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Defining an optimal surface chemistry for pluripotent stem cell culture in 2D and 3D

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DEFINING AN OPTIMAL SURFACE CHEMISTRY FOR
PLURIPOTENT STEM CELL CULTURE IN 2D AND 3D

By

Michael R. Zonca Jr.

A Dissertation
Submitted to the University at Albany, State University of New York
in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy

College of Nanoscale Science and Engineering (CNSE)
2013
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Abstract

Surface chemistry is critical for growing pluripotent stem cells in an undifferentiated state. There is great potential to engineer the surface chemistry at the nanoscale level to regulate stem cell adhesion. However, the challenge is to identify the optimal surface chemistry of the substrata for ES cell attachment and maintenance. Using a high-throughput polymerization and screening platform, a chemically defined, synthetic polymer grafted coating that supports strong attachment and high expansion capacity of pluripotent stem cells has been discovered using mouse embryonic stem (ES) cells as a model system. This optimal substrate, N-[3-(Dimethylamino)propyl] methacrylamide (DMAPMA) that is grafted on 2D synthetic poly(ether sulfone) (PES) membrane, sustains the self-renewal of ES cells (up to 7 passages). DMAPMA supports cell attachment of ES cells through integrin β1 in a RGD-independent manner and is similar to another recently reported polymer surface. Next, DMAPMA has been able to be transferred to 3D by grafting to synthetic, polymeric, PES fibrous matrices through both photo-induced and plasma-induced polymerization. These 3D modified fibers exhibited higher cell proliferation and greater expression of pluripotency markers of mouse ES cells than 2D PES membranes. Our results indicated that desirable surfaces in 2D can be scaled to 3D and that both surface chemistry and structural dimension strongly influence the growth and differentiation of pluripotent stem cells. Lastly, the feasibility of incorporating DMAPMA into a widely used natural polymer, alginate, has been tested. Novel adhesive alginate hydrogels have been successfully synthesized by either direct polymerization of DMAPMA and methacrylic acid blended with alginate, or photo-
induced DMAPMA polymerization on alginate nanofibrous hydrogels. In particular, DMAPMA-coated alginate hydrogels support strong ES cell attachment, exhibiting a concentration dependency of DMAPMA. This research provides a new avenue for stem cell culture and maintenance using an optimal organic-based chemistry.
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>AIBN</td>
<td>Azobisisobutyronitrile</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>APMAAm</td>
<td>Aminopropylmethacrylamide</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAI</td>
<td>Cell attachment index</td>
</tr>
<tr>
<td>CAM</td>
<td>Cell adhesion molecules</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-Diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DI</td>
<td>Deionized</td>
</tr>
<tr>
<td>DMAPMA</td>
<td>N-[3-(Dimethylamino)propyl] methacrylamide</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EBs</td>
<td>Embryoid bodies</td>
</tr>
<tr>
<td>E-cad-Fc</td>
<td>E-cadherin Fc domain</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonic stem</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>GAGs</td>
<td>Glycosaminoglycans</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>HFIP</td>
<td>1,1,1,3,3,3 hexafluoro-2-propanol</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HMDS</td>
<td>Hexamethyldisilazane</td>
</tr>
<tr>
<td>hPSCs</td>
<td>Human pluripotent stem cells</td>
</tr>
<tr>
<td>IGF1</td>
<td>Insulin-like growth factor 1</td>
</tr>
<tr>
<td>iPSCs</td>
<td>Induced pluripotent stem cells</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia inhibitory factor</td>
</tr>
<tr>
<td>MAA</td>
<td>Methacrylic acid</td>
</tr>
<tr>
<td>MBAA</td>
<td>N-N’ methylenebisacrylamide</td>
</tr>
<tr>
<td>MEFs</td>
<td>Mouse embryonic fibroblasts</td>
</tr>
<tr>
<td>MSCs</td>
<td>Mesenchymal stem cells</td>
</tr>
<tr>
<td>NSCs</td>
<td>Neural stem cells</td>
</tr>
<tr>
<td>Oct4</td>
<td>Octamer-binding transcription factor 4</td>
</tr>
<tr>
<td>PANi</td>
<td>Polyaniline</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCL</td>
<td>Polycaprolactone</td>
</tr>
<tr>
<td>PDX-1</td>
<td>Pancreatic and duodenal homeobox 1</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PEO</td>
<td>Poly(ethylene oxide)</td>
</tr>
<tr>
<td>PES</td>
<td>Poly(ether sulfone)</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(L-lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PLL</td>
<td>Poly(L-lysine)</td>
</tr>
<tr>
<td>PLLA</td>
<td>Poly(L-lactic acid)</td>
</tr>
<tr>
<td>PMEDSAH</td>
<td>Poly[2-(methacryloyloxy) ethyl dimethyl- (3-sulfopropyl) ammonium hydroxide]</td>
</tr>
<tr>
<td>PMGI</td>
<td>Polymethylglutarimide</td>
</tr>
<tr>
<td>PMVE-alt-MA</td>
<td>Poly(methyl vinyl ether-alt-maleic anhydride)</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-glycine-aspartic acid</td>
</tr>
<tr>
<td>RGDS</td>
<td>Arginine-glycine-aspartic acid-serine</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radio-immunoprecipitation assay</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SAMs</td>
<td>Self-assembled monolayers</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SF</td>
<td>Silk fibroin</td>
</tr>
<tr>
<td>SSEA-1</td>
<td>Stage-specific embryonic antigen 1</td>
</tr>
<tr>
<td>SSEA-4</td>
<td>Stage-specific embryonic antigen 4</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline and Tween 20</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N′,N′- tetramethylethylenediamine</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Transforming growth factor beta 1</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>---------------------------------------</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>XPS</td>
<td>X-ray photoelectron spectroscopy</td>
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 OVERVIEW

I. Objectives and Significance

The long-term goal of this research is to identify an optimal, synthetic chemistry for pluripotent stem cell culture and to effectively modify 3D culture systems with the optimal modified chemistry. To achieve this goal, a novel high-throughput approach is utilized to synthesize and screen a library of surface chemistries in 2D and select the optimal chemistry for stem cell growth using mouse embryonic stem (ES) cells as the model cell line. Secondly, the 2D results are transferred to 3D synthetic polymeric system through both photo-induced and plasma-induced graft polymerization. Lastly, approaches to incorporate the optimal surface chemistry to natural polymeric systems are established to develop adhesive alginate hydrogels that supports pluripotent stem cell attachment and maintenance in 3D.

The hypothesis of this thesis is that an organic-based chemistry that supports mouse ES cell attachment and maintenance can be determined using a high-throughput approach. Once identified in 2D, modifications can be performed to make the optimal chemistry a functional 3D culture system. To test this hypothesis, the following studies will be undertaken and are shown in the accompanying figure (Scheme 1):

Study 1. High-throughput screening of surface chemistries for pluripotent stem cell attachment and maintenance

Summary: Surface attachment is necessary for growing pluripotent stem cells in an undifferentiated state. Engineering the surface chemistry has great potential to regulate stem cell adhesion. The challenge is to identify the optimal surface chemistry of the substrata for pluripotent stem cell attachment and maintenance. Using a rapid high-
throughput polymerization and screening platform with a comprehensive library of 66 monomer-grafting poly(ether sulfone) (PES) membrane surfaces, a chemically-defined, synthetic polymer-grafted coating that supports strong attachment and high expansion capacity of mouse ES cells is revealed. This identified substrate, N-[3-(Dimethylamino)propyl] methacrylamide (DMAPMA), sustains the self-renewal of ES cells (up to 7 passages).

**Significance:** This monomer-based, chemically-defined, scalable, sustainable, covalently grafted and controllable polymeric substrate provides a new opportunity to manipulate surface chemistry for pluripotent stem cell expansion, long-term self-renewal and differentiation.

**Study 2. Creating chemically modified electrospun 3D fibrous matrices for improved stem cell pluripotency**

**Summary:** DMAPMA is grafted to 3D PES fibrous matrices through both photo-induced and plasma-induced graft polymerization. Confirmation of grafting is done through X-ray photoelectron spectroscopy (XPS) and Fourier transform infrared spectroscopy (FTIR). Mouse ES cell proliferation and metabolic activity on 3D fibrous matrices are compared to 2D membranes, with 3D culture exhibiting greater metabolic activity. Additionally, these 3D modified fibers exhibit higher cell expansion and greater expression of pluripotency markers of mouse ES cells than 2D membranes, as demonstrated by immunocytochemistry and Western Blot analysis.

**Significance:** These studies show that optimal surfaces identified in 2D can be scaled to 3D and suggests that both surface chemistry and structural dimension strongly influence growth and differentiation of pluripotent stem cells.
**Study 3. Synthesis of adhesive alginate hydrogels for stem cell expansion**

**Summary:** The feasibility of further incorporating the optimal chemistry into a natural polymeric alginate hydrogel system is further investigated. Two approaches are taken in order to incorporate DMAPMA into alginate hydrogel systems. In the first approach, DMAPMA is combined with methacrylic acid (MAA) to create a solid polymer: DMAPMA-MAA. This polymer is dissolved and blended with alginate, which then can be used to form alginate microbeads or microstrands for adhesive stem cell culture, or in the presence of polyethylene oxide (PEO), electrospun to make adhesive alginate-DMAPMA-MAA nanofibers. In both cases, the addition of DMAPMA to alginate hydrogels was confirmed by FTIR and enhanced ES cell growth.

**Significance:** Alginate hydrogels are one of the most widely used natural biomaterials in cell culture, tissue engineering and cell therapy since they are biocompatible, FDA-approved and easy to use. Additionally, alginate nanofibers are promising since they mimic the extracellular matrix due to their structural similarity to glycosaminoglycans (GAGs). However, due to the lack of recognition sites, alginate is not ideal for culturing adherent cells. The incorporation of DMAPMA with alginate would establish a novel, adhesive alginate 3D culture system, providing a new avenue for utilizing alginate hydrogels.

**II. Organization of the Thesis**

This OVERVIEW section explains the long-term goals, objectives, and significance of this project. Next, CHAPTER 1 introduces the background, including the
potential of chemically defined substrates for stem cell culture, the need for synthetic chemistry for pluripotent stem cell culture, the state-of-the-art of both peptide-based and organic-based chemistries for pluripotent stem cells, and modifications of micro- and nanostructured systems for pluripotent stem cells, leading to the challenges and opportunities of defining the optimal synthetic chemistry for pluripotent stem cell culture in 2D and 3D. In CHAPTER 2, a high-throughput screening method is utilized to determine a surface chemistry for mouse ES cell attachment and maintenance in 2D. Characterization of the surfaces is done through atomic force microscopy and contact angle measurements. ES cell growth on the identified surface chemistry is validated by scanning electron microscopy (SEM) observation of cell morphology, expression of pluripotency markers, the ability of ES cells to form embryoid bodies (EBs) and maintenance of differentiation potential, and long-term self-renewal of ES cells. It reveals that the selected surface chemistry, DMAPMA supports strong attachment, high expansion capacity, and long-term self-renewal of ES cells. In CHAPTER 3, 3D PES fibrous matrices are formed by electrospinning, which are then modified with the optimal chemistry, DMAPMA. These modified fibrous matrices are characterized by XPS. ES cells that are cultured on modified fibers exhibits greater pluripotency compared to unmodified fibers, as shown through confocal microscopy of immunocytochemistry and Western blot analysis. In CHAPTER 4, the selected surface chemistry, DMAPMA is further incorporated into alginate hydrogel systems to develop a novel adhesive alginate hydrogel system. Confirmation of the incorporation of the polymer is done through FTIR. It exhibits a concentration dependency of DMAPMA for cell expansion. In CHAPTER 5, the potential reasons for DMAPMA-modified surfaces promoting cell
attachment are analyzed. Lastly, the complete thesis is summarized in the SUMMARY, where conclusions and future directions of the work are discussed.

Scheme 1. Flowchart of the research tasks to define the optimal surface chemistry for pluripotent stem cell culture in 2D and 3D.
CHAPTER 1

Opportunities and Challenges of Chemically Modified Micro- and Nanostructures in Pluripotent Stem Cell Culture

1.1. Introduction

Pluripotent stem cells include embryonic stem (ES) cells, embryonic germ cells, embryonic carcinoma cells, and induced pluripotent stem cells (iPSCs). Pluripotent stem cells are distinct in that they can exhibit indefinite proliferation and pluripotency, meaning that they can differentiate into cells comprising all three germ layers[1, 2]. These pluripotent stem cells exhibit great potential to be used in the medical field as possible treatments for spinal cord injuries, Parkinson's disease, Alzheimer’s disease, heart disease, diabetes, amyotrophic lateral sclerosis, and other diseases. In addition, these cells are important sources and tools for understanding developmental biology and disease mechanisms, developing and screening drug candidates and performing toxicity testing. In order to maximize stem cell potential it is critical to maintain pluripotent stem cells in an undifferentiated state upon expansion, which is essential for controlling their differentiation into a desired lineage with high efficiency[3-5]. Surface chemistry plays an important role in facilitating cell attachment which is critical for the expansion of undifferentiated cells that are anchor-dependent, replating of differentiating cells and maintenance of terminally differentiated cells in order to obtain the necessary quantities of cells needed for clinical applications or drug screening and testing purpose.

The timeline for pluripotent stem cell culture has been summarized in Figure 1.1. In 1981, mouse ES cells were first isolated from the inner cell mass of the blastocyst and

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1 Part of this chapter is previously appeared as: M.R. Zonca Jr. and Yubing Xie. “Chemically Modified Micro- and Nanostructured Systems for Pluripotent Stem Cell Culture” BioNanoScience 2(4): 287-304 (2012), with copyright permission from Springer.
established in culture as cell lines by two individual groups, Evans and Kaufman, and Martin[1, 6].
Initially, ES cells needed to be cultured on a feeder layer which was a confluent layer of mitomycin C-treated or γ-irradiated human STO fibroblasts or mouse embryonic fibroblasts (MEFs), or even chemically fixed MEFs, which have been treated with glutaraldehyde or formaldehyde, then freeze-dried to immobilize proteins on the membranes of the MEFs[7] (Fig. 1.2a and b)[8]. To avoid xenogenic contamination caused by the feeder layer, feeder-free culture of mouse ES cells was devised by the addition of leukemia inhibitory factor (LIF) to culture medium[9]. Due to ethical and practical difficulties, human ES cell lines were not established until 1998 when Thomson and colleagues first successfully cultured human ES cells on mouse embryonic fibroblast feeder layers[2]. Thereafter, around 200 ES cell lines have been established and various culture systems have been developed[10]. In 2001, feeder-free culture of human ES cells was developed by growing undifferentiated ES cells on Matrigel (Fig. 1.2c and d) or laminin-coated surfaces in media conditioned by MEFs[11]. The discovery that fibroblasts could be reprogramed to a pluripotent ES-like state using a combination of transcription factors enabled the derivation of mouse iPSCs[12] in 2006 and human iPSCs in 2007[13-15]. This 2012 Nobel-Prize winning technology and discovery provides a new avenue for pluripotent stem cell-based disease study and treatment.

Matrigel, a basement membrane matrix is the most widely used approach to feeder-free culture of human pluripotent stem cells. To avoid batch-to-batch variation as well as pathogenic contamination associated with Matrigel, extracellular matrix (ECM) proteins are used to coat tissue culture plates for maintaining ES cells in an undifferentiated state, including fibronectin[16], vitronectin[17], collagen, gelatin and
laminin (Fig. 1.2e and f)[18, 19]. In particular, human recombinant laminin-511 surface has been found to most effectively support long-term self-renewal of human ES cells and iPSCs[19].

Culturing cells on the surface of a tissue culture plate or flask (2D culture) is suitable for laboratory experiments, but 3D culture is essential in order to achieve large-scale expansion. In order to achieve high density cell growth and large scale cell expansion, microcarriers (e.g., dextran, polystyrene, gelatin, collagen, and alginate) coated with MEF, Matrigel or ECM proteins are used for ES cell attachment in suspension and/or in bioreactors[20-27]. Alternatively, 3D hydrogels have been used to immobilize or encapsulate human ES cells for feeder-free culture including hyaluronic acid, alginate and chitosan[28-30]. To better mimic the nanostructure of the ECM, nanofibrous matrices have been used for feeder-free ES cell culture (Fig. 1.2g and h). In 2010, chemically defined substrates have been reported for long-term self-renewal and growth of human ES cells[31-36] (Table 1.1). The ability to synthesize and identify substrate chemistries will allow us to incorporate the optimal chemistry to 3D micro- and nanostructured systems to develop better culture systems for pluripotent stem cells (Tables 1.2 and 1.3).
1.2. Potential of Chemically Defined Substrates for Culture of Pluripotent Stem Cells

1.2.1. Importance of Cell Attachment for Pluripotent Stem Cells

Cell attachment plays an essential role in the control of stem cell self-renewal and pluripotency. Studies have been performed where human ES cells were cultured with various microcarriers to study attachment. Surfaces that showed high cell attachment also exhibited a great deal of cell expansion and maintenance of pluripotency demonstrated by expressing pluripotency markers and maintaining the ability to spontaneously differentiate into cells comprising all three germ layers[22]. Additionally,
cell attachment plays an important role in influencing cell shape and organization, cell motility, and differentiation[37]. Cell adhesion to supporting cells and/or to the ECM has been generally used to maintain ES cells in an undifferentiated state[38], which is essential for maintaining the “stemness” of ES cells. The use of MEFs or Matrigel to mimic adhesion in a stem cell niche has several pitfalls for human ES cell culture, including batch to batch variation and potential cross-species and/or pathogenic contamination since MEFs are of mouse origin and Matrigel is derived from mouse tumor cells[39, 40]. Well-defined substrates are one of the important steps toward a fully defined culture system for stem cell-based drug discovery and/or stem cell-based therapy.

1.2.2. Cell-Cell and Cell-ECM binding

Cell-cell interaction molecules have been investigated for long-term self-renewal of pluripotent stem cells. E-cadherin is one of the cell-surface proteins which are called cell adhesion molecules (CAM), and concentrated in adherens junctions. It plays a critical role in cell-cell contact formation, cell-cell adhesion signaling and in particular, regulating pluripotency of stem cells[41]. Cells cultured on surfaces coated with a protein consisting of the E-cadherin extracellular domain and the IgG Fc domain (E-cad-Fc) were shown to retain their pluripotency[42]. Studies performed by this group on human ES cells and iPSCs showed that E-cad-Fc supports stem cell pluripotency, as shown through the expression of pluripotency markers Oct4, SSEA4 and alkaline phosphatase[43]. Additionally, cells grown on this substrate were similar to those grown on Matrigel in terms of their cell morphology, rate of proliferation, and maintenance of an undifferentiated phenotype.
The mechanism for cell attachment to surfaces is also a field generating a lot of interest. Integrins play an important role in cell binding. They are receptors that mediate cell adhesion through cell-cell or cell-ECM interactions. The ECM proteins responsible for cell adhesion and proliferation through integrins, namely collagens, fibronectins, and laminins are being studied fully for their implications in *ex vivo* culture and therapies. There are 24 integrin receptors in mammals, consisting of one of eighteen α units, and one of eight β units (Fig. 1.3). The α and β units are paired and form dimers. Some integrins are expressed at certain stages of development, whereas some, like β1 are omnipresent[44]. About half of integrin α subunits contain an inserted (I) domain which consists of approximately 200 amino acids. The I domain is a major ligand-binding site[45]. Similarly, integrin β subunits contain a conserved domain that resembles the I domain, and is called the I-like domain. This region is also associated with ligand-binding and directly binds ligands to integrins that do not contain an I domain[45]. Binding assays have been performed to investigate which integrins are responsible for cell adhesion in a variety of cell types (such as stem cells and cancer cells), and one of the major findings was that the β1 integrin plays a major role in cell adhesion and growth[46-48]. There are several rationales as to why β1 integrin plays such an integral role, however, one of the main reasons is that β1 integrin is expressed in early development in both human and mouse cell lines[48, 49]. Combining this with the fact that β1 integrin is ubiquitous, this integrin is vital to cellular functions.
ES cells have prominent expression of α5, α6, αv, β1, and β5 integrin subunits[48, 50]. The β1 unit in particular is vital, as it can associate with several alpha units and is responsible for a variety of cell functions, including adhesion and maintenance. Integrin heterodimer αvβ5 is a vitronectin/RGD receptor, α5β1 is a fibronectin/RGD receptor, and α6β1 is a laminin receptor. It has been reported that fibronectin, laminin, short RGD peptides, peptides for integrins α5β1, α6β1, or αvβ5 alone are not sufficient for maintaining ES cells in an undifferentiated state[32, 48]. Fibronectin is a universal ECM protein that plays a major role in cellular rearrangement[51]. It has been demonstrated that production and interaction with this protein has led to the maintenance of pluripotency for mouse ES cells[51]. Laminins are basement membrane proteins, and are comprised of an alpha unit, a beta unit, and a gamma unit which are critical for cell adhesion. A particular combination of laminins, laminin-511 has been shown to support the self-renewal of mouse ES[52] as well as human ES cells and iPSCs[19].
Additionally, adhesion blocking assays and cell adhesion assays revealed that β1 integrins have a critical role in the binding of human ES cells to laminin-511[19].

In addition to integrins, interactions through cell-surface glycosaminoglycans (GAGs) have been found to promote human ES cell adhesion and support self-renewal of human ES cells as well. GAGs are polysaccharide components of the ECM. They are made of unbranched polysaccharide chains which are composed of repeating disaccharide units and are highly negative charged. GAGs can serve as co-receptors and play an important role in cell-cell and cell-ECM interactions. Klim et al. has demonstrated that cell-surface GAGs participated in the binding of human ES cells to heparin-binding sequences, suggesting their role in human ES cell adhesion and self-renewal[32]. Additionally, it has been shown that feedback of the ECM helps explain the relationship between cell behavior and substrate stiffness[53].

1.2.3. Needs of Synthetic Chemistry for Pluripotent Stem Cells

Chemicals and other soluble factors can interact with integrins and other signaling proteins in cells, and can cause a range of cellular functions. Surface chemistries have great potential to provide substrata for cell adhesion, control protein adsorption and folding, mimic cell-ECM binding, support the long-term maintenance of pluripotent stem cells and regulate their fate decision[54-57]. As mentioned above, chemistries of ECM proteins such as fibronectins, laminins, and vitronectins are studied to examine cell attachment, as these proteins are associated with that function. Since these biological macromolecules are associated with high cost, lot-to-lot variation, and a short shelf life, synthetic chemicals which can mimic cell-ECM interactions are greatly needed for the
development of cost effective, well-defined approaches to pluripotent stem cell culture[31].

