Cell Behavior Control with Functional Nanoplatforms Based on Biocompatible Polymers

생체적합성 고분자 기반 기능성 나노플랫폼을 이용한 세포 거동 연구

2018년 2월

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Based on Biocompatible Polymers

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Abstract

Cell Behavior Control with Functional Nanoplatforms Based on Biocompatible Polymers

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Cell behaviors, from cell adhesion and proliferation to stem cell differentiation, can be easily affected by the surrounding environments. For organizing artificial surrounding environments to control cell behavior, especially biocompatible polymers are widely utilized due to their excellent biocompatibility and easily modifiable to endow functionality. Biocompatible polymers, including synthetic and natural polymers, refer to the polymer that has the ability to function in intimate contact with living tissue without allergic reactions or other adverse effects. Regulating structures and physiochemical
properties of polymers constituting cell scaffolds enable control of cell behavior. This dissertation presents the basic studies of factors influencing the cell behavior, by manipulating cell-to-cell interactions through nanoporous membranes and modification of surfaces that cells grow with nanoparticles or polymer brushes. In particular, we do not only observe cellular adhesion and proliferation according to substrates that cells grow, but also identify the crucial components that influence differentiation of stem cells.

In Chapter 1, a brief background of engineering cell behavior using biocompatible polymers is introduced. In Chapter 2, we have developed nanothin membrane with tunable pore size and thickness using biocompatible polymer, cellulose acetate. By changing of various process parameters, we controlled the membrane features. With utilizing developed membranes, we investigated the effects of pore size in membrane to cell-cell interactions, i.e., paracrine diffusion, direct cell-cell contact and migration, when cells are cocultured in membrane-based separation system. Furthermore, gold nanoparticles enclosed with cyclic RGD (Arg-Gly-Asp) peptides were employed to modify nanoporous membranes for studying effect of nanotopographical cues on stem cell proliferation and differentiation.

Based on study of Chapter 2, we have further modified nanothin coculture membranes with thermoresponsive functionality for the generation of transfer-printable stem cell-derived cardiac sheets in Chapter 3. We compared the developed membranes with the conventional coculture membranes. Developed nanothin and highly porous (NTHP) membranes are ~ 20-fold thinner and ~ 25-
fold more porous than the conventional coculture membranes, thus, differentiation of the cocultured stem cells was dramatically enhanced in NTHP membrane-based coculture system compared to conventional coculture methods. Also, the thermoresponsive functionality of the NTHP membranes enabled the efficient generation of homogeneous, ECM-preserved, highly viable, and transfer-printable sheets of cardiomyogenically differentiated cells.

In Chapter 4, we developed biodegradable, nanothin and highly porous (BNTHP) membranes by changing of materials to biodegradable polymer, poly(lactic-co-glycolic) acid (PLGA). Then we established a novel coculture system with 3D cellular geometry through cellular layer-by-layer (cLbL) arrangement using BNTHP membranes. The cLbL coculture platform facilitated higher extent of cellular cross-talks between cocultured cells, resulting in more efficient differentiation of stem cells compared to the conventional bilayer coculture systems. Besides, attributing to biodegradable and highly flexible features of BNTHP membranes, conversion of cell-attached membranes into directly implantable 3D cell constructs is producible, which enables avoiding harmful enzymatic harvesting of the cells.

In Chapter 5, we developed antifouling and antimicrobial surfaces by combination of polymer brush and Ag nanoparticles (AgNPs). With the development of biomedical field, prevention of coverage with foulants and microbial contamination of implanted biomedical devices is essential to maintain their functionality. We utilized poly(sarcosine) (PSar), which has an excellent biocompatibility and has recently been highlighted as potentially new biopolymers to replace poly(ethyleneglycol), as antifouling polymer brushes.
Catechol moiety in polymer performs dual roles; anchor groups of polymer brush and reductant for formation of AgNPs. Attributing to dual function of catechol moiety, antifouling PSar brush and antimicrobial AgNPs can adhere stably on metal oxide surfaces. Antifouling and antimicrobial activity was confirmed by protein and bacteria adhesion measurements. Moreover, cytotoxicity test was also conducted proving that this platform can be useful for functional modification of biomaterials surface to preserve their performance by reducing the risk of bacterial infection.

Finally, in conclusion, we summarized the overall research work presented in this thesis. We strongly believe that study presented in this thesis would provide relevant information from basic control of cell behavior to tissue engineering. In detail, newly developed cell coculture platform would be a promising tool in not only cardiac or chondrogenic differentiation but also other tissue engineering applications and may bring significant advancement in stem cell therapy. Moreover, polymer brush with AgNP conjugated platforms showing highly viability of human cells could thus be applied in transplanted device surface without adverse effects.

**Keywords**: biocompatible polymer • membrane • stem cell • coculture • polymer brush • antifouling

**Student Number**: 2013-20971
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Chapter 1. Introduction

1.1. Factors Influencing Cell Behavior

There is a myriad of factors of the 3D microenvironment that influence cell behavior. Cells are highly sensitive to their environment, thus cell behaviors from adhesion, proliferation to stem cell differentiation can be regulated by controlling microenvironment. Mechanical properties (e.g., scaffold stiffness, external force etc.), chemical signals (e.g., growth factors, cytokines etc.) and extra cellular matrix (ECM) architectures (e.g., composition, alignment etc.) affect cell behaviors, and dynamic dependence exists between all three factors (Figure 1.1).\(^1\) In introduction, representative studies that demonstrated regulating cell behaviors by controlling specific factors are presented.

1.1.1. Mechanical Properties

Cells probe their microenvironment to sense and respond to the stiffness of the microenvironment by pulling on the ECM. Such processes are dependent on ECM adhesions that allow to transmit force between the ECM and the cellular cytoskeleton, in which myosin-based contractility acts as a primary regulator of cellular contractile forces.\(^2\) Since mechanical properties is also affect the ECM architectures ultimately, classification of factors influencing cell behavior into mechanical properties, chemical signals, and ECM
Figure 1.1. Important factors of 3D microenvironment that influence cell behavior.
architectures, is ambiguous. We focused on the effect of scaffold stiffness on cell behaviors.

Cell spreading is the initial kinetic process following adhesion events once the cell touches the substrate, therefore, cell spreading is guided by interfacial stiffness. In the same vein, various previous studies indicated that stiffer substrates generally promote cell spreading, whereas soft substrates induce a more rounded cell shape. Since substrate stiffness influence the cytoskeletal organization, it affects cell morphology. Also, changes in cell morphology and spreading are accompanied by changes in cell differentiation. It has been demonstrated that certain stiffness induces optimally differentiate into adipocyte, myocyte, osteocyte etc.

Not only differentiation is affected by substrate stiffness, but cell migration is also affected by stiffness. For example, Pelham and co-workers showed that epithelial cells and fibroblasts migrate faster on soft substrates compared to stiff substrates. Moreover, when fibroblasts were cultured on substrates with a stiffness gradient, they tended to migrate into direction of the stiff side. Various studies have investigated that substrate stiffness affects cell behavior including spreading, differentiation and migration. The cytoskeleton of the cell plays a crucial role in transmitting feedback from the mechanical properties to cell behavior.

1.1.2. Chemical Signals

Chemical signal is any substance that affects cell metabolism or behavior of the individual and it can be used between body parts, between cells, and between individual organisms, and among them, intercellular chemical signals is introduced in this part. Signaling molecules such as growth factors and
cytokines, as well as small molecules like neurotransmitters modulate cell behavior.

Fibroblast growth factors (FGF) are multifunctional proteins involved in processes of proliferation and differentiation of a variety of cells and tissues through their stimulatory functions.\[^9\] Previous study showed that fibrin-bound FGF-2, also known as basic FGF (bFGF) stimulated the growth and increased proliferation of endothelial cells \textit{in vitro}.\[^10\] Cell migration was also observed with concentration gradient of bFGF. Smooth muscle cell preferred migrating to direction of increased bFGF concentration, demonstrating control over cell mobility.\[^11\] Vascular endothelial growth factor (VEGF) is the other angiogenic protein capable of regulating new blood vessel formation. VEGF-enriched scaffolds were critical for \textit{in vitro} endothelial cell survival and migration within the 3D scaffold environment.\[^12\]

Furthermore, generally, differentiation of stem cell is induced by adding certain artificial growth factors or cytokines.\[^13, 14\] A representative example of bone remodeling \textit{in vitro} using bioactive molecules was reported in previous studies,\[^15-18\] which is the motif that supported osteoblast differentiation derived from bone morphogenetic protein (BMP)-2, known to influence the development and cartilage. Also, soluble chondroinductive factors, such as transforming growth factor (TGF) - β1, insulin-like growth factor 1 (IGF-1), have been reported to promote chondrogenic differentiation of stem cells.\[^19-21\]

1.1.3. ECM Architectures

ECM is the extracellular part of tissue that usually provides structural support to cells. In the complex ECM microenvironment cells encounter various types of ECM architecture, such as composition, density and alignment. To control
ECM architectures, alteration in micro- and nano-scale topological features of substrates is a simple method for observation of cell behavior dependent on ECM architectures. In this context, the focus of this part is the topological effect to cell behavior, which ultimately results in change of ECM topology, alignment, and composition.

According to previous studies, growth characteristics and morphology of primary human dermal fibroblasts were found to be significantly modulated by the microstructure of the substrate.[22] These changes in growth and adhesion formation will impact on cytoskeletal tension, as like mentioned in ‘mechanical properties’ part. Therefore, surface topological structure can affect stem cell differentiation.[23-25] For example, human mesenchymal stem cells (hMSCs) were differentiated toward osteoblast lineage by nanotube surfaces,[26] and neuronal differentiation of human neural stem cells (hNSCs) was regulated by nano grooves and pillars.[27] Furthermore, micro- and nanofibers are considered as mimicking native ECM, numerous studies utilizing micro/nano fibers for cell study have been published.[28, 29]

1.2. Biocompatible Polymers for Engineering Cell Behavior in Vitro

Scaffolds for cell culture should be based on biomaterials with adequate properties, such as biocompatibility, bioactivity, and biodegradation. Among the materials, polymers are suitable for fabrication of diverse types of scaffold due to their not only excellent biocompatibility, but also tunability of morphology, mechanical properties, and biodegradability. They can be classified as natural or synthetic polymers (Figure 1.2).[30] Natural polymers,
Biocompatible Polymers

Synthetic Polymers
- Poly(caprolactone)
- Poly(ethylene glycol)
- Poly(lactic acid)
- Polypeptoid

Natural Polymers
- Collagen
- Fibrin
- Polysaccharide
- Hyaluronic Acid
- Alginate

**Figure 1.2.** Various biocompatible polymers utilized for cell studies.
such as proteins and polysaccharides, exhibit several advantages, e.g., degradability, negligible toxicity. While, synthetic polymers are reported to include highly controlled degradation rate and excellent reproducible mechanical and physical properties compared with natural polymers.

1.2.1. 3D Hydrogel Scaffolds

Polysaccharide gel formation is generally of two types; hydrogen bonded and ionic. Hydrogen-bonded gels are typical of molecules such as agarose and chitosan, whereas ionic crosslinked gels are characteristics of alginates and carageenans. Injectable, biocompatible 3D hydrogel scaffolds are important biomaterials for tissue engineering and drug delivery. Hydrogels derived from natural polysaccharide are ideal scaffolds as they resemble the ECM of tissues including various glycosaminoglycans (GAGs).

Tan et al. reported chitosan and hyaluronic acid composite hydrogel for chondrocytes culture. Biocompatible and biodegradable hydrogel developed in this study provides a potential opportunity for cartilage tissue engineering applications. Also, macroporous cellulosic hydrogels consist of hydroxypropylcellulose were applied to various cell types culture. Biocompatibility was demonstrated even in vivo environment with minimal inflammatory response in subcutaneous implantation.

1.2.2. Polymer-Based Coculture System

Cell coculture concepts were developed in the 1980s to study cell-cell communication. So far established coculture systems can be classified into direct and indirect coculture system. In direct coculture system, different types of cells are typically mixed and cocultured on tissue culture plate dishes (TCPS)
or in 3D materials, such as hydrogels or 3D fiber, sponge scaffolds consist of polymers.\textsuperscript{[36, 37]} However, in the case of mixed coculture system, cross-contamination of cells and the difficulty in specific cell isolation prevent their widespread use.

To work around the limitations of mixed coculture, indirect coculture system using polymer porous membrane was developed. Transwell\textsuperscript{®} is commercially developed porous membrane, which is often used for indirect coculture. Transwell membrane is track-etched permeable membrane that is made with polyethylene terephthalate (PET), polycarbonate (PC), or polytetrafluoroethylene (PTFE). Transwell enables easy isolation of a specific cell line and allows for the study of indirect interactions between cells through soluble paracrine factors, however, it prevents direct cell-cell contact due to centimeter-scale distance between cocultured cells. Therefore, for direct coculture, the cells are seeded on the porous membrane of the Transwell insert and the other cells are seeded on the opposite side of the porous membrane, respectively (Figure 1.3).\textsuperscript{[38]}

These various coculture systems have been established to study proper cell-cell interactions between cocultured cells. For example, coculture system allow to study metastatic cancer cell behavior when cancer cells are coexist with diverse stromal cells.\textsuperscript{[39]} Furthermore, adaptation of the coculture system to promote differentiation of stem cells was introduced in a lot of research.\textsuperscript{[40, 41]}

1.2.3. Surface Modification with Polymers

Polymer is useful materials to modify surface properties such as hydrophilicity,\textsuperscript{[42]} rigidity,\textsuperscript{[43]} and charge density,\textsuperscript{[44]} since polymer properties
Figure 1.3. Different types of coculture system. Coculture systems can be categorized into direct and indirect coculture systems, which includes cell-cell direct contacts with paracrine factor, and only paracrine factor, respectively.
could be easily tailored by adjustment of molecular weight, functional group, or pH etc. Specifically, simple coating of polymers can be used for surface modification, however, for more solid and stable coating, layer-by-layer (LbL) assembly technique or polymer brush platform has been introduced.

The LbL assembly technique is one of the most versatile methods to prepare multifunctional polymer surfaces formed by various intermolecular interactions among paired species, such as hydrogen bond, electrostatic interactions, and hydrophobic forces etc.\textsuperscript{[45]} Also, polymer brushes have been widely studied and applied to a variety of areas for surface modification due to their diverse functionality and physical stability by surface-confined architectures.\textsuperscript{[46]}

Cellular functions can be controlled through surface modification with polymers. First, cell adhesion and repulsion has been regulated by polymers anchored on surfaces. In general, polymer types that resist cell adhesion are either hydrophilic\textsuperscript{[47]} or zwitterionic,\textsuperscript{[48]} mainly due to a tightly held hydration area. Low fouling hydrophilic polymers include polyethylene glycol (PEG), polyamides, and polysaccharides etc.\textsuperscript{[49]} Whereas, to promote cell adhesion on surfaces, integrin-specific peptide sequence of arginine-glycine-aspartic acid (RGD) has been widely used.\textsuperscript{[50]} Furthermore, stem cell fate can be determined with polymeric coatings due to tunable features of polymers and the possibility of immobilization of elements affecting stem cells fate, such as BMP-2 or alendronate.\textsuperscript{[18, 51]}
Chapter 2. Development and Modification of Nanothin and Highly Porous Membranes to Control Cell Behavior

2.1. Introduction

The microenvironment surrounding cells, such as soluble factors, extracellular matrix and direct cell-cell contacts etc, regulate their behaviors. Therefore, to control their behaviors in vitro, understanding cell-cell interactions in response to changes in their microenvironment is essential. Interactions between cells can be classified into four representative signaling types; autocrine, paracrine, endocrine signaling and signaling across gap junctions, which are sorted by acting distance of the signaling molecules. Cells can affect itself or nearby cells through secretion of the signaling molecules, called as cytokines. Before signaling molecules degrade by external fluids or enzyme, they should be transferred to target cells. Also, a cell can target a adjacent cell by connecting gap junctions with direct contact, meanwhile, a cell can target a distant cell through hormone secretion into blood, called as endocrine signaling.

When two different types of cells interact with each other, i.e., co-cultivated heterogenous cells, porous membrane is adequate tool to study cell-cell interactions, since we can control the interactions between cocultured cells via porous membrane, especailly paracrine interactions and gap junctions, by controlling thickness as well as pore size of the membrane.
In this chapter, we developed free-standing, nanothin and nanoporous membranes to study cell-cell interactions using biocompatible polymer, i.e., cellulose acetate. The nanoporous structure of the membranes employed in this study was realized by the vapor-induced phase separation (VIPS) mechanism during the spin-coating process. Attributing to spin-coating process, we could obtain membranes with nano-thickness. Therefore, we named membranes developed in this study NTHP (nanothin and highly porous) membranes. Surface morphologies were changed by diverse parameters, such as solvent type, relative humidity, and polymer solution concentration etc., and we characterized surface morphologies and analyzed pore sizes. Then we investigated change of cell-cell interactions, particularly signaling molecules diffusion, cell-cell direct contact and cell migration, through NTHP membranes with different pore sizes.

Furthermore, NTHP membranes were modified with cyclic RGD (cRGD)-coated gold nanoparticles (AuNPs) to detect change of stem cell behaviors according to nanofeatured surfaces. AuNPs density was controlled with the number of drop or spin casting, and cRGD-coated AuNPs were covalently immobilized onto membranes via Michael addition. We observed cell adhesion and stem cell differentiation according to AuNPs density on NTHP membranes.

NTHP membranes developed in this study can be applied to not only for useful coculture system, but also for studying nanotopology effect simultaneously. Therefore, we expect that this system would provide versatile tools to study factors influencing cell behaviors, such as direct cell contact, cytokines, and nanotopology etc.
2.2. Experimental Section

Fabrication and Modification of NTHP Membrane. Cellulose acetate (CA) with average number of molecular weight of 30,000 g/mol (39.8 wt% acetyl labeling extent) was purchased from Aldrich. CA was dissolved in a good solvent such as THF, acetone, and ethyl acetate. To induce porous structure in CA thin films, the spin-casting of CA solutions was performed in homemade closed chamber with controlled relative humidity (RH) as shown in Figure 2.1. Since water is non-solvent for CA polymers, the porous structure in membranes can be developed by VIPS mechanism. RH can be controlled in the closed chamber packed with different types of supersaturated salt solutions (Table 2.1).

To investigate the solvent type effects on membrane’s morphology, CA was dissolved in a good solvent such as THF, acetone, ethyl acetate and acetic acid at 4 wt%. Membranes were deposited onto silicon (Si) wafers, which were previously cleaned by dipping in a piranha solution, a mixture of 70 vol% H₂SO₄ and 30 vol% H₂O₂, for 20 minutes at room temperature, then rinsed with deionized (DI) water and dried with a nitrogen stream. NTHP membranes with 100 nm, 380 nm, and 860 nm of pore size were obtained with a spinning rate of 3000 rpm and 1000 rpm, respectively, for 25 seconds.

We modified NTHP membranes using the method proposed by Ku et al. to enhance cell adhesion.[55] NTHP membranes were immersed in dopamine hydrochloride (Sigma-Aldrich) solution for 2 hours. Dopamine hydrochloride was dissolved in 10 mM Tris buffer at 2 mg/ml, and the pH was adjusted to 8.5 using diluted NaOH solution.

