RICE UNIVERSITY

Development of Bioactive Polyurethaneureas
to Support Endothelialization

by

Ho-Wook Jun

A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE

Doctor of Philosophy

APPROVED, THESIS COMMITTEE:

Jennifer West, Associate Professor,
Bioengineering, Chair

Antonios Mikos, Professor,
Bioengineering

Kyriakos Zygourakis, Professor
Chemical Engineering

Jeffrey Hartgerink, Assistant Professor
Chemistry

Houston, Texas
April, 2004
INFORMATION TO USERS

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleed-through, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

UMI®

UMI Microform 3122493

Copyright 2004 by ProQuest Information and Learning Company. All rights reserved. This microform edition is protected against unauthorized copying under Title 17, United States Code.

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346
ABSTRACT

Development of bioactive polyurethaneureas to support endothelialization

By

Ho-Wook Jun

Vascular diseases are responsible for the majority of deaths in the United States. Synthetic materials have been developed for blood vessel substitutes but not suitable for small diameter vascular applications such as coronary artery bypass grafting (CABG). Polyurethaneureas (PUU) have been widely used for biomedical applications due to their excellent mechanical properties and relatively good biocompatibility. However, like other synthetic materials, they are generally thrombogenic on exposure to blood. Endothelialization of synthetic grafts is a good strategy to improve graft patency. However, the graft patency is dependent on retention of endothelial cells on exposure to physiological shear stress. In this study, we developed bioactive polyurethaneureas to support endothelialization. First, we have demonstrated that endothelial cell behaviors could be altered by the surface YIGSR peptide concentrations. Bioactive polyurethanureas (PUUYIGSR) have been developed by incorporating YIGSR peptide sequences into polymer main chain, and improved endothelialization has been observed on the surface. In addition, PEG- and YIGSR-modified polyurethaneureas (PUUYIGSR-
PEG) have been developed, and enhanced endothelialization and improved thromboresistance have been obtained simultaneously. Our bulk modification strategy allowed us to fabricate microporous scaffolds without interfering bioactivity of incorporated peptide sequences. Microporous scaffolds have been also used as a carrier of vascular endothelial growth factor (VEGF). The synergistic effects of peptide sequences, microporous structure, and incorporated VEGF on endothelialization have been observed. Additionally, nitric oxide (NO) releasing polyurethanes (PUBD-NO) have been developed by incorporating NO donor into the polymer main chain. NO was successfully released from the PUBD-NO in controlled manner and reduced platelet adhesion and smooth muscle cell proliferation but improved endothelialization proliferation.
Acknowledgements

It has been five years to finish this work.

Most of all, I acknowledge the enthusiastic supervision, patience, and support of Dr. Jennifer West for last five years. I also greatly appreciate Dr. Antonios Mikos, Dr. Kyriakos Zygourakis, and Dr. Jeffrey Hartgerink for serving on my committee.

In my office at Rice, I have been surrounded by knowledgeable and friendly members of West group. I thank to all of them, especially; to Liz for spending most of daytime by sitting next; to SooHong for sharing a pleasant coffee break; for Lakeshia for collaboration of nitric oxide study.

Funding was provided by NIH (R01-HL 60485).

Special thanks to my parents and parents-in-law for their devoted support, constant encouragement, and their earnest prayer.
Dedication to

My lovely wife, KyoungA Cecilia

My playful son, HyunWoong Joseph

My cute daughter, HyunJin Claire

Who

Inspired me to do this.
Table of Contents

Abstract i

Acknowledgements iii

List of Figures viii

Chapter 1. Background

1.1. Clinical significance of vascular grafts 1

1.2. Endothelialization 4

1.3. Bioactive biomaterials 7

1.4. Polyurethane and polyurethaneurea 12

1.5. Conclusions 21

Chapter 2. Surface modification with YIGSR peptide to alter endothelialization

2.1. Introduction 22

2.2. Materials and Methods 24

2.3. Results and Discussion 31

2.4. Conclusions 40
Chapter 3. Development of a YIGSR peptide-modified polyurethaneurea to enhance endothelialization

3.1. Introduction 41

3.2. Materials and Methods 44

3.3. Results 58

3.4. Discussion 74

3.5. Conclusions 77

Chapter 4. Modification of polyurethaneurea with PEG and YIGSR peptide to enhance endothelialization without platelet adhesion

4.1. Introduction 78

4.2. Materials and Methods 80

4.3. Results 88

4.4. Discussion 99

4.5. Conclusions 102

Chapter 5. Endothelialization on a microporous bioactive YIGSR/PEG-modified polyurethaneurea

5.1. Introduction 103

5.2. Materials and Methods 106
List of Figures

**Figure 1.1.** The Vascular diseases can be caused by various ways such as deposition of fatty plaques, damaged intima, or accumulation of cholesterol. It leads to CAD, heart attach, and stroke.