The importance of synthetic chemistry on self-renewal and differentiation of ES cells has been demonstrated. Modification of surface chemistry of the tissue culture polystyrene by oxygen plasma etching could maintain the pluripotency of human ES cells in conditioned medium as evidenced by the expression of stem cell markers (Oct4, TRA1-60, and SSEA-4), maintenance of a stable karyotype, multi-germ layer differentiation in vitro, as well as generation of pharmacologically responsive cardiomyocytes[58]. Surface chemical gradients created by plasma deposition from hydrophobic octadiene to hydrophilic acrylic acid could influence cell adhesion, self-renewal and differentiation status through the density of carboxylic acid groups[59]. Increasing the hydrophobicity through surface chemistry properties of self-assembled monolayers of various lengths of alkanethiolates on gold substrates could facilitate uniform embryoid body formation, leading to efficient stem cell differentiation[60]. In order to better mimic cell-ECM proteins, a combinatorial library of chemistries from acrylate and methacrylate monomers have been synthesized at the nanoliter scale and used to characterize human ES cell-biomaterials interactions[61]. In general, two divergent approaches have been used to synthesize and identify surface chemistries for ES cell culture. One focuses on introducing peptide-derived synthetic chemistries to provide ligands binding to surface receptors in ES cells and the other focuses on seeking binders from a library of possible organic chemistries. Synthetic chemistries for pluripotent stem cell culture are summarized in Figure 1.4.
1.3. High-throughput Screening of Synthetic Chemistries for Pluripotent Stem Cells

1.3.1. Peptide-based Surface Chemistries for Pluripotent Stem Cells

Peptide-derived chemistries have been investigated for their potential to sustain ES cell maintenance. Specifically, the Arginine-Glycine-Aspartic Acid (RGD) sequence and its associated integrins comprise a major cell adhesion recognition system. Additionally, two ECM proteins associated with cell adhesion, fibronectin and laminin contain RGD sequences in their domains. Currently, there is a lot of ongoing work in this
field to create similar sequences for cell binding. Melkoumian et al. designed acrylate surfaces and coated them with peptides derived from laminin, fibronectin, vitronectin, and bone sialoprotein for the purpose of supporting the self-renewal and subsequent differentiation of human ES cells[33]. They were able to show that cells cultured on these chemically defined, synthetic membranes colonized as well as cells grown on Matrigel. Derda et al. established a phage display method in which various sequences were grown on self-assembled monolayers (SAMs) and implemented it to discern sequences that support cell adhesion and growth of undifferentiated human ES cells. Briefly, sequences were screened by phage display in which wild-type or environmental phages were tested to see if they change plaque color due to the presence of peptide-specific interactions. The sequences that contained peptide specific interactions were further screened to observe how they supported ES cell proliferation on SAMs. The results indicated that ES cell proliferation was supported by peptide-specific sequences and that phage display was a reliable method to obtain these sequences. In particular, synthetic surfaces presenting phage-derived peptides (TVKHRPDALHPQ or LTTAPKLPKVTR) support human ES cells in a chemically defined medium to express pluripotency markers at levels similar to cells grown on Matrigel[62]. Poly (ethylene-co-vinyl alcohol) membranes were modified with the laminin peptide GYIGSR in order to support the adhesion, differentiation, and migration of rat neural stem/precursor cells[63]. Work done by Klim et al. utilized a high-throughput screening platform in which over 500 substrates were screened based on 18 peptides. In addition to investigating substrates that contained peptides derived from laminin, heparan-binding sequences were looked at as they can interact with GAGs, promoting human ES cell adhesion and supporting
undifferentiated human ES cell growth in a chemically defined medium[32]. Out of all the substrates screened, the most effective substrata for cell adhesion and propagation were those that can bind anionic sugar molecules similar to heparan. This work shows the significant role of GAGs in ES cell adhesion and proliferation. In particular, the GAG heparan sulfate was immobilized onto electrospun poly(L-lactic-co-glycolic acid) (PLGA) microfibers, and this proved to maintain mouse ES cell culture and differentiation[64]. It was also demonstrated that hydrogels comprised of a GAG binding peptide, GKKQFRHRNRKG, were suitable for human ES cell self-renewal[65].

1.3.2. Synthetic Organic Chemistries for Pluripotent Stem Cells

High-throughput screening methods have been implemented to screen for chemistries that support ES cell maintenance. Villa-Diaz et al. synthesized six polymer coatings by surface-initiated graft polymerization and identified a synthetic zwitterionic polymer coating, poly[2-(methacryloyloxy) ethyl dimethyl-(3-sulfopropyl) ammonium hydroxide] (PMEDSAH) that supports long-term growth of human ES cells without differentiation for 15 passages[34]. Mei et al. created microarrays consisting of combinatorial polymers synthesized from 22 biocompatible, acrylate-based monomers by UV radiation and showed substrates generated from monomers with high acrylate content support clonal growth of fully disassociated human ES cells and iPSCs. This work outlines an approach to develop a chemically defined culture system for human pluripotent stem cells independent of a feeder-layer and serum-free. The screening method implemented allowed for many polymers and replicates to be tested. Additionally, hit monomers were further studied for their ability to maintain ES cell pluripotency for long term culture through integrin αvβ3 and αvβ5 engagement with
adsorbed vitronectin[35]. A similar, high-throughput microarray screening process was implemented by Brafman et al. A synthetic polymer, poly(methyl vinyl ether-alt-maleic anhydride) (PMVE-alt-MA) was selected from a microarray of 91 polymers of varying chemical compositions, functional groups, and molecular weights that supported self-renewal of human pluripotent stem cells (hPSCs) for over five passages[36]. Irwin et al. designed a hydrogel network from aminopropylmethacrylamide (APMAAm) and a crosslinker, N,N’ methylenebisacrylamide (MBAA). These modified surfaces supported long-term culture and pluripotency of both H1 and H9-hOct4-pGZ cells[66]. Compared with peptide-based substrates, organic chemistry-based, polymeric substrates should be better defined, more reproducible, less expensive and easier to handle and store with longer shelf lives.

1.4. Chemical Modification of Micro- and Nanostructured Systems for Pluripotent Stem Cells

1.4.1. Nanofibers for Pluripotent Stem Cells

Many types of 3D scaffolds are being investigated to mimic the ECM of native tissue. Electrostatic spinning, or electrospinning, is a technique that has been widely used in cell culture. First developed in the late 1800’s, and studied in detail in the early 20th century, this technology allows for the formation of nanofibers. Electrospinning is unique in that it allows both natural and synthetic polymers to be used to create these fibers. Tissue engineering is a field of great interest for utilizing these electrospun nanofibers. Since the fibers are able to imitate the native tissue in 3D, cells will grow as if they are enveloped by the tissue. Currently, there is a great deal of research examining various types of fibers and how they interact with ES cells to push differentiation into
specific cell lineages. Alternatively, self-assembly and phase separation have been used for synthesizing nanofibers[67]. The chemical coating plays an important role in stem cell fate decision. For example, Matrigel coating to PLGA nanofibers promoted differentiation of mouse ES cells into mesodermal cell lineages and germ cells, but inhibited the derivation of endodermal cell lineages[68].

1.4.1.1. Self-renewal of pluripotent stem cells

Nur-E-Kamal et al. found that the nanofibrillar polyamide matrix, Ultra-Web, could enhance proliferation and the self-renewal of mouse ES cells in comparison to conventional culture methods. This enhancement was correlated with the activation of the small GTPase Rac and phosphoinositide 3-kinase (PI3K) signaling pathways and increase of Nanog expression which is required for the maintenance of pluripotency[69]. Additionally, covalently bound FGF-2 to the nanofibrillar polyamide surface could greatly activate FGFR, extracellular signal-regulated kinase (ERK1/2), and c-fos, enhancing the stability and bioactivity of the cells[70]. Polycaprolactone (PCL)-collagen or PCL-gelatin nanofibrous scaffolds could support large colony formation of human ES cells but require the presence of MEFs[71]. Mouse ES cells were cultured on electrospun thermoplastic polymethylglutarimide (PMGI) nanofibers which support their growth and maintenance of pluripotency without the need of MEFs[72]. Surface modification of poly(ether sulfone) (PES) nanofibers using collagen grafting showed enhanced proliferation, infiltration and teratoma formation and maintenance of pluripotency of mouse ES cells[73].

Although these scaffolds consist of nanofibrous architectures, the difference in substrate chemistry exhibits distinct performance for ES cell growth. For example,
polyamide or PMGI alone could support enhanced growth of undifferentiated mouse ES cells while PES needs a collagen coating. Both polyamide and PMGI contain amine groups and display peptide bonds, which have the potential to mimic peptide sequences for enhanced cell binding to the substrate.

1.4.1.2. Neuronal differentiation

As mentioned previously, a great deal of ES cell research is focused on the treatment of debilitating diseases, such as Parkinson’s disease, spinal cord injuries, and other diseases to the central nervous system. In work done by Yang et al., a synthetic polymer, poly (L-lactic acid) (PLLA) was electrospun to create an aligned scaffold to grow mouse cerebellum stem cells onto. Since neural stem cells (NSCs) are integral in nerve repair, their maintenance and interactions with the PLLA fibers was extensively studied with various types of microscopy[74]. It was concluded that PLLA nanofibers exhibited a greater differentiation rate compared to microfibers, however, alignment was crucial for NSC elongation and neurite outgrowth, which is important for the formation of neurons. Based on these success of neuronal differentiation of adult stem cells, PCL nanofibers have recently been shown to induce differentiation into specific neural lineages, including astrocytes, neurons, and oligodendrocytes[75]. Additionally, similar to what was exhibited with PLLA fibers, when aligned, neurite outgrowth was enhanced. PCL nanofibers coated with poly-L-ornithine/laminin supported human ES cell adhesion and differentiation to neural precursors[76]. This study confirmed that aligned nanofibers significantly enhanced the neuronal differentiation of neural precursors and found that the effect of fiber diameter was not significant in the range of 250 - 1000 nm being examined. PCL blended fibers have also been investigated[77]. When PCL fibers were
mixed with the protein collagen, the biocompatibility of PCL was enhanced, while creating fibers with smaller diameters and higher porosities. In addition, collagen contains amine groups, and when combined with PCL and electrospun, these amino groups are found on the surface of the 3D scaffolds. Since amine groups are present in the proteins associated with cell adhesion, namely, fibronectin and laminin, these scaffolds showed an increase in the proliferation of nerve-like cells. It has been demonstrated that nanofibrillar structures along with neurogenic growth factors could promote neurogenic differentiation and function of human ES cells[78].

Rahjouei et al. showed that polyamide matrices could generate extendable and self-renewable neural progenitor cells[79]. Additionally, Wang investigated Tussah silk fibroin nanofibers to culture human ES cell-derived neural precursors[80]. The effects of fiber diameter (400 nm vs. 800 nm) and orientation (random vs. aligned) on cell viability, neuronal differentiation and neurite outgrowth were investigated. It was shown that 400 nm aligned TSF nanofibers were more suitable for human ES cell-derived neural precursor development.

Conductive polymers, such as polyaniline (PANi) have been incorporated with PLLA fibers for the formation of neural scaffolds[81]. Since PANi is conductive, it has the capability to change the viscosity as well as conductivity of the solution. This was shown when comparing PLLA fibers to PLLA/PANi fibers, which had smaller diameters. Additionally, the conductance of the PLLA/PANi fibers was similar to cell stimulation values. The addition of carbon nanotubes to PLLA during the electrospinning process could also introduce conductivity to nanofibers for enhancing mouse ES cells differentiation to mature neurons[82]. Palmgren et al. investigated the use of a bioactive
nanofiber gel to promote the differentiation of neurons. The gel was comprised of self-assembling peptide amphiphile molecules. The efficacy of nanofibers for promoting \textit{in vivo} neuronal differentiation was demonstrated by injecting tau-GFP mouse embryonic stem cells into the auditory nerve in rats and applying bioactive isoleucine-lysine-valine-alanine-valine peptide amphiphile nanofiber gel around the injection site[83].

These studies demonstrated that nanofibers provide a versatile platform for enhanced neuronal differentiation of ES cells by providing: 1) various substrate chemistries as scaffolding materials (e.g., PLLA, PCL, polyamide, silk, peptide amphiphiles), 2) surface coatings or mixtures for electrospinning to enhance cell adhesion (e.g., collagen, poly-L-ornithine, laminin), 3) fiber alignment to promote neuronal elongation and neurite outgrowth, 4) incorporation of conductive components to enhance neuronal function, and 5) bioactive nanofibers to promote both \textit{in vitro} and \textit{in vivo} neuronal regeneration.

\textbf{1.4.1.3. Osteogenic differentiation}

Bone tissue engineering is another field seeing extensive study in the medical arena. Compared to neural cell scaffolds, these scaffolds must have much larger elastic moduli to promote bone cell differentiation. Mechanical properties of 3D scaffolds can impact stem cell fate. Engler et al.[84] showed that mesenchymal stem cells (MSCs) exhibit branched morphology similar to neurons grown on Matrigel when cultured on soft collagen gels. Similarly, cells cultured onto stiffer matrices exhibited morphologies similar to muscle and bone. For studies in 3D, MSCs were cultured onto a polyethylene glycol-silica composite gel[85]. The substrate stiffness was controlled by the amount of fumed silica in the gel, and this methodology may provide more insight for cellular
interactions in the human body. Methacrylamide chitosan systems have also been
developed to determine the optimal elastic modulus for neural stem/progenitor cell
proliferation and differentiation[86]. Other scaffold properties have been shown to
influence cell differentiation, including content and porosity; however the elastic
modulus proved to be a crucial factor when determining the fate of human ES cells[87].
Lower modulus substrates tended to yield cells that behaved similar to neurons, while
harder elastic moduli yielded cells that resembled skin and bone.

The differentiation of MSCs into chondrocytes has been observed on electrospun
PCL fibers containing chondrogenic medium containing TGF-β1[88]. This is one of the
pioneering works to utilize electrospun nanofibers in stem cell research. The nature of the
3D constructs not only aided in the formation of chondrocytes, but also in the expression
of a cartilaginous matrix as well as cartilage-specific ECM genes. Self-assembled
peptide nanofibers were used for differentiation of mouse ES cells and MEF to
osteoblast-like cells which expressed osteopontin and collagen type I with high alkaline
phosphatase activity and calcium phosphate mineralization[89].

Additionally, self-assembled peptide nanofibers were observed to support the
maintenance of ES-like colonies during osteogenic differentiation[90]. Smith et al.
fabricated PLLA nanofibrous matrices using a phase separation method and demonstrated
the importance of nanofibrous architecture in promoting osteogenic differentiation of
both mouse and human ES cells by comparing ES cell differentiation on nanofibrous
matrices to 2D flat films and 3D solid-wall scaffolds[91-94].

The presence of collagen on the nanofibers played a significant role in cell
proliferation, as these rates were higher on nanofibers coated with collagen than those
fibers sans collagen[95]. Chitosan, a linear polysaccharide comprised of random units of D-glucosamine and N-acetyl-D-glucosamine is another compound being investigated for use as a coating on synthetic polymers. Additionally, chitosan is biocompatible, non-toxic, and exhibits cell affinity due to the amine groups present on the compound. Since chitosan is a natural polymer, researchers have combined it with synthetic polymers in order to increase its stability upon electrospinning. Cells grown on chitosan/PCL fibers exhibited good cell attachment, as well as a spreading morphology, indicating that these conditions are optimal for cell maintenance[96]. Other amine-based compounds have been investigated as well. In particular, dopamine, of the catecholamine family has been shown to promote cell adhesion. Dopamine is a single-molecule consisting of a DOPA-like catechol group and a lysine-like alkylamine group, which can polymerize to surfaces. Although the exact polymerization mechanism is not known, one prevailing hypothesis is that the catechol group is oxidized, thus forming covalent interactions with substrates[97]. The use of polydopamine as a coating on fiber-based scaffolds is of interest, due to the strong binding interactions, as well as high cell affinity due to the presence of the amine functional group. Rim et al. has shown that polydopamine not only aids in the proliferation of hMSCs, but it also assisted in the up-regulation of osteogenic genes, as measured by RT-PCR[98]. These results are particularly encouraging in the growing field of bone engineering, as in this case, surface modification using polydopamine greatly enhanced osteogenic formation.

1.4.1.4. Cardiac differentiation

Cardiovascular diseases are the number one cause of death globally, and approximately 30% of global deaths were attributed to cardiovascular diseases in 2008.
according to the World Health Organization. MSCs have been utilized as a source for cardiac cell therapy, due to their ability to differentiate into cardiomyocytes. Guan et al. constructed a block copolymer diol, and then polymerized a urea functional group to it. This substrate served as a tissue construct for cardiomyocyte differentiation. This substrate was soft in nature, with an elastic modulus of $5.5 \pm 0.9 \text{ MPa}$[99]. Of particular interest was the tissue construct methodology. In addition to electrospinning the modified copolymer substrate, simultaneous electrospraying of MSCs was performed. This was done to observe the effects of high voltage on cell proliferation, multipotency, and cell death. Not only did the cells survive at a high rate, but they were shown to differentiate into cardiac cells, and the constructs exhibited myocardium-like properties[99].

Pluripotent stem cells have also been studied for cardiac cell therapy as well. Electrospun PLGA/Collagen nanofibers were found to better support differentiation of EB outgrowths into cardiomyocytes than PLGA nanofibers, as shown by immunocytochemistry and scanning electron microscopy[100]. Work performed by Ikonen et al. [101] showed that hydrophilic self-assembled nanofiber hydrogels supported the growth and culture of both rat cardiomyocytes and human embryonic stem-cell-derived cardiomyocytes. These results indicate that these types of biomaterials can potentially be used for cardiac tissue engineering in the future.

1.4.1.5. Hepatic differentiation

Liver diseases, specifically hepatitis are particularly debilitating globally. Of the 5 types of hepatitis (A-E), hepatitis B and C can lead to chronic disease in hundreds of millions of people according to the World Health Organization. In particular, hepatitis B
is particularly devastating, as it is 50-100 times more infectious than HIV and approximately 25% of adults that contracted the illness during childhood die from either liver cancer or cirrhosis. A method for culturing and replating hepatocytes is greatly needed. Meng et al.[102] showed that a matrix modified with poly (N-p-vinylbenzyl-4-O-β-D-galactopyranosyl-D-gluconamide) and E-cadherin-IgG Fc assisted in the specific differentiation of mouse ES cells into hepatocytes. E-cadherin has a role in the spheroid shape of hepatocytes when they adhere onto the matrix. Additionally, it was shown that cells grown on PVLA tended to aggregate after 3 days in culture, and there were characteristic liver markers in both protein and genetic measurements. This methodology sheds light on the ability to differentiate mouse ES cells and maintain them as hepatocytes. Nanofibrous structures could also enhance the hepatic function derived from human ES cells[103].

1.4.1.6. Adipogenic differentiation

Obesity is chronic illness that has become a worldwide problem in recent years. Fatty and unhealthy foods, as well as a lack of exercise are some of the key issues surrounding this illness. Overall, worldwide obesity has doubled since 1980, and in 2008, 1.5 billion adults were overweight according to the World Health Organization. It is the fifth leading cause of death worldwide, as approximately 2.8 million people die annually from complications of being overweight or obese. Additionally, individuals that are obese have a greater chance of contracting other illnesses such as cardio vascular diseases, cancer, and diabetes. With obesity and diabetes on the rise, understanding adipocyte growth and function is paramount. In work done by Kang et al.[104], mouse ES cells were grown on electrospun PCL nanofibers. The ES cells were initiated to
undergo adipogenesis. The PCL scaffold served as an appropriate matrix for the differentiation of ES cells into adipocytes, as verified by oil red O staining, immunocytochemistry and immunoblot analysis of adipocyte markers and functional analysis of adipocytes. This work serves as a platform for the study of early events during adipogenesis and 3-D adipocyte models for drug development and testing, leading knowledge and technology to combat obesity, diabetes, and metabolic disorders.

1.4.2. Nanofilms for Pluripotent Stem Cells

1.4.2.1. PLL/HAlnanofilms

Blin et al.[105] recently showed that alternating multilayers of poly (L-lysine) (PLL) and hyaluronan (HA) could be built on glass coverslips. They were able to culture ES cells on these films to test for cell adhesion and differentiation potential for two different conditions: native or cross-linked. It was shown that ES cells retained their pluripotency after being cultured on native films, as confirmed by RT-PCR. Altogether, the multilayer technology for developing nanofilms holds great promise for pluripotent stem cell culture. Future applications may include surface coatings and/or scaffolds.

1.4.2.2. Silk nanofilms

Silk is a natural polymer that is showing great promise in tissue engineering. Derived from spiders and silkworms, silk is comprised mainly of silk fibroin (SF) and sericin. It has appropriate mechanical properties, as well as biocompatibility, non-toxicity, and cell-controlled degradability that make it an attractive option in tissue engineering. Many silk preparation methods have been recorded in the literature; however, silk films are the most common. Wang et al.[106] prepared silk films to be used as 3D scaffolds. Adult mesenchymal stem cells were then seeded onto these films.
with appropriate growth factors for proliferation and chondrogenesis. Stem cells grown on the silk scaffolds exhibited dense cartilaginous ECM, as shown through genotypic and phenotypic tests. Additionally, these constructs were similar to native cartilage tissue. A second instance in which silk films were utilized was for adenosine release. The release of adenosine has been shown to lower seizure activity in the rat-kindling model[107]. Uebersax et al.[108] has shown that mouse ES cells grown on SF constructs were able to differentiate from the glial phase into astrocytes efficiently. Additionally, SF constructs exhibited adenosine release in the absence of cell-binding domains, such as RGD. This indicates that these binding domains do not need to be included on SF for either adenosine release or differentiation; therefore the use of these SF films as a substrate for further studies is a viable option. Silk scaffolds have been prepared in different manners as well. In work done by Chen et al. porous silk scaffolds were fabricated by knitting. Collagen was then added to the silk mesh, acidified, then freeze-dried to form collagen micro-sponges[109]. After human ES cell-derived MSCs were seeded onto the scaffold, they were exposed to a mechanical stress in order to mimic the properties of tendons. The cells successfully differentiated into tendon cells, as verified morphologically, histologically, and genetically through PCR. Functionalized SF-based patches have also been used as scaffolds for the differentiation of rat mesenchymal stem cells into cardiomyocytes[110]. It was shown that after 7 days, stem cells cultured on these patches were morphologically similar to cardiomyocytes. This was further confirmed by immunofluorescence staining, as well as real-time PCR for cardiac-specific genes. Overall, these preparations exhibit other avenues to utilize silk scaffolds for pluripotent stem cell culture and tissue engineering.
1.4.3. Microsystems for Pluripotent Stem Cells

1.4.3.1. Microcarriers

Microcarriers including dextran, polystyrene, gelatin, collagen, and alginate coated with MEF, Matrigel or ECM proteins were used for ES cell attachment in suspension and/or in bioreactors[20-27]. Biocompatibility and biodegradability are critical for microcarrier usage in pluripotent stem cell culture. For this reason, various polymers have been investigated for microcarrier usage. PLGA when loaded with retinoic acid has been shown to induce embryonal carcinoma cell differentiation[111]. Microcarriers are an alternative for loading soluble factors for stem cell pluripotency. Additionally, commercially available microcarriers, such as Cytodex3 and Cultispher S have been shown to support mouse embryonic stem cells expansion under stirred conditions[112]. The cells cultured in these carriers retained their pluripotency using serum-free medium.