To obtain cRGD-coated AuNPs, first, 80 nm sized AuNPs were modified with carboxyl-PEG-SH (Mw 5000, Sigma-Aldrich), and then carboxyl group of surfaces were reacted with amine group of cRGD via EDC coupling. The
Figure 2.1. Homemade equipment for VIPS process during spin coating and process parameters affecting morphology of polymer membrane fabricated by VIPS process.
Table 2.1. Equilibrium relative humidity of different types of saturated salt solutions at room temperature.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Lithium Chloride</th>
<th>Magnesium Chloride</th>
<th>Magnesium Carbonate</th>
<th>Magnesium Nitrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>11.30 ±0.35</td>
<td>32.78 ± 0.16</td>
<td>43.16 ± 0.33</td>
<td>52.89 ± 0.22</td>
</tr>
<tr>
<td><strong>Temperature (°C)</strong></td>
<td><strong>Sodium Chloride</strong></td>
<td><strong>Potassium Chloride</strong></td>
<td><strong>Potassium Nitrate</strong></td>
<td><strong>Potassium Sulfate</strong></td>
</tr>
<tr>
<td>25</td>
<td>75.29 ± 0.12</td>
<td>84.34 ± 0.26</td>
<td>93.58 ± 0.55</td>
<td>97.30 ± 0.45</td>
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surface zeta-potential and hydrodynamic diameters of cRGD-coated AuNPs were characterized with dynamic light scattering (DLS; DLS-7000, Ohtsuka Electronics). For 1000 particles (pts)/400 μm², concentration with 0.95x10¹⁰ pts/ml cRGD-coated AuNPs solution was drop casted once, and 1.9x10¹⁰ pts/ml for 2500 pts/400 μm². In case of 7000 pts/400 μm², concentration with 3.98x10¹⁰ pts/ml cRGD-coated AuNPs solution was drop casted three times. After AuNPs coating, NTHP membranes were washed with PBS buffer and DI water several times to remove physically anchored particles.

**Characterization of NTHP Membranes.** The surface morphologies of NTHP membranes were characterized by scanning electron microscopy (SEM; JSM-6701F, JEOL) and atomic force microscopy (AFM; JPK, Nanowizard 3). The thickness of membranes were measured by a step height measurement (AlphaStep IQ (Rev. A1-1), KLA-Tencor). The porosity of each membrane was analyzed using an imaging processing software, Image J (National Institutes of Mental Health). AuNPs were characterized with energy-dispersive X-ray spectroscopy (EDS; X-MaxN, JEOL).

**Cell Culture.** Human bone marrow-derived mesenchymal stem cells (MSCs) were purchased from a commercial source (Lonza), and H9C2 cells, a rat cardiac myoblast cell line were purchased from Korean Cell Line Bank. Prior to coculture, both MSCs and H9C2 cells were cultured in growth medium consisting of high-glucose Dulbecco’s modified Engle’s Medium (DMEM; Gibco-BRL) containing 10% (v/v) fetal bovine serum (FBS; Gibco-BRL) and 1% (v/v) penicillin-streptomycin (PS; Gibco-BRL) at 37 °C in humid air with 5% CO₂.

**Coculture of MSCs and H9C2 Cells.** For the coculture of MSCs and H9C2 cells using NTHP membrane, H9C2 cells were plated at a density of 2x10³ cells/cm² on TCPS dish, and MSCs were plated at a density of 3x10³ cells/cm².
on NTHP membrane. On the following day, MSCs-seeded membrane was layered on top of H9C2-seeded TCPS dish. To prevent the layered membrane from moving, stainless ring was placed on top of the membrane. The medium was changed every 2 days.

**Dye Transfer Assay through Gap Junctions.** For dye transfer assay, MSCs were dual labelled with DiI (6.25 μg/ml) and calcein-AM (10 μmol/L; Sigma-Aldrich) prior to coculture. Images were photographed after 48 hours of coculture using a confocal microscope (SP8 X STED, super-resolution confocal microscope, Leica).

**Protein Diffusion Rate Measurements.** To determine the rate of protein diffusion through NTHP membrane, a protein diffusion chamber was designed. Developed NTHP membrane with different average por size was set to partition the protein diffusion chamber into two secluded compartments filled with 10 mL of 10 μg/mL bovine serum albumin (BSA; Sigma-Aldrich) solution on one side and phosphate buffer saline (PBS; Sigma-Aldrich) of equal volume on the other side. Samples were collected from the PBS chamber at various time points, and the concentrations of BSA in the samples were quantified with Bradford protein assay (n=3 per group) (Sigma-Aldrich).

**Cell Migration through NTHP Membrane.** Prior to coculture, MSCs were pre-labelled with DiO (6.25 μg/ml) and H9C2 with DiI (6.25 μg/ml). After coculture for 1 week, images of the cocultured MSCs and H9C2 cells were photographed using a fluorescence microscope (IX71 inverted microscope, Olympus). To assess the purity of the collected cells, cells adhered on transferred membrane were trypsinized and evaluated by fluorescence-activated cell sorting (FACS AriaII, BD Biosciences, USA) and fluorescence microscopy (IX71 inverted microscope, Olympus) (n=10 images per group).
2.3. Results and Discussion

2.3.1. Development of Nanothin and Highly Porous (NTHP) Membranes via Vapor-Induced Separation Process

Porous structures in polymeric materials with micrometer or submicrometer dimensions can be created using different approaches, such as use of templates, phase inversion, and breath figures etc.\textsuperscript{[56]} The phase separation induced by nonsolvent or thermal change has been widely utilized to prepare porous polymer membranes, and specifically, phase separation of polymer solution layers induced by nonsolvent vapor was investigated in this study. When a nonsolvent in the liquid phase comes in contact with a polymer solution, mass exchange between the polymer solution and non-solvent occurs rapidly, forming macrovoids in the membrane.\textsuperscript{[57]}

To induce the nanoscale pore architecture, the mass exchange rate was slowed down by using the vapor phase of the non-solvent (\textit{i.e.}, water), with controlled relative humidity (RH), during the spin-coating process. RH has a decisive effect on the number and size of pores as it results in different composition paths as shown in the ternary phase diagram (Figure 2.2).\textsuperscript{[58]} In general, two mechanisms are well known for liquid-liquid demixing of polymer solutions; nucleation/growth (NG) and spinodal decomposition (SD).\textsuperscript{[59]} At low RH homogeneous phase retained as blue composition path, and NG occurs at about RH 65%. As humidity gets higher, polymer systems depart from homogeneous and stable region to metastable region which is located between the bionodal and spinodal lines in phase diagram. At very high RH, \textit{i.e.}, RH 85%, SD occurs which results in a bicontinuous morphology.
Figure 2.2. VIPS composition paths of NTHP membranes in a ternary phase diagram and the corresponding AFM images obtained at different RH.
2.3.2 Variation of Process Parameters for Tuning Pore Size and Thickness of NTHP Membranes

As solvent changes in ternary system, phase diagram is also altered including bionodal and spinodal curves. Therefore, despite same RH 65 % condition, surface morphologies of membranes are different as shown in Figure 2.3. We can control surface morphology including pore size and pore density by change various process parameters. The rate of the pore and matrix formation is usually dependent on kinetic part as well as thermodynamic part at the same time.\[^{[59]}\]

Moreover, we adopted spin-coating to induce nanothin film, thus, we supposed that formation of porous membrane is rarely affected by thermodynamic factors. We have changed exposure time in humid chamber followed by spin-coating and observed the surface morphology as Figure 2.3. For the longer time exposed before spin-coating, the bigger size of pores were formed. It results from that the polymer solution layers absorb larger amount of water vapor as increased exposure time, which induces bigger pores in membranes.

Thickness of NTHP membranes can be simply modified with spinning rate or polymer solution concentration. When polymer solution concentration has increased, thickness of membranes grows drastically (Figure 2.4). In contrast, surface morphologies changed slightly. The viscosity of polymer solution increases with the polymer concentration, and it seems that the high viscosity of polymer solution lowers the diffusion coefficients which plays a key role in the kinetics of phase separation.\[^{[59]}\] We speculated that diffusion barrier resists polymers to transport across the disconnected domains. In other words, for higher concentration, which is more viscous, smaller amount of nonsolvent added in polymer solution layer than that for lower concentration resulting in formation of smaller pores. However, pore size difference is relatively insignificant compared to thickness changes.
Figure 2.3. Different surface morphologies of spin-coated membranes depending on solvent types and exposure time followed by spin-coating.
Figure 2.4. Thickness control of NTHP membranes and the corresponding surface morphologies characterized by AFM.
Among the various process parameters in designing VIPS-based membranes,[52] for cell-cell interaction study, the pore size of the NTHP membrane was tuned by controlling the RH, spin-rate and different types of solvent in polymer solution. NTHP membranes with distinctively different sizes of well-defined pores (i.e., an average of 100 nm, 380 nm, and 860 nm pore size, respectively, with similar thickness) can be generated by changing the process parameters (Figure 2.5, Table 2.2). Both SEM and AFM images were used to determine the average pore sizes of the NTHP membranes. Pore size represents the average value of the shortest and the longest axes of each pore.

2.3.3. Effects of Membrane Structural Features on Cell-Cell Interactions in Coculture

We investigated how membrane structural features affect interactions between cocultured cells when MSCs and H9C2 were cocultured using NTHP membranes. We utilized NTHP membranes with 100, 380, 860 nm pore size to study cell-cell interactions. First, to confirm whether the cocultured MSCs and H9C2 were undergoing active direct cell-to-cell crosstalk via gap junctions, a calcein-AM dye transfer assay was performed (Figure 2.6). MSCs, on the top side of each membrane, were dual-labelled with DiI (red) and calcein-AM (green). DiI is a dye that labels cell membrane and cannot be transferred through direct cell-cell contact. Calcein–AM is non-fluorescent when it exists outside cells. However, once calcein-AM penetrates cells, it is cleaved to fluorescent calcein by esterase within the cells.[60] The fluorescent calcein is cell membrane-impermeant and can pass only through direct cell-to-cell gap junction channels.[61] Thus, without direct cell-to-cell connections, calcein-AM
Figure 2.5. Morphologies of NTHP membranes with different pore sizes and their pore size distributions.
Table 2.2. The average thickness, pore size and porosity of NTHP membranes with various pore sizes. *p < 0.05 compared to the other groups.

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<td>100 nm</td>
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<tr>
<td>Membrane Thickness (nm)</td>
<td>400 ± 12*</td>
</tr>
<tr>
<td>Pore Size (nm)</td>
<td>100 ±30*</td>
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<tr>
<td>Porosity (%)</td>
<td>45 ± 4*</td>
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Figure 2.6. Confocal images of calcein-AM (green) dye transfer between cocultured MSCs and H9C2 cells via gap junctions across NTHP membranes. Bars, 50 μm.
cannot diffuse from MSCs into the underlying H9C2 cells during the dye transfer assay. Thus, only cells that form direct contact would show calcein-AM transfer from MSCs (on top of the membrane) to H9C2 cells (on bottom of the membrane). The results showed that calcein-AM was not transferred to H9C2 across NTHP-100 membrane system (arrowheads in Figure 3.6(ii)), indicating cells were not forming direct cell-cell contacts. In contrast, confocal images of the top and side views of NTHP membranes with pore size of 380 nm and 860 nm, respectively, indicate calcein transfer from dual-labelled MSCs (red and green) on the top of NTHP membrane to H9C2 cells (green only, arrows in (iv) and (vi)) on the bottom, demonstrating direct contact between MSCs and H9C2 layer. These data demonstrate that pore size below 100 nm were not able to induce formation of direct cell contact mediating via gap junctions between cocultured cells, whereas, NTHP-380, 860 membrane allows dynamic and direct MSCs-H9C2 contact providing enough pore size for gap junction formation.

Next, to compare the rate of protein diffusion through the NTHP membrane with different pore sizes, empirical studies on protein diffusion were conducted. For protein diffusion modelling test, a system with two chambers and part for insertion of the membrane was designed. Each membrane was set to partition the protein diffusion chamber into two secluded compartments. One compartment was filled with 10 mL of 10 μg/mL BSA solution, while the other with PBS buffer of equal volume. BSA was allowed to diffuse into the pure PBS chamber only through the separating porous membrane (Figure 2.7). The result showed that the BSA diffusion rate had no significant difference with pore size of NTHP membranes, which was likely due to the comparable porosity and thickness among the membranes. According to previous study of one-dimensional mathematical model for diffusion molecules, diffusion rate is
affected by concentration of molecules and diffusion coefficient.\cite{62} Furthermore, diffusion coefficient is a function of membrane depth and porosity.\cite{63} Thus, cytokine diffusion rate was not affected by the pore sizes of NTHP membranes empirically and theoretically.

Finally, we investigated whether cell migration occurs through pores in membranes. Cell migration was confirmed by observation of homogeneity of MSCs after coculture. The homogeneity of the collected MSCs after one week of coculture was assessed using FACS (Figure 2.7A) and fluorescence microscopy (Figure 2.7B). NTHP-100 membranes prevent the formation of direct cell-to-cell contact; therefore, it is certain that cell migration through below 100 nm is impossible. Thus, we tested cell migration through NTHP membranes with 380 nm and 860 nm-pore sizes. The quantitative analysis by FACS and qualitative observation through fluorescence microscopy revealed that the homogeneity of the collected MSCs from the NTHP-380 membrane was high; however, the impurity of the collected MSCs from NTHP-860 membrane system was much higher compared to NTHP-380 system. These data were consistent with a previous study that indicated cellular cross-migrations in cocultured populations separated by membranes with pore sizes around 1 μm.\cite{64} In other words, NTHP membranes with 860 nm only permit cells to migrate through nanopores in membrane.

2.3.4. Decoration of Nanoparticles (NPs) on NTHP Membranes

NTHP membrane with 100 nm average pore size was further modified with AuNPs to observe nanotopological effect to cell behavior. RGD motif is cell adhesive peptide that mimic the ECM and enables controlling cell attachment and behavior.\cite{65} To provide more in vivo-like environment by introducing ECM
Figure 2.7. Illustration of protein diffusion-modeling chamber and quantitative analysis of the rate of BSA molecule diffusion through the membrane according to various pore sizes of NTHP membranes.
Figure 2.8. Confirmation of cell migration through NTHP membranes. (A) Homogeneity of the collected MSCs analyzed by FACS analysis. MSCs are expressed in green and H9C2 cells in red. (B) Fluorescent images and quantification of the collected and re-plated MSCs. Bars, 100 μm. *p < 0.05 compared to the other groups.
materials, we decorated NTHP membranes with AuNPs enclosed with cRGD peptides. Size and zeta potential analysis demonstrated the surface modification of AuNPs (Figure 2.9A). cRGD-coated AuNPs were covalently anchored on NTHP membranes via Michael reaction. NTHP membranes were immersed in polydopamine solution for 2 hours to use Michael reaction between catechol group on NTHP membrane and thiol groups in cRGD-coated AuNPs (Figure 2.9B). We confirmed that AuNPs were anchored on the surface stably by EDS analysis (Figure 2.9C).

AuNPs density was controlled with particle concentration of solution and coating method. Drop casting is widely used method for particle assembly on substrates, and previous studies used spin-coating method to modify LbL films with protein-covered AuNPs.\[66, 67\] Therefore, we adopted spin-coating and drop-casting for modification of NTHP membranes. Spin-coating of cRGD-modified AuNPs solution led to sparse AuNPs-decorated membranes. The maximum particle density resulted from spin coating was 500 pts/400 μm², i.e., 900 nm of average spacing. However, denser AuNPs can be achieved by drop-casting method. Repetition number and solution concentration of spin-coating or drop-casting was changed to control AuNPs density as shown in Figure 2.10.

2.3.5. The Effect of Nanotopography of NTHP Membranes on Stem Cell Proliferation and Differentiation

We selected AuNP-conjugated NTHP membranes with three different particle densities, i.e., 1000 pts/400 μm², 2500 pts/400 μm², 7000 pts/400 μm² for cell behavior study. Their average distance between particles are 630, 400, and 238 nm, respectively, and surface morphologies were characterized by SEM (Figure 2.11A). Despite polydopamine coating followed by modification of AuNPs and AuNPs solution coating, nanoporous structures retained as shown in SEM
Figure 2.9. Modification of NTHP membranes with cRGD-coated AuNPs. (A) Characterization of cRGD-coated AuNPs fabricated via EDC coupling. (B) Michael addition of thiol groups to catechol in polydopamine. (C) AuNPs confirmation by EDS analysis.
Figure 2.10. Schematic representation of AuNPs modification on NTHP membranes using spin-coating and drop-casting method. SEM images of AuNPs-conjugated NTHP membrane surfaces with different AuNPs densities.
Figure 2.11. The effect of nanotopography on hMSCs. (A) Selected AuNPs-conjugated NTHP membrane surfaces for cell study. (B) Number of live cells and osteogenesis of MSCs in vitro at different cRGD-AuNPs density.
images. We expected that randomly dispersed cRGD-AuNPs grafted onto NTHP membranes, specifically ligand spacing, affect cell adhesion spreading as previous studies.\cite{24, 50} hMSCs were seeded on to AuNPs-conjugated NTHP membranes with different AuNPs densities (i.e., bare, 1000, 2500 and 7000 pts/400 μm² AuNPs on NTHP membranes), then incubated for 10 days.

Number of live cells was measured following incubation, however, there was no significant difference among different density of AuNPs-conjugated surfaces (Figure 2.11B). This is because polydopamine also promotes cell adhesion, therefore cRGD on AuNPs effects was relatively reduced compared to previous studies. In previous research, surface was coated with cell repelling polymer, such as PEG, excluding AuNPs-conjugated area to study impact of RGD ligands arrangement on cell adhesion. In contrast, our NTHP membranes modified with cRGD-coated AuNPs are cell adhesive at overall surface, since polydopamine adhered tightly to ECM materials promoting cell adhesion.\cite{68}

In recent studies, abundant evidence suggests that nanotopographic features instruct behaviors of MSCs.\cite{25} In particular, nanofiber scaffolds, nanoparticles or nanopit array demonstrated significantly promoted osteogenesis of MSCs.\cite{69, 70, 71, 72} In the same vein, we observed osteogenic differentiation of MSCs cultured on AuNPs-conjugated NTHP membranes. After 10 days of cell seeding, the alkaline phosphatase (ALP) activity and osteocalcin (OCN) expression were detected to evaluate the osteogenic differentiation of MSCs on the different density of AuNPs-conjugated surfaces. In previous research reported that differentiation to osteoblasts is defined by an increase in ALP activity, and acquisition of expression of several non-collagenous bone matrix proteins such as OCN as the latest marker of the mature osteoblast.\cite{73} ALP supports to prepare the ECM for the deposition before the onset of mineralization that coincide with OCN expression. ALP activity was increased on cRGD-AuNPs-
modified surfaces compared to bare NTHP membranes. Among them, the ALP activity was significantly increased in 1000-NTHP membranes compared to all other membranes. However, tendency for expression of OCN was different with ALP results. 2500-NTHP showed slightly increased OCN expression compared to 1000-NTHP, and there was no expression of OCN on bare, 7000-NTHP membranes. These disparities may attribute to insufficient cues of nanotopography in our system to induce mature osteoblast. Also, when AuNPs are exposed to light, the oscillating electromagnetic field of light induces collective coherent oscillation of the free electrons of the metal. The amplitude of the oscillation reaches maxium at a specific frequency, called surface plasmon resonance (SPR).\[74\] The SPR induces a strong absorption of UV-Vis region, specifically plasmon peaks for 96 nm-sized AuNPs at 564 nm,\[75, 76\] which overlaps emission wavelength of green dyes. Therefore, the fluorescence intensity of OCN marker (green) could be impeded by AuNPs.

