Tailoring Cell-Material Interactions via Poly(acrylic acid) Brushes to Enhance Nonviral Substrate-Mediated Gene Delivery

Amy Mantz

University of Nebraska-Lincoln, amy.mantz@gmail.com
TAILORING CELL-MATERIAL INTERACTIONS VIA POLY(ACRYLIC ACID) BRUSHES TO ENHANCE NONVIRAL SUBSTRATE-MEDIATED GENE DELIVERY

by

Amy Mantz

A DISSERTATION

Presented to the Faculty of
The Graduate College at the University of Nebraska
In Partial Fulfillment of Requirements
For the Degree of Doctor of Philosophy

Major: Biological Engineering

Under the Supervision of Professor Angela K. Pannier

Lincoln, Nebraska

July, 2019
Nonviral gene delivery modifies gene expression by transferring exogenous genetic material into cells and tissues, typically through a bolus of complexes formed by electrostatic interactions between cationic lipid or polymer vectors with negatively charged nucleic acids (e.g. DNA). Although nonviral gene delivery is safer, more cost-effective, and more flexible compared to viral systems, nonviral transfection suffers from low efficiency due to extracellular and intracellular barriers. Much research has focused on tuning physiochemical properties of the complexing vectors to improve transfection, yet the cell-material interface may prove a better platform to immobilize DNA complexes for substrate-mediated delivery (SMD) and modulate the cellular response to improve transfection to overcome transfection barriers, especially in *ex vivo* or site-specific applications (e.g. biomedical implants). Natural and synthetic substrate modifications have both been investigated to improve transfection via SMD, but synthetic polymer films are often considered more reproducible and tunable compared to natural substrate modifications. While synthetic polymers films have been shown improve the efficacy of SMD (e.g. self-assembled monolayers or polyelectrolytes multilayers), these films have issues with degradation and impeded release of the DNA cargo and, moreover, are not typically studied in the context of clinically relevant metals (i.e. titanium (Ti)). In this
dissertation, polymer films formed with pH-responsive poly(acrylic acid) (PAA) brushes were investigated to resolve these issues by grafting to a Ti substrate, immobilizing DNA complexes through electrostatic interactions with the PAA brushes, and modulating cellular response via conjugated adhesion moieties (i.e. RGD) and adsorbed free PEI. We showed our PAA-RGD platform increased transfection in cells cultured on PEI-DNA complexes immobilized to PAA-RGD compared to PAA alone. Investigations into further tuning the PEI vector and the RGD ligand showed that reduced cytotoxicity and increased proliferation, focal adhesion formation, and endocytic pathway activation may have improved our transfection success, suggesting that PAA-RGD brushes have the potential to immobilization of therapeutic DNA complexes for applications such as Ti biomedical devices, implantable sensors, and diagnostics tools.
DEDICATION

To
Kyle and Dog
(and Cat)
ACKNOWLEDGMENTS

First, thank you to Angie for your guidance and providing me an example of the researcher I could be. Thank you for contributing to my college experience and for allowing me to develop personally and professionally in your lab. Thank you to Mathias for giving advice and guidance throughout my degree, as well as providing me with international collaboration opportunities. Thank you to Petra Uhlmann for hosting me at her institution in Dresden and for giving me the opportunity to learn new techniques in polymer science. Thank you to Alice Rosenthal for collaborating with me while I worked in Dresden and showing me her amazing city and friend group. Thank you to the Pannier Lab members who helped me with science and provided entertainment with conversations about work and other subject matter. Thank you for letting me confide my worries and anxieties about research and my life, especially to Sarah Plautz, Taylor Laughlin, Albert Nguyen, and Sophie Walsh.

Thank you to my family who provided support and first stimulated my interest in science. Without you, I would not be on the path that I am today. Thank you to my friends who listened to me practice lectures (even when you didn’t know what I was talking about) and gave me a much-needed break with your companionship.

Finally, most importantly, thank you to my husband Kyle. Without you, I couldn’t have done any of this. Your constant support and confidence in my abilities in science have been unwavering and I love you more than words could say.
PREFACE

The work presented in Chapter 2 contains portions of a previously published article and has been reprinted and adapted with permission from Mantz, A., & Pannier, A. K. Biomaterial substrate modifications that influence cell-material interactions to prime cellular responses to nonviral gene delivery. Experimental Biology and Medicine (244:2), pp. 100–113. Copyright © 2019, SAGE Publishing. DOI: 10.1177/1535370218821060

The work presented in Chapter 3 has been previously published and has been reprinted and adapted with permission from Rosenthal et al. J. Phys. Chem. B, 122(25), 6543-6550. Copyright 2018, American Chemical Society. This work was completed together in collaboration with Alice Rosenthal, of the Leibniz Institute of Polymer Research, Dresden, Germany.

The work presented in Chapter 4 has been previously published in Frontiers in Chemistry, an open access journal, where the authors retain the copyright and are free to reproduce and disseminate their work. This work was completed together in collaboration with Alice Rosenthal, of the Leibniz Institute of Polymer Research, Dresden, Germany.
LIST OF ABBREVIATIONS

Amil Amiloride
ANOVA Analysis of variance
AOI Angle of incidence
BCA Bicinchinonic acid
bPEI Branched polyethylenimine
BSA Bovine serum albumin
Cdc42 Cell division control protein 42
CHCl₃ Chloroform
cm Centimeter
CMV Cytomegalovirus
CPZ Chlorpromazine
cRGDyK cyclic RGD
DAPI 4’,6-diamidino-2-phenylindole
DMEM Dulbecco’s Modified Eagle’s Media
DMSO dimethyl sulfoxide
DNA deoxyribonucleic acid
DOPE dioleyl phosphatidylethanolamine
DOSPA 2,3-dioleoyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-l-propanaminium trifluoroacetate
DOTAP N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate
DOTMA N-[1-(2, 3-dioleoyloxy) propyl]-N, N, N-trimethylammonium chloride
EDC N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride
ECM Extracellular matrix
eGFP Enhanced green fluorescence protein
EthD-1 Ethidium homodimer 1
EtOH Ethanol
GE Generalized spectroscopic ellipsometry
Gen  Genistein

GLAD  Glancing angle deposition

GRGDS  linear RGD

HA  Hyaluronan

hMSCs  Human mesenchymal stem cells

kDa  Kilodalton

LF2000  Lipofectamine 2000®

LPEI  Linear polyethylenimine

LUC  Luciferase protein

MES  2-(N-morpholino)ethanesulfonic acid

mg  Milligram

mL  Milliliter

mMSCs  Murine mesenchymal stem cells

MW  Molecular weight

N/P  Nitrogen to phosphorus ratio

NHS  N-hydroxysuccinimide

NIH  National Institutes of Health

NIH/3T3  Mouse fibroblast cell line

PAA  Poly(acrylic acid)

PBS  Phosphate buffered saline

PEI  Polyethylenimine

PEG  Polyethylene glycol

PEM  Polyelectrolyte multilayer

PGMA  Poly(glycidyl methacrylate)

PLL  Poly-L-lysine

pnSCTF  Pre-nucleated slanted columnar thin film

PNIPAM  Poly(N-isopropylacrylamide)
P2VP  Poly(2-vinyl pyridine)
PDMAEMA Poly(N,N-dimethylaminoethyl methacrylate)
PS  Polystyrene
Rac1  Ras-related C3 botulinum toxin substrate 1
RGD  Arginine-glycine-aspartic acid
RGE  control
RLU  Relative light unit
SAM  Self-assembled monolayer
SEM  Standard error of the mean or scanning electron microscopy
Si  Silicon
SiO₂  Silicon dioxide
SMD  Substrate-mediated gene delivery
STFs  Sculptured thin films
TCPS  Tissue culture polystyrene
Ti  Titanium
TiO₂  Titanium dioxide
µg  microgram
WST  water-soluble tetrazolium
**TABLE OF CONTENTS**

**CHAPTER 1**
Introduction – Dissertation Overview ................................................................. 1
  1.1 Motivation and Objectives ...................................................................... 1
  1.2 Dissertation Outline ............................................................................. 3

**CHAPTER 2**
Biomaterial Substrate Modifications that Influence Cell-Material Interactions to Prime Cellular Responses to Substrate-Mediated Nonviral Gene Delivery................................................................. 6
  2.1. Introduction .......................................................................................... 6
  2.2. Nonviral Gene Delivery ....................................................................... 7
    2.2.1. Cationic Lipid and Polymers .............................................................. 8
    2.2.2. Delivery Methods of Nonviral Complexes ....................................... 10
    2.2.3. Barriers to Successful Nonviral Gene Delivery ................................. 12
      2.2.3.1. Internalization .......................................................................... 12
      2.2.3.2. Intracellular Trafficking and Nuclear Localization ......................... 15
    2.2.4. Priming the Cellular Response to Transfection ................................. 17
  2.3. Modifications to Surface Chemistry that Influence Cellular Responsiveness to Gene Delivery ............................................................................................................ 20
    2.3.1. Natural Material Coatings and Chemical Side Groups to Prime Bolus Gene Delivery ...................................................................................................... 20
    2.3.2. Natural Material Coatings to Prime Substrate-Mediated Gene Delivery ................................................................. 26
    2.3.3. Polymer Films to Prime Substrate-Mediated Gene Delivery ............. 28
  2.4. Polymer Brushes ..................................................................................... 30
    2.4.1. Poly(acrylic acid) Brushes as a Platform for SMD ............................... 32
  2.5. Conclusions .......................................................................................... 35

**CHAPTER 3**
Biofunctionalization of Titanium Substrates using Nanoscale Polymer Brushes with Cell Adhesion Peptides ............................................................................................. 38
  3.1. Introduction .......................................................................................... 38
  3.2 Materials and Methods .......................................................................... 41
    3.2.1. Deposition of Ti on Si Substrates ....................................................... 41
    3.2.2. Preparation of Poly(acrylic acid) (PAA) brushes on Ti Surfaces .......... 41
    3.2.3. Covalent Binding of RGD Peptides to PAA Brushes .......................... 42
    3.2.4. Ellipsometric Measurements and Modeling of PAA Brushes ............ 43
    3.2.5. Quantification of RGD Amount via Ellipsometric Modeling ............ 46
3.2.6. AFM Measurements of Flat Ti and PAA Brushes ........................................ 47
3.2.7. Dynamic Contact Angle Measurements .................................................. 48
3.2.8. Cell Culture and Adhesion Assay ............................................................ 48
3.2.9. Statistical Analysis .................................................................................. 49
3.3. Results and Discussions .............................................................................. 50
  3.3.1. Investigation of PAA grafting and pH-dependent swelling behavior ........ 50
  3.3.2. Immobilized RGD amount on PAA brushes ............................................ 54
3.4. Conclusions ................................................................................................. 59

CHAPTER 4
Free Polyethyleneimine Enhances Substrate-Mediated Gene Delivery on
Titanium Substrates Modified with RGD-Functionalized Poly(acrylic acid)
Brushes ............................................................................................................... 61
4.1. Introduction .................................................................................................... 61
4.2. Materials and Methods .................................................................................. 65
  4.2.1. Preparation of PAA brushes on Ti Surface and Covalent Bonding of
         RGD/RGE Peptides ....................................................................................... 65
  4.2.2. DNA complex formation and characterization ......................................... 67
  4.2.3. Ellipsometric measurements for characterization of PAA brushes and DNA
         complex immobilization ............................................................................... 68
  4.2.5. Cell culture and substrate-mediated gene delivery .................................... 71
  4.2.6. Cell adhesion of NIH/3T3 fibroblasts cultured on PAA brushes with
         immobilized complexes ............................................................................... 72
  4.2.7. Assessing the contribution of free bPEI on transfection success with SMD .. 73
  4.2.8. Cell viability of NIH/3T3 fibroblasts cultured on PAA brushes with immobilized
         filtered and unfiltered complexes ................................................................. 73
  4.2.9. Statistical analysis .................................................................................. 74
4.3. Results ........................................................................................................... 74
  4.3.1. PAA brush film characterization ............................................................... 74
  4.3.2. Substrate-mediated gene delivery ............................................................. 76
  4.3.3. Immobilization and release of DNA-bPEI complexes ............................. 79
  4.3.4. Cellular adhesion and viability on DNA-bPEI complexes immobilized on
         substrates ..................................................................................................... 82
  4.3.5. Investigating the effect of free bPEI on substrate-mediated gene delivery ... 84
4.4. Discussion ...................................................................................................... 88
4.5. Conclusions .................................................................................................. 96

CHAPTER 5
Priming the Cellular Response for Substrate-Mediated Gene Delivery on
Titanium Substrates Modified with RGD-Functionalized Poly(acrylic acid) Brushes ................................................................. 98

5.1. Introduction ........................................................................................................................................ 98

5.2. Materials and Methods ..................................................................................................................... 102

5.2.1. Preparation of PAA brushes on Ti surfaces and covalent bonding of peptides .................................................. 102

5.2.2. DNA complex formation and immobilization ..................................................................................... 104

5.2.3. Ellipsometric measurements for characterization of peptide conjugation and complex immobilization to PAA brushes ............................................................................................................. 105

5.2.4. Cell culture ...................................................................................................................................... 107

5.2.5. Substrate-mediated gene delivery to NIH/3T3 fibroblasts cultured on PAA brushes with immobilized PEI-DNA complexes ................................................................................................................. 107

5.2.6. Cellular proliferation of NIH/3T3 fibroblasts cultured on PAA brushes with immobilized PEI-DNA complexes ........................................................................................................................................................................ 108

5.2.7. Immunofluorescent staining of focal adhesions and actin stress fibers of NIH/3T3 fibroblasts cultured on PAA brushes with immobilized 25 kDa bPEI-DNA complexes ........................................................................................................ 109

5.2.8. Endocytic pathway inhibition of NIH/3T3 fibroblasts cultured on PAA brushes with immobilized 25 kDa bPEI-DNA complexes ........................................................................................................... 110

5.2.9. Statistical analysis ............................................................................................................................ 111

5.3. Results ............................................................................................................................................... 111

5.3.1. Characterization of the PAA-RGD brush platform for SMD ........................................................................... 111

5.3.1.1. Conjugation of peptides to PAA brushes ................................................................................................. 112

5.3.2. The efficiency of substrate-mediated gene delivery for NIH/3T3 fibroblasts cultured on PAA brushes in response to GRGDS concentration .............................................................................................................. 113

5.3.3. The transgene expression of substrate-mediated gene delivery for NIH/3T3 fibroblasts cultured on PAA brushes in response to the conjugated peptide and PEI vector ........................................................................................................ 115

5.3.3.1. Immobilization of PEI-DNA complexes ...................................................................................................... 117

5.3.3.2. Assessing cellular proliferation in response to SMD on PAA brushes ................................................................................................................................. 119

5.3.4. Assessing cellular focal adhesion formation and cytoskeletal arrangement in response to substrate-mediated gene delivery on PAA brushes ............................................................................................................. 121

5.3.5. Assessing endocytic pathways in response to substrate-mediated gene delivery on PAA brushes ........................................................................................................................................................................... 125

5.4. Discussion ........................................................................................................................................... 127

5.5. Conclusions ....................................................................................................................................... 135
CHAPTER 6
Conclusions and Future Directions .............................................................. 137

6.1. Conclusions .......................................................................................... 137

6.2. Future Directions .................................................................................. 141

6.2.1. Further Tuning the Presentation of Free PEI and the RGD Ligand on PAA Brushes .................................................................................. 141

6.2.1.1. Future Direction: Modulating Transfection via the Use of Two PEI Constituents for Separate Functions as Free and Complexing Polymers .................................................................................. 142

6.2.1.2. Future Direction: Conjugation of Multiple Ligands to Mimic the ECM .................................................................................. 144

6.2.2. Future Direction: Binary Polymer Brushes for Multiple Stimuli-Response ... 146

6.2.3. A Combinatorial Approach of Chemical Substrate Modifications with Physical Substrate Modifications .................................................................................. 149

6.2.3.1. Future Direction: Combinatorial Physical and Chemical Cues for Enhanced Cellular Response to Transfection via SMD .................................................................................. 151

6.3. Final Conclusions .................................................................................. 154

References .................................................................................................... 156

APPENDIX A
Supporting Information for Chapter 5 ............................................................ 182

A.1. Methods .................................................................................................. 182

A.1.1. Cellular Adhesion of NIH/3T3 Fibroblasts on PAA-GRGDS Brushes .... 182

A.1.2. AFM-IR of GRGDS conjugated to PAA brushes .................................. 182

A.1.3. DNA complex characterization and immobilization .......................... 183

A.3 Figures .................................................................................................. 186

APPENDIX B
Supporting Information for Chapter 6 ............................................................ 190

B.1 Supplemental Text .................................................................................. 190

B.2 Supplemental Results and Discussion ...................................................... 190

B.2.1. Bolus investigations on STFs .............................................................. 190

B.2.2. SMD Investigations on STFs .............................................................. 195

B.3 Tables .................................................................................................. 199

B.4 Figures .................................................................................................. 201
LIST OF FIGURES

Figure 2-1: Bolus (A) and substrate-mediated delivery (SMD) (B) methods of transfection

Figure 2-2: Endocytic pathways involved in nonviral gene delivery

Figure 2-3: Integrin binding and focal adhesion assembly

Figure 2-4: “Grafting from” (A) and “grafting to” via physisorption (B) or chemisorption (C) techniques of polymer brush formation.

Figure 3-1: “Grafting to” of PAA brushes to Ti substrates.

Figure 3-2: Structural formula of GRGDS

Figure 3-3: A representative image acquired by AFM in a tapping mode of the roughness of Ti evaporated onto Si wafer.

Figure 3-4: Triplicate images of the roughness of PAA brushes on Ti acquired by AFM in a tapping mode (A) and measured for the average roughness of PAA brushes on Ti (B).

Figure 3-5: pH-dependent swelling behavior of PAA brushes grafted to Ti substrates.

Figure 3-6: Representative images of the adhesion of NIH/3T3 fibroblasts cultured on Flat Ti surfaces with PAA brushes, Flat Ti surfaces with 1.0 mg/mL RGD-modified PAA brushes, and Flat Ti control, stained with calcein at 24 hours (A-C) and 48 hours (D-F).

Figure 3-7: Quantification of the adhesion of NIH/3T3 fibroblasts cultured on Flat Ti grafted with PAA brushes, PAA brushes modified with RGD at 1.0 mg/mL, and Flat Ti control.

Figure 4-1: bPEI-DNA complex immobilization on PAA brushes at pH 7.2

Figure 4-2: Zeta potential and sizing of bPEI-DNA complexes with varied N/P ratios

Figure 4-3: Substrate-mediated gene delivery of bPEI-DNA complexes in NIH/3T3 fibroblasts with varied N/P ratios

Figure 4-4: DNA complexes immobilized to PAA brushes compared to Flat Ti

Figure 4-5: DNA complexes released from PAA and PAA-RGD brush substrates, compared to Flat Ti
Figure 4-6: Adhesion and viability quantification of NIH/3T3 fibroblasts cultured on PAA brushes with bPEI-DNA complexes

Figure 4-7: Substrate-mediated gene delivery of filtered bPEI-DNA complexes in NIH/3T3 fibroblasts

Figure 4-8: Viability quantification of NIH/3T3 fibroblasts cultured on PAA brushes with filtered bPEI-DNA complexes

Figure 4-9: Substrate-mediated gene delivery of filtered bPEI-DNA complexes in NIH/3T3 fibroblasts with the addition of free bPEI

Figure 5-1: Substrate-mediated gene delivery of bPEI-DNA complexes in NIH/3T3 fibroblasts with varied peptide concentrations

Figure 5-2: Substrate-mediated gene delivery of NIH/3T3 fibroblasts cultured on PEI-DNA complexes immobilized to PAA brushes conjugated with different RGD peptides

Figure 5-3: Complex immobilization of PEI-DNA complexes with different PEI vectors

Figure 5-4: Proliferation of NIH/3T3 fibroblasts cultured on PAA brushes with PEI-DNA complexes

Figure 5-5: Representative images of NIH/3T3 fibroblasts stained with TRITC phalloidin for actin filaments (red), Alexa Fluor® 488 for vinculin (green), and nuclei counterstained with DAPI (blue)

Figure 5-6: Quantification of actin stress fibers and focal adhesions in NIH/3T3 fibroblasts cultured on 25 kDa bPEI-DNA complexes immobilized to modified PAA brushes (with cRGDyK and RGE, or no peptides)

Figure 5-7: Endocytic inhibition of NIH/3T3 fibroblasts cultured on 25 kDa bPEI-DNA complexes immobilized to modified PAA brushes (with cRGDyK and RGE, or no peptides)

Figure 6-1: PAA brushes on 100 nm Ti pnSCTFs and Flat Ti

Figure A-1: Viability staining of NIH/3T3 fibroblasts cultured on PAA brushes with RGD peptides

Figure A-2: AFM-IR of PAA and PAA-GRGDS

Figure A-3: Zeta potential and sizing of PEI-DNA complexes with different PEI vectors
**Figure A-4:** PEI-DNA complexes formed with Cy®5 labeled DNA plasmids immobilized to PAA brushes

**Figure B-1:** Titanium nanostructures formed via GLAD

**Figure B-2:** Transfection Investigations of NIH/3T3 fibroblasts cultured on STFs

**Figure B-3:** SEM investigations of NIH/3T3 fibroblast podia production cultured on STFs

**Figure B-4:** DNA complexes immobilized to PAA brushes on pnSCTFs compared to brushes on Flat Ti

**Figure B-5:** Transfection of NIH/3T3 fibroblasts on DNA complexes immobilized to PAA brushes on pnSCTFs compared to PAA brushes on Flat Ti
LIST OF TABLES

Table 3-1: Modeled PAA Brush Parameters

Table 3-2: Modeled Dry and Swollen PAA Brush Parameters and Swelling Degrees

Table 3-3: Modeled Swollen PAA Brush Parameters, Functionalized with Peptides

Table 4-1: PAA Brushes Formed on Ti substrates

Table 4-2: PAA Brushes Swelling in OptiMEM

Table 4-3: PAA Brushes with Covalently Bound Peptide

Table 5-1: Swelling of PAA Brushes

Table 5-2: PAA Brushes with Covalently Bound Peptide

Table B-1: Deposition of STFs via GLAD

Table B-2: Podia Features Measured Quantitatively

Table B-3: Spearman's Correlation of Transfection Success Compared to Height and Podia Production

Table B-4: PAA Brushes Grafted to 100 nm pnSCTFs
LIST OF EQUATIONS

(3-1) $\Gamma_{GRGDS} = d_{brush} \frac{n_{comb} - n_{brush}}{(d_n/d_c)} + d_{add} \frac{n_{comb} - n_{amb}}{(d_n/d_c)}$

(4-1) $\Gamma_{peptide/complexes} = d_{brush} \frac{n_{comb} - n_{brush}}{(d_n/d_c)} + d_{add} \frac{n_{comb} - n_{amb}}{(d_n/d_c)}$

(5-1) $\Gamma_{peptide/complexes} = d_{brush} \frac{n_{comb} - n_{brush}}{(d_n/d_c)} + d_{add} \frac{n_{comb} - n_{amb}}{(d_n/d_c)}$
1.1 Motivation and Objectives

Gene delivery transfers exogenous genetic material into cells and tissues to modify gene expression and can be used for a variety of applications including the treatment of genetic disorders, improving tissue engineering and diagnostic platforms, and functionalizing medical implants (4, 5). Viral vectors such as lentivirus or adenovirus are considered the most effective systems to deliver nucleic acids due to high efficiency, innate endosomal release mechanisms, and stable transgene expression (6). Although viral gene delivery systems have had clinical success (7), the use of viral vectors has safety concerns and many other disadvantages, including gene size limitations, insertional mutagenesis, and immunogenicity (8). As an alternative to viral gene delivery, nonviral gene delivery has emerged as a more cost-effective option with the ability to deliver larger genetic cargoes, improved scalability, and lower immunogenicity (9, 10), but the bolus delivery of nonviral gene delivery complexes (formed with cationic lipids or polymers and negatively charged nucleic acids) suffers from low efficiency (11, 12).

Although research in the field of nonviral gene delivery has focused on tuning physiochemical properties of the vectors to improve bolus delivery (4, 10, 13), the cell-material interface may prove a better platform to immobilize DNA complexes for substrate-mediated delivery (SMD) and modulate the cellular response to improve transfection (14), especially in ex vivo or site-specific
applications (i.e. biomedical implants). The cellular response can be modulated by the chemistry of the cell-material interface and tuned by incorporating synthetic or natural materials that are components of the extracellular matrix (ECM), mimic the components of the ECM, or immobilizing nucleic acids for delivery (i.e. SMD) (15-18). Since natural materials show high batch variability based on their source, synthetic polymer films are considered a more attractive option to modulate the cellular response to transfection via SMD as these films are tunable and reproducible. Synthetic polymers films have been investigated to improve the efficacy of SMD by releasing DNA or DNA complexes adsorbed to or encapsulated within polymer films (e.g. self-assembled monolayers (SAMs) or polyelectrolytes multilayers (PEMs) formed layer-by-layer (19-25)), but these films have issues with degradation and impeded release, and are not typically studied in the context of clinically relevant metals (i.e. titanium (Ti) (1-3)). Thus, the objective of this dissertation was to investigate polymer films formed with pH-responsive poly(acrylic acid) (PAA) brushes that may resolve these issues by immobilizing DNA complexes through electrostatic interactions with the highly negative brushes (at physiological pH) and by modulating cellular response via conjugated adhesion moieties (i.e. RGD (26-28)) and the presentation of adsorbed free PEI. Since traditionally PAA brushes have been grafted to silicon substrates (29, 30), this dissertation begins with reporting the first investigation into grafting PAA brushes to a Ti substrate and then describes our studies using these brushes as a platform for SMD, as outlined in the next section.
1.2 Dissertation Outline

The objective of this project is to discuss substrate modifications to alter cell-material interactions to improve the functionality of biomaterials and enhance nonviral gene delivery. Chapter 2 outlines the background of this project including nonviral gene delivery, current gene delivery techniques and vectors, and the common extracellular and intracellular barriers that impede transfection. Next, chemical substrate modifications to biomaterials that may enhance transfection success are introduced, focusing on the cellular behaviors and features that are produced in response to the cell-material interface. Furthermore, background information on the chemical modification of substrates with polymer films and their use in SMD are discussed. Finally, polymer films made from PAA brushes and the polymer brush “grafting to” process (utilized in the studies of this dissertation) are introduced.

The investigations of this dissertation are found in Chapters 3 through 5, which focus on PAA brushes grafted to Ti and functionalized with RGD peptides as a platform to improve SMD. The first investigation presented in Chapter 3 describes the efficacy of the “grafting to” process to functionalize Ti substrates with PAA brushes. The pH-dependent swelling and deswelling behavior of PAA is monitored with ellipsometry, which is also used to measure the covalent bonding of RGD peptides to PAA brushes (PAA-RGD). A brief investigation into the cellular response by measuring cellular adhesion to the modified substrate showed that PAA-RGD maintained the biocompatibility of the substrate. Thus, Chapter 4 investigates the use of the PAA-RGD substrates for SMD. First,
immobilization and release of the complexes formed with DNA and branched polyethylenimine (bPEI) are measured by scintillation counting of radioactively labeled DNA plasmids, and ellipsometry is utilized to monitor the total immobilized mass (unlabeled DNA and bPEI, free and complexed). Finally, unlabeled DNA complexes are used in transfection studies to show the influence on transfection success by culturing cells on PAA-RGD compared to a scrambled RGE sequence or Flat Ti, as well as assessing the adjuvant-like effect of free bPEI on transfection.

Finally, the investigations in Chapter 5 focus on tuning the effects of RGD presentation and adjuvant-like free PEI to influence transfection success via SMD on PAA brushes. PAA-RGD on Ti substrates were prepared using two different types of RGD motifs (linear GRGDS and cyclic cRGDyK (104)), then investigations were performed to understand the cellular response and its effect on transfection outcomes of NIH/3T3 fibroblasts cultured on PAA-RGD brushes loaded with PEI-DNA complexes with different types of PEI (i.e. 2 kDa bPEI, 25 kDa bPEI, 2.5 kDa linear PEI (LPEI) and 25 kDa LPEI). Cytotoxicity of the PEI-DNA complexes was assessed using a proliferation assay of cells cultured on PEI-DNA complexes immobilized to PAA brushes in comparison cells cultured on PEI-DNA complexes immobilized to tissue culture polystyrene (TCPS). Then the cellular response was probed via visualization of integrin binding to the presented RGD peptides, which was quantified through immunohistochemistry staining of the focal adhesion protein vinculin, as focal adhesions form via integrin binding (105). Furthermore, stress actin fiber formation around the aforementioned focal
adhesions was investigated through staining of the cytoskeleton (106). Finally, as these processes (i.e. proliferation, integrin binding, focal adhesion formation) can subsequently affect endocytosis, we investigated the effect on transfection when cells were treated with inhibitors for the common endocytic pathways for transfection: macropinocytosis, and clathrin- and caveolae-mediated endocytosis (31). Thus, transfection was monitored via flow cytometry to quantify the transfection efficiency of cells cultured on PAA-RGD substrates loaded with complexes and treated with inhibitors compared to cells cultured on PAA-RGD substrates loaded with complexes without inhibitors.

Collectively, the work in this dissertation aimed to demonstrate the influence of cell-material interactions on SMD transfection outcomes and the capability of modulating this response via substrate modifications. Results from this dissertation suggest that PAA brushes can modify Ti substrates for controlled cell-material responses and DNA complex immobilization, which could be used for applications involved with gene delivery ranging from the improvement of functionality and integration of biomedical implants to novel biosensor assays.
CHAPTER 2
Biomaterial Substrate Modifications that Influence Cell-Material Interactions to Prime Cellular Responses to Substrate-Mediated Nonviral Gene Delivery

2.1. Introduction

Nonviral gene delivery has the potential to improve applications in gene therapy, tissue engineering, and regenerative medicine through the transfer of therapeutic, exogenous genetic material into cells, but the process of transfection has had limited success due to low efficiency in comparison to gene delivery with viral vectors. Many extracellular and intracellular barriers limit the process of transfection and designing nonviral vectors to overcome these barriers has not had sufficient success in improving gene delivery. However, a novel solution to improving nonviral gene delivery is priming the cellular response to improve transfection. Thus, this chapter introduces nonviral gene delivery and the critical cellular barriers to transfection success that may be modulated by the cellular response. The cellular response can be controlled or modulated by biomaterial modifications at a cell-material interface by chemically modifying a substrate to promote biocompatibility, to modulate certain cellular behaviors (e.g. adhesion, proliferation, and migration), and to deliver therapeutic materials to cells (e.g. nucleic acids formed into complexes for nonviral gene delivery). Therefore, this chapter also introduces methods to chemically modify a substrate through natural and synthetic material coatings that may enhance the cellular response to transfection and, moreover, affect the presentation of the genetic cargo to the cell
via immobilizing nucleic acids to substrates (i.e. substrate-mediated gene delivery), with specific emphasis on polymer films (i.e. self-assembled monolayers, polyelectrolyte multilayer films) and an introduction to polymer brush films, which are used throughout this dissertation.

2.2. Nonviral Gene Delivery

Gene delivery is the transfer of exogenous genetic material into somatic cells to modify their gene expression, with applications including tissue engineering (32), regenerative medicine (33), sensors and diagnostics (34, 35), and gene therapy (36). Viral vectors such as lentivirus or adenovirus are considered the most effective systems to deliver nucleic acids due to high efficiency, innate endosomal release mechanisms, and stable transgene expression, which is applicable for long-term therapy (6). Although viral gene delivery systems have had clinical success (7), the use of viral vectors poses safety concerns among many other disadvantages, including gene size limitations, insertional mutagenesis, and immunogenicity (8). As an alternative to viral gene delivery, nonviral gene delivery has emerged as a more safe and cost-effective option with the ability to deliver larger genetic cargoes, improved scalability, lower immune response, and flexible delivery methods (i.e. physical or chemical) (9, 10). Physical nonviral delivery methods allow for facilitated movement of nucleic acids across the cellular membrane by creating transient openings through the use of electroporation (37), ultrasound (38), gene guns (39), and magnetofection (40). Although the use of physical delivery methods is
feasible, disadvantages are still apparent: nucleic acid degradation can occur in the extracellular and cytosolic environments, physical delivery methods themselves can cause cellular damage, and logistical concerns arise regarding \textit{in vivo} applications and treatments (e.g. electrode placement for electroporation) (6). Given the challenges with physical delivery, both \textit{in vitro} and \textit{in vivo}, chemical delivery methods for nonviral gene delivery are more commonly used (11, 12). Chemical nonviral gene delivery is typically accomplished by electrostatically complexing cationic lipids or polymers with negatively charged nucleic acids, often as DNA plasmids. Forming a complex condenses the DNA, protecting the nucleic acid from degradation by nuclease. The formed complexes elicit a lower immune response and show lower toxicity compared to viral vectors (12), both \textit{in vivo} and \textit{in vitro}. Transfection success with these nonviral complexes can be affected by the vector, often a cationic lipid or polymer, and the delivery method (i.e. a bolus or substrate-mediated).