1.4.3.2. Hyaluronic acid hydrogels

Three-dimensional hydrogels have been used to immobilize or encapsulate human ES cells for feeder-free culture including hyaluronic acid, alginate and chitosan[28-30]. Natural polymer systems have been investigated due to properties such as mechanical strength and controllable biodegradation. Dextran-based hydrogels encapsulated with regulatory factors have been synthesized and studied for their use in enhancing differentiation of hESCs[113]. Hyaluronic acid (HA) is another option for hydrogel based systems, as it is biocompatible, and is found in the ECM of connective tissue. HA receptors are also involved in signaling pathways, which are critical for a variety of cell functions[114]. Recently, Jha et al. has utilized an HA hydrogel matrix system
comprised of HA particles to control cellular adhesion and direct the differentiation of human MSCs by controlling the composition and structure of the HA hydrogel[115]. Work done by Lei et al. has shown that mouse MSCs encapsulated in modified HA hydrogels mimicked cell-matrix interactions, including cell proliferation, migration and spreading[116]. HA hydrogels have also been shown to support the maintenance of both mouse ES cells and human iPSCs[117]. Mechanical properties of hydrogels were also shown to influence cell behavior[87].

1.4.3.3. **Alginate hydrogels**

Alginate, a polysaccharide derived from brown algae, is an attractive natural polymer being used in cell culture, tissue engineering and cell therapy and shows great potential for pluripotent stem cell culture. Alginate can be crosslinked when its comprising monomers, β-D-mannuronic acid (M) and α-L-guluronic acid (G) form polymers in the presence of divalent ions, such as Ca$^{+2}$, and Sr$^{+2}$[118]. The use of Zn$^{+2}$ as a crosslinking ion has also been investigated, as it is capable of forming MM and MG blocks, as opposed to calcium, and strontium, which typically form GG blocks[119]. Work has been done investigating the effects of various crosslinking cations on the release profiles of growth factors, including vascular endothelial growth factor (VEGF)[120]. Alginate microparticles are also being explored as drug delivery vehicles. Oral administration of drugs is the preferred route of delivery, as it offers patients with a greater convenience and less pain. However, protein and peptide drugs degrade due to the acidity of the digestive enzymes in the gastrointestinal tract[121]. To circumvent this issue, Yu et al. created a composite delivery system comprised of alginate, chitosan, and pectin encapsulating bovine serum albumin (BSA) as a model drug. The various
compositions of microparticles exhibited high pH sensitivity, shedding light on site-specific drug delivery via these microparticles[122]. Drug-alginate interactions can be tuned to control the release of the drug from the alginate drug, due largely to charge polarization of the molecule[123]. The ability to incorporate drugs and growth factors in alginate microparticles, microbeads and/or microcapsules offer a promising platform for controlled release of growth factors for pluripotent stem cell maintenance and differentiation.

Alginate is a biocompatible, inert, FDA-approved, natural polymer and can mimic the hydrogel component of the ECM. It can be easily incorporated with ECM proteins (e.g., collagen, fibronectin, laminin) and RGD sequences into the hydrogel matrix or as coatings[124, 125]. Extensive studies have demonstrated the feasibility, safety and effectiveness of using alginate hydrogels for implantation and tissue engineering[126]. It has been demonstrated in our lab that alginate hydrogels can mimic stem cell microenvironments, allow the maintenance of the stemness and pluripotency of mouse ES cells, release chemokines and cytokines from neighboring cells to the microenvironment, and enable co-culture of different type of cells[127-129]. A 3D miniaturized array consisting of mouse ES cells encapsulated in 60 nL alginate gel spots has been developed for high-throughput screening of conditions for the identification of signals to control stem cell fates[130].

Alginate hydrogels are very effective in supporting stem cell proliferation and self-renewal[29, 30] and differentiation. Siti-Ismail has shown that human ES cells encapsulated in alginate hydrogels can realized prolonged feeder-free maintenance for as long as 260 days[29]. Alginate can be used for producing large quantities of EBs with a
controlled size[131]. Alginate hydrogel microcapsules have been used for propagation and direct differentiation of pluripotent stem cell into definitive endoderm[132], hepatocytes[133], insulin producing cells[134, 135], osteogenic lineages[136], cardiac cells[137], and neural lineages[138, 139].

Alginate hydrogels can be easily adapted into other systems or devices. Human ES cell-derived MSCs were encapsulated in alginate microbreads which were incorporated into macroporous calcium phosphate cement and induced osteogenic differentiation. These microbeads dramatically enhanced gene expression of osteogenic marker at day 21[140]. The incorporation of alginate microcapsules with nanoporous PCL membrane enclosed chambers has shown increased immunoprotection ability of microcapsules[141]. Additionally, encapsulation of cells in alginate hydrogels has been recognized as a powerful tool to integrate propagation and cryopreservation[27]. Surface-based vitrification showed better survival rates of intact human ES cell colonies than slow-freezing of cells encapsulated in alginate hydrogels[142]. Arginine-glycine-aspartic acid-serine (RGDS)-modified alginate could increase the survival rate of human ES cells during cryopreservation. This demonstrated the need to chemically modify alginate to increase cell attachment[143]. These findings indicate that incorporation of surface chemistries which can enhance cell attachment to alginate hydrogels may help improve the efficiency of cryopreservation.

1.5. Opportunities and Challenges of Defining Synthetic Chemistry for Pluripotent Stem Cell Culture

The pluripotent stem cell culture field as a whole has dramatically progressed in decades. The importance of surface chemistry on cell attachment and long-term self-
renewal of pluripotent stem cells in a well-defined system has recently been recognized through high-throughput screening of a library of surface chemistries. The use of polymers, both synthetic and natural, has greatly changed the stem cell culture and tissue engineering field, and will help shape it for the future. The 2D chemically defined approach is suitable for laboratory culture, but to simulate interactions in vivo, 3D feeder-free, micro- and nanostructured systems have been developed to support pluripotent stem cell self-renewal and to direct their differentiation to the desired lineage.

Synthetic nanofibers have been used for creating 3D scaffolds in a variety of cases, most often to differentiate pluripotent stem cells into specific lineages for disease treatment that is showing a great deal of promise in medical arena. These fibers have been shown to induce differentiation of stem cells into neurons, osteoblasts, cardiomyocytes, hepatic cells and adipocytes. Among nanofibers being tested, only polyamide and polymethylglutarimide showed the ability to support the self-renewal of mouse ES cells. None of these nanofibers could support the self-renewal of human ES cells in the absence of MEF. In order to make other nanofibrous materials suitable for human pluripotent stem cell culture, it is necessary to identify the optimal chemistry for propagation and differentiation of pluripotent stem cells with high efficiency and coat nanofibers with the “hit” chemistry. Natural polymers, including silk and alginate have been used as films and hydrogels respectively and both have great promise due to their biocompatibility, biodegradability and capability to mimic the ECM of organs. Similar, the ability to incorporate the optimal chemistry into these natural polymers will dramatically enhance pluripotent stem cell-substrate interactions and further strengthen their ability for pluripotent stem cell culture.
To address these challenges in defining the optimal surface chemistry for pluripotent stem cell culture, the following studies have been performed as detailed in Chapters 2-4:

**Study 1:** High-throughput screening of a comprehensive library to identify the optimal chemistries for pluripotent stem cell culture using mouse ES cells as a model system

**Study 2:** Incorporation of the selected chemistry into 3D synthetic fibrous system for pluripotent stem cell culture

**Study 3:** Incorporation of the selected chemistry into 3D natural alginate hydrogel systems for pluripotent stem cell culture
### Table 1.1 Summary of chemically-defined substrates for pluripotent stem cells.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Fabrication process</th>
<th>ES cell lines</th>
<th>Culture conditions</th>
<th>Performance</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMEDSAH</td>
<td>Graft polymerization</td>
<td>hESCs and H9</td>
<td>MEF-conditioned media, xeno-free</td>
<td>25 passages</td>
<td>Villa-Diaz</td>
</tr>
<tr>
<td>PMVE-alt-MA</td>
<td>Glass slides cleaned, silanized, and functionalized with polyacrylamide gel.</td>
<td>HUES1, HUES9, iPSCs</td>
<td>H1299, iPSC media: non-essential amino acids, glutamine, 120 mM 2-mercaptopoethanol</td>
<td>Supported long-term growth of hPSCs for 5 passages</td>
<td>Brafman</td>
</tr>
<tr>
<td>Acrylate-based monomers</td>
<td>Combinatorial chemistry</td>
<td>hESC, hIPSC</td>
<td>DMEM supplemented with 15% FBS. MEF-conditioned media, xeno-free, feeder-free</td>
<td>Supported long-term culture for 1 month</td>
<td>Mei</td>
</tr>
<tr>
<td>Synthetic peptide acrylate surfaces</td>
<td>EDC/NHS chemistry</td>
<td>H1, H7 hESCs</td>
<td>Defined media: X-VIVO + growth factors, xeno-free</td>
<td>&gt; 10 passages. Supported differentiation into functional cardiomyocytes</td>
<td>Melkoumian</td>
</tr>
<tr>
<td>APMAAm</td>
<td>Photoinitiated addition polymerization</td>
<td>H1 and H9- hOct4-pGZ hESCs</td>
<td>Defined media: mTeSR®1 with BSA and 5 mM Rock inhibitor</td>
<td>Maintained pluripotency for 22 passages</td>
<td>Irwin</td>
</tr>
<tr>
<td>Synthetic peptide substrates</td>
<td>Peptide-substituted alkanethiol conjugates that formed SAMs on gold</td>
<td>hESCs iPSCs</td>
<td>Defined mTeSR® media supplemented with Y-27632 (ROCK inhibitor)</td>
<td>Maintained pluripotency for 2-3 months</td>
<td>Klim</td>
</tr>
<tr>
<td>Laminin-511</td>
<td>Cells grown on human recombinant laminin-511</td>
<td>hESCs iPSCs</td>
<td>Defined media: O3 and H3</td>
<td>Supported self-renewal for at least 4 months</td>
<td>Rodin</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Cells grown on purified fibronectin</td>
<td>mESCs</td>
<td>DMEM supplemented with 15% FBS, 2mM glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin/ 10 µg/ml streptomycin, 1.3 x10-3 M 1-thioglycerol, 1000 U/ml LIF</td>
<td>An optimal concentration of fibronectin led to the self-renewal of mESCs</td>
<td>Hunt</td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td>Immobilized onto electrospun PLGA microfibers</td>
<td>mESCs</td>
<td>Knockout DMEM supplemented with 10% FBS, 1% non-essential amino acids, 1% L-glutamine, 0.1% 2-mercaptopoethanol, 1000 U/ml LIF</td>
<td>Enhanced growth factor signaling. Also have therapeutic potential in wound repair, and tissue engineering.</td>
<td>Meade</td>
</tr>
<tr>
<td>PDMS</td>
<td>Photolithography and casting</td>
<td>mESCs</td>
<td>DMEM-GlutaMax-1 medium supplemented with 20% FBS, 1% non-essential amino acids, 0.1 mM 2-mercaptopoethanol, 1% penicillin-streptomycin, 1000 U/ml LIF</td>
<td>Nanopatterned PDMS led to the up-regulation of self-renewal-associated proteins</td>
<td>Jeon</td>
</tr>
<tr>
<td>Synthetic polymers</td>
<td>Fabrication process</td>
<td>ES cell lines</td>
<td>Culture conditions</td>
<td>Performance</td>
<td>References</td>
</tr>
<tr>
<td>------------------------------------------</td>
<td>---------------------</td>
<td>------------------------</td>
<td>-------------------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>Polyamide (Ultra-Web)</td>
<td>Electrospinning</td>
<td>Mouse ES cells</td>
<td></td>
<td>Promoted self-renewal through activation of Rac and PI3K signaling pathways</td>
<td>Nur-E-Kamal, 2006</td>
</tr>
<tr>
<td>Peptide</td>
<td>Self-assembly</td>
<td>Mouse ES cells and MEFs</td>
<td></td>
<td>Promoted osteogenic differentiation</td>
<td>Garreta, Mari-Buye</td>
</tr>
<tr>
<td>Polyurethane acrylate mold</td>
<td>Photolithography</td>
<td>H9</td>
<td>Feeder layer of MEF-media contained DMEM + growth factors, 0.1 mM 2-mercaptoethanol, 4ng/ml human FGF-2</td>
<td>Supported neuronal differentiation</td>
<td>S. Lee</td>
</tr>
<tr>
<td>Poly(L-lactic acid) (PLLA)</td>
<td>Electrospinning</td>
<td>C17.2 mouse stem cells</td>
<td>DMEM + 15% fetal calf serum + 5% horse serum</td>
<td>Promoted the growth of neurites</td>
<td>F. Yang</td>
</tr>
<tr>
<td></td>
<td>Phase separation,</td>
<td>D3 mouse ES cells</td>
<td>DMEM supplemented with 10% FBS, 10-4 M β-mercaptoethanol, 0.224 μg/ml L-glutamine</td>
<td>Enhanced osteogenic differentiation</td>
<td>L. Smith, 2009</td>
</tr>
<tr>
<td></td>
<td>followed by freeze-dry cycles</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BG01 hESCs</td>
<td>Expanded on MEF, cultured in DMEM/F-12 media</td>
<td>Enhanced osteogenic differentiation</td>
<td>L. Smith, 2010</td>
</tr>
<tr>
<td>Poly(L-lactic acid)/ poly(lactic co-glycolic acid) blends</td>
<td>Salt-leaching</td>
<td>H9 clone hESCs</td>
<td>Feeder layer in knock-out media</td>
<td>Mechanical stimuli promoted differentiation into each of the 3 germ layers</td>
<td>Zoldan</td>
</tr>
</tbody>
</table>

**Table 1.2** Nanostructured systems for pluripotent stem cell culture
<table>
<thead>
<tr>
<th>Polycaprolactone</th>
<th>Electrosprining</th>
<th>CCE mouse ES cells</th>
<th>Feeder-free</th>
<th>Supported adipogenic differentiation</th>
<th>X Kang</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CE3 and RW4 mESCs</td>
<td>DMEM + 10% New born calf serum + 10%FBS + 0.3mM nucleosides</td>
<td>Induced differentiation of mESCs into neurons, oligodendrocytes and astrocytes</td>
<td>J Xie</td>
</tr>
<tr>
<td>Polycaprolactone/Calcium-deficient hydroxyapatite</td>
<td>Electrosprining</td>
<td>R1 mESCs</td>
<td>DMEM+ 15% FCS, 20 mM Hepes</td>
<td>mESCs remained undifferentiated and pluripotent</td>
<td>Bianco</td>
</tr>
<tr>
<td>Polyethersulfone</td>
<td>Electrosprining</td>
<td>mESCs</td>
<td>DMEM supplemented with 20% Knockout serum + antibiotics</td>
<td>Feeder-free culture method was established for mESCs</td>
<td>Hashemi</td>
</tr>
</tbody>
</table>
### Table 1.3 3D Microsystems for stem cell culture.

<table>
<thead>
<tr>
<th>Natural polymers</th>
<th>Fabrication process</th>
<th>ES cell lines</th>
<th>Culture conditions</th>
<th>Performance</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyaluronic acid</td>
<td>Photopolymerization</td>
<td>H9, H13 and H1 hESCs</td>
<td>MEF-conditioned media</td>
<td>Maintained undifferentiated morphology for 20 days; capacity of angiogenic differentiation after 1 week</td>
<td>Gerecht</td>
</tr>
<tr>
<td>Alginate</td>
<td>Microencapsulation</td>
<td>CEE14</td>
<td>DMEM supplemented with 10% FCS, 1% penicillin/streptomycin, 0.1% β-mercaptoethanol 1000U/ml LIF</td>
<td>Supported differentiation of pancreatic cells within alginate beads</td>
<td>N Wang</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E14/Tg2a murine ESCs</td>
<td>1:1 mixture of HepG2 medium and ES maintenance media, then osteogenic media for differentiation</td>
<td>Supported osteogenic differentiation after 21 days</td>
<td>Y Hwang</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Envy hESCs</td>
<td>DMEM supplemented with 20% knockout serum replacer, 4ng/ml basic fibroblast growth factor</td>
<td>Directed hESC differentiation into endoderm lineage</td>
<td>Chayosumrit</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCE mESCs</td>
<td>DMEM supplemented with 15% FBS, 10 ngd/ml LIF</td>
<td>Supported differentiation of all three lineages</td>
<td>Raof, 2011</td>
</tr>
<tr>
<td>Alginate/Gelatin</td>
<td>Microencapsulation</td>
<td>H1 hESCs</td>
<td>Serum-free (20% knockout serum replacement), change media every 3-4 days</td>
<td>Maintained hESC pluripotency for 260 days</td>
<td>Siti-Ismail</td>
</tr>
<tr>
<td>Alginate/Chitosan</td>
<td>Thermally induced phase separation + solvent sublimation</td>
<td>BG01V hESCs</td>
<td>5% knockout serum replacement + 15% FBS, change media daily</td>
<td>Maintained hESC pluripotency for 21 days</td>
<td>Z Li</td>
</tr>
<tr>
<td>Collagen/Matrigel</td>
<td>Casting molds</td>
<td>ES-D3 mESCs</td>
<td>DMEM supplemented with 15% BSA, 1000 U/ml LIF</td>
<td>Supported differentiation into cardiomyocytes</td>
<td>J Zhou</td>
</tr>
<tr>
<td>Silk</td>
<td>Micro-sponge formation</td>
<td>H9</td>
<td>DMEM supplemented with 10% FBS and 5 ng/ml FGF2</td>
<td>Supported differentiation into tendons</td>
<td>JL Chen</td>
</tr>
</tbody>
</table>
CHAPTER 2
Substrate Chemistry Screening for Pluripotent Stem Cell Attachment and Maintenance

2.1. Introduction

A chemically defined robust synthetic substrate is desirable for the development of a uniform culture system for pluripotent stem cell expansion without spontaneous differentiation[31]. With the aim of synthesizing a completely defined substrate for expanding ES cells in an undifferentiated state, several surfaces have recently been reported in the literature including peptide-derived substrata[32, 33] or wholly synthetic coatings, such as polymers, using both rational[34] and high throughput combinatorial approaches[35, 36]. The challenge is to identify the optimal surface chemistry of the substrata for ES cell attachment and maintenance.

Maintaining pluripotency during expansion of embryonic stem cells is necessary for exploiting the full potential of stem cells for regenerative medicine, developmental biology and drug discovery[144]. The nanostructured surface plays a critical role in maintaining stem cell pluripotency[54, 145, 146]. In particular, engineering the surface chemistry at the nanoscale has great potential to manipulate and regulate cell behaviors [55-57]. Patterning extracellular matrix proteins have been shown to control neural stem cell differentiation[147]. Additionally, micropatterning biodegradable substrates have been shown to modulate kinase pathways in human mesenchymal stem cells[148]. A fully defined, cost effective, scalable, robust, reproducible, off-the-shelf synthetic

\[ \text{Part of this chapter is previously appeared as: M.R. Zonca Jr., P. Yune, C. Heldt, G. Belfort and Y. Xie “High-Throughput Screening of Substrate Chemistry for Embryonic Stem Cell Attachment, Expansion and Maintaining Pluripotency” Macromolecular Bioscience 13(2): 177-190 (2013), with copyright permission from John Wiley and Sons.} \]
substrate is desirable for the development of a simple, broadly applicable, uniform culture system for pluripotent stem cell expansion without differentiation and/or directing differentiation of ES cells[31]. Since cell attachment plays an essential role in the control of stem cell pluripotency[38], the search for surface chemistries that affect such behavior and prevent formation of unwanted embryoid bodies (EBs) is critical. Both rational and combinatorial approaches have been used to search for surfaces with desirable properties. Recent developments in cell culture techniques have led to the development of chemically-defined media and feeder-free ES cell culture systems that employ animal- or human-derived ECM proteins that coat the culture substrata, including ECM (e.g., Matrigel, Geltrex) and individual ECM proteins (i.e., fibronectin, vitronectin, laminin, collagen, gelatin)[11, 19, 149-151], and 3D polysaccharide scaffolds for feeder-free cultures[28-30, 152]. All these biological materials are naturally derived and are characterized by heterogeneity, high lot-to-lot variability, low reproducibility and limited scalability. Hence, chemically defined surfaces are in great demand for precise control of self-renewal and differentiation of ES cells. To simulate the natural environment, Matrigel was developed and is currently the most widely used substrate[39, 66]. It comprises basement membrane components (laminin, collagen IV, heparan sulfate proteoglycans and entactin) extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcomas and matrix degrading enzymes, inhibitors, numerous growth factors, and a broad variety of other proteins[40, 66]. Thus, Matrigel is complex, inconsistent from batch to batch, has the potential to be pathogenic as it is derived from an animal, and could bind to a variety of cell–surface receptors complicating reproducibility[32].
Clearly, we need a simpler, more reproducible, less expensive scalable substrate than Matrigel.

With the aim of synthesizing a completely defined substrate for expanding ES cells in an undifferentiated state, several surfaces have recently been reported in the literature including those that expose selected peptides to the ES cells[32, 33] or those that are wholly synthetic such as polymers[34-36, 66]. Rational approaches using selected binding peptides and variants thereof (RGD tripeptide, bone sialoprotein, vitronectin, fibronectin, laminin, heparin) with cell culture supplement like ROCK inhibitor[32] have been very successful in maintaining pluripotency with expansion, but suffer from high cost and difficulty of scale-up. With respect to polymeric substrates, both rational[66] and high throughput combinatorial approaches[35, 36] have been used. Most of these studies have used the expression of known human ES cell markers (Oct4, SSEA-4, Tra-1-60, Nanog and/or SOX2) and the potential of differentiation into cell lineages of all three germ layers (ectoderm, mesoderm and endoderm) to confirm the maintenance of pluripotency after culturing ES cells on these synthetic substrates. Some have used specific blocking agents (e.g., enzyme chondroitinase ABC to catalyze the hydrolysis of glycosaminoglycans, antibodies against vitronectin-binding integrin α_vβ_3 and α_vβ_5) to search for the dominant cell binding protein. Most have also used different media to grow and maintain the cells, such as defined basal medium mTeSR2 supplemented with anti-ROCK, StemPro which contains bFGF, IGF1, Heregulin and Activin A or X-VIVO 10 supplemented with bFGF and TGF-β1, while others have used MEF conditioned media or basal media supplemented with 10-15% FBS. All these
differences, including the use of different cell lines, seriously complicate comparison of performance between different surfaces.

Here we present a simple and novel high-throughput approach to identify the optimal, synthetic, covalently-grafted polymeric substratum for mouse ES cell attachment, self-renewal, expansion and maintenance of pluripotency among the library of 66 surface chemistries. We validated the optimal surface chemistry by comparing it with those reported in the literature for human ES cell adhesion and expansion. We further investigated the mechanism by which the surface chemistry interacts with ES cells. A flowchart of our work is shown in Figure 2.1.
Figure 2.1 High-throughput synthesis and screening of surface chemistries for ES cell attachment and long-term maintenance. (a) Schematic illustration of experimental setup, (b) Chemical reactions during photo-induced monomer graft polymerization.
2.2. Experimental Section

2.2.1. Membranes and Monomers

Poly(ether sulfone) (PES) 96-membrane plates (Seahorse Labware, Chicopee, MA) were used as base membranes for creating the library of surface chemistry via photo-induced graft polymerization. Sixty-six water- or ethanol-soluble vinyl monomers (Sigma-Aldrich, St. Louis, MO) were used as-received without further purification (Table 2.1). PES sheets were kindly provided by Dr. Eberhard Wuenn (Sartorius Stedim Biotech, Goettingen, Germany) as the PES (precursor) substrates for long-term maintenance of self-renewal studies.