Thy-1 (CD 90) is hMSC specific marker protein (red) and Thy-1 was expressed in all groups, particularly in bare NTHP and 7000-NTHP, which showed no expression of OCN marker. The hMSCs cultured on 1000, 2500-NTHP membranes were differentiated toward osteogenic lineage resulting in loss of hMSCs’ characteristics. Passivation of exposed polydopamine coating with cell-repelling polymers, as mentioned above, would enhance osteogenesis and enable emphasizing nanotopography effects.
2.4. Conclusion

In the present study, fabrication method of NTHP membranes and application of NTHP membranes to cell culture was introduced. Features of NTHP membranes, such as pore size and thickness can be controlled by changing process parameters during VIPS process. By using this process, the pore size of NTHP membranes can be finely tuned in nanoscale with simple changes of process parameters and conditions. MSCs and H9C2s were cultured on NTHP membranes, and cell-to-cell interaction through pore of membrane was investigated. In the case of coculture with ~100 nm pore-sized membranes, direct cell-to-cell contacts between heterogeneous cocultured cells were not possible. And ~900 nm pore-sized membranes allow cell migration through pores in membrane, while cell migration was not occurred through ~400 nm pore-sized membranes. In addition, diffusion rate of soluble factors across the membrane had not big difference with pore size, which was likely due to the comparable porosity. Finally, NTHP membranes were modified with cRGD-coated AuNPs to demonstrate nanotopographical effects on MSCs. AuNPs decorated-NTHP membranes showed similar proliferation compared to bare NTHP membranes, however AuNPs conjugated-NTHP membranes showed better osteogenic differentiation of MSCs than bare NTHP membranes.
3.1. Introduction

Stem cell therapy has been spotlighted as a promising cure for various types of diseases and tissue regeneration. The induction of stem cell differentiation into the desirable cell type prior to implantation has been proposed to improve the therapeutic outcomes,[77, 78] whereas, transplantations of naïve stem cells have showed low differentiation efficiency in vivo.[79] The differentiation of stem cells can be regulated by the microenvironment surrounding the stem cells, the so-called stem cell niche.[80] Several approaches, such as introducing bioactive molecules or scaffolds that provide the biomimetic microenvironments, have been reported to promote the differentiation of stem cells.[13, 81, 82] However, such artificial microenvironments may be insufficient in mimicking the complex nature of the native microenvironments to regulate the differentiation of stem cells.[83] Instead, coculturing of stem cells with the desired type of differentiated cells has been reported highly effective for controlling the fate of the stem cells.[84-86] The coculture strategy induces effective differentiation by providing naturally occurring cell-to-cell cross-

* This chapter is based on paper from S. Ryu,† J. Yoo,† Y. Jang, J. Han, S. J. Yu, J. Park, S. Y. Jung, K. H. Ahn, S. G. Im, K. Char, B.-S. Kim, ACS Nano (2015), 9, 10186-10202.
talk in native tissue microenvironment either through paracrine factors or direct cell-cell contact. Furthermore, coculture strategy can often be more cost-effective than dosing the culture medium with specific biomolecules including growth factors. Several studies have been reported in which effective stem cell differentiation into osteoblast, cardiomyocyte, neuronal and glial cells was achieved through coculture.

The features of membranes used for coculture are crucial in order to achieve the best outcome. Not only should the membrane act as a physical barrier that prevents the mixing of cocultured cell populations, but it should also allow effective interactions between the cocultured cells. Unfortunately, conventional membranes, such as track-etched PET membranes, used for the coculture do not sufficiently meet these requirements. In addition, cell harvesting using proteolytic enzymes following the coculture impairs cell viability and ECM produced by the cultured cells. To overcome these problems, newly developed NTHP membrane which is further modified with thermoreponsive property was applied to stem cell differentiation as coculture membrane for the generation of transfer-printable cardiac sheets.

It was found that membranes with ~100 nm pores prevented cell-cell direct contacts while membranes with ~900 nm pores allowed for such interactions but were ineffective in providing a physical barrier for the separation of cocultured heterogeneous cells. The NTHP membranes with optimized pore size at 380 nm are ~20-fold thinner and ~25-fold more porous than the conventional Transwell membranes. Due to the ultra-thin and highly porous properties of NTHP membranes, effective direct cocultured cellular contacts and paracrine factor diffusion across the membrane were realized in this study, resulting in efficient differentiation of stem cells compared to Transwell membrane without cross-contamination of cocultured cells.
Furthermore, initiated chemical vapor deposition (iCVD) process was employed to graft a thermoresponsive layer conformally onto the NTHP membranes without altering the porous structure of the membranes. Freestanding and thermoresponsive NTHP membrane can generate a transfer-printable cell sheet in response to temperature change. The NTHP membrane would allow an easy harvesting of the differentiated cell sheets without harmful enzyme treatment. Moreover, it can print the differentiated cell sheets to form multilayered cell sheets or tissue for implantation. The ECM of the cell sheets are preserved with this technique, which would enhances the therapeutic efficacy of the cells following implantation. In short, the NTHP membrane with thermoresponsive functionality can be used as an effective coculture tool for differentiating stem cells and producing various types of multilayered cell sheets applicable in regenerative medicine.
3.2. Experimental Section

**Fabrication and of NTHP Membrane.** Cellulose acetate (CA) with average number of molecular weight of 30,000 g/mol (39.8 wt% acetyl labeling extent) was dissolved in THF at 4 wt% as proposed in Chapter 1. CA solutions were deposited on piranha-treated Si wafers by spin-coating in closed chamber with controlled RH. The NTHP membrane with pore size of 380 nm was utilized in this study, since it functions as an effective physical barrier preventing cell cross-contamination and allows to induce direct cell contacts at the same time. For the NTHP membranes used in our experiment, we set up the RH to 65±5%. The freestanding NTHP membranes were obtained by peeling off the membranes from the Si wafers in water after efficiently drying of the samples. A PET film was used as a frame for easier handling of the NTHP membranes. The PET-frame was fixed with UV-curable poly(urethane acrylate) (PUA, 311-RM, Minuta Tech, Korea), which acts as a glue. The PUA is irradiated with UV (λ=365 nm) for 3 hrs.

**PNIPAAm (poly(N-isopropylacrylamide)) Grafting onto NTHP Membrane for Temperature-Responsive Property.** Glycidyl methacrylate (GMA) monomer (97%) and the tert-butyl peroxide (TBPO) initiator (98%) were purchased from Sigma-Aldrich and used without further purification. The polymerized GMA films were deposited onto the NTHP membranes in an iCVD reactor (Daeki Hi-Tech Co.). The GMA monomer was heated to 35 °C and the vaporized GMA was fed into the reactor at a flow rate of 1.9 sccm for coating pGMA film on the NTHP membrane. The vaporized TBPO initiator was fed into the reactor via metering valves at a flow rate of 0.8 sccm at room-temperature. In order to keep the NTHP membrane at 25 °C during the pGMA coating, NTHP membranes were placed on a stage cooled by a recirculating...
chiller. The filament temperature was maintained at 180 °C. The growth rate of film deposition was monitored in situ using a He-Ne laser (JDS Uniphase, Milpitas, USA). Amine-terminated PNIPAAm (Mn = 2500, Sigma-Aldrich Co.) was dissolved in DI water at a concentration of 1 g/30 mL. The PNIPAAm solution was reacted with the pGMA deposited NTHP membrane through epoxy-amine addition reaction in a shaker at 50 °C for 12 h at 55 rpm, and then it was washed several times with DI water. Prior to cell seeding, membranes were sterilized with 70% (v/v) ethanol and UV treated for 1 h on a clean bench.

Characterization of NTHP Membranes. The surface morphologies of NTHP membranes were characterized by SEM and AFM. The thicknesses of the NTHP membranes were measured by a step height measurement (AlphaStep IQ (Rev. A1-1), KLA-Tencor). The porosity of each membrane was analyzed using an image processing software, Image J (National Institutes of Mental Health, USA). Fourier transform infrared spectroscopy (FT-IR, IFS 66VS, BRUKER, Billerica, USA) spectra were obtained in normal absorbance mode and averaged over 64 scans in order to confirm PNIPAAm functionalization. X-ray photoelectron spectroscopy (XPS, MultiLab 2000, Thermo) was also utilized to determine surface chemical composition of thermoresponsive NTHP membrane. Water contact angle measurements were performed on a contact angle meter (Phoenix 150, SEO) using a 2.5 μl DI water droplet. Contact angle meter was equipped with an environment chamber to enable control the temperature range from 25 °C to 250 °C.

Cell Culture and Coculture of MSCs/H9C2 Cells. Experimental methods relevant to cell culture and coculturing of MSCs/H9C2 are same as Chapter 2. Briefly, prior to co-culture, both MSCs and H9C2 were cultured in growth medium consisting of high-glucose Dulbecco’s modified Engle’s Medium (Gibco BRL, USA) containing 10% (v/v) fetal bovine serum (Gibco-BRL) and
1% (v/v) penicillin-streptomycin (Gibco-BRL) at 37 °C in humid air with 5% CO2. For the co-culture of MSCs and H9C2 using NTHP membranes, H9C2 were plated at a density of 2x10^3 cells/cm^2 on TCPS dishes, and MSCs were plated at a density of 3x10^3 cells/cm^2 on NTHP membranes. On the following day, an MSCs-seeded NTHP membrane was layered on top of an H9C2-seeded TCPS dish. To prevent the layered membrane from moving, a square stainless ring was placed on top of the NTHP membrane. Two coculture methods in Transwell system, indirect coculture and direct coculture, were set as comparison groups. In indirect coculture, H9C2 were seeded on a Transwell insert and MSCs were seeded on the lower six-well plate compartment. In direct coculture, MSCs were seeded on the bottom side of Transwell membrane. On the following day, H9C2 were seeded on the other (top) side of the Transwell membrane. For non-co-cultured control groups, MSCs were cultured on TCPS dishes or NTHP membranes. The medium was changed every 2 days.

**Proximity Evaluation of Cocultured Cells.** The proximities between MSCs and H9C2 co-cultured on the two sides of the NTHP membranes and the Transwell membranes were observed by pre-labelling the MSCs with DiO (6.25 μg/ml; Sigma-Aldrich Co.) and H9C2 with DiI (6.25 μg/ml; Sigma-Aldrich Co.), prior to co-culture. Images were taken using a confocal microscope (SP8 X STED, super-resolution confocal microscope, Leica, Germany) photographed after 48 hours of co-culture.

**Differentiation of MSCs After Coculture for 1 Week.** The expressions of cardiac-specific genes in MSCs were evaluated with qRT-PCR. RNA was extracted from MSCs and reverse-transcribed into cDNA. Expression of a cardiac transcription factor (MEF2C), a cardiac structural marker (MLC2v), a gap junction marker (connexin 43), and cardiac ion channel markers (CACNA1D, HCN2) were evaluated using StepOnePlus real-time PCR system.
(Applied Biosystems) with FAST SYBR Green PCR master mix (Applied Biosystems) for 45 cycles. Each cycle consisted of the following temperatures and times: 94 °C for 3 seconds and 60 °C for 30 seconds (n=3 per group). For protein detection, western blot analysis on sarcomeric α-actininin and cardiac troponin T were performed. Additionally, immunocytochemistry for connexin-43, sarcomeric α-actininin, and cardiac troponin T was performed. The numbers of sarcomeric α-actininin-positive cells and cardiac troponin T-positive cells were digitally quantified (n=10 images per group) using Image Pro Plus software (Media Cybernetics).

**Transfer-Printing of Cell Sheet.** The cell sheet printing was achieved by transferring an MSC-seeded, thermoresponsive NTHP membrane onto a new culture dish and changing the temperature. The MSC-seeded membrane was turned upside down and incubated for 4 hr at 37 °C, allowing the cell sheet to adhere on the new culture dish. Then, cell sheet printing from the thermoresponsive NTHP membrane was induced by incubation for 30 minutes at 20 °C. Cell sheet printing was observed under a light microscope (IX71 inverted microscope, Olympus). To compare the viability of the harvested cells via transfer-printing with thermoresponsive NTHP membrane compared to that of trypsinized cells, live and dead cells of the collected cells were assessed by calcein-AM and ethidium homodimer, respectively, using a two-color fluorescence live/dead assay kit (Molecular Probes) (n=10 images per group). To analyze the extracellular matrices of the transfer-printed cell sheet, immunocytochemical staining for fibronectin and laminin was performed. For examination of bilayered cell sheets, prior to transfer-printing, each cell sheet was pre-labelled by DiI (6.25 μg/ml; Sigma-Aldrich Co.) or DiO (6.25 μg/ml; Sigma-Aldrich Co.). The consecutive transfer-printing technique with thermoresponsive NTHP membrane was performed to stack the DiI-labelled
cell sheet on the DiO-labelled cell sheet. The z-stack 3D confocal images of the bilayered cell sheets were obtained using a confocal microscope (SP8 X STED, super-resolution confocal microscope, Leica).

**Western Blot Analysis.** Cell lysate was prepared using cell lysis buffer (Cell Signaling, U.S.A). The total concentration of the protein was determined with Bradford protein assay (Sigma-Aldrich Co.) and further performed through 10% (w/v) SDS-polyacrylamide gel electrophoresis. Proteins were transferred to Immobilon-P membrane (Millipore Corp., USA), blocked with 5% skim milk solution for 1 hr at room-temperature, and incubated with antibodies against sarcomeric α-actininin (Abcam, UK), cardiac troponin T (Abcam), fibronectin (Abcam), and laminin (Abcam) overnight at 4 °C. The membrane was incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, USA) for 1 hr at room-temperature. The blots were developed using a chemiluminescence detection system (Amersham Bioscience, USA).

**Cell Staining.** Cells and cell sheets were fixed in 4% paraformaldehyde solution, permeabilized with 0.1% Triton X-100, and then blocked with 1% BSA for 1 hr for actin filament staining. The actin filaments of the cells were stained by tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin (1:40; Molecular Probes). For immunocytochemical staining, cells were fixed in 4% paraformaldehyde solution, permeabilized with 0.6% Triton X-100 and then blocked with 10% donkey serum (Jackson ImmunoResearch Laboratories). Then, the samples were reacted with antibody against cardiac troponin T (produced in mouse, 1/400; Abcam), connexin 43 (produced in rabbit, 1:1000; Abcam), sarcomeric α-actininin (produced in rabbit, 1/200; Abcam), fibronectin (produced in rabbit, 1/500; Abcam), and laminin (produced in rabbit, 1/100; Abcam). For cardiac troponin T, and connexin 43 staining, the samples were detected with TRITC-conjugated secondary
antibodies (Jackson ImmunoResearch Laboratories). For sarcomeric α-actinin, fibronectin, and laminin staining, the samples were detected with fluorescein isothiocyanate-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). The samples were mounted in DAPI for nuclear staining. Fluorescence images were captured using a fluorescence microscope (IX71 inverted microscope, Olympus, Japan).

**Statistical Analysis.** Quantitative data were expressed as the means ± standard deviations. The statistical analysis was performed using one-way analysis of variance (ANOVA) with the Tukey’s significant difference post hoc test using SPSS software (SPSS Inc., USA). A value of p<0.05 was considered to denote statistical significance.
3.3. Results and Discussion

3.3.1. Fabrication and Characterization of Optimized NTHP Membranes with Thermoresponsive Functionality

Figure 3.1 illustrates the procedure for the fabrication of the thermoresponsive NTHP membrane. To harvest transfer-printable cell sheets from NTHP membranes, the surface of the membrane was grafted with a thermoresponsive polymer, PNIPAAm.\[^{94}\] Previous studies reported that changes in hydration of PNIPAAm polymer in response to temperature can alter the cellular adhesiveness/non-adhesiveness of PNIPAAm-grafted surface.\[^{94-97}\] Above the lower critical solution temperature (LCST) in aqueous solution of 32 °C, PNIPAAm-grafted polymer become mildly hydrophobic since the conformation of the PNIPAAm polymer chains collapses. The collapsed state of PNIPAAm polymer chains promotes adsorption of cell-adhesive proteins (e.g., fibronectin) on the PNIPAAm-surface and allows subsequent cellular adhesion. Below the LCST, the PNIPAAm-grafted polymer rapidly swells and becomes highly hydrophilic. The entropic repulsion of the protein adsorption initiates the detachment of the cells.\[^{98}\] To conformally graft a PNIPAAm layer onto NTHP membranes, while preserving the porous structure of the membranes, the iCVD method was adopted. iCVD is an effective method of coating various types of functional polymer thin films uniformly onto complex surfaces, including nano-patterned substrates and nanoporous membranes without altering the substrate structure and properties.\[^{99}\] The NTHP membranes were initially coated with an epoxy-containing linker layer composed of pGMA,\[^{100}\] followed by a grafting reaction with amine-terminated PNIPAAm using an epoxy-amine addition reaction. Due to its free-standing capability, the
Figure 3.1. Fabrication procedure of thermoresponsive NTHP membranes using VIPS mechanism via spin coating and iCVD process.
The thermoresponsive NTHP membrane is readily transferable as showed in digital photograph.

### 3.3.2. Characterization of NTHP Membranes

Next, we characterized developed NTHP membranes and commercial Transwell membranes. The surface morphologies of commercial Transwell and our NTHP membranes (with comparable pore size of 400 nm and 380 nm, respectively) were examined and compared by SEM and AFM (Figure 3.2). While the thickness and porosity of the Transwell membrane were 10 μm and 2%, respectively, those of the NTHP membrane were 380 nm and 54%, respectively. This highlights the nanothin and highly porous architecture of the developed NTHP membrane. Attributing to iCVD process, we can obtain PNIPAAm-grafted NTHP membranes without damaging porous structures in membranes (Figure 3.3). This method successfully preserved the highly porous architecture of the NTHP membrane even after PNIPAAm engraftment.

The chemical compositions of deposited polymer films, *i.e.*, PNIPAAm layers, were examined by FT-IR (Figure 3.4A). In the FT-IR spectrum of GMA monomer and iCVD pGMA polymer, The red arrows denote the C=C vinyl group in the GMA monomers, which disappeared in the iCVD pGMA polymer, confirming the completion of free-radical polymerization of pGMA. The gray box represents characteristic peaks of the epoxy pendant groups (760, 847, and 908 cm⁻¹), which were clearly detected in the spectrum of the pGMA polymer, as well as the GMA monomers. The observation clearly indicates that the iCVD polymerization of GMA monomers was successfully performed without disrupting the epoxy functionality of the monomer. Also, the FT-IR spectrum of the PNIPAAm-immobilized surface clearly showed the increase of the
Figure 3.2. SEM and AFM images of (i, ii) a Transwell membrane and (iii, iv) an NTHP membrane. Bars, 2 μm. Thickness and porosity of a Transwell membrane and an NTHP membrane (with comparable pore size of 400 nm and 380 nm, respectively). *p < 0.05.
**Figure 3.3.** AFM images of NTHP membranes before and after the grafting of PNIPAAm showing the preserving of highly porous architecture.
secondary amides N-H stretching vibrations (3370-3270 cm⁻¹), compared to the pristine pGMA deposited surfaces, confirming the PNIPAAm functionalization onto the NTHP membranes (gray box of inset spectra). Furthermore, in comparison with PNIPAAm powder, the decreased primary amine peak intensity of the PNIPAAm-immobilized surface clearly illustrated that the primary amine functionality in the amine-terminated PNIPAAm was consumed by the epoxy-amine addition reaction with the surface pGMA linker layer (blue box).