\textit{2.2.1. Cationic Lipid and Polymers}

Many different forms of natural and synthetic cationic lipids and polymers have been used as vectors for chemical nonviral gene delivery to form lipoplexes or polyplexes, respectively (9). Lipids used to form lipoplexes have a common structure consisting of a hydrophobic tail connected with a linker structure to a positively charged polar hydrophilic head, which can bind to the negatively charged phosphates groups on nucleic acids. Some commonly used lipids include (N-[1-(2, 3-dioleyloxy) propyl]-N, N, N-trimethylammonium chloride)
(DOTMA), (N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate) (DOTAP), (2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-l-propanaminium trifluoroacetate) (DOSPA), and (dioleyl phosphatidylethanolamine) (DOPE) (41-43). Lipid based systems are known for their flexible design and synthesis, as well as inexpensive production, but lipoplexes often exhibit high toxicity related to the densely charged polar heads (44) and lipoplexes have also been shown to cause toxicity in vivo by aggregating within the blood and inducing an inflammatory response (12). In contrast to lipoplexes, polyplexes form more stable and condensed complexes that are typically smaller in size than lipoplexes, which is considered to be optimal for transfection success (45). One of the most commonly used polymers is cationic polyethylenimine (PEI) (46), and its efficacy as a transfection agent is attributed to the high density of non-protonated amine groups (at physiological pH) that may aid in endosomal release (47). The transfection efficiency of the complexes formed with PEI is dependent on the molecular weights (MWs) (48), structures (i.e. linear (LPEI) versus branched (bPEI)) (49, 50), and the nitrogen/phosphate (N/P) ratio (46, 49). While PEI is often considered the gold standard for transfection agents, PEI systems suffer from toxicity issues associated with free polymer in the complexing solution of complexes formed at nitrogen/phosphate (N/P) ratios >3 (51) or from the intracellular release of the PEI from the DNA plasmid (52); yet, contradictory studies have shown that free polymer may also increase overall gene transfection and that toxicity is also dosage-dependent (45, 51, 53-56). Thus, the efficacy of the polyplexes may be
tuned through the properties and dosage of PEI (both free and complexed), which may, as previously stated, be affected by the method of delivery of the complexes.

2.2.2. Delivery Methods of Nonviral Complexes

For the delivery of nonviral complexes, there are two common methods: a bolus or SMD (Figure 2-1). In \textit{in vitro} bolus delivery, complexes are added to the media of cultured cells which can cause issues with mass transport limitations (i.e. diffusion through the media), aggregation and degradation of the complexes, or nuclease degradation (4). In \textit{in vivo} bolus delivery, the complexes can be administered to cells through either infusions or injections (local or systemic) that can be used to distribute the genetic cargo throughout the body. Although it may be clinically useful for treating disorders without a specific target area due to its ease of administration, systemic bolus gene delivery of nonviral complexes can

\textbf{Figure 2-1:} Bolus (A) and substrate-mediated (B) delivery methods of transfection.
result in unwanted reactions and side effects, hypersensitivity, and potentially rapid clearance by the kidneys or liver (57). These issues caused by systemic delivery may be resolved through localized injections, which also can lower the required dosage and are used for site-specific applications, but even with local injection (in vivo or in vitro) delivery still may result in complex aggregation or degradation within the extracellular environment (58). Thus, an alternative administration route for site-specific and ex-vivo applications has been investigated to overcome the extracellular and intracellular barriers to transfection, i.e. SMD.

Also termed reverse transfection or solid-phase delivery, SMD is a method of immobilizing nonviral complexes to the substrate via covalent attachment or nonspecific adsorption before culturing cells on the substrate (14, 19, 20, 59). Compared to bolus delivery, SMD has been shown to limit complex aggregation, lower the necessary DNA dosage, as well as increase transgene expression and the number of transfected cells by increasing the local concentration of DNA within the microenvironment around the cell, thereby overcoming a mass transport barrier to gene delivery efficiency (19, 20, 58-63). Furthermore, the lower dosage of DNA complexes used in SMD allows for the reduction of cytotoxicity to cells cultured on the substrate, an issue frequently cited as affecting the efficacy of transfection (10, 64). Thus, SMD may be an optimal delivery technique for applications with a substrate (e.g. biomedical implants, diagnostic sensors, or tissue engineering constructs). Regardless of delivery
type (bolus or SMD), transfection efficiency is still hindered by the intracellular barriers to nonviral gene delivery.

2.2.3. Barriers to Successful Nonviral Gene Delivery

While nonviral gene delivery circumvents many disadvantages associated with viral systems and choosing the optimal vector and delivery method may enhance transfection, nonviral gene delivery suffers from low efficiency due to the inability to effectively overcome extracellular and intracellular barriers that impede transfection (65). Extracellular barriers that prevent the complexes from entering the cell include mass transport limitations (i.e. diffusion to the cell in the extracellular environment), complex degradation, and aggregation of complexes in the extracellular environment (66), yet intracellular barriers (i.e. internalization, trafficking and uncomplexing, nuclear localization, and transcription and translation) are considered to be even more critical to transfection success (10, 31, 67, 68).

2.2.3.1. Internalization

Once complexes overcome extracellular barriers, the positively charged complexes can then interact with the negatively charged cell membrane to be
internalized into the cell (12). Internalization of the complexes is typically accomplished through one of three different endocytic pathways determined by properties of the complex (e.g. size, targeting moieties, etc.): macropinocytosis, clathrin-mediated, and caveolae-mediated endocytosis (Figure 2-2) (13).

**Figure 2-2**: Endocytic pathways involved in nonviral gene delivery. The DNA complex must interact with the cell membrane to be taken up by the cell, usually through endocytic pathways including macropinocytosis (marked by membrane ruffling), and clathrin- and caveolae-mediated endocytosis. The endocytic pathways must form vesicles to transport the DNA complex (i.e. macropinosome, clathrin-coated vesicle, or caveosome, respectively). While being transported through the cell, the DNA complex must escape the vesicle and avoid lysosomal degradation, and then traffic through the cytosol to reach the nucleus. Nuclear transport is required for the DNA complex to enter the nucleus, whether by diffusion through a compromised nuclear envelope or through the nuclear pores. Within the nucleus, transcription must occur, and then the mRNA will enter the cytosol to be translated into a therapeutic protein.
Internalization of complexes by macropinocytosis occurs when an actin-formed membrane ruffle fuses with the plasma membrane and complexes are engulfed into a large invagination (>0.2 µm) (69) called a macropinosome. After complexes are engulfed, the macropinosome matures from an early to a late macropinosome, which is a leaky vesicle that may facilitate escape of the complexes into the cytosol (31), possibly allowing complexes to avoid lysosomal degradation (70). Macropinocytosis is responsible for internalization of large complexes (>0.2 µm), yet most studies have identified receptor-mediated endocytosis (e.g. clathrin- or caveolae-mediated) as the primary mechanism responsible for internalization of DNA complexes under 500 nm in diameter (71). Studies have shown clathrin-mediated endocytosis (72) to be used for larger complexes (100-200 nm) as compared to caveolae-mediated endocytosis, which typically internalizes smaller particles (50-100 nm) (73). Clathrin-mediated endocytosis is modulated by cell division control protein (Cdc42), a protein from the Rho family of GTPases that can modulate many cellular processes such as focal adhesion formation (Figure 2-3), migration, cytoskeletal remodeling, and vesicle trafficking (74, 75). In clathrin-mediated endocytosis, complexes are internalized into clathrin-coated vesicles that bud from the plasma membrane of the cell (76). After DNA complexes are engulfed within a clathrin-coated vesicle, the invaginations form into early endosomes, mature into late endosomes, and ultimately fuse with lysosomes where complexes may be degraded (70), unless endosomal escape is accomplished via vector-mediated rupturing of the endosome through acidification (77) or destabilization of the membrane (68). Like
clathrin-mediated endocytosis, in caveolae-mediated endocytosis, complexes are engulfed within a vesicle and the process is modulated by focal adhesions (78). In caveolae-mediated endocytosis, after an invagination forms around the complexes, the vesicles progress into early caveolin-coated endosomes, then transform into caveosomes (77). Similar to the macropinosome in macropinocytosis, DNA complexes are not degraded by caveosomes (77); however like in all endocytic mechanisms, for successful transfection, the complex must still escape from the caveosome to continue to be trafficked through the cell to the nucleus for transgene expression.

2.2.3.2. Intracellular Trafficking and Nuclear Localization

Once the DNA escapes from a macropinosome, endosome or caveosome into the cytosol, the DNA must then be trafficked through the cytosol to the nucleus as an intact DNA complex or as a DNA plasmid that has been disassociated with from the vector (79-81). Thus, exposure to nucleases within the cytosol present another barrier to transfection (13, 82), as nucleases may degrade the complex or the plasmid during the process of trafficking. Trafficking can be facilitated by elements of the cytoskeleton (Figure 2-3) including microtubules (66), a cytoskeletal component formed by protein filaments, and actin stress fibers (83), bundles of actin that anchor to focal adhesions and extend throughout the cell. Microtubules and stress fibers are critical cytoskeletal elements with known roles in the regulation of cellular adhesion and shape (74). The family of Rho GTPases has been shown to mediate the assembly and
disassembly of microtubules and stress fibers, as well as transmit mechanical signals that affect the trafficking of vesicles (which may contain complexes) (31) along microtubules or stress fibers to the nucleus (75). After the complexes reach the nucleus, nuclear entry of DNA or DNA complexes can be facilitated through disruption of the nuclear envelope during cell division (82); thus, proliferation, a process enabled by cell division, has been shown to enhance gene delivery (84). In nondividing cells, nuclear entry may also be facilitated by the nuclear pore, which is 25-30 nm in diameter and allows for the diffusion of molecules under 40 kDa but large molecules may enter the nucleus through this pore via active transport (85, 86). Typically the DNA is disassociated from the nonviral vector.

**Figure 2-3:** Integrin binding and focal adhesion assembly. Focal adhesions may form when integrins bind to the RGD motif of extracellular matrix (ECM) proteins like fibronectin. Intracellular signaling proteins, such as focal adhesion kinase (FAK), bind to the intracellular complex to form the base of a focal adhesion and transmit signals by Rho GTPases, which may stimulate intracellular trafficking along cytoskeletal elements such as stress fibers.
after escaping a macropinosome, endosome or caveosome into the cytoplasm (79). The cytoplasm is filled with various DNA-binding proteins, polyamines, and other polycations that may complex with the plasmids to neutralize the charge and condense the DNA plasmid (82). Thus, complexing with one of these innate proteins may reduce the size of the plasmid, which could aid in nuclear transport. However, if a formed DNA complex is trafficked to the nucleus, DNA can also be released from the complexing vector in the nucleus (87). Once the DNA transfers into the nucleus, the final barriers to transfection include transcription of the transgene in the nucleus, translation of the transcript to a transgenic protein, and subsequent transgenic protein folding, which can be significantly downregulated in response to the delivery of nonviral complexes (88, 89). Thus, there is a need to overcome these critical barriers that impede transfection success, which may be accomplished by modulating the cellular response to nonviral gene delivery.

2.2.4. Priming the Cellular Response to Transfection

Efforts to improve nonviral gene delivery have focused on overcoming the aforementioned barriers by modifying the previously described nonviral vectors (section 2.2.1) through methods such as conjugating cell targeting ligands to increase cellular uptake (90-92), conjugating moieties that disrupt the endosome through charge interactions (93), or immobilizing nuclear localization signaling peptides to traffic complexes to the nucleus (94), yet vector modification has had limited success in improving nonviral gene delivery efficiency in vitro or in vivo (10). A novel solution that may prove more effective than vector modifications to
improve nonviral transfection efficiency is stimulating or “priming” cells before transfection to modulate and mitigate the cellular response to nonviral gene delivery. For example, the addition of dexamethasone (a glucocorticoid) to in vitro culture media prior to the delivery of DNA complexes has been shown to enhance transfection efficiency and transgene expression in human mesenchymal stem cells (hMSCs), possibly by promoting normal cellular metabolism during transfection, as demonstrated by improved proliferation observed in cells treated with dexamethasone before transfection in comparison to untreated, transfected cells (95). Further, several other clinically-approved drug groups (e.g. steroids, GABAA modulators, antioxidants) have been shown to promote transfection efficiency by modulating the cellular oxidative stress (96) commonly induced by bPEI-mediated gene delivery.

While the aforementioned studies demonstrate that the addition of pharmacological agents to the cell medium can prime the cell response to transfection, cell priming can also come from substrate-derived cues. There are many nonviral gene delivery substrate applications where priming via a cell-material interface could enhance the cellular response to transfection. For example, some applications include coating a vascular stent with poly(lactic-co-glycolic acid) (PLGA) bilayer nanoparticles and DNA encoding vascular endothelial growth factor to prevent restenosis (97), loading a collagen patch with bPEI and DNA encoding a platelet-derived growth factor to increase wound healing (98), or coating a Ti bone implant with poly-(d,l-lactide) and polymer vectors complexed with DNA plasmids encoding bone morphogenetic protein-2
(BMP-2) to encourage osseointegration (99). To use a cell-material interface to
prime cells for more efficient transfection, substrate properties can be tuned
through chemical modifications such as the addition of natural coatings, ligands,
or functional side groups (which are the focus of this dissertation). The cell-
material interface is known to influence cell behaviors innately controlled by the
extracellular matrix (ECM) proteins (100) that adsorb onto a culture surface or
exist natively in tissue. These cellular behaviors include morphology (101-103),
adhesion (104, 105), and migration (106, 107), which have all been shown to
influence transfection success (108). For example, migration involves the
production of cytoplasmic protrusions like filopodia, long filamentous actin
protrusions from the cell that, in addition to propelling the cell forward, have been
shown to “carry” complexes into the intracellular environment of the cell body
(109). Likewise, membrane ruffles are actin-based features used by the cell to
guide motility but have also been shown to be associated with macropinocytosis
as described above (110), suggesting these features could aid in internalization
of DNA complexes. Furthermore, the processes of cell adhesion and migration
require focal adhesion assembly and disassembly (111), which may affect
endocytosis through the activation of Rho GTPases such as Cdc42, which
modulates clathrin-mediated endocytosis as described above (74); in addition,
focal adhesions anchor actin stress fibers that may facilitate intracellular
trafficking of DNA complexes. Given these insights into ECM-induced cellular
behaviors that are intricately related to transfection success, substrate
modifications to biomaterials can be used to mimic the extracellular cues from
the native ECM to enhance nonviral gene delivery. Thus, the remainder of this chapter highlights current research using chemical substrate modification techniques that can modulate cell-material interactions to prime the cellular response to improve nonviral gene delivery and explores how the cellular response was investigated in this dissertation, informed by the conclusions of the cited studies.

2.3. Modifications to Surface Chemistry that Influence Cellular Responsiveness to Gene Delivery

The surface chemistry of a substrate significantly impacts cell-material interactions (112, 113), typically translated to the cell through the ECM proteins that bind to the substrate (Figure 2-3) (114). The composition, conformation, and density of protein adsorption are controlled by surface chemistry (via hydrophobicity, surface energy, or end-functionalization), and in turn, the cell response is mediated by integrin binding to the adsorbed proteins (113). Cellular responses shown to be influenced by surface chemistry include adhesion (115, 116), morphology (117), and migration (118), which are all cellular behaviors shown to be important in transfection success (108). Surface chemistry can be tuned through natural and synthetic material coatings, modifying or adding chemical side groups, or by immobilizing nucleic acids to substrates to affect the presentation of the genetic cargo to the cell (i.e. SMD).

2.3.1. Natural Material Coatings and Chemical Side Groups to Prime Bolus Gene Delivery
Natural material coatings are an attractive option for substrate modifications due to their innate biocompatibility, sustainable production, and ability to integrate with cells and tissues (119). For example, chitosan is a cationic polysaccharide derived from deacetylated chitin from crustaceans well known for its use in nonviral gene delivery to form complexes with DNA plasmids (120, 121), but there is also promising evidence that chitosan surface coatings can alter cell-material interactions resulting in enhanced transfection success. Hsu et al. (122) showed that cell priming via substrate modifications with chitosan or hyaluronan-modified chitosan (chitosan-HA) improved the cellular response to transfection by increasing endocytosis via RhoA activation, a Rho GTPase that may facilitate intracellular trafficking (123). Moreover, the addition of HA, an essential component of native ECM (115), further primed the cells, potentially through upregulating caveolae-mediated endocytosis (Figure 2-2) (124), a more advantageous uptake pathway for transfection that may avoid lysosomal degradation of the DNA complex. Other studies have demonstrated that transfection can be influenced by coating substrates with natural ECM proteins such as collagen I/IV, vitronectin, laminin, and fibronectin (Figure 2-3) (108). Investigations with murine mesenchymal stem cells (mMSCs) transfected with LPEI-DNA complexes showed that cells cultured on fibronectin had increased transgene expression compared to cells cultured on all other coatings (collagen I/IV, vitronectin, laminin) and thus fibronectin has often been investigated as a protein coating to enhance nonviral gene delivery (108). For example, a series of investigations were performed to understand the effect of culturing mMSCs on
fibronectin coated onto TCPS and then transfected with LPEI-DNA complexes encoding EGFP and LUC by Dhaliwal et al. (125). First, given that integrins can bind to the arginyl-glycyl-aspartic acid (RGD) ligand on fibronectin (126) and such binding can modulate endocytic pathway activation, investigations were performed to show the influence of fibronectin on the internalization pathways used by cells (125). A significant decrease in transfection and internalization after inhibiting clathrin-mediated endocytosis in cells cultured on fibronectin compared to cells with no treatment was observed (i.e. an order of magnitude of decrease in transgene expression and 92% reduction of internalization), suggesting that fibronectin coatings (presumably through integrin binding to fibronectin) (126) improved transfection of adhered cells through specific endocytic pathway activation (Figure 2-2), which could possibly be mediated by cytoskeletal organization. Therefore, the authors investigated the role of the cytoskeleton in transfection outcomes, which showed that disrupting actin stress fibers and actin-myosin activity with inhibitors led to increased transgene expression in cells cultured on fibronectin, thus suggesting that untreated cells cultured on fibronectin had a large actin network with high cellular contractility (from actin-myosin interactions) (127) that impeded transfection success, suggesting a more moderate level of contractility and actin organization is optimal for successful transfection (125).

Since cellular adhesion signaling via Rho GTPases between cell surface receptors (i.e. integrins) and fibronectin modulate the cellular contractility and stress fiber formation (Figure 2-3), in a separate paper, mMSCs were cultured on
fibronectin coated onto TCPS and used to measure the endogenous activation of several Rho GTPases including RhoA, Ras-related C3 botulinum toxin substrate 1 (Rac1), and Cdc42 levels (128), which showed that the cells cultured on fibronectin had significant Rho GTPase activation, which correlated to transfection success. Furthermore, inhibiting Rho GTPase activity resulted in a significant decrease in the formation of actin stress fibers and transfection success in cells cultured on fibronectin, suggesting Rho GTPase activity may increase the formation of stress fibers that can mediate intracellular trafficking and thus, transfection. Together, these studies provide compelling evidence that transfection success in cells cultured on fibronectin may be attributed to integrin binding, cytoskeletal dynamics (i.e. stress fibers, cellular contractility), and the activation of Rho GTPases, especially Cdc42 that modulates clathrin-mediated endocytosis (74).

In a separate investigation, the RGD ligand on fibronectin (and other ECM proteins), to which integrins bind and form the base of focal adhesions (Figure 2-3), was bound to alginate hydrogels using carbodiimide chemistry, with varied density (3-60x10⁹ peptides/mm²) and spacing between clustered islands of RGD (36-120 nm) (129). Cellular proliferation and stress fiber formation (presumably mediated by focal adhesion formation (130)) were analyzed, which showed increased actin stress fiber formation and proliferation with increasing RGD density in MC3T3-E1 preosteoblasts cultured on hydrogels while culturing cells on RGD islands with increased spacing showed the inverse. Furthermore, similar to proliferation and actin stress formation, DNA internalization and transfection
increased in cells on gels with increasing RGD density, which suggests that higher RGD densities may improve transfection through upregulating proliferation, and increased actin stress fiber formation, which may aid in endocytosis and intracellular trafficking of the DNA complex. The results of all of these studies suggest that fibronectin (or the RGD motif) is pivotal in affecting gene transfection success through cell-material interactions and that integrin binding may be the initiator to this type of priming, with downstream effects that can influence focal adhesion formation, stress fiber formation, and subsequent endocytic pathways and intracellular trafficking.

While natural coatings on biomaterials (e.g. chitosan, ECM proteins, RGD ligands) may enhance transfection efficiency by stimulating endocytic pathways, natural materials also show high batch variability based on their source. Therefore, modifying the substrate synthetically with functional groups that resemble those found in natural materials has also been investigated to enhance gene delivery. Synthetic additions to the substrate can be specifically manufactured with homogeneity; e.g. self-assembled monolayers (SAMs) have highly defined chemistries that can present surface functional groups that may affect protein and cell attachment (131). Kasputis and Pannier (132) investigated NIH/3T3 fibroblasts cultured on SAMs on gold substrates modified with -CH₃ and -COO⁻ terminal functional groups, which are hydrophobic and hydrophilic, respectively. Transfection was performed using bolus delivery of complexes formed with lipid based Lipofectamine 2000 (LF2000) or bPEI and DNA plasmid. Delivery of both types of complexes resulted in increased transfection (by ~2-
fold) in cells cultured on hydrophilic substrates (i.e. SAMs with carboxyl terminal functional groups) compared to hydrophobic substrates (i.e. SAMs with methyl terminal functional groups). In addition to transfection, cellular viability, shape, cytoskeletal features, and focal adhesions were analyzed as a function of surface chemistry and then correlated to transfection success. Successful transfection performed with LF2000 complexes was significantly correlated to the viability of cells induced by surface chemistry, but no other morphological factors. Conversely, successful transfection performed with bPEI complexes was highly correlated with substrate-induced cellular behaviors including cell density, spreading, cytoskeletal organization, and focal adhesions. The authors proposed that cytoskeletal reorganization was strongly affected by focal adhesions in response to the substrate environment, therefore successful transfection may have been facilitated by cytoskeletal elements that attach to focal adhesions that in turn contribute to endocytosis and intracellular trafficking (i.e. stress fibers) (Figure 2-3). These bolus studies on natural coatings and functional group modifications suggest that such features may help to overcome intracellular barriers of transfection (i.e. endocytosis, intracellular trafficking), but the cell-material interface can also be used to overcome the extracellular barrier of mass transport by allowing primed cells to directly interact with complexes through substrate-mediated gene delivery (SMD), which will be further discussed in the next section.
2.3.2. Natural Material Coatings to Prime Substrate-Mediated Gene Delivery

While cell-surface interactions have been shown to influence bolus delivery, surface functionalization could facilitate SMD by influencing both DNA loading and cell priming and may be more beneficial for substrate applications of gene delivery, as described above. Often SMD substrates are coated with natural or synthetic materials to enhance the immobilization of nucleic acids and modulate the cellular response to the genetic material. For example, Bengali et al. (60) studied the effect of coating TCPS substrates with different ECM and serum proteins (fetal bovine serum, fibronectin, collagen I, laminin, and bovine serum albumin (BSA)) to prime cells for enhanced SMD transfection efficiency, using complexes formed with bPEI and a DNA plasmid. First, the authors showed that the loading of DNA complexes onto the substrate was not affected by protein coatings. However, when NIH/3T3 fibroblasts were seeded on the aforementioned protein-coated substrates with immobilized DNA complexes, there were significantly more DNA complexes taken up by cells cultured on fibronectin and collagen I compared to the other protein coatings. When SMD transfection was analyzed on these surfaces, cells cultured on fibronectin had the highest level of reporter gene expression compared to cells cultured on the other proteins or control surfaces. Given these observations, the authors hypothesized that the presence of fibronectin on the surface may promote integrin binding (Figure 2-3), which may lead to the assembly of focal adhesions and cytoskeletal rearrangement, which can, in turn, affect endocytosis and downstream intracellular trafficking of the complexes (Figure 2-2). To test part of this
hypothesis, the authors investigated endocytic pathways in cells cultured on the protein-coated substrates with immobilized DNA complexes by inhibiting caveolae- and clathrin-mediated endocytosis (Figure 2-2) with genistein and chlorpromazine, respectively. For all protein coatings investigated, internalization of DNA complexes and the resulting transfection were both decreased significantly when cells were cultured with genistein (compared to cells cultured with chlorpromazine), suggesting that culturing cells on protein coatings may upregulate caveolae-mediated endocytosis. Thus, in this study, similar to those described earlier in this chapter in the context of bolus delivery, protein coatings, and in particular fibronectin, may have primed the cells (presumably through integrin binding, focal adhesion formation, and cytoskeletal rearrangement) that resulted in biasing toward the potentially more efficient caveolae-mediated endocytosis mechanism (77) to enhance transfection. Substrate modifications with natural coatings can mimic ECM cues and modulate cellular behaviors that influence transfection efficiency, but, as previously stated, synthetic polymer coatings are a more attractive option than natural coatings. Synthetic polymers are considered more reproducible than natural coatings, and the chemically defined nature of the polymer films allows for more tunability to modulate the cellular response to transfection. Moreover, the addition of polymer films has previously been shown to enhance SMD by both promoting complex immobilization for SMD and enhancing cellular responsiveness to DNA transfer (19, 62), which will be described in the next section.
2.3.3. Polymer Films to Prime Substrate-Mediated Gene Delivery

Polymer films are layered polymer materials that have been deposited onto a substrate to thicknesses ranging from nanometric (e.g. a monolayer) to up to several micrometers. Polymers can be deposited as one species of monomer or a blend of multiple species into copolymers (e.g. block, alternating, periodic). Common techniques to produce polymer films include self-assembled monolayers (SAMs), polyelectrolyte multilayer films (PEMs), or polymer brushes (utilized in this dissertation). SAMs and PEMs have been investigated as substrate modifications to enhance the efficacy of nonviral gene delivery via SMD by encapsulating or embedding DNA or DNA complexes within layers of a polymer film and releasing the DNA cargo through degradation or diffusion processes (20, 23-25, 133-139). Polymer films release the therapeutic materials either through interactions with physiological triggers (23, 134-136) or the application of an electric charge (24, 140, 141) to produce a rapid (<1 day) or sustained release depending on the properties of the films (e.g. amount of layers, polymer, complex formation). The DNA cargo delivered from polymer films is typically polyplexes that are embedded or immobilized as formed complexes or naked DNA that is condensed by degradation products (23), but enhanced transfection via SMD has also been shown in cells cultured on polymer films with lipoplexes (25, 133). In general, while these SMD studies focus primarily on substrate biocompatibility and the effectiveness of cellular transgene expression, the DNA complex-material interactions are cited as the cause of enhanced transfection success (19, 20). For example, investigations into SAMs as a
substrate for SMD have shown that surface modification with hydrophilic entities, such as polyethylene glycol (PEG)-like moieties (i.e. oligo(ethylene glycol) (EG) groups (20) or carboxylic acid from 11-mercaptoundecanoic acid (MUA) (19)), affected DNA complex immobilization to substrates and subsequent transgene expression was modulated through complex properties (surface charge, aggregation, complex–cell interaction). Thus, the cited studies in this section show that cells cultured on polymer films may have improved transfection efficacy via SMD.

Despite these promising results with polymer films, there are still many issues with polymer film formation for SMD using traditional methods like SAMs and PEMs, such as low loading capabilities of complexes (142) and film instability (143-146). Furthermore, the encapsulation or embedding of the DNA cargo with the polymer film may inhibit delivery via issues with mass transport (147), which is a commonly cited extracellular barrier to transfection that other SMD techniques have been shown to ameliorate. Moreover, the erosion mechanism of these polymer films may release acidic degradation products, which can have detrimental effects on transfection success (148); therefore, a polymer film that allows for complex immobilization and release from the substrate may be more desirable compared to embedded or encapsulated DNA cargoes released by the erosion of the polymer construct. Furthermore, SAMs and PEMs for SMD are often applied to glass substrates (133-136), TCPS (25), silicon (149), or gold (20, 150), and few studies use clinically relevant materials like stainless steel (24, 137) or Ti. To resolve these issues with current polymer
film techniques, polymer brushes are an attractive option to enhance SMD using stimuli-responsive polymer brushes to: 1) immobilize DNA complexes onto clinically relevant materials (i.e. Ti), 2) release those complexes without the production of degradation products, and 3) prime the cellular response to transfection through presentation of ECM ligands, described further detail in the next section.

2.4. Polymer Brushes

Polymer brushes are chains of polymer molecules densely bound to a substrate at multiple attachment points and the polymer chains extend normal to the grafted surface by volume exclusion effects (151, 152). In comparison to other polymer films, polymer brushes have a higher density of available functional groups for modification and their hydrophilic branched structure in aqueous solutions is more likely to mimic the in vivo ECM environment (153). Polymer brushes are used as stimuli-responsive “smart” coatings that respond to various physical, chemical, or biological cues, including pH, temperature, ionic strength, chemical agents, light, electrical field, and magnetic fields (154-156). The stimuli-response is dependent on their chemistry and some commonly used polymer brushes include poly(N-isopropylacrylamide) (PNIPAM), poly(2-vinyl pyridine) (P2VP), Poly(N,N-dimethylaminoethyl methacrylate) (PDMAEMA), and poly(acrylic acid) (PAA) (157). Within biomedical science, polymer brushes have been investigated for applications including biosensors (158), diagnostics (159), drug delivery (160), tissue engineering (161), implant coatings (162), as well as
platforms for studying processes relevant to understanding cell-material interactions, such as protein adsorption (163). Polymer brushes can be fabricated by a variety of approaches and chemistries (164) including physisorption methods like those previously discussed in this chapter (i.e. PEMs (23, 24, 165, 166)), or chemical approaches, typically using the “grafting from” or the “grafting to” approach.

For the “grafting from” approach (Figure 2-4A), the substrate surface is modified with initiator sites and exposed to a solution of monomers, which then polymerize typically through radical polymerization strategies (167). The “grafting from” approach typically produces thick, dense brush layers with accurately controlled architectures (168-171), but the chemical reactions are complex and the characterization of the brushes is difficult (157, 172). In the “grafting to” method polymer chains are synthesized and characterized prior to addition to the substrates via physical adsorption (Figure 2-4B) or chemical reaction between reactive groups on the surface (or an anchoring layer) and functional end groups of the polymer (Figure 2-4C) (157, 164), which allows for precise control of the molecular weight and dispersity of the polymer chains. Furthermore, polymer brushes chemically “grafted to” a substrate have higher stability compared to physically adsorbed polymers with weak interactions between the substrate like hydrogen bonds or van der Waals interactions (157, 173, 174); thus, the investigations in this dissertation focused on polymer brushes formed using the “grafting to” process on a Ti substrate as a platform to immobilize DNA complexes and to prime cells for transfection via SMD. These investigations were
performed using pH-responsive poly(acrylic acid) brushes, which are described in the next section.

2.4.1. Poly(acrylic acid) Brushes as a Platform for SMD

Poly(acrylic acid) (PAA) is a weak polyelectrolyte that reacts to pH changes by swelling and deswelling in a brush conformation (175), caused by the confinement of counterions within the brush layer and charge repulsions of deprotonated carboxyl groups in aqueous solutions above the isoelectric point of pH=2.1 (176). PAA brushes can be produced with both “grafting from” and “grafting to” approaches (153, 177). In the “grafting to” approach, PAA brushes form into polydisperse pseudo-brushes are termed “Guiselin brushes” (29, 30, 177-179), whereas “loops” and “tails” are attached with multiple anchor points on a substrate (Figure 2-4B) with only a small amount of carboxylic groups attached to the anchoring layer. These Guiselin brushes have many non-reacted carboxyl...
groups open to functionalize with cell adhesions peptides, i.e. RGD, using NHS/EDC chemistry (180). Although PAA brushes are considered cell repellent (153, 181, 182) and have often been used to prevent bacterial adhesion to substrates (183, 184), PAA brushes with the RGD motif have been used to enhance cell attachment in cell instructive studies to modulate behaviors such as adhesion, spreading, migration, proliferation and cytoskeletal organization (185, 186), all of which have been cited previously in this chapter for their ability to influence transfection success. Furthermore, despite their highly hydrophilic nature at physiological pH, PAA brushes have been shown to adsorb protein to the substrate, which may improve cell adhesion and is dependent on the pH-responsive conformation of the brushes (29). For example, in a study of PAA brushes produced in an array, cells cultured on PAA brushes regions with adsorbed fibronectin (a protein known to contain the RGD motif (187)) had actin polymerization and cytoskeletal reorganization but not in cells cultured on bare PAA brushes (i.e. without adsorbed protein) (153). Finally, PAA brushes have also been used to adsorb growth factors to the substrate (179), which can also alter the cellular response of cultured cells (179). For example, in the study by Psarra et al., hepatocyte growth factor (HGF) and basic fibroblasts growth factor (FGF) were functionalized to PAA through physical adsorption or covalent binding via EDC/NHS chemistry (179). The latter method showed a more pronounced effect on cultured cells, whereas HGF had a greater biological effect arresting cell growth of human hepatoma cells when the growth factor was chemisorbed to PAA than HGF physically adsorbed to PAA and, similarly, FGF
chemisorbed to PAA guided cellular differentiation of cultured stem cells than those cultured on FGF physically adsorbed to PAA. Furthermore, cells cultured on PAA with immobilized growth factors had decreased proliferation (as the expected response to the presented growth factor) and formation of lamellipodia (an actin protrusion associated with migration (188)). Together, these cited studies suggest that PAA brushes are tunable and can be used to modulate the cellular response; however, no previous investigations have been performed on the “grafting to” process of PAA brushes onto a clinically relevant material, i.e. Ti. Previously, PAA brushes films have been shown to successfully and stably modify substrates on materials including silicon (177, 189) and kaolinite (190) and metals like nickel (191) and gold (192); thus, this dissertation presents a novel investigation into chemically grafting PAA brushes to Ti via an annealed anchoring layer of poly(glycidyl methacrylate) (PGMA) (Figure 2-4C).

The main advantage of utilizing PAA brushes to modify Ti and improve transfection via SMD to cultured cells is their electrostatic interaction with DNA complexes that allow complexes to immobilize to the brushes. As previously stated, increasing deprotonation of the carboxyl groups on the brushes occurs as the pH value alkalizes, resulting in a highly negative charge at physiological pH (176), suggesting that electrostatic interactions to immobilize positively charged PEI-DNA complexes (which are optimal for transfection) would be strongest at the optimal pH for cell culture. Moreover, in addition to immobilizing the formed complexes to the substrate, cationic free PEI may also be immobilized and neutralized, thus decreasing its cytotoxicity and enabling the free PEI to
modulate the cellular response to transfection, as previously suggested in this chapter. Finally, PAA brushes have already been investigated as a platform for drug delivery (e.g. antibiotics (193)), suggesting that the delivery of DNA complexes from the substrate is feasible. While delivery of the DNA complexes and free PEI from PAA brushes may improve transfection via the cellular response, as previously stated, PAA brushes can be conjugated with the cell adhesion moiety RGD, which has also been shown to improve the cellular response to transfection (129, 194-196). Thus, PAA-RGD brushes are a chemical substrate modification for clinically relevant Ti that may increase the success of nonviral gene delivery in cultured cells via the presentation of immobilized DNA complexes and enhanced cell-material interactions stimulated by free PEI and the RGD ligand.