**Figure 1.2.** Synthetic ePTFE vascular grafts. Synthetic materials have been successfully used for large diameter vascular grafts but have experienced significant limitations for small diameter vascular grafts.

**Figure 1.3.** The structure of artery and vein. Blood vessels consist of intimal endothelial cell layer, medial smooth muscle cell layer, and outer connective tissue layer. The endothelial cell layer prevents thrombosis and regulates vascular smooth muscle cell tone.

**Figure 1.4.** Structure of laminin. YIGSR is located at B1 chain and RGD is located in A chain.

**Table 1.1.** Adhesive peptide sequences in extracellular matrix.

**Figure 1.5.** Surface modifications of biomaterials with cell adhesive peptide sequences. Ligand-receptor binding affects cellular behaviors such as proliferation, migration, and extracellular matrix production. Thus, it allows us to design biomimic materials.

**Figure 1.6.** Phase separation of polyurethane and polyurethanurea. (a) micro phase separation of hard segments of polyurethane (b) AFM tapping mode phase image of polyurethanurea.

**Figure 2.1.** Immobilization of YIGSR peptide to aminophase glass surface. Acetylated YIGSR peptide was coupled to aminophase surface at various concentrations.

**Figure 2.2.** Effect of YIGSR surface concentration on endothelial cell attachment. *P < 0.05, # P < 0.01, compared to aminophase. Empty column; aminophase, vertical column; YIGSR (0.23 nmol/cm²), horizontal column; YIGSR (0.34 nmol/cm²), diagonal column; YIGSR (0.61 nmol/cm²), solid column; YIGSR (1.53 nmol/cm²).
Figure 2.3. Effect of YIGSR surface concentration on cell spreading. *P < 0.05, #P < 0.01, compared to aminophase. Empty column; aminophase, vertical column; YIGSR (0.23 nmol/cm²), horizontal column; YIGSR (0.34 nmol/cm²), diagonal column; YIGSR (0.61 nmol/cm²), solid column; YIGSR (1.53 nmol/cm²).

Figure 2.4. Effect of YIGSR surface concentration on strength of endothelial cell adhesion. *P < 0.05, compared to aminophase glass not modified with YIGSR peptides.

Figure 2.5. Effect of YIGSR surface concentration on proliferation of endothelial cell. *P < 0.05, # P < 0.01, compared to aminophase glass not modified with YIGSR peptides.

Figure 2.6. Effect of YIGSR surface concentration on migration of endothelial cell. *P < 0.05, # P < 0.01, compared to aminophase glass not modified with YIGSR peptides.

Figure 2.7. Effect of YIGSR surface concentration on production of ECM proteins by endothelial cell. *P < 0.05, # P < 0.01, compared to aminophase glass not modified with YIGSR peptides.

Figure 2.8. Effect of YIGSR surface concentration on platelet adhesion. *P < 0.05, # P < 0.01, compared to aminophase glass not modified with YIGSR peptides.

Figure 3.1. The distribution of peptide sequences incorporated into the hard segment domains in polyurethaneurea matrix.

Figure 3.2. Synthesis of polyurethaneurea (PUUPPD). Prepolymer was synthesized from MDI and PTMO and then extended with PPD.

Figure 3.3. Synthesis of a peptide-modified polyurethaneurea (PUUYIGSR). Prepolymer was synthesized from MDI and PTMO and then extended with PPD and peptide.

Figure 3.4. In vitro anastomotic site was simulated.

Figure 3.5. ¹H NMR spectra of PUUYIGSR. Tyrosine peak was identified in a.

Table 3.1. Electron spectroscopy for chemical analysis (ESCA) measurements; atomic concentration of PUUPPD and PUUYIGSR films at variable take-off angles.
Figure 3.6. FT-IR spectra of PUUYIGSR.

Figure 3.7. DSC thermogram of PUUYIGSR.

Figure 3.8. AFM tapping mode phase images of the surfaces of polyurethaneurea at $r_{sp} = 0.88$. Phase images are 500 X 500 nm.

Figure 3.9. Attachment and phase contrast micrographs of BAECs on polyurethaneurea films. (a) number of adherent cells after 4 and 24 hr incubation. Data represent the mean of three samples. * $p < 0.05$, # $p < 0.01$, compared to PUUPPD. Shaded columns represent PUUPPD and filled columns PUUYIGSR. Phase contrast micrographs of BAECs on (b) PUUPPD and (c) PUUYIGSR films after 4 hr incubation. Scale bar = 50 $\mu$m.