The XPS analysis also showed the increase in nitrogen surface compositions at pGMA-PNIPAAm-grafted NTHP membranes, supporting the FT-IR result that the PNIPAAm was covalently functionalized on the NTHP membranes (Figure 3.4B and Table 3.1). A sharp, reversible phase transition of PNIPAAm-grafted surface was monitored by measuring the water contact angle change with repeated temperature variation (Figure 3.4C), demonstrating the thermoresponsive property of the PNIPAAm-grafted NTHP membranes.

3.3.3. Enhanced Differentiation of Mesenchymal Stem Cells (MSCs) by NTHP Coculture System

Figure 3.5A illustrates the NTHP-based coculture system for generating transfer-printable differentiated cell sheets originating from cocultured stem cells. MSCs were seeded on the PNIPAAm-grafted surface of NTHP membranes at 37 °C. H9C2 cells (H9C2), a cardiomyoblast cell line, were seeded on culture dishes. Following this, the MSC-seeded NTHP membrane was layered on top of the H9C2-seeded culture dish for coculture. To secure the membrane stacking on H9C2 and prevent membrane displacement during coculture, a square stainless steel ring was placed on top of the membrane. MSCs were cocultured with H9C2 to induce the cardiac differentiation of
Figure 3.4. Surface characterization of thermoresponsive NTHP membranes. (A) FT-IR spectra of PNIPAAm-grfated NTHP membranes. (B) Surface chemical composition analyzed by XPS. (C) Water contact angle measurements of the PNIPAAm-grfated surfaces at different temperatures.
Table 3.1. XPS analysis of surface atomic composition (%).

<table>
<thead>
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<th></th>
<th>C 1s</th>
<th>O 1s</th>
<th>N 1s</th>
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<tbody>
<tr>
<td>NTHP membrane</td>
<td>62.37</td>
<td>37.63</td>
<td>0</td>
</tr>
<tr>
<td>NTHP membrane + iCVD pGMA</td>
<td>70.68</td>
<td>29.32</td>
<td>0</td>
</tr>
<tr>
<td>NTHP membrane + pGMA + PNIPAAm</td>
<td>74.07</td>
<td>16.91</td>
<td>9.02</td>
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MSCs by simulating the niche of MSCs with paracrine factors secreted from H9C2 and direct MSCs-H9C2 contact across the NTHP membrane. After 1 week of coculture, the NTHP membrane, originally seeded with MSCs, was transferred from the H9C2-cultured dish onto a new culture dish. At a lower temperature of 20 °C, a sheet of cardiac-differentiated cells was printed on to the new dish. Figure 3.5B briefly illustrates the advantages of the new coculture system using NTHP membranes over the conventional coculture methods using ‘Transwell’ membranes. In general, there are two coculture methods in the ‘Transwell’ system: indirect coculture and direct coculture. In the indirect coculture method, H9C2, which provide important cues for the cardiac differentiation of MSCs, are seeded on the porous membrane of the Transwell insert, while the MSCs are seeded on the bottom chamber. This coculture system induces stem cell differentiation only via indirect intercellular communication, which is the diffusion of cytokines secreted by H9C2 across the porous membrane. Direct cell-cell contact is absent in this system. The other method is the direct coculture method, in which H9C2 are seeded on the porous membrane of the Transwell insert and MSCs are seeded on the opposite side of the porous membrane. This system is expected to allow both the diffusion of cytokines secreted by H9C2 and direct cell-cell contact through the micro-thin, porous Transwell membrane. In contrast, we expect that our NTHP membrane system benefits from a nanothin, highly porous membrane, which enables dynamic crosstalk between H9C2 and MSCs through faster cytokine diffusion and more effective cell-cell contact compared to the Transwell system.

To confirm the higher extents of cellular interactions between cocultured cells in NTHP-based coculture system than in Transwell coculture system, first, we observed the cell-to-cell proximity between MSCs and H9C2s (Figure 3.6A). The side view of the co-cultured MSCs and H9C2 separated by a Transwell
Figure 3.5. Schematic illustrations of (A) the thermo-responsive NTHP membrane-based generation of transfer-printable sheets of differentiated cells through coculture, and (B) a comparison of NTHP membrane-based coculture with the conventional Transwell coculture systems.
membrane showed that the distance between the cocultured cells was as far as 10 μm, which corresponds to the thickness of Transwell membrane (Figure 3.6Aii). In contrast, in the NTHP membrane system, the cocultured MSCs and H9C2 were in close contact, protruding through the physical barrier of the nanoporous membrane (Figure 3.6Aiii and iv). To investigate whether the cocultured MSCs and H9C2 were undergoing active direct cell-to-cell crosstalk via gap junctions, a calcein-AM dye transfer assay was performed (Figure 3.6B). MSCs were dual-labelled with DiI (red) and calcein-AM (green). DiI is a cellular dye that labels the membrane of the cell and cannot be transferred through direct cell-cell contact. On the other hand, calcein-AM is a small molecular-size dye that can be actively transferred to the adjacent cells through direct cell-cell contact via gap junctions.\textsuperscript{[103]} The result indicated that calcein-AM-transferred H9C2 was not observed in the Transwell system (Figure 3.6Bi and ii). This is likely due to the large thickness and low porosity of the Transwell membrane, which does not allow physical direct contact between the cocultured MSCs and H9C2. On the contrary, extensive calcein-AM transfer (green color only) was observed in the co-cultured cells in the NTHP membrane system (Figure 3.6Biii and iv). These data indicate that the NTHP membrane allows for direct contact interactions between MSCs and H9C2 via gap junctions.

Next, to compare the rate of protein diffusion through the NTHP membrane versus the Transwell membrane, mathematical modeling and empirical studies on protein diffusion across the membranes were conducted. To evaluate the effects of thickness and porosity of the membrane on the interphase transfer of protein molecules, we employed a simplified one-dimensional mathematical model that was established in a previous study.\textsuperscript{[62]}
\[
\frac{\partial C}{\partial t} = D_{\text{eff}} \frac{\partial^2 C}{\partial x^2} \quad (D_{\text{eff}} = D_0 f(C_0, C_s) h[\epsilon, \zeta, L(X)])
\]  
(A)

where \( C \) is the concentration of proteins, \( D_0 \) the diffusion coefficient in water, \( D_{\text{eff}} \) the effective diffusion coefficient of proteins which is influenced by the physical properties of the solute, \( f(C_0, C_s) \), and the microstructural properties of the membrane, \( h[\epsilon, \zeta, L(X)] \). In addition, a hindrance factor of the diffusion coefficient,\(^{104}\) which accounts for the decrease in the rate of diffusion in less porous material, was proposed as

\[
\frac{D_{\text{eff}}}{D_0} \approx \frac{2\theta}{3-\theta}
\]  
(B)

Equation (A) combined with equation (B) was solved numerically under proper boundary conditions. The results indicated that protein diffusion occurred faster when the membrane thickness is reduced and the membrane porosity is increased (Figure 3.6C). A protein diffusion chamber was designed to compare the rate of protein diffusion through the NTHP membrane and the Transwell membrane (Figure 3.6D). The result showed that the BSA diffusion rate was significantly higher through the NTHP membrane than through the Transwell membrane, likely due to the smaller thickness and higher porosity of the NTHP membrane compared to the Transwell membrane.\(^{62,63}\)

We investigated whether the cardiac differentiation of MSCs could be achieved through coculture with H9C2 in the NTHP membrane system, as coculturing with cardiomyoblasts has been shown to promote cardiac-lineage differentiation.\(^{105}\) Previous studies have reported that naïve MSCs transplantation for myocardial infarction treatment is insufficient to achieve the best therapeutic outcome because transplanted MSCs rarely differentiate into
Figure 3.6. Assessments of direct intercellular interactions and the rate of protein diffusion across membranes in cocultures using Transwell membrane versus NTHP membranes. (A) Confocal images of cocultured MSCs (green) and H9C2 (red). (B) Confocal images of calcein-AM (green) dye transfer between cocultured MSCs and H9C2 via gapjuction of the cells. (C) Protein concentration at the outlet of the membranes with different depth and porosity, respectively. (D) Illustration of protein diffusion chamber experiment and quantitative analysis of BSA molecule diffusion through Transwell and NTHP membranes. *p < 0.05
Figure 3.7. Enhanced cardiac differentiation of MSCs in NTHP membrane-based coculture system. (A) The mRNA expression of cardiac transcription factor (MEF2C), cardiac structural marker (MLC2v), gap junction marker (connexin 43), and cardiac ion channel markers (CACNA1D and HCN2) in MSCs after coculture with H9C2 for one week. (B) Western blot analysis for cardiac structural proteins, sarcomeric cardiac protein markers of MSCs and quantification for the cardiac specific marker-positive MSCs after co-culture with H9C2 for 1 week. Bars, 50 μm. *p < 0.05 versus non-co-cultured MSCs. #p < 0.05 versus Transwell (indirect). $p < 0.05 versus Transwell (direct).
cardiomyocytes in vivo and the physiological activity of naïve MSCs is inharmonious with the heart tissue, imposing the risk of arrhythmia.\cite{78, 106-108} The transplantation of MSCs that express cardiac biomarkers was reported to have better reparative effects.\cite{109, 110} Thus, cardiac differentiation of MSCs ex vivo prior to transplantation is a high-priority requirement for high therapeutic efficacy of MSCs for cardiac repair.

The expressions of cardiac-associated genes and proteins were assessed after one week of culturing MSCs in various conditions: non-cocultured, cocultured with H9C2 using the Transwell indirect coculture system (Transwell (Indirect)), cocultured with H9C2 using the Transwell direct coculture system (Transwell (Direct)), and cocultured with H9C2 using the NTHP coculture system (NTHP). Gene expressions of a cardiac transcription factor (MEF2C), a cardiac structural marker (MLC2v), a gap junction marker (connexin 43), and cardiac ion channel markers (CACNA1D, HCN2) were significantly higher in the NTHP group (Figure 3.7A). Western blot analysis (Figure 3.7B) and immunocytostaining (Figure 3.7C) of non-cocultured MSCs showed no expression of the cardiac structural proteins, sarcomeric α-actinin (SA) and cardiac troponin T (cTnT). The same results were observed in Transwell (Indirect). Meanwhile, Transwell (Direct) showed slightly increased expression of SA protein. In contrast, NTHP demonstrated significantly higher expressions of both SA and cTnT proteins compared to other groups. Also, NTHP showed a higher expression of connexin-43 compared to the other groups. Statistically, the percentages of MSCs that were positive for cardiac-specific markers were significantly higher in the NTHP group than the other groups (Figure 3.7C). Collectively, these results showed that NTHP membranes improve the cardiac differentiation efficiency of MSCs compared to other coculture systems, which
is likely due to the dynamic cell-to-cell interactions between the cell populations in this membrane system.

3.3.4. Generation of Multilayered Transfer-Printable Stem Cell-Derived Cardiac Sheets

The poor survival of the implanted cells hampers the functional benefits of the cell therapy.\textsuperscript{[111]} As an approach that can overcome such problem, cell sheet engineering has attracted considerable interest. The transferable and thermoresponsive features of NTHP membranes allow the generation of transfer-printable, multilayered sheets of differentiated cells after coculture (Figure 3.8A). The temperature change-mediated detachment of cell sheet from thermoresponsive NTHP membranes was examined under light microscopy (Figure 3.8B).

Detachment of cell sheet from a thermoresponsive substrate is reported to be an advanced approach to harvesting cultured, adherent cell layers compared to the conventional method using trypsin.\textsuperscript{[112, 113]} Trypsin, a proteolytic enzyme that degrades ECM proteins, is known to impair the viability of the trypsinized cells as ECM proteins are crucial components for cell survival.\textsuperscript{[114, 115]} Our data showed that cells harvested in the NTHP group by trypsin treatment showed a poor cell-viability, while transfer-printed cell sheets in the NTHP membrane system showed higher cell viability (Figure 3.8C).

Additionally, whilst the actin cytoskeleton staining of the trypsinized cells showed dissociated cells after cell harvest, transfer-printed cell sheet exhibited the formation of an intact cell sheet (Figure 3.8D). Notably, the ECM proteins of the cell sheets such as fibronectin and laminin were preserved more in the NTHP membrane-generated cell sheet compared to cells harvested by trypsin.
treatment. The preservation of ECM of the cell sheet has been reported to facilitate cell survival and engraftment after implantation.\textsuperscript{93, 115}

Prior to transfer-printing, each cell sheet was pre-labelled by DiI (red) or DiO (green). The transfer-printing technique with thermoresponsive NTHP membrane was performed by stacking a DiI-labelled cell sheet on a DiO-labelled cell sheet, resulting in the fabrication of a bilayered cell sheets (Figure 3.7E). The z-stack 3D confocal images of the bilayered cell sheets showed intact incorporation between the layers. The data confirmed that transfer-printed cell sheets generated with thermoresponsive NTHP membranes showed a higher cell-viability and more preserved ECM compared to the conventional cell-harvesting method.
Figure 3.8. Generation of bilayered cell sheets by transfer-printing technique in the thermoresponsive NTHP membrane-based cocultures, and the characterization of cell sheets. (A) Schematic diagram of the generation of multilayered cell sheets with transfer-printing technique. (B) Light microscopic images of detaching cell sheet from NTHP membrane. (C) Cell viability assessment after trypsinization or transfer-printing as evaluated live/dead assay. (D) Immunofluorescent images of expressed actin cytoskeleton (F-actin) and ECM proteins. (E) Z-stack confocal images of bilayered (first layer (red) and second layer (green)) cell sheets generated by transfer-printing technique using NTHP membranes. Bars, 100 μm.
3.4. Conclusion

In summary, we developed a nano-thin, highly porous and thermoresponsive membrane for the generation of transfer-printable, multilayered sheets of differentiated cells from stem cells through coculture. The feasibility of using NTHP membranes as an effective substrate for coculture was confirmed by demonstration of the direct cell-cell contact between the MSCs and H9C2 on the NTHP membrane, fast protein diffusion across NTHP membrane, and enhanced cardiac differentiation of MSCs post-coculture with the NTHP membrane system. Additionally, the thermoresponsive NTHP membrane enabled the facile engineering of multilayered cell sheets by the transfer-printing technique. The NTHP membrane system may provide a new platform for engineering various types of multilayered cell sheets or tissues those are differentiated from stem cells through the coculture, serving as a promising modality for cell therapy.
4.1. Introduction

Although innumerable studies have been reported on cartilage restoration, cartilage is regarded as one of the most difficult tissue to develop therapies and current researches focus on the healthy cartilage tissue regeneration with stem cells. \cite{116, 117} Inducing chondrogenic differentiation of mesenchymal stem cells (MSCs) using bioactive molecules or scaffolds are general and they show practical effect. \cite{118, 119} However, they do not sufficiently convey the complexity of \textit{in vivo} microenvironment, \cite{120-123} and there remained unfulfilled expectations such as hypertrophic fate of MSCs. As an alternative strategy, coculture systems are designed to mimic complex cellular interactions which stem cells encounter within \textit{in vivo} native microenvironments.\cite{124} In respect that coculture of chondrocytes and MSCs not only promotes the effective chondrogenic differentiation but also prevents MSCs hypertrophy, it is highly promising method to induce chondrogenic differentiation of MSCs.\cite{125-129}

Despite the highly advantageous strategy of membrane-based coculture system, such as easy separation of cocultured cells and effective cell-cell
interactions, there are some major drawbacks of current membrane-based coculture systems for application in stem cell-based therapy. The membranes of current coculture systems, which have micro-scale thickness and low porosity, do not support active interactions between the cocultured cells, resulting in relatively low efficiency of stem cell differentiation. Also, the 2D bilayer geometry of conventional coculture systems is inadequate to mimic the 3D dynamic cell-cell interactions between cocultured cells, which is crucial in promoting the differentiation of stem cells. Moreover, non-biodegradability of conventional coculture membrane necessitates proteolytic enzymatic treatment to detach and collect cells for in vivo implantation. Enzymatic harvesting severely damages the ECM produced by the differentiated cells and cellular viability, impairing cellular function following in vivo delivery of these cells.

Here, we introduce a cellular layer-by-layer (cLbL) coculture platform using biodegradable, nanothin and highly porous (BNTHP) membranes developed for stem cell differentiation and implantation. In BNTHP-based cLbL coculture system, MSC-seeded BNTHP membrane was sandwiched between top and bottom layers of chondrocyte-seeded BNTHP membrane. We hypothesize that cLbL coculture platform using ultra-thin, nanoarchitected BNTHP membranes would provide 3D coculture geometry that better enhances cellular interaction occurring in nano-scale between the heterogeneous cell populations. The augmented cellular interactions between cocultured cells in cLbL coculture would improve stem cell differentiation efficiency compared to bilayer cocultures. Despite its nanoscale thickness, strong mechanical property of the BNTHP membranes as a cell substrate facilitates easy manipulation of each cell layer, thus allowing cellular layer-by-layer sandwich coordination and collection of the MSC layer following coculture. Since membranes are
Degraded naturally, cell-laden scaffolds can be implanted directly allowing avoidance of the problematic enzymatic harvest of cells following coculture. High flexibility of the BNTHP membranes allows in situ folding of the 2D membranes into 3D construct of the differentiated cells along with the membranes prior to in vivo implantation. In short, BNTHP membranes act as effective physical barrier during coculture allowing dynamic interaction between cocultured cells, in addition, they function as a supporter of extracellular matrix (ECM) produced by cultured cells during in vivo experiments. BNTHP-based cLbL coculture system developed in this study provides effective and useful model for cartilage engineering and even for various tissue regeneration applications.
4.2. Experimental Section

Preparation of BNTHP Membrane. PLGA (LA/GA molar ratio 75:25) with weight-average molecular weight (Mw) of 66,000-107,000 g/mol (Sigma, USA) was dissolved in a good solvent, THF, at 4 wt% and BNTHP membranes were prepared by VIPS technique. The polymer solution was prepared by filtering through a 0.4 μm polytetrafluoroethylene filter prior to use. While the phase separation is occurring, polymer solution-casted silicon (Si) substrate was spin-coated with 1000 rpm spinning rate for 25 seconds. The spin-coating process was conducted in the closed chamber in order to control the RH with various types of supersaturated salt solution as proposed in previous studies.\textsuperscript{133, 134} Water vapor was used as a non-solvent in ternary phase system, and the solvent, THF, was all evaporated and the polymer was precipitated during spin-coating process. Si substrates were cleaned by dipping substrates in piranha solution (a mixture of 70 vol% H\textsubscript{2}SO\textsubscript{4} and 30 vol% H\textsubscript{2}O\textsubscript{2}) for 20 minutes at room-temperature before casting the solution onto substrates. The self-standing BNTHP membranes were obtained by peeling off the membrane from Si substrates in aqueous environment. The edges of the spin-coated BNTHP membranes on Si substrate were scratched to expose the hydrophilic Si substrate to water,\textsuperscript{135} leading to spontaneous detachment of the BNTHP membranes in water without sacrificial layer. Subsequently, the freely floating BNTHP membrane in water was collected and framed with the PET frame for easy handling of the membrane. The external and inside diameters of PET frame are 2.1 cm and 1.5 cm respectively, and its thickness is about 310 μm. BNTHP membrane spontaneously wrapped up the PET frame when brought to air atmosphere. Once dried, it remained adsorbed to the frame ever since.
Characterization of BNHTP Membranes. The surface morphologies of BNHTP membranes both in air and water were characterized by AFM (JPK, Nanowizard 3 & diInnova, Veeco). The morphological structures of initial and degraded BNHTP membranes were also characterized by SEM (JSM-6701F, JEOL) following Pt coating using a sputter coater. The pore size and porosity of each membrane was analyzed using an image processing software, Image J (National Institutes of Mental Health). AFM images were adjusted to black/white binary phase, and the portion of black area to the total area was considered as the porosity of membrane. We assumed the pores in the membrane were in circular shape, thus the pore diameters were calculated from the value of the area of each circle.