2.5. Conclusions

In summary, nonviral gene delivery suffers from low efficiency, but intracellular barriers to transfection may be overcome by modulating the cellular response with modifications to the surface chemistry of the substrate to which cells adhere. The chemistry of the cell-material interface can be tuned by incorporating materials that are components of the ECM (e.g. collagen, fibronectin, RGD), mimic the components of the ECM (e.g. chitosan or functional groups), or immobilizing nucleic acids (i.e. SMD). In this chapter, some of the underlying mechanisms that may modulate cellular responsiveness to transfection (via bolus or SMD) through cell-material interactions with chemical
modifications of the substrate have been suggested (e.g. integrin binding to RGD, focal adhesion formation, cytoskeletal remodeling, intracellular trafficking, endocytic mechanisms). These behaviors can be modulated by substrate modifications with natural or synthetic coatings to influence transfection efficiency, but natural materials show high batch variability based on their source. Thus, synthetic polymer films, which are more reproducible and tunable, are considered a more attractive option to modulate the cellular response to transfection via SMD.

Synthetic polymers films have been investigated to understand the cellular response to the substrate and to improve the efficacy of SMD by releasing DNA or DNA complexes adsorbed to or encapsulated within polymer films (e.g. self-assembled monolayers (SAMs) or polyelectrolytes multilayers (PEMs) formed layer-by-layer). Polymer films formed with PAA brushes may resolve the issues that have arisen with the use of SAMs and PEMs, as they stably attach to the substrate (which may be feasible on clinically relevant Ti), may entrap complexes through electrostatic interactions rather than embedding, and can be used to modulate cell-material interactions via adhesion moieties (i.e. RGD) and the presentation of free PEI. Investigations in this dissertation begin with the development of the method of grafting PAA-RGD brushes to Ti (chapter 3), which is then characterized as a platform for transfection via SMD in cells cultured on PAA-RGD with immobilized PEI-DNA complexes (chapter 4) and culminating in studies that seek to tune the RGD peptide and PEI vector to optimize the cellular response to transfection (chapter 5). The results of this dissertation suggest that
many substrate applications may be improved by the addition of PAA-RGD
brushes ranging from enhancing the delivery of immobilized genetic cell signaling
and differentiation cues to cells cultured onto Ti implants to developing *in vitro*
assays, diagnostics, or functional genomic arrays, which could be used in the
fields of gene therapy, tissue engineering, and regenerative medicine.
3.1. Introduction

The grafting of polymer brushes to substrates is a promising method to modify surface properties such as corrosion resistance, wettability and the affinity toward proteins and cells for applications in microelectronics (26), biomedical devices (27, 197) and sensors (198). By grafting polymer chains to a substrate in close proximity, polymer chains are forced to stretch away from the surface via excluded volume effects and hence form brushes (151, 152). Polymer brushes can be prepared by the “grafting from” or the “grafting to” approach. For the “grafting from” approach, the substrate surface is modified with initiator sites and exposed to a solution of monomers, which then polymerize typically through radical polymerization strategies. For the “grafting to” method, preformed polymer chains are grafted to surfaces via a chemical reaction between reactive groups on the surface and functional end groups of the polymer (164). The “grafting from” approach is able to produce homogeneous brushes with high grafting densities, but the chemical reactions are complex and the characterization of the so-produced polymers is difficult (157, 172). On the other hand, the “grafting to” approach enables precise control of the molecular weight and dispersity of the polymer chains because polymers with desired properties can be synthesized and characterized prior to addition to the surface. The Guiselin brushes produced
by “grafting to” are annealed in such a way that the attachment is realized by very few grafting points (2-3 per chain); which guarantees that the produced loops and tails are large enough so that the grafted polymer layer behaves like a brush (29, 177). Although the grafting densities are lower for the “grafting to” approach, this method allows for facile modification of surfaces with homogeneous polymer brushes of sufficient grafting density that offer a well-defined structure as well as higher stability compared to physically adsorbed polymers with weak interactions between the substrate like hydrogen bonds or van der Waals interactions (157, 173, 174).

There are a variety of polymer brushes that can be added to substrates using either technique, including responsive polymer brushes that react to external stimuli such as temperature or pH and lead to a change of chain conformation, generally shown by a swelling-deswelling behavior in solution (199). For example, poly(acrylic acid) brushes (PAA) are weak polyelectrolytes that are known for their pH-dependent deprotonation of carboxyl groups along the chain. PAA “pseudo” brushes are grafted by more than one point of attachment of the chain to a substrate, forming loops and tails (178). The grafting procedure is controlled so that these PAA films show brush like swelling behavior (29, 30, 179). The numerous accessible carboxyl groups in addition to serving as grafting points, also allow the covalent immobilization of moieties in a controlled amount, for example for specifically tailored biomaterials.

One of the most commonly employed biomaterials is titanium (Ti) and its alloys for applications ranging from dental implants to biosensors (2, 3). Although
Ti has many desirable properties like mechanical strength and biocompatibility (2, 3, 200-203), tailoring the biomaterial for optimal cellular and tissue responses must be performed through modification of surface chemistry (16, 204-206). For example, one of the main failures of biomedical implants is the slow corrosion of implanted metal substrates resulting in potential infections and implant failure (207); which could be prevented by coating the metal with corrosion resistant polymers. Furthermore, functionalization of Ti and other metals with polymer brushes could be used to prevent nonspecific protein adsorption to the substrate (16) or to present bioactive factors that modulate cell behaviors (208, 209). The addition of responsive polymers to Ti surfaces could enable the controlled release of biomolecules or drugs, which could improve biomaterial performance and wound healing after implantation for many applications in medical devices (179). Although functionalization of PAA has been well characterized for silicon (Si) substrates (29, 30), a “grafting to” method has not been reported on Ti substrates. Furthermore, the ability of PAA to be further conjugated with bioactive moieties (i.e. Arg-Gly-Asp (RGD) (185, 204, 210)) has not been quantified on Ti substrates or evaluated for cellular response. In this work, we describe a method of grafting reactive PAA brushes to Ti substrates as a first step towards bioactive surfaces, as well as demonstrate the cellular response to PAA brushes on Ti before and after conjugation of the brushes with RGD.
3.2 Materials and Methods

3.2.1. Deposition of Ti on Si Substrates

Ti substrates were fabricated in an ultra-high deposition vacuum chamber by electron beam evaporation of Ti pellets (Super Conductor Materials, Inc., Tallman, NY) onto Si wafer substrates (University Wafer, South Boston, MA). A substrate was mounted normal to the flux and rotated at 2 rpm counterclockwise while material was deposited at 0.15 nm/s for a total deposition of 100 nm, both monitored using a quartz crystal microbalance.

3.2.2. Preparation of Poly(acrylic acid) (PAA) brushes on Ti Surfaces

Polymer brushes were prepared according to the previously reported “grafting-to” method (Figure 3-1, a-c) for grafting preformed PAA brushes to Si surfaces, with slight modifications as described here (29, 155, 177, 211). For the “grafting to” method, a thin anchoring layer is used to graft preformed polymer chains to the Ti surface in a brush-like manner (164). Prepared Ti surfaces were rinsed with ethanol absolute (EtOH, VWR, France) and dried with a N$_2$ flux to cleanse the surface and remove debris. Subsequently, the Ti substrate was activated with oxygen plasma for one minute (Plasma Cleaner PDC-002 with PlasmaFlo PDC-FMG-2, Harrick Plasma, USA). After activation, a solution composed of 0.02 wt% of poly(glycidyl) methacrylate (PGMA, M$_n$=17,500 g/mol, M$_w$/M$_n$=1.7, Polymer Source, Inc., Canada) in chloroform (CHCl$_3$, Fisher Scientific, UK) was spin-coated (Spin150 spin coater, Polos, Putten, Netherlands). The PGMA layer was annealed for 10 min at 110°C under vacuum,
resulting in a thin reactive anchoring layer with epoxy groups for the adjacent grafting step. A poly(acrylic acid) (PAA, Mn=26,000 g/mol, Mw/Mn=1.12, Polymer Source, Inc., Canada) solution was prepared at 1.0 wt% in ethanol absolute and spin-coated onto the grafted PGMA layer. The PAA layer was annealed at 80 °C for 30 minutes under vacuum to the react the epoxy groups of PGMA with some COOH-groups along the chain of PAA, grafting the PAA chains in loops and tails via ester bonds. The annealing temperature was chosen below the glass transition temperature at 105 °C of PAA in order to minimize the amount of grafting points and achieve highly swellable polymer brush films. Excess polymer was extracted by stirring the samples in ethanol for 30 minutes at room temperature and drying them with a N₂ flux.

3.2.3. Covalent Binding of RGD Peptides to PAA Brushes

All materials used for peptide conjugation were purchased from Sigma-Aldrich (St. Louis, MO). Buffers were prepared using boric acid, phosphate buffered saline tablets (PBS) and 2-(N-morpholino)ethansulfonic acid (MES). The linear RGD-containing peptide GRGDS (Sigma-Aldrich) was covalently bound to PAA brushes on Ti surfaces via activation of the PAA carboxy (COOH) groups with N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC) and N-Hydroxysuccinimide (NHS) for direct conjugation of COOH groups with the primary amines (NH2) of the peptides (180). For conjugation, PAA brush Ti substrates were equilibrated in 0.1 M MES at pH 6 for 10 min. After aspiration of this buffer, brushes were reacted with 0.5 ml of a 5 mM EDC solution and 0.5 ml
of a 2 mM NHS solution in 0.1 M MES buffer (pH 6) by gently shaking for 40 min. Subsequently, a 1.0 mg/mL solution of GRGDS (Figure 3-2) in 0.1 M Borate buffer (pH 8) was added to the activated PAA brush substrates. After gentle shaking at room temperature for 16 h, the peptide solution was aspirated and the GRGDS modified samples were washed three times by stirring in 0.1 M PBS buffer, pH 7.4 for three minutes.

3.2.4. Ellipsometric Measurements and Modeling of PAA Brushes

Ellipsometric measurements were acquired using a Woollam RC2 or a M2000 spectroscopic ellipsometer (both from J.A. Woollam, Co., Inc., Lincoln, NE, USA) to confirm surface parameters. For dry brushes measured with the RC2, the scan was performed at four angles of incidence (AOI; 45°, 55°, 65°, 75°). Measurements with the M2000 were performed at an AOI of 70°. For measurements in liquid, a batch cuvette (TSL Spectrosil, Hellma, Muellheim, Germany) was used and measurements were performed at an AOI of 70°. The ellipsometric data, Δ (relative phase shift) and tanΨ (relative amplitude ratio), were recorded at wavelengths (λ) of 380-1700 nm, except for measurements in liquid, which were performed at λ=380-1200 nm. Experimental data were modeled in CompleteEASE® software (Version 4.64, J.A. Woollam Co., Inc., Lincoln, NE, USA).
Figure 3-1: “Grafting to” of PAA brushes to Ti substrates. After activation of the Ti layer, a PGMA anchoring layer is bound to the surface, which enables further covalent binding of PAA chains through functional groups. RGD peptides are also covalently bound to remaining COOH groups of PAA using carbodiimide chemistry.

Figure 3-2: Structural formula of GRGDS
To evaluate the dry and the swollen thicknesses \((d_{\text{PAA}}, d_{\text{brush}})\) of the PAA brushes, a multilayer box model with distinct interfaces was used to analyze the ellipsometric data. The optical properties (dielectric function) of the opaque Ti layer on the Si substrate was modeled using a Lorentz oscillator function\(^{(212)}\) with five oscillators and served as the substrate. A native and optically transparent TiO\(_2\) layer was included in the box model as an additional layer and its model thickness was determined before and after plasma activation. For the optical properties (optical constants \(n(\lambda)\)) of TiO\(_2\) a software implemented Cauchy-Urbach dispersion formulae \((n(\lambda)=A+B/\lambda^2+C/\lambda^4, \ k(\lambda)=\alpha e^{\beta(E-E_{\text{edge}})}\) with fixed parameters for \(A=2.335, B=0.0238 \ \mu m^2\) and \(C=0.00672 \ \mu m^4\), \(\alpha=0.0155, \ \beta=1.243 \ (eV)^{-1}\) and \(E_{\text{edge}}=3.1 \ eV\) was used. The anchoring PGMA layer and the PAA brush layer were modeled as separate layers in the box model. For PGMA, the Cauchy parameters were estimated as \(A=1.516\) and \(B=0.004 \ \mu m^2\) for a thick dry PGMA layer and applied as fixed values in the Cauchy relation. For the dry PAA brush layers with film thicknesses below 10 nm, \(n\) was fixed to 1.522 because of the strong correlation between its thickness parameters \(d\) and \(n\) in this region \((213)\). For determination of the swollen PAA brush thicknesses in different pH solutions as well as before and after activation with GRGDS, \(n\) was modeled by a Cauchy dispersion \(n(\lambda)=A+B/\lambda^2\). The swelling degree was calculated as \(d_{\text{brush}}/d_{\text{PAA}}\).
3.2.5. Quantification of RGD Amount via Ellipsometric Modeling

Modeling RGD-peptide bioconjugation on swollen, soft polymer brush films from ellipsometric data requires a different analysis model than for smooth, rigid surfaces, given there is no sharp interface between the peptide and the polymer brush, since the peptide is assumed to penetrate into the brush (185). Hence, a composite polymer-peptide box layer with the thickness $d_{\text{comb}}$ was modeled, leading to an average Cauchy dispersion $n_{\text{comb}}(\lambda)$ for both components that does not distinguish between the incorporation mode (primary: on the PGMA layer; secondary: at the brush-solution interface; ternary: along the polymer chains). To estimate the GRGDS amount on PAA brushes, the PAA brush swelling before and after GRGDS binding was measured using the box modeling of ellipsometric data. In principle, a change in in-situ roughness of the GRGDS modified PAA brushes has to be considered. However, for in-situ ellipsometry measurements roughness values (or refractive index gradients) are rarely explicitly modeled for swollen brush layer thicknesses much smaller than 100 nm, since the box model is usually the best-fit model in this thickness regime, and changes in in-situ roughness are reflected in the in-situ refractive index (163). The amount of the peptide ($\Gamma$) was then calculated with the modified de Feijter approach (Equation 3-1) (29):

$$
\Gamma_{\text{GRGDS}} = d_{\text{brush}} \frac{n_{\text{comb}} - n_{\text{brush}}}{\left(\frac{dn}{dc}\right)} + d_{\text{add}} \frac{n_{\text{comb}} - n_{\text{amb}}}{\left(\frac{dn}{dc}\right)} \quad \text{(Equation 3-1)}
$$
By using the ellipsometric box modeling, the initial swollen PAA brush model was comprised of the components brush thickness $d_{\text{brush}}$ and refractive index $n_{\text{brush}}$. Upon peptide incorporation, the uptake of a certain peptide amount is considered and represented by the component $n_{\text{comb}}$, where ‘comb’ describes a combined biomolecule and brush layer. Additionally, a top layer consisting of solely hydrated peptide molecules was added to the model. The thickness of the top layer $d_{\text{add}}$ equals the thickness change after peptide incorporation (i.e. $d_{\text{add}} = d_{\text{comb}} - d_{\text{brush}}$). Consequently, the molecular concentration change in the biomolecule-brush layer is represented by $n_{\text{comb}} - n_{\text{brush}}$ and in the biomolecule-ambient layer by $n_{\text{comb}} - n_{\text{amb}}$. Equation 1 represents a virtual two-layer approach for calculation only and it is valid for both positive and negative $d_{\text{add}}$. For the peptides the refractive index increment $dn/dc = 0.185 \text{ cm}^3/\text{g}$ was used (214).

3.2.6. AFM Measurements of Flat Ti and PAA Brushes

For physical characterization of Flat Ti and PAA brushes, a Dimension 3100 with Nanoscope IIIa controller (Vecco Instruments, USA) was used. The AFM images were recorded in the tapping mode. Tips of the type BSTap (Budget Sensors, Bulgaria) with a resonance frequency of 300 kHz and a spring constant of 40 N/m were used. The processing and evaluation of the data were conducted with the software NanoScope Analysis (version 1.5; Bruker AXS).
3.2.7. Dynamic Contact Angle Measurements

The wettability of PAA brush modified Ti surfaces was determined before and after binding of GRGDS with an OCA20 (DataPhysics Instruments GmbH, Filderstadt, Germany). Advancing water contact angles were determined from dynamic dispensing/redispensing measurements with a volume of the sessile drop of 5 µL to 10 µL at a 0.2 µL/s suspension rate by using the goniometer technique. Low suspension rates were chosen to assure the mechanical equilibrium of all interface tensions between single measurement steps. Receding contact angles could not be measured due to a pinning of the contact line.

3.2.8. Cell Culture and Adhesion Assay

To evaluate cell adhesion to PAA brush modified Ti surfaces, prepared substrates were cut with a diamond-tipped scribe (EURO TOOL, Grandview, MO) into pieces that fit into Falcon™ 48 well tissue culture plates (Fisher Scientific, Asheville, NC). The surfaces were bathed in 70% ethanol for ~ 5 minutes and then transferred to a new sterile 48 well plate to air dry for 30 minutes in a sterile biosafety cabinet. Surfaces were rinsed with 1XPBS (Fisher Scientific) at pH 7.4 to remove any residual ethanol before cell seeding.

Murine fibroblast NIH/3T3 cells (ATCC, Manassas, VA) were expanded and cultured in Dulbecco’s Modified Eagle’s Media (DMEM; ATCC) completed with 10% Calf Serum (Colorado Serum Co., Denver, CO) and 1% Penicillin/Streptomycin (Fisher Scientific). Fibroblasts were grown at 37°C and
5% CO₂ and passaged every two days with 0.05% Trypsin (Fisher Scientific). Cells were seeded onto the sterile substrates at a density of 50,000 cells/mL. Cell adhesion was measured at 24- and 48-hours following cell seeding with a calcein stain (Life Technologies, Carlsbad, CA). Briefly, Ti surfaces with adhered cells were transferred into new well plates prior to the assays. Substrates for staining were rinsed with PBS and then stained for 20 min in phenol-free DMEM (Fisher Scientific) with 2 µM Calcein-AM. Substrates were imaged with a Leica DMI 3000B fluorescence microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany) and five images per well of three replicate wells were acquired using a 5x objective. Image analyses were performed using NIH ImageJ Processing Software to quantify cell counts.

3.2.9. Statistical Analysis

All ellipsometric values are reported with corresponding standard error values with three replicates. All cell experiments were performed in triplicate. A two-tailed unpaired t-test with a Tukey's Post Test was conducted using Prism 5.0 graphing and statistical analysis software (Graph Pad, La Jolla, CA) at 95% confidence level (α=0.05) to make statistical comparisons between modified Ti substrates, as well as the control Flat Ti.
3.3. Results and Discussions

3.3.1. Investigation of PAA grafting and pH-dependent swelling behavior

The objective of this work was to develop and verify a method to graft reactive PAA brushes to Ti substrates and then demonstrate the ability to further functionalize the brushes with a cell adhesion peptide, for future applications in biomedical devices and scaffolds. While “grafting-from” approaches have been reported for polymer brushes to Ti surfaces (215-218), to the best of our knowledge, there has been no method reported for a “grafting-to” approach of PAA to Ti. The “grafting-to” method makes it feasible to easily prepare homogeneous PAA “pseudo” brushes with predetermined molecular weight.

By using ellipsometry, dry thicknesses were measured and modeled after each step of the polymer brush preparation to follow the grafting process. The data in Table 3-1, which represents three different Ti surfaces functionalized with PAA, demonstrate that Ti surfaces can be reproducibly grafted with PAA brushes by using the “grafting to” approach (Figure 3-1, a-c). A Ti surface was deposited to a thickness of 100 nm and determined to have a roughness of around 0.6 nm as determined by AFM (Figure 3-3). These surfaces showed an average TiO$_2$ thickness ($d_{TiO_2}$) of 0.5 nm after oxygen plasma activation (Table 3-1), when an active oxide layer is added to the Ti surfaces. The addition of this oxide layer is critical to further PAA functionalization, as hydroxyl groups (OH) of the activated TiO$_2$ layer react with the epoxy groups of the anchoring layer PGMA to form covalent ether bonds (219). During the grafting process, the average thickness of the PGMA ($d_{PGMA}$) anchoring layer was found to be 2.1 nm (Table 3-1).
Remaining epoxy groups of the PGMA layer enabled binding of PAA polymer chains via ester bonds, leading to PAA “pseudo” brushes, where the polymers are grafted with more than one anchoring point to the surface. The average thickness measured by ellipsometry of the final PAA brushes ($d_{PAA}$) was 4.7 nm (Table 3-1). The roughness of PAA brushes was determined by AFM (tapping mode) as an average from three height images (Figure 3-4A) and found to be 0.35 nm (Figure 3-4B). The AFM height images and roughness values indicate that smooth homogenous PAA brush surfaces were obtained. The PGMA and PAA final thicknesses reported in this current work, on Ti, are comparable to PGMA and PAA thicknesses on flat Si surfaces (177, 211). For example, Aulich et al. obtained a PGMA thickness of 2.7 nm and PAA thickness of 5.1 nm using a “grafting to” approach on Si substrates (177).

**Figure 3-3:** A representative image acquired by AFM in a tapping mode of the roughness of Ti evaporated onto Si wafer. (scale bar=400 nm).
Figure 3-4: Triplicate images of the roughness of PAA brushes on Ti acquired by AFM in a tapping mode (A) and measured for the average roughness of PAA brushes on Ti (B).
Table 3-1: Modeled PAA Brush Parameters

<table>
<thead>
<tr>
<th>replicate</th>
<th>$d_{\text{TiO}_2}$ [nm]</th>
<th>$d_{\text{PGMA}}$ [nm]</th>
<th>$d_{\text{PAA}}$ [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.4±0.1</td>
<td>2.0±0.1</td>
<td>5.5±0.1</td>
</tr>
<tr>
<td>2</td>
<td>0.6±0.1</td>
<td>2.2±0.1</td>
<td>4.3±0.1</td>
</tr>
<tr>
<td>3</td>
<td>0.4±0.1</td>
<td>2.2±0.1</td>
<td>4.4±0.1</td>
</tr>
</tbody>
</table>

Next, the ability of the PAA brushes on the Ti surfaces to maintain pH-swelling behavior (Figure 3-5) was measured and modeled via ellipsometry by the pH-dependent thickness change of the swollen PAA layer. For PAA brushes, at pH values close to the isoelectric point of PAA (pH=2.1 (176)), the polymer chains arrange in a compact conformation, but an increase of the pH (more basic) results in extended polymer chains (175). Here, at a pH of 7.2, the PAA brushes exhibited an average swelling degree of 6.0 (Table 3-2), due to deprotonation of COOH groups and expansion of brushes (220). At a more acidic pH value of 3.6, the average swelling degree was reduced to 1.7 (Table 3-2), due to protonation of COO- groups, and formation of hydrogen bonds, which results in a more compact conformation of PAA chains (221). Similar swelling-deswelling behavior was observed for PAA brushes with $M_N$=26,500 g/mol grafted to Si substrates, showing swelling degrees of ~2.8 at pH 3.6 and ~6.0 at pH 7.2 in buffer solutions with $c_{\text{Na}^+}$=0.01 M (213). The pH-responsive swelling for the third sample could not be evaluated due to a high window effect of the cell used for the swelling measurement. However, it can be assumed that the swelling of this sample is in agreement with the other two samples. Consequently, it is
demonstrated that PAA brushes can be covalently grafted to Ti substrates and retain their functionality, including pH-dependent swelling behavior.

Figure 3-5: pH-dependent swelling behavior of PAA brushes grafted to Ti substrates. Above its isoelectric point (IEP=2.1), PAA carries a net negative charge. With increasing pH, PAA becomes more negatively charged and the polymer chain is more extended.

Table 3-2: Modeled Dry and Swollen PAA Brush Parameters and Swelling Degrees

<table>
<thead>
<tr>
<th>replicate number</th>
<th>d_PAA [nm]</th>
<th>d_brush at pH 7.2 [nm]</th>
<th>η_brush</th>
<th>swelling degree</th>
<th>d_brush at pH 3.6 [nm]</th>
<th>η_brush</th>
<th>swelling degree</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.5±0.1</td>
<td>30.6±0.4</td>
<td>1.37±0.01</td>
<td>5.6</td>
<td>9.4±0.1</td>
<td>1.42±0.01</td>
<td>1.7</td>
</tr>
<tr>
<td>2</td>
<td>4.3±0.1</td>
<td>27.6±1.6</td>
<td>1.36±0.01</td>
<td>6.4</td>
<td>6.7±0.1</td>
<td>1.41±0.01</td>
<td>1.6</td>
</tr>
</tbody>
</table>

3.3.2. Immobilized RGD amount on PAA brushes

Next, PAA brushes grafted to Ti were functionalized with GRGDS to demonstrate the ability to confer bioactivity. Other reports have demonstrated the covalent binding of RGD peptides to different kinds of polymer brushes (e.g. poly(2-hydroxyethyl methacrylate), poly(ethylene glycol) methacrylate) or poly[oligo(ethylene glycol)methacrylate]), all prepared by the “grafting-from” approach (216, 222). Furthermore, an approach reported by Psarra et al. describes the covalent binding of RGD peptides via EDC/NHS chemistry to PAA brushes on Si, in which the PAA brushes were prepared by the “grafting-to”
approach (185). Here-in, we use the same reaction conditions as reported by Psarra et al. with an increased GRGDS concentration \(c_{\text{GRGDS}}\) of 1 mg/mL.

After conjugation with RGD, the ability of the PAA-RGD brushes on Ti to still exhibit swelling behavior was measured using ellipsometry. Higher swollen thicknesses are observed for PAA brushes in 0.1 M PBS compared to water as a result of an increased ionic strength in the osmotic regime. An increasing condensation of counter-ions inside the brush forces the polymer chains to stretch owing to the osmotic pressure of the trapped counter-ions (211, 223).

By using a modified de Feijter equation (163), it is possible to model the conjugated peptide amount on the brushes from the ellipsometric data of the swelling measurements, since an increase of RGD molecules inside the brush will affect the refractive index and the thickness of the brush (163). After peptide binding, the swollen thicknesses \(d_{\text{comb}}\) and the refractive indices \(n_{\text{comb}}\) were higher than for the unmodified brush \(d_{\text{brush}}; n_{\text{brush}}\) (Table 3-3). This behavior was also observed in a previous report of PAA-RGD brushes on Si (224), in which the increased thickness and refractive index was attributed to penetration of the GRGDS peptide into the brush, which causes an extended brush swelling. Given that, RGD amounts \(\Gamma_{\text{GRGDS}}\) of 1.2-1.5 \(\mu\text{g/cm}^2\) \((2.4 \cdot 10^9 \text{ to } 3.1 \cdot 10^9 \text{ mol/cm}^2)\) (Table 3-3) were estimated with the modified de Feijter equation for \(c_{\text{GRGDS}}=1\) mg/mL. Psarra et al. obtained a similar \(\Gamma_{\text{GRGDS}}\) of 1.4 \(\mu\text{g/cm}^2\) \((2.9 \cdot 10^9 \text{ mol/cm}^2)\) for \(c_{\text{GRGDS}}=0.5\) mg/mL. (185) The RGD conjugation density achieved in this current work is sufficient to induce the adhesion and spreading of cells onto surfaces (130).
Table 3-3: Modeled Swollen PAA Brush Parameters, Functionalized with Peptides. Thicknesses and refractive indices of the swollen PAA brush (in 0.1 M PBS, pH 7.4) before and after covalent binding of GRGDS (c=1 mg/ml) to PAA brushes were used to calculate the immobilized peptide amount using a modified de Feijter approach.

<table>
<thead>
<tr>
<th>replicate</th>
<th>swelling before GRGDS binding</th>
<th>swelling after GRGDS binding</th>
<th>GRGDS amount</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$d_{\text{brush}}$ [nm]</td>
<td>$n_{\text{brush}}$</td>
<td>$d_{\text{comb}}$ [nm]</td>
</tr>
<tr>
<td>1</td>
<td>32.0±2.0</td>
<td>1.36±0.01</td>
<td>48.0±0.5</td>
</tr>
<tr>
<td>2</td>
<td>28.1±2.0</td>
<td>1.37±0.01</td>
<td>48.6±0.6</td>
</tr>
<tr>
<td>3</td>
<td>18.8±1.5</td>
<td>1.39±0.01</td>
<td>37.6±0.3</td>
</tr>
</tbody>
</table>

Our results show that GRGDS can be covalently bound to PAA brushes on Ti by using an EDC/NHS bioconjugation method with similar amounts of peptide that have been shown on PAA brushes on Si substrates (185). We also report significantly higher RGD peptide amounts compared to investigations using self-assembled monolayers or “grafting from” polymer brushes on Si and Ti substrates, where RGD amounts ranged in the picomolar to nanomolar (216, 222, 225).

To confirm that the conjugation and presentation of the RGD groups conjugated to the PAA brushes were functional, NIH/3T3 fibroblast cells were cultured on PAA and PAA-RGD modified substrates for 24 and 48 hours. Cells grown on PAA brushes alone did not sufficiently adhere to the substrate (Figure
3-6A, D), possibly due to the hydrophilicity of the PAA brushes (contact angles: <10°; data not shown), which can inhibit the protein adsorption necessary for subsequent cell attachment (153). Indeed, the rounded morphology of cells at both 24 (Figure 3-6A) and 48 hours (Figure 3-6D) on PAA brushes alone indicates poor cell adhesion (226, 227) and can be attributed to lack of focal adhesions formed with proteins on the substrate that allow for healthy cell spreading behaviors (228, 229). However, with the immobilization of GRGDS peptides onto PAA brushes, cells were able to adhere and proliferate (Figure 3-6B, E), comparable to cellular adhesion on bare Flat Ti (Figure 3-6C, F). Cells adhered to PAA-RGD brushes were slightly rounded at 24 hours (Figure 3-6B), but extended to a spread morphology characteristic of healthy, adhered NIH/3T3 fibroblasts at 48 hours (Figure 3-6E), suggesting that the RGD ligands conjugated to PAA brushes remained active and available to cells, as RGD ligands are known to support adhesion and spreading behaviors of cells, especially when enhancing a biomaterial (187, 208, 209, 230-232).
Figure 3-6: Representative images of the adhesion of NIH/3T3 fibroblasts cultured on Flat Ti surfaces with PAA brushes, Flat Ti surfaces with 1.0 mg/mL RGD-modified PAA brushes, and Flat Ti control, stained with calcein at 24 hours (A-C) and 48 hours (D-F) (scale bar=200 µm).

Figure 3-7: Quantification of the adhesion of NIH/3T3 fibroblasts cultured on Flat Ti grafted with PAA brushes, PAA brushes modified with RGD at 1.0 mg/mL, and Flat Ti control. Cells were stained with calcein and adhesion was quantified at 24 hours (A) and 48 hours (B); significance was seen at 48 hours with more cells on Flat Ti with 1.0 mg/mL RGD compared to PAA brushes alone (n=15; *, P≤0.05).
Quantification of the number of cells on each substrate was determined through image analysis of the calcein staining, measured as live cells per cm$^2$.

While there was no significant difference in the cell adhesion between substrates modified with PAA, PAA-RGD, or Flat Ti at 24 hours (Figure 3-7A), after 48 hours (Figure 3-7B), the amount of cells on PAA brushes without biomolecule modification was almost equal to the 24 hour quantification (Figure 3-7A), indicating that the proliferation of the cells on PAA brushes without biomolecule modification was inhibited as expected by the hydrophilic nature of the polymer (153). However, there was a significant increase in the numbers of cells adhered to PAA brushes modified with 1.0 mg/mL RGD compared to unmodified PAA brushes (n=15; *, p≤0.05). Furthermore, the amount of cells adhered to bare Flat Ti was similar to 1.0 mg/mL RGD modified PAA brushes (Figure 3-7B), indicating that the addition of RGD to PAA improved cell adhesion of NIH/3T3 fibroblasts (187). Investigations of the cellular response show that, although hydrophilic PAA can prevent cellular attachment (233), the conjugation of RGD to PAA brushes can restore biocompatibility, which will be critical for future applications of these substrates.