Table 2.1 List of library of monomers used to synthesize and screen surface chemistries for ES cell attachment.

<table>
<thead>
<tr>
<th>#</th>
<th>Name</th>
<th>Structure</th>
<th>FW</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>Methyl methacrylate</td>
<td></td>
<td>100.12</td>
<td>HPO Methacrylate</td>
</tr>
<tr>
<td>2*</td>
<td>Ethyl methacrylate</td>
<td></td>
<td>114.14</td>
<td>HPO Methacrylate</td>
</tr>
<tr>
<td>3*</td>
<td>Butyl methacrylate</td>
<td></td>
<td>140.20</td>
<td>HPO Methacrylate</td>
</tr>
<tr>
<td>4*</td>
<td>Isobutyl methacrylate</td>
<td></td>
<td>142.20</td>
<td>HPO Methacrylate</td>
</tr>
<tr>
<td>5*</td>
<td>tert-Butyl methacrylate</td>
<td></td>
<td>142.20</td>
<td>HPO Methacrylate</td>
</tr>
<tr>
<td>6*</td>
<td>Hexyl methacrylate</td>
<td></td>
<td>170.25</td>
<td>HPO Methacrylate</td>
</tr>
<tr>
<td>7*</td>
<td>2-Ethylhexyl methacrylate</td>
<td></td>
<td>198.30</td>
<td>HPO Methacrylate</td>
</tr>
<tr>
<td>8*</td>
<td>2-(Methylthio)ethyl methacrylate</td>
<td></td>
<td>160.23</td>
<td>PEG</td>
</tr>
<tr>
<td>9*</td>
<td>Allyl methacrylate</td>
<td></td>
<td>126.15</td>
<td>HPO Methacrylate</td>
</tr>
</tbody>
</table>

Monomers dissolved in reagent grade ethanol are marked with a star (*) after monomer number. All other monomers are dissolved in DI water.
<table>
<thead>
<tr>
<th></th>
<th>Chemical Name</th>
<th>Molecular Formula</th>
<th>Molecular Weight</th>
<th>Functional Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>2,2,2-Trifluoroethyl methacrylate</td>
<td></td>
<td>168.11</td>
<td>Other</td>
</tr>
<tr>
<td>11</td>
<td>2-(Methacryloyloxy)ethyl acetoacetate</td>
<td></td>
<td>214.22</td>
<td>Other</td>
</tr>
<tr>
<td>12</td>
<td>2-Butoxyethyl methacrylate</td>
<td></td>
<td>186.25</td>
<td>PEG</td>
</tr>
<tr>
<td>13</td>
<td>3-(Acryloyloxy)-2-hydroxypropyl methacrylate</td>
<td></td>
<td>214.22</td>
<td>Hydroxyl</td>
</tr>
<tr>
<td>14</td>
<td>Cyclohexyl methacrylate</td>
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<td>168.23</td>
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<td>15</td>
<td>Phenyl methacrylate</td>
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<td>162.19</td>
<td>Aromatic</td>
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<td>Glycidyl methacrylate</td>
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<td>Hetero Ring</td>
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<tr>
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<td>Tetrahydrofurfuryl methacrylate</td>
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<td>Hetero Ring</td>
</tr>
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<td>Benzyl methacrylate</td>
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<td>Aromatic</td>
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<td>Isobornyl methacrylate</td>
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<td>222.32</td>
<td>HPO Methacrylate</td>
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<td>Trimethylsilyl methacrylate</td>
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<td>158.27</td>
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<tr>
<td>21</td>
<td>3-(Trimethoxysilyl)propyl methacrylate</td>
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<td>248.35</td>
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<td>2-Isocyanatoethyl methacrylate</td>
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<td>2-Ethoxymethyl methacrylate</td>
<td></td>
<td>158.19</td>
<td>PEG</td>
</tr>
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<td>24</td>
<td>Hydroxypropyl methacrylate</td>
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<td>144.17</td>
<td>Hydroxyl</td>
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<td>3-Chloro-2-hydroxypropyl methacrylate</td>
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<td>Hydroxyl</td>
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<td>Ethylene glycol phenyl ether methacrylate</td>
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<td>Aromatic</td>
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<td>Ethylene glycol dimethacrylate</td>
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<td>198.22</td>
<td>PEG</td>
</tr>
<tr>
<td>28</td>
<td>Ethylene glycol dicyclopentenyl ether methacrylate</td>
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<td>262.34</td>
<td>HPO Methacrylate</td>
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<tr>
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2.2.2. Synthesis of Monomer-modified PES Membranes

A high-throughput platform (HTP) approach was applied to modify the PES membranes in a 96-well plate using photo-induced graft polymerization as described previously[153]. Briefly, the membranes were irradiated with UV light at 300 nm, forming reactive radical sites. Monomers dissolved in ethanol or water were covalently bonded to the radical sites for subsequent free-radical polymerization. Membrane wells incubated with ethanol were used as the control for monomers dissolved in ethanol while those incubated with water were used as the control for monomers dissolved in water.
Each 96-membrane plate accommodated 22 monomers and two controls in four replicates. In this way, a library of 66 surface chemistries was established in three 96-membrane plates with four replicates for each monomer grafted surface. These modified plates were stored in deionized water. Prior to cell culture, these 96-membrane plates were first sterilized with 70% ethanol for 5 min, followed by washing with sterilized PBS three times.

2.2.3. ES Cell Culture

Mouse CCE embryonic stem (ES) cells were obtained from StemCell Technologies (Vancouver, BC, Canada)[154, 155]. ES cells were cultured in gelatin-coated tissue culture flasks and fed with ES maintenance media, which is comprised of the following components: Dulbecco’s Modified Eagle’s Medium (DMEM with 4.5 g/l D-glucose) supplemented with 15% (v/v) fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.1 mM MEM non-essential amino acids, 10 ng/ml murine recombinant leukemia inhibitory factor (LIF; StemCell Technologies, Vancouver, Canada), 0.1 mM monothioglycerol, 2 mM L-glutamine, and 1 mM sodium pyruvate (Sigma Aldrich, St. Louis, MO) as described previously[104]. LIF is typically used to maintain mouse ES cells in an undifferentiated state. Thus, for the formation of EBs and spontaneous differentiation experiments, growth medium without LIF was used. For the cell attachment assay and cell expansion assay, five thousand CellTracker Green-labeled or unlabeled mouse ES cells were seeded onto each well in 96-membrane plates and cultured in a CO₂ incubator at 37 ºC for 24 hours. In order to examine the long-term maintenance of self-renewal ability on the optimal chemical substrate, 20,000 ES cells were seeded onto each disk of substrates, which are grafted with the optimal chemistry
and fitted in the 24-well plate, and cultured in ES maintenance media for 7 days without any subculture or subcultured for 7 passages.

2.2.4. Cell Attachment Analysis

A high-throughput cell attachment analysis was established. Mouse ES cells were first labeled with Cell Tracker Green™ CMFDA (5-chloromethylfluorescein diacetate, Molecular Probes, Life Science Technologies) as described previously[156]. Briefly, Mouse ES cell pellets were stained with Cell Tracker Green™ CMFDA working solution (5 µM) in DMEM and incubated at 37°C for 30 minutes to label ES cells with green fluorescence. The stained cells were then seeded onto sterilized 96-membrane plates at a density of 5,000 cells/membrane and incubated at 37 °C, 5% CO₂ for 24 hours. Then, these 96-membrane plates were washed with PBS three times to remove non-attached and loosely attached cells on each membrane. The fluorescence intensity of Cell Tracker Green™-labeled ES cells, which remained attached on 96-membrane plate, was measured using a Tecan Infinite M200 plate reader (Tecan US, Research Triangle Park, NC) under the excitation wavelength of 494 nm and emission wavelength of 528 nm. The integration time was 20 µs and a total of 25 flashes were taken. The Cell Attachment Index (CAI) was defined and calculated by dividing the fluorescence intensity of ES cells in each monomer-grafted membrane to the average fluorescence intensity of ES cells on corresponding control membranes. The experiment was repeated in duplicate. In order to reduce the systematic errors, the arrangement of monomers in each 96-membrane plate was changed every time. The codes for each set of three 96-membrane plates were shown as Figures 2.2a-c.
Figure 2.2a-c Plate setup for monomer grafting of ES cell attachment for three separate experiments. Yellow and blue blocks correspond to ethanol and water soluble monomers, respectively.
Figure 2.2 Continued
2.2.5. Resistance and Permeability Measurements

Permeability and resistance measurements were performed on all 66 membranes as described previously[153]. Initially, the resistance of each membrane was measured by mounting the 96-well plate onto a vacuum manifold. A constant transmembrane pressure (9.8 psi) was applied by a vacuum pump. The water and PBS flux were measured, followed by the addition of 300 µl of foulant solution. After vacuum application for 4 minutes, the wells were emptied and PBS flux was measured again, followed by a DI water measurements. Additionally, during filtration, a receiver plate was placed underneath the 96-well plate in order to collect the permeate from each well to measure solute concentration and volume. These values were further used to calculate the flux properties of each membrane.

2.2.6. Sessile Contact Angle Measurements

The sessile contact angle of each surface was measured using a SIT-66 goniometer (Dage-MTI, Michigan, IN). Briefly, 20 µl of distilled water came into contact with the surfaces, which were randomly cut from membrane sheets. Sessile contact angle measurements were performed three times, and the values were averaged.

2.2.7. Atomic Force Microscopy (AFM) Measurement of Surface Roughness

Surface roughness was characterized by atomic force microscopy (Veeco Biocatalyst, Bruker, Billerica, MA). Random pieces of each membrane were cut from membrane sheets, and a 2 µm x 2 µm scan size was performed on each membrane in triplicate. A SiN cantilever was used to take the measurements, with a nominal spring constant (k) of 0.4 N/m. The value for the surface roughness was the average of these three measurements.
2.2.8. Scanning Electron Microscopy (SEM)

Mouse ES cells were seeded onto representative membranes #57, 44, 33 and a water control at a density of \(2 \times 10^5\) cells/membrane, which was secured in a transwell insert. After overnight culture, samples were washed with PBS, fixed with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) containing 0.1 M sucrose (Sigma Aldrich). After dehydration in serial gradients of ethanol, the samples were dried by adding hexamethyldisilazane (HMDS) using graded HMDS in ethanol (25%, 50%, 75%, 100%, 100%, and 100%), (Ted Pella, Inc., Redding, CA). Samples were observed using an environmental scanning electron microscope (Nova Nano SEM 600, FEI, Hillsboro, OR).

2.2.9. MTT Assay and Cell Expansion Capacity

Cells were seeded at 5,000 cells/well in a 96-well plate grafted with 10 monomers and replicated in 8 wells for each monomer as shown in Table 2.1. ES Maintenance media was changed every 2 days, and after 7 days an MTT assay was performed to investigate cell expansion. To make a cell number standard curve, 5,000, 10,000, 20,000 and 40,000 ES cells were seeded in control wells of PES membrane in the same 96-well filter plate on Day 7. Briefly, 10 µl of MTT Labeling Reagent was added to each well and the plate was incubated for 4 hours at 37 ºC. After incubation, 100 µl of solubilization reagent was added to each well and the plate was incubated overnight. Absorption readings were taken at 560 nm and 700 nm (as the reference) on a Tecan Infinite M200 plate reader (Tecan US, Research Triangle Park, NC). The absorption of each well was converted to cell number by the use of a standard curve. Cell expansion
was quantified in fold by dividing the average cell number on day 7 by the initial cell number.

2.2.10. Optical Microscopy Observation of Cell Morphology

The morphology of ES cells and embryoid bodies were observed under Nikon inverted TS 100 F microscope (Micro Video Instruments, Avon, MA).

2.2.11. Immunocytochemistry of Pluripotency Markers

The maintenance of stem cell pluripotency after culturing on the optimal membrane (#57) was characterized using immunocytochemistry analysis of the stem cell marker, Oct4 and SSEA-1. After an overnight culture, mouse ES cells were washed with PBS, trypsinized, and collected followed by seeding onto glass coverslips. These ES cells were incubated in maintenance medium containing LIF to allow cell adhesion, then fixed with 4% paraformaldehyde. Fixed cells were then rinsed with PBS, permeabilized in 0.1% Triton-X-100, and blocked with 5% FBS in PBS. ES cells were incubated with primary antibody for anti-Oct4 produced in rabbit (Sigma Aldrich, St. Louis, MO) detected by the anti-rabbit Alexa Fluor® 488 and co-stained with anti-SSEA-PE (Sigma Aldrich, St. Louis, MO). Cells were also tri-stained with 4',6-diamidino-2-phenylindole (DAPI) to reveal the nuclei in blue and to reveal the total cell population and verify the co-localization of Oct-4 in nuclei. Samples were observed using an epifluorescence microscope (Nikon ECLIPSE 80i, Nikon, Melville, NY) with filter sets for FITC and Texas Red and UV to reveal the Oct4 in green, SSEA-1 in red, and nuclei in blue.

2.2.12. Formation of Embryoid Bodies

In order to test the ability to form EBs, a hanging drop approach was used to facilitate the formation of EBs. Mouse ES cells grown on the optimal membranes were
trypsinized and collected after culturing overnight. One thousand ES cells in 20 µl stem cell culture medium without LIF were placed on the lid of a petri dish. Ten milliliters of PBS was added to the petri dish to maintain the humidity. The lid was placed over the petri dish, allowing the hanging drop to form. After 2 days, the hanging drops were observed under a Nikon inverted TS 100 F microscope (Micro Video Instruments, Avon, MA) to examine the formation of EBs.

2.2.13. Examination of Differentiation Potential in vitro

Pluripotent embryonic stem cells have the ability to form cell types of all three germ layers (ectoderm, mesoderm, and endoderm). To evaluate the differentiation potential, mouse ES cells were grown on the optimal membrane in LIF-free growth medium for 7 days to allow for spontaneous differentiation. Samples were fixed, permeabilized, and blocked, as described above. ES cells were incubated with three primary antibodies, for ectoderm (anti-nestin), mesoderm (anti-Myf-5), and for endoderm (anti-PDX-1) (Santa Cruz Biotechnology, Santa Cruz, CA), detected by secondary antibodies, Alexa Fluor® 594, Alexa Fluor® 488, and Alexa Fluor® 647 (Invitrogen, Carlsbad, CA)[127, 157]. Samples were observed using an epifluorescence microscope (Nikon ECLIPSE 80i, Nikon, Melville, NY) using the filter sets for FITC, Texas Red, and Alex 647.

2.2.14. Integrin Blocking Assay

Briefly, 100,000 ES cells were incubated with 10 µg/ml of the antibodies against integrin β1, αβ5, α5 and α6 (BD Biosciences, Sparks, MD) in PBS, respectively, for 30 minutes at 37 °C. The antibodies were monoclonal, anti-human, and were produced in either mice or rats. ES cells incubated with PBS were used as control. After blocking
with integrin antibodies, cells in PBS were plated onto the membranes and incubated for 1 hour at 37 °C. After the incubation period, the membranes were washed three times with 1X PBS and the supernatants containing unbound cells were collected. After centrifugation at 1200 rpm for 5 minutes, the supernatant was removed and the unbounded cells were counted using a TC10 automated cell counter (Bio-Rad, Hercules, CA). The percentage of unbound ES cells was calculated and normalized with control.

2.2.15. Statistical Analysis

Data analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey multiple comparison post test. Data was expressed as the mean ± standard deviation, and p < 0.05 was considered significant.

2.3. Results

2.3.1. High-Throughput Screening of Substrate Chemistry for Mouse ES Cell Adhesion

This high throughput platform has been successfully used to discover new and confirm previously known protein resistant surfaces[153, 158, 159]. First, a comprehensive library of 66 monomers were chosen to represent 9 different chemical functional groups – hydrophobic methacrylates (#s 1-28 from Table 2.1), amines (#s 51, 52 and 55-58), hetero rings (#s 16, 17 and 38-40), aromatics (#s 15, 18, 26 and 37), hydroxyl (#s 13, 21, 24, 25, 54, 64 and 66), zwitterionic (#s 59-63), acids (#s 42-50 and 65), PEGs (#s 8, 12, 23, 27 and 29-35) and others (#s 10, 11, 20, 22, 36, 41 and 53). The 66 monomers were covalently grafted onto light sensitive poly(ether sulfone) membranes in three 96-well filter plates through photo-induced graft polymerization using our high throughput synthesis and screening method. PES membranes at the base of each filter
well were activated with ~300 nm UV light in the presence or absence (control) of added monomer dissolved in ethanol or water. Each 96-well-filter plate contained 22 monomers and two controls (without monomer) in four replicates. After UV-irradiation at ~300 nm for 30 seconds, monomer-graft induced polymerization occurred through free-radical polymerization, resulting in surface coating of the PES membranes[153, 160, 161]. Reproducibility and scalability of the high-throughput synthesis method was previously validated[153, 159]. Thus, a simple, fast, high-throughput platform was used to produce a library of polymer-coated membranes with a variety of 66 surface chemistries.

Pluripotent mouse CCE ES cells in ES maintenance media were used as a model to test the propensity of cells to attach and retain pluripotency when placed onto a library of 66 modified PES surfaces on three 96-well membrane/filter plates (in quadruplets). The mouse ES cells were labeled with Cell Tracker Green™ CMFDA (5-chloromethylfluorescein diacetate, Invitrogen, Carlsbad, CA) as described previously[156], and seeded at a density of 5,000 cells/well into each filter well of three 96-filter plates. After culturing overnight with ES cells, each membrane was washed with PBS three times to remove non-attached and loosely attached cells. The fluorescence intensity of Cell Tracker Green™ CMFDA-labeled ES cells that remained attached on the membrane was measured using a fluorescent plate reader (Tecan Infinite M200, Tecan US, Research Triangle Park, NC) with an excitation wavelength of 494 nm and an emission wavelength at 528 nm. The cell attachment index is obtained by dividing the fluorescence intensity of ES cells in each filter well with modified surfaces by the average fluorescence intensity on corresponding control membranes. The
experiment was repeated twice with different arrangements of surface chemistries on the 96-well filter plate (Figures 2.2a-c). The average CAI and standard deviation was calculated based on the aggregated data of all three sets of experiments.

The cell adhesion results are summarized in Figure 2.3a and are plotted with decreasing CAI values versus different grafted surface chemistries. The surface chemistries exhibiting the highest and lowest CAI values were amines (#s 57, 55, and 56 from Table 2.1) and an acid (#44), and PEGs (#s 33 and 32), respectively. The optimal membranes, #57, N-[3-(Dimethylamino)propyl]methacrylamide (DMAPMA), and #44, 2-Acrylamidoglycolic acid, with the largest CAI values were significantly higher than the control surfaces (p<0.05), while poly(ethylene glycol) methyl ether methacrylate (PEG, #33) exhibited the lowest CAI. In order to compare the performance of different groups of chemistries with respect to mouse ES cell attachment, a histogram of surface chemistries was generated based on the categories and values of CAI, e.g. < 0.95, 0.95-1.0, 1.0-1.2, > 1.2 (Figure 2.3b). Amine and acid functional groups favored cell adhesion (CAI > 1.2) and mainly occupied the top two categories, and PEGs did not support cell adhesion (CAI < 0.95) and mainly occupied the two lowest categories. This is as expected since amines are known to bind cells and PEGs effectively repel proteins that may facilitate cell binding[162, 163]. Additionally, most of the methacrylates and all zwitterionic groups in our library had CAI values between 1.0-1.2. In Figure 2.3c, the relationship between permeability and CAI is observed. For our studies, we are interested in chemistries that exhibit high CAI values, and low permeability values.
Figure 2.3 a) Cell attachment index for ES cells grown on monomer-modified PES membranes with 66 surface chemistries. Membranes 44 and 57 were statistically significant with p<0.05 in comparison to PES control. b) Histogram of monomer chemistries by monomer types. c) Cell attachment index plotted vs. permeability for all 66 monomer-modified membranes.
2.3.2. Characterization of Chemically Modified PES Surfaces

Three modified membranes (#s 57, 44 and 33), which gave rise to the highest and lowest CAI values, were chosen for surface characterization. The PES membrane treated with distilled water was used as a control. These surfaces were characterized by measuring their wettability ($\cos \theta_s$, where $\theta_s$ is the sessile contact angle), as well as their roughness ($t$, nm). This method measures the contact angle formed between the solid-liquid and liquid-gas interfaces. Table 2.2 summarizes the average of 3 independent measurements for each surface. From these results, we see that the chemically modified PES surfaces are more wettable (hydrophilic) than the PES control surface. Membrane #44 is more hydrophilic than others while the values of contact angle for membrane # 57 and 33 are similar. It has been shown that hydrophobicity of substrates affect cell adhesion[37]. Our results indicated that the hydrophobicity is not the only factor influencing ES cell adhesion. Surface roughness after chemical modification of PES membrane using AFM was also measured (Table 2.2). We used a SiN cantilever to probe the membrane surfaces. The AFM image showed that the membrane #57 which was grafted with DMAPMA exhibited the smoothest topography (compare Figure 2.4d to 2.4a-c). Again, the values given are the averages of 3 independent measurements for each surface. It is possible that the high apparent wettability of #44 was due to it much greater roughness[164].
Figure 2.4 Atomic force microscopy was used to quantify surface roughness. (a) Water control surface. (b) PEG (#33) surface. (c) 2-acrylamidoglycolic acid (#44) surface. (d) DMAPMA (#57) surface.
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<th>Wettability Cos $\theta_s$ (→)</th>
<th>RMS Surface Roughness $t$ (nm)</th>
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<td>Poly(ethylene glycol) methyl ether methacrylate MW = 475 g/mol (#33)</td>
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<td>PES Membrane (Control)</td>
<td>54.1 ± 0.4</td>
<td>0.586</td>
<td>16.3 ± 1.8</td>
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### 2.3.3. Morphology of ES Cells Attached on Chemically Modified PES Surfaces

To further confirm mouse ES cell attachment on the modified/grafted PES membranes, three modified membranes (#s 57, 44 and 33) were chosen in order to examine the cell morphology. These three surfaces represent high (#s 57 and 44) and low (#33) cell attachment in the cell attachment assay (see Figure 2.3a). Since the three membranes were modified with water-soluble monomers, water-treated PES membranes were used as a control. The membranes were scaled-up from a 96-filter well (area 50 mm$^2$/well) to a disk of 16 mm in diameter (area = 200 mm$^2$), and were fit into wells of 24-well plates. $2 \times 10^4$ ES cells were seeded onto each membrane and cultured overnight.
at 37°C followed by triplicate washing with PBS. The experiment was run in duplicate and repeated twice. First, cell morphology was observed using an environmental scanning electron microscope (Nova Nano SEM 600, FEI, Hillsboro, OR). DMAPMA had the greatest cell attachment and PEG exhibited the lowest cell attachment. Mouse ES cells grown on DMAPMA formed large cell colonies with striking outlines, which is characteristic of undifferentiated ES cells (Figure 2.5a). Membrane #44 exhibited cell adhesion with a well-spread morphology (Figure 2.5b). Cells grown on membrane #33 showed cell size smaller than normal ES cells and did not spread at all (Figure 2.5c). The PES membrane served as control and exhibited limited cell attachment (Figure 2.5d). These results support the contention that DMAPMA promotes the strongest cell attachment. In Figure 2.5e, we show that at least two different ES cell lines (CCE and GFP) exhibited high and low CAI values with DMAPMA and PEG, respectively, confirming earlier results with only one ES cell line (CCE) (Figure 2.3a).
Figure 2.5 SEM images of mouse ES cells attached to a) membrane #57, b) membrane #44, c) membrane #33 and d) PES control. e) Cell attachment index on different synthetic surfaces for both CCE and GFP mouse ES cells. Scale bar for image a) is 5 μm, b-d) 10 μm.
2.3.4. Validation of ES Cell Attachment and Expansion on DMAPMA

In order to compare our results from mouse ES cells with those reported in the literature with human ES cells, we grafted and measured the CAI values of DMAPMA, PEG and a series of other eight organic monomers, which are similar to or exactly the same as those reported as hits by others[34-36, 66] (See Table 2.3). The results show that DMAPMA has the highest value of 1.5±0.3 for the CAI, while all the others vary between 1.4±0.3 and 0.9±0.1 for ethylene glycol dicyclopentenyl ether acrylate and pentaerythritol triacrylate or PEG (#33), respectively (see Table 2.3). Interestingly, Villa-Diaz et al. have reported that zwitterionic PMEDSAH polymer coating support long-term human ES cell maintenance[34] and Mei et al. have demonstrated that polymer with high acrylate content support the clonal growth from a single disassociated human ES cells[35].