Differential Scanning Calorimetry Measurements To measure the $T_g$, DSC (DSC 4000, PerkinElmer) equipped with a low-temperature environmental chamber was used. DSC thermograms were obtained by heating 5-7 mg samples in aluminum pans from 15 to 100°C with 10°C/min heating rate in a nitrogen atmosphere (flow rate 20 mL/min). DSC thermograms were obtained during the second heating cycle for pristine and partially degraded BNHTP membranes.

Weight and Thickness Reduction Monitoring of BNHTP Membranes The weight loss of BNHTP membranes were detected by quartz crystal microbalance with dissipation (QCM-D) (Q-Sense D 300, Q-Sense) and calculated with Sauerbrey’s equation as previous studies. The QCM-D measurements were performed to monitor the changes in frequency of Au sensor crystal (QSX301) which is spin-coated with PLGA adopting the same condition to fabricate BNHTP membranes. The changes of resonance frequency of a crystal quartz reflect the mass changes in a layer deposited on the quartz sensor. QCM-D simultaneously measures the absolute resonant
frequency of the crystal for all four overtones (n=1, 3, 5 and 7, i.e., 5, 15, 25, and 35 MHz), however, Δf_1 was typically noisy due to insufficient energy trapping. Thus, frequency changes in the third overtone Δf_3/3 were chosen based on reports from previous studies.[136, 139, 140] PBS was injected into QCM-D chamber where the PLGA-coated Au sensor crystal was mounted. After the balance was established in 0.1M PBS as a baseline, the measurement of degradation was initiated. The released mass was calculated using Sauerbrey’s equation:

$$\Delta m = -C \frac{\Delta f_n}{n} \ (C = 17.7 \ ng \cdot cm^{-2} \cdot Hz^{-1} \ at \ f = 5 MHz)$$

showing the relationship between frequency shift and mass change is linear. The initial weight of BNTHP membrane was calculated to be 0.06 mg and the remaining mass was obtained by addition of the mass change. The thickness of the BNTHP membranes was measured by a step height measurement (AlphaStep IQ (Rev. A1-1), KLA-Tencor, USA) as previous studies.[141, 142]

**Mechanical Property Tests of BNTHP Membranes** The tensile strength and elongation of BNTHP membranes were measured by dynamic mechanical analyzer (DMA, DMA Q800, TA Instruments). To handle the nanothin membrane for DMA analysis, we used PET film as the stanchion for the part of clamps. The PET film preserved the nanothin membrane from any shear or microscopic damage during DMA analysis. Prior to the measurement of the mechanical properties of initial BNTHP membranes, the membranes were completely dried, and degraded BNTHP membrane samples were placed into pH 7.4 PBS at 37 °C for 2 weeks and then dried. The isothermal strain-stress curve was obtained with approximately 10 mm of the sample between the tension film clamps of the DMA at RT with a 0.001 N preload force, and 0.5 N/min ramp rate.
**Gel Permeation Chromatography Measurements** The average molecular weights and molecular weight distributions of the initial and degraded BNTHP membranes were determined by the GPC (YL9100, Young Lin Instruments). The samples were dissolved in THF with a 2 mg/mL polymer concentration and before the GPC measurements, a calibration curve was prepared with poly(styrene) standards.

**Cell Preparation** Human bone marrow-derived MSCs were purchased from a commercial source (Lonza, lot number 458207, tissue acquisition number 28450, isolated from 32-year-old female donor). Articular cartilage tissue slices from knee joints (generously provided by Prof. Ho Jae Han’s laboratory) of 2-3 months New Zealand white rabbits (Hallim Experimental Animals Ltd.) were treated with 0.2% collagenase D (Sigma) in a high-glucose DMEM (Gibco BRL) overnight at 37°C in humid air with 5% CO₂. After treated for 16 hours overnight, the chondrocytes were enzymatically released. The cell yield was 5x10⁵ cells/knee joint. The released cells were plated on TCPS (Corning) at density 1x10⁴ cells/cm² for assessment of the chondrogenic phenotypes at various passage numbers. Prior to coculture, MSCs and chondrocytes were cultured in growth medium consisting of high-glucose Dulbecco’s modified Engle’s Medium (DMEM; Gibco BRL) containing 10% (v/v) fetal bovine serum (FBS; Gibco-BRL) and 1% (v/v) penicillin-streptomycin (PS; Gibco-BRL) at 37°C in humid air with 5% CO₂.

**Culture of MSCs and Chondrocytes** Five groups were compared to elucidate the effects of cLbL culture system on chondrogenic differentiation of the MSCs; non-cocultured MSCs cultured on BNTHP membranes, non-cocultured MSCs in pellet without BNTHP membranes, cocultured MSCs with BNTHP-based indirect system, cocultured MSCs with BNTHP-based direct system membranes, and cocultured MSCs with BNTHP-based cLbL system. For non-
cocultured groups, MSCs were monocultured either on BNTHP membrane or in pellet form. For pellet formation, 1x10^5 MSCs in 15 mL polypropylene tube (Corning) were centrifuged at 150g for 5 minutes according to a previous study. For coculture groups, chondrocytes at passage 1 were plated at a density of 2.5x10^3 cells/cm^2 on either TCPS or BNTHP membrane. MSCs at passage 5 were plated at a density of 7.5x10^3 cells/cm^2 on BNTHP membrane. 24 hours after cell seeding, MSCs and chondrocytes were stacked for coculture.

A chondrogenic medium consisting of serum-free high-glucose DMEM (Gibco BRL), 50 mg/mL ascorbic acid (Sigma), 100 nM dexamethasone (Sigma), 1% (v/v) ITS (Gibco-BRL) and 1% (v/v) penicillin-streptomycin (PS; Gibco-BRL) supplemented with 10 ng/mL TGF-β3 (PeproTech) was changed every 2 days.

**Cell-Cell/Cell-ECM/Cell-Paracrine Factor Interactions between Cocultured MSCs and Chondrocytes**

MSCs and chondrocytes were pre-labelled with DiI (6.25 μg/mL; Sigma) and DiO (6.25 μg/mL; Sigma), respectively and cocultured via BNTHP-based direct and cLbL coculture systems. After 48 hours of coculture, images for colocalizations of MSCs and chondrocytes were captured using a confocal microscope (SP8 X STED, super-resolution confocal microscope, Leica) and visualized with LAS AF software (Leica). For the dye transfer assay, either MSCs or chondrocytes on TCPS were incubated in DiI working solution (6.25 μg/mL; Sigma) for 2 hours at 37 °C and then rinsed with culture medium three times to wash off excess DiI. Next, the cells were incubated in serum-free culture medium containing calcein-AM (10 μmol/L; Sigma) for 30 minutes at 37 °C, and rinsed with PBS three times to wash off the extracellular calcein-AM. Subsequently, the dual-labelled cells were trypsinized and plated on BNTHP membranes. Meanwhile, chondrocytes on TCPS were incubated in DAPI working solution (0.1 μg/mL; Sigma) for 30 minutes, rinsed with culture medium three times, and re-plated onto new dishes.
at 37 °C. For the BNTHP-based direct coculture, dual-labelled MSCs-seeded BNTHP membrane was layered on culture dish seeded with DAPI-labelled chondrocytes. For the BNTHP-based cLbL coculture, additional BNTHP membrane seeded with un-labelled MSCs was prepared. Then, dual-labelled chondrocytes-seeded BNTHP membrane was sequentially layered with un-labelled MSCs-seeded BNTHP membrane followed by dual-labelled chondrocytes-seeded BNTHP membrane. Images were captured after 48 hours of coculture using a confocal microscope to investigate the calcein dye transfer. The dye transfer assay was performed based on previous studies. In separate experiments with identical coculture system of unlabeled MSCs and chondrocytes, Western blot analyses for integrin β1, DDR2, and connexin 43 expressed by MSCs collected from either BNTHP-based direct bilayer or cLbL coculture system were performed (n=3 per group). In addition, Western blot analyses on intracellular signaling pathway of ERK, JNK, p38 and their phosphorylation forms expressed by the MSCs collected from either BNTHP-based direct or cLbL coculture system were performed to analyze the extent of interactions between MSCs and chondroinductive paracrine factors secreted by chondrocytes, such as TGF-β1, BMP-2, IGF-1, and FGF-2 based on previous reports (n=3 per group).

**Chondrogenic Differentiation of MSCs** The chondrogenic gene expressions of MSCs were evaluated using qRT-PCR. RNA in MSCs was extracted and reverse-transcribed into cDNA. Expression of SOX9, collagen II, and collagen X were evaluated using StepOnePlus real-time PCR system (Applied Biosystems, USA) with FAST SYBR Green PCR master mix (Applied Biosystems). Each gene expression was normalized by GAPDH expression. The PCR consisted of 45 cycles of denaturing (95 °C, 10 seconds), annealing (60 °C, 15 seconds), and elongation (72 °C, 15 seconds) (n=3 per group). All of
the data were analyzed using the $2^{-\Delta\Delta Ct}$ method. For protein detection, Western blot analyses and immunocytochemistry were performed.

Generation and Subcutaneous Implantation of Chondrogenic-Differentiated Cells-Laden BNTHP Membrane Constructs into Athymic Mice After 2 weeks of coculture/monoculture in a chondrogenic medium consisting of serum-free high-glucose DMEM (Gibco BRL), 50 mg/mL ascorbic acid (Sigma), 100 nM dexamethasone (Sigma), 1% (v/v) ITS (Gibco-BRL) and 1% (v/v) PS (Gibco-BRL) supplemented with 10 ng/mL TGF-β3 (PeproTech), chondrogenic differentiated cells-attached BNTHP membranes were collected, washed with PBS to remove the excessive chondrogenic culture media, separated from the PET frame, and formed into 3D constructs through centrifugation at 500 g for 5 minutes. Each 3D construct consists of three layers of chondrogenic differentiated cells-laden BNTHP membrane. The 3D constructs were subcutaneously implanted into six-week-old female BALB/c athymic mice (Orient Bio). Nine mice were tested in each group. Each mouse was implanted with samples from four different sample groups (non-cocultured, indirect, direct, and cLbL group) in separate subcutaneous pocket region to eliminate bias between individuals. The mice were anesthetized using ketamine (100 mg/kg) and xylazine (10 mg/kg). Based on previous studies, when chondrogenic differentiated cells are implanted subcutaneously into athymic mice, the optimal time point for chondrogenic phenotypic assessment of the formed cartilage tissue is 4 weeks.[146,149,150] Thus, after 4 weeks of implantation, the animals were euthanized by CO₂ asphyxiation, and the implanted constructs were harvested for gross observation, immunohistochemical analyses, and Western blot analyses. The animal study was approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-160720-12).
**Western Blot Analysis** Cell and tissue lysate from the explanted constructs was prepared with cell lysis buffer (Cell Signaling). The total concentration of the protein was quantified with the Bradford protein assay (Sigma) prior to samples loading on a 10% (w/v) SDS-polyacrylamide gel. Proteins were transferred to Immobilon-P membrane (Millipore Corp.), blocked with 5% skimmed milk solution for 1 hour at RT, and incubated with antibodies against integrin β1, DDR2, connexin 43, β-actin, pp38, p38, pJNK, JNK, pERK, ERK, SOX9, collagen II, collagen X, and aggrecan with suggested dilution rate overnight at 4 °C. The membrane was incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 hour at RT. The blots were developed using a chemiluminescence detection system (Amersham Bioscience). All antibodies were purchased from Abcam.

**Histological Analysis** Cells from all of the *in vitro* groups (i.e. non-cocultured, pellet, indirect, direct, and cLbL groups) were fixed in 4% paraformaldehyde solution (PFA, Sigma) and permeabilized with 0.6% Triton X-100 and blocked with 10% donkey serum (Jackson ImmunoResearch Laboratories). Prior to permeation, cells from the *in vitro* pellet group were embedded in optimal cutting temperature compound (O.C. T. compound, Scigen) and sectioned at a thickness of 10 μm. The samples from all groups were then stained with antibody against SOX9 and collagen II. SOX9 was detected with fluorescein isothiocyanate-conjugated secondary antibodies (FITC, Jackson ImmunoResearch Laboratories). Collagen II was detected with tetramethylrhodamine isothiocyanate-conjugated secondary antibodies (TRITC, Jackson ImmunoResearch Laboratories). The explanted constructs were fixed in 4% PFA (Sigma), dehydrated in a graded alcohol series, and embedded in paraffin. The constructs were sectioned at a thickness of 4 μm and stained with antibody against aggrecan (Abcam) and collagen II (Abcam).
Aggrecan was detected with TRITC and collagen II was detected with FITC. The cell nuclei were counterstained DAPI (Vector Laboratories). Fluorescence images were captured using a fluorescence microscope (IX71 inverted microscope, Olympus). All antibodies were purchased from Abcam. To examine degradability of the BNTHP membranes inside the constructs, constructs of cLbL coculture group were retrieved at 1, 2, 3, and 4 weeks following implantation. The constructs were fixed, dehydrated, embedded, and sectioned as stated above. The cross-sectioned constructs were stained with H&E staining.

**Statistical Analysis** Quantitative data were expressed as the means ± standard deviations. The statistical analysis was performed using one-way analysis of variance (ANOVA) with the Tukey’s significant difference post hoc test using SPSS software (SPSS Inc.). A value of p<0.05 was considered as statistically significant. For surface characterization of BNTHP membranes, three samples of each time point were prepared and two different regions of each membrane were analyzed to calculate the average pore size and porosity of membranes. Both the thickness and mechanical property of BNTHP membranes were analyzed using six samples. For biological analyses, the experiments were repeated three times. Independent samples were tested in triplicate.
4.3. Results and Discussion

4.3.1. Fabrication and Application of BNTHP Membranes in cLbL Coculture

Figure 4.1 illustrates the fabrication procedure of the BNTHP membranes and BNTHP-based cLbL coculture for generating chondrogenic-differentiated 3D constructs derived from stem cells. The BNTHP membranes were made from PLGA, a FDA-approved, biocompatible, and biodegradable material, suitable for biomedical applications.[30, 151] Human bone marrow-derived MSCs were utilized in this study to evaluate the efficacy of the cLbL coculture platform for potential clinical tissue engineering applications using patients-derived MSCs. As human primary chondrocytes are hardly available for research, numerous previous studies have demonstrated successful chondrogenic differentiation of MSCs and cartilage tissue engineered through coculture of MSCs with xenogeneic chondrocytes,[126, 128, 129, 152-158] and in this study, rabbit chondrocytes were cocultured with human MSCs. The cLbL coculture of MSCs between upper and lower layers of chondrocytes exhibited enhanced dual-sided direct cell-cell and cell-matrix contact along with more paracrine factor diffusion across the BNTHP membrane, better mimicking the complex 3D cell-cell crosstalk of the in vivo environment. After 2 weeks in coculture, the BNTHP membranes with chondrogenic differentiated cells that originated from stem cells were collected and separated from the PET frame, and converted into a 3D construct through centrifugation. Subsequently, the 3D constructs of the chondrogenic differentiated cells and BNTHP scaffolds were implanted into athymic mice. The developed membranes should be durable enough during 2-week in vitro coculture time period and yet to be promptly degraded following in vivo implantation. PLGA is known for its high
Figure 4.1. Schematic illustration of the fabrication of BNTHP membranes, and their application in cLbL coculture platform for chondrogenic differentiation of MSCs and generation of in vivo-implantable 3D constructs of chondrogenic differentiated cells and BNTHP membranes.
biodegradability compared to other biodegradable materials, such as PCL (polycaprolactone).\textsuperscript{[159, 160]} Also, previous study reported that greater amounts of ECM proteins in serum-containing media, such as fibronectin and vitronectin, adsorb onto PLGA than PCL. Such ECM proteins promote better cellular adhesion onto the substrate. In this respect, we chose PLGA to develop BNTHP membranes.\textsuperscript{[160]}

4.3.2. Characterization of Physiochemical Properties and Biodegradation of BNTHP Membranes

Previous studies have shown that micro- and nano-sized pores can be formed by VIPS in thin films.\textsuperscript{[58, 161]} This principle was applied to produce the BNTHP membranes in our study. Various process parameters (i.e., solvent volatility, initial polymer concentration, relative humidity, etc.) have been used to control the morphology of polymer membranes that were obtained through VIPS process as introduced in Chapter 2.\textsuperscript{[52]} Among them, we controlled the relative humidity to alter the pore size of BNTHP membrane in Figure 4.2. Since water vapor was employed as a non-solvent, the nanoporous architecture of the membranes was created by controlling the mass exchange rate by regulating the relative humidity. In addition, the spin-coating process was adopted to achieve nanothin thickness of membranes under closed environmental conditions that maintained constant RH.\textsuperscript{[162]} We selected a RH of around 45\% as this allowed formation of well-defined pores with an optimum pore size for cell coculture membranes based on our previous work (Chapter 2 and 3).\textsuperscript{[130]}

The suitability of BNTHP membranes for cell coculture was evaluated by atomic force microscopy (AFM) and differential scanning calorimetry (DSC) (Figure 4.3). The AFM images of these BNTHP membranes in liquid state (water) showed that the nanoporous architecture of these membranes was
Figure 4.2. Molecular structure of PLGA and Different VIPS composition paths of BNTHP membranes at ternary phase diagram and the corresponding AFM images with different RH.
preserved in the aqueous environment emphasizing their capacity as stable coculture membranes. The thermal properties of the BNTHP membranes were measured by DSC. The glass transition temperature ($T_g$) of BNTHP membranes was above 37°C, which is consistent with results obtained in previous studies on PLGA-based thin films. The DSC data also indicated that the BNTHP membranes are in the glassy state in a cell culture condition at 37°C, demonstrating the retention of their nanoporous architecture at this temperature due to fairly frozen chain structure of polymers under $T_g$. In addition, an optical image of a PET-framed BNTHP membrane in Figure 1B displayed notable optical transparency as well as the free-standing feature of the prepared BNTHP membranes.