3.4. Conclusions

Here we have described a facile method to modify biocompatible Ti substrates with PAA brushes in order to amplify their substrate functionality. PAA brushes offer a high binding capacity due to the high density of COOH groups and since PAA is a polyelectrolyte, it has the ability to bind moieties via
electrostatic interactions. By using the “grafting to” approach, PAA brushes were successfully and reproducibly grafted to Ti surfaces, with PAA brush thicknesses comparable to that on Si surfaces. Furthermore, PAA brushes retained their pH-dependent swelling behavior. Next, the abundant COOH groups of the PAA “pseudo” brushes were used for covalent binding of the RGD containing peptide GRGDS as a model bioactive functional group, resulting in higher RGD amounts as compared to “grafting from” brushes. Hydrophilicity and swelling of the RGD functionalized PAA brushes were conserved, therefore the PAA brushes are still responsive and able to interact with biomolecules at least electrostatically. While NIH/3T3 cell adhesion and proliferation were significantly decreased on PAA brush functionalized Ti surfaces, functionalization of the PAA brushes with the peptide GRGDS enabled cell adhesion comparable to Flat Ti surfaces at both 24 and 48 hours. Furthermore, after 48 hours a significantly higher amount of cells adhered to PAA-RGD brush modified Ti surfaces compared to PAA brush modified Ti surfaces. Consequently, the biocompatibility of the Ti substrates was conserved by modification of the PAA brushes with RGD peptides. Indeed, the ability to functionalize Ti substrates with reactive PAA brushes can be employed in various future applications including biomedical devices and implants, biosensors, and diagnostics. Future investigations will also study the effect of long-term cell culture with respect to pH-dependent swelling behavior of PAA brushes.
CHAPTER 4
Free Polyethylenimine Enhances Substrate-Mediated Gene Delivery on Titanium Substrates Modified with RGD-Functionalized Poly(acrylic acid) Brushes

4.1. Introduction

Nonviral gene delivery is the delivery of exogenous genetic material to cells or tissues, generally to produce a therapeutic protein, with applications in gene therapy, tissue engineering and regenerative medicine, and biomedical implants. Nonviral gene delivery is often performed using cationic polymer or lipid vectors complexed with DNA plasmids through electrostatic interactions. The formed complexes are typically delivered using a bolus method, which can be limited by mass transport to the cells and leaves the complexes susceptible to processes such as degradation and aggregation, thereby limiting gene transfer (12). Substrate-mediated gene delivery (SMD), also known as reverse transfection or solid-phase delivery, is a method of immobilizing DNA complexes to the substrate via covalent attachment or nonspecific adsorption. Compared to bolus delivery, SMD has been shown to limit complex aggregation and require a lower dose of DNA, as well as increase transgene expression and the number of transfected cells by increasing the local concentration of DNA within the microenvironment around the cell and overcoming a mass transport barrier to gene delivery efficiency (19, 20, 58-63). Although a promising delivery method, past investigations into SMD have focused on using tissue engineering scaffolds like poly(lactide-co-glycolide) (PLG) (32, 234) or traditional culturing substrates...
such as tissue culture polystyrene (TCPS), with or without protein coatings (22, 59, 235), but few SMD studies have focused on the modification of commonly used metal biomaterials (236, 237). For example, titanium (Ti) is one of the most commonly used biomaterials (3), with many applications that could benefit from nonviral SMD such as enhancing the integration of bone implants by delivering genes to increase osseointegration (237, 238), gene-eluting stents to accelerate re-endothelialization (239), or developing implantable sensors protected by the local delivery of anti-inflammatory and anti-fibrosis genes (240), but to date there have been few studies published using SMD on Ti.

Along with the limited scope of biomaterials investigated for nonviral SMD, further tuning of the substrate to enhance DNA complex interactions and cell-material interactions are necessary to make SMD more efficient and therapeutically relevant. Polymer brushes are an attractive substrate modification for SMD, as the brushes have stimuli-responsive and bioactive properties (186, 241), can be engineered for controlled cellular response through covalent binding of adhesions peptides (116, 179, 185, 242) (chapter 3), and can be used to control the adsorption of proteins and release of biomolecules (153, 179, 243-245). Polymer brushes are formed by grafting polymer chains adjacent on a substrate, which forces the chains to stretch from the substrate (151, 152). There are two common approaches for grafting polymer brushes, “grafting from” and “grafting to”. For the “grafting from” approach, a substrate is modified with initiator sites and then exposed to monomers, which are polymerized on the surface, often by radical polymerization strategies. In this
“grafting from” approach, homogenous brushes are formed with high brush density but are more difficult to produce and characterize (157). For the “grafting to” method, polymer chains are formed before grafting to the substrate and added to the surface via chemical reactions between reactive groups on the surface and a functional end group of the polymer (164). With the “grafting to” approach, “pseudo”-brushes with more than one grafting point per chain can be prepared with swelling properties not distinguishable from end-grafted brushes (29, 177). Although less dense compared to “grafting from” brushes, the “grafting to” approach, in general, produces homogeneous polymer brushes with a well-defined structure and higher stability compared to physically adsorbed polymers (157, 173, 215).

While methods to produce polymer brushes on silicon and other materials (i.e. gold, stainless steel) are well known (29, 30, 153, 184, 246, 247), in our recent paper (116) (chapter 3), we showed for the first time that the poly(acrylic acid) (PAA) brush “grafting to” process is feasible on Ti substrates and the pH-responsive deprotonation of the PAA brushes is maintained. Furthermore, following the addition of the RGD-containing peptide GRGDS to the brushes (PAA-RGD), cell adhesion of NIH/3T3 fibroblasts was significantly enhanced compared to cells cultured on unmodified PAA brushes. Swollen deprotonated brushes have been shown to produce negatively charged polymer chains at a pH of 7.2 (197), and that charge was further decreased by the inclusion of RGD peptides (185). Therefore, given the negative charge of the PAA brushes and the inclusion of the RGD peptide (Figure 4-1), we propose Ti substrates modified
with PAA-RGD are an ideal platform for SMD, as PAA brushes could improve loading of cationic DNA complexes through charge interactions and mediate cell adhesion via the RGD peptide. In this work we expand on our previous study by showing, for the first time, the feasibility of immobilizing complexes formed with branched polyethylenimine and DNA plasmids (bPEI-DNA) onto PAA-RGD brushes (Figure 4-1), and characterize their release and transfection ability, as well as propose a potential benefit of PAA-RGD brushes to allow for the presentation of free bPEI to cells to improve gene delivery.

**Figure 4-1:** bPEI-DNA complex immobilization on PAA brushes at pH 7.2. Complex formation with DNA plasmid encoding for enhanced green fluorescent protein (eGFP) and luciferase (LUC) and branched polyethylenimine (bPEI) at a N/P ratio of 20 complexes with an overall positive charge (6 mV). These positively charged bPEI-DNA complexes can interact with negatively charged, swollen PAA-RGD brushes (pH 7.2) on the substrate to transfect NIH/3T3 fibroblasts cultured on the substrate.
4.2. Materials and Methods

4.2.1. Preparation of PAA brushes on Ti Surface and Covalent Bonding of RGD/RGE Peptides

Throughout this study the substrates investigated include PAA brushes on Ti (abbreviated as PAA), PAA brushes modified with GRGDS on Ti (abbreviated as PAA-RGD), PAA brushes modified with the control peptide RGES on Ti (abbreviated as PAA-RGE), and Ti with no modification (termed Flat Ti) as a control. Ti substrates (100 nm Ti, Grade 2, on a Si wafer) were purchased from Platypus Technologies (Madison, WI) and used for flat controls. Ti substrates for polymer brush functionalization were produced by Fraunhofer IWS (Dresden, DE) by sputtering Ti pellets (Grade 2) on Si wafer (Silicon Materials, Germany) or fabricated in an ultra-high deposition vacuum chamber by electron beam evaporation of Ti pellets (Super Conductor Materials, Inc., Tallman, NY) onto Si wafer substrates (University Wafer, South Boston, MA) (116) (chapter 3). Samples were functionalized with polymer brushes according to our previously reported “grafting-to” method (116) (chapter 3). Briefly, the Ti substrate was activated with oxygen plasma for 1 min (Plasma Cleaner PDC-002 with Plasmaflo PDC-FMG-2, Harrick Plasma, USA). After activation, a solution composed of 0.02 wt % of poly(glycidyl) methacrylate (PGMA, Mn = 17,500 g/mol, Mw/Mn = 1.7, Polymer Source, Inc., Canada) in chloroform (CHCl₃, Fisher Scientific, UK) was spin-coated (Spin150 spin coater, Polos, Putten, Netherlands). The PGMA layer was annealed for 10 min at 110 °C under vacuum, resulting in a thin reactive anchoring layer with epoxy groups for the adjacent grafting step. A PAA (Mn = 26,000 g/mol, Mw/Mn = 1.12, Polymer
Source, Inc., Canada) solution was prepared at 1.0 wt % in ethanol (EtOH) and spin-coated onto the grafted PGMA layer. The PAA layer was annealed at 80 °C for 30 min under vacuum to react the epoxy groups of PGMA with COOH groups along the chain of PAA, grafting the PAA chains in loops and tails via ester bonds. Excess polymer was extracted by stirring the samples in ethanol for 30 min at room temperature and drying with a N₂ flux. Peptide conjugation based on the carboxyl-amine-reaction (EDC-NHS coupling) was performed as previously described (116) (chapter 3) and all materials used for peptide conjugation were purchased from Sigma-Aldrich (St. Louis, MO). Briefly, buffers were prepared using boric acid, phosphate-buffered saline (PBS), and 2-(N-morpholino)ethanesulfonic acid (MES). The linear RGD-containing peptide GRGDS (or RGE-containing peptide RGES) was covalently bound to PAA brushes on Ti surfaces via activation of the PAA carboxy groups with N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) for direct conjugation of carboxyl groups with the primary amines of the peptides. For conjugation, PAA brushes on Ti substrates were equilibrated in 0.1 M MES at pH 6 for 10 min. After aspiration of this buffer, brushes were reacted with 0.5 mL of 5 mM EDC solution and 0.5 mL of 2 mM NHS solution in 0.1 M MES buffer (pH 6) by gently shaking for 40 min. Subsequently, a 1.0 mg/mL solution of GRGDS (or RGES) in 0.1 M borate buffer (pH 8) was added to the activated PAA brush substrates. After gentle shaking at room temperature for 16 hours, the peptide solution was aspirated and the
GRGDS (or RGES)-modified samples were washed three times by stirring in 0.1 M PBS buffer at pH 7.4 for 3 min.

4.2.2. DNA Complex Formation and Characterization

Plasmid (pEGFP-LUC) that encodes both the enhanced green fluorescent protein (EGFP) and firefly luciferase protein (LUC) under the direction of a CMV promoter, was used in all studies in this work. Plasmids were purified from bacteria culture using Qiagen (Valencia, CA) reagents and stored in Tris–EDTA buffer solution (10 mM Tris, 1 mM EDTA, pH 7.4) at −20 °C. For DNA complex formation, 25 kDa branched polyethylenimine (bPEI; Sigma-Aldrich) was dissolved in reduced serum medium OptiMEM (Fisher Scientific) and then added dropwise to DNA in OptiMEM, vortexed for 10 sec, and incubated for 15 min at room temperature. Complexes were formed at nitrogen/phosphate (N/P) ratios of 3, 5, 10, or 20 in OptiMEM with 2 μg of DNA, and delivered in a volume of 3 mL for the spectroscopic ellipsometry measurement and 300 μL for all other studies, resulting in a DNA amount of 1 μg/cm² used for immobilization to substrates in all studies.

The size and zeta potential of the bPEI/DNA complexes were determined by dynamic light scattering and Laser Doppler micro-electrophoresis, respectively, using a Zetasizer Nano ZS90 (Malvern Instruments Ltd, UK). Size measurements were taken at 25°C at a scattering angle of 90° and size reported as the Z-average diameter (d. nm). Zeta potential measurements were also taken
at 25°C using folded capillary cells with the measurement mode set to automatic and the values reported in mV.

4.2.3. Ellipsometric Measurements for Characterization of PAA brushes and DNA Complex Immobilization

Ellipsometric measurements were acquired using a Woollam RC2 or a M2000-VI spectroscopic ellipsometer (both from J.A. Woollam, Co., Inc., Lincoln, NE, USA) to confirm brush parameters, as previously described (116) (chapter 3). Briefly, for dry brushes the ellipsometric data, Δ (relative phase shift) and tan Ψ (relative amplitude ratio), were recorded at wavelengths (λ) of 380–1700 nm and four angles of incidence (AOI: 45°, 55°, 65°, 75°). To confirm brush swelling and functionality (indicative of deprotonation), substrates were first sterilized with EtOH and then the pH-reactive brush swelling was performed by adding OptiMEM (pH 7.2) to dry PAA brushes. Brush swelling within OptiMEM was measured at AOI 70° with a batch cuvette (TSL Spectrosil, Hellma, Muellheim, Germany), at wavelengths λ = 400–1200 nm. The brush film thickness was quantified via the change in Ψ and Δ, which was used to calculate the swelling degree (swollen brush thickness divided by dry brush thickness). Brush swelling was also measured before and after the addition of RGD and RGE peptides, as well as before and after complex immobilization, to determine the amount of peptide and complexes immobilized. These measurements were all performed in situ. Experimental data were modeled in CompleteEASE software (Version 4.64, J.A. Woollam Co., Inc., Lincoln, NE, U.S.A.) as described in our previous work (116) (chapter 3). The amount of the peptides RGD and RGE at the PAA brush
surface were calculated with a modified de Feijter approach (Equation 4-1) (186):

$$
\Gamma_{\text{peptide/complexes}} = d_{\text{brush}} \frac{n_{\text{comb}} - n_{\text{brush}}}{d_n/d_c} + d_{\text{add}} \frac{n_{\text{comb}} - n_{\text{amb}}}{d_n/d_c}
$$

(4-1)

In this approach changes in the layer parameters in-situ refractive index and in-situ thickness \((n_{\text{comb}}, d_{\text{comb}})\) after covalent peptide immobilization are referenced to the swollen state of the surface \((n_{\text{brush}}, d_{\text{brush}})\) before immobilization, which are the parameters of the swollen PAA brushes (Equation 4-1). The amount of DNA complexes immobilized to the Flat Ti substrate was calculated by the de Feijter equation (248), while amounts of complexed DNA on PAA and PAA-RGD brushes were calculated again with the modified de Feijter approach (Equation 4-1), referencing the in-situ layer parameters \((n_{\text{comb}}, d_{\text{comb}})\) of the combined complexes and brushes to the parameters \((n_{\text{brush}}, d_{\text{brush}})\) of the swollen PAA brushes or the parameters of the swollen PAA-RGD brush, respectively. The refractive index increment \(d_n/d_c = 0.185 \text{ cm}^3/\text{g}\) was used for the RGD peptides (116) (chapter 3) and \(d_n/d_c = 0.183 \text{ cm}^3/\text{g}\) for the DNA complexes (249).

4.2.4. DNA complex immobilization and release measured by radiolabeled DNA

Plasmid radiolabeled with \([\alpha-^{32}\text{P}]d\text{ATP}\) (Perkin Elmer, Akron, OH) was used to measure the immobilization of DNA complexes on Flat Ti, PAA, and PAA-RGD substrates. To label the DNA plasmid, a nick translation kit (Invitrogen, Waltham, MA) was used following the manufacturer’s protocol. The radiolabeled
DNA was diluted with unlabeled DNA to a final concentration (0.806 µg/µL) and used to form DNA complexes, as described above. First, the substrates were prepared by cutting with a diamond-tipped scribe into pieces that fit into Falcon™ 48 well tissue culture plates (Fisher Scientific). Images of each substrate used for immobilization studies were taken prior to complex immobilization and analyzed with NIH ImageJ Processing Software to determine the surface area (cm²). Next, the substrates were bathed in 70% EtOH and then transferred to a new sterile well plate to air dry in a sterile biosafety cabinet. Complexes (300 µl in OptiMEM as described above) were immobilized by incubation on substrates for 2 hours. After complex immobilization, the complex solution was removed and the substrates were washed twice with PBS. The quantity of DNA immobilized was determined by immersing substrates in a scintillation cocktail (5 mL, Thomas Scientific, Swedesboro, NJ) for measurement with a Packard Tri-Carb 1900 TR Liquid Scintillation Counter. Counts per minute were correlated to the DNA amount using a standard curve and the amount of DNA immobilized to each sample was normalized to the surface area (cm²).

The release profiles of immobilized DNA complexes from PAA, PAA-RGD, or Flat Ti were determined by incubation of the DNA-loaded substrates with either reduced serum OptiMEM, serum-containing cell growth media, or conditioned growth media (from flasks of NIH/3T3 fibroblasts cultured for 48 hours) at 37 °C in a humid chamber. At time 0, substrates with immobilized complexes were moved to a fresh well before adding the media to the substrates. At predetermined time points (0.5, 4, 24, and 48 hours), the total volume of
media was removed and counts per minutes were measured using a Packard Tri-Carb 1900 TR Liquid Scintillation Counter. An equal volume of fresh warmed media was then added to each substrate and the release was allowed to continue. At the final time point, the DNA remaining on the samples was also determined. The amount of DNA released from the substrate was determined from the measured counts per minutes using a standard curve with known amounts of DNA. The percentage of DNA released was calculated by dividing the cumulative counts released (at each time point) divided by the total counts initially on the substrate (determined by mass balance); thus, the release curves represent the percentage of DNA released relative to the initial amount bound to each surface.

4.2.5. Cell Culture and Substrate-Mediated Gene Delivery

Transfection studies were performed with murine fibroblast NIH/3T3 cells (ATCC, Manassas, VA) cultured in Dulbecco’s Modified Eagle’s Media (DMEM) completed with 10% Calf Serum (Colorado Serum Co., Denver, CO) and 1% Penicillin/Streptomycin. Fibroblasts were cultured at 37°C and 5% CO₂ and passaged every two days with 0.05% Trypsin-EDTA. For transfection studies, substrates were cut and sterilized (as described above), bPEI-DNA complexes were formed and immobilized for 2 hours onto the four substrate conditions (Flat Ti, PAA, PAA-RGD, and PAA-RGE), after which the solution containing the DNA complexes were removed and then substrates were rinsed with OptiMEM before cells were seeded onto the substrates at a density of 50,000 cells/mL. Cells were
cultured for 48 hours at 37°C and 5% CO₂ and then the substrates were
transferred into a new well plate and lysed using 200 μL of 1X reporter lysis
buffer (Promega, Madison, WI). Transfection levels were quantified by
measuring the luciferase activity using the Luciferase Assay System (Promega)
and a luminometer (Turner Designs, Sunnyvale, CA). Luciferase activity
(measured as relative light units, or RLUs) was normalized to the total protein
amount determined with a Pierce BCA protein assay (Pierce, Rockford, IL), as
seen in previous investigations.

4.2.6. Cell Adhesion of NIH/3T3 Fibroblasts Cultured on PAA brushes with
Immobilized Complexes

To determine the effect of complex immobilization on the cellular response
of NIH/3T3 fibroblasts cultured on bPEI-DNA complexes immobilized to PAA,
PAA-RGD, PAA-RGE, and Flat Ti, calcein staining (Life Technologies, Carlsbad,
CA) was used to visualize cellular adhesion and quantify the cell counts per area
(cm²) at 48 hours following cell seeding. Briefly, surfaces with adhered cells were
transferred into new well plates prior to the assays. Substrates for staining were
rinsed with PBS and then stained for 20 min in phenol-free DMEM (Fisher
Scientific) with 2 μM Calcein-AM. Substrates were imaged with a Leica DMI
3000B fluorescence microscope (Leica Microsystems CMS GmbH, Wetzlar,
Germany) and five images per well of three replicate wells were acquired using a
5x objective. Image analyses were performed using NIH ImageJ Processing
Software to quantify cell counts.
4.2.7. Assessing the Contribution of Free bPEI on Transfection Success with SMD

To assess the contribution of free bPEI on transfection success in NIH/3T3 fibroblasts cultured on bPEI-DNA complexes immobilized to PAA, PAA-RGD, PAA-RGE, and Flat Ti, complexes were first formed as previously described, and then filtered to remove free (uncomplexed) bPEI using a Vivaspin®6 Centrifugal Concentrator (Vivaproducts, Inc., Littleton, MA). Complexes were filtered by centrifuging the solution at 3000 g for 3 minutes at 4°C. The DNA complexes trapped in the filter were eluted using an equal volume of OptiMEM. These filtered complexes were immobilized onto the substrates (PAA, PAA-RGD, PAA-RGE, and Flat Ti) and cells were cultured on these substrates and transfection was assessed, as described above.

To further understand the effect of free bPEI on transfection success in NIH/3T3 fibroblasts cultured on bPEI-DNA complexes immobilized to PAA, PAA-RGD, PAA-RGE, and Flat Ti, SMD transfection was performed with a controlled dosage of free bPEI. Filtered complexes formed as previously described received an addition of 1 or 5 µg of free bPEI during immobilization to the substrate, and then transfection was performed and assessed as described above.

4.2.8. Cell Viability of NIH/3T3 Fibroblasts Cultured on PAA Brushes with Immobilized Filtered and Unfiltered Complexes

To understand the effect of immobilized complexes (and free bPEI) on the cellular response, the metabolic activity of cultured NIH/3T3 fibroblasts was assessed using a Water Soluble Tetrazolium (WST-1) salt cell proliferation assay kit (Roche, Indianapolis, IN), according to manufacturer’s protocol, to quantify the
cell viability at 48 hours following cell seeding. Briefly, cells cultured on PAA-RGD, PAA-RGE, PAA, and Ti substrates (immobilized with unfiltered or filtered complexes) were transferred into new well plates prior to the assays. Cells were washed with 1× PBS and incubated at 37 °C in WST-1 solution (10 vol% WST-1 reagent in phenol-free Dulbecco's Modified Eagle Medium) for 3 hours. After incubation, absorbance values were measured on an Epoch Microplate spectrophotometer (BioTek, Winooski, VT) at 430 nm and corrected with 690 nm as a reference wavelength, and then normalized per area (cm²).

4.2.9. Statistical Analysis

All experiments were performed in triplicate on duplicate days, and values are reported from one representative experiment as means with standard error of the mean. Statistical comparisons were performed with Prism 5.0 graphing and statistical analysis software (Graph Pad, La Jolla, CA) at 95% confidence level (α=0.05), with the statistical tests used specified in the figure legends.

4.3. Results

4.3.1. PAA Brush Film Characterization

The objective of this paper was to apply SMD to a Ti substrate functionalized with PAA brushes, further functionalized with RGD (or control RGE) peptides (Figure 4-1). First, the PAA brush parameters and pH swelling behavior were measured and modeled with spectroscopic ellipsometry to confirm the brush film thickness and swelling functionality of PAA brushes before the
immobilization of bPEI-DNA complexes. Similar to our previous study where we functionalized Ti with PAA brushes (116) (chapter 3), the average film thickness for the activated oxide groups ($d_{TiO2}$[nm]) after plasma activation, the PGMA anchoring layer ($d_{PGMA}$[nm]), and PAA brush thickness ($d_{PAA}$[nm]) were 0.8±0.6 nm, 1.9±0.3 nm, and 5.5±0.3 nm, respectively (Table 4-1). After the addition of OptiMEM (pH 7.2; the reduced serum media used for complex immobilization), PAA brushes swelled to an average thickness of 23±3.0 nm (average swelling degree of 4.0±1.0, Table 4-2), which is similar to the swelling in 0.1 M PBS (pH 7.4) reported in our previous study (116) (chapter 3). Swelling measurements were also used to calculate RGD and RGE conjugation densities using Equation 5-1 (1.3±0.2 and 1.0±0.2 µg/cm², respectively; Table 4-3), which is similar to the RGD density we reported in our previous study (116) (chapter 3).

Table 4-1: PAA Brushes Formed on Ti Substrates. The "grafting-to" process was monitored with spectroscopic ellipsometry at each step of the PAA brush formation. The first step is plasma activation for 1 min to form oxide groups ($d_{TiO2}$[nm]), and then a PGMA anchoring layer was spin-coated onto the activated substrate and annealed at 110 °C for 10 min under vacuum ($d_{PGMA}$[nm]). Next, a layer of PAA was spin-coated onto the PGMA anchoring layer and annealed at 80 °C for 30 min under vacuum. Finally, the excess polymer was extracted with EtOH for 30 min at room temperature ($d_{PAA}$[nm]). Average values for each thickness are reported for three replicate substrates.

<table>
<thead>
<tr>
<th>Replicate</th>
<th>$d_{TiO2}$ [nm]</th>
<th>$d_{PGMA}$ [nm]</th>
<th>$d_{PAA}$ [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.2</td>
<td>2.3</td>
<td>5.1</td>
</tr>
<tr>
<td>2</td>
<td>0.7</td>
<td>1.8</td>
<td>5.7</td>
</tr>
<tr>
<td>3</td>
<td>1.4</td>
<td>1.7</td>
<td>5.6</td>
</tr>
<tr>
<td>Average</td>
<td>0.8±0.6</td>
<td>1.9±0.3</td>
<td>5.5±0.3</td>
</tr>
</tbody>
</table>

Table 4-2: PAA Brushes Swelling in OptiMEM. Swelling was performed to measure the increase in brush film thickness and calculate the swelling degree.
The first measurement of the dry PAA brushes on Ti was performed in the cuvette ($d_{\text{PAA}}$ [nm] in cell). Next, the brushes were swollen by adding OptiMEM (pH 7.2) to PAA brushes ($d_{\text{brush}}$ in OptiMEM [nm]). The swelling degree was calculated as a ratio of swollen thickness to dry thickness. Three replicate samples were measured, and the average is given with the standard deviation of the data.

<table>
<thead>
<tr>
<th>Replicate number</th>
<th>$d_{\text{PAA}}$ [nm] in cell</th>
<th>$d_{\text{brush}}$ in OptiMEM [nm]</th>
<th>swelling degree</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.2</td>
<td>26.0</td>
<td>5.0</td>
</tr>
<tr>
<td>2</td>
<td>6.7</td>
<td>23.2</td>
<td>3.4</td>
</tr>
<tr>
<td>3</td>
<td>6.3</td>
<td>19.7</td>
<td>3.1</td>
</tr>
<tr>
<td>Average</td>
<td>6.1±0.8</td>
<td>23±3.0</td>
<td>4.0±1.0</td>
</tr>
</tbody>
</table>

**Table 4-3**: PAA Brushes with Covalently Bound Peptide. Brush swelling of PAA brushes before and after covalent binding of GRGDS or RGES (c= 1 mg/ml) to PAA brushes were used to calculate the immobilized peptide amount ($\Gamma_{\text{RGD/RGE}}$ [µg/cm²]) using a modified de Feijter approach. Three replicate samples were measured, and the average is given with the standard deviation of the data.

<table>
<thead>
<tr>
<th>Replicate</th>
<th>$\Gamma_{\text{RGD}}$ [µg/cm²]</th>
<th>$\Gamma_{\text{RGE}}$ [µg/cm²]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.5</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>3</td>
<td>1.2</td>
<td>0.9</td>
</tr>
<tr>
<td>Average</td>
<td>1.3±0.2</td>
<td>1.0±0.2</td>
</tr>
</tbody>
</table>

**4.3.2. Substrate-Mediated Gene Delivery**

After assessing the brush formation and swelling behavior, the ability of the substrates modified with PAA brushes to support SMD was measured in NIH/3T3 fibroblasts and reported as transgene expression normalized to the total amount of protein. Transfection was investigated by as a function of the N/P ratio used to form bPEI-DNA complexes, which resulted in complexes with increasingly positive zeta potential and smaller diameter as the N/P ratio increased (Figure 4-2), as expected. Transfection success increased for cells cultured on all substrates as the N/P ratio increased (Figure 4-3). Transfection
with complexes formed at the lowest N/P ratio of 3 showed no significant
difference in transfection success comparing all substrates (Figure 4-3A). While
there was no significant difference in transfection measured in cells on all
substrates with immobilized complexes formed at an N/P ratio of 5, transfection
was increased by one order of magnitude for cells cultured on PAA-RGD
compared to those cultured on PAA-RGE, PAA, and Flat Ti (Figure 4-3B).
Finally, forming complexes at the higher N/P ratios of 10 and 20 resulted in a
significant increase in transfection success (by up to an order of magnitude) in
cells cultured on complexes immobilized to PAA-RGD compared to those
cultured on PAA (Figure 4-3C; **, P≤0.01; Figure 4-3D; *, P<0.05). Given that
complexes formed at the N/P ratio of 20 exhibited the highest transgene
expression, further investigations on immobilization, release, and transfection
were performed using this parameter.

![Figure 4-2: Zeta potential and sizing of bPEI-DNA complexes with varied N/P
ratios. bPEI-DNA complexes were formed with 2 µg of DNA at N/P of 3, 5, 10, or
20, and the zeta potential and size of the complexes were determined by
dynamic light scattering and Laser Doppler micro-electrophoresis, respectively,
at room temperature. Statistical differences between the measurements for zeta
potential (and complex diameter) were analyzed using one-way ANOVA with
Tukey’s post-test. The zeta potential measurements showed a significant]
increase in the charge of all complexes formed at the higher N/P ratios (5, 10, 20) compared to those formed at N/P of 3 (****; P≤0.0001), as well as a significant increase in the charge of complexes formed at N/P of 10 and 20, compared to those formed at a N/P of 5 (*; P<0.05, and **; P≤0.01, respectively). The sizing of the complexes showed that complexes formed at a N/P ratio of 3 were significantly larger than those formed at all other N/P ratios (5, 10, 20) (**; P≤0.001, **; P≤0.01, and ***; P≤0.001 respectively), and complexes formed at a N/P ratio of 20 were significantly smaller than those formed at a N/P of 5 and 10 (****; P≤0.0001).

Figure 4-3: Substrate-mediated gene delivery of bPEI-DNA complexes in NIH/3T3 fibroblasts with varied N/P ratios. NIH/3T3 fibroblasts were cultured onto bPEI-DNA complexes formed with 2 µg of DNA at N/P of 3, 5, 10, or 20, and immobilized to the substrate for transfection. SMD studies were analyzed using one-way ANOVA with Tukey’s post-test, and cells cultured on complexes at N/P ratio of 3 or 5 has no statistical significance in transfection success for all substrates (A & B), whereas cells cultured on immobilized complexes at N/P ratio of 10 showed a statistically significant increase in transfection of cells cultured on PAA-RGD compared to those cultured on PAA (**, P≤0.01) (C), and cells cultured on immobilized complexes at N/P ratio of 20 had a statistically significant...
increase in transfection of cells cultured on PAA-RGD compared to those cultured on PAA (*, P≤0.05) (D).

4.3.3. Immobilization and Release of DNA-bPEI Complexes

To determine if the amount of DNA adsorbed to each substrate was the primary determinant for increased transfection success in cells cultured on PAA-RGD, the immobilization and release of bPEI-DNA complexes were analyzed. DNA complexes were loaded onto PAA, PAA-RGD, and Flat Ti substrates and the adsorbed amounts were measured by monitoring radiolabeled DNA plasmids with scintillation counting or total organic mass (bPEI, free and complexed to DNA, as well as DNA) with spectroscopic ellipsometry modeling. For immobilization determined with radioactivity, the amount of DNA adsorbed to PAA, PAA-RGD, and Flat Ti was 0.055±0.007 µg/cm², 0.048±0.008 µg/cm², and 0.055±0.008 µg/cm², respectively (Figure 4-4A); these amounts were not significantly different among the three substrates. For ellipsometry monitoring, the total mass of organic material adsorbed to the substrates was 0.93±0.03 µg/cm² for PAA, 0.97±0.09 µg/cm² for PAA-RGD, and 0.053±0.003 µg/cm² for Flat Ti, which showed a significant increase of adsorbed mass (i.e. DNA and free and complexed bPEI) on PAA-RGD and PAA compared to Flat Ti (Figure 4-4B; ****, P ≤ 0.0001).
Figure 4-4: DNA complexes immobilized to PAA brushes compared to Flat Ti. For complexes formed at an N/P ratio of 20, the amount of material immobilized onto substrates measured by (A) radiolabeled DNA via scintillation counting and (B) total mass (bPEI and DNA plasmid, free and complexed) by spectroscopic ellipsometry. Statistical analyses were completed using one-way ANOVA with Tukey’s post-test. There were no significant differences in the amount of DNA immobilized as measured by radioactivity (A), but there was a statistically significant difference between the amount of total mass on PAA-RGD and PAA substrates compared to Flat Ti (****; P≤0.0001) (B). A dotted line marks the expected mass of bPEI-DNA complexes immobilized to the substrate based on the N/P ratio and quantification of DNA by radioactivity (B).

To determine the effect of electrostatic and hydrophobic interactions between bPEI-DNA complexes and the substrates, and the contribution of complex release from substrates to transfection profiles, DNA release was quantified at multiple time points up to 48 hours, using three different media: reduced serum OptiMEM, serum-containing cell growth media, or conditioned growth media (from flasks of cultured cells). The average percentages of total DNA released in OptiMEM from PAA-RGD, PAA, and Flat Ti after 48 hours were 7.0±1.5%, 14±2.6%, and 13±2.3%, respectively (Figure 4-5A), and there was no significant difference in the release of bPEI-DNA complexes from any of the substrates. The average percentage of total DNA released in serum-containing growth media for PAA-RGD, PAA, and Flat Ti at 48 hours were 15±1.0%,
26±2.9%, and 19±2.6%, respectively (Figure 4-5B), and the release of bPEI-DNA complexes from PAA substrates was significantly increased (11±3.1%;*, P≤0.05) compared to the release from PAA-RGD at the final time point when statistics were performed. Finally, the average percentage of total DNA released in conditioned growth media for PAA-RGD, PAA, and Flat Ti at 48 hours were 11±1.2%, 16±1.0%, and 17±1.3%, respectively (Figure 4-5C) and the release of bPEI-DNA complexes from Flat Ti was significantly increased (5.0±1.6%;*, P≤0.05) compared to the release from PAA-RGD.

**Figure 4-5:** DNA complexes released from PAA and PAA-RGD brush substrates, compared to Flat Ti. The amount of DNA released from the substrates with OptiMEM (A), serum-containing growth media (B), or conditioned DMEM media (C) at 37°C was measured by radiolabeled DNA via scintillation counting. Release experiments were analyzed using one-way ANOVA with Tukey’s post-tests at the final timepoint, which showed a statistically significant difference between PAA-RGD and PAA (*, P ≤ 0.05) for release with growth media (B), and
a statistically significant difference between PAA-RGD compared to Flat Ti (\(^*, P \leq 0.05\)) for release with conditioned media (C).