In order to examine cell expansion capacity, DMAPMA, PEG and a series of other eight organic monomers as shown in Table 2.1, were grafted onto PES membranes in one 96-well filter plate through photo-induced graft polymerization as described in Materials and Methods and replicated in eight wells. CCE ES cells (5,000 cells/well) were seeded on these membranes and propagated for 7 days followed by an MTT assay to measure cell viability. The cell expansion capacity was quantified as the fold increase of cell propagation which is calculated by dividing the viable cell number on day 7 by the initial cell number. The results show that DMAPMA has the highest cell expansion capacity with a value of 11.5±5.6 for the fold increase of cell propagation, which is statistically significantly higher than all other substrate chemistries (p<0.05). All the others vary between 2.3±0.5 and 7.3±3.1 for PEG (#33) and 2-(methacryloyloxy) ethyl
dimethyl-(3-sulfopropyl)ammonium hydroxide (#60) which is similar to PMEDSAH, respectively (see Table 2.3). Another recently reported polymer surface, aminopropylmethacrylamide (APMAAm) which shares a similar chemical backbone as DMAPMA, has shown to support long-term self-renewal of human ES cells for over 20 passages[66]. Altogether, we discovered that DMAPMA supported the highest cell attachment and expansion capacity with mouse ES cells in comparison with those surface chemistries reported in the literature and PEG gave rise to the lowest cell attachment and expansion capacity.

Table 2.3 Chemical structures and cell attachment indices for surfaces reported in literature and novel surfaces discovered in this work.

<table>
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<th>Author</th>
<th>Literature Surfaces</th>
<th>Our Validation</th>
<th>Cell Expansion (Fold of cell number increase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irwin et al.</td>
<td>N-(3-Aminopropyl)methacrylamide hydrochloride (APMAAm)</td>
<td>N-[3-((\text{Dimethylamino}))propyl]methacrylamide (#57)</td>
<td>1.45±0.30</td>
</tr>
<tr>
<td>Brafman et al.</td>
<td>poly(methyl vinyl ether-alt-maleic anhydride) (PMVE-alt-MA)</td>
<td>((R))-(\alpha)-Acryloyloxy-(\beta,\beta)-dimethyl-(\gamma)-butyrolactone (#40)</td>
<td>1.13±0.19</td>
</tr>
<tr>
<td>Mei et al. September 2010</td>
<td>Poly(propylene glycol) diacrylate (Mn = 540)</td>
<td>Poly(propylene glycol) diacrylate (Mn = 800)</td>
<td>1.22±0.18</td>
</tr>
<tr>
<td>-------------------------</td>
<td>---------------------------------------------</td>
<td>---------------------------------------------</td>
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</tr>
<tr>
<td>Ethylene glycol dicyclopentenyl ether acrylate</td>
<td>Ethylene glycol dicyclopentenyl ether acrylate</td>
<td>1.35±0.28</td>
<td>3.9±1.0</td>
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<tr>
<td>4-tert-Butylcyclohexyl acrylate</td>
<td>4-tert-Butylcyclohexyl acrylate</td>
<td>0.98±0.13</td>
<td>3.7±1.3</td>
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<td>2-Hydroxy-3-phenoxypropyl acrylate</td>
<td>2-Hydroxy-3-phenoxypropyl acrylate</td>
<td>1.10±0.23</td>
<td>3.6±0.9</td>
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<tr>
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<td>Pentaerythritol triacrylate</td>
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<td>3.2±0.6</td>
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<td>Villa-Diaz et al. June 2010</td>
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<td>Poly(ethylene glycol)methyl ether methacrylate (#33)</td>
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<td>1.05±0.16</td>
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<td>Villa-Diaz et al. June 2010</td>
<td>Poly(ethylene glycol)methyl ether methacrylate (PEGMA)</td>
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<td>Trimethylolpropane triacrylate</td>
<td>Trimethylolpropane triacrylate</td>
<td>1.05±0.16</td>
<td>3.1±1.0</td>
</tr>
</tbody>
</table>

**Notes:**
- Measured values are given with uncertainty.
2.3.5. Maintenance of Pluripotency of ES Cells on DMAPMA

Next, we focused on the maintenance of pluripotency of ES cells grown on DMAPMA through the expression of pluripotency markers of mouse ES cells, the ability to form EBs and the potential of differentiation to cell lineages of all three germ layers (ectoderm, mesoderm and endoderm)[129, 157]. After culturing mouse CCE ES cells on DMAPMA for 2 days, ES cells were passaged onto a new DMAPMA membrane and a gelatin-coated glass coverslip to create hanging drops to form embryoid bodies (EBs) in two days. Immunocytochemistry of pluripotency markers was performed. Mouse ES cells grown on DMAPMA and glass coverslips retained expression of pluripotency markers: SSEA-1 and Oct4 (Figures 2.6a-d). This indicated that all the mouse ES cells that attached to the membrane or were passaged from the membrane remained undifferentiated. ES cells grown on DMAPMA were collected and allowed to form EBs by the hanging drop technique. These ES cells retained the ability to form EBs, which is a necessary stage for in vivo-like differentiation. To test whether these ES cells retained their differentiation potential after growing on DMAPMA, EBs were cultured in a reduced concentration of LIF for 3 days. Outgrowths of EBs were able to spontaneously differentiate into multiple cell lineages as revealed by the expression of markers for the nucleus and all three germ layers: the nucleic acid stain DAPI, the skeletal muscle marker Myf-5 for mesoderm, the pancreatic marker PDX-1 for endoderm, and the neural marker nestin for ectoderm and (Figures 2.6e-h, respectively). A merged image is shown in Figure 2.6i. A 63x zoomed image of the same EB is shown in Figures 2.6j-n, with the same differentiation markers. Thus, mouse ES cells maintained their differentiation
potential for ectoderm, mesoderm, and endoderm after growing on DMAPMA.

Altogether, DMAPMA supports the maintenance of pluripotency of mouse ES cells.

**Figure 2.6** Maintenance of the pluripotency of mouse ES cells on DMAPMA. (a) Optical image of ES cells one day after passaging. (b-c) Expression of ES cell pluripotency markers, Oct4 (b), SSEA-1 (c) and merged image (d). Confocal microscopy images showing (e) DAPI localization, (f) Myf-5 staining, (g) PDX-1 staining and (h) nestin expression. A merged image is depicted in (i). Confocal microscopy images with a 63x zoom showing (j) DAPI staining, (k) Myf-5 staining, (l) PDX-1 staining and (m) nestin staining. A merged image is shown in (n).
2.3.6. Maintenance of Mouse ES cells on DMAPMA-modified Surfaces

We further tested the ability of DMAPMA and PEG to support the maintenance of self-renewal ability during 7-day cultivation. Mouse ES cells were also grown on gelatin-coated glass coverslips (control), since they are the standard method for culturing/maintaining mouse ES cells in the short-term (1-3 days). During the first three days, mouse ES cells established colonies and grew in size, while forming EB-like structures during culturing on the same gelatin-coated surface for 7 days (Figure 2.7a). After culturing for 7 days, ES cells grown on DMAPMA formed a dense cell layer (Figure 2.7b) and exhibited morphology of undifferentiated ES cells (Figure 2.7c) while ES cells grown on the PEG surface exhibited few attached cells. In particular, ES cells grown on gelatin-coated glass coverslips showed that cells detached from the culture surface and form EBs, indicating spontaneous differentiation. The maintenance of pluripotency of mouse ES cells after culturing on DMAPMA for 7 days was further confirmed by the expression of pluripotency marker, Oct4, and the ability to form EBs. Altogether, these results demonstrate that DMAPMA supported the self-renewal and pluripotency of ES cells for 7 days, or at least 4 days longer than with a gelatin-coated surface.
2.3.7. Long-term Self-renewal and Passaging of Mouse ES Cells

In order to test whether DMAPMA supports the long-term self-renewal of ES cells, ES cells were cultured on DMAPMA-grafted PES membranes in maintenance medium and passaged onto a new DMAPMA-grafted substrate every 2-3 days. In order to monitor cell morphology routinely, during each passage ES cells were plated onto gelatin-coated glass coverslips. Cell morphology was observed one day after passaging from DMAPMA-grafted substrates for passage 1-6 (Figures 2.8a-f). We noticed that the number of ES cells decreased with the increase in cell passage. This is because we could not harvest all ES cells from the DMAPMA-grafted substrate for the next passage due to the strong cell binding capacity of DMAPMA. What is worth mentioning is that after 6 passages of culture on the DMAPMA-grafted substrate, mouse ES cells could strongly attach to gelatin-coated tissue culture plate and grow into a large cell colony from a few.
cells after 7 days (Figure 2.8g). In particular, these ES cells retained the ability to form EBs by the hanging drop technique, which is one of the hallmarks of the maintenance of stem cell pluripotency (Figure 2.8h). As evidenced in Figure 2.5a (the rightmost panel), it is unachievable to conventionally culture on and attach mouse ES cells to gelatin-coated surface for 7 days. Our results indicate that DMAPMA-grafted substrates can be used for long-term maintenance (7-day cultivation without subculture) and long-term self-renewal (7 passages) of mouse ES cells.

![Figure 2.8](image)

**Figure 2.8** Optical images of ES cells during long-term self-renewal. (a-f) Morphology of ES cells one day after each passage. (g) Cell propagation after 7 days of cultivation for passage 6. (h) After 7 passages, ESCs retained the capacity to form embryoid bodies by the hanging drop technique. Scale bar for all images is 50 µm.

### 2.3.8. Adhesion through Integrin β1

To investigate the mechanism of ES cells binding with DMAPMA, we selected and tested a series of known integrin cell binding agents (antibodies against integrin β1, αvβ5, α5 and α6) to search for the dominant cell binding protein. Mouse ES cells have prominent expression of α5, α6, αv, β1, and β5 integrin subunits[48, 50]. Integrin heterodimer αvβ5 is a vitronectin/RGD receptor, α5β1 a fibronectin/RGD receptor, and
α6β1 a laminin receptor. Our data show that blocking β1 integrin resulted in a significant decrease of cell adhesion to DMAPMA (48% ± 5), whereas blocking αvβ5, α5 and α6 integrin subunits had little significant effect on cell adhesion. It has been reported that fibronectin, laminin, short RGD peptides, peptides for integrin α5β1, α6β1, or αvβ5 alone are insufficient for maintaining human ES cells in an undifferentiated state[32, 48]. Laminin-511 is one of the most efficient ECM proteins for long-term self-renewal of human ES cells where integrin β1 has a critical role in the binding of human ES cells to laminin-511[19]. Our results suggest that DMAPMA provided a unique surface for ES cell adhesion via RGD-independent integrin β1, indicating the feasibility of developing new and simple, non-ECM protein-based, organic chemical surfaces for effective ES cell culture. It has been shown that surface chemistry could alter the adsorption kinetics and structure of adsorbed fibronectin, thereby modulating focal adhesion composition and signaling[165, 166]. Recent findings by Trappmann et al. have shown the relationship between cell behavior and substrate stiffness is translated through the feedback of the ECM, which is substrate-dependent[53]. The way that ECM molecules are tethered to a substrate affects stem cell spreading and differentiation[167]. For example, if the ECM is more loosely bound, it cannot provide the necessary mechanical feedback that integrin complexes require to cluster and signal[53]. The ability to use a comprehensive library of organic chemistry and high-throughput platform will not only allow us to identify the optimal chemistry for expanding/propagating and differentiating ES cells, but also enable further understanding on how different surface chemistries could amplify the effect of topography and stiffness for most efficient self-renewal and differentiation of ES cells.
2.4. Discussion

Using a novel high throughput synthesis and screening platform, we have discovered that photo-induced graft polymerized DMAPMA from a library comprising 9 different chemical groups (or 66 total monomers) exhibits unique mouse ES cell binding properties. This surface:

- Possessed higher hydrophilicity and lower surface roughness than PES control surface.
- Exhibited a significantly higher degree of cell attachment (CAI value) and expansion capacity (fold of propagation value) than any other of the polymers or even than those reported in the literature for human ES cell adhesion.
- Maintained pluripotency of mouse ES cells for 7 days as measured by the expression of pluripotency markers (Oct4 and SSEA-1), formation of EBs and in vitro differentiation to cell lineages expressing markers of ectoderm, mesoderm and endoderm; maintained long-term self-renewal of mouse ES cells for 7 passages.
- Facilitated ES cell adhesion through RGD-independent integrin β1, since the chemical structure of DMAPMA is unique in that it contains peptide bonds and possesses a secondary amine and a tertiary amine, connected by a propyl alkane functional group.

The ability to synthesize, screen and identify polymeric surfaces for ES cell attachment, maintenance and expansion in high-throughput is of great scientific and commercial interest. Compared with natural materials-derived culture matrices (e.g.,
Matrigel, peptide-based, polysaccharide-based substrates), these monomer-modified surfaces are chemically defined, easy to synthesize in large scale, reproducible, cost-effective, stable with long-term storage capability and easily available off-the-shelf. The correlation of high cell attachment index to cell expansion capacity and long-term maintenance of self-renewal ability and pluripotency of ES cells represents a simple way to screen surface chemistries for ES cell culture. Strong cell–substrate adhesion is required to support the cytoskeletal rearrangements and intracellular tensions during differentiation[37]. Therefore, our high throughput platform has great potential to screen surface chemistries for efficient ES cell differentiation to the desired cell lineage. Altogether, the methods and results presented here indicate that our high-throughput platform could be used to synthesize and identify surface chemistries for ES cell maintenance in a simple, easy, fast, reproducible, and scalable way. It provides a new avenue for understanding and manipulating surface chemistry for stem cell self-renewal and differentiation.

2.5. Conclusions

In this study, we have used a high-throughput platform to screen surface chemistries for stem cell culture and discovered a “hit” chemistry, DMAPMA, from a library that consisted of 66 monomers, that supports both ES cell expansion and pluripotency. We have investigated this substrate for long-term maintenance of ES cells, and found that it binds to ES cells through an integrin β1 mechanism. We tested three other integrins that are associated with mouse ES cell binding (α5, α6 and αvβ5) and integrin β1 exhibited the greatest effect on cell binding. This novel approach provides for the development of chemically defined culture system for pluripotent stem cell
culture. Future work on 3D matrices is being pursued with DMAPMA, including synthetic PES nanofibrous matrices, as well as natural alginate nanofibrous matrices and hydrogels.
CHAPTER 3

Creating Three-Dimensional DMAPMA-Modified Nanofibrous Matrices for Improved Stem Cell Pluripotency

3.1. Introduction

Surface chemistry and spatial dimension play important roles in maintaining stem cell pluripotency and controlling stem cell behavior, e.g., attachment, colony size, retention of stem cell markers, differentiation potential\[168\]. High-throughput synthesis and screening platforms have been used to discover optimal chemistries for the attachment, self-renewal and maintenance of human pluripotent stem cells\[32-36, 62\]. From our initial chemical library of 66 monomers, we have identified an optimal, synthetic, organic chemistry, DMAPMA that promotes strong attachment and long-term self-renewal of mouse ES cells\[169\]. In particular, the DMAPMA-grafted surface exhibits the highest cell expansion capacity for ES cells among the chemistries tested, including those synthetic chemistries reported in the literature\[169\]. This result is confirmed by the report that aminopropylmethacrylamide, another amide chemistry that also possesses a terminal amine, supports the long-term self-renewal of human ES cells for over 20 passages in chemically defined media\[66\]. In addition, the grafting density of the surface chemistry affects cell attachment, colony size and morphology, and pluripotent marker expression\[170, 171\]. However, all these studies are limited to ES cell culture on a 2D surface.

Among 3D cultures, nanofibers have been widely used for stem cell culture on the nanofibrous matrices\[168\]. Nanofibers can imitate the architecture of the extracellular

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3 Part of this chapter has been accepted as M.R. Zonca Jr., P.S. Yune, J.K. Williams, M. Gu, A.M. Unser, G. Belfort and Y. Xie “Enhanced Stem Cell Pluripotency in Surface-Modified Electrospun Fibrous Matrices.” Macromolecular Bioscience.
matrix, enhance stem cell survival and proliferation, direct stem cell fate decision, promote stem cell-derived tissue organization, and serve as a delivery vehicle for stem cell secretome[74, 172, 173]. Natural or synthetic nanofibers used for ES cell culture include chitosan[174], Tussah silk fibroin[80], self-assembled peptide amphiphiles[89], polyamide[69], polyurethane[175, 176], PCL[75, 104], PCL/Collagen or PCL/gelatin[71], PLGA[68], PLLA[93], PLLA/carbon nanotubes[82], PMGI[72], and PES[73]. Nanofibers can be fabricated through self-assembly[177], electrospinning[104, 178], phase separation[93], and a combination of thermally induced phase separation with reverse solid freeform fabrication[179]. Electrospinning is one of the most popular approaches to fiber formation as well as a potential way to encapsulate cells[180] since it is versatile, easy to set up and low in cost, can form small diameter fibers (~100s nm) with high surface areas and has a great selection of materials and broad applications[181]. This method utilizes high voltages, which creates an electric field between the polymeric solution and the ground allowing the polymeric solution to be drawn out of a syringe and into fibers. Additionally, there is a great deal of research examining fiber interactions with undifferentiated ES cells, and how these fibers induce differentiation of the ES cells[174]. ES cells have self-renewing capabilities, due to their ability to proliferate indefinitely, as well as remain pluripotent after division. These capabilities make ES cells an attractive option for understanding diseases, with regards to drug discoveries and therapeutic developments. Several synthetic polymeric nanofibers have recently been shown to enhance the self-renewing capabilities of pluripotent stem cells. Both PCL/collagen and PCL/gelatin nanofibrous scaffolds supported the formation of large colonies of human ES cells. However, these fibers required the presence of MEFs in
order for the human ES cells to colonize[71]. PCL and PCL/hydroxyapatite nanofibers supported mouse ES cells to maintain their pluripotency, however, the initial cell adhesion was low. Mouse ES cells were shown to grow and maintain their pluripotency on nanofibers synthesized from PMGI without the need of MEFs[72]. Additionally, PES nanofibers that were modified with collagen maintained the pluripotency of mouse ES cells[73]. Mouse ES cells cultured on polyamide nanofibers indicated that the nanofibrous structure could activate Rac and PI3K signaling pathways, leading to enhanced proliferation and self-renewal capacity in comparison to conventional 2D culture[69]. In addition to maintaining self-renewal capability, nanofibers are being investigated to promote the differentiation of ES cells into specific lineages, e.g., neuronal[75, 76, 78, 80, 82, 176], osteogenic[89, 90, 93], adipogenic[104], smooth muscle[94], and hepatic[102, 103]. Nanofibers have also demonstrated the ability to enhance cell survival, migration and differentiation in vivo[83] and to support ES cell-derived smooth muscle cell phenotype in vivo[94]. In particular, 3D culture of ES cells on nanofibers exhibits enhanced osteogenic differentiation and mineralization[91, 92] and superior neuronal function when compared with those cultured on 2D surfaces[79]. Although electrospun nanofibers are increasingly used in pluripotent stem cell research, the surface chemistry of these nanofibers is not optimal for stem adhesion and growth. Cell growth relies heavily on the chemistry of the polymer being used or coating with ECM proteins. Thus, it is important to transfer optimal surface chemistry from two- to three-dimensions in regulating pluripotent stem cell cultures[64, 182].

In this work, we demonstrate the advantages of directly modifying nanofibers with DMAPMA for enhanced 3D ES cell culture and maintenance of pluripotency. We
first fabricated fibrous matrices from a PES solution by electrospinning and then chemically modified these nanofibers with DMAPMA through photo-induced graft polymerization. The DMAPMA-grafted fibers were characterized by SEM, X-ray photoelectron spectroscopy (XPS) and Fourier Transform Infrared Spectroscopy (FTIR). Lastly, we examined cell proliferation and expression of pluripotency markers of ES cells on DMAPMA-modified fibers, as shown in the flowchart in Figure 3.1. These modified fibrous matrices are expected to maintain mouse ES cells in a pluripotent state upon expansion in a 3D microenvironment. The ability to incorporate the optimal surface

![Flowchart](image)

**Figure 3.1** Flowchart showing the experimental process of this work.
chemistry into nanofibers provides a new avenue to advanced 3D culture that supports
the self-renewal and differentiation of pluripotent stem cells and functionality of their
derivatives.

3.2. Experimental Section

3.2.1. Materials

Poly(ether sulfone) powder was supplied by BASF (Florham Park, NJ).

1,1,1,3,3,3 hexafluoro-2-propanol (HFIP) (Sigma-Aldrich, St. Louis, MO) was used as a
solvent for dissolving the PES powder along with N-[3-(dimethylamino) propyl]
methacrylamide (DMAPMA) purchased from Sigma-Aldrich.

