of mammalian cells onto the SLB that is inherently non-adhesive to cells. After growing the cells on functionalized SLBs, different cellular activities such as cell

attachment, cell spreading, and proteins secretion can be analyzed. For instance, the systematic comparison of the cell attachment and function on full-length ECM components and short peptides derived from these proteins shed light on their adhesive potency and effectiveness in triggering specific signaling reactions. Such information would help to develop the optimal approach for regulating cell adhesion to biomaterials with specific cellular activities.

7.2.2. To Control Stem Cell Differentiation by Physical Interaction with Extracellular Space.

Stem cells are a group of cells in the body that are able to replicate themselves and differentiate into various types of cells [2]. In recent years, scientists have been focused on approaches to differentiate stem cells into specialized cells for tissue engineering applications such as stem cell therapy [2,3]. The fate of stem cell is regulated by a variety of microenvironmental parameters which can be categorized into mechanical cues such as the rigidity of the substrate shear stress, mechanical strain, matrix topography, and rigidity and biochemical cues including soluble factors such as growth factors and insoluble ECM proteins. It has been reported that physical interactions between stem cells and ECM can control stem cell fate [2,4]. ECM signalling molecules can induce stem cell fate by controlling stem cell shape which is an important factor for stem cell differentiation [5,6]. In addition, the mechanical stiffness of ECM has a major effect on stem cell differentiation [2,7].

We have shown that the cellular morphology is dependent on the rigidity of the platform and the cells are more rounded on the SLB-based surfaces compare to the glass one (Chapter 4). Indeed, the projected surface area of cells was greater on the platform with higher rigidity. Additionally, we also demonstrated that the rigidity of SLBs is tuneable and is increased by incorporation of high fraction of Chol in the bilayer (Chapter 4). These findings suggest that using SLB platform membrane viscosity can be controlled to modulate cell shape. The possibility of controlling the shape of the cell may offer an opportunity to control the fate of stem cells grown on the ECM-SLB bilayer. Therefore,

we propose to study the stem cell proliferation and differentiation on ECM-SLB of various viscosities. Additionally, different types of ECM signalling molecules and growth factors with different surface density can be applied to build the right combination of mechanical and biochemical properties. Thus, dECM-SLB can serve as a platform for studying stem cell-matrix interactions.

7.2.3. Studying Cell-Receptor Interactions in the Cell–ECM-SLB Interface

Cell-cell interactions occur via extensive formation of membrane protein complexes at the cell interface. The process is dynamics and involves large scale rearrangements of protein complexes over lengths scales of tens of micrometers [8]. Intercellular engagement play crucial roles in physiological and pathological process such as differentiation [9], adhesion [8,10], embryogenesis [11,12], cancer [13], and diabetes [14]. SLBs functionalized with proteins have been shown to be an enabling platform for studying cell-cell interactions, thanks to the fluidity of the underlying supported bilayers.

In particular, SLBs have been extensively employed to study cell-cell interaction in immunosystem [15]. For instance, the function of FC receptor (typical immune cell receptor) was investigated using SLBs. In this study, SLBs was modified with specific antibodies contain FC region and exposed to the live cells with FC receptors. This interaction regulates different functions such as adhesion.

The next step was using SLBs to investigate the effect of ligand lateral mobility on cell adhesion. In this strategy, the action of membrane-anchored CD58 or lymphocyte function-associated antigen 3 (LFA-3) was compared with its transmembrane form. The anchored form of LFA-3 is mobile while the transmembrane form is immobile in SLBs. It has been shown that the mobile form CD58 was 50-times more potent and stable compare to the immobile form. Due to the mobility of the anchored CD58 they can accumulate under the cells that result in higher cell surface receptor-ligand interactions [16].

In this context, the fact that lipids in the platforms developed in this work are laterally mobile enables studying cell-receptor interaction studies in ECM-derived protein functionalized substrate, which more closely mimics the *in vivo* extracellular environment. The ECM-derived SLB platform should provide a diverse set of ligands which perhaps better mimics the cell surface environment and interfacing cells with such platform, leading to responses which would not be triggered otherwise. For instance, the interaction of cell with membrane-anchored CD58 or FC receptors can be studied in the presence of ECM proteins using the ECM-SLB platform. Therefore, we envisage that performing the above-mentioned research using the dECM platform may lead to better understanding the *in vivo* biological responses leading to an increase in our knowledge of these systems.

Taken together, this platform could thereby serve as a more relevant option for studying cellular processes that occur at the membrane interface.

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Appendix

1. Papain Extraction Buffer

40 mg cysteine HCl

0.2 M sodium phosphate buffer ($\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$), pH 6.4

400 mg sodium acetate

200 mg EDTA, disodium salt

5 mg papain enzyme

List of Publications

1. **Vafaei S**, Tabaei SR, Groves JT, Cho NJ. "Dynamic Cellular Interactions with Extracellular Matrix Triggered by Biomechanical Tuning of Low-Rigidity, Supported Lipid Membranes adhesion studies". *Advanced Healthcare Materials* (2017); 6(10).
2. **Vafaei S**, Tabaei SR, Cho NJ. "Optimizing the Performance of Supported Lipid Bilayers as Cell Culture Platforms based on Extracellular Matrix Functionalization". *ACS Omega* 2017; 2(6), 2395-2404
3. **Vafaei S**, Tabaei SR, Guneta V, Choong C, Cho NJ. "Supported Lipid Bilayer Functionalized with Decellularized Extracellular Matrix Components". **2017, Submitted to Langmuir.**
4. Tabaei SR, Gillissen JJ, **Vafaei S**, Groves JT, Cho NJ. "Size-Dependent, Stochastic Nature of Lipid Exchange between Nano-Vesicles and Model Membranes". *Nanoscale* 2016; 8(27), 13513-13520.
5. Tabaei SR, Guo F, Rutaganira F, **Vafaei S**, Chong I, Shokat K, Glenn JS, Cho NJ. "Multi-step compositional remodeling of supported lipid membranes by interfacially active phosphatidylinositol kinases". *Analytical Chemistry* 2016; 88(10), 5042-5045.
6. . Tabaei SR, Jackman JA, Kim MC, Yorulmaz S, **Vafaei S**, Cho NJ. "Biomembrane Fabrication by the Solvent-Assisted Lipid Bilayer (SALB) Method." *Journal of Visualized Experiments* 2015; 106, e53073
7. Tabaei SR, **Vafaei S**, Cho NJ. "Fabrication of Charged Membranes by the Solvent-Assisted Lipid Bilayer (SALB) Formation Method on SiO₂ and Al₂O₃". *Physical Chemistry Chemical Physics* 2015; 17(17) 11546-52

Poster Presentation

1. **Vafaei S**, et al. Functionalized Supported Lipid Bilayer as a Platform to Study Cell-Extracellular Matrix Interactions" *Tethered Membrane (TethMem)* 2015); Singapore.