Next, we investigated the degradation of BNTHP membranes in vitro in phosphate-buffered saline (PBS) solution, pH 7.4, at 37°C for 2 weeks, i.e., coculture period. Several studies have reported in vitro degradation of porous PLGA foams or microthin films. However, to the best of our knowledge, we believe that this is the first study to quantitatively characterize the physicochemical degradation profiles of nanothin membranes with nanoarchitecture. The degradation rate of biodegradable polymer scaffolds is influenced by various factors, including the type of polymer, environmental parameters, and surface area to volume ratio. Therefore, the systematic characterization of the degradation of newly developed BNHTP membranes is essential for bioengineering applications of BNTHP membranes. The weight loss of the BNTHP membrane was monitored by QCM-D and calculated from Sauerbrey’s equation (Figure 4.4A). The blue curves are the changes in QCM-D frequency which represent the changes in mass of BNTHP membrane in PBS. The black curves represent the calculated values of real-time remaining mass of BNTHP membrane. The weight of a BNTHP membrane first significantly

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Figure 4.3. The suitability test of BNTHP membranes for cell coculture. (A) AFM images of BNTHP membranes in aqueous state. (B) DSC graph showed thermal stability of BNTHP membranes at 37 °C, i.e., cell culture temperature. (C) Photograph showed the transparency of BNTHP membranes.
Figure 4.4. Characterization of biodegradation of BNTHP membranes. (A) Real-time weight loss of the BNTHP membranes measured by QCM-D. (B) Thickness decrease of BNTHP membranes after 1 and 2 weeks. *p < 0.05 between two groups; n = 6 per group. (C) Changes in mechanical property of BNTHP membranes at different time points. *p < 0.05 between two groups; n = 6 per group.
decreased upon exposure to PBS, followed by gradual weight loss over the next 2-week period. We adopted QCM-D to observe mass change of BNTP membranes, since it is difficult to measure the changes in weight of nanothin membrane, which mass changes were within the margin of error. The BNTHP membranes showed significant decrease in film thickness after the 2-week-time period when compared with the initial thickness (Figure 4.4B). The results demonstrated that films degrade faster at early part than latterly, i.e., fewer thickness decrease between 1 week and 2 week-time point. These results are in agreement with previous study. Lu et al. demonstrated that thick films degrade faster than corresponding thin films due to the greater extent of the autocatalytic effect.\textsuperscript{[163]} In the same vein, reduce of thickness is larger at 1-week period than between 1 and 2-week period.

Mechanical properties, including Young’s modulus and elongation-at-break, of the BNTHP membranes are shown in Figure 4.4C. The Young’s modulus of the BNTHP membrane did not show any significant change over the 2-week-time period compared to the initial modulus. However, the level of elongation-at-break of the BNTHP membrane after 2 weeks of incubation was significantly lower than the initial elongation-at-break value. Although the BNTHP membrane showed a notably decreased elongation degree after the 2-week incubation period, such a change did not impose any significant influence during coculture since the PET-framed BNTHP membranes were rarely deformed, far away from the threshold of the attainable elongation degree, i.e. 4%. Together, the data on the physicochemical changes of the BNTHP membranes after 2 weeks of incubation in the PBS aqueous environment at 37°C confirmed the degradability of these membranes.

Also, the changes in average molecular weight and molecular weight distribution of the BNTHP membranes before and after degradation were
measured by gel permeation chromatography (GPC) (Figure 4.5A). The molecular weight of the PLGA-based BNTHP membrane decreased after 2 weeks of incubation and the polydispersity index (PI) was also slightly increased, verifying the degradability of the BNTHP membrane after 2 weeks of incubation in PBS at 37 °C.[164] However, despite the degradation features of the BNTHP membranes, in reality, the BNTHP membranes used for the coculture retained their robust and transferable features after 2 weeks of incubation compared to the initial state (Figure 4.5B).

Additionally, AFM images of Figure 4.5C shows that the pore size of BNTHP membranes was increased as the degradation proceeding. The porous structure of the BNTHP membranes after 2 weeks at 37°C was well preserved, as confirmed with AFM and scanning electron microscope (SEM). Changes in pore size and pore distribution with 2 weeks of gradation time was analyzed (Figure 4.6). The physicochemical properties, including average molecular weight, polydispersity index, film thickness, pore size, and porosity for the initial- and 2-week-incubation of BNTHP membranes treated at 37°C in PBS aqueous environment are shown in Table 4.1. The data revealed that the biodegradable BNTHP membranes were initially 540 nm thick with 27% porosity and an average pore size of 393 nm. After 2 weeks of treatment under aqueous solution, biodegradation of the BNTHP membrane was observed with changes in thickness, pore size, and porosity.

### 4.3.3. Higher Intercellular Interactions in cLbL Coculture Compared to Conventional Coculture

Figure 4.7A briefly illustrates the various BNTHP-based coculture systems and other control groups compared in this study showing the extent of cellular
Figure 4.5. Molecular weight and pore size change in biodegradation of BNTHP membranes. (A) Gel permeation chromatograms of BNTHP membranes showed alteration in molecular weight distribution 2 weeks after the membranes cultured in 37 °C aqueous solution. (B) Photographs of robust and transferable features of BnTHP membranes at initial time point and 2 weeks after culture at 37 °C culture medium. (C) AFM images of BNTHP membrane as the degradation proceeding.
Figure 4.6. Changes in pore size distribution and porosity of BNTHP membranes 2 weeks after cultured in 37 °C aqueous solution as evaluated by AFM and SEM images.
Table 4.1. Quantification of physicochemical properties of BNTHP membranes at different time points. *p < 0.05 compared to another group.

<table>
<thead>
<tr>
<th>Time</th>
<th>Mn (Da)</th>
<th>Mw (Da)</th>
<th>PI</th>
<th>Thickness (nm)</th>
<th>Average Pore Size (nm)</th>
<th>Porosity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 week</td>
<td>44,100</td>
<td>79,500</td>
<td>1.8</td>
<td>540 ± 4*</td>
<td>393 ± 5*</td>
<td>27 ± 3*</td>
</tr>
<tr>
<td>2 weeks</td>
<td>30,800</td>
<td>61,100</td>
<td>2.0</td>
<td>438 ± 10*</td>
<td>528 ± 9*</td>
<td>34 ± 5*</td>
</tr>
</tbody>
</table>
interaction of the MSC and chondrocytes in coculture. The experimental culture system consists of five groups; the non-coculture groups, i.e. MSCs culture on BNTHP membranes (non-cocultured) or MSCs in pellet culture (pellet), and the coculture groups using different BNTHP membranes dispositions, i.e. indirect coculture (indirect), direct coculture (direct), and cLbL coculture (cLbL). Non-cocultured MSCs served as the negative control. Non-cocultured MSCs in pellet culture, a widely used method to induce chondrogenic differentiation,\cite{118, 165} served as a positive control. Even though coculture of MSCs with chondrocytes in pellet has been reported to show immense potential for chondrogenesis in some studies,\cite{128, 144} separation of MSCs from the pellet of MSCs-chondrocytes is challenging.\cite{128, 144} Thus, coculture of MSCs with chondrocytes in pellets was excluded from the experimental groups.

In BNTHP-based indirect coculture, the MSCs-attached BNTHP membrane was layered on top of the chondrocytes-attached TCPS. A round, 1-mm high stainless steel ring was placed between MSCs-attached BNTHP membrane and chondrocytes-attached TCPS to eliminate cell-cell contact. Thus, cellular communication in indirect coculture system occurred solely through diffusion of soluble paracrine factors between MSCs and chondrocytes. In BNTHP-based direct coculture, the MSCs-seeded BNTHP membrane was directly layered on top of the chondrocytes-attached TCPS, forming direct cell-cell contact and diffusion of the soluble factors between the cocultured cells. In the cLbL coculture, the MSCs-attached BNTHP membrane was directly layered between chondrocytes-attached BNTHP membrane (top) and chondrocytes-attached TCPS (bottom). To prevent the layered membranes from moving, a round stainless steel ring was placed on top of the cells-attached BNTHP membranes. The detailed arrangements of BNTHP membranes and stainless steel ring are indicated in Figure 4.7B.
Figure 4.7. Schematic illustration and of culture methods for chondrogenic differentiation of MSCs, and arrangements of BNTHP membrane-based cocultures. (A) Illustrations of the various BNTHP membrane-based coculture systems and other comparative control groups evaluated in this study. (B) Top and side view of various BNTHP membrane-based coculture systems. Blue arrows represent stainless rings, and red arrows represent PET-framed BNTHP membranes.
We hypothesized that the cLbL configuration coculture system provides enhanced dual-sided direct cell-cell contact and paracrine factor diffusion between the cocultured cells, mimicking more \textit{in vivo}-like 3D cellular interactions.

Next, we examined the proximity and intercellular interactions between the cocultured cells in the cLbL system compared to the direct bilayer system. MSCs and chondrocytes were labelled with red fluorescent DiI and green fluorescent DiO, respectively (Figure 4.8Ai and iv). The side view of both direct (Figure 4.8Aiii) and cLbL coculture (Figure 4.8Avi) obtained by confocal microscopy showed that MSCs and chondrocytes were in close contact despite the presence of BNTHP membranes and PET-frames among the cellular layers. The flexible, nanothin, and highly porous properties of BNTHP membranes enabled the close contact of two types of cell populations. Importantly, colocalization of the contacting MSCs and chondrocytes were analyzed with confocal microscopy and expressed in yellow pixels, showing significantly greater contact area of the cells in the cLbL coculture (Figure 4.8Av) compared to the direct bilayer coculture (Figure 4.8Aii).

To investigate further whether close proximity between MSCs and chondrocytes allowed formation of functional gap junctions between the cocultured cells through the BNTHP membranes and transfer of biomolecules \textit{via} the gap junctions, the calcein-AM dye transfer assay was performed (Figure 4.8B). Cells plated on the top layer of each coculture system (MSCs for the direct bilayer coculture and chondrocytes for the cLbL coculture) were dual-labeled with DiI (red) and calcein-AM (green). Meanwhile, chondrocytes plated on the bottom-most TCPS were labeled with DAPI (blue) (illustrated in figure 4.8B). DiI is a cell membrane-labelling fluorescence dye and cannot be transferred to the adjacent cells through direct cell-cell contact.\textsuperscript{[130]} Calcein-AM,
Figure 4.8. Direct cell-cell interactions between MSCs and chondrocytes in the cLbL BNTHP coculture compared to the direct bilayer BNTHP coculture. (A) z-stacked confocal images showing direct contact of MSCs (red) and chondrocytes (green) in the BNTHP-based direct and cLbL cocultures. (B) Illustration and confocal images of calcein-AM dye transfer between MSCs and chondrocytes through cellular gap junction in direct cell-cell contacts in the BNTHP-based direct or cLbL cocultures.
Figure 4.9. Evaluation of cell-cell, cell-ECM and cell-paracrine factor interactions between MSCs and chondrocytes in cLbL BNTHP coculture compared to the direct bilayer BNTHP coculture. (A) Western blot analyses of MSCs 3 days postcoculture for evaluation of cell-cell and cell-ECM interactions. *p < 0.05 between two groups; n = 3 per group. (B) Schematic diagram of cellular signaling involved in chondrogenic differentiation of MSCs induced by chondroinductive paracrine factors secreted by cocultured chondrocytes, and Western blot analyses of MSCs day postcoculture for evaluation of the signaling molecule expression. *p < 0.05 between two groups; n = 3 per group.
on the other hand, is a non-fluorescent molecule present outside the cells and becomes fluorescent calcein once it penetrates the cells.\cite{61} Of note, the fluorescent calcein molecule is cell membrane-impermeant and can be transferred to the adjacent cells only through gap junction channels formed by the directly contacting cells.\cite{61, 144} Thus, without direct cell-cell contact between top and underlying cell layers, calcein-AM in the top cellular layer would not transfer to the bottom cellular layer. Surprisingly, 48 hours after coculture, the bottom-most DAPI-labeled chondrocyte layers in both direct and cLbL system exhibited DAPI/calcein colocalization (arrows in Figure 4.8Bi and 4.8Biv). In the cLbL coculture, MSCs between two chondrocyte-layers (arrowheads in Figure 4.8Biv) also showed green fluorescence. These data collectively indicate that the cell layers in both direct bilayer and cLbL cocultures allowed direct cell-cell contact between adjacent cell-layers, formation of functional gap junctions, and transfer of intracellular molecules between adjacent cell-layers through the BNTHP membranes.

Next, we compared the extent of direct cell-cell and cell-ECM interactions between MSCs and chondrocytes in the cLbL coculture and the direct bilayer coculture by Western blot analysis. The expression of a gap junction channel, connexin 43,\cite{144} which can directly link MSCs to chondrocytes, and receptors to ECMs of chondrocytes, integrin β1\cite{166} and discoidin domain receptor 2 (DDR2)\cite{167}, were significantly higher in MSCs of the cLbL coculture compared to MSCs of the direct bilayer coculture (Figure 4.9A). These data indicate that MSCs in the cLbL coculture formed more extensive cell-cell and cell-ECM interactions with chondrocytes compared to MSCs in the direct bilayer coculture. Also, to compare the MSC interactions with chondroinductive paracrine factors secreted by cocultured chondrocytes in the cLbL coculture to
those in the direct bilayer coculture, we investigated cellular signaling in the MSCs, which were induced by chondroinductive soluble factors secreted by the cocultured chondrocytes. Soluble factors, such as TGF-β1,[145, 146] BMP-2,[168] IGF-1,[169] and FGF-2,[148] have been reported as effective chondroinductive factors secreted by chondrocytes,[146, 170] inducing chondrogenic differentiation of MSCs. These factors trigger the activation of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinases (JNK), and p-38 mitogen-activated protein kinases (p38),[145, 171] which subsequently promote chondrogenic differentiation (illustration in Figure 4.9B).[145, 148, 172] Conforming to reports from previous studies, MSCs in the cLbL coculture showed upregulated phosphorylation of ERK, JNK, and p38 compared to those in the direct bilayer coculture (Figure 4.9B). These data show that interaction of MSCs with chondroinductive paracrine factors secreted by the cocultured chondrocytes in the cLbL coculture were much more extensive than those in the direct bilayer coculture.

Collectively, the data indicated partial infiltrations of the ECM and cell through the highly porous BNTHP membrane, thus allowing MSCs-chondrocyte interactions through the ECMs and direct cell-cell contact between the two cell layers. The 393-nm pore size of the BNTHP membrane was adequate enough to support formation of the gap junction channel in the regions of the directly contacting cells across the pores of BNTHP membrane as the width of the gap junction channels channel reported to be 9~10 nm.[173] Also, the cLbL coculture supported greater cell-cell, cell-ECM, and cell-paracrine factor interactions between the cocultured MSCs and chondrocytes compared to direct bilayer coculture.
4.3.4. Superior Chondrogenic Differentiation of MSCs Through cLbL Coculture

Chondrogenic differentiation of the MSCs was evaluated by analyzing the expression of chondrogenic biomarkers after 2 weeks in coculture with chondrocytes. To comprehensively compare and elucidate the effects of cLbL culture system on chondrogenic differentiation of the MSCs, MSCs from the cLbL coculture (cLbL) were compared with MSCs cultured alone on BNTHP membranes (non-cocultured), MSCs cultured in pellets (pellet), MSCs from the indirect coculture using BNTHP membranes (indirect), and MSCs from the direct bilayer coculture using BNTHP membranes (direct). The cLbL group showed extensive upregulations in chondrogenic transcription factor (SOX9)\textsuperscript{[174]} and collagen II (chondrogenic ECM)\textsuperscript{[175]} at the gene and protein levels compared to the other groups, even compared to the pellet positive control group, a widely used method to induce chondrogenic differentiation of MSCs (Figure 4.10A, B, and C). Moreover, the cLbL coculture system supported superior chondrogenic marker expression of MSCs compared with the direct bilayer coculture system, which was likely attributed to the augmented cell-cell, cell-ECM, and cell-paracrine factor interactions between the cocultured MSCs and chondrocytes in the cLbL coculture (Figure 4.8 and 4.9). Even though the indirectly cocultured MSCs expressed an elevated level of collagen II compared to the non-cocultured MSCs which is consistent with the results of a previous study,\textsuperscript{[129]} SOX9 expression was not elevated (Figure 4.10A). These data suggest that interactions with chondroinductive soluble paracrine factors secreted by chondrocytes without direct cell-cell/cell-ECM interactions were not sufficient to promote effective chondrogenic differentiation of MSCs.
Interestingly, the coculture groups showed suppressed expression of collagen X, a hypertrophy marker, compared to the pellet positive control group (Figure 4.10A and B). These data were consistent with results from previous studies, which reported that MSCs cocultured with chondrocytes in direct cell-cell/cell-matrix contact underwent efficient chondrogenic differentiation with significantly suppressed hypertrophy. Even though MSC pellet cultures, which mimic mesenchymal condensation during developmental chondrogenesis, have been considered an effective method for stimulating chondrogenic differentiation of MSCs, high expression of cartilage hypertrophy phenotype in chondrogenic differentiated MSCs in pellet cultures has been an unsolved problem for application of this method in cartilage tissue engineering. The implantation of cells expressing hypertrophic marker causes cellular ossification that leads to cartilage malfunction. Therefore, prevention of hypertrophy in chondrogenic differentiated MSCs is particularly important for effective chondrogenesis and cartilage repair. Collectively, the data demonstrate the BNTHP-based cLbL coculture platform facilitated a much higher chondrogenic differentiation efficacy of MSCs and more suppressed hypertrophic collagen X expression compared to the widely used pellet culture method.

4.3.5. Implantation of 3D Constructs of Differentiated Cells-Laden BNTHP Membranes

Following coculture, the in vitro differentiated cell-laden BNTHP membranes were subcutaneously implanted into athymic mice for 4 weeks to determine whether the cells with chondrogenic phenotypes and suppressed hypertrophy could form cartilaginous tissues in vivo. The BNTHP membranes with robust, nanothin, and highly flexible properties were folded into 3D structures, thus
Figure 4.10. Enhanced in vitro chondrogenic differentiation of MSCs through BNTHP-based cLbL coculture with chondrocytes for 2 weeks as compared to other control groups. (A) mRNA expressions of a SOX9, collagen II, and a hypertrophic marker (collagen X) in MSCs cultured under various culture conditions compared to noncocultured groups, $^*p < 0.05$ compared to noncocultured group, $^#p < 0.05$ between two groups, n=3 per group. (B) Western blot analyses of MSCs cultured under various culture conditions for SOX9, collagen II, and collagen X of MSCs. (C) Immunocytochemistry images of SOX9 and collagen type II in MSCs cultured under various culture conditions. Cell nuclei were counterstained with DAPI. SOX9 expression is indicated with arrows.
Figure 4.11. Preparation of 3D cells-laden BNTHP constructs through centrifugation, and their implantation into athymic mice.
facilitating in situ formation of the differentiated cell-BNTHP constructs through quick and simple centrifugation (Figure 4.11). The biodegradability of BNTHP membranes benefited the membranes as in vivo implantable scaffolds. Moreover, this property of the BNTHP membranes eliminated the proteolytic enzyme treatment procedure to harvest the differentiated cells after coculture. The membranes used in the conventional coculture systems are mainly non-biodegradable.\textsuperscript{[130]} Following coculture, the use of proteolytic enzymes to harvest cells from the non-biodegradable membranes may damage the ECMs, imposing major drawbacks on cell viability and function.\textsuperscript{[92, 93, 130-132]} The biodegradability of BNTHP membranes overcomes such limitations and allows implantation of the cells together with the membranes, thus avoiding the cell- and ECM-destructive cell harvest procedure. Implantation of cells cultured in pellets was eliminated in the animal studies as the cells expressed a high level of hypertrophic marker (Figure 4.10A and B), which would be clinically unsuitable for cartilage engineering.\textsuperscript{[123]} Moreover, as BNTHP membranes were not used in the culture, the cells from pellet cultures cannot be implanted.