3.2.2. Fabrication of Poly(ether sulfone) Fibrous Matrices

Micro- and nanofibers are widely utilized as matrices in stem cell culture and
tissue engineering, as they are able to mimic the dimensionality (microporous structure)
of the extracellular matrix in 3D. The challenge is to find the right combination of
suitable surface chemistry and compatible dimensionality. Electrospinning is a well-
known industrial technique that has recently become popular for fabricating fibers,
synthetic membranes and microporous matrices, as it allows for precise control of fiber
size by varying the polymer concentration, voltage, flow rate, and the distance from the
needle tip to the collector plate. This technique also allows for the formation of both
synthetic and natural based fibers and allows fabrication of relatively small diameter
fibers (~100 nm) with large surface area per unit volume[181]. We dissolved PES
powder in HFIP at 3 different concentrations, 2.5, 5, and 7.5% w/v. The voltage and tip
to collector distance were kept the same for all 3 concentrations: 15 kV and 14 cm
respectively. Flow rates were determined by the ability of the solution to be electrospun
and chosen as follows: 40, 25 and 10 µl/min for the 2.5%, 5% and 7.5% PES/HFIP solutions, respectively. Improper flow rates would not allow for the electrospinning jet to form. The trend we observed was that lower concentrations necessitated higher flow rates due to their lower viscosity. The solutions were electrospun onto 5-mm or 12-mm glass coverslips, or aluminum foil sheets to collect the fibers for cell culture experiments.

3.2.3. Synthesis of Monomer-Modified PES Fibers via Photo-Induced Graft Polymerization

A high-throughput platform approach that utilizes photo-induced graft polymerization[153], was applied to modify the PES fibers on glass coverslips. Briefly, the fibers were irradiated with UV light at 300 nm, forming reactive radical sites. We were then able to covalently graft DMAPMA via free radical polymerization. These modified fibers were stored dry until use. Prior to culturing the cells in the fibrous matrices, the modified fibers were sterilized with 70% ethanol for 5 min, followed by washing with sterilized PBS three times.

3.2.4. Synthesis of Monomer-Modified PES Fibers via Plasma-Induced Graft Polymerization

A high-throughput platform approach that utilizes atmospheric pressure plasma-induced graft polymerization[183, 184] was applied to modify the PES fibers on glass coverslips. Briefly, the fibers were pre-soaked in 5 ml of 0.2 M DMAPMA monomer solution for 30 minutes and then exposed to an APP plasma source (Model ATOMFLO, Surfx Technologies LLC, Culver City, CA) at a helium flow rate of 30 L min$^{-1}$ and an oxygen flow rate of 0.5 L min$^{-1}$ for 8 minutes with a source to fiber distance of 3 mm, forming reactive radical sites. The plasma source was operated at 150 W and driven by a
radio frequency power of 27.12 MHz. An XYZ Robot (Surfx Technologies LLC, Culver City, CA) was used to control the plasma source over the fibers with a scan speed of 6 mm s\(^{-1}\). This allowed us to covalently graft DMAPMA via free radical polymerization. These modified fibers were stored dry until use. Prior to culturing the cells in the fibrous matrices, the modified fibers were sterilized with 70% ethanol for 5 min, followed by washing with sterilized PBS three times. Characterization of the fibers was done via a Tensor 27 FTIR instrument from Bruker (Bruker Corporation, Billerica, MA). The instrument features a single-beam KBr beamsplitter, and can detect compounds in the range of 4000- 400 cm\(^{-1}\), with resolution of 1 cm\(^{-1}\). OPUS software was used for data processing. Analysis was done on fibers both before and after modification with DMAPMA.

### 3.2.5. Scanning Electron Microscopy

The average sizes of modified and unmodified fibrous matrices were characterized by scanning electron microscopy. The samples were observed directly, with no fixation or dehydration necessary, and observed using an environmental scanning electron microscope (Nova NanoSEM 600, FEI, Hillsboro, OR). The average size of a particular concentration of fibers was determined by averaging 20 random fibers.

### 3.2.6. X-ray Photoelectron Spectroscopy

Modified and unmodified fibrous matrices were characterized using a ThermoFisher ThetaProbe x-ray photoelectron spectroscopy, equipped with an Al x-ray source (1486.6 eV) and a crystal monochromator, with energy resolution of 0.48 eV on clean Ag. The surveys (broad scans) were acquired using pass energy of 300 eV with a 1.0 eV step size, and the narrow energy spectra were acquired using pass energy of 125
eV and a 0.1 eV step size. For chemistry determination, all energies are charge-corrected using the C1s peak at 284.6 eV.

3.2.7. Fourier Transform Infrared Spectroscopy

Characterization of the fibers was done via a Tensor 27 FTIR instrument from Bruker (Bruker Corporation, Billerica, MA). The instrument features a single-beam KBr beamsplitter, and can detect compounds in the range of 4000-400 cm\(^{-1}\), with resolution of 1 cm\(^{-1}\). OPUS software was used for data processing.

3.2.8. ES Cell Culture

Mouse CCE embryonic stem cells were obtained from StemCell Technologies (Vancouver, BC, Canada)[154, 155]. ES cells were cultured in gelatin-coated tissue culture flasks and fed with ES maintenance media, which is comprised of the following components: Dulbecco’s Modified Eagle’s Medium with 4.5 g/l D-glucose supplemented with 15% (v/v) fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.1 mM MEM non-essential amino acids, 10 ng/ml murine recombinant leukemia inhibitory factor (StemCell Technologies, Vancouver, Canada), 0.1 mM monothioglycerol, 2 mM L-glutamine, and 1 mM sodium pyruvate (Sigma Aldrich, St. Louis, MO) as described previously[104]. LIF is typically used to maintain mouse ES cells in an undifferentiated state. Immunocytochemistry followed by confocal microscopy and Western Blot analysis were used to determine how DMAPMA-modified fibers in a matrix mat maintained ES cell pluripotency. The cells were fed every other day to limit the amount of LIF added to the culture. For the MTT assay, cells were seeded at 7,500 cells/well and grown on the fibers in a 96-well plate for 3 days. For the immunocytochemistry experiments, ES cells were seeded at 20,000 cells/well and grown on fibers in a 24-well plate. For Western
Blot experiments, four coverslips were placed in a petri dish and 80,000 cells were seeded on top of the four coverslips in order to harvest sufficient lysate. For all experiments, the cells were grown in a 5% CO₂ atmosphere at 37°C.

3.2.9. MTT Assay

Cells were seeded on 2.5%, 5% and 7.5% DMAPMA-modified and unmodified PES fibers that were electrospun on top of 5 mm glass coverslips and fitted in a 96-well plate at a density of 7,500 cells/well. Additionally, DMAPMA-grafted 2D membrane surfaces and coverslips coated with gelatin served as controls. ES maintenance media was changed every 2 days, and after 3 days an MTT assay was performed to investigate cell expansion. Briefly, 10 µl of MTT labeling reagent was added to each well and the plate was incubated for 4 hours at 37 ºC. After incubation, 100 µl of solubilization reagent was added to each well and the plate was incubated overnight. Absorbance readings were taken at 560 nm and 700 nm (as the reference) on a Tecan Infinite M200 plate reader (Tecan US, Research Triangle Park, NC). Cell growth index was calculated by dividing the absorbance value of each sample by that of ES cells grown on conventional gelatin-coated coverslips. To quantify the cell number, a standard curve was created using absorbance values associated with cell seeding densities at 10,000, 20,000, 30,000 and 40,000 cells/well. A linear, best-fit line was generated from this data, with an R² value of 0.994.

3.2.10. Optical Microscopy and SEM Observation of Cell Morphology on Fibers

The morphology of ES cells grown on fibers was observed under a Nikon inverted Eclipse TS100F microscope (Nikon, Melville, NY). To reveal the ultrastructure of ES cells grown on fibrous matrices, samples were washed with PBS, fixed with 3%
glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) containing 0.1 M sucrose (Sigma Aldrich). After dehydration in serial ethanol of different concentrations (50%, 70%, 80%, 95%, and 100%), the samples were dried by adding graded HMDS in ethanol (25%, 50%, 75%, 100%, and 100%) (Ted Pella, Inc., Redding, CA). Samples were observed using an environmental SEM (FEI Nova Nano SEM 600).

3.2.11. Immunocytochemistry of Pluripotency Markers

The maintenance of stem cell pluripotency after culturing on both modified and unmodified electrospun fibers was characterized using immunocytochemistry analysis of the stem cell markers, Oct4, SSEA-1, and alkaline phosphatase (ALP). After culture for 5 days, mouse ES cells were washed with PBS, and fixed with 4% paraformaldehyde in PBS. Fixed cells were then rinsed with PBS, permeabilized in 0.1% Triton-X-100, and blocked with 5% FBS in PBS. ES cells were incubated with primary antibody for anti-Oct4 produced in rabbit (Sigma Aldrich, St. Louis, MO) detected by the anti-rabbit Alexa Fluor® 488 and co-stained with anti-SSEA-phycoerythrin (PE) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-ALP produced in goat (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) detected by the anti-goat Alexa Fluor® 647. Cells were also stained with DAPI to reveal the nuclei in blue fluorescence for identifying the total cell population and verifying the co-localization of Oct-4 in nuclei. Samples were observed using a Leica SP5 confocal microscope (Leica Microsystems, Mannheim, Germany) with a 405 nm laser to reveal cell nuclei in blue, a 488 nm laser to reveal the Oct4 expression in green, SSEA-1 expression in yellow, and a 633 nm laser to reveal ALP expression in red. Confocal images were captured using the same laser intensity and
gain settings in order to compare the signaling intensity across samples and processed using Leica LasAF software.

3.2.12. Western Blot Analysis

Confirmation of protein expression levels was obtained through Western blot analysis. After 5 days of cultivation, the mouse ES cells grown on DMAPMA-modified and unmodified PES fibers were lysed with a RIPA buffer-protease inhibitor cocktail. The lysate was collected and Bicinchoninic acid (BCA) analysis was performed in order to calculate lysate concentrations. Pre-prepared graded polyacrylamide gels (4-12%, Life Technologies, Carlsbad, CA) were washed with deionized water and then loaded into a gel electrophoresis system (Life Technologies). The inner and outer chambers of the gel electrophoresis system were loaded with 1X NuPAGE Buffer (Life Technologies). Lysates were combined with a loading dye composed of a 10:1 (volume) ratio of NuPAGE LDS Buffer (Life Technologies) and 0.5 M dithiothreitol (DTT). The samples were then boiled for 10 minutes, vortexed, cooled, and then loaded into the polyacrylamide gels. An equivalent mass of each lysate was added to each well, along with 5 µl of SeeBlue Standard, to be used as a molecular weight ladder (Life Technologies). Once loaded, the gel was run at 200 V for 45 minutes. The gel was then transferred to a nitrocellulose membrane using an iBlot Gel Transfer Device (Life Technologies). The reactive sites on the membrane were then blocked in 5% milk for 30 minutes to minimize non-specific binding. For primary antibody additions, a 1:1000 dilution of antibody was prepared in 5% bovine serum albumin in Tris-Buffered Saline and Tween 20 (TBST). The membrane was then placed at 4°C and shaken overnight. The next day, the membranes were washed three times for 15 minutes each in 1X TBST
solution (10 mM Tris, 150 mM NaCl, 0.1% Tween 20). For secondary antibody additions, a 1:10000 dilution of antibody in 5% milk in TBST solution was prepared. The samples were placed on a shaker at room temperature for 1 hour. The membranes were then washed three times for 15 minutes each in 1X TBST solution to remove any residual antibody solution. For exposure, a 1:1 ratio of SuperSignal West Femto Stable Peroxide Buffer and SuperSignal West Femto Luminol/Enhancer Solution were added to the membrane. A Fluor Chem E imager (Protein Simple, Santa Clara, CA) was used to obtain pictures of the gels. Quantitation analysis of the gels was conducted using Image J software (NIH).[185]

3.3. Results

3.3.1. Modification of Electrospun Poly(ether sulfone) Fibers using DMAPMA

Based on our ability to successfully graft DMAPMA onto PES membranes in 2D, we chose PES for our 3D synthetic fiber work. We selected three concentrations (2.5, 5 and 7.5%) of PES dissolved in HFIP based on solution viscosity. Greater concentrations of PES were tried initially, however, due to their high viscosity, fibers did not form after electrospinning. Fiber morphology was observed with an environmental SEM and images are shown in Figure 3.2a-c. Fibers formed from the 2.5% PES solution did not stick to glass coverslips, only to aluminum foil and exhibited beading. PES solutions of 5% and 7.5% formed fibers on both glass coverslips and aluminum foil. Additionally, fibers formed from 5% PES beaded, while fibers formed from 7.5% PES were of uniform and micrometer diameter. We calculated the fiber size by averaging 20 random fibers for each concentration. The fiber diameter increased from $235 \pm 64$ nm, to $429 \pm 195$ nm.
and to 2464 ± 264 nm, with increased PES concentration from 2.5, to 5 to 7.5%, respectively.

Based on our previous findings that DMAPMA-grafted PES membrane substrates exhibited the highest cell attachment among a library of 66 chemistries[169], we further examined the feasibility of modifying PES fibrous matrices using the photo-induced graft polymerization. We irradiated the fibers with UV light (~300 nm) for 30 seconds[161, 164]. Monomer-graft induced polymerization then occurred through free-radical polymerization, resulting in surface coating of the PES fibers. SEM images showed film-like coating on the surface DMAPMA-grafted nanofibers electrospun from 2.5 and 5% PES solution (Figure 3.2d&e). After DMAPMA grafting, electrospun microfibers from 7.5% PES solution showed distinct fiber morphology (Figure 3.2f) which is similar to that before the chemical modification (Figure 3.2c). For all three sets of PES fibers, the DMAPMA modification did not change the fiber diameter significantly (Figure 3.3). Additionally, we noticed that after DMAPMA grafting, the fibrous matrices became transparent and more robust. We further confirmed the grafting of DMAPMA to the fibers through XPS. The presence of a nitrogen 1s peak (N1s) between 390-410 eV is characteristic for nitrogen, confirming the presence of DMAPMA on the modified samples, but not in the unmodified samples (Figure 3.4). Additionally, FTIR measurements of our modified samples (Figure 3.5) showed the presence of an amide peak in the range of 1630 cm⁻¹, indicating that DMAPMA has modified the PES fibers.
Figure 3.2 Scanning electron microscopy (SEM) images of unmodified (a-c) and modified (DMAPMA, d-f) PES electrospun fibers at various concentration. (a, d) 2.5% PES. (b, e) 5% PES. (c, f) 7.5% PES. Scale bar: 2 µm (a, b); 10 µm (c-f).

Figure 3.3 The relationship between fiber diameter (nm) and concentration of PES in HFIP. Empty (○) and solid (●) circles are for unmodified and modified PES fibers, respectively.
We further examined the feasibility of chemically modified fibrous matrices using plasma-induced graft polymerization. This alternative polymerization method has several advantages over photo-induced graft polymerization. Namely, plasma combines UV and ion bombardment without the use of vacuum, it discharges with low breakdown voltage, and properties of the grafted polymer phase are controllable[183, 184]. The plasma treatment duration was optimized for both 5% and 7.5% PES fibers. FTIR spectroscopy was used to confirm the grafting of DMAPMA onto the fibers, and is shown in Figure

**Figure 3.4** X-ray photoelectron spectroscopy (XPS) spectra of modified (full lines) and unmodified (dashed lines) PES fibers for (a) 2.5% PES. (b) 5.0% PES. (c) 7.5% PES dissolved in HFIP. The nitrogen 1s peak (N1s) between 390-410 eV indicates the grafting of DMAPMA to PES fibers.
3.5. In both cases, the power used to graft the DMAPMA to the fibers was 150W. It was revealed that the samples exposed to the plasma for 10 minutes showed the greatest signal for the 5% fibers, and there was no drastic change for the 7.5% samples, so to be consistent, a 10 minute plasma exposure was chosen for both sets of samples.
Figure 3.5 FTIR spectra of a) DMAPMA monomer, b) 7.5% unmodified fibers, c) 7.5% DMAPMA-modified fibers and d) 5% DMAPMA-modified fibers. The presence of the amide peak (~1650 cm⁻¹) in the 7.5% and 5% DMAPMA modified fibers and the absence of this peak in the unmodified fibers indicates that DMAPMA has been grafted to our PES fibers.
Figure 3.5 Continued
3.3.2. Cell Proliferation on DMAPMA-modified Fibers

For cell proliferation studies, 2.5% PES/HFIP fibers were electrospun onto aluminum foil since fibers at this concentration did not adhere to glass coverslips. The 5 and 7.5% PES/HFIP fibers were electrospun onto 5-mm Vectabond-coated glass coverslips that were fit into a 96-well plate. Mouse ES cells were seeded at a density of 7,500 cells/well and supplemented with 100 µl of ES maintenance media. After 3 days, an MTT assay was performed to determine cell proliferation. The cell growth index was calculated by normalizing the absorbance of ES cells grown on various fibers to ES cells grown on gelatin-coated glass coverslips which was used for conventional mouse ES cell culture and shown in Figure 3.6a. The cell growth index of ES cells grown on 2.5% PES nanofibers was slightly higher than 1.0, indicating that nanofibers from 2.5% PES in HFIP could support better ES cell growth compared to conventional gelatin-coated surface. However, ES cells grown on nanofibers with DMAPMA-modified 2.5% PES from HFIP did not show enhanced cell growth. The cell growth index of ES cells grown on both nanofibers that were DMAPMA-modified and unmodified and electrospun from 5% PES from HFIP exceeded 1.5, which is much higher than cell growth on conventional gelatin-coated surface. The cell growth index of ES cells grown on DMAPMA-modified microfibers electrospun from 7.5% PES from HFIP reached 1.7 as well and was higher than that of unmodified microfibers. Additionally, we found that fibers with DMAPMA-modified from 5% and 7.5% PES from HFIP support better ES cells growth than DMAPMA-modified 2D flat sheet membranes. According to the standard curve, the cell number on DMAPMA-modified PES fibers was determined, as shown in Figure 3.6b. We observed that DMAPMA-modified fibers from 7.5% PES had over 50,000 cells after
3 days in culture while the 2D DMAPMA-modified membrane yielded ~32,000 cells and the conventional gelatin-coated coverslips yielded ~24,000 cells.

Upon examination with an inverted optical microscope, we observed rounded colonies of ES cells grown on fibers modified with DMAPMA from 5% and 7.5% PES from HFIP after 3 days (Figure 3.7a and b), which indicated these ES cells remained undifferentiated, as opposed to unmodified fibers (Figure 3.7c and d). However, we noticed beading occurred in the fibers from 5% PES from HFIP (Figure 3.7c), which is not optimal, as beading decreased fiber surface area and also made imaging of samples with cells tedious. Thus, lower concentrations of PES showed a greater tendency to bead, compared with higher PES concentrations. Hence, 7.5% PES from HFIP was chosen for the remainder of our experiments. We further observed ES cell growth on 7.5% PES fibrous matrices using SEM and found that ES cells grown on DMAPMA-modified microfibers formed a dense colony-like structure while cells grown on unmodified fibers exhibited spread-out, monolayer-like structure, similar to differentiating cells, indicating that DMAPMA-modified fibers likely prevent spontaneous differentiation (Figure 3.8).
Figure 3.6 Cell proliferation of mouse ES cells grown on DMAPMA-modified PES fibers compared with a 2D PES membrane. (a) The cell growth index on unmodified (white column) and modified with DMAPMA (grey column) PES fibers was calculated by normalizing to the conventional 2D mouse ES cell culture on gelatin-coated coverslip. (b) The cell number of mouse ES cells grown on DMAPMA-modified PES fibers and 2D membranes using conventional gelatin-coated glass coverslips as the control.
**Figure 3.7** Optical images of mouse ES cell grown on DMAPMA-modified (a, b) and unmodified PES fibrous matrices (c, d). (a, c) 5% PES fibers. (c, d) 7.5% PES fibers. Scale bar: 100 µm.

**Figure 3.8** SEM images of mouse ES cell grown on fibrous matrices that were DMAPMA-modified (a, b) and unmodified (c, d) from 7.5% PES dissolved in HFIP. Scale bar: 20 µm (a, c) and 5 µm (b, d).
3.3.3. Maintenance of Pluripotency of ES Cells on DMAPMA-modified Fibers

We next focused on the maintenance of pluripotency for ES cells grown on fibers with DMAPMA-modified 7.5% PES from HFIP, compared with unmodified fibers. ES cells were cultured for 5 days on these DMAPMA-modified and unmodified fibers, while changing the media every other day. Using confocal microscopy we measured immunocytochemical markers for stem cell pluripotency. These include Oct4, SSEA-1 and ALP. DAPI-stained nuclei provided an estimate of the total cell population. ES cells grown on DMAPMA-modified fibers showed a greater expression of pluripotency markers, Oct4 (pseudo-colored in green), SSEA-1 (pseudo-colored in yellow) and ALP (pseudo-colored in red) than those grown on unmodified fibers (Figure 3.9a and b). Additionally, we observed that unmodified PES fibers were stained in the presence of SSEA-1-PE. This may be due to the fact that SSEA-1 is a phycoerythrin (PE) conjugated antibody and the nonspecific binding of PE dye to PES fibers gave rise to the fluorescence signal that is pseudo-colored in yellow. In particular, almost all ES cells grown on DMAPMA-modified PES fibers strongly expressed Oct4 in cell nuclei (see the top panels in Figure 3.9a), indicating that these ES cells remained in an undifferentiated state and maintained ES cell pluripotency.

To confirm our immunocytochemistry results, we performed Western Blots in order to quantify the level of protein expression. As with our immunocytochemistry experiments, cells were again grown on both modified and unmodified fibers from 7.5% PES from HFIP for a period of 5 days. After 5 days, the cells were lysed with a RIPA buffer-protease inhibitor cocktail. The lysate was collected and BCA analysis was performed in order to calculate lysate concentrations. Western Blots were obtained and
are shown in Figure 3.10. We measured the expression of Oct4 normalized to the housekeeping gene GAPDH, and the results indicated that a greater expression of Oct4 was seen for ES cells grown on modified fibers, compared with those cells grown on unmodified fibers (Figure 3.10a). We were able to quantify protein expression by using ImageJ software (NIH), which allowed us to plot the individual lanes of the Western Blot as a curve. By calculating the area beneath the curve for both the Oct4 and GAPDH, we quantified the expression of this transcription factor (Figure 3.10b). Our results confirmed that the DMAPMA-modified fibrous matrix supported the expression of pluripotency marker Oct4 in ES cells even in the presence of a reduced amount of LIF, an essential growth factor to keep mouse ES cells undifferentiated in conventional 2D cell culture. Additionally, we further examined whether ES cells grown on DMAPMA-modified fibers exhibited a change in phenotype in comparison to the cells that were initially seeded. For this experiment, 80,000 mouse ES cells were seeded onto 4 12-mm glass coverslips deposited with DMAPMA-modified 5% and 7.5% PES fibrous matrices, respectively. After culturing for 5 days, cells were harvested for protein extraction followed by Western blot analysis of Oct4 expression using GAPDH as a control (Figure 3.11). The relative expression of Oct4 normalized to GAPDH is 1.9 for DMAPMA-modified 7.5% PES nanofibers and 1.6 for DMAPMA-modified 5% PES nanofibers, which is at the same level (1.9) as initially seeded cells, confirming no changes in pluripotent phenotype of stem cells after long-term culture on DMAPMA-modified nanofibers.
Figure 3.9 Confocal images of mouse ES cells grown on fibrous matrices that were (a) DMAPMA-modified and (b) unmodified from 7.5% PES dissolved in HFIP, and expressing pluripotency markers, Oct4 (in green), SSEA-1 (pseudo-colored in yellow), and alkaline phosphatase (ALP, pseudo-colored in red), and co-stained nuclei with DAPI to reveal the total cell population. Scale bar: 50 µm.
Figure 3.10 Western blot analysis of the expression of the pluripotency marker, Oct4 in mouse ES cells grown on 7.5% DMAPMA modified fibers and unmodified PES fibers for 5 days. Undifferentiated mouse ES cells grown on a gelatin-coated coverslip were used as the positive control. (a) Representative blot. UMF: unmodified PES fibers. MF: modified PES fibers. (b) Relative expression of Oct4 by normalizing to GAPDH.