4.3.4. Cellular Adhesion and viability on DNA-bPEI Complexes Immobilized on Substrates

The cellular responses of NIH/3T3 fibroblasts cultured onto PAA brushes with immobilized bPEI-DNA complexes were assessed, including the number of cells adhered per area (cm\(^2\)) and cellular morphology. Morphologically, the cells were spread with filamentous extensions characteristic of fibrotic cells on all substrates investigated (Figure 4-6A-D). No evidence of cytotoxicity was visually detected from these investigations. The number of live cells per area (cm\(^2\)) was higher on PAA-RGD compared to all other surfaces, which was significant compared to the number of cells adhered to PAA (\(***, P \leq 0.001\)) and Flat Ti (\(**, P \leq 0.01\)) (Figure 4-6E). Cell viability assays were performed in cells cultured on PAA-RGD, PAA-RGE, PAA, and Ti substrates with immobilized complexes (N/P 20), which showed no statistical differences in the viability of cells cultured on PAA-RGD, PAA-RGE, PAA, or Flat Ti (Figure 4-6F) after 48 hours.
Figure 4-6: Adhesion and viability quantification of NIH/3T3 fibroblasts cultured on PAA brushes with bPEI-DNA complexes. Measurements of the adhesion and viability of NIH/3T3 mouse fibroblasts were acquired using calcein staining and water-soluble tetrazolium (WST-1), respectively, with cells cultured on PAA brushes with bPEI-DNA complexes immobilized to the substrate, 48 hours following cell seeding. For assessment of adhesion, cells were stained with calcein (2 µM) for 15 minutes before imaging. Cells cultured on all substrates exhibited healthy spreading and morphologies, as seen in representative images for PAA-RGD (A), PAA-RGE (B), PAA (C), and Flat Ti (D) (Scale bar= 200 µm). Images were quantified for the live cells per area (cm²) using NIH ImageJ.
Processing Software. Statistical analysis was performed using one-way ANOVA with Tukey’s post-tests, which showed a statistically significant difference between the number of live cells/cm² on PAA-RGD compared to those on PAA (***, P≤0.001) and Flat Ti (**, P ≤0.01) (E). WST-1 quantification of cell viability after 48 hours was measured at an absorbance of λ=430 nm and normalized to the area (cm²) and statistical analysis using a one-way ANOVA with Tukey’s post-tests showed no statistical differences (F).

4.3.5. Investigating the Effect of Free bPEI on Substrate-Mediated Gene Delivery

Given that DNA adsorption studies suggested that all surfaces loaded the same amount of DNA and ellipsometric measurements suggested there was additional organic matter (i.e. free bPEI) adsorbed to the substrates with PAA brushes, the role of free bPEI on SMD on polymer brush-modified substrates was investigated. To study the effect of free bPEI on transfection, free (i.e. uncomplexed) bPEI was filtered out from the formed bPEI-DNA complexes prior to immobilization to substrates for SMD. After the removal of free bPEI, there were no significant differences in transfection for NIH/3T3 fibroblasts cultured on any of the substrates (Figure 4-7). Furthermore, comparing the results of transfection using complexes (formed at N/P 20) with or without free bPEI (Figure 4-3D vs. 6) showed that transfection mediated by filtered complexes was nearly two orders of magnitude lower than transfection mediated by unfiltered complexes with the free bPEI. Furthermore, similar to the cell viability measured on substrates with immobilized (unfiltered) complexes (Figure 4-6F), there were no statistical differences in the viability of fibroblasts cultured on PAA-RGD, PAA-RGE, PAA, or Flat Ti with immobilized filtered complexes (Figure 4-8).
Figure 4-7: Substrate-mediated gene delivery of filtered bPEI-DNA complexes in NIH/3T3 fibroblasts. Fibroblasts were cultured onto filtered bPEI-DNA complexes immobilized onto the substrate for transfection. Filtered samples were centrifuged through a Vivaspin6 filter to remove free bPEI and the complexes were eluted from the filter. SMD studies were analyzed using one-way ANOVA with Tukey’s post-test, and the results showed no significant difference between transfection success in cells cultured on immobilized filtered complexes on any substrate.

Figure 4-8: Viability quantification of NIH/3T3 fibroblasts cultured on PAA brushes with filtered bPEI-DNA complexes. The measurement of the viability of NIH/3T3 mouse fibroblasts was acquired using water-soluble tetrazolium (WST-1) with cells cultured on PAA brushes with filtered bPEI-DNA complexes immobilized to the substrate, 48 hours following cell seeding. WST-1
quantification of cell viability was measured at an absorbance of $\lambda=430$ nm and normalized to the area ($\text{cm}^2$). Statistical analysis was performed using one-way ANOVA with Tukey’s post-tests, which showed no statistical differences.

To further elucidate the effect of free bPEI on transfection success, the addition of free bPEI was controlled by adding free bPEI to filtered complexes during immobilization to the different substrates. Two different amounts of free bPEI (1 or 5 µg) were added onto the substrate with the filtered complexes and immobilization was allowed to proceed for 2 hours as described above. By defining the mass of DNA required in the final complex solution, the desired N/P ratio, and using the molecular weight of bPEI and the pEGFP-LUC plasmid, we were able to calculate the approximate masses of bPEI needed to form complexes at various N/P ratios. Furthermore, based on previous literature suggesting an N/P ratio of 3 results in fully complexed DNA with little to no excess free bPEI (51), we were able to estimate the mass of complexed and free PEI present in the complex solution when forming complexes at varying N/P ratios. Using these calculations, the dose of free bPEI added was determined by subtracting the calculated mass of complexed bPEI required to complex 0.05 µg DNA (0.13 µg) from the calculated total mass of bPEI required (0.89 µg) for complexes formed at a N/P ratio of 20. Additionally, the calculated difference between the total mass immobilized to PAA (0.93 µg) and PAA-RGD (0.97 µg), both measured by ellipsometry (Figure 4-4B), and the mass accounted for by the complexed DNA and PEI (0.07 µg, calculated based on the mass of radiolabeled DNA measured plus the mass of PEI required to fully complex that mass of DNA) suggests an amount of approximately 1 µg of free PEI in solution (0.86 and 0.90
µg, respectively; Figure 4-4). For all substrates, although there were no statistical differences, increasing the amount of free bPEI increased the normalized transgene expression in a dose-dependent manner by one order of magnitude in cells cultured on substrates dosed with 5 µg compared to those dosed with 1 µg (Figure 4-9B vs A), except those on PAA. When investigating the substrate response by dose, there was no significant difference in transfection success for cells cultured on all substrates dosed with 1 µg of free bPEI (Figure 4-9A), but substrates dosed with 5 µg of free bPEI showed one order of magnitude higher transfection for cells cultured on PAA-RGD, PAA-RGE, and Flat Ti when compared to those on PAA (Figure 4-9B).

Figure 4-9: Substrate-mediated gene delivery of filtered bPEI-DNA complexes in NIH/3T3 fibroblasts with the addition of free bPEI. Fibroblasts were cultured onto filtered bPEI-DNA complexes immobilized to the substrate for transfection. At the time of complex immobilization, 1 (A) or 5 (B) µg of bPEI (1 µg/µL) was added concurrently to the substrates. SMD studies were analyzed using one-way ANOVA with Tukey’s post-test, and there was no significant difference between transfection success in cells cultured with additional 1 or 5 µg free PEI.
4.4. Discussion

The objective of this work was to investigate the immobilization of DNA complexes to substrates functionalized with polymer brushes, taking advantage of the high negative surface charge of the brushes to attract and load cationic complexes, while also presenting cell-binding ligands to potentially influence the cellular response. Previous studies have indicated that the chemical properties of the substrate (e.g. self-assembly monolayers, polymer films, protein coatings) affect DNA complex binding and the efficiency of SMD (17, 19, 20, 22, 59, 60, 133-135, 235, 250), but many of those studies have focused on substrates like TCPS or glass, rather than biomaterials with possible clinical applications such as Ti. In this paper, we investigated the ability of chemically modified Ti substrates (with PAA brushes with or without peptide modifications) to support SMD. Building off our previous work, where we showed that PAA brushes grafted to Ti maintain swelling functionality and the addition of the RGD peptide enhances cell attachment compared to unmodified PAA (116) (chapter 3), herein we hypothesized that the highly negative charge of PAA (197) could allow for improved DNA complex adsorption and that cells cultured on PAA-RGD would have increased transfection success with SMD.

After determining that brushes were grafted and modified similarly to our previous investigation (Tables 4-1:3) (116) (chapter 3), the ability of PAA-RGD brushes to support SMD in NIH/3T3 fibroblasts. Fibroblasts were chosen given their frequent use in transfection studies (61, 132, 150, 251), and their role in wound healing. Fibroblasts cultured on PAA-RGD with immobilized bPEI-DNA
complexes (for N/P ratios of 5, 10, 20) had the highest transfection compared to cells cultured on all other surfaces (PAA-RGE, PAA, Flat Ti). Transfection was significantly increased in cells cultured on PAA-RGD compared to cells cultured on PAA on surfaces when complexes formed at N/P 10 and N/P 20 were immobilized (Figure 4-3); complexes formed at these ratios exhibited the smallest diameters and highest positive charges (Figure 4-2), two attributes that have previously been shown to produce high transfection success (252). Using the highest N/P ratio, experiments were then performed to investigate the amount of DNA immobilized on and released from the substrate to determine if enhanced SMD on PAA-RGD substrates could be attributed to increased DNA adsorption (and thus dose presented to the cells), which is often what contributes to improved transfection success seen in SMD (14). Given that complexes formed at a N/P ratio of 20 exhibited the highest overall positive charge compared to those formed at lower N/P ratios (Figure 4-2) and both PAA brushes (197) and the RGD peptide GRGDS (185) have a negative charge under physiological conditions (pH 7.2; Figure 4-1), we hypothesized that PAA brushes would increase the amount of DNA loading. However, there was no increase in the amount of radiolabeled DNA immobilized onto PAA and PAA-RGD substrates compared to Flat Ti (Figure 4-4A). Furthermore, the amount of immobilized radiolabeled DNA measured on PAA substrates was within the range, albeit low, of previously reported studies using other substrates for SMD (17, 19, 20, 59, 133), further suggesting that PAA brushes do not increase the DNA loading capacity of the substrate. After analyzing the immobilization of complexes with
radiolabeled DNA onto PAA, PAA-RGD, and Flat Ti substrates, the release of DNA was similarly measured with radioactivity using three different media conditions (OptiMEM, growth media, and conditioned media) to investigate the effect of electrostatics and competitive protein binding on the release of DNA from PAA brushes. The release profiles here are comparable to previously reported studies (20, 59, 133, 134), suggesting that the brushes provide sufficient release for transfection success. Three release media were used with different amounts of serum components (OptiMEM<growth culture media<conditioned media) and cellular metabolites (i.e. conditioned media) that aid release (20). We hypothesized the amount of DNA released should correlate to the respective increase in serum/metabolites, yet the release profiles were similar regardless of release media (Figure 4-5), suggesting that the combined effect of competitive protein binding and electrostatics were similar for all media types. In addition, within each media condition, while there were some significant differences in release profiles among the different substrates, it is unlikely that the difference in the amount of DNA released accounted for the difference in transfection outcomes seen in Figure 4-3D.

Finally, cellular adhesion and viability in the presence of immobilized complexes on the substrates were investigated, as these cellular behaviors are known to influence transfection success (20, 59, 132). Cellular adhesion was enhanced significantly in cells cultured on PAA-RGD compared to those cultured on PAA and Flat Ti (Figure 4-6), which confirms results from our previous work (116) (chapter 3) and is expected due to the known effect of RGD on cell
adhesion (187). However, it should be noted that in comparison to cellular adhesion on these substrates without complexes (116) (chapter 3), cell adhesion was increased in this work on all substrates (i.e. PAA-RGD, PAA-RGE, PAA, Flat Ti) with immobilized complexes (Figure 4-6A-D), suggesting that immobilized bPEI-DNA complexes can increase cellular adhesion, even on nonfouling substrates (i.e. PAA-RGE, PAA). Similar observations have been made on other nonfouling substrates used for SMD (20), and the promotion of cell adhesion on immobilized complexes has been attributed to possible interactions between serum proteins and the immobilized positively charged complexes (20), which subsequently can promote adhesion. Along with the increase in positive charge of the substrate by the cationic complexes, the addition of peptides has been shown to alter the charge of the substrate (185), which also may explain the similarity in cell adhesion on PAA-RGD and PAA-RGE substrates (Figure 4-6E), due to increased protein adsorption to the complexes immobilized on both substrates, allowing for cell adhesion.

Although complex immobilization significantly increased the number of adhered cells onto PAA-RGD compared to PAA and Flat Ti (Figure 4-6E), there was no significant difference in the viability of cells cultured on PAA-RGD, PAA-RGE, PAA, or Flat Ti (Figure 4-6F). While the presence of RGD was shown to improve cell adhesion and transfection, cell viability was shown to be similar and high on all substrates (Figure 4-6F). Many previous investigations of RGD-modified substrates have shown that cellular adhesion and viability are often related (253-255). However, the difference between the results for adhesion and
viability assays reported here is presumably due to the processing required for each technique, as adhesion staining has more wash steps in comparison to the WST-1 assay, which presumably results in only the most adhered cells remaining for image analysis. Like the investigations of DNA immobilization and release, the investigations of the cellular response also do not sufficiently explain the difference in transfection outcomes seen in Figure 4-3D.

Given that traditional indicators of successful SMD transfection (DNA immobilization and DNA release from the substrate, and the cellular response) did not explain the differences seen in SMD transfection among the different substrates, and adsorption measurements made using radiolabeled DNA only account for the mass of DNA adsorbed to the substrates (i.e. bPEI cannot be accounted for using radiolabeled DNA), we explored ellipsometric methods to measure and model the total amount of adsorbed mass (DNA and bPEI, both free and complexed). Using ellipsometry we showed that there was a significant increase in total mass immobilized onto the substrates modified with PAA and PAA-RGD compared to the mass on Flat Ti (Figure 4-4B). Given that it requires approximately 0.02 µg of 25 kDa bPEI to fully complex 0.05 µg DNA (based on the calculations as described above using the molecular weight of bPEI and DNA and N/P ratio of 3), which would result in a theoretical total mass of 0.07 µg for the fully formed complexes used in the adsorption studies (Figure 4-4B, dotted line), and there was nearly no difference in the amount of DNA measured on Flat Ti (Figure 4-4A) and total mass measured on Flat Ti (Figure 4-4B); we hypothesize ellipsometric measurements are underestimating the total mass on
the Flat Ti, which has been shown in previous investigations with complex immobilization monitored by ellipsometry (256). However, even with an underestimation, the increased mass measured on PAA-RGD and PAA compared to Flat Ti is large and may be from the adsorption of complexed bPEI but also free bPEI, as free bPEI in the complexing solution has been previously suggested as a component of the immobilized material in SMD (20). Based on the assumptions that a N/P of 3 will have no free bPEI (51) and calculations to determine the polymer present in a solution formed for complexes at an N/P of 20, we estimate a mass of approximately 0.76 µg of free bPEI was present in the complex solution used for taking ellipsometric measurements, which is similar to the change in mass for substrates modified with PAA and PAA-RGD compared to Flat Ti (0.86 and 0.90 µg, respectively; Figure 4-4).

In bolus studies, free bPEI has been proposed to increase overall gene transfection efficiency by up to hundreds of fold (45, 51, 53-56). Specifically, free bPEI has been suggested to reduce charge interactions that repeal complexes from the cellular membrane, reduce lysosomal entrapment of complexes, assist translocation of complexes through the nuclear membrane, enhance transcription, and facilitate translocation of mRNA (257). The role of free bPEI has not been significantly investigated for SMD, given that traditional SMD methods usually perform a rinse after immobilization of DNA complexes to remove loosely bound complexes (19, 20, 22). Therefore, rinsing the substrates would presumably result in free bPEI also being washed away from the surface before performing SMD, as seen on bare Flat Ti in this current study (Figure 4-
However, the highly negative PAA brushes could allow for the capture of the positively charged free bPEI to the substrates, which may improve subsequent transfection. Therefore, we hypothesized that the increase in transfection seen in cells cultured on bPEI-DNA complexes immobilized to PAA-RGD may be related to free bPEI attracted to the brushes. To test this, we investigated the effect of free bPEI on transfection success by performing transfection with filtered complexes (i.e. free bPEI removed) and complexes formed with different N/P ratios to tune the amount of free bPEI in the complexing solution, which has previously been shown to dramatically affect transfection success \((45, 55)\). The removal of all free bPEI through a size-exclusion membrane resulted in a substantial decrease in transfection by two orders of magnitude compared to transfection performed with unfiltered complexes (Figure 4-7 vs. 2) and transfection was not different amongst the investigated substrates (Figure 4-7), which supported our hypothesis that the presence of free bPEI may enhance transfection. These results are similar to those for bolus delivery studies that show the presence of free bPEI enhances transfection success \((45, 51, 53-56)\), thereby suggesting that free bPEI could also enhance transfection success in SMD on PAA-RGD, possibly through bPEI adsorption and subsequent release from the PAA-RGD surface. To further investigate the role of free bPEI in SMD, investigations were performed using the filtered complexes immobilized to the substrate, but with the addition of free bPEI (1 or 5 µg) to the complexing solution during immobilization. As previously stated, the doses of free bPEI were determined by the estimated amount of free bPEI in the complexing solution,
which was calculated to be about 0.76 µg, and the difference in mass calculated for substrates modified with PAA and PAA-RGD compared to Flat Ti (0.86 µg and 0.90 µg, respectively; Figure 4-4). Therefore, a dose close to the calculated amount (1 µg) and a dosage in excess (5 µg) were chosen as free bPEI amounts to immobilize with filtered complexes. Transfection outcomes were then assessed, which showed an increase in transfection success for all surfaces, except for PAA, in a dose-dependent manner (Figure 4-9), further validating the importance of free bPEI for enhancing transfection. In addition to studies with filtered complexes, the dose of free bPEI can also be controlled simply by forming complexes at various N/P ratios. Complexes formed at a N/P of 3 have been shown to have little to no free bPEI (51) and showed low SMD transfection success in our investigation. Conversely, complexes at higher ratios (i.e. 5, 10, 20) have been shown to have more free bPEI (45), and in our investigations showed an increase in transfection levels that corresponded with the increase of the N/P ratio, thereby supporting our hypothesis that transfection is influenced by the presence of free bPEI on the substrates. Furthermore, viability was also studied on substrates with immobilized filtered complexes (Figure 4-8), which showed, like in viability assays on substrates with immobilized unfiltered complexes (Figure 4-6F), there was no statistical difference in viability as a function of substrate modification. More importantly, cell viability was not statistically different on filtered complexes (Figure 4-8) compared to unfiltered complexes (Figure 4-6F), which suggests that free bPEI (which is present in
unfiltered complexes immobilized on substrates in Figure 4-6F), does not negatively impact the cellular response to the substrate.

Finally, in addition to free bPEI, the RGD ligand on PAA-RGD may be aiding SMD transfection success with complexes at higher N/P ratios (Figure 4-3B-D), given the transfection was enhanced in cells cultured on complexes immobilized to PAA-RGD substrates compared to PAA-RGE. Fibroblasts (e.g. the NIH/3T3 cell line) are known to express integrin α5β1 (258), which is known to aid cell adhesion through binding to RGD (130, 259, 260), and supports the results of our previous work (116) (chapter 3) and work shown here (Figure 4-6E) that show an increased number of cell attached to PAA-RGD compared to the control surfaces. Furthermore, the inclusion of the RGD ligand may activate signaling cascades that regulate cell processes pivotal for transfection, such as endocytosis and internalization (187, 210). Integrin binding to RGD ligands has been shown to improve bolus nonviral gene delivery (129) and SMD (235), and via the RGD motif on fibronectin coatings for both types of delivery (60, 125, 128). However, the role of the RGD ligand in our system here requires further investigation to understand its role in transfection success.

4.5. Conclusions

In our previous study, we showed that PAA brushes can be “grafted-to” Ti substrates and RGD can be conjugated to these brushes to support cell adhesion (116) (chapter 3). Herein, we investigated those PAA-RGD modified Ti substrates as a platform for improving SMD to NIH/3T3 fibroblasts using
immobilized bPEI-DNA complexes. From our studies, we found that transfection was significantly increased on PAA-RGD modified substrates, but this improvement in transfection could not be attributed to the amount of DNA immobilized to the surface or the DNA release profile. Instead, we found that substrates modified with PAA brushes adsorb more overall mass, which may be attributed to immobilization of free and complexed bPEI, as measured with spectroscopic ellipsometry. To confirm the role of free bPEI in SMD on PAA-RGD substrates, transfection investigations were performed with filtered complexes and controlled dosages of free bPEI. The results of these transfection investigations with filtered complexes suggest that free bPEI is beneficial to transfection success and PAA brushes allow for the adsorption and presentation of free bPEI in a SMD format. To our knowledge, this paper is one of the first reports using polymer brushes grafted to a Ti substrate for SMD and the conclusions from our findings suggest that these substrates can enhance the cellular response to transfection via SMD. Therefore, future studies will investigate the adjuvant-like effect of free bPEI in cells cultured on PAA-RGD brush substrates through further optimization of the dosage and complex formation, as well as investigations into the intracellular mechanisms affected by RGD and free bPEI that are involved in transfection efficiency (i.e. endocytosis, trafficking). Overall, the findings of this article suggest that the modification of Ti with PAA-RGD may be a future platform for applications that could be improved by gene delivery such as biomedical devices, implantable sensors, and diagnostics tools.
CHAPTER 5
Priming the Cellular Response for Substrate-Mediated Gene Delivery on Titanium Substrates Modified with RGD-Functionalized Poly(acrylic acid) Brushes

5.1. Introduction

Nonviral gene delivery is the transfer of exogeneous genetic material to cells, which is typically accomplished through the delivery of plasmid DNA complexed with either cationic lipid or polymer vectors (12, 261), with applications in gene therapy, regenerative medicine, and tissue engineering. Nonviral vectors are often considered safer than their viral counterparts with respect to stimulation of the immune response (132), yet transfection with nonviral complexes has lower efficiency compared to viral systems. The barriers that impede transfection success in vitro (and in vivo) are often attributed to nucleic acid degradation that can occur in the extracellular environments, mass transport limitations, vector cytotoxicity, and aggregation of the complexes that prevent interactions with the cellular membrane, and issues with intracellular barriers such as internalization, intracellular trafficking, and nuclear import (66). Moreover, the typical in vivo delivery method for nonviral transfection, systemic bolus administration of nonviral complexes, can result in unwanted side effects and potentially rapid clearance of the DNA cargo by the kidneys or liver (57), which is not effective for site-specific applications of gene delivery, i.e. a biomedical implant or stent (137, 236), or a tissue engineering construct (32, 262-264). Thus, an alternative administration route for site-specific applications
has been investigated to overcome the extracellular and intracellular barriers to transfection, i.e. substrate-mediated gene delivery (SMD).

In the process of SMD (also termed “reverse transfection” (265) or “solid phase delivery”(266)), DNA complexes are immobilized to a culture substrate through electrostatic interactions or covalent bonding prior to cell seeding (20, 59), which has been shown to increase the local concentration of the DNA in the microenvironment of the cell and decrease the necessary dosage for transfection (14, 17, 19, 20, 34, 59, 267). SMD is often performed on substrates modified with natural material coatings (e.g. chitosan (133), extracellular matrix proteins like fibronectin, collagen I, and laminin (59, 60)), which have been shown to improve the efficacy of gene delivery by enhancing the immobilization of nucleic acids and priming the cellular response to the genetic material. Yet, there are drawbacks to these substrate modifications; for example, natural material coatings often show high batch variability based on their source (268). Therefore, synthetic coatings (i.e. self-assembled monolayers (SAMs), or polyelectrolyte films (PEMs) (19, 20, 24, 25, 135-138, 269)) have also been investigated to improve transfection success via SMD (19, 20, 24, 25, 135-138, 269). Although the well-defined properties of SAMs and PEMs can immobilize the DNA cargo and prime the cellular response to transfection via chemical cues, these types of films can be difficult to produce, may impede the release of the DNA cargo, and produce toxic degradation products (146, 270). Moreover, previous nonviral SMD investigations with polymer films often focus on using tissue engineering scaffolds like poly(lactide-co-glycolide) (PLG) (32, 234, 271) or traditional culturing substrates
such as tissue culture polystyrene (TCPS), with or without natural protein coatings (22, 59, 60, 235), rather than clinically relevant materials for biomedical implants, i.e. metals such as titanium (Ti) or stainless steel (2, 24, 137).

Thus, there is a need to produce a polymer film on Ti to enhance transfection via SMD that effectively immobilizes and releases the DNA cargo and primes the cellular response to transfection. Recently, our lab has introduced poly(acrylic acid) (PAA) brushes, as a substrate modification to enhance transfection via SMD (116, 267) (chapter 3, 4) in cells cultured on a Ti substrate. The well-defined polymer structures of PAA brushes possess pH-responsive properties (30, 116, 179, 197, 267) (chapter 3, 4), stably attach to the substrate (i.e. no degradation products (157)), entrap complexes through electrostatic interactions to allow for release of genetic cargo (rather than embedding within layers (29, 116, 243, 267)) (chapter 3, 4), and tailor cell-material interactions by the conjugation of cell adhesion moieties such as the linear peptide GRGDS (PAA-GRGDS) (116, 185, 186, 267) (chapter 3, 4). Previous investigations of our platform have shown the efficacy of SMD in NIH/3T3 fibroblasts cultured on PAA-GRGDS brushes immobilized with complexes formed with 25 kDa branched polyethylenimine (bPEI) and plasmid DNA for transfection in vitro (116, 267) (chapter 3, 4). In these studies, we demonstrated that increased transgene expression may be due to the cellular response to the presence of the linear GRGDS motif and the presentation of electrostatically immobilized free (i.e. uncomplexed) 25 kDa bPEI.
Thus, given that we have shown Ti substrates modified with PAA-GRGDS are a platform for SMD, herein we aimed to investigate each component of the system (i.e. the RGD ligand, free PEI) for its ability to enhance transfection via the cellular response and a potential synergistic priming effect from the two. Specifically, we examined the role of RGD by tuning the surface density (129) and the integrin binding affinity of the motif (i.e. cyclic, linear (186, 272)). Previous investigations of the RGD motif have been well characterized for its ability to stimulate cellular adhesion through integrin binding (273) and which is critical for many intracellular responses that can improve transfection (i.e. endocytosis, cytoskeletal remodeling, and intracellular trafficking (73, 108, 111, 125, 274, 275)). Cyclic RGD has exhibited increased integrin binding affinity and cellular adhesion in comparison to cells cultured on linear RGD, suggesting that conjugating cyclic cRGDyK to PAA brushes will further enhance transfection compared to our previous investigations with linear GRGDS. We also examined the role of free (and complexed) PEI (which is often considered cytotoxic (46, 50, 276)) and has not been significantly investigated for SMD, given that traditional SMD methods usually perform a rinse to remove loosely bound DNA complexes (19, 20, 22), thereby removing the free PEI. However, the highly negative charge of PAA brushes at physiological pH (116, 267) (chapter 3, 4) may sequester and neutralize the cationic free polymer; thus, the neutralized presentation of free PEI to the cellular membrane may allow the improved cellular response to transfection by modulation intracellular processes (i.e. endocytosis, intracellular trafficking, nuclear import (51, 53, 54, 257)). Specifically, free PEI has been
suggested to reduce lysosomal entrapment of complexes, assist nuclear import of complexes, enhance transcription, and facilitate translocation of mRNA (257). Since bolus investigations have shown that the effect of free PEI on improving transfection success is dependent on the molecular weight (MW) and branching of the PEI (48-50, 252, 277); thus, we examined forming PEI-DNA complexes with branched and linear conformations, each with low and high MWs.

Herein, we characterized the conjugation of these ligands (i.e. cRGDyK, GRGDS, and control RGES) to PAA brushes, as well as the immobilization of PEI-DNA complexes, using 2 and 25 kDa bPEI and 2.5 and 25 linear PEI (LPEI) as complexing vectors. Then, after analyzing the transfection profiles of cells cultured on PAA brushes with conjugated peptides and determining the optimal PEI vector and peptide parameters, the ability of the substrate to prime the cellular response for transfection success was assessed by investigating cellular proliferation, focal adhesion formation, cytoskeletal organization, and endocytic pathway stimulation.

5.2. Materials and Methods

5.2.1. Preparation of PAA brushes on Ti Surfaces and Covalent Bonding of Peptides

To form the PAA substrates, Ti substrates (100 nm Ti, Grade 2, on a Si wafer) were purchased from Platypus Technologies (Madison, WI) and functionalized with polymer brushes according to our previously reported “grafting-to” method (116, 267) (chapter 3, 4). Briefly, the Ti substrate was activated with oxygen plasma for 1 min (Plasma Cleaner PDC-002 with
Plasmaflo PDC-FMG-2, Harrick Plasma, USA). After activation, a 0.02 wt % solution of poly(glycidyl) methacrylate (PGMA, Mn = 17,500 g/mol, Mw/Mn = 1.7, Polymer Source, Inc., Canada) in chloroform (CHCl₃, Fisher Scientific, UK) was spin-coated (Spin150 spin coater, Polos, Putten, Netherlands) and annealed for 10 min at 110°C under vacuum. After the addition of the PGMA anchoring layer, a PAA (Mn = 26,000 g/mol, Mw/Mn = 1.12, Polymer Source, Inc., Canada) solution at 1.0 wt % in ethanol (EtOH) was spin-coated and annealed at 80°C for 30 min under vacuum. Finally, the excess polymer was extracted in ethanol for 30 min at room temperature and dried with a N₂ flux.

Next, peptide conjugation based on the carboxyl-amine-reaction (EDC/NHS coupling) was performed as previously described (116, 267) (chapter 3, 4) and all materials used for peptide conjugation were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Briefly, the linear RGD-containing peptide GRGDS or cyclic RGD-containing cRGDyK (Peptides International, Louisville, KY), or RGE-containing peptide RGES (Genscript, Piscataway, NJ)) was covalently bound to PAA brushes on Ti surfaces via activation of the PAA carboxyl groups with N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) for direct conjugation to the primary amines of the peptides. For conjugation, PAA brushes on Ti substrates were equilibrated in 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES) buffer at pH 6 for ~10 min. After aspiration of the MES buffer, brushes were reacted with 5 mM EDC solution and 2 mM NHS solution in 0.1 M MES buffer (pH 6) by gently shaking for 40 min. After
removing the EDC/NHS solution, a 1.0 mg/mL solution of GRGDS or cRGDyK, or RGES or 1.5 mg/mL solution of GRGDS in 0.1 M borate buffer (pH 8) was added to the activated PAA brush substrates. After gentle shaking at room temperature for 16 hours, the peptide solution was aspirated and the peptide modified samples were washed in 0.1 M phosphate buffered saline (PBS) buffer at pH 7.4 for 10 min. Throughout this study the substrates investigated include PAA brushes modified with GRGDS or cRGDyK on Ti (abbreviated as PAA-GRGDS, PAA-cRGDyK), PAA brushes modified with the control peptide RGES on Ti (abbreviated as PAA-RGE), and PAA brushes on Ti (abbreviated as PAA) as a control.

5.2.2. DNA Complex Formation and Immobilization

Next, the formation of PEI-DNA complexes for transfection studies was investigated. For all studies, the formed DNA complexes contained a plasmid that encoded both the enhanced green fluorescent protein (EGFP) and firefly luciferase protein (LUC) under the direction of a CMV promoter (pEGFP-LUC), with the exception of immunofluorescence staining that was performed using a plasmid that only encoded LUC. Plasmids were purified from bacterial culture using Qiagen (Valencia, CA) reagents and stored in Tris–EDTA buffer solution (10 mM Tris, 1 mM EDTA, pH 7.4) at −20°C. For DNA complex formation, 25 kDa bPEI (Sigma-Aldrich)(or 2 kDa bPEI, 2.5 kDa LPEI, or 25 kDa LPEI (PolyScience, Warrington, PA)) was dissolved in reduced serum medium OptiMEM (Fisher Scientific) and then added dropwise to DNA in OptiMEM,
vortexed for 10 sec, and incubated for 15 min at room temperature. Complexes were formed at nitrogen/phosphate (N/P) ratio of 20 (to ensure high levels of free PEI in solution (20)) in OptiMEM with 2 μg of DNA and delivered at a DNA concentration of 1 μg/cm² for immobilization and transfection studies.

To characterize immobilization of the complexes and perform transfection experiments, the formed PEI/DNA complexes (with 2 kDa bPEI, 25 kDa bPEI, 2.5 kDa LPEI, or 25 kDa LPEI) were allowed to adsorb to the substrates for 2 hours and then rinsed with OptiMEM to remove any loose (i.e. not adsorbed) complexes or free PEI. Then, ellipsometry and transfection were performed as described in the following sections.

5.2.3. Ellipsometric Measurements for Characterization of Peptide Conjugation and Complex Immobilization to PAA Brushes

To assess the conjugation of peptides to the PAA brushes (and the immobilization of complexes), ellipsometric measurements were acquired using a Woollam RC2 (J.A. Woollam, Co., Inc., Lincoln, NE, USA) to confirm brush parameters, as previously described (116). Briefly, for dry brushes, the ellipsometric data, Δ (relative phase shift) and tan Ψ (relative amplitude ratio), were recorded at wavelengths (λ) of 400–1200 nm and four angles of incidence (AOI: 45°, 55°, 65°, 75°). To confirm brush swelling and functionality (indicative of carboxyl deprotonation (177, 186)), substrates were first sterilized with EtOH and then the pH-reactive brush swelling was performed by adding PBS (pH 7.4) to dry PAA brushes. Brush swelling within PBS was measured at AOI 70° with a batch cuvette (TSL Spectrosil, Hellma, Muellheim, Germany), at wavelengths $\lambda =$
400–1200 nm. The film thickness was quantified via the change in Ψ and Δ (for dry and swollen brushes), which was measured before and after the addition of the peptides (cRGDyK, GRGDS, RGES) and complex immobilization.