Figure 3.10. Spreading of BAECs on polyurethaneurea films. (a) cell surface area, (b) percent of cells that were spread. Data represent the mean of thirty samples for cell surface areas and ten samples for percent of cell spreading. * $p < 0.01$, compared to PUUPPD. Shaded columns represent PUUPPD and filled columns PUUYIGSR.

Figure 3.11. Competitive inhibition of attachment and spreading of BAECs by soluble YIGSR peptides. (a) competitive inhibition of attachment. Data represent the mean of four samples. * $p < 0.02$, compared to PUUPPD, # $p < 0.01$, compared to untreated PUUYIGSR. (b) cell surface area and (c) percent of cells that were spread. Data represent the mean of thirty samples for cell surface areas and ten samples for percent of cell spreading. * $p < 0.05$, compared to PUUPPD. # $p < 0.001$, compared to untreated PUUYIGSR.

Figure 3.12. Proliferation of BAECs on polyurethaneurea films. * $p < 0.001$ compared to PUUPPD.

Figure 3.13. Migration of endothelial cells on polyurethaneurea films. * $p < 0.001$, compared to PUUPPD.

Figure 3.14. Migration of BAECs across simulated anastomotic sites; from collagen I surfaces to polyurethaneurea surfaces. Data represent the mean of six samples. * $p < 0.02$, compared to PUUPPD.
Figure 3.15. Extracellular matrix (ECM) production of endothelial cells on polyurethaneurea films. * $p < 0.05$, compared to PUUPPD.

Figure 3.16. The effect of the YIGSR peptide in the polyurethaneurea structure on the platelet adhesion. Platelets were fluorescently labeled with mepacrine. Data represent the mean of ten samples. * $p < 0.001$, compared to Collagen I.

Figure 4.1. The environment change leads to surface reorganization and minimize the interfacial free energy.

Figure 4.2. Synthesis of PEG and peptide modified polyurethaneurea. PEG was used as a soft segment and YIGSR peptide was incorporated into the hard segment.

Figure 4.3. $^1$H NMR spectra of PUUYIGSR-PEG. Tyrosine peaks were identified in a.

Figure 4.4. FTIR spectra of PUUYIGSR-PEG.

Figure 4.5. DSC thermogram of PUUPPD, PUUPPD-PEG, and PUUYIGSR-PEG. A second Tg was found in PEG-modified polyurethaneurea.

Table 4.1. Electron spectroscopy for chemical analysis (ESCA) measurements; atomic concentration of PUUPPD-PEG and PUUYIGSR-PEG films at variable take-off angles.

Figure 4.6. Attachment and spreading of BAECs on polyurethaneurea films after 4 hr incubation. (a) number of adherent cells. * $p < 0.005$ compared to PUUPPD, # $p < 0.001$, compared to PUUPPD PEG. (b) cell surface area and (c) percent of cells that were spread. # $p < 0.001$ compared to PUUPPD, * $p < 0.001$ compared to PUUPPD-PEG.

Figure 4.7. Phase contrast micrographs of BAECs on (a) PUUPPD-PEG and (b) PUUYIGSR-PEG after 4 hr incubation. The length of the scale bar = 50 µm.

Figure 4.8. Phase contrast micrographs of competitive inhibition of attachment and spreading of BAECs incubated with soluble YIGSR peptides at three different concentrations. (a) PUUYIGSR-PEG (b) + 0.01, (c) + 0.1, and (d) + 1 YIGSR. The length of the scale bar = 50 µm.
Figure 4.9. Competitive inhibition of attachment and spreading of BAECs by soluble YIGSR peptides after 4 hr incubation. BAECs were incubated with soluble YIGSR peptides at three different concentrations (0.01, 0.1, and 1 mM). (a) a number of adherent cells. * p < 0.001 compared to PUUPPD-PEG, # p < 0.001 compared to untreated PUUYIGSR-PEG. (b) cell surface areas and (c) cell spreading. * p < 0.001 compared to PUUPPD-PEG, # p < 0.001 compared to untreated PUUYIGSR-PEG.

Figure 4.10. Migration of BAECs on PUUYIGSR-PEG after (a) 24 hr and (b) 48 hr.

Figure 4.11. The number of adherent platelets on the surfaces of collagen I, PUUPPD, PUUPPD-PEG, and PUUYIGSR-PEG. Data represent the mean of five samples. * p < 0.001 compared to Collagen I, # p < 0.001 compared to PUUPPD. Platelets were fluorescently labeled with mepacrine.

Figure 5.1. Fabrication of microporous scaffolds using salt leaching and gas forming method. Sodium bicarbonate was used as a effervescent salt.

Figure 5.2. Migration of endothelial cells through the scaffolds. Cell seeded scaffold was placed into a transwell cell culture insert. After 7 days of culture, a number of cells migrated through the scaffold were counted.