Four weeks after implantation, the constructs were retrieved. The gross observations of the tissue constructs of the three coculture groups demonstrated off-white tissues with cartilage-like appearances compared to reddish tissues in the non-coculture group (Figure 4.12A). Hematoxylin & eosin (H&E)-stained cross-sections of the tissue constructs indicated existence and gradual degradation of BNTHP membranes (arrows in Figure 4.12B) in the construct at the 1, 2, and 3-week time point. While BNTHP membrane was observed in the construct at 1, 2, and 3 week time points, at the 4-week time point, BNTHP was not detected. At 4-week time point, the constructs exhibited tissue formation with no evidence of the BNTHP membranes, demonstrating high biodegradability of the membranes (Figure 4.12B). Next,
immunohistochemical staining of the cross-sections of the constructs for collagen II and aggrecan (cartilage-specific proteoglycan core protein)\[^{180}\] was performed. The tissues of the cLbL group expressed significantly higher levels of collagen II and aggrecan compared to the other groups (Figure 4.12C). Western blot analyses confirmed that constructs of the cLbL group expressed the highest levels of aggrecan and lowest levels of collagen X (Figure 4.12D). Compared to other groups, constructs in the cLbL group demonstrated the highest chondrogenic phenotypes with suppressed hypertrophy. Coculture of MSCs and chondrocytes has been proposed as a promising method to induce mature chondrogenesis of MSCs with suppressed hypertrophy.\[^{127, 128, 177}\]

Our coculture system developed enhanced chondrogenic differentiation of MSCs and suppressed hypertrophy compared to other coculture systems. Also, compared to simpler TGF-β3 growth factor only stimulation (i.e. non-cocultured MSCs), MSCs cocultured with chondrocytes via cLbL coculture platform showed much more efficient in vivo chondrogenesis (Figure 4.12C and D). We postulated that once the cells-laden BNTHP 3D construct was implanted, the laden cells secreted extracellular matrices and replaced the BNTHP membranes as in vivo cellular scaffolds along with degradation of the membranes, leading to formation of 3D unfolded, compact cartilaginous tissues in vivo. Thus, through in vivo implantation and chondrogenic phenotype assessment of the 3D constructs, the data collectively emphasized the outstanding performance of the BNTHP-based cLbL coculture platform for generation of cartilage-like tissues from MSCs.
Figure 4.12. Chondrogenic phenotypes assessment of tissues formed by implantation of the 3D constructs of differentiated cells-laden BNTHP membranes into subcutaneous space of athymic mice for 4 weeks. (A) Gross images of the constructs explanted at 4 weeks. (B) In vivo degradation of BNTHP, as evaluated by H&E-stained cross-sections of the constructs explanted at 1, 2, 3, and 4 weeks after implantation. (C) Immunohistological staining for aggrecan and collagen II of the cross-sectioned constructs 4 weeks after implantation. Nuclei were counterstained with DAPI. (D) Western blot analyses of the constructs for aggrecan and collagen X 4 weeks after implantation. *p < 0.05 compared to noncocultured group, #p < 0.05 between two groups; n = 3 per group.
4.4. Conclusion

In conclusion, we developed cLbL coculture of stem cells and chondrocytes using BNTHP membranes for the generation of readily implantable 3D constructs of chondrogenic differentiated cells and BNTHP membranes. The BNTHP-based cLbL coculture platform demonstrated augmented interactions between MSCs and chondrocytes in three-dimensional manner compared to BNTHP-based direct bilayer coculture. BNTHP-based cLbL coculture platform showed notably enhanced chondrogenesis with suppressed hypertrophy of MSCs. Moreover, the highly flexible and biodegradable features of BNTHP membranes enabled the generation of readily implantable 3D constructs of chondrogenic differentiated cells and BNTHP scaffolds through a simple centrifugation procedure. Such properties of the BNTHP-based cLbL coculture platform enabled avoidance of enzymatic harvesting of the cultured cells which often impairs the cells, ECMs and, in turn, functional tissue formation of the harvested cells. The BNTHP-based cLbL coculture platform may serve as a prospective modality for stem cell differentiation into chondrocyte and cartilage tissue engineering.
Chapter 5. Antifouling Catechol-Functionalized Polysarcosine Brushes Conjugated with AgNPs for Antimicrobial Activity on Metal Oxides

5.1. Introduction

Implantable medical devices have become important in the practice of modern medicine. However, implanted devices may cause adverse effects, including inflammation, fibrosis and infection. Shortly after implantation, biomaterials are covered with a layer of proteins, predominantly albumin, fibrinogen, fibronectin and so on.\cite{181} It is generally believed that the composition and state of adsorbed proteins may play a pivotal role prior to accumulation of inflammatory cells, and subsequently promote immune reactions.\cite{182, 183} Moreover, bacterial infection of implanted devices has led to serious problems since it can cause fatal disease that threaten patients’ health along with substantial healthcare costs. Inhibiting bacterial adhesion is crucial to prevent implant-associated infection, since biofilm formed by colonization of certain bacterial species is extremely resistant to immune system and antibiotics,\cite{184} resulting in several cases of implant failure.\cite{185}

For antifouling coatings, polymer brushes have been widely studied and applied to a variety of areas for surface modification, including electrode modification for electronic devices or particle modification for catalytic purposes,\cite{186} due to their diverse functionality and physical stability by surface-confined architectures. Especially, numerous researches in biomedical
applications of polymer brushes have been reported with biocompatible and biodegradable properties.\cite{46}

Among polymer brushes that have excellent potential for bio-related applications, poly(sarcosine) (PSar) has recently received attention as the simplest form of polypeptoids, that possesses similar protein resistance and chemical properties to poly(ethyleneglycol) (PEG). Owing to excellent biocompatibility of polypeptoids, PSar has been readily applied to biomedical filed in substitution for PEG\cite{187,189} such as nanocarrier for drug delivery or bio-imaging etc.\cite{190,192}

In this study, we utilized hydrophilic property of PSar for antifouling application, based on well-demonstrated previous works using hydrophilic polymer brushes for antifouling performance.\cite{49,193} We developed PSar brush platforms on titanium (Ti) surfaces with catechol anchor groups via grafting-to method, which is easier to modify rough and/or non-flat surfaces such as real-implanted devices than grafting-from technique. Ti is one of the most commonly used biomaterials due to its biocompatibility, durability and corrosion resistance, however, sterile Ti can be easily exposed to fungus when it is exposed to air.\cite{194,195} In order to prevent adhesion of such foulants on Ti surfaces, we designed catechol as anchoring groups by synthesizing block copolymer of poly(glutamic acid-b-sarcosine) followed by substitution of glutamic acid for catechol moiety. Adherable property of catechol, especially onto metal oxide surfaces including titanium oxide, has been approved in previous studies.\cite{196,198}

Messersmith and co-workers synthesized PSar by iterative submonomer solid-phase synthesis method and demonstrated nonfouling properties of PSar brushes.\cite{47} However, by nucleophilic ring-opening polymerization (ROP) under living conditions, which is utilized in this study, PSar can be polymerized
up to a degree of polymerization of several hundreds in contrast with solid-phase synthesis method. Since grafting density and polymer chain lengths determine the performance of antifouling polymer brushes,[48,199] we confirmed the chain lengths effect to extent of surface coverage and antifouling properties on Ti surfaces.

Surface properties to prevent bacterial adhesion and/or kill adhered bacteria are of great importance of biomedical implant devices for their performance. Silver nanoparticles (AgNPs) have been highlighted as a promising approach for the development of antimicrobial systems.[200] Attributing to their robust and wide-spectrum antimicrobial efficiency, AgNPs are employed for developing nanohybrids such as AgNPs/polymer[201] or AgNPs/graphene[202] composites.

Successful surface modification with biocompatible polymer brushes for preventing adhesion of foulants and AgNPs for bactericidal activity in a single platform will have a potential for implantable biomedical devices.[203-206] Hence, we further utilized the rest of catechol groups, which was not covalently adhered to titanium oxide surfaces attributing to long chain lengths of the anchor groups, for performing additional functions as both reductants and stabilizing agents for the interfacial AgNPs.[207] In short, we showed enhanced resistance to protein adsorption by catechol-grafted hydrophilic PSar brush and antimicrobial activity by catechol-induced reduction of Ag⁺ in the AgNPs-PSar-modified surfaces. To the best our knowledge, this is the first study that utilizing catechol moiety as dual roles, i.e., anchor groups of polymer brush and reductant for formation of AgNPs. Psar brush conjugated with AgNPs platform developed in this study does not require harsh conditions, therefore, it offers an effective surface coating method in biomedical fields.
5.2. Experimental Section

**PSar Brush Modification on Titnaium Oxide Surfaces.** Almost 50 nm of titanium was deposited on silicon wafers by thermal evaporation process and the surface layer was naturally oxidized.\textsuperscript{208} Prior to modification of surfaces, the substrates were exposed to oxygen plasma with 120 mTorr and 100W for 3 minutes to obtain a clean TiO\textsubscript{2} surface layer as previous study.\textsuperscript{209} Cleaned substrates were immersed in solution of 1 mg/mL PSar dissolved in mixture of 5 vol\% DMSO (dimethlysulfoxide) and 0.1 M MOPS (3-(N-morpholino) propanesulfonic acid) buffer, pH 6.0 at room temperature for 6 hours. After the modification, substrates were cleaned with solvent and DI water thoroughly to remove the physically adsorbed polymers. PSar with different chain lengths was dissolved at acidic condition (MOPS buffer, pH 6.0) to reduce the conversion of catechol to quinone which hinder the interaction with metal oxide, and to prevent the catechol cross-linking.\textsuperscript{47, 210} We have designed three different chain lengths of PSar, \emph{i.e.,} PSar(A), PSar(B), and PSar(C), which have 62, 124, and 244 repeat units of Sar, respectively. In addition, we added 5 vol\% of DMSO for fully dissolved PSar, especially PSar(A) with the shortest chain length, and we conjecture that since the relative chain length of hydrophilic sarcosine in PSar(A) is short, it is not fully dissolved in pure MOPS buffer. For the characterization of PSar-modified surfaces, each TiO\textsubscript{2} substrates was immersed in solution of PSar(A) and (B), (C) for 6 hours followed by washing step with solvent and DI water several times.

**Characterization of PSar-Modified Surfaces.** For quartz crystal microbalance with dissipation (QCM-D, Q-Sense D 300, Q-Sense) measurements, 50 nm TiO\textsubscript{2} coated sensors were purchased from Q-Sense. Following every QCM-D measurements, crystals were immersed in 0.1 M
aqueous HCl solution for 3 hours and solution of 2 wt% SDS (sodium dodecyl sulfate) successively, followed by sonicated in ethanol for 15 minutes and UV/ozone treatment for 10 minutes to reuse several times. After the balance was established in solvent (mixture of 5 vol% of DMSO and 0.1 M MOPS buffer) as a baseline, solution of 1 mg/mL PSar with different chain lengths was injected. When adsorption of PSar reached equilibrium, which means that further decrease of frequency was not occurred, the solvent was re-injected to the chamber to wash out the physically adsorbed PSar to the surfaces. Fourier transform infrared spectroscopy (FT-IR, TENSOR27, Bruker) spectra were obtained in ATR (attenuated total reflectance) mode and averaged over 64 scans. Energy-dispersive X-ray spectroscopy (EDS, JEOL, JED-2300) and X-ray photoelectron spectroscopy (XPS, Sigma Probe, Thermo) was utilized to determine surface chemical composition of PSar-modified surfaces. The surface morphologies of substrates were characterized by scanning electron microscopy (SEM, JSM-7600F, JEOL) and atomic force microscopy (AFM, JPK, Nanowizard 3). Water contact angle was measured using a contact angle meter with a 2 μl DI water droplet.

**Protein Adsorption Test.** Cleaned TiO₂ crystal was immersed in each PSar solution with different chain length followed by QCM-D measurements of protein adsorption. Protein solution was injected into QCM-D chamber where the PSar-coated sensor crystal was inserted. Fibrinogen was dissolved in pH 7.4, 10 mM HEPES buffer, 150 mM NaCl at a concentration of 2 mg/ml. Human serum was used without further purification or dilution and BSA was dissolved in PBS buffer at concentration of 1 mg/ml. All of the protein adsorption measurements by QCM-D were performed at 37 °C. For the visualization of protein adsorption, substrates were immersed in fluorescein isothiocyanate conjugated-bovine serum albumin (FITC-BSA) solution (1
mg/mL in PBS) and incubated for 24 hours at 37 °C. Fluorescence images were photographed using a fluorescence microscope (IX71 inverted microscope, Olympus). The fluorescence intensities of microscopy images were quantified by image processing software, Image J (National Institutes of Mental Health).

**Preparation of AgNPs-Conjugated Surfaces and Measurements of Their Antibacterial Activity.** PSar-modified surfaces were rinsed with DI water several times. For *in situ* AgNPs formation, substrates were immersed in the 10 mM of silver nitrate solution for 10 hours and thoroughly washed with DI water. *E. coli* DH5α was inoculated in 2 ml of LB media and grown overnight. The cell density (OD 600) was determined by UV-visible spectrometry. The cells were washed twice with PBS and diluted to 5x10⁵ CFU/ml. The cell suspension was added onto the surface of each surface and incubated at 30 °C for 4 hours. The whole set of experiments were performed in triplicate. Agar plates to test growth inhibition was prepared by streaking 100 μl of 5x10⁸ CFU/ml using swabs. The bare, Psar-coated and AgNPs -conjugated substrates were placed onto the agar surface with sterilized tongs. The plate was incubated at 37 °C for 18 hours.

Surface chemical composition and morphology were analyzed by XPS and SEM, respectively. To obtain SEM image of bacteria, each substrate was dipped into the bacterial suspension for 18 hours at 30 °C, and after that, the attached bacteria were fixed in 2.5 % (v/v) glutaraldehyde for 30 min following washing with PBS buffer. The fixative was removed by thorough washing, and the samples were dehydrated by successive soaking in mixture of ethanol and distilled water (*i.e.*, 20%, 50%, 70%, 90%, and 100% (v/v) ethanol for 20 min each solution).

**Cytotoxicity Test of AgNPs-PSar-Modified Surfaces.** To confirm biocompatibility of PSar- and AgNPs-PSar-modified surfaces on mammalian
cells, viability of human dermal fibroblasts in direct contact with each surface were evaluated using CCK-8 assay and live-dead imaging. Human dermal fibroblasts (HDF) were purchased from Lonza Inc. (Rockland, ME, USA) and cultured with Dulbecco's Modified Eagle's Medium (DMEM, high glucose, Gibco-BRL, Gaithersburg, MD) that was supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco-BRL), 100 units mL$^{-1}$ of penicillin (Gibco-BRL), and 100 μg mL$^{-1}$ of streptomycin (Gibco-BRL). HDFs were seeded at a density of 1 × 10$^4$ cells cm$^{-2}$ on to 24 well tissue culture plate (Corning, NY, USA). Twelve hours after seeding, small pieces of bare, PSar- and AgNPs-PSar-modified substrates were directly placed on top of the cell layers as previous study.$^{[212]}$ The cells were incubated at 37 °C and 5% CO$_2$ for 12 h, 18 h, 24 h. After the incubation time, HDFs were rinsed thrice with PBS and CCK-8 solution (Dojindo Laboratories, Japan) with fresh medium was added to each well for quantitative analysis. The absorbance was measured at 450 nm using a microplate reader (Infinite® 200 PRO, Tecan, Swiss). The cell viability was calculated as the percentage of viable cells relative to the untreated group (n = 4 per group). Live and dead cells were detected by calcein-AM and ethidium homodimer-1 respectively, using a two-color fluorescence live/dead assay kit (Molecular Probes, USA). HDFs were incubated in calcein-AM/ethidium homodimer-1 solution for 5 min at 37 °C and rinsed with PBS. The stained cell were examined using a fluorescence microscope.
5.3. Results and Discussion

5.3.1. Surface Modification of Metal Oxide with Catechol Anchor Groups Tethered-PSar Brush

We have designed three different types of PSar, \( i.e., \) PSar(A), (B) and (C), which have different portion of hydrophilic blocks as shown in Figure 5.1 to confirm chain lengths effect. It has been reported in many studies that higher tethered surface chain densities and longer polymer chain lengths are regarded as the main determinants for enhancing resistance to protein adsorption.\(^{[213,214]}\) However, for grafting-to systems, grafting density is strongly dependent on the molar mass of polymer chains.\(^{[215]}\) Therefore, we have investigated the correlation of chain lengths with surface coverage to identify critical chain length in resisting protein adsorption. Figure 5.2A shows the XPS measurement determining the chemical composition of the coatings (Table 5.1). Ti signal decreased, while C and N signal increased following the modification proving the stably tethered polymer brush even after the thorough washing step. The C1s signals can be deconvoluted into four components: C-H, C-C, C=C (\( C_A, 284.5 \text{ eV} \)); C-O, C-N (\( C_B, \sim 286 \text{ eV} \)), C=O (\( C_C, \sim 288 \text{eV} \)), and O=C-O, O=C-N (\( C_D, \sim 289 \text{eV} \)). The N1s signals are depicted by two curves: Ti-N in bare TiO2 (\( N_A, 397.82 \text{ eV} \)) and amide nitrogen (\( N_B, \sim 400 \text{ eV} \)).\(^{[216]}\) After coating, the relatively strong \( C_B \) and \( C_D \) signals appeared (Figure 5.2B). In addition, higher \( N_B \) signals, which resulted from amide bonds in PSar brushes, were also observed on coated surfaces, and noticeable decrease of exposed Ti atomic % was observed at PSar-coated surfaces. The \( N_B/Ti \) ratios increased in all PSar-coated surfaces, but related amount of amides of surfaces was different, proving the chain lengths affect grafting density. The longer of PSar chain lengths had
Figure 5.1. Molecular structure of PSar-based block copolymer with different chain lengths. PSar(A), PSar(B), and PSar(C), have 62, 124, and 244 repeat units of Sar, respectively.

Poly(Glutamic acid_{33-b-Sarcosine_{m}})

PSar(A); m = 62
PSar(B); m = 124
PSar(C); m = 244

DOPA : 70% Functionalization of Glutamate

Figure 5.1. Molecular structure of PSar-based block copolymer with different chain lengths. PSar(A), PSar(B), and PSar(C), have 62, 124, and 244 repeat units of Sar, respectively.
Figure 5.2. XPS spectra for bare/Psar-modified surface analysis according to different chain length of Psar.
Table 5.1. Surface elemental composition of bare/PSar-modified surfaces analyzed by XPS (atomic %).

<table>
<thead>
<tr>
<th></th>
<th>Ti</th>
<th>O</th>
<th>C</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare TiO$_2$</td>
<td>13.7</td>
<td>56.1</td>
<td>28.5</td>
<td>1.7</td>
</tr>
<tr>
<td>PSar(A) Coating</td>
<td>0.3</td>
<td>37.9</td>
<td>48.4</td>
<td>13.4</td>
</tr>
<tr>
<td>PSar(B) Coating</td>
<td>6.0</td>
<td>36.9</td>
<td>45.0</td>
<td>12.1</td>
</tr>
<tr>
<td>PSar(C) Coating</td>
<td>8.1</td>
<td>39.4</td>
<td>41.6</td>
<td>10.9</td>
</tr>
</tbody>
</table>
the less coating efficiency in XPS measurement, which is in agreement with FT-IR spectrum (Figure 5.3A). PSar(A) shows the strongest peaks that represent sarcosine and catechol. Furthermore, the adsorption of PSar on TiO$_2$ surfaces was also detected by QCM-D that provide information about the adsorbed PSar masses onto surfaces in situ. Figure 5.3B shows the frequency shift for the adsorption of the three different chain lengths of PSar as a function of adsorption time up to 2 hours. The QCM-D frequency change for the PSar(A) polymer is approximately 150% higher in comparison to PSar(C), confirming larger amount of adsorption was occurred as polymer has short chain lengths which shows consistent tendency with XPS and FT-IR results. We speculated that this might be due to the steric hindrance of long chain in PSar, resulting in mushroom-like brush structure that hinder the dense grafting density.