Experimental data were modeled in CompleteEASE software (Version 4.64, J.A. Woollam Co., Inc., Lincoln, NE, U.S.A.) as described in our previous work (116, 267) (chapter 3, 4). To determine the density of the conjugated peptides (GRGDS, cRGDyK, and RGES) on the PAA brushes substrates (in µg/cm²) and the density of the immobilized PEI-DNA complexes on the PAA brush substrates (in µg/cm²), these measurements were calculated with a modified de Feijter approach (Equation 5-1) (186):

\[
\Gamma_{\text{peptide/complexes}} = d_{\text{brush}} \frac{n_{\text{comb}} - n_{\text{brush}}}{\frac{dn}{dc}} + d_{\text{add}} \frac{n_{\text{comb}} - n_{\text{amb}}}{\frac{dn}{dc}} \tag{5-1}
\]

In this approach, the in-situ refractive index and in-situ thickness before peptide conjugation \((n_{\text{brush}}, d_{\text{brush}})\) and after peptide conjugation \((n_{\text{comb}}, d_{\text{comb}})\) were measured for swollen PAA brushes (Equation 5-1), therefore \(n_{\text{amb}}\) was the in-situ refractive index for the ambient solution (i.e. OptiMEM). The refractive index increment \(\frac{dn}{dc} = 0.185 \text{ cm}^3/\text{g}\) was used for the peptides (116) and \(\frac{dn}{dc} = 0.183 \text{ cm}^3/\text{g}\) for the DNA complexes (249). Values are reported as means for triplicate measurements with the standard deviation.
5.2.4. Cell Culture

Transfection studies were performed with murine fibroblast NIH/3T3 cells (ATCC, Manassas, VA) cultured in Dulbecco’s Modified Eagle’s Media (DMEM) completed with 10% Calf Serum (Colorado Serum Co., Denver, CO) and 1% Penicillin/Streptomycin. Fibroblasts were cultured at 37°C and 5% CO₂ and passaged every two days with 0.05% Trypsin-EDTA.

5.2.5. Substrate-Mediated Gene Delivery to NIH/3T3 fibroblasts Cultured on PAA Brushes with Immobilized PEI-DNA Complexes

To determine if increasing the concentration of the linear RGD peptide (1.0 mg/mL to 1.5 mg/mL) conjugated to PAA brushes on Ti increased SMD transfection success, PEI-DNA complexes were formed with 25 kDa bPEI and immobilized in a volume of 300 µL for 2 hours onto the substrate conditions (Flat Ti, PAA, PAA-GRGDS, and PAA-RGE), after which the solution containing the DNA complexes was removed and then substrates were rinsed with OptiMEM before cells were seeded onto the substrates at a density of 50,000 cells/mL. Cells were cultured for 48 hours at 37°C and 5% CO₂ and then the substrates were transferred into a new well plate and trypsinized to collect pooled samples and analyze transfection efficiency using flow cytometry with the FACSCalibur platform (BD Biosciences, Sparks, MD). Experiments were performed in triplicate replicates on triplicate days and 2000 total events per condition (with pooled samples). The percentage of GFP+ cells was presented as a fold change with respect to cells cultured on PAA brushes.
After assessing the effect of increasing the concentration of GRGDS on transfection, further SMD studies were performed comparing different kinds of RGD peptides (at a concentration of 1 mg/mL) and different PEI-DNA complexes formed with 2 kDa bPEI, 25 kDa bPEI, 2.5 kDa LPEI, or 25 kDa LPEI. The PEI-DNA complexes were immobilized for 2 hours onto the substrate conditions (PAA, PAA-GRGDS, PAA-cRGDyK, and PAA-RGE), and then seeded with NIH/3T3 fibroblasts. After cells were cultured for 48 hours at 37°C and 5% CO₂, substrates were transferred into a new well plate and lysed using 200 μL of 1X reporter lysis buffer (Promega, Madison, WI). Transfection levels were quantified by measuring the luciferase activity (measured as relative light units, or RLUs) using the Luciferase Assay System (Promega) and a luminometer (Turner Designs, Sunnyvale, CA) and then normalized per area (cm²).

5.2.6. Cellular Proliferation of NIH/3T3 Fibroblasts Cultured on PAA Brushes with Immobilized PEI-DNA Complexes

To assess the possible cytotoxic effect of immobilized PEI-DNA complexes to cells cultured on PAA brushes, the metabolic activity of cultured NIH/3T3 fibroblasts was assessed using a Water Soluble Tetrazolium (WST-1) salt cell proliferation assay kit (Roche, Indianapolis, IN), according to manufacturer’s protocol, at 48 hours following cell seeding. Briefly, cells were cultured on PEI-DNA complexes (formed with 2 kDa bPEI, 25 kDa bPEI, 2.5 kDa LPEI, or 25 kDa LPEI) immobilized to PAA substrates or tissue culture polystyrene (TCPS). After 48 hours, the PAA substrates were transferred into
new well plates prior to the assays. Both the cells cultured on PAA substrates with immobilized PEI-DNA complexes and cells cultured on tissue culture polystyrene (TCPS) with immobilized PEI-DNA complexes were washed with 1XPBS and incubated at 37°C in WST-1 solution (10%/vol WST-1 reagent in phenol-free DMEM (Fisher Scientific)) for 4 hours. After incubation, absorbance values were measured on an Epoch Microplate spectrophotometer (BioTek, Winooski, VT) at 430 nm and corrected with 690 nm as a reference wavelength. Finally, the readings were normalized with respect to the control cells cultured without complexes.

5.2.7. Immunofluorescent Staining of Focal Adhesions and Actin Stress Fibers of NIH/3T3 Fibroblasts Cultured on PAA Brushes with Immobilized 25 kDa bPEI-DNA Complexes

To establish correlations between cellular adhesion, the cytoskeletal arrangement, and successful transgene expression, focal adhesions, and intracellular filamentous actin were visualized and quantified with immunofluorescent cell staining and confocal microscopy imaging. First, NIH/3T3 fibroblasts were cultured on 25 kDa bPEI-DNA complexes immobilized to PAA, PAA-cRGDyK, and PAA-RGE for 4 hours. Then, cell culture media was removed, and the samples were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in 1X PBS for 15 min. For staining, anti-vinculin conjugated to Alexa Fluor® 488 (Abcam, Cambridge, United Kingdom) was used to mark focal adhesions, cytoskeletal actin stress fibers were stained with TRITC-conjugated phalloidin (Sigma-Aldrich), and cell nuclei were counterstained with
DAPI (Sigma-Aldrich). After staining, the samples were stored in 1X PBS until imaging. To collect the images, the substrates were placed on glass slides and covered with a cover slip before imaging using a Nikon A1R-NiE Confocal Microscope (Nikon, Inc., Minato, Tokyo, Japan). Three images per condition (in duplicate experiments) were analyzed to obtain statistically relevant n-values of cells (n>150 (132)), which was quantified using ImageJ (NIH) and used to calculate cell density (in cells/image). The average cell density was used to normalize the average number of stress fiber bundles and focal adhesions per cell.

5.2.8. Endocytic Pathway Inhibition of NIH/3T3 Fibroblasts Cultured on PAA Brushes with Immobilized 25 kDa bPEI-DNA Complexes

To determine if culturing NIH/3T3 fibroblasts onto 25 kDa bPEI-DNA complexes immobilized to PAA, PAA-cRGDyK, and PAA-RGE increased internalization of the complexes via specific endocytic pathways, transfection was performed as described above with the addition of chemical inhibitors (concurrently with cell seeding) for macropinocytosis, and clathrin-mediated or caveolae-mediated endocytosis: amiloride (Amil; 1 mM (278)), chlorpromazine (CPZ; 10 µg/mL (71)), and genistein (Gen; 200 µM (71)), respectively. After cells were cultured on 25 kDa bPEI-DNA complexes immobilized to PAA, PAA-cRGDyK, and PAA-RGE for 24 hours, and flow cytometry was performed as previously described. The percentage of GFP+ cells was presented as a fold change with respect to cells cultured with the same conditions in the absence of these inhibitors.
5.2.9. Statistical Analysis

All experiments were performed in triplicate on duplicate days and values are reported from one representative experiment as means with a standard error of the mean, unless otherwise stated. Statistical comparisons were performed with Prism 5.0 graphing and statistical analysis software (Graph Pad, La Jolla, CA) at 95% confidence level ($\alpha=0.05$), with the statistical tests used specified in the figure legends.

5.3. Results

5.3.1. Characterization of the PAA-RGD Brush Platform for SMD

Before performing SMD studies and probing the cellular response to transfection on PAA brush substrates, the PAA grafting process and swelling functionalities were confirmed with ellipsometry. Similar to our previous studies where we functionalized Ti with PAA brushes (116, 267) (chapter 3, 4), the average film thickness of the dry PAA brushes was 6.0±0.58 nm (Table 5-1). After the addition of PBS (pH 7.4), PAA brushes swelled to an average thickness of 37±0.95 nm (with an average swelling degree of 6.4±0.93, Table 5-1), which was also similar to the swelling in 0.1 M PBS reported in our previous study (116) (chapter 3).
Table 5-1: Swelling of PAA Brushes. Three replicate samples were measured, and the average is given with the standard deviation of the data.

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Dry brush thickness [nm]</th>
<th>Brush thickness in PBS (pH 7.4) [nm]</th>
<th>Swelling Degree</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.0</td>
<td>38</td>
<td>5.4</td>
</tr>
<tr>
<td>2</td>
<td>5.7</td>
<td>37</td>
<td>6.5</td>
</tr>
<tr>
<td>3</td>
<td>5.2</td>
<td>37</td>
<td>7.3</td>
</tr>
<tr>
<td>Average</td>
<td>6.0±0.58</td>
<td>37±0.95</td>
<td>6.4±0.93</td>
</tr>
</tbody>
</table>

5.3.1.1. Conjugation of Peptides to PAA Brushes

After confirming the brush swelling functionality was maintained, conjugation of the different peptides (cyclic cRGDyK, linear GRGDS (at 1.0 and 1.5 mg/mL), and RGE) was performed and the density of the adsorbed peptides was measured with spectroscopic ellipsometry and calculated using Equation 5-1 (in µg/cm²). For the peptides conjugated to the PAA brushes using a solution of 1 mg/mL, the density of the peptides immobilized to the PAA brushes for cRGDyK, GRGDS, and RGE (Table 5-2; 0.94±0.050, 1.0±0.18, and 0.97±0.25 µg/cm², respectively) was similar to our previously reported study and other investigations with PAA brushes (116, 186). Furthermore, the GRGDS peptide conjugated to the PAA brushes at 1.5 mg/mL had 1.7 times more GRGDS conjugated to the substrates compared to those conjugated with GRGDS peptides with 1.0 mg/mL (Table 5-2, 1.7±0.10 and 1.0±0.18 µg/cm², respectively).
Table 5-2: PAA Brushes with Covalently Bound Peptide. Brush swelling measurements of PAA brushes (before and after covalent binding of cyclic cRGDyK, linear GRGDS (at 1.0 and 1.5 mg/mL), and RGE to PAA brushes) were used to calculate the immobilized peptide amount ($\Gamma_{\text{peptide}}$ [$\mu g/cm^2$]) using a modified de Feijter approach. Three replicate samples were measured, and the average is given with the standard deviation of the data.

<table>
<thead>
<tr>
<th>Replicate</th>
<th>$\Gamma_{\text{cRGDyK}}$ [$\mu g/cm^2$]</th>
<th>$\Gamma_{\text{GRGDS1}}$ [$\mu g/cm^2$]</th>
<th>$\Gamma_{\text{GRGDS1.5}}$ [$\mu g/cm^2$]</th>
<th>$\Gamma_{\text{RGE}}$ [$\mu g/cm^2$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.93</td>
<td>0.85</td>
<td>1.7</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>0.99</td>
<td>1.2</td>
<td>1.8</td>
<td>1.2</td>
</tr>
<tr>
<td>3</td>
<td>0.89</td>
<td>1.0</td>
<td>1.6</td>
<td>0.71</td>
</tr>
<tr>
<td>Average</td>
<td>0.94±0.050</td>
<td>1.0±0.18</td>
<td>1.7±0.10</td>
<td>0.97±0.25</td>
</tr>
</tbody>
</table>

5.3.2. The Efficiency of Substrate-Mediated Gene Delivery for NIH/3T3 Fibroblasts Cultured on PAA Brushes in Response to GRGDS Concentration

After assessing the swelling behavior and brush functionalization with peptides, the ability of the substrates modified with PAA brushes to support transfection via SMD in NIH/3T3 fibroblasts was assessed. In our previous paper, linear GRGDS was conjugated to the PAA brushes at a concentration of 1.0 mg/mL (267) (chapter 4). Given that increasing the density of RGD ligands has been shown to increase transfection success (267) (chapter 4), we first investigated the effect of increasing the concentration of GRGDS conjugated to the substrate to 1.5 mg/mL. Since our previous investigation used complexes formed with 25 kDa bPEI with 2 $\mu g$ of DNA at a N/P ratio of 20 (267) (chapter 4), these parameters were also used in the current investigation for comparison. Transfection efficiency was determined using flow cytometry to measure the transfected populations (GFP+), which showed, when transfection in cells cultured on all substrates (PAA-GRGDS at 1.0 mg/mL, PAA-GRGDS at 1.5 mg/mL, PAA-RGES at 1.0 mg/mL, PAA-RGES at 1.5 mg/mL) were compared to
transfection in cells cultured on PAA, the fold changes were 1.6±0.30, 1.1±0.30, 1.2±0.18, and 1.1±0.41, respectively (Figure 5-1). Since the fold change comparing substrates modified with peptides at 1.5 mg/mL was lower than those with peptides at 1.0 mg/mL (Figure 5-1), these results suggesting that increasing the concentration of GRGDS conjugated to the PAA brushes does not improve transfection in cultured cells in the context of this system. Thus, we investigated transfection in cells cultured on PAA brushes conjugated with another type of RGD (cRGDyK) and immobilized with complexes formed with different PEI vectors (with 2 kDa bPEI, 25 kDa bPEI, 2.5 kDa LPEI, or 25 kDa LPEI).

Figure 5-1: Substrate-mediated gene delivery of bPEI-DNA complexes in NIH/3T3 fibroblasts with varied peptide concentrations. NIH/3T3 fibroblasts were cultured onto bPEI-DNA complexes formed with 2 µg of DNA at N/P of 20 and immobilized to the PAA substrates conjugated with 1.0 mg/mL or 1.5 mg/mL concentration of peptides (GRGDS or RGE) for transfection. Transfection efficiency was determined using flow cytometry to measure the transfectioned populations (GFP+) and expressed as a fold change in comparison to transfection efficiency of cells cultured on PAA brushes (with no peptide). SMD
studies were analyzed using one-way ANOVA with Tukey’s post-test, which showed no statistical differences.

5.3.3. The Transgene Expression of Substrate-Mediated Gene Delivery for NIH/3T3 Fibroblasts Cultured on PAA Brushes in Response to the Conjugated Peptide and PEI Vector

For the transfection investigations on PAA brushes immobilized with other structures of RGD (cyclic cRGDyK and linear GRGDS) and four types of PEI vectors (with 2 kDa bPEI, 25 kDa bPEI, 2.5 kDa LPEI, or 25 kDa LPEI), all complexes were formed at a N/P ratio of 20 and immobilized to the substrates before culturing NIH/3T3 fibroblasts for transfection. For the cells that were cultured on PAA brushes immobilized with complexes formed with 2 kDa bPEI, cells that were cultured on PAA brushes conjugated with cRGDyK had the highest transfection success (8.4x10⁷ RLU/mg) compared to cells cultured on PAA-GRGDS, PAA-RGE, and PAA alone (5.0x10⁷, 2.0x10⁷, and 1.3x10⁷ RLU/mg, respectively) (Figure 5-2A). For the cells that were cultured on PAA brushes immobilized with complexes formed with 25 kDa bPEI, cells that were cultured on PAA brushes conjugated with cRGDyK had the highest transfection success (1.1x10⁸ RLU/mg) compared to cells cultured on PAA-GRGDS, PAA-RGE, and PAA alone (5.6x10⁷, 3.9x10⁷, and 8.5x10⁶ RLU/mg, respectively) (Figure 5-2A). The fold change comparing transfection of cells cultured on PAA-cRGDyK and those cultured on PAA alone was 6.5 and 13 times for transfection with complexes formed with 2 kDa bPEI and 25 kDa bPEI, respectively. Even so, there were still no significant statistical differences in normalized transgene expression in cells cultured on PAA brushes bPEI-DNA (at both MW) complexes immobilized to cRGDyK and those cultured on RGE or PAA (Figure 5-2A, B).
In contrast, for the cells that were cultured on PAA brushes immobilized with complexes formed with 2.5 kDa LPEI, cells that were cultured on PAA brushes conjugated with cRGDyK had no differences in transgene expression (7.0x10^5 RLU/mg) compared to cells cultured on PAA-GRGDS and PAA-RGE (7.9x10^5 and 7.8x10^5 RLU/mg, respectively) (Figure 5-2C). Transfection was still higher in cells that were cultured on PAA brushes conjugated with cRGDyK compared to those on PAA alone (5.7x10^4 RLU/mg), but it was not significant and had a fold change of 12. Similarly, for the cells that were cultured on PAA brushes immobilized with complexes formed with 25 kDa LPEI, cells that were cultured on PAA brushes conjugated with cRGDyK had no differences in transgene expression (3.7x10^7 RLU/mg) compared to cells cultured on PAA-GRGDS and PAA-RGE (2.0x10^7 and 5.3x10^7 RLU/mg, respectively) (Figure 5-2D). Transfection was still higher in cells that were cultured on PAA brushes conjugated with cRGDyK compared to those on PAA alone (1.0x10^7 RLU/mg), but it was not significant and had a fold change of 3.7.
Figure 5-2: Substrate-mediated gene delivery of NIH/3T3 fibroblasts cultured on PEI-DNA complexes immobilized to PAA brushes conjugated with different RGD peptides. NIH/3T3 fibroblasts were cultured onto PEI-DNA complexes (A) 2 kDa bPEI, B) 25 kDa bPEI, C) 2.5 LPEI, or D) 25 kDa LPEI) formed with 2 µg of DNA at N/P of 20, and immobilized to the PAA substrates, conjugated with either cyclic cRGDyK, linear GRGDS, or RGE, for transfection. SMD studies were analyzed using one-way ANOVA with Tukey’s post-test, which showed no statistical differences.

5.3.3.1. Immobilization of PEI-DNA Complexes

Next, to determine if transfection success was affected by the total adsorbed organic mass of the complexes (PEI, free and complexed, and DNA), of complexes adsorbed to each substrate, the density of immobilized PEI-DNA complexes was monitored via ellipsometry. First, the different PEI vectors (with 2 kDa bPEI, 25 kDa bPEI, 2.5 kDa LPEI, or 25 kDa LPEI) were used to form PEI-DNA complexes (at N/P 20 and 2 µg DNA), the total adsorbed mass substrates.
was calculated using spectroscopic ellipsometry measurements and Equation 5-1 (in µg/cm²). For ellipsometry monitoring, the total mass of organic material adsorbed to the substrates was 1.0±0.070 µg/cm² for complexes formed with 2 kDa bPEI, 1.0±0.015 µg/cm² for complexes formed with 25 kDa bPEI, 0.58±0.0050 µg/cm² for complexes formed with 2.5 kDa LPEI, and 0.62±0.015 µg/cm² for complexes formed with 25 kDa LPEI, which showed a significant decrease of adsorbed mass (i.e. DNA and free and complexed PEI) when complexes were formed with 2.5 kDa LPEI and 25 kDa LPEI compared to those formed with 2 kDa bPEI and 25 kDa bPEI (Figure 5-3A; **, P ≤ 0.01). The total adsorbed mass immobilized to the polymer brushes for complexes formed with 2 kDa bPEI and 25 kDa bPEI was comparable to our previous reported values (267) (chapter 4), but the total adsorbed mass immobilized to the polymer brushes was lower for complexes formed with 2.5 kDa LPEI and 25 kDa LPEI.

Figure 5-3: Complex immobilization of PEI-DNA complexes with different PEI vectors. For ellipsometric quantification of PEI-DNA complex immobilization,
complexes were formed with 2 µg of DNA at N/P of 20 using 2 kDa bPEI, 25 kDa bPEI, 2.5 kDa LPEI, or 25 kDa LPEI as a vector. Statistical differences between the measurements for the total immobilized mass were analyzed using one-way ANOVA with Tukey’s post-test. There was no significant difference for the MW for each type (bPEI and LPEI), but a significantly higher total mass was measured in substrates immobilized with 2 kDa bPEI and 25 kDa bPEI compared to those formed with 2.5 kDa LPEI and 25 kDa LPEI(**; P≤0.01).

5.3.3.2. Assessing Cellular Proliferation in Response to SMD on PAA Brushes

Next, to begin assessing the cellular response to transfection, we assessed the effect of free PEI and the PEI-DNA complex on cellular proliferation. First, NIH/3T3 fibroblasts were cultured on PAA brushes immobilized with PEI-DNA complexes formed at an N/P of 20 for each vector (2 kDa bPEI, 25 kDa bPEI, 2.5 kDa LPEI, and 25 kDa LPEI) and their metabolic activity was measured after 48 hours with a WST-1 assay (Figure 5-4). when cells were cultured on PEI-DNA complexes immobilized to TCPS (Figure 5-4A), there was a significant decrease in metabolic activity for fibroblasts cultured on 2 kDa bPEI and 2.5 kDa LPEI, and 25 kDa LPEI, in comparison to cells cultured on 25 kDa bPEI complexes (**; P≤0.01, * and P≤0.05, respectively).

![Figure 5-4](image-url)

**Figure 5-4:** Proliferation of NIH/3T3 fibroblasts cultured on PAA brushes with PEI-DNA complexes. Measurements of the metabolism of NIH/3T3 mouse fibroblasts were acquired using a water-soluble tetrazolium (WST-1) assay with
cells cultured on TCPS (A) or PAA brushes (B) with PEI-DNA complexes (2 kDa bPEI, 25 kDa bPEI, 2.5 kDa LPEI, and 25 kDa LPEI) immobilized to the substrate, 48 hours following cell seeding. WST-1 quantification of cell proliferation was measured at an absorbance of λ=430 nm and corrected with a reference wavelength of 690 nm and normalized by the measurement for cells cultured on substrates without PEI-DNA complexes. A statistical analysis using a one-way ANOVA with Tukey’s post-tests showed when cells were cultured on complexes immobilized to TCPS (A), there was a significant decrease in metabolic activity for fibroblasts cultured on 2 kDa bPEI, and 2.5 kDa LPEI, and 25 kDa LPEI in comparison to cells cultured on 25 kDa bPEI complexes (**; P≤0.01 and *; P≤0.05, respectively). Yet, no statistical differences for cells cultured on PAA brushes with immobilized PEI-DNA complexes (2 kDa bPEI, 25 kDa bPEI, 2.5 kDa LPEI, and 25 kDa LPEI) (B).

In comparison to the results of culturing cells on PEI-DNA complexes immobilized to PAA brushes, when cells were cultured on PAA brushes with immobilized PEI-DNA complexes (2 kDa bPEI, 25 kDa bPEI, 2.5 kDa LPEI, and 25 kDa LPEI), there was no statistical difference in the metabolic activity of the cells (Figure 5-4A) on surfaces with any of those types of complexes. Moreover, cells cultured on PAA brushes immobilized with the complexes formed with 2 kDa bPEI and 25 kDa bPEI had increases to their metabolic activity (Figure 5-4A) compared to those cultured on PAA brushes without complexes. Therefore, given that fibroblasts cultured on complexes formed with 25 kDa bPEI exhibited the highest transgene expression, which was most apparent on PAA brushes functionalized with cRGDyK (Figure 5-2B), further investigations on the cellular response were performed by culturing cells on 25 kDa bPEI-DNA complexes immobilized to those substrates (cRGDyK) in comparison to control PAA and PAA-RGE brushes.
5.3.4. Assessing Cellular Focal Adhesion Formation and Cytoskeletal Arrangement in Response to Substrate-Mediated Gene Delivery on PAA Brushes

To investigate the cellular adhesion response, confocal microscopy was used to quantify the immunofluorescence staining of vinculin (a protein component recruited to focal adhesions (279)), as well as the cell density (measure with DAPI-stained nuclei) and the actin stress fibers that attach to focal adhesion sites (123). In NIH/3T3 fibroblasts cultured on 25 kDa bPEI-DNA complexes immobilized to PAA brushes compared to those cultured on 25 kDa bPEI-DNA complexes immobilized to PAA-cRGDyK and to PAA-RGE, the most apparent difference was the cellular morphology. Fibroblasts cultured on the latter two substrates (PAA-cRGDyK and PAA-RGE) were much more spread with actin in a uniform direction (Figure 5-5C vs. 5-6A, B), while those cultured on 25 kDa bPEI-DNA complexes immobilized to PAA brushes spreading in multiple different directions, and the actin and vinculin staining was less apparent in these cells (Figure 5-5C). Finally, the DAPI stain also stained the DNA plasmids in the formed complexes, showing that the plasmid DNA of the complexes was apparent in the substrates with PAA alone (i.e. no peptide).
**Figure 5-5:** Representative images of NIH/3T3 fibroblasts stained with TRITC phalloidin for actin filaments (red), Alexa Fluor® 488 for vinculin (green), and nuclei counterstained with DAPI (blue). Immunofluorescent staining of NIH/3T3 fibroblasts cultured on 25 kDa bPEI-DNA complexes immobilized to PAA-cRGDyK (A), PAA-RGE (B), and PAA (C) was imaged with confocal microscopy using a 60x water immersion objective. Scale bar=20 µm.

After qualitative analysis of the images showed distinct cellular morphologies dependent on the culturing substrates (i.e. 25 kDa bPEI-DNA complexes immobilized to either PAA-cRGDyK, PAA-RGE, and PAA), the cell density, focal adhesion per cell, and actin stress fibers per cell were quantified. Similar to the results of viability staining in previous investigations (116, 267) (chapter 3, 4), the cell density was not significantly different when the cells were cultured on 25 kDa bPEI-DNA complexes immobilized to PAA-cRGDyK, PAA-RGE, or PAA (Figure 5-6A), although a wider distribution of average cells per image was seen for those cultured on PAA-RGE and PAA.
After analyzing the cell density, focal adhesions were measured by the presence of vinculin (280) and normalized by the cell count as the average number of focal adhesions per cell. Focal adhesion formation was found to be most abundant in cells cultured on 25 kDa bPEI-DNA complexes immobilized to PAA-cRGDyK, which was quantified as 26±2.9 focal adhesion per cell (Figure 5-6B), which was significantly higher than cells cultured on PAA-RGE (*; P≤0.05, 15±2.9 focal adhesion per cell) and those cultured on PAA (***; P≤0.001, 9.7±1.4 focal adhesion per cell). Given that actin stress fibers can form around focal adhesion sites (281), the cytoskeletal arrangement of actin stress fibers was also quantified. The actin stress fibers of cells cultured on 25 kDa bPEI-DNA complexes immobilized to PAA-cRGDyK, PAA-RGE, or PAA were differentiated from the rest of the cytoskeleton as the bright regions of TRITC stain (282) and then averaged by the cell count. The results of the immunofluorescence imaging showed that fibroblasts cultured on 25 kDa bPEI-DNA complexes immobilized to PAA-RGE had the most actin stress fibers per cell at 5.1±1.2 (Figure 5-6C), which was significantly higher than cells cultured on 25 kDa bPEI-DNA complexes immobilized to PAA-cRGDyK (*; P≤0.05, 2.2±0.34 actin stress fibers per cell) or PAA (***; P≤0.001, 0.37±0.14 actin stress fibers per cell). Finally, since the DAPI stained the DNA plasmids in the formed complexes, we quantified an estimate of the complexes that were not internalized by the cell as DNA plasmids per image area (Figure 5-6D), which showed no complexes for PAA-cRGDyK (10±5.0), which was less than the amount of complexes
immobilized to PAA-RGE (170±52) and significantly less than the amount of complexes immobilized to PAA (*; P≤0.05, 650±230).

Figure 5-6: Quantification of actin stress fibers and focal adhesions in NIH/3T3 fibroblasts cultured on 25 kDa bPEI-DNA complexes immobilized to modified PAA brushes (with cRGDyK and RGE, or no peptides). Images of NIH/3T3 fibroblasts stained with TRITC phalloidin for actin filaments (red), Alexa Fluor® 488 for vinculin (green), and nuclei counterstained with DAPI (blue) were quantified using ImageJ Processing (NIH) to measure the cell density in cells per image (A), the focal adhesions per cell (B), and the actin stress fibers per cell (C). The DNA plasmids of the complexes immobilized to the substrate (i.e. complexes that were not taken up by the cell) were also quantified per image (D). A statistical analysis using a one-way ANOVA with Tukey’s post-tests showed no statistical differences for cell density in cells cultured on 25 kDa bPEI-DNA complexes immobilized to PAA-cRGDyK, PAA-RGE, or PAA (A). For the cellular features of focal adhesions (marked by vinculin) and actin stress fibers, there were significantly more focal adhesions per cell in those cultured on 25 kDa bPEI-DNA complexes immobilized to PAA-cRGDyK (B) compared to those
cultured on PAA-RGE (\*; P≤0.05) and to those cultured on PAA (***; P≤0.001), but there were significantly more actin stress fibers per cell in those cultured on 25 kDa bPEI-DNA complexes immobilized to PAA-RGE compared to those cultured on PAA-cRGDyK (\*; P≤0.05) and to those cultured on PAA (***; P≤0.001).

5.3.5. Assessing Endocytic Pathways in Response to Substrate-Mediated Gene Delivery on PAA Brushes

Finally, after assessing cellular adhesion and the cytoskeletal arrangement, the cellular response was studied via internalization of the complexes through endocytosis. For these investigations, NIH/3T3 fibroblasts were seeded on 25 kDa bPEI-DNA complexes immobilized to PAA-cRGDyK, PAA-RGE, or PAA and chemical inhibitors for three common types of endocytosis (i.e. macropinocytosis, and clathrin-mediated or caveolae-mediated endocytosis) were added concurrently: amiloride (Amil; 1 mM (278)), chlorpromazine (CPZ; 10 µg/mL (71)), and genistein (Gen; 200 µM (71)).

After culturing the cells for 24 hours, the transfection efficiency was assessed with flow cytometry and compared to cells seeded without inhibitors on corresponding control substrates (i.e. 25 kDa bPEI-DNA complexes immobilized to PAA-cRGDyK, PAA-RGE, or PAA). The reduction of transfection efficiency in cultured cells was significantly dependent on the culturing substrate (i.e. PAA-cRGDyK, PAA-RGE, or PAA) (Figure 5-7; \*; P≤0.05) but, for each culturing substrate, there were no statistical differences relative to the type of inhibitor used. For cells cultured on 25 kDa bPEI-DNA complexes immobilized to PAA-cRGDyK, the fold changes in transfection efficiency comparing cells transfected in the presence of inhibitors to uninhibited cells were 0.19±0.083 for macropinocytosis, 0.43±0.091 for clathrin-mediated endocytosis, and 0.25±0.013
for caveolae-mediated transfection, respectively (Figure 5-7). For cells cultured on 25 kDa bPEI-DNA complexes immobilized to PAA-RGE, the fold changes in transfection efficiency comparing cells transfected in the presence of inhibitors to uninhibited cells were 0.12±0.030 for macropinocytosis, 0.44±0.19 for clathrin-mediated endocytosis, and 0.15±0.050 for caveolae-mediated transfection, respectively (Figure 5-7). Finally, for cells cultured on 25 kDa bPEI-DNA complexes immobilized to PAA, which was the substrate where cultured cells were the least affected by the inhibitors (Figure 5-7), the fold changes in transfection efficiency comparing cells transfected in the presence of inhibitors to uninhibited cells were 0.65±0.31 for macropinocytosis, 0.87±0.42 for clathrin-mediated endocytosis, and 1.4±0.83 for caveolae-mediated transfection, respectively (Figure 5-7).

![Figure 5-7: Endocytic inhibition of NIH/3T3 fibroblasts cultured on 25 kDa bPEI-DNA complexes immobilized to modified PAA brushes (with cRGDyK and RGE, or no peptides). The transfection of NIH/3T3 fibroblasts was inhibited via the addition of chemicals that modulate endocytosis (i.e. amiloride (Amil; 1 mM), chlorpromazine (CPZ; 10 µg/mL), and genistein (Gen; 200 µM) for macropinocytosis, and clathrin- and caveolae-mediated endocytosis, respectively). A statistical analysis using a two-way ANOVA with Tukey’s post-](image-url)
tests showed that the culturing substrates (i.e. PAA-cRGDyK, PAA-RGE, PAA) significantly altered the endocytic pathways utilized by the cultured cells (*:P≤0.05) but there were no statistical differences in the effect of the inhibitors on reducing transfection.

5.4. Discussion

The objective of this work was to tune the ability of PAA brushes to prime the cellular response for SMD transfection through the functionalization of cellular adhesion peptides and the immobilization of different PEI-DNA complexes. After confirming that the PAA brush characteristics (i.e. grafting thickness, swelling behavior) and peptide immobilization (Tables 6-1 and 6-2) were consistent with previously reported results (116, 186, 267) (chapter 3,4), the efficacy of SMD was tested in a model cell line for transfection, NIH/3T3 fibroblasts (132, 150, 251). First, the concentration of the linear GRGDS peptide conjugated to the PAA brushes was investigated, the same peptide used in our previous investigation (267) (chapter 4). Without complexes, substrates conjugated with PAA-GRGDS at 1.5 mg/mL had significantly more cells adhered to the substrate compared to those with PAA-GRGDS at 1.0 mg/mL (and PAA-GRGDS at 0.5 mg/mL; Figure A-1 in Appendix), suggesting that there may be increased transfection in cells cultured on PAA-GRGDS at 1.5 mg/mL. Yet, when fibroblasts were cultured on 25 kDa bPEI-DNA complexes immobilized to PAA brushes conjugated with GRGDS at a higher concentration of 1.5 mg/mL had no significant increase in transfection efficacy in comparison to those on 25 kDa bPEI-DNA complexes immobilized to PAA brushes conjugated with GRGDS at a lower concentration of 1.0 mg/mL. Previous investigations have shown that the RGD density can significantly impact the cellular response to a substrate, whereas cells (e.g.
endothelial cells, fibroblasts) cultured on substrates with higher RGD densities have shown more focal adhesion formation and higher cell spreading and migration rate in comparison to those cultured lower RGD densities (130, 231, 283-286), all of which are cell behaviors that have been shown to improve transfection success (268).