Figure 3.11 Western blot analysis of the expression of the Oct4 pluripotency marker in mouse ES cells grown on DMAPMA-modified fibers for 5 days in comparison to that in cells before seeding (Day 0). 5% MF: DMAPMA-modified fibers electrospun from 5% PES solution. 7.5% MF: DMAPMA-modified fibers electrospun from 7.5% PES solution.
3.4. Discussion

In this study, we grafted DMAPMA, which was earlier identified in a 2D high throughput screening study, onto nanofibrous matrices for 3D ES cell culture and maintenance of pluripotency. Previously, we showed that DMAPMA-grafted surfaces exhibited the strongest cell attachment and highest expansion capacity compared with any of the other polymeric surfaces tested from a library of 66 monomers that comprised nine different chemical functional groups (e.g., hydrophobic methacrylates, amines, hetero rings, aromatics, hydroxyl, zwitterionic, acids, PEGs, others), and those reported in the literature[169]. Electrospun nanofibers from PES from HFIP were used here to form a modified (with grafted DMAPMA) nanofibrous matrix. Graft induced photo-polymerization was used here to modify the PES fibers[153, 169]. PES nanofibers are biocompatible and can be used for ES cell culture[186]. Additionally, PES-based fibers are mechanically strong. For example, the modulus of core-shell PES-PCL fibers increased from 7.1 MPa for pure PCL fibers to 30.6 MPa for PES-PCL fibers[187]. In order to support stem cell self-renewal and differentiation, PES nanofibers were previously grafted either with collagen or coated with laminin[73, 188-190]. The ability here to graft desirable monomers (DMAPMA) onto light-sensitive PES fibers that form 3D matrices, allows one to now substitute the currently used complex natural extracellular matrix that contains undefined components (proteins and other macromolecules) with a well-defined inexpensive organic coating. It is a simple, reproducible, low cost, scalable 3D substrate for ES cell adhesion, self-renewal and differentiation.
PES was electrospun into fibrous matrices in the presence of several solvents, including dimethyl sulfoxide, dimethylformamide, DMF/N-methyl-pyrrolidinone, DMF/toluene, and HFIP[190-193]. We chose HFIP as a solvent due to its strong volatility. PES was electrospun into fibers whose diameters increased from nanometers to micrometers with increasing PES concentration. This result is consistent with the trend reported elsewhere[190]. PES electrospun fibrous matrices were modified with DMAPMA (confirmed with XPS) using photo-induced graft polymerization, and then utilized the modified fibrous matrices to maintain ES cell pluripotency. The chemical structure of DMAPMA is unique in that it possesses peptide bonds and also a secondary and tertiary amine, connected by a propyl alkane functional group[169]. In this study, we demonstrated that DMAPMA-modified fibrous matrices promoted higher cell proliferation than 2D DMAPMA-modified membranes. We and others also report that nano/microfibrous matrices provide better microenvironments for ES cell survival, migration, proliferation, and differentiation than 2D surfaces[69, 79, 91, 104]. This also highlights the critical role of fibrous matrices in 3D stem cell culture. In particular, we found that DMAPMA-modified microfibers exhibited greater protein levels of the pluripotency markers of ES cells than the unmodified fibrous matrix, indicating the significance of surface chemistry regulating stem cell fate.

Our approach is generic and not limited to grafting only DMAPMA. The ability to incorporate our photo-induced graft polymerization method onto 3D substrates has a great potential for the use of surface chemistry in ES cell culture. Taken together, the methods and results presented here indicate that our platform could be used to synthesize and modify 3D substrates for any well-defined culture system.
3.5. Conclusions

In this study, we have covalently grafted DMAPMA to electrospun fibers using photo-induced graft polymerization. We synthesized fibrous matrices by varying the concentration of PES in the solvent HFIP. These fibrous matrices can be chemically modified by DMAPMA. These modified fibers supported enhanced ES cell growth in comparison with both DMAPMA-modified 2D membranes and conventional gelatin-coated 2D surfaces. In particular, the chemically modified fibers developed here support the expression of ES cell pluripotency markers at a much higher level than unmodified fibers, which was confirmed through immunocytochemistry of ES cell pluripotency markers, as well as Western Blot analysis. This work provides a new opportunity to functionalize fibrous matrices for advanced 3D cell culture of hard-to-grow cell types and maintenance of stem cell pluripotency via a simple synthetic chemistry.
CHAPTER 4

Synthesis of DMAPMA-Modified Alginate Hydrogels for Pluripotent Stem Cell Culture

4.1. Introduction

Alginate hydrogels are biocompatible, bioabsorbable, easy to synthesize and use, and FDA-approved materials for biomedical applications. Versatile alginate hydrogels, (e.g., microparticles, microspheres, microbeads, microcapsules, microtubes, microstrands, microfibers, nanofibers, gel slabs) have been widely used for drug delivery[121-123, 194], cell encapsulation[195, 196], cell replacement therapy[138] and tissue engineering[197-199].

Alginate is a naturally occurring polymer, consisting of 1,4-linked β-D-mannuronic acid (M units) and α-L-guluronic acid (G units)[118, 200, 201] and is derived from brown seaweed. The M and G units vary in distribution and proportion along the alginate chain. Since there are no regular repeat units in alginate, regions along the chain can be described as M blocks, G blocks, or a combination of both[118]. Divalent cations, such as Ca$^{+2}$ and Sr$^{+2}$[118], Ba$^{+2}$[202] as well as Zn$^{+2}$[119] have been used to successfully crosslink alginate to form hydrogels. Calcium and strontium tend to form GG blocks of alginate, while zinc typically leads to the formation of MM and MG blocks.

Alginate nanofibers provide benefits to mimic the nanoscale geometry as well as the hydrogel component of the extracellular matrix (ECM). On its own, sodium alginate cannot be electrospun easily, due to the strong repulsive force of the polyanions[203]. However, when poly(ethylene oxide) (PEO) is blended with sodium alginate, the
repulsive force is reduced, allowing fibers to form[203]. It was found that the molecular weight of PEO plays a critical role in fiber formation as well[204].

The applications of alginate hydrogel nanofibers in stem cell research have been hindered by the lack of cell recognition sites to alginate[205, 206]. Currently, alginate relies on chemical modification with natural macromolecules (e.g., polylysine, collagen, laminin, fibronectin, chitosan)[207-211] or RGD peptides[124, 125]. These surface coatings are either less effective or expensive for stem cell culture. We have demonstrated that N-[3-(dimethylamino)propyl]methacrylamide (DMAPMA) is a simple, organic compound that promotes attachment and maintenance of mouse ES cells[169, 212]. In this work, we will further investigate the feasibility of incorporating DMAPMA into natural, polymeric, alginate nanofibers- in order to create adhesive alginate hydrogel systems for pluripotent stem cell culture. We have two approaches that will be studied: incorporation of DMAPMA with methacrylic acid to form a polymer which will be blended with an alginate-PEO solution to form electrospun fibers, and using DMAPMA as a surface coating on alginate nanofibers through photo-induced polymerization.

4.2. Experimental Section

4.2.1. Materials

Sodium alginate, poly(ethylene oxide) (PEO), DMAPMA, methacrylic acid (MAA), and the thermal initiator, azobisisobutyronitrile (AIBN) were all purchased from Sigma-Aldrich (St. Louis, MO). Deionized water (DI) with a resistivity of 18 MΩ cm was used to dissolve the polymeric solutions via a filtration system from Millipore (Billerica, MA).
4.2.2. Synthesis of Alginate-DMAPMA-MAA Nanofibers

4.2.2.1. Synthesis of DMAPMA-MAA Polymer

For the initial synthesis of the DMAPMA-MAA polymer, 0.028 moles (5 ml) of DMAPMA was combined with 0.059 moles (5 ml) of methacrylic acid in the presence of 0.740 moles (30 ml) of 100% methanol. To this solution, 0.0002 moles (40 mg) of AIBN was added to serve as a thermal initiator of the polymerization reaction. The reaction was carried out in a sand bath at 60º C overnight. The resultant polymer was washed three times with methanol to remove impurities (50 ml, 100 ml, and 75 ml methanol respectively), and the remaining solid was left in a fume hood to air dry. A second synthesis was performed in which the same molar quantities of DMAPMA, MAA, and methanol were used; however the molar quantity of AIBN was increased to 0.00087 moles (143 mg). This increase in AIBN led the resulting ratio of (DMAPMA+MAA)/AIBN to be equal to 100, as opposed to 435 as before. Additionally, this reaction was carried out with an oxygen purge prior to the reaction taking place.

4.2.2.2. Preparation of Alginate-PEO-DMAPMA-MAA Solution

The DMAPMA-MAA polymer was dissolved in DI water at pH 7 after rapid shaking at 225 rpm in a 37º C incubator at a concentration of 1.5%. Alginate at a concentration of 1.5% and PEO at a concentration of 3% were then directly added to this solution. The resulting solution was vortexed until homogeneity was reached. Additionally, two concentrations of DMAPMA-MAA were prepared: 0.5 and 1%. These solutions were then combined with 1.5% alginate and 3% PEO respectively.
4.2.2.3. Electrospinning of Alginate-PEO-DMAPMA-MAA Nanofibers

The solutions of the DMAPMA-MAA polymer combined with 1.5% alginate and 3% PEO were electrospun with the following conditions: an applied voltage of 20 kV, a tip to collector distance of 15 cm, and a 22 gauge needle. The flow rates for both solutions were 10 μl/min. The fibers were collected onto 12-mm Vectabond-coated glass coverslips for cell culture experiments.

4.2.2.4. Crosslinking of Alginate-PEO-DMAPMA-MAA Nanofibers

The electrospun fibers were crosslinked in the presence of 200 μl of 1.5% CaCl₂ for 2 minutes at room temperature. After the CaCl₂ solution was removed, the fibers were washed three times with sterile DI water for cell culture experiments.

4.2.3. Synthesis of DMAPMA-coated Alginate Hydrogels

4.2.3.1. Electrospinning of Alginate-PEO Nanofibers

Alginate at 1.5% was combined with PEO at 3% and then electrospun at a voltage of 20 kV, a flow rate of 10 μl/min, and a tip to collector distance of 15 cm. A 22 gauge needle was used for flow.

4.2.3.2. Surface Coating of DMAPMA on Alginate Nanofibers

Surface coatings were performed on the alginate nanofibers by combining DMAPMA with ammonium persulfate (APS- photo initiator), N, N, N’, N’ tetramethylethylenediamine (TEMED- crosslinking accelerator), and N, N methylenebisacrylamide (MBAA- crosslinker) in various concentrations. A final solution volume of 200 μl was used for all samples. After coating, the samples were irradiated with UV light (λ = 254 nm, P = 30 W) for one hour.
4.2.4. Fourier Transform Infrared Spectroscopy (FTIR)

Characterization of the fibers was done via a Tensor 27 FTIR instrument from Bruker (Bruker Corporation, Billerica, MA). The instrument features a single-beam KBr beamsplitter, and can detect compounds in the range of 4000-400 cm$^{-1}$, with resolution of 1 cm$^{-1}$. OPUS software was used for data processing. Analysis was done on fibers both before and after crosslinking.

4.2.5. Scanning Electron Microscopy (SEM)

The average size of the alginate modified and unmodified fiber mats were characterized by scanning electron microscopy. The samples were observed directly, with no fixation or dehydration necessary, and observed using an environmental scanning electron microscope (Nova Nano SEM 600, FEI, Hillsboro, OR). The average size of a particular concentration of fibers was determined by averaging 20 random fibers.

4.2.6. ES Cell Culture

Mouse CCE embryonic stem (ES) cells were obtained from StemCell Technologies (Vancouver, BC, Canada)[154, 155]. ES cells were cultured in gelatin-coated tissue culture flasks and fed with ES maintenance media, which is comprised of the following components: Dulbecco’s Modified Eagle’s Medium (DMEM with 4.5 g/l D-glucose) supplemented with 15% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.1 mM MEM non-essential amino acids, 10 ng/ml murine recombinant leukemia inhibitory factor (LIF; StemCell Technologies, Vancouver, Canada), 0.1 mM monothioglycerol, 2 mM L-glutamine, and 1 mM sodium pyruvate (Sigma Aldrich, St. Louis, MO) as described previously[104]. LIF is typically used to maintain mouse ES cells in an undifferentiated state. Thus, for the MTT assay, confocal
microscopy, and SEM analysis, where we are observing how alginate-modified fibers alone could maintain ES cell pluripotency, the cells were fed every other day to limit the amount of LIF.

4.2.7. MTT Assay for Cell Proliferation

Cells were seeded onto 0%, 0.5%, and 1% DMAPMA-MAA modified alginate fibers that were electrospun on top of 12 mm glass coverslips and fitted in a 24-well plate at a density of 20,000 cells/well. Additionally, there were coverslips coated with gelatin to serve as controls. ES maintenance media was changed every 2 days, and after 5 days an MTT assay was performed to investigate cell expansion. Briefly, 100 µl of MTT labeling reagent was added to each well and the plate was incubated for 4 hours at 37 ºC. After incubation, 750 µl of solubilization reagent was added to each well and the plate was incubated overnight. Absorbance readings were taken at 560 nm and 700 nm (as the reference) on a Tecan Infinite M200 plate reader (Tecan US, Research Triangle Park, NC). Cell growth index was calculated by dividing the absorbance value of each sample by that of ES cells grown on conventional gelatin-coated coverslips.

4.3. Results

4.3.1. Synthesis of Alginate-DMAPMA-MAA Nanofibers for Embryonic Stem Cell Expansion

4.3.1.1. Synthesis of DMAPMA-MAA polymer

Initially, we tried to directly synthesize the DMAPMA-MAA copolymer using a thermal initiation method. DMAPMA and MAA were polymerized in methanol at a molar ratio of 1:2:26.4 (DMAPMA/MAA/methanol) using AIBN as a thermal initiator, at 60º C overnight. This ratio equated to an equal volume of DMAPMA and methacrylic
acid being used (5 ml) with an excess of methanol (30 ml). FTIR analysis was performed on the solid DMAPMA-MAA polymer, and the resulting spectrum is shown in Figure 4.1. As a comparison, analysis of both DMAPMA and MAA monomers were performed, and the spectra are shown in the same figure. The presence of functional peaks in the region of 2700-3400 cm\(^{-1}\) in the synthesized polymer indicates that both DMAPMA and MAA have been incorporated into the polymer. The characteristic amide peak (~1630-1680 cm\(^{-1}\)) is also present in the DMAPMA monomer and synthesized DMAPMA-MAA polymer, confirming the incorporation of DMAPMA into the resulting polymer.

![FTIR Spectra of DMAPMA-MAA Polymer](image)

**Figure 4.1** FTIR spectra of synthesized DMAPMA-MAA copolymer (green), along with its 2 constituent monomers, DMAPMA (blue), and MAA (red).

### 4.3.1.2. Electrospinning of Alginate-DMAPMA-MAA Blended Nanofibers

Next, the ability to electrospin DMAPMA-Alginate nanofibers was examined. An equal volume of 1.5% alginate-PEO was combined with a 1.5% DMAPMA-MAA solution and vortexed until homogeneity occurred. This uniform solution was then
electrospun, however, it was revealed under SEM that a substantial amount of beading had occurred, as shown in Figure 4.2. It was determined that the solution viscosity was too low for it to be electrospun, so a different approach to form the nanofibers was performed. The DMAPMA-MAA solid was dissolved into an appropriate amount of DI water to make the concentration either 0.5% or 1%. After the solution was homogenous, alginate (1.5%) and PEO (3%) were incorporated into the mixture and the solution was stirred until homogeneity was reached. This solution was then electrospun and fibers formed with very little beading. The effect of DMAPMA-MAA concentration on nanofiber formation was further investigated, and SEM images were taken before and after crosslinking with 1.5% CaCl$_2$ (Figure 4.3). The diameters of the nanofibers for both concentrations before and after crosslinking were calculated and are shown in Table 4.1. Additionally, FTIR spectra showed that DMAPMA-MAA fibers with a concentration of 0.5% did not retain their functionality after crosslinking compared to DMAPMA-MAA fibers with a concentration of 1% (compare Figure 4.4a to 4.4b).

![Figure 4.2](image1.png)  
**Figure 4.2** SEM images of a blended solution of 1.5% DMAPMA-MAA polymer and 1.5% Alginate. Both images show a high degree of beading upon electrospinning the fibers. Scale bar for a) is 20 µm and b) 1 µm.
Figure 4.3 SEM images of alginate modified fibers. All fibers were 1.5% alginate-3% PEO with incorporation of either 0.5% or 1% DMAPMA-MAA. Images shown are done before and after crosslinking with 1.5% CaCl\(_2\). Scale bars are a,b,d) 10 µm; c) 5 µm.

Table 4.1 Average diameter of DMAPMA-MAA nanofibers before and after crosslinking. Diameters are the average of 20 random fibers.

<table>
<thead>
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<th>Condition</th>
<th>Average Fiber Diameter (nm)</th>
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<tr>
<td>0.5% DMAPMA-MAA before crosslinking</td>
<td>144 ± 24</td>
</tr>
<tr>
<td>0.5% DMAPMA-MAA after crosslinking</td>
<td>298 ± 92</td>
</tr>
<tr>
<td>1% DMAPMA-MAA before crosslinking</td>
<td>131 ± 51</td>
</tr>
<tr>
<td>1% DMAPMA-MAA after crosslinking</td>
<td>217 ± 51</td>
</tr>
</tbody>
</table>
Figure 4.4 FTIR spectra of a) 1.5% Alginate-3% PEO-1% DMAPMA-MAA fibers and b) 1.5% Alginate-3% PEO-0.5% DMAPMA-MAA fibers before and after crosslinking with 1.5% CaCl$_2$. The presence of the amide peak (~1630 cm$^{-1}$) in both spectra after crosslinking indicates that the DMAPMA is still intact.
4.3.1.3. Cell Adhesion and Proliferation on Electrospun Alginate-DMAPMA-MAA Blended Nanofibers

To investigate cell adhesion and proliferation, mouse ES cells were seeded onto electrospun Alginate-DMAPMA-MAA fibers at a concentration of 20,000 cells/well. The cells were allowed to grow for 5 days, with the maintenance media being changed every other day in order to reduce the amount of LIF the cells were receiving. After 5 days, an MTT assay was performed in order to determine the cell proliferation. Initial results were not promising, as cells grown on Alginate-DMAPMA-MAA modified fibers did not exhibit greater cell proliferation than cells cultured onto gelatin-coated coverslips (the control group), as shown in Figure 4.5. We attributed this issue to the fact that the majority of the functional groups of DMAPMA might not be present on the outer surface of the alginate-DMAPMA-MAA fibers. In addition, the percentage of DMAPMA in the blended polymer may be below the optimal concentration for the greatest cell attachment.

The molar ratio of monomers to AIBN initially was 435 during the synthesis of the DMAPMA-MAA polymer, which may have led to the consumption of the thermal initiator too quickly, and caused incomplete DMAPMA polymerization. The residue of the monomer in the hydrogel nanofibers may cause a cytotoxicity effect. An experiment showing that mouse ES cells encapsulated inside the alginate-DMAPMA-MAA hydrogel supported adhesive, undifferentiated cell growth (data not shown), suggests that the alginate-DMAPMA-MAA hydrogel is not toxic to cells but might not contain sufficient DMAPMA on the surface of the hydrogel. This result indicates that the low cell attachment and growth on the alginate-DMAPMA-MAA nanofibers might be caused by insufficient DMAPMA on the surface of the blended hydrogel nanofibers. More work
needs to be carried out to determine the optimal DMAPMA content in the alginate-DMAEMA-MAA blended hydrogel nanofibers for the best cell attachment and expansion.

4.3.2. DMAPMA Modification of Electrospun Alginate Nanofibers

4.3.2.1. Surface coating and photo-initiation of DMAPMA-functionalized Alginate Nanofibers

In an effort to synthesize alginate nanofibers that support strong cell attachment and high cell expansion, an alternative approach to photo-initiation of DMAPMA-coated alginate nanofibers was further investigated, inspired by polyacrylamide polymerization. We have electrospun our alginate-PEO nanofibers, and then crosslink them with a

![Figure 4.5 Cell attachment as quantified by MTT assay. Cells were initially seeded at 20,000 cells in 400 µl media, and were allowed to grow on the fibers for 5 days.](image)
solution of 1.5% calcium chloride to form alginate hydrogel nanofibers. After washing, a solution of DMAPMA (monomer), TEMED, APS, and deionized water were added directly to the alginate nanofibers. The solution reacted for 10 minutes, was removed, and the hydrogel nanofibers were washed with DI water. We examined four different conditions: 4% DMAPMA-0.5% TEMED-0.8% APS; 4% DMAPMA-0.1% TEMED-0.1% APS; 10% DMAPMA-1.25% TEMED-2% APS; 10% DMAPMA-0.25% TEMED-0.25% APS. The concentrations used by Sun et al. served as a guideline for us to initially achieve polymerization[213], in which APS was used as a photo-initiator, MBAA as a crosslinker and TEMED as an accelerator for polyacrylamide, and calcium sulfate was used as a crosslinker for alginate to form highly stretchable and tough alginate-acrylamide hydrogels. A diagram indicating how these polymer chains react is shown in Figure 4.6. Our 4% DMAPMA solutions simulated conditions similar to our initial grafting conditions of 2D membranes. For cell studies, these fibers were sterilized with 70% ethanol for 5 minutes, and then washed three times with deionized water. FTIR analysis was performed to see if the characteristic peaks of DMAPMA were present after polymerization, and the results are shown in Figure 4.7.

![Diagram of polymer networks](image)

**Figure 4.6** In the alginate-polyacrylamide gel, the two types of polymer networks are linked by amine groups on the polyacrylamide chains and carboxyl groups on the alginate chains, from Sun et al. Copyright granted by Nature.
We demonstrated that DMAPMA was successfully incorporated into all four modified conditions. We seeded 20,000 ES cells onto all of the scaffolds, and let them grow for 3 days, while limiting the amount of LIF the cells received. We observed that the cells adhered to the scaffolds over the 3 day period, however, cell proliferation was greatest for the 10% DMAPMA-1.25% TEMED-2% APS polymerized alginate hydrogel, as demonstrated by an MTT assay (Figure 4.8).