To confirm that concentration of PSar solution, i.e., 1 mg/mL, is high enough to cover the surfaces, we monitored the frequency change for the adsorption of PSar(A) solution according to concentration (Figure 5.4). From the results, we became assured that 1 mg/mL concentration achieved complete surface coverage for surface modification, since even 0.005 mg/mL of PSar solution shows the same frequency shift with 1 mg/mL following washing step. Surface morphology was characterized by SEM, however, there was no significant change in micro-scale when surface was coated with PSar although EDS data shows polymer coating of surfaces with existence of carbon (Figure 5.5A). While surface morphology was not altered in micro-scale since polymer was coated uniformly on surfaces, surface morphology and roughness in nanoscale was changed as shown in AFM images (Figure 5.5B). Root mean square roughness of surfaces was increased in PSar(A)-modified surfaces. Static water contact angle measurement was utilized to analyze the wettability of PSar-coated surfaces, and the contact angle on the surfaces coated with PSar
Figure 5.3. Characterization of PSar-modified surfaces. (A) FT-IR spectra of each PSar-coated surface by using bare TiO$_2$ as a background. (C) Real-time QCM-D measurements of PSar adsorption on TiO$_2$ quartz. Frequency shifts are shown as a function of adsorption time (2 hours). After adsorption time, the surface was subsequently washed with solvent to remove weakly physisorbed polymers.
Figure 5.4. Adsorption of PSar(A) to titanium oxide surface corresponding to concentration of PSar(A) solution.
Figure 5.5. Surface characterization and comparison of PSar-modified surfaces and bare surfaces. (A) EDS analysis showing existence of carbon on PSar-modified surfaces. (B) Surface morphology and roughness changes in polymer coated TiO$_2$. (C) Contact angle measurements of bare and PSar-coated surfaces.
obviously decreased as compared to the bare TiO$_2$ surfaces (Figure 5.5C).
These results in Figure 5.2 ~ Figure 5.5 support the successful surface
modification with PSar, especially PSar with the shortest chain lengths; PSar(A).

5.3.2. Chain Length Effects of PSar Brush on Antifouling Behavior

Resistance of the four types of surfaces (i.e., PSar(A), (B), (C)-modified and
bare TiO$_2$ surfaces) to nonspecific protein adsorption was monitored by QCM-
D in contact with fibrinogen (Fg), human serum albumin (HSA), and bovine
serum albumin (BSA) solution as shown in Figure 5.6A. All of the PSar-coated
surfaces showed better antifouling efficiency than the bare TiO$_2$ surfaces. The
adsorbed amounts of proteins gradually decreased in order of PSar(C), (B) and
(A) surfaces with increasing surface coverage with PSar brush, which is
consistent with previous studies.$^{[47, 216]}$ To evaluate the performance of protein
resistance with visualization, fluorescence images of substrates were obtained
following exposed to FITC-BSA for 24 hours at 37 °C, along with
quantification of fluorescent intensities. All PSar-modified surfaces
demonstrated enhanced protein resistance compared to bare TiO$_2$, showing
lower fluorescent intensities (Figure 5.6B). Figure 5.6 proved that PSar(A)
coatings, with the shortest chain lengths, showed the most efficient antifouling
performance due to the complete coverage of surfaces as confirmed by XPS,
FT-IR and QCM-D in Figure 5.3. Therefore, we studied further modification
of PSar-coated surfaces for antibacterial activities, especially, with PSar(A)-
coated surfaces.
Figure 5.6. Resistance to protein adsorption of PSar-modified surfaces. (A) Nonspecific protein adsorption measured by QCM-D. Different chain lengths of PSar was coated on quartz followed by insertion of protein solution in QCM-D chamber. Frequency changes were shown when fibrinogen, human serum, BSA solution were injected, respectively. (B) After FITC-BSA immobilization, representative fluorescence images and their fluorescence intensity of different modified surfaces.
5.3.3. *In Situ* Formation of AgNPs on PSar-Modified Surfaces using Catechol Moiety

The catechol moiety of DOPA or dopamine is known to induce mineralization nanoparticles as a green reducing agent in previous researches.\cite{217-219} PSar used in this study has catechol anchoring group with ~20 repeat units, which exceeds seven times to that of previous study.\cite{47} We demonstrated that the rest of catechol moiety, which is not tethered to the surface, could facilitate introduction of AgNP-loaded Psar-modified surfaces by reducing the Ag$^+$ ions (Figure 5.7A). Redox couple between catechol and Ag$^+$ in solution induces growth of an Ag at surfaces stably. The samples dipped in AgNO$_3$ solution for 10 hours generated well-defined AgNPs on PSar-modified surfaces as observed in SEM images (Figure 5.7B). We also checked that extended reaction time in AgNO$_3$ solutions result in increased particle size and well-defined formation of AgNPs. Immobilization of AgNPs was not observed in the samples treated with AgNO$_3$ solution without PSar pre-coating, which function as reducing agent. XPS was adopted to characterize the chemical composition of the samples. Characteristic peaks of Ag$_{3d}$ (~370 eV) in sequentially modified surfaces, *i.e.*, PSar and AgNO$_3$, were shown in XPS data, confirming *in situ* formation of AgNPs (Figure 5.7C).

5.3.4. Antibacterial Activities of AgNPs-Conjugated PSar Surfaces

To evaluate the antibacterial activities of AgNPs-modified PSar surfaces, the viability of *E. coli* attached on the surface were determined by agar plating. The bare, PSar-coated and AgNPs-conjugated PSar surfaces were incubated with *E. coli* suspension (5×10$^5$ CFU/ml) for 4 hours. After that, the bacterial
Figure 5.7. Characterization of AgNPs-PSar-modified surfaces. (A) Illustration of TiO2 surfaces modified with PSar brush and AgNPs and mechanism for formation of AgNPs by oxidation of catechol. (B) SEM images of AgNPs-PSar-modified surfaces prepared by treating with silver nitrate. (C) XPS spectra of each surface following silver nitrate treatment to bare and PSar-modified surfaces.
suspension was extracted for colony forming unit counting by plating methods in series dilutions (Figure 5.7A). For the TiO$_2$ substrates that was not treated further modification, agar plates were full of bacterial colonies after 24 hours. On the contrary, AgNPs-conjugated PSar surfaces showed 100 % antibacterial activity for *E. coli*. Only Psar-modified surfaces, without AgNO$_3$ treatment, showed reduced viability of bacteria compared to bare surfaces, however, the efficiency is low compared to previous works.$^{[47, 205, 220]}$ We suppose the reason for this is that long anchoring groups reduce the antimicrobial effect while inducing loosely packed PSar brushes. Although the PSar-coated substrates lack in antimicrobial activity, it was found that further modification with AgNPs by utilizing the rest catechol groups significantly improve the antibacterial performance close to 100 %.

The effect of surface coating on the attachment of *E. coli* was also investigated by SEM analysis (Figure 5.7B). The SEM images in Figure 5B showed the results of bare, Psar-, AgNPs-PSar-modified substrates incubated with *E. coli* for 18 hours, respectively. While a large number of bacteria was adhered on bare surfaces, few or no bacteria was observed in the AgNPs-PSar-modified surfaces that indicates AgNPs-conjugated surface exhibited excellent antibacterial activity, which accords with agar plating results. In addition, antibacterial effect was evaluated by bacterial inhibition ring assay against *E. coli* (Figure 5.7C). The AgNPs-conjugated PSar surfaces exhibited visible inhibition zones, indicating bactericidal mechanism of the incorporated AgNPs.

Biocompatibility test with mammalian cells *in vitro* should be required for a new coating applied on medical implant devices. Therefore, we tested cytotoxicity of human dermal fibroblasts on each substrate, which are often used for *in vitro* biocompatibility studies of potential implant materials because fibroblast cell line is among a set of well-characterized lines mandated for use
Figure 5.8. Antibacterial activities of AgNPs-PSar-modified surfaces. (A) Photographs and the number of *E. coli* colonies with bare, PSar-modified, and AgNPs-PSar-modified surfaces. (B) SEM images showing the number and morphologies of *E. coli* on bare, PSar-modified, and AgNPs-PSar-modified surfaces. (C) Inhibition zones of the growth of *E. coli* samples.
Figure 5.9. Viability of human dermal fibroblast during 24 hours when cells were contact with bare, PSar- and AgNPs-PSar-modified substrates at 37 °C. The number of live cells relative to the number of non-treated live cells was determined by CCK-8 assay. Fluorescent images of live & dead assay of fibroblasts in contact with bare, PSar-, and AgNPs-PSar-modified surfaces after 24 hours. Live cells are visualized as green and the dead cells as red.
by the FDA as part of cytotoxicity testing protocols for approving new medical devices.[221-223] And there was no significant difference in viability when cells were exposed to bare, PSar- and AgNPs-PSar-modified substrates (Figure 5.8). Also, live/dead cell assay confirmed that AgNPs-containing substrates showed high cellular viability, similar to the control groups of tissue culture plate dishes (no treat in Figure 5C). These results demonstrated that the polymer and AgNPs conjugated to PSar did not cause significant cytotoxicity to human cells proving their excellent biocompatibility.[218, 219, 224, 225] Nie et al. reported that polymers coated with AgNPs function as protective coatings shielding the direct exposure of AgNPs, and moreover, negatively charged coatings control the slow release of Ag\(^{+}\) ions resulting in improved cell viability.[219] Since the PSar block copolymers that we synthesized contain negative charges in glutamates, AgNPs-PSar-modified surfaces present appropriate cytocompatibility attributing to shielding effect.
4.4. Conclusion

In this study, we present a facile and effective strategy for surface modification with antifouling and antibacterial effect via catechol chemistry. We synthesized block copolymer of poly(glutamic acid-b-sarcosine) followed by substitution of glutamic acid for catechol moiety. We investigated effect of chain lengths in hydrophilic brushes, i.e., PSar brushes, and the consequential resistance to protein adsorption. PSar brushes decreased protein adsorption significantly. We also utilized catechol moiety as reductant for formation of AgNPs that have been successfully modified on PSar-coated titanium oxide surfaces. The antibacterial activity and biocompatibility of AgNP-PSar-modified surfaces were confirmed. The antifouling PSar brushes conjugated with antibacterial AgNPs platform developed in this study hold potential for prevention of bacterial infection in biomedical devices.
This dissertation presents the development of polymer-based nanoplatforms for controlling cell behavior, including cell-cell interactions, cell adhesion, and stem cell differentiation.

In Chapter 1, a brief background of engineering cell behavior using biocompatible polymers, by regulating mechanical properties, chemical signals and ECM architectures, is introduced. In Chapter 2, we have developed nanothin membrane with tunable pore size and thickness using biocompatible polymer, cellulose acetate. We could controlled the membrane features by changing of various process parameters. The pore size controllability in nanoscale of the membranes facilitates tuning of cell-cell interactions, i.e., paracrine diffusion, direct cell-cell contact and migration, when cells are cocultured in membrane-based separation system. Moreover, AuNPs encircled with cyclic RGD (Arg-Gly-Asp) peptides were employed to modify nanoporous membranes for studying effect of nano-topographical cues on stem cell differentiation.

Based on strategy of controlling cellular interaction introduced in Chaper 2, Chapter 3 demonstrates the development of NTHP membranes for the generation of transfer-printable, stem cell-derived, multilayered cardiac sheets. The nonothin and highly porous features of NTHP membranes act as effective substrates for coculture, forming direct cell-cell contact between MSC-H9C2, allowing fast protein diffusion across the membrane, and preventing cellular cross-migrations between the cocultured cell populations simultaneously.
Therefore, enhanced cardiac differentiation of MSCs following coculture could be achieved with NTHP membrane-based coculture system. In addition, thermoresponsive NTHP membranes enabled the facile engineering of multilayered cell sheets by the transfer-printing technique.

Chapter 4 demonstrates the development of cLbL coculture platform using BNTHP membranes for the generation of readily implantable 3D constructs of chondrogenic differentiated cells and BNTHP membranes. We systematically characterized the physicochemical properties and degradation of BNTHP membranes via QCM-D, AFM, SEM, and DMA during coculture period, i.e., 2 weeks. The BNTHP-based cLbL coculture platform demonstrated augmented interactions between MSCs and chondrocytes in three-dimensional manner compared to BNTHP-based direct bilayer coculture. BNTHP-based cLbL coculture platform showed notably enhanced chondrogenesis with suppressed hypertrophy of MSCs. Moreover, the highly flexible and biodegradable features of BNTHP membranes enabled the generation of readily implantable 3D constructs of chondrogenic differentiated cells-laden BNTHP scaffolds through a simple centrifugation procedure, enabling avoidance of enzymatic harvesting of the cultured cells.

In Chapter 5, with combination of antifouling PSar brush and antibacterial AgNPs, highly biocompatible and easily produced antibacterial surfaces have been developed in this study. We designed PSar block copolymer brushes to present hydrophilic ends at the surface and adhesive catechol groups at the substrate side. The catechol moieties perform dual roles as anchor groups of polymer brush to implant surfaces as well as reductants for formation of AgNPs. Attributing to the dual functions of catechol groups in the designed PSar brushes, we demonstrated that titania (TiO$_2$) surfaces, which are mainly used in biomedical implants due to their biocompatibility, corrosion resistance and high
fatigue limit, can be easily modified with PSar brushes and antimicrobial AgNPs. The AgNPs-containing PSar brush coating exhibits improved antifouling to prevent protein adhesion and antimicrobial activity as compared with as-received TiO₂ surfaces, while displaying good cytocompatibility to mammalian cells. This platform can be utilized for functional modification of biomaterial surfaces to reduce undesirable fouling and the risk of bacterial infection.

The development of novel nanoplatforms including polymer membrane-based coculture system and polymer brushes, will serve as efficient and biocompatible systems that can regulate cell behaviors. The customized and tunable NTHP membrane-based coculture platform can be utilized from paracrine singaling assays to stem cell engineering according to pore size by regulating cell-to-cell interactions. Therefore, these platforms might prove useful for engineering various types of functional tissues by coculturing stem cells and the desired type of differentiated cells. Moreover, combination of biocompatible polymer and antibacterial AgNPs might offer an effective surface coating method in biomedical implant devices preventing adhesion of foulants and bacteria.
Bibliography


1 3 8


요약 (국문초록)

세포의 흡착, 증식 및 줄기세포의 분화와 같은 세포 거동은 주변 환경의 영향을 쉽게 받는다. 따라서 생체적합성 고분자를 이용하여 세포 주변 환경을 조성함으로써 세포의 거동을 조절할 수 있으며, 생체적합성 고분자는 뚜렷한 생체적합성과 기능성을 부여하기 쉽다는 장점으로 인하여 세포 거동 조절에 널리 사용되고 있다. 전반 고분자 및 합성 고분자를 포함한 생체적합성 고분자는 세포 조직과 직접적인 접촉을 하여도 어떠한 알려지 반응이나 부작용이 일어나지 않는 고분자를 나타낸다. 이러한 생체적합성 고분자의 분자 구조나 물리화학적 특성을 조절하여 만든 세포 지지체는 세포 거동을 조절하기에 적합하다. 본 학위논문에서는 다공성 박막의 모양 및 두께 조절, 표면 치환 등을 통해 구조적 특징의 변화를 체계적으로 분석하였고, 이를 이용하여 세포간 상호작용 조절 및 세포의 흡/탈착에 미치는 영향에 대한 연구를 진행하였다.

제 1 장에서는 생체적합성 고분자를 이용한 세포 거동 조절에 관한 기존 문헌들과 배경지식에 대해 간략히 소개하였다. 제 2 장에서는 생체적합성 고분자인 셀룰로오스 아세테이트를 이용하여 기공 사이즈 및 두께 조절이 가능한 다공성 박막을 제조하였다. 다양한 공정 파라미터들을 변화시킴으로써 박막의 기공사이즈를 변화시키고, 다공성 박막을 기반으로 세포가 공배양 될 경우, 기공 사이즈가 세포간 상호작용 (간극연접 형성을 통한 직접적인 접촉 및 사이토카인을 이용한 신호 전달, 세포 이동)에 미치는 영향을 연구하였다. 또한 단백질로 둘러싸인 금나노입자를 다공성 박막에 도입함으로써 나노지형이 세포 거동을 어떻게 변화시키는지 살펴보았다.
제 2장에서 진행한 연구를 바탕으로, 제 3장에서는 다공성 박막에 온도감응성을 부여하여 심근세포와 줄기세포 공배양에 적응함으로써 심근 분화 유도된 줄기세포 시트를 얻을 수 있었다. 기존의 상용화된 공배양 막과 본 연구에서 개발된 NTHP (nanothin and highly porous) 막을 비교하였다. 우리는 기존의 공배양 막보다 약 20배 더 얇고 25배 이상 기공도가 높은 막을 개발 및 줄기세포의 공배양에 적응함으로써 기존 공배양 시스템보다 향상된 분화율을 얻을 수 있었다. 또한 NTHP 막의 온도감응성을 이용하여 이동-부착 가능하며 세포외기질이 보존되어 세포 생존율이 높은 심근 분화된 세포 시트를 효율적으로 얻을 수 있었다.

제 4장에서, 우리는 생분해성, 나노 두께 및 높은 다공성의 BNTHP (biodegradable nanothin and highly porous) 막을 사용하여 세포 적층 공배양 (cellular layer-by-layer, cLbL) 플랫폼을 개발하였다. cLbL 공배양 플랫폼은 생체 내 삼차원 미세 환경을 보다 정확히 모사하고, 나노 크기에서 발생하는 다층 세포간 상호작용을 통해 이중층 배양 시스템보다 높은 줄기세포 분화 효율을 보였다. 또한, BNTHP 막은 생분해성, 생체 적합성 및 유연성이 뛰어난 특성을 가지기 때문에, 보다 용이하게 막에 부착된 세포를 삼차원 세포 구조물로 전환 및 이식이 가능하여 세포에 유해한 효소적 수확 방법을 피할 수 있었다.

마지막으로 제 5장에서는, 생체적합성 고분자 브리쉬와 음 나노입자를 도입함으로써 향균성과 오염방지 기능을 갖는 표면을 구현하였다. 생의학분야가 발전함에 따라, 생체 이식 장치의 기능 유지 및 감염 위험 방지를 위하여 표면 오염 방지는 필수적이다. 우리는 근래 뛰어난 생체적합성을 갖춘 고분자 브리쉬들과 결합한 여백을 보존하는 폴리사코산 (polysarcosine)으로 오염방지 고분자 브리쉬를 구현하였다. 폴리사코산에 달린 카테르 작용기는
폴리머의 한쪽 부분이 표면에 부착하여 브러쉬를 형성할 수 있도록 도울 뿐만 아니라, 환원력을 지니고 있어 은 나노입자를 표면에 부착할 수 있게끔 도와준다. 이러한 카테콤 기의 기능으로 인하여 표면에 오염 방지를 표면과 향균성을 부여할 수 있었다. 오염방지 기능과 향균성은 다양한 단백질 흡착 및 박테리아 흡착 실험을 통하여 확인하였다. 뿐만 아니라, 고분자 브러쉬 및 은 나노입자 하이브리드 플랫폼이 세포 독성을 떠지 않는 것을 확인함으로써 잠재적으로 생체내 이식 장치 표면 코팅에 적합 가능한 플랫폼이라는 것을 증명하였다.

주요어 : 생체적합성 고분자・막・줄기세포・공배양・고분자 브러쉬・오염방지

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