The concentrations of conjugated GRGDS ligands in this work were calculated at a density of 1.0-1.7 µg/cm², which is within the range of expected values comparable to previously reported functionalized polymer substrates (187, 287-289); yet, close packing of the RGD ligand on the PAA brushes conjugated at a higher concentration may provide insufficient spacing for integrin engagement in cultured cells (284). Furthermore, according to AFM measurements, the GRGDS ligands are evenly distributed across the PAA brushes (Figure A-2 in Appendix), and given that the presentation of the RGD ligands in clustered forms has been reported as more effective at promoting cellular adhesion and subsequent transfection (196, 231, 288, 289) rather than evenly distributed RGD ligands, increasing the concentration of GRGDS conjugated to PAA brushes may not have had an effect on subsequent SMD transfection because of the uniform presentation of the ligand. Therefore, after analyzing the effect of the conjugated GRGDS concentration on transfection in NIH/3T3 fibroblasts, we tested a cyclic RGD ligand (cRGDyK), which has been shown to have protease resistance, high stability, and high affinity for cellular integrins (186, 272), which may improve transfection by upregulating intracellular processing regulated by integrin binding (i.e. focal adhesion formation,
endocytosis, and intracellular trafficking). The transgene expression for all conditions was comparable to our previous investigation (16) (chapter 4) and transfection was highest in cells cultured on 25 kDa bPEI-DNA complexes immobilized to PAA-cRGDyK compared to cells cultured on all other PEI-DNA complexes (i.e. 2 kDa bPEI, 25 kDa LPEI, 2.5 kDa LPEI) immobilized to all other PAA brush substrates (PAA-GRGDS, PAA-RGE, PAA).

After analyzing the effect of the RGD ligand presentation, transfection was assessed with respect to the PEI vector. The MW and branching of PEI vectors are considered the two properties that will dictate the physical properties of PEI-DNA complexes and transfection success (49). In general, branched polymers (i.e. bPEI) and higher MW are considered better transfection vectors because they can more effectively condense the DNA into smaller particles (48, 49); thus, transfection is typically performed using 25 kDa bPEI (46). Yet, in our studies, the size of the complex was not significantly different dependent on the vector in our studies (Figure A-3 in Appendix), which may be due to the salt concentration in the media (i.e. OptiMEM) which can produce larger PEI-DNA complexes (>500 d.nm) that are still able to transfect cells (290, 291)). Given that transfection is typically performed using 25 kDa bPEI (46) and that linear structure and lower molecular weights have been shown to reduce cytotoxicity while maintaining transfection success in cultured cells (252), we examined the four PEI vectors of 2 kDa bPEI, 25 kDa bPEI, 2.5 kDa LPEI, and 25 kDa LPEI to form complexes for immobilization studies and transfection.
Although different N/P ratios can be used to optimize transfection with each of these vectors, a N/P ratio of 20 was chosen for our studies to maintain a high level of free PEI in solution to adsorb to the brushes (20). When the total adsorbed mass immobilized to the PAA brushes for complexes formed with 2 kDa bPEI and 25 kDa bPEI was calculated, the value (1.0 µg/cm²) was comparable to our previous reported values (267) (chapter 3) and the complexes formed with 2.5 kDa LPEI and 25 kDa LPEI had significantly less estimated total absorbed mass on the PAA substrate than those formed with 2 kDa bPEI and 25 kDa bPEI. In our previous paper, we calculated that complexing 0.050 µg of DNA required 0.020 µg of 25 kDa bPEI, suggesting a theoretical total of 0.070 µg of fully formed complexes were adsorbed to the substrate (267) (chapter 4). For the investigations, the amount of DNA immobilized to the substrate was estimated using Cy5-labeled DNA plasmids (Table A-1, Figure A-4 in Appendix), which was 0.050 µg/cm² for complexes formed with 25 kDa bPEI and 25 kDa LPEI and 0.030 µg/cm² for complexes formed with 2 kDa bPEI and 2.5 kDa LPEI. Therefore, we calculated that complexing 0.030 µg of DNA required 0.010 µg of 2 kDa bPEI or 2.5 kDa LPEI, suggesting a theoretical total of 0.040 µg of fully formed complexes were adsorbed to the substrate. Thus, the amount of free PEI immobilized to PAA brushes was estimated as 0.96 µg (1.0-0.040), 0.93 µg (1.0-0.070), 0.54 µg (0.58-0.040), and 0.55 µg (0.62-0.070) for complexes formed with 2 kDa bPEI, 25 kDa bPEI, 2.5 kDa LPEI, and 25 kDa LPEI, respectively. Thus, complexes formed with LPEI may have had less free PEI adsorbed to the substrate and that may have contributed to the lower transfection success in cells.
cultured on 2.5 kDa LPEI and 25 kDa LPEI compared to those cultured on complexes formed with 2 kDa bPEI and 25 kDa bPEI. Given that lower MWs and LPEI is known to produce less transfection in cultured cells (45, 48) and less free polymer may be presented with immobilized complexes formed with 2.5 kDa LPEI and 25 kDa LPEI, the resulting higher levels of transfection in cells cultured on 25 kDa bPEI-DNA complexes (compared to those formed with all other PEI vectors) was consistent with reports that term 25 kDa bPEI as the “gold standard” vector for transfection (46, 292). Moreover, cells cultured on PAA brushes immobilized with the complexes formed with 2 kDa bPEI and 25 kDa bPEI had slightly higher proliferation compared to cells cultured on PAA brushes immobilized with the complexes formed with 2.5 kDa LPEI and 25 kDa LPEI. The slight increase in proliferation of cells cultured on complexes formed with 2 kDa bPEI and 25 kDa bPEI may have contributed to the increase in transfection success, as proliferation is commonly associated with successful internalization and nuclear entry due to the compromised integrity of the nucleus in dividing cells (132). The results of the proliferation assay that immobilizing the PEI-DNA complexes (and adsorbed free PEI) to PAA brushes does not cause cytotoxicity (possibly even improving proliferation as shown in cells cultured on bPEI-DNA complexes at both MWs) is exceptional, as vector cytotoxicity in cells cultured on TCPS and other substrates has often been cited as a significant barrier to transfection success with PEI-DNA complexes (56, 293, 294).

Given the results for transfection and proliferation, we further investigated the cellular response of cells cultured on 25 kDa bPEI-DNA complexes
immobilized to PAA-cRGDyK in comparison to cells cultured on 25 kDa bPEI-DNA complexes immobilized to control PAA-RGE and PAA. First, the cell density showed that cells cultured on 25 kDa bPEI-DNA complexes immobilized to PAA-cRGDyK had a similar cell count for each image and the most focal adhesions per cell compared to cells cultured on PAA-RGE and PAA, which indicates that the cells were more adhered to the substrates, presumably through integrin binding to the RGD ligand (187, 273). In contrast, cells on 25 kDa bPEI-DNA complexes immobilized to PAA-RGE had a wide distribution of cell densities from 1 cell per image to 60 cells per image, which suggested there were islands of confluent cells and areas of empty culture space on these substrates (i.e. PAA-RGE) rather than evenly distributed cultured cells. Healthy growth of fibroblasts is typically in an even monolayer (295), and cellular aggregation is commonly mediated by cell-cell adhesion that can increase stress fiber formation (296), which agreed with our images that showed cells cultured on 25 kDa bPEI-DNA complexes immobilized to PAA-RGE had vinculin staining on cells that appeared to be adhered to one another rather than the substrate or focal adhesions and these cells also had the most actin stress fibers per cell. Thus, given that vinculin can also mark adheren junctions for intercellular adhesion (297) and that stress fibers are also known to stabilize protein complexes at those junctions (298), transfection in cells cultured on 25 kDa bPEI-DNA complexes immobilized to PAA-RGE may have been stimulated through the intercellular interactions and intracellular trafficking along the stress fibers (125), rather than cell-material interactions. Finally, actin staining also showed that the morphology of cells that
were cultured on 25 kDa bPEI-DNA complexes immobilized to PAA were flattened and spread but did not have the structural organization of cells cultured on PAA-cRGDyK and PAA-RGE, suggesting that there was less cell adhesion to PAA brushes (226), as expected, but the immobilized complexes (and free PEI) still enable cell adhesion to occur on PAA brushes that are typically cell-repellent without peptides (116, 153) (chapter 3).

Along with the results for the cellular response, DAPI stain for the nucleus also stained the DNA plasmids in the formed 25 kDa bPEI-DNA complexes, as it binds strongly to adenine-thymine rich regions in DNA (299). In images of the stained cells, the plasmid DNA of the complexes is especially apparent in the substrates with PAA alone (i.e. no peptide), suggesting that there is low internalization of the complexes from cells cultured on PAA alone, which may contribute to their low levels of transfection. Furthermore, the conclusion that there is significantly less internalization of 25 kDa bPEI-DNA complexes into cells cultured on PAA compared to those cultured on PAA-cRGDyK is supported by the results of inhibiting endocytosis, which had a minimal effect on transfection efficiency in cells cultured on PAA in comparison to those cultured on PAA-cRGDyK and PAA-RGE.

Along with the low effect of the inhibitors on cells cultured on PAA alone, investigations into the endocytic pathways showed that the effect of the inhibitors was more significant in cells cultured on substrates with peptides (i.e. PAA-cRGDyK, PAA-RGE), as RGD peptides are known to enhance endocytosis and transfection (73, 108, 111, 125, 196, 274, 275). Chlorpromazine (which causes
clathrin to localize and accumulate in late endosomes, thereby preventing endosomal escape of complexes (300)) was the least effective at inhibiting transfection efficiency, suggesting that clathrin-mediated endocytosis was not the most efficient pathway for transfection in our system, in agreement with previous reports that clathrin-mediated endocytosis is optimal for lipid-based transfection rather than polymers such as PEI (71, 300-302). Moreover, genistein, which prevents vesicle formation in caveolae-mediated endocytosis (303), had more success at reducing transfection efficiency than chlorpromazine, which is supported by previous investigations that have cited caveolae-mediated endocytosis as a more efficient endocytic pathway for transfection (compared to clathrin-mediated endocytosis) (77), especially with polyplexes in both bolus (109, 300, 304) and substrate-mediated (60) delivery formats. Therefore, the investigations into the endocytic mechanisms suggest that the PEI vector (free and complexed) influenced the internalization pathway in tandem with the cRGDyK ligand.

Overall, the most effective inhibitor for cells cultured on 25 kDa bPEI-DNA complexes immobilized to substrates (i.e. PAA-cRGDyK, PAA-RGE, PAA) was amiloride. Amiloride has been cited as effective at decreasing macropinocytosis by lowering submembranous pH and preventing signaling from the RhoGTPases Cdc42 and Rac1 (278), which are known for contributing to focal adhesion formation (111, 128, 305). Macropinocytosis, clathrin-, and caveolae-mediated endocytosis have all been shown to be modulated by focal adhesion formation (305, 306). Thus, substrates that promote focal adhesion formation in cultured
cells (i.e. PAA-cRGDyK) may increase transfection success in those cultured cells through increased endocytosis via all three common pathways, but especially via macropinocytosis. Finally, another reason that macropinocytosis may have been the most optimal pathway for transfection may simply be related to the size of the complexes (i.e. ~600 nm, Figure A-3 in Appendix), as receptor-mediated pathways such as clathrin- and caveolae-mediated endocytosis typically takes up complexes under 200 nm in diameter (72). Thus, although the results of the investigations into the cellular response (i.e. proliferation, focal adhesion formation, and endocytosis) strongly suggested that cells can be primed by the substrate to increase transfection success, factors such as the characteristics of the complex (i.e. size, charge) are important to consider in investigations to improve transfection.

5.5. Conclusions

In our previous studies, we showed that PAA brushes can be “grafted-to” Ti substrates and RGD can be conjugated to these brushes to support cell adhesion, and those PAA-RGD modified Ti substrates can be used as a platform to immobilize 25 kDa bPEI-DNA complexes to transfect NIH/3T3 fibroblasts via SMD. Given that the presence of the RGD ligand and the presence of the free PEI may have synergistically contributed to enhanced transfection via SMD in fibroblasts cultured on 25 kDa bPEI-DNA complexes immobilized to PAA-GRGDS substrates compared to transfection in cells cultured on substrates without the RGD ligand and without free PEI, herein we investigated tuning these
factors through the concentration and binding affinity of the RGD ligand (i.e. cyclic vs. linear) and the branching and the MW of the PEI to prime the cellular response to transfection. After determining the optimal priming conditions (complexes formed with 25 kDa bPEI immobilized to PAA brushes conjugated with cRGDyK), the cellular response was investigated. Increased proliferation of cells cultured on 25 kDa bPEI-DNA complexes immobilized to PAA brushes may have increased the nuclear availability to the complexes, which may have contributed to the transfection success. Furthermore, the presentation of 25 kDa bPEI-DNA complexes and free bPEI adsorbed to the PAA brushes may have mitigated the cytotoxicity effect of culturing cells on PEI-DNA complexes. Further investigations into the cellular response show that cells cultured on PAA-cRGDyK had increased focal adhesion formation, presumably related to integrin binding of the RGD ligand, which may have led to increased endocytosis of the complexes (especially via macropinocytosis), although further tuning of the RGD density and presentation (i.e. clustering) may enhance the improvement in transfection. Overall, the findings of this chapter suggest that the modification of Ti with PAA brushes is a tunable method to affect the efficacy of nonviral gene delivery, that there is a synergistic effect of free PEI and the RGD ligand on the cellular response to transfection, and that PAA-cRGDyK may have future applications to modify substrates that could be improved by gene delivery including biomedical devices, implantable sensors, and diagnostics tools.
CHAPTER 6
Conclusions and Future Directions

6.1. Conclusions

Cell-biomaterial interactions that occur on a substrate can modulate the cellular processes related to successful nonviral gene delivery, such as adhesion and proliferation (307-309), migration (310, 311), and endocytosis (312, 313). Along with priming cellular responsiveness to gene delivery, biomaterial interfaces can be used to immobilize formed DNA complexes through electrostatic interactions or covalent binding in a process termed “substrate-mediated gene delivery” or SMD (19, 20, 59) to enhance transfection by presenting DNA within the microenvironment of the cell. Nonviral SMD investigations have not previously been performed on clinically relevant metallic biomaterials (e.g. titanium (Ti) (2)), but Ti is used ubiquitously in medical devices and implants whose integration and functionality could be further improved with gene delivery as shown in a previous viral investigation into SMD on Ti (314)

Given that nonviral gene delivery is safer but less efficient compared to viral vectors, there is a need for a cell-material interface that modulates the cellular response and immobilization of nonviral DNA complexes onto Ti biomedical implants and devices. Thus, a novel platform for SMD (chapters 3, 4, and 5) was investigated by chemically altering the cell-material interface through grafting of stimuli-responsive poly(acrylic acid) brushes (PAA) to Ti, and conjugating the PAA brushes with arginyl-glycyl-aspartic acid (RGD) ligands, which showed enhanced transfection facilitated by the cellular response to the interface, as well
as the ability of the brushes to sequester adjuvant-like free PEI. These substrates may immobilize therapeutic DNA complexes for applications such as Ti biomedical devices, implantable sensors, and diagnostics tools.

In chapter 3 of this dissertation, we described the development and characterization of a simple method of grafting PAA brushes to Ti substrates, which amplified the substrate functionality through the high density of COOH groups that deprotonate in response to pH-stimuli and allow for the ability to conjugate cell adhesion moieties via EDC/NHS chemistry. PAA brushes were reproducibly grafted to Ti surfaces with brush thicknesses comparable to that on traditionally studied Si substrates and retained their pH-dependent swelling behavior (179, 185, 189). Since PAA brushes are known to be nonfouling, the abundant COOH groups on the PAA brushes were used for covalent binding of the RGD-containing peptide GRGDS (PAA-RGD) as a model bioactive functional group for cell adhesion, which conjugated at a density comparable to study with PAA brushes on traditional Si substrates (177, 179, 185, 197, 213). The cellular response to the RGD ligand on the PAA-RGD brushes on Ti was quantified by assessing cell adhesion of NIH/3T3 fibroblasts, which showed that PAA-RGD substrates enabled cell adhesion comparable to Flat Ti surfaces at both 24 and 48 hours after cell seeding suggesting that biocompatibility was conserved with PAA-RGD brushes on Ti substrates. The results of these studies suggested that PAA-RGD is a viable platform to investigate for cell culture and SMD applications on Ti substrates, and this study was the first to report success of grafting PAA brushes to a Ti substrate.
In chapter 4, with an optimized system for culturing cells on clinically relevant Ti, we then studied PAA-RGD modified Ti substrates as a platform for improving SMD to NIH/3T3 fibroblasts using immobilized 25 kDa branched PEI (bPEI)-DNA complexes. Cells cultured on PAA-RGD with immobilized bPEI-DNA complexes had significantly increased transfection compared to cells cultured on PAA with immobilized bPEI-DNA complexes, which was not attributed to the amount of DNA immobilized to the surface or the DNA release profile (measured through radioactively-labeled DNA), two features that previously have been shown to influence SMD with other substrates (17, 19, 59). However, ellipsometric measurements processed using the de-Feijter equation (248) showed that PAA-RGD brushes (and PAA alone) adsorbed more overall mass compared to unmodified Ti substrates, which may be attributed to immobilization of free and complexed bPEI through electrostatic interactions with the charged COOH groups. Thus, it may be a combinatorial effect of the free PEI and the RGD ligand that enhanced transfection in cells cultured on PAA-RGD with immobilized bPEI-DNA complexes compared to those cultured on PAA alone and those cultured on Flat Ti. Previously, free PEI has been attributed to aiding transfection via bolus delivery (45, 51, 54, 257) but, in traditional SMD, free PEI is often washed away. Given that the density of deprotonated carboxyl groups on PAA-RGD brushes become highly negative at physiological pH, electrostatic interactions may have neutralized the highly cationic free PEI. Therefore, transfection investigations were performed with cells cultured on PAA-RGD surfaces with immobilized filtered complexes (i.e. complexes with free PEI
removed) to which controlled dosages of free bPEI were added, and the results of these studies suggested that free bPEI had an adjuvant-like effect on SMD transfection in cells cultured on PAA-RGD. Again, given that free polymer is typically rinsed away before SMD transfection on traditional culture substrates with weaker electrostatic interactions, the ability to prime cells with free PEI for transfection via SMD is a novel capability of PAA-RGD brushes on Ti.

Given that the presence of the RGD ligand and the presence of the free PEI both contributed to enhanced SMD on PAA substrates, we next investigated their synergistic effect on transfection. Since the RGD ligand is able to stimulate endocytosis and free PEI can aid in intracellular trafficking of complexes (187, 257), the cellular response to both of these elements of our system may be the cause of enhanced transfection in cells cultured on PAA-RGD with immobilized bPEI-DNA complexes. Moreover, the cellular response to the RGD peptide and PEI vector can be tuned by altering the binding affinity of the RGD ligand (i.e. cyclic vs. linear (186, 285, 315)), and the branching and the molecular weight (MW) of the PEI (45, 51, 54). Thus, in chapter 5, the intracellular mechanisms affected by RGD and free PEI that are involved in transfection efficiency were investigated using two types of RGD (linear GRGDS and cyclic cRGDyK) covalently bound to PAA brushes and complexes formed with four types of PEIs (linear (LPEI) at 25 and 2.5 kDa, branched (bPEI) at 25 and 2 kDa). Transfection investigations showed that the highest transfection occurred in cells cultured on 25 kDa bPEI-DNA complexes immobilized to PAA brushes conjugated with cRGDyK, as expected since cyclic RGD is a more activated form than linear
RGD (315) and 25 kDa bPEI is the gold standard for polymer transfection (46). When investigating the cellular response, fibroblasts that were cultured on 25 kDa bPEI-DNA complexes immobilized to PAA-cRGDyK were shown to have increased focal adhesion formation and stress actin fiber formation (in comparison to cells cultured on 25 kDa bPEI-DNA complexes immobilized to PAA), which may both affect internalization and intracellular trafficking (108, 125). Furthermore, comparing the transfection levels of cells cultured on 25 kDa bPEI-DNA complexes immobilized to PAA-cRGDyK with inhibitors for the three common endocytic pathways (i.e. macropinocytosis, clathrin- and caveolae-mediated endocytosis (71)) to untreated cells cultured on 25 kDa bPEI-DNA complexes immobilized to PAA-cRGDyK, showed transfection success in cells cultured on 25 kDa bPEI-DNA complexes immobilized to PAA-cRGDyK may have been increased by endocytosis via all three common pathways (71), but especially via macropinocytosis. Overall, the findings of this chapter suggest that the modification of Ti with PAA brushes is a tunable method to affect the efficacy of nonviral SMD and PAA-cRGDyK may have future applications to improve the efficacy and integration of Ti biomedical devices, implantable sensors, and diagnostics tools.

6.2. Future Directions

6.2.1. Further Tuning the Presentation of Free PEI and the RGD Ligand on PAA Brushes

Based on the conclusions of our investigations, there are two factors in our system that could be further optimized to affect the cellular response to SMD
transfection on PAA brushes: the presentation of free polymer and the
conjugated RGD ligand. Thus, a future direction of this project is tuning these
elements, which will be described in the next sections.

6.2.1.1. Future Direction: Modulating Transfection via the Use of Two PEI
 Constituents for Separate Functions as Free and Complexing Polymers

One of the novel aspects of the PAA-RGD brush system as a platform for
SMD is the ability to sequester and present free PEI to cultured cells. Previously,
the adjuvant-like quality of free polymer has only been shown in bolus
investigations (45, 51, 53, 54, 257), but here we show its effect in SMD and also
demonstrate that the presentation of free PEI on PAA brushes showed a
decrease in cytotoxicity. However, the effect of free PEI on transfection was only
tested in a small range of doses and MWs. It has previously been shown that the
a wide range of free PEI chains (at different lengths) can significantly impact
transfection success (51, 54), but the cytotoxicity was apparent in the studies as
free PEI increased. Since our PAA brushes seem to mitigate the issues of
cytotoxicity (possibly by neutralizing the free PEI through electrostatic
interaction), higher dosage of free PEI may be feasible to enhance transfection in
our system. Thus, a future direction of this project would be to further optimize
the free PEI dosage, and the branching and MW of the PEI, to maintain a
balance of high transfection and low toxicity, starting with two possible methods.
First, since a lesser amount of free polymer was estimated on substrates with
LPEI-DNA complexes (at both MW) in chapter 5, more free LPEI could be
immobilized by adding an extra dosage of LPEI to adsorb to the substrate after
complex immobilization. Second, since 25 kDa bPEI-DNA complexes are
considered the best transfecting vector but adding more free bPEI may be more cytotoxic than adding LPEI (45, 50, 55), and since LPEI has been shown to affect the cellular response to transfection in a similar fashion to bPEI, adding the less cytotoxic LPEI as a free polymer to filtered 25 kDa bPEI-DNA complexes may create synergistic delivery with low toxicity and high transfection. For both investigations, the pH-responsive characteristics of the PAA brushes may also be used to further enhance the loading capacity for the substrate for both PEI-DNA complexes and free PEI. Finally, these are only two of the many options for investigating the combinatorial effect of free and complexed PEI but other polymer vectors may also be investigated for their effect on transfecting cells cultured on PAA brushes via SMD, such as poly(amidoamine) (PAMAM) dendrimers, poly(l-lysine) (PLL), and poly(β-amino ester) (44).

In addition to studying the addition of free and complexed PEI, the formation of PEI-DNA complexes was investigated for their charge and size and their effect on transfection. In this dissertation complexes were formed in OptiMEM, a reduced serum media buffered with HEPES and sodium bicarbonate and it has been shown that forming complexes in other medias can change their properties (i.e. size, charge (290)), and, combined with the ionic strength of the media used to immobilize complexes (which in turn would affect protonation of the PAA brushes (223)) could be investigated to optimize the formation of PEI complexes with different media (e.g. NaCl, tris buffer saline) to form a smaller, positively charged complex that will be better internalized and trafficked for transfection (45); thus, suggesting the size and the charge of a complex
modulate the cellular response to transfection. Since free PEI may also modulate intracellular trafficking of the formed PEI-DNA complex (53, 257), the cellular response may be primed by a combination of these properties of the complex (i.e. size, charge, and free PEI). Moreover, immobilized complexes can be presented in tandem with modifications that mimic the extracellular matrix (ECM) cues and enhance the cellular response to transfection, which will be discussed in the next section.

6.2.1.2. Future Direction: Conjugation of Multiple Ligands and Patterned RGD

One of the goals of substrate modifications is to recapitulate the ECM to provide cues that direct cellular processes that maintain tissue homeostasis, growth and repair functions (316), which are vital to the success of applications such as gene delivery, tissue engineering, and regenerative medicine. In this dissertation, PAA brushes were conjugated with the RGD ligand that is found on many different proteins within the ECM (i.e. fibronectin, vitronectin, fibrinogen, some collagens, and many others (317)). Previous investigations have shown that the density of the RGD can significantly impact the cellular response to a substrate, whereas cells (e.g. endothelial cells, fibroblasts) cultured on substrates with higher RGD densities have shown more focal adhesion formation and higher cell spreading and migration rate in comparison to those cultured lower RGD densities (130, 283-286, 318), all of which are cell behaviors that have been shown to improve transfection success (268). Along with the density of the RGD ligand, the presentation of these ligands (i.e. clustering) has also been shown to
improve cell adhesion and transfection (129, 194, 288, 289). For example, a study by Gojgini et al. showed that tuning the clustering of RGD ligands on substrates (and their concentration) significantly altered transgene expression (194). Moreover, in a system similar to our PAA-RGD platform, poly(oligo(ethylene glycol) methacrylate) (poly(OEGMA)) brushes grafted from Ti were used to present recombinant constructs of fibronectin-binding domains in clusters, which enhance cell adhesion of stem cells, osteogenic signaling and differentiation into bone (without transfection) (319). Thus, a future direction of this project would be to use our PAA brushes, which are formed with simpler “grafting to” approach compared to the “grafting from” approach by Petrie et al. (319), and investigate tuning the RGD concentration and presentation of the RGD ligand (i.e. clustering) to enhance transgene expression in cultured cells and to deliver genes that improve differentiation of stem cells.

Although RGD has frequently been shown to enhance transfection success by stimulating the cellular response through integrin binding (129, 196, 285), RGD is only one type of cell adhesion peptide found on the proteins in the ECM. Many different cell adhesion peptides can be derived from ECM proteins such as collagen I and IV, elastin, laminin, osteopontin, and vitronectin (320), and the most well-characterized peptides (other than RGD) are isoleucine-lysine-valine-alanine-valine (IKVAV), tyrosine-isoleucine-glycine-serine-arginine (YIGSR), and AG73 from laminin (321, 322). All three peptides (IKVAV, YIGSR, AG73) have been shown to induce integrin binding, focal adhesion formation, proliferation, migration, and endocytosis (323-326), all behaviors that affect
transfection success (268). Furthermore, YIGSR and AG73 have already been shown to enhance transfection (albeit conjugated to complexes rather than a substrate) (327, 328). Thus, given that the ECM contains many different proteins (e.g. laminin, tenascin, vitronectin, fibrillin, osteonectin, and others (329)) using a combination of ligands should be implemented and could have a synergistic effect on improving the cellular response to transfection. Moreover, using a combination of the aforementioned ligands to modify polymer brushes would maintain the nonfouling properties of the brushes (compared to immobilizing proteins to a substrate), yet stimulate the expected effect of the ligand (273). The addition of multiple types of cell adhesion peptides has been shown in hydrogels and other tissue engineering scaffolds (320, 322, 330-332), and this modification has also been used on a 2D platform to directly functionalize Ti (333). In this investigation, Fraioli et al. showed that Ti was able to be covalently bound with two synergistic motifs (RGD and PHSRN), and cellular adhesion and differentiation were increased in stem cells cultured on the substrates modified with both peptides compared to those bare Ti substrates or fibronectin. Thus, since cellular adhesion peptides have been shown to improve the cellular response to transfection (129, 327, 328), another future application of this project may be conjugating multiple cellular adhesion peptides (i.e. RGD, IKVAV, YIGSR, AG73) to PAA brushes to modulate the cellular response and further improve transfection.

6.2.2. Future Direction: Binary Polymer Brushes for Multiple Stimuli-Responses
In addition to varying the type of ligand and their density presented on polymer brushes in SMD, two different polymers with different stimuli-responses may be grafted to the substrate to form a binary polymer that enables further alteration of the physiochemical characteristics of the substrates (e.g. wettability, biocompatibility, surface charge, chemical composition) (155, 211, 334, 335) to tune complex immobilization and cell-material interactions. There, a future direction of this project may be to graft binary polymer brush films to the substrate to further tune the chemistry of the substrate and modulate the cellular response. The addition of binary films can be accomplished by randomly adsorbing the different polymers to the substrate (336), or in an ordered fashion by patterning a substrate (e.g. photolithography mask (337)) or by forming the brushes on a gradient stage (338). The two polymers used to create binary brushes may be reactive to the same stimuli; for example, weak anionic and cationic polyelectrolytes, poly(acrylic acid) (PAA) and poly(2-vinylpyridine) (P2VP) are two pH-reactive polymers that have been used to graft binary polymer brush films onto silicon (Si), whereas the pH environment for swollen brushes is a pH of 10 and 2, respectively (213, 334). More commonly, binary brush films are formed to respond to different stimuli unique to each polymer to increase the functionality of the brush layer (155, 185, 197, 335). In this regard, thermoresponsive polymers are often used with pH-stimuli responsive polymers, and some of these thermoresponsive polymers include poly(2-(dimethylamino)ethyl methacrylate), hydroxypropylcellulose, poly(vinylcaprolactame), and polyvinyl methyl ether but the most studied is
PNIPAM (339). PNIPAM exhibits temperature-dependent water solubility and undergoes a reversible phase transition due to having a lower critical solution temperature (LCST) of 32°C (197, 340) (i.e. close to physiological temperature). The LCST controls the hydrophilicity of the substrate where the substrate is hydrophobic at higher temperatures (i.e. encouraging protein adsorption and cell attachment above 32°C (341)) and hydrophilic at a lower temperature i.e. cell repellent below 32°C (341); thus, PNIPAM has often been used for “cell sheet engineering”, i.e. producing detachable monolayers of cells for tissue engineering applications (342-347). For our application with nonviral gene delivery, blending PNIPAM with PAA brushes onto a Si substrate has been shown to further increase the negative charge of the polymer brushes at pH 7 (197), suggesting that electrostatic interactions with free polymer and PEI-DNA complexes may be enhanced, thus allowing for an increase of immobilized total mass. Given that the “grafting to” of PNIPAM has been shown to be feasible on Ti (348) and that polymer brush films formed with PAA and PNIPAM have been previously documented (211), forming binary brushes with PAA and PNIPAM by grafting onto Ti may be feasible. Although PNIPAM been shown to be cell-repellent (348), these brushes, like PAA, can be conjugated with peptides to enhance the cellular adhesion to the substrate (186). Therefore, a future direction of this project may be utilizing binary brushes for the patterned presentation of RGD clusters or the presentation of different cell adhesion peptides (i.e. RGD, IKVAV, YIGSR, AG73) that are uniquely conjugated to different polymer brushes.
Thus, as a culmination of all these ideas (i.e. free polymer, ECM ligands, and brush composition), a future direction of this project would be to form a binary brush film with PAA/PNIPAM grafted to Ti (conjugated with cell adhesion peptides) as an effective culture substrate to differentiate human mesenchymal stem cells (hMSCs). As a SMD platform, PAA/PNIPAM could immobilize and then deliver PEI-DNA complexes (with adjuvant-like free PEI) while stimulating cellular behaviors that enhance transfection through conjugated ligands to the cultured hMSCs, subsequently differentiating the cells into a specific cell type through the nature of the delivered genes; for example, osteoblasts to aid in integration of a hip or knee implant or cardiomyocytes to prevent restenosis of a cardiac stent. Modification of the substrate with polymer brush films is a highly tunable process (i.e. free polymer, ECM ligands, and brush composition) with many possible applications to increase the functionality of biomaterials for applications in regenerative medicine and tissue engineering, which may also be further enhanced through the addition of topographical features to the substrate as discussed in the next section.

6.2.3. A Combinatorial Approach of Chemical Substrate Modifications with Physical Substrate Modifications

Similar to chemical modifications, physical modifications of the substrate impact cell-material interactions while maintaining bulk material properties such as biocompatibility and hardness (349, 350). Physical characteristics of the substrate have been shown to affect cellular adhesion, spreading, migration, proliferation, and morphology, presumably by spatially confining adsorbed ECM
proteins and cells (103, 351-354) or by mediating cytoskeletal tension (281, 355-357) and thus combining physical modifications with the chemical modification described in this dissertation (PAA-RGD brushes) may be effective at further enhancing transfection in cultured cells. Physical modifications often are performed through the addition of topographical features (358, 359), which are designed to mimic the physical cues of the ECM (100, 329) to influence cell-material interactions. Micro- and nanotopographical features have been shown to be innately patterned on native ECM and basement membrane by proteins forming complex hierarchically structured microscale and nanoscale pores, grooves, ridges, and fibers (100). Physical surface modifications can be accomplished by etching (360), lithography or imprinting (361), or depositing (362) topographical features in a variety of ordered and disordered architectures, such as columns, grooves, islands, pits, pores, wires, and more (363).