Figure 4.7 FTIR spectra of DMAPMA, TEMED and APS polymerizations. The spectra showed the DMAPMA characteristic spectra for all four conditions.
4.3.2.2. Effects of DMAPMA Concentration on Cell Adhesion and Proliferation

Expanding on our previous results with photo-initiated DMAPMA modified alginate hydrogels, we wanted to investigate cell behavior as a function of DMAPMA concentration. With these new constructs, we added in the crosslinker, N, N’ methylenebisacrylamide (MBAA) to potentially strengthen them. These DMAPMA-modified alginate hydrogels were also cured under UV light ($\lambda = 254$ nm, $P = 30$ W) for one hour[213]. Initially, we compared a 10% DMAPMA-1.25% TEMED-2% APS-1.35% MBAA gel slab to a 20% DMAPMA-2.5% TEMED-4% APS-2.7% MBAA gel slab. In this case, the polymer was formed on top of sterilized glass coverslips. A gelatin-coated coverslip were used as a control in this experiment. After seeding the constructs with 20,000 cells/well, and allowing the cells to attach overnight, cell proliferation was measured via an MTT assay, as shown in Figure 4.9.

Figure 4.8 Cell proliferation, as quantified by MTT assay. These results are from two separate 3-day experiments. Cells were initially seeded at 20,000 cells in 400 $\mu$l media, and were allowed to grow on the scaffolds for 3 days.
The trend of increasing DMAPMA concentration leading to greater cell proliferation led us to further increase the concentration of DMAPMA up to 30%. We had two formulations for 30% DMAPMA alginate hydrogels: 30% DMAPMA-2.5% TEMED-4% APS-2.7% MBAA (high concentration) and 30% DMAPMA-0.5% TEMED-0.5% APS-0.5% MBAA (low concentration). These values were scaled up based on the typical formulations that were used in the literatures for forming polyacrylamide gel for electrophoresis or cell culture. For both of these cases, the solution was added on top of alginate nanofibers, and exposed to UV light for 1 hour. ES cells were then seeded directly on top of the modified nanofibers overnight, and cell proliferation was quantified the next day by an MTT assay. We observed that the 30% DMAPMA with the higher concentration of TEMED and APS was far superior to the lower concentration, as well as unmodified alginate nanofibers and gelatin coated coverslips (Figure 4.10 a-b).
result is likely due to the greater concentration of photoinitiator and accelerator in the solution, which may lead to the formation of more durable hydrogels with a higher DMAPMA content.

**Figure 4.10a-b** Cell proliferation of ES cells on 30% DMAPMA-modified fibers. These are the results of two separate experiments with these conditions.
4.4. Discussion

In this study, we demonstrated that DMAPMA can be combined with methacrylic acid to form a DMAPMA-MAA copolymer. This polymer can then be added to an alginate-PEO solution and electrospun to create blended Alginate-DMAPMA-MAA nanofibers. DMAPMA can also be used as a surface coating, when photo-initiated in the presence of APS, TEMED and MBAA. By treating alginate nanofibers with this solution, we were able to create surfaces that showed high cell attachment and proliferation. We demonstrated through cell proliferation assays that a higher concentration of initiators presumably creates more DMAPMA. This could be independently confirmed through FTIR, in which the ratio of the amide peak to the C=C peak could be taken for the various concentrations. A greater ratio would indicate a larger quantity of DMAPMA.

Polyacrylamide is comprised of acrylamide repeat units, which share a similar chemistry (amide) to DMAPMA. Polyacrylamide has been widely used in stem cell research. For example, polyacrylamide hydrogels have been used for investigating human ES cell organization and fate decision[214], central nervous system regeneration[215], and differentiation of umbilical cord stem cells[216], demonstrating its versatility in the field. A combination polymer consisting of polyacrylamide-alginate has shown very unique mechanical properties-highly stretchable and tough[213], with a fracture toughness ~ 9000 J/m²[217]. This polymer showed low cytotoxicity of D1 mouse MSCs, minimal inflammation of tissue surrounding the gels, as well as low degradation of the gels upon exposure to culture conditions[217]. Structurally, DMAPMA and polyacrylamide both possess amide functional groups, which may explain why these
polymers exhibit high cell attachment. Additionally, DMAPMA contains a tertiary amine, so there is potential for this compound to form hydrogen bonds with the cysteine-rich regions of integrin β1, which is a known binding protein for ES cells. The chemical structure and behavior of DMAPMA with ES cells give great potential for DMAPMA-alginate nanofibrous matrices. We predict that DMAPMA-modified alginate hydrogels could not only be adhesive but also be highly stretchable and mechanically strong, similar to alginate-polyacrylamide hydrogels[216].

The ability to incorporate DMAPMA into alginate provides a new avenue to generate adhesive and mechanically strong alginate hydrogels and broadens the impact of alginate in biomedical research. Wang et al. demonstrated the feasibility and effectiveness of using alginate hydrogels for implants and cell therapies[126]. It has been previously demonstrated that alginate hydrogels can mimic stem cell microenvironments, allow the maintenance of the stemness and pluripotency of mouse embryonic stem cells, release of chemokines and cytokines from neighboring cells to the microenvironment, and enable co-culture of different type of cells[127, 128, 218]. Alginate hydrogels are effective for supporting stem cell proliferation and self-renewal[29, 30], as well as differentiation. For example, alginate hydrogels have been used for the propagation and direct differentiation of pluripotent stem cells into definitive endoderm[132], hepatocytes[133], insulin producing cells[134, 135], osteogenic lineages[136], cardiac cells[137], and neural lineages[138, 139]. Additionally, alginate can be used for producing embryoid bodies (EBs) with controlled sizes in large scale[131].
Further characterization of the DMAPMA-modified hydrogels will be measured, including elasticity and degradation. By tuning the elasticity, we could potentially create scaffolds that would allow better directed differentiation of stem cells.

Electrospun core-shell nanofibers have great potential in the tissue engineering field. Ma et al. demonstrated that fibroblasts could be cultured onto core-shell fibers and that these fibers were not toxic to the cells. Additionally, the cells exhibited ideal morphology (spindle-like) after 72 h of culture[219]. Incorporating biological molecules with alginate to enhance its properties has been demonstrated. Chitosan, a polysaccharide consisting of glucosamine, has been combined with alginate to create a chitosan sheath with polyanion-polycation complexes[220], wound dressings[221], and with collagen and hydroxyapatite for bone tissue engineering applications[222]. Additionally, ECM proteins (e.g., collagen, fibronectin, laminin) and RGD sequences have been incorporated into alginate to make it adhesive to cells. These macromolecules are very expensive and cell-type dependent. The ability to use the optimal synthetic organic chemistry, e.g., DMAPMA as surface coating offers a new way to produce chemically defined, scalable, and sustainable polymeric substrates and/or hydrogels and in particular, provides inexpensive and effective alternative to RGD sequences, ECM proteins or other macromolecule coatings.

4.5. Conclusions

In this work, we have synthesized a polymer consisting of DMAPMA-MAA. We have further incorporated this polymer into an alginate solution to make scaffolds via two strategies: first, by directly combining the polymers together and electrospinning to make blended DMAPMA-MAA-alginate nanofibers, and secondly, by adding the DMAPMA
monomer in the presence of a photoinitator onto the electrospun alginate nanofibers and curing them by UV irradiation. Mouse ES cells grown on UV-initiated DMAPMA-coated alginate hydrogel exhibited greater cell attachment and proliferation than those grown on blended DMAPMA-MAA-alginate nanofibers. Additionally, we have demonstrated that there is a concentration dependency of DMAPMA for the UV-treated DMAPMA-functionalized alginate hydrogels. It offers a novel approach to creating adhesive alginate hydrogels, opening up wider applications of alginate hydrogels in cell culture, tissue engineering and cell therapy.
CHAPTER 5

Potential Cell-Surface Chemistry Interactions

5.1. Cell-Surface Chemistry Interactions

Initially, our 66 monomers were chosen as they all contained vinyl groups which could undergo free radical polymerization. These monomers covered a comprehensive spectrum of chemical groups. We selected monomers from 9 different chemical groups since we were approaching this high-throughput screening from an exploratory perspective, as oppose to a rational perspective. Conventionally, screens have been performed using peptides or chemistries which are known for attachment[32, 33]. In this study, DMAPMA-grafted surfaces have been identified and validated to support strong cell attachment, high cell expansion, and maintenance of stem cell pluripotency using mouse ES cells as a model. The potential interactions between DMAPMA and our ES cells will be addressed further in this section.

5.1.1. DMAPMA Grafting and Polymerization

DMAPMA is unique in that it possesses a terminal tertiary amine and an amide in its chemical structure. In our library of 66 monomers tested, this chemistry was the only compound that contained both of these functional groups. Similar chemistries in our library that possessed terminal tertiary amines (2-dimethylamino ethyl methacrylate-#55 and 2-diethylamino ethyl methacrylate-#56) had slightly lower CAI values. There were also chemistries that possessed amides (N-isopropylacrylamide-#51, N-tert-butylacrylamide-#52, diacetone acrylamide-#53 and N-trishydroxymethyl methyl acrylamide-#54) and they generally had CAI values around 1.0. In our initial screen, chemistries #55 and #56 were behind only chemistries #44 and #57, indicating that the
terminal tertiary amine may play an important role in cell binding. The structures of chemistries #55-57 are shown in Figure 5.1.

![Chemical structures of chemistries #55, 56 and 57 from our monomer library.](image)

After the PES has been irradiated with UV light, breaking the C-S bond, the radical DMAPMA monomers undergo chain growth via free radical polymerization to graft onto each PES active site. This chemical synthesis scheme is demonstrated in Figure 5.2. In this way, the tertiary amines are terminal, not in the backbone of the polymer, and are more available for cell binding.
Although the degree of grafting was not directly measured in this study, it has been shown that FTIR spectroscopy can be utilized to determine this measurement by taking a ratio of a peak corresponding to a functional group and a typical C=C peak[223, 224].

Our collaborator, Dr. Georges Belfort has determined the concentration of the monomers to be 0.2 M, thereby allowing for the maximum degree of grafting for each chemistry[223, 224].

**Figure 5.2** Chain growth of DMAPMA via free radical polymerization.
Additionally, we took permeability measurements of all 66 monomers in which we measured and analyzed the flow of water and PBS on our membranes before and after cell adhesion (Figure 5.3). Our results showed that DMAPMA had one of the lowest permeability values, along with other amide chemistries, compounds #44, 48 and 54, suggesting there may be more grafting of these chemistries compared to the other chemistries on PES membranes with higher permeability. However, more grafting does not necessarily lead to high cell attachment indices, as indicated by the CAI values of chemistries 48 and 54, indicating that the surface chemistry may play an important role in influencing cell adhesion. Additionally, we demonstrated that several PEGs, compounds 32-34 exhibited high permeability values before after cell adhesion, and similarly had CAI values between 0.85-0.95. Altogether, our results indicate that the terminal tertiary amine plays a critical role in ES cell adhesion.
Figure 5.3 Permeability values of all 66 monomers a) before cell adhesion and b) after cell adhesion.
5.1.2. DMAPMA-Cell Interactions Through Integrin β1

Integrin β1 has cysteine-rich repeats[225] that can potentially react with the tertiary amine group of DMAPMA. Work done by Das et al.[226] suggested that due to its bulkiness, DMAPMA would typically be in the trans conformation. When DMAPMA was reacted with acrylic acid, it was speculated that a complex formed by hydrogen bonding between the free hydroxyl group of the acid and the terminal amine (Figure 5.4).
We speculate that the thiol groups on the cysteine rich regions of integrin β1 may react with the tertiary amine group of DMAPMA via hydrogen bonding, similar to the mechanism described by Das. This proposed mechanism is depicted in Figure 5.5.

Figure 5.4 Proposed reaction mechanism of DMAPMA with acrylic acid, from Das et al. Copyright permission granted from Elsevier.

Figure 5.5 Proposed reaction mechanism of DMAPMA with the cysteine rich regions of integrin β1.
5.2. Three-Dimensional Cell-Surface Interactions

After concluding that the DMAPMA-grafted membranes supported mouse ES cell attachment and maintenance in 2D, we then created modified fiber matrices to confirm the capacity to transfer 2D effects into 3D systems. We demonstrated that DMAPMA-modified 3D PES fibers supported slightly higher cell expansion than DMAPMA-modified 2D membranes. Compared to 2D membranes, PES fibers could provide a higher surface-to-volume-ratio for DMAPMA to graft. Therefore, the DMAPMA grafting density for 3D fiber matrices could be higher than 2D surfaces. For these electrospun fiber matrices, similar volumes of solution were pumped through the syringe, however the packing density of the matrix was not measured in this study. Profilometry measurements could be utilized in order to determine the packing density to make this consistent for each sample. This value is critical as a greater amount of DMAPMA-grafted fibers can lead to multiple binding sites between the DMAPMA matrix and ES cells, which can enhance cell attachment.

All of our 3D studies were performed using random fibers, as opposed to aligned fibers. We do speculate that changing from random to aligned fibers will have an effect on cell morphology as well as potentially differentiation. From a chemical modification point of view, aligned fibers would provide more DMAPMA-grafting than random fibers. With the case of morphology, cells will tend to align and spread along the fibers. We are also interested in examining DMAPMA coated fibers vs. ECM coated fibers, such as laminin and fibronectin. For these studies, we would perform similar cell proliferation assays to determine metabolic activity, and also confirm cell pluripotency through immunocytochemistry of pluripotency markers. To further confirm pluripotency, the
hanging drop technique could be implemented to form embryoid bodies from cells that were cultured on top of these scaffolds.

In summary, we proposed a mechanism for the chain growth of DMAPMA when it undergoes free radical polymerization, as well as a mechanism for its interaction with integrin β1. We speculate the terminal tertiary amines that are present on the DMAPMA polymer are primarily responsible for DMAPMA-cell interactions. We were able to scale from 2D surfaces to 3D fibrous matrices and demonstrated that DMAPMA-modified 3D PES fibers supported slightly higher cell expansion than DMAPMA-modified 2D membranes. In the future, the packing density of these matrices will be determined in order to better understand the DMAPMA-cell interactions in 3D.
SUMMARY

I. Conclusions

The ability to identify an optimal synthetic organic chemistry for a uniform culture system for pluripotent stem cell expansion is critical for many avenues of stem cell research, including therapeutic development, drug discovery and developmental biology. Nanoscale engineering of the surface chemistry and topography has been shown to manipulate cell behavior and is critical in maintaining stem cell pluripotency. Additionally, spatial dimensionality plays a key role in cell behavior, including attachment, colony size, and differentiation potential. Among 3D culture systems, nanofibers have been shown to serve as ideal platforms, as they can mimic the extracellular matrix, direct stem cell fate decisions, and enhance stem cell proliferation. Both natural and synthetic nanofibers have been used for 3D culture.

In this thesis, we have confirmed the hypothesis that a synthetic, organic chemistry can support pluripotent stem cell attachment and maintenance in 2D and 3D. First, we have successfully identified a “hit” chemistry, DMAPMA that possessed a terminal tertiary amine group and peptide bond for pluripotent stem cell culture using a novel high-throughput synthesis and screening approach, and demonstrated that DMAPMA supported a higher degree of cell attachment (CAI value) and expansion capacity (fold of propagation value) than any other surface chemistries in the library or even than those reported in the literature for human ES cell adhesion. This surface maintained pluripotency of mouse ES cells for 7 days as measured by the expression of pluripotency markers, formation of EBs and in vitro differentiation to cell lineages expressing markers of ectoderm, mesoderm and endoderm. It also maintained long-term
self-renewal of mouse ES cells for 7 passages. We have been able to successfully transfer
these results to 3D by grafting DMAPMA to electrospun poly(ether sulfone) fibers
through both photo-induced and plasma-induced graft polymerization and demonstrated
that our modified fibers exhibited greater expression of the pluripotency marker, Oct4
than unmodified fibers. Lastly, we have been able to incorporate this chemistry with
methacrylic acid into a natural, polymeric, alginate-based hydrogel system directly or
through UV-induced DMAPMA polymerization. Using a UV-induced DMAPMA
polymerization technique, we were able to coat electrospun alginate nanofibers with
DMAPMA and demonstrate that these DMAPMA-modified alginate hydrogels supported
mouse ES attachment and expansion in a DMAPMA concentration dependent manner.

II. Future Directions

In the future, it is necessary to further characterize these alginate-modified gels in
order to utilize them appropriately. For example, by varying the elastic modulus of the
substrate, we may be able to induce differentiation of a specific lineage in ES cells.
Additionally, we would like to test our ideal synthetic chemistry with human iPS or ES
cells to observe if the same type of behavior is observed that we saw with mouse ES
cells.

II.I. Biomechanical characterization and manipulation of alginate-modified
hydrogels

These alginate-modified gels can be utilized as substrates for stem cell
differentiation by altering their composition, which will effectively change their
elasticity. It has been demonstrated that substrate elasticity influences stem cell
differentiation, as cells cultured on stiffer substrates tended to differentiate into bone,
while cells cultured on softer substrates differentiated into neurons. We will characterize the mechanical properties of these hydrogels using atomic force microscopy, and use those results to serve as a basis for the design and synthesis of substrates that can be specific for directing ES cell differentiation.

II.II. Use of human ES cells

All of this work was performed with mouse ES cells. In order to demonstrate how DMAPMA could potentially work as a scaffold to be used for drug discovery or therapeutic development, experiments using human ES and/or iPS cells need to be performed to better gauge their interaction with the optimal surface chemistry. Additionally, it is of paramount importance to use the established chemistry library and high-throughput screening platform for the identification of the optimal surface chemistry for directed stem cell differentiation.

II.III. Synthesis of versatile DMAPMA-modified alginate hydrogels

Other various versatile modified alginate hydrogels can be synthesized to serve as vehicles for drug delivery and tissue engineering. In our lab, we have synthesized DMAPMA-modified alginate microstrands that have encapsulated ES cells and examined their morphology over time. We observed that normal morphology was retained with cells encapsulated in modified alginate microstrands, compared to unmodified microstrands (data not shown). Incorporating DMAPMA into alginate microtubes, microfibers, microbeads, microcapsules, microparticles or microspheres may provide another avenue for diverse future therapeutic development.
II.IV. DMAPMA as a surface coating material alternative to biomacromolecules

We have demonstrated that DMAPMA can be used as a surface coating for nanofibers. However, further formulations are necessary in order to obtain the proper concentration so that better cell attachment and function can be achieved, as well as the nanofiber topography still being intact. In particular, DMAPMA has a great potential to serve as an alternative to RGD sequences or ECM proteins for surface coating to support hard-to-grow cells. More biological studies are required to better understand the optimal synthetic organic chemistry-cell interactions, leading to better design of the substrate for stem cell and other hard-to-grow cell cultures.
References


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APPENDIX

Curriculum Vitae

EDUCATION

College of Nanoscale Science and Engineering, University at Albany, State University of New York, Albany, N.Y.
Ph.D. in Nanoscale Engineering, December 2013
Thesis Title: Defining an Optimal Surface Chemistry for Pluripotent Stem Cell Culture in 2D and 3D

Rensselaer Polytechnic Institute, Troy, N.Y.
B.S. in Biochemistry/Biophysics, Cum Laude, December 2004

EMPLOYMENTS

Graduate Research Assistant, College of Nanoscale Science and Engineering, University at Albany, State University of New York, August 2008-Present

- Researched the effects of surface chemistry on mouse embryonic stem cell attachment for cell expansion, maintenance of cell pluripotency, and differentiation potential
- Engineered synthetic-based functionalized electrospun fibers to create a 3D culture system for mouse embryonic stem cells
- Optimizing a 3D culture system for stem cells by incorporating our optimal surface chemistry into a natural polymer (alginate)
- Investigating the effects of various concentrations of nanoparticles on differentiated mouse embryonic stem cells
- Fabricating polycaprolactone scaffolds for retinal pigment epithelial stem cell growth


- Worked independently and as a team member on taking R&D –scale methods for II-VI, III-V, and IV-VI semiconductor nanocrystal synthesis and scaling to manufacturing quantity, consistency, and quality
- Responsible for quality control of products and responsible for keeping detailed lab notebooks documenting the procedures that were used
- Directly involved with several new product launches from lab to mass fabrication
- Responsible for inventory of all products
- Worked directly with the marketing department to ensure customer satisfaction
- Helped train new employees on manufacturing techniques
- As a member of the manufacturing team, my work translated into revenue generating products that generated in excess of $1M
Samaritan Hospital, Troy, N.Y., Customer Service Specialist, May 2004-February 2005

- Responsible for both clerical and clinical aspects of the laboratory
- Worked in the chemistry department and was responsible for running blood tests

**SKILLS**

- **Cell and Molecular Biology:** Stem cell culture, MTT assay, microplate reader analysis, immunocytochemistry, mRNA extraction, cDNA synthesis, RT-PCR analysis, Western Blot analysis
- **Materials Characterization:** Optical microscopy, scanning electron microscopy (SEM), confocal microscopy, atomic force microscopy, X-ray photoelectron spectroscopy (XPS), infrared spectroscopy, UV-Vis spectroscopy, nuclear magnetic resonance (NMR)
- **Materials Synthesis and Fabrication Experience:** Electrospun various polymeric materials to make nano/micro fibers, electrostatic generation of microbeads and cell encapsulation, and microfluidic synthesis of hydrogel microstrands; Modified alginate with an optimal surface chemistry for ES cell maintenance; photo-induced polymerization; microfabrication using contact aligner and spin-coater
- **Modeling/Computer Analysis:** GraphPad, ImageJ, Proficient in Microsoft Office

**PUBLICATIONS**

Peer-reviewed journal publications

**M.R. Zonca, Jr.,** P.S. Yune, J. Williams, M. Gu, A.M. Unser, G. Belfort and Y. Xie

**M.R. Zonca, Jr.,** P.S. Yune, C.L. Heldt, G. Belfort and Y. Xie

**M.R. Zonca, Jr.** and Y. Xie

B. Falk, **M.R. Zonca, Jr.** and J.V. Crivello

**M.R. Zonca, Jr.,** B. Falk and J.V. Crivello


Manuscripts submitted or in preparation


**TEACHING EXPERIENCE**

- Helped develop the Nanochemistry Lab for the undergraduate course, Chemical Principles of Nanoscale Science and Engineering at CNSE and was a teaching assistant in 2010, 2011, and 2012.
- Supervised two high school students on research projects for individual Intel Science Talent Search (STS) competition, *one of whom became the semifinalist*.
- Supervised six minority high school students from the Troy School District for a 2-hour lab activity.
- Developed a nanobiotechnology lab module for NanoHigh program and Engineering Exploration program targeting urban high school students, and Tech Valley Summer Camp targeting middle school students.
- Disseminated research findings to K-12 students through NanoCareer Day program and to general public through NanoCommunity Day program at CNSE; Worked as a teacher/mentor at Evident Technologies with visiting students from local high schools and with Tech Valley Summer Camp.

**PRESENTATIONS**


**HONORS**

- NSF Fellowship (2009-2012)
- Dean’s List, Rensselaer Polytechnic Institute (7 semesters)
- Arthur G. Schultz Award for Research in Organic Chemistry (May 2003)
- Founders Award of Excellence (Highest Award Given at Rensselaer Polytechnic Institute, Oct. 2003)
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