Topographical features on biomaterials have been shown to affect cellular behaviors, including motility (310, 364), focal adhesion formation (365-368), and actin fiber alignment (312, 369-371), which may affect transfection success as often discussed in this dissertation. In studies on the effect of nanotopography on the cellular response, along with multiple cell types and materials used, the parameters of the nanostructures vary extensively for factors such as height, spacing between structures, arrangement, and diameter. Some of these studies indicate that the diameter of the structures is the important factor to dictate cell behavior. One such study indicated Ti nanotubes with a 15 nm diameter showed an increase in migration, proliferation, and adhesion in hMSCs compared to
nanotubes with 20-100 nm diameter, and structures that were wider than 70 nm were shown to impair the proliferation and migration of the hMSCs (372). Others have studied the role of nanofeature height, showing increased proliferation and adhesion of primary human fibroblasts on shorter polymer islands (13 nm) compared to structures with a height of 95 nm (373). When investigating the effect of the cellular response to nanotopography on subsequent transfection outcomes, many studies suggest the interplay of integrin activation, focal adhesion formation, and cytoskeletal arrangement as critical determinants of cellular transfectability (312, 367, 368, 374-376). Given that behavioral enhancement is contingent upon multiple features of nanostructures (e.g. parameters, materials), it may be crucial to study platforms with many topographical features, combined with chemical modifications as discussed in this dissertation, to identify key determinants to improve nonviral gene delivery transfection success (376).

6.2.3.1. Future Direction: Combinatorial Physical and Chemical Cues for Enhanced Cellular Response to Transfection via SMD

The findings of the cited studies above show promising evidence that nanotopography can be used to stimulate the cellular response to transfection, but combining chemical and physical modifications to the substrate has been shown to be effective to enhancing the cellular response to a biomaterial and to transfection (100, 236, 367, 368, 374, 377-379). For example, hMSCs cultured on substrates modified with polyurethane (PU) microgrooves showed high levels of transfection compared to those cultured on flat PU substrates (and
investigated other chemistries and topographies, e.g. chitosan-HA, electrospun PU fibers), which was associated with the activation of integrins and correlated to the migration rate of the cultured cells (368). Thus, using our chemical modification of grafting PAA-RGD brushes onto topographical Ti features could enhance the cellular response to transfection, but there is a need to study the mechanisms of this response through an ordered topography using a technique that can produce variable topographical parameters (i.e. height, spacing), which may be feasible with sculptured thin films (STFs) formed by glancing angle deposition (GLAD) (228). STFs consist of nanostructures made by physical vapor deposition and the nanotopography produced by GLAD is determined by the angle of incidence relative to the vapor flux, substrate rotation, and deposition, to produce precisely ordered columns in many different conformations (228). STFs can be made with highly reproducible film thickness, slanting angles, and spacing with many different materials (362, 380, 381). Although other fabrication methods can produce ordered structures (e.g. lithography) (382), the advantages of GLAD include nanolevel spacing (228, 383), low cost (381, 383), and a wide range of specific geometries (362). Therefore, one of the future directions of this project is adding topographical modifications to the substrate using GLAD in a combinatorial approach with PAA-RGD brushes to produce highly ordered, reproducible Ti nanotopography (Figure 6-1) to study the cellular response to the culturing substrate and the relationship of cell-material interactions to subsequent transfection outcomes.
In a previous study, the cellular response to nanotopography was investigated using Ti STFs, which showed that STFs promote cell adhesion and proliferation of NIH/3T3 fibroblasts and murine stem cells (mMSCs) compared to bare controls (Flat Ti), and these cellular responses were most enhanced on pnSCTFs with an intercolumnar spacing of ~100 nm (228). Preliminary investigations on bare STFs (i.e. no PAA brushes), with varied heights (373), showed that fibroblasts cultured on STFs showed enhanced transfection in comparison to cells cultured on Flat Ti, which was dependent on the STF parameters (i.e. height, intercolumnar spacing) (Figure B-1, Figure B-2, Table B-1 in Appendix). SEM investigations into the cellular response to Ti STFs showed that the production of podia associated with transfection (membrane ruffles (312) and filopodia (109)) was significantly altered by the nanotopography of the culturing substrate (Table B-2; Figure B-3 in Appendix), which is discussed in greater detail in Appendix B. The results of these preliminary studies showed a strong correlation between cellular response (i.e. podia production) and transfection success (Table B-3 in Appendix), but the enhancement was marginal with a bolus delivery of a lipoplex in cells cultured on bare STFs. Therefore, a combinatorial approach of chemically grafting PAA brushes to Ti STFs may be
effective to further enhance the efficacy of transfection via SMD. Previous work in our lab has shown that Si STFs are able to be grafted with PAA brushes that maintain their swelling functionality (30), given that there was no difference in PAA brush grafting to flat Si or flat Ti substrates (116), suggesting that functionalizing Ti STFs with PAA is feasible, which was confirmed by preliminary measurements (Table B-4 in Appendix). Preliminary transfection studies suggested that a combinatorial approach of physically and chemically modifying a substrate with Ti STFs grafted with PAA-RGD brushes may further enhance transfection via SMD (Figure B-4, Figure B-5; discussed further in Appendix B), but further investigations are necessary to understand the immobilization and release of PEI-DNA complexes from these substrates. Moreover, the cellular response to cells cultured on these Ti STFs grafted with PAA-RGD brushes are required to understand the effect on transfection in comparison to cells that are cultured on PAA-RGD on Flat Ti.

6.3. Final Conclusions

The results of this dissertation suggest that grafting PAA brushes to Ti, and conjugating RGD ligands to those brushes, can effectively enhance SMD in fibroblasts cultured on those substrates in comparison to those cultured on bare Ti or on PAA brushes on Ti (i.e. no peptide). Future mechanistic studies with PAA-RGD brushes to understand the cellular response to transfection (e.g. integrin binding, endocytosis, intracellular trafficking) of more clinically applicable cells, such as hMSCs, are necessary. Furthermore, tuning of the platform with
specific free PEI dosages, peptides from the ECM, and a secondary polymer (i.e. PNIPAM), would result in a more complex binary brush film to improve transfection. Furthermore, the addition of topographical features for a combinatorial stimulation of the cellular response to physical and chemical priming cues may also be used to enhance transfection outcomes in applications of regenerative medicine and tissue engineering e.g. to deliver therapeutic genes to promote healing, in applications of medical device and to decrease the inflammatory response, and to differentiate hMSCs into specific cell types to increase the integration of biomaterial implants and diagnostic sensors.
References

45. Dai Z, Gjetting T, Mattebjerg MA, Wu C, Andresen TL. Elucidating the interplay between DNA-condensing and free polycations in gene transfection through a mechanistic study of linear and branched PEI. Biomaterials. 2011;32(33):8626-34.


236. Shekhar S, Lee B, Roy A, Candiello J, Kumta PN. Surface mediated non-viral gene transfection on titanium substrates using polymer electrolyte and


373. Dalby MJ, Yarwood SJ, Riehle MO, Johnstone HJ, Affrossman S, Curtis AS. Increasing fibroblast response to materials using nanotopography:


APPENDIX A
Supporting Information for Chapter 5

A.1. Methods

A.1.1. Cellular Adhesion of NIH/3T3 Fibroblasts on PAA-GRGDS Brushes

To assess if cellular adhesion increased in response to the RGD concentration, cultured NIH/3T3 fibroblasts were stained with calcein from a LIVE/DEAD™ Viability and Cytotoxicity Kit (Life Technologies, Carlsbad, CA) to quantify the cell counts per area (cm²) at 48 h following cell seeding. Briefly, surfaces with adhered cells were transferred into new well plates prior to the assays. Substrates for staining were rinsed with PBS and then stained for 20 min in phenol-free DMEM (Fisher Scientific) with 2 μM Calcein-AM. Substrates were imaged with a Leica DMI 3000B fluorescence microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany) and three images per well of three replicate wells were acquired using a 5x objective. Image analyses were performed using NIH ImageJ Processing Software to quantify cell counts per area (cm²).

A.1.2. AFM-IR of GRGDS Conjugated to PAA Brushes

To confirm the conjugation of the peptides and increased concentration of GRGDS conjugated to the PAA brushes, a nanoIR2 (Anasys Instruments, Inc.) was used to collect localized nanoIR spectra, as well as chemical IR imaging at a constant wavelength. Contact mode nIR2 probes (Model: PR-EX-nIR2, Anasys Instruments) with a resonance frequency of 13 ± 4 kHz and spring constant of 0.07–0.4 N/m were used. The pulsed tunable IR source has a pulse length of
~10 ns and the chemical composition of unmodified PAA brushes was quantified by looking at a characteristic peak for the carboxylic acid (1715 cm\(^{-1}\)) and covalent binding of RGD peptides was shown by monitoring the peak for amide I (1650 cm\(^{-1}\)) (185, 384, 385). The processing and evaluation of the data were conducted with the software NanoScope Analysis (version 1.5; Bruker AXS).

**A.1.3. DNA Complex Characterization and Immobilization**

To characterize the complexes, the size and zeta potential of the formed PEI/DNA complexes (with 2 kDa bPEI, 25 kDa bPEI, 2.5 kDa LPEI, or 25 kDa LPEI) were determined by dynamic light scattering and Laser Doppler micro-electrophoresis, respectively, using a Zetasizer Nano ZS90 (Malvern Instruments Ltd, UK). Size measurements were taken at 25°C at a scattering angle of 90° and size reported as the Z-average diameter (d. nm). Zeta potential measurements were also taken at 25°C using folded capillary cells with the measurement mode set to automatic and the values reported in mV. Values are reported as means for triplicate measurements with the standard error of the mean.

Next, to characterize immobilization of the complexes, PEI/DNA complexes (with 2 kDa bPEI, 25 kDa bPEI, 2.5 kDa LPEI, or 25 kDa LPEI) were formed with DNA plasmids labeled with Cy®5 using a Label IT® Nucleic Acid Labeling Kit (Mirus Bio LLC, Madison, WI) and then formed complexes were allowed to adsorb to PAA brush substrates for 2 hours. After the complexes had immobilized to the PAA brushes, a rinse step was performed (with OptiMEM) to remove any loose (i.e. not adsorbed) complexes or free PEI. Finally, the
immobilized DNA plasmids (in formed PEI-DNA complexes) were imaged using a Cytation 1 cell Imaging System (Biotek, Winooski, VT) configured with a 4x objective and a light cube for Cy®5. Images were processed using ImageJ (NIH) and complex immobilization was calculated as DNA plasmids/cm² and converted to µg/cm² using the molecular weight of the DNA plasmid (4.2x10⁶ g/mol).
A.2 Table

Table A-1: Density of PEI-DNA complexes immobilized to PAA brushes. PEI-DNA complexes (with 2 kDa bPEI, 25 kDa bPEI, 2.5 kDa LPEI, or 25 kDa LPEI) were formed with Cy®5 labeled DNA plasmids and then formed complexes were allowed to adsorb to PAA brush substrates for 2 hours. To quantify the density of DNA plasmids (in formed DNA complexes) adsorbed to PAA brushes, the area of a DNA plasmid (i.e. ~50 nm diameter (386)) was calculated and used to estimate the plasmids/cm², then converted to µg/cm² using the molecular weight of the DNA plasmid (4.2x10⁶ g/mol).

<table>
<thead>
<tr>
<th>PEI vector</th>
<th>Plasmids/cm²</th>
<th>µg/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 kDa bPEI</td>
<td>2.11E+08</td>
<td>0.05</td>
</tr>
<tr>
<td>2 kDa bPEI</td>
<td>1.17E+08</td>
<td>0.03</td>
</tr>
<tr>
<td>25 kDa LPEI</td>
<td>1.95E+08</td>
<td>0.05</td>
</tr>
<tr>
<td>2.5 kDa LPEI</td>
<td>1.26E+08</td>
<td>0.03</td>
</tr>
</tbody>
</table>
A.3 Figures

**Figure A-1:** Viability staining of NIH/3T3 fibroblasts cultured on PAA brushes with RGD peptides. Cells were cultured on substrates for 48 hours and then stained with 2 µM calcein-AM and quantified as the amount of cells adhered per area (cm²). Statistical differences were analyzed using one-way ANOVA with Tukey’s post-test. There were significantly more cells adhered to PAA-GRGDS at a concentration of 1.5 mg/mL in comparison to all other substrates (i.e. GRGDS at 0.5 mg/mL and 1.0 mg/mL, RGE at all concentrations, and PAA) (***, P≤0.001; **, P<0.05, and *, P<0.01, respectively).
Figure A-2: AFM-IR of PAA and PAA-GRGDS. AFM-IR measurements were taken of carboxyl and amide I (1715 cm⁻¹ and 1650 cm⁻¹, respectively) to quantify GRGDS conjugation. PAA brushes without RGD had almost no amide groups and thus had the lowest mean value for the histogram ratio. As the GRGDS concentration increased, so the measurement mapping at 1650 cm⁻¹ and the mean of the histogram ratio, although the values were similar.
Figure A-3: Zeta potential and sizing of PEI-DNA complexes with different PEI vectors. PEI-DNA complexes were formed with 2 µg of DNA at N/P of 20, and the zeta potential (A) and size (B) of the complexes were determined by dynamic light scattering and Laser Doppler micro-electrophoresis, respectively, at room temperature. Statistical differences between the measurements for zeta potential (A) and complex diameter (B) were analyzed using one-way ANOVA with Tukey’s post-test. The zeta potential measurements (A) showed a significant increase in the charge of all complexes formed with 25 kDa bPEI, 25 kDa LPEI, and 2.5 kDa LPEI compared to those formed with 2 kDa bPEI (****; P≤0.0001), as well as a significant increase in the charge of complexes formed with 25 kDa bPEI and LPEI, compared to those formed with 2.5 kDa LPEI (**; P≤0.01, and *; P<0.05, respectively). The sizing of the complexes (B) showed that, regardless of the PEI vector, the PEI-DNA complexes formed at a N/P ratio of 20 did not have significantly different sizes.
**Figure A-4:** PEI-DNA complexes formed with Cy®5 labeled DNA plasmids immobilized to PAA brushes. PEI-DNA complexes (with 2 kDa bPEI (A), 25 kDa bPEI (B), 2.5 kDa LPEI (C), or 25 kDa LPEI(D)) were formed with Cy®5 labeled DNA plasmids and then formed complexes were allowed to adsorb to PAA brush substrates for 2 hours. After a rinse step was performed (with OptiMEM) to remove any loose (i.e. not adsorbed) complexes or free PEI. The immobilized DNA plasmids (in formed PEI-DNA complexes) were imaged using a Cytation 1 cell Imaging System (Biotek, Winooski, VT) configured with a 4x objective and a light cube for Cy®5. (Scale bar=0.05 cm).
APPENDIX B
Supporting Information for Chapter 6

B.1 Supplemental Text

Chapter 7 was focused on discussing the future directions of this project in regards to modifying the parameters of the polymer brush films or the ligands presented to prime the cellular response to nonviral gene delivery via SMD, as well as some possible physical modifications to the substrate (i.e. sculptured thin films (STFs)). Previous investigations with STFs were performed with nanostructures at a height of 100 nm and shorter columnar height has been previously identified as an important variable in controlling the cellular response (313, 373) and this appendix describes the cellular response to bare STFs (i.e. without the addition of PAA brushes or complexes for SMD) and the efficacy of bolus nonviral gene delivery after incorporating varied columnar heights.

B.2 Supplemental Results and Discussion

B.2.1. Bolus Investigations on STFs

For preliminary investigations, STFs were fabricated and modeled as previously published (228). Briefly, for slanted columnar thin film (SCTF, Figure B-1) depositions, Ti was evaporated with a deposition thickness of 2200 Å and an 85° vapor flux angle and vertical columnar thin films (VCTFs, Figure B-1) were deposited with a deposition thickness of 2400 Å, 86° vapor flux, and 3 rpm counter-clockwise substrate rotation, both of which resulted in 100 nm thick films. SCTFs with wider column spacing referred to as pre-nucleated SCTFs (pnSCTFs, Figure B-1), were prepared by depositing SCTFs on a pre-nucleated
Ti adhesion layer. The Ti nucleation layer was deposited with a 100 Å thickness and 0º vapor flux, followed immediately by either a 1500, 750, or 375 Å deposition of Ti at an 85º vapor flux for a thickness of 100, 50, or 25 nm, respectively. Finally, Flat Ti substrates (Figure B-1) were deposited at a 0º vapor flux angle and rotated at 2 rpm in a counterclockwise direction to produce a smooth film. Immediately following the fabrication of STFs, generalized ellipsometry (GE) measurements of STFs were acquired using a Woollam RC2 spectroscopic ellipsometer (J.A. Woollam, Co., Inc., Lincoln, NE) to confirm deposited film parameters using the previously published procedure for ellipsometric measurement modeling and acquisition (228, 387-389). Briefly, spectroscopic data was acquired at multiple discrete wavelengths between 400 and 1600 nm, four angles of incidence (AOI: 45º, 55º, 65º, and 75º), and 0-360º rotation (measured every 12º) in the polar azimuth plane, measured in standard ambient temperature and pressure conditions. Spectral Mueller-matrix data obtained by GE was modeled and analyzed with WVASE32 software (J.A. Woollam Co.) using an anisotropic Bruggeman effective medium approximation (AB-EMA) approach, which allows for the determination of geometrical thin film parameters as well as fractions of multiple constituents (228, 390). Triplicate substrates were analyzed for the average height of the film (nm), theta slanting angle of the column (º), and the fraction of Ti material (%) (Table B-1).

First, transfection investigations with fibroblasts were performed using Lipofectamine 2000 (LF2000)-DNA complexes with bolus delivery. When cells were cultured on STFs at a height of 100 nm and varied columnar orientations,
there was no significant difference in transfection success between the different topographies (Figure B-2A). Previous research in our lab showed that NIH/3T3 cells primed with 100 nm pnSCTFs were the most proliferative compared to cells on other STF types (228), but the cellular response to nanostructures has been shown to be significantly altered by structural height (309, 373, 391, 392); thus, we also tested the pnSCTFs at heights of 50 and 25 nm (Figure B-1). Changing the height of the pnSCTFs resulted in higher transfection for all cells cultured on nanotopography compared to Flat Ti (Figure B-2B), and a statistically significant increase of transfection in fibroblasts cultured on 50 nm pnSCTFs compared to those cultured on Flat Ti (Figure B-2B; *, p≤0.05). Therefore, pnSCTFs at varying heights were used to investigate the cellular response to transfection and nanostructures.

Next, to test our hypothesis that varied nanostructures that stimulate different actin features involved in gene delivery, cells were cultured on pnSCTFs and Flat Ti and then observed at 18 hours (i.e. when the addition of complexes is performed for bolus transfection) for podia production (i.e. membrane ruffles and filopodia) that may affect transfection (109, 229, 393). SEM was performed to determine the podia types stimulated in cells primed with STFs to differentiate the cellular response to STF presentation (Figure B-3). A difference in podia production in NIH/3T3 cells primed with STFs was seen when compared to cells primed with Flat Ti and certain unique morphologies were identified. For example, when fibroblasts were primed with 25 nm pnSCTFs (Figure B-3A, E, I), the frequency of cells with filopodia cultured on 25 nm pnSCTFs was highest at
80% (Table B-2; n=60) compared to all other topographies (i.e. 50 and 100 nm pnSCTFs, and Flat Ti) (Table B-2; 77, 73, and 63%, respectively). In addition, there were more filopodia per cell in NIH/3T3 cells primed with 25 nm pnSCTFs (Table B-2; 12±1.1 filopodia per cell) compared to cells cultured on all other topographies (i.e. 50 and 100 nm pnSCTFs, and Flat Ti) (Table B-2; 7.9±0.57, 6.0±0.90, and 7.1±0.95 filopodia per cell, respectively), which was all significant (§§, p≤0.01; §§§, p≤0.0001; §, p≤0.05; respectively). Previous investigations have suggested that improved endocytosis (and subsequent transfection) may be associated with filopodia, either by the assembly and disassembly of focal adhesions (83) (and thus endocytosis and intracellular trafficking) or by “carrying” complexes into the intracellular environment of the cell body (109). Thus, the increase of filopodia on cells cultured on 25 nm pnSCTFs may have caused the non-statistical improvement of transfection success compared to cells cultured on Flat Ti (Figure B-2B). Next, fibroblasts primed with 50 nm pnSCTF (Figure B-3B, F, J) had the highest frequency of cells with membrane ruffles at 63% (Table B-2; n=60) compared to all other topographies (i.e. 25 and 100 nm pnSCTFs, and Flat Ti) (Table B-2; 27, 48, and 20%, respectively) and membrane ruffles are a podia type specifically related to a commonly investigated endocytic pathway, macropinocytosis (69). Thus, the significantly higher level of transfection in cells cultured on 50 nm pnSCTFs compared to those on Flat Ti (Figure B-2B) may be related to enhanced endocytosis, similar to the previously described study by Teo et al. (312). Furthermore, fibroblasts cultured on 50 and 100 nm pnSCTFs (Figure B-3C, G, K) had the shortest filopodia lengths with an average length of
3.1±0.38 and 3.4±0.41 µm, respectively (Table B-2) and cells cultured on 100 nm pnSCTFs had the least amount of filopodia per cell at 6.0±0.90 (Table B-2).

Once again, filopodia may “sweep” complexes toward the cell body for improved endocytosis (109), which may be more enhanced by shorter filopodia that could sufficiently maintain electrostatic interactions to bring the complexes to the cell body compared to longer filopodia. Thus, shorter filopodia produced by cells cultured on 100 nm pnSCTFs may have aided the process of endocytosis, thereby causing the non-statistical improvement in transfection success compared to cells cultured on Flat Ti (Figure B-2B). Finally, fibroblasts adhered to Flat Ti (Figure B-3D, H, L) showed the significantly longer filopodia extensions (Table B-2; 11±1.3 µm) compared to all other topographies (i.e. 25, 50, and 100 nm pnSCTFs) (Table B-2; ***, p≤0.001; ****, p≤0.0001, ****, p≤0.0001, respectively). On Flat Ti, fibroblasts showed filopodia extending far from the peripheral edges of the cell body, which may be due to lack of topographical interactions that provide a place for the cells to ‘grab’ (371) and assemble stable focal adhesions. Thus, NIH/3T3 cells primed with Flat Ti may form more transient attachment to the substrate (188, 394), suggesting that fibroblasts would be more motile when culture on Flat Ti, which has been correlated with increased endocytosis (368); thus, culturing NIH/3T3 fibroblasts on Flat Ti may have influenced transfection through cellular motility.

Using the analysis of the SEM micrographs and transfection studies, podia production was correlated to STF height and transfection success to show their relationships. For example, membrane ruffles had a high correlation to STF
height (0.800; Table B-3); which indicates that the height of the STF substrate will influence the amount of cells that produce membrane ruffles. Relationships between the podia (filopodia vs. membrane ruffles) had low correlation values (0.400; Table B-3). A filopodium forms a focal adhesion to adhere to the substrate, after which the actin branches into a lamellipodium, finally forming a membrane ruffle after the lamellar adhesion to the substrate is broken (395), therefore the low correlation results are sensible. Furthermore, Spearman’s correlation showed that transfection success was perfectly correlated to membrane ruffle production (Table B-3), which demonstrates improving transfection via induction of specific podia types associated with endocytosis (i.e. macropinocytosis (69)) may be feasible. Furthermore, previous investigations have shown that receptor-mediated endocytic pathways (i.e. clathrin-mediated and caveolae-mediated) can be stimulated by nanotopography (396-399); thus, future investigations on STFs would investigate other types of endocytic pathways to correlate endocytosis, nanotopography, and podia production. Collectively, these preliminary results suggest that ordered nanotopography can prime cellular podia production, possibly stimulating macropinocytosis, which in turn can improve transfection efficiency of bolus gene delivery.

B.2.2. SMD Investigations on STFs

Since transfection can be enhanced by transfecting cultured cells via SMD instead of bolus delivery (268), and SMD can be enhanced via PAA brush modifications (267), further investigations were performed using Ti STFs grafted
with PAA brushes conjugated with linear RGD. For preliminary investigations, the fabrication of 100 nm pnSCTFs was performed as described previously in Appendix B and grafted with PAA brushes as described in chapters 3, 4, and 5. Next, GE measurements were performed to characterize the bare pnSCTFs, as well as PAA brush grafting and swelling. Briefly, spectroscopic data was acquired at multiple discrete wavelengths between 400 and 1600 nm for dry brushes at AOI of 45º, 55º, 65º and 75º with 0-360º rotation (by 12º) in the polar azimuth plane, and 400 and 1200 nm for swollen brushes in phosphate buffer solution (PBS) at pH 7.4 at AOI of 70º with no rotation in the polar azimuth plane, measured in standard ambient temperature and pressure conditions. Spectral Mueller-matrix data was, again, modeled and analyzed with WVASE32 software (J.A. Woollam Co.) using an AB-EMA approach, with the addition of a Cauchy layer for PAA brushes around the STFs and ambient PBS optical constants for the swelling measurements. PAA brushes grafted to the Ti pnSCTFs in a reproducible fashion with a dry brush EMA percentage of 19.3±1.50% within the structures (Table B-4), which is similar to a previous investigation in our lab that Si SCTFs had an average brush fraction of 25.4±3.52% (30). After grafting of the brushes, the height and the theta angle (with respect to normal) of the structures was slightly decreased (Table B-4; 12.2 nm and 2.20º, respectively), suggesting a slight bend in the nanostructures after grafting. This decrease of the Ti pnSCTF height and slanting angle was less substantial than those formed with Si SCTFs (i.e. 16.5±1.74 nm and 6.86±1.21º, respectively (30)), which suggests that Ti
pnSCTFs (which have an increase in the space between nanostructures compared to SCTFs (228)) are more stable than Si SCTFs.

Although the total mass of complex immobilization can be modeled with QCM/GE, the process can also be monitored with the immobilization of radioactively labeled DNA (267). Therefore, preliminary experiments were performed to determine the amount of DNA immobilized to 100 nm pnSCTFs (with and without PAA brushes, modified by linear RGD). The amount of immobilized DNA (in µg/cm²) was measured on modified substrates for all investigated substrates (i.e. bare pnSCTFs, PAA on pnSCTFs, PAA-RGD on pnSCTFs, and PAA-RGD on Flat Ti) (Figure B-4; 0.0697±0.0168 µg/cm², 0.0249±0.00512 µg/cm², 0.0630±0.00223 µg/cm², and 0.102±0.0131 µg/cm², respectively), although PAA on pnSCTFs had significantly less DNA compared to PAA-RGD on Flat Ti (**; P≤0.01), which suggests that PAA brushes (without linear RGD modification) on STFs may have inhibited the amount of DNA immobilized to the brushes on the substrates. The results of the amount of DNA immobilized on the substrates seemed to correspond to transfection efficiency for pnSCTFs with PAA or PAA-RGD and PAA-RGD on Flat Ti, although there were no statistical differences (Figure B-5). Since cyclic RGD is a more activated form of RGD (186) that can further enhance transfection, future investigations would be performed with PAA brushes grafted to Ti STFs with covalently bound with cyclic RGD to investigate transfection as well as the cellular response to the substrates. Finally, significant investigations into the cellular response to PAA-RGD brushes grafted to Ti STFs are needed to understand the combinatorial
priming effect of culturing cells on these substrates (e.g. podia production, integrin binding, cytoskeletal arrangement, endocytosis, intracellular trafficking).
### B.3 Tables

**Table B-2:** Deposition of STFs via GLAD. Parameters measured include slanting angle (Theta), nanostructure porosity (STF Fraction) by altering factors such as the amount of material deposited, flux angle, and substrate rotation. The data are expressed as mean±standard error of the mean (SEM) (n=3).

<table>
<thead>
<tr>
<th>STF Type</th>
<th>Thickness (nm)</th>
<th>Theta (° w.r.t normal)</th>
<th>STF Fraction %</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCTF</td>
<td>120±8.2</td>
<td>40±1.2</td>
<td>13±1.1</td>
</tr>
<tr>
<td>VCTF</td>
<td>96±5.5</td>
<td>0 (fixed)</td>
<td>15±0.53</td>
</tr>
<tr>
<td>pnSCTF (25 nm)</td>
<td>23±1.0</td>
<td>26±3.3</td>
<td>14±2.5</td>
</tr>
<tr>
<td>pnSCTF (50 nm)</td>
<td>47±2.6</td>
<td>36±5.0</td>
<td>18±1.3</td>
</tr>
<tr>
<td>pnSCTF (100 nm)</td>
<td>91±3.3</td>
<td>43±6.9</td>
<td>14±1.7</td>
</tr>
<tr>
<td>Flat Ti</td>
<td>100±1.2</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table B-2:** Podia Features Measured Quantitatively. Fibroblasts cultured on STFs (n=60) had a high frequency of cells with filopodia when cultured on 25 nm pnSCTFs and the frequency decreased as the height of the structures increased, with the lowest frequency of filopodia-presenting cells occurring in those cultured on Flat Ti. Cells presenting membrane ruffles were most frequently seen in those cultured on 50 nm pnSCTFs. The average length of filopodia (n=26) and amount of filopodia per cell were significantly higher in cells cultured on Flat Ti and 25 nm pnSCTFs, respectively. Data are means ±SEM (***, p≤0.001; ****, p≤0.0001; compared to Flat Ti; §, p≤0.05; §§, p≤0.01; §§§, p≤0.0001; compared to 25 nm pnSCTFs).

<table>
<thead>
<tr>
<th>STF Type</th>
<th>25 nm pnSCTF</th>
<th>50 nm pnSCTF</th>
<th>100 nm pnSCTF</th>
<th>Flat Ti</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of Cells with (n=60)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Filopodia</td>
<td>80</td>
<td>77</td>
<td>73</td>
<td>63</td>
</tr>
<tr>
<td>Membrane Ruffles</td>
<td>27</td>
<td>63</td>
<td>48</td>
<td>20</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Filopodia Length</td>
<td>5.7±0.61***</td>
<td>3.1±0.38****</td>
<td>3.4±0.41****</td>
<td>11±1.3</td>
</tr>
<tr>
<td>Filopodia per Cell</td>
<td>12±1.1</td>
<td>7.9±0.57§§</td>
<td>6.0±0.90§§§§</td>
<td>7.1±0.95§</td>
</tr>
</tbody>
</table>
**Table B-3**: Spearman’s Correlation of Transfection Success Compared to Height and Podia Production.

<table>
<thead>
<tr>
<th></th>
<th>STF Height</th>
<th>Transfection Level</th>
<th>Filopodia</th>
<th>Membrane Ruffles</th>
</tr>
</thead>
<tbody>
<tr>
<td>STF Height</td>
<td>0.8</td>
<td>0.2</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Transfection Level</td>
<td>0.8</td>
<td>0.4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Filopodia</td>
<td>0.2</td>
<td>0.4</td>
<td></td>
<td>0.4</td>
</tr>
<tr>
<td>Membrane Ruffles</td>
<td>0.8</td>
<td>1</td>
<td></td>
<td>0.4</td>
</tr>
</tbody>
</table>

**Table B-4**: PAA Brushes Grafted to 100 nm pnSCTFs. Data are means ±SEM (n=10).

<table>
<thead>
<tr>
<th></th>
<th>Before Brushes</th>
<th>After Brushes</th>
</tr>
</thead>
<tbody>
<tr>
<td>STF Height (nm)</td>
<td>99.2±1.56</td>
<td>87.1±1.38</td>
</tr>
<tr>
<td>Theta (°)</td>
<td>38.0±1.55</td>
<td>40.2±1.90</td>
</tr>
<tr>
<td>STF Height (nm)</td>
<td>87.1±1.38</td>
<td>40.2±1.90</td>
</tr>
<tr>
<td>Theta (°)</td>
<td>40.2±1.90</td>
<td>19.3±1.50</td>
</tr>
<tr>
<td>Brush EMA (%)</td>
<td>19.3±1.50</td>
<td></td>
</tr>
</tbody>
</table>
B.4 Figures

Figure B-2: Titanium nanostructures formed via GLAD. Titanium sculptured thin films (Ti STFs) were formed into A) slanted columnar thin films (SCTFs), B) vertical columnar thin films (VCTFs), C) pre-nucleated slanted columnar thin films (pnSCTFs), and D) a control flat film of titanium (Flat Ti). The Ti STFs were all grown to a thickness of 100 nm, with two additional heights for pnSCTFs (25 and 50 nm).
Figure B-2: Transfection Investigations of NIH/3T3 fibroblasts cultured on STFs. A) The slanting angle and STF fraction of STFs formed at 100 nm thickness did not affect transfection success of cells cultured on nanostructures compared to those cultured Flat Ti. B) Decreasing the height of pnSCTFs altered transfection success, which showed that NIH/3T3 fibroblasts cultured on 50 nm pnSCTFs had significantly improved transfection compared to cells cultured on Flat Ti (*; P≤0.05). Data are means±SEM (n = 3).
Figure B-3: SEM investigations of NIH/3T3 fibroblast podia production cultured on STFs. Fibroblasts were cultured on pnSCTFs at heights of 25 nm (A, E, I), 50 nm (B, F, J), and 100 nm (C, G, K), as well as Flat Ti (D, H, L). Micrographs of the cells showed that cells on STF substrates had filopodia (black arrows) and membrane ruffles (white arrows), which have been shown to aid in transfection. Scale bars are 20 µm (A, E, J), 5 µm (B, C), 10 µm (D, F-H), 30 µm (I), 2 µm (K), and 1 µm (L).
Figure B-4: DNA complexes immobilized to PAA brushes on pnSCTFs compared to brushes on Flat Ti. PAA brushes grafted to pnSCTFs or Flat Ti were modified with linear RGD. For complexes formed at an N/P ratio of 20 with bPEI, the amount of material immobilized onto substrates measured via scintillation counting. Statistical analyses were completed using one-way ANOVA with Tukey’s post-test. There was a statistically significant difference between the amount of DNA immobilized to PAA on pnSCTFs compared to PAA-RGD on Flat Ti (**; P≤0.01).

Figure B-5: Transfection of NIH/3T3 fibroblasts on DNA complexes immobilized to PAA brushes on pnSCTFs compared to PAA brushes on Flat Ti. PAA brushes grafted to pnSCTFs or Flat Ti were modified with linear RGD. For complexes formed at an N/P ratio of 20 with bPEI, the amount of material immobilized onto substrates measured by radiolabeled DNA via scintillation counting. Statistical analyses were completed using one-way ANOVA with Tukey’s post-test and there was no statistically significant difference.