Figure 5.3. SEM images of surface and cross section of the scaffolds. Highly interconnected pore structure was observed through the scaffold matrix. Length bar = 100 μm. (a) surface and (b) cross section of PUUPPD-PEG and (c) surface and (d) cross section of PUUYIGSR-PEG.

Figure 5.4. Mechanical properties of the PUUPPD-PEG and PUUYIGSR-PEG scaffolds. (a) tensile strength (MPa), * p<0.01 and (b) maximum elongation (%), * p< 0.02.

Figure 5.5. SEM images of endothelial cells cultured for 3 days in PUUPPD-PEG and PUUYIGSR-PEG scaffolds. Higher density cell colonies were found in PUUYIGSR-PEG scaffolds.

Figure 5.6. Hematoxylin-stained endothelial cells on PUUPPD-PEG and PUUYIGSR-PEG scaffolds. Endothelial cells were cultured for 3 days. Higher cell density was observed throughout the PUUYIGSR-PEG scaffolds.
**Figure 5.7.** Migration of endothelial cells through the PUUPPD-PEG and PUUYIGSR-PEG scaffold matrices after 7 days of culture. * p< 0.005.

**Figure 5.8.** DNA and Hydroxyproline production in PUUPPD-PEG and PUUYIGSR-PEG scaffold matrices after 3 days of culture. (a) DNA concentration (ng) per scaffold, * p< 0.001 and (b) hydroxyproline production (ng) per DNA (ng), * p< 0.05.

**Figure 6.1.** TVEGF functions through binding and activating VEGF-receptors. Receptors for VEGF are VEGF R1 (Flt-1), VEGF R2 (KDR), and VEGF R3 (Flt-1). Endothelial cells also express additional receptors such as Neuropilin-1 and -2.

**Figure 6.2.** The dose response of endothelial cells to VEGF. Four known concentrations of VEGF were exposed to endothelial cells for 48 hr. * p < 0.05 compared to control.

**Figure 6.3.** Release profile of VEGF from the scaffolds. Cumulative VEGF release (%) for 24 hr (a) and 14 days (c). VEGF release profiles at each time period for 24 hr (b) and 14 days (d).

**Figure 6.4.** Increase of number of endothelial cells exposed to conditioned medium containing released VEGF at each time period for 48 hr. * p < 0.05 compared to corresponding control (normalized to 100%).

**Table 6.1.** Bioactivity of the released VEGF. There was no significant effect of VEGF on endothelialization after 5 days.

**Figure 6.5.** DNA and hydroxyproline production of endothelial cells in PUUYIGSR-PEG and PUUYIGSR-VEGF scaffold after 3 days of culture. (a) DNA concentration (ng) per scaffold, * p < 0.01 and (b) hydroxyproline production (ng) per scaffold, * p < 0.05.

**Figure 6.6.** Histological sections of VEGF-loaded scaffolds implanted subcutaneously for 14 days. Stained with CD31 antibody and hematoxylin. Arrow indicates blood vessels.

**Figure 7.1.** Nitric oxide is an important molecule and involves in a wide range of physiological processes.
Figure 7.2. The formation of diazeniumdiolates from amines and spontaneous release of NO.

Figure 7.3. Synthesis of polyurethane (PUBD). Prepolymer was synthesized from MDI and PTMO and then extended with BD.

Figure 7.4. Synthesis of NO releasing polyurethane (PUBD-NO). Prepolymer was synthesized from MDI and PTMO and then extended with BD and peptide sequences containing diazeniumdiolates.

Figure 7.5. $^1$H NMR spectra of PUBD-NO which was synthesized by incorporating lysine sequences. Lysine peaks were identified in a.

Figure 7.6. The elastic modulus and the tensile strength of PUBD and PUBD-NO. *$p < 0.01$ compared to PUBD.

Figure 7.8. Proliferation of BAEC for PUBD and PUBD-NO films. * $p < 0.001$ compared to PUBD. Proliferating cells appear red and non-proliferating cells appear blue.

Figure 7.9. Proliferation of SDSMC for PUBD and PUBD-NO films. * $p < 0.001$ compared to PUBD. Proliferating cells appear red and non-proliferating cells appear blue.

Figure 7.10. The number of adherent platelets on collagen I, PUBD, and PUBD-NO. * $p < 0.001$ compared to collagen I, # $p < 0.001$ compared to PUBD.
Chapter 1. Background

1.1. Clinical significance of vascular grafts

Vascular diseases, especially atherosclerosis, are responsible for the majority of deaths in the United States (Edward et al., 1998). Atherosclerosis is caused by deposition of fatty plaques on the inner walls of blood vessel. These deposits reduce blood flow and cause coronary artery disease (CAD), heart attack, and stroke (Nikalson, 1999). A common procedure used to direct blood flow around occluded segments is bypass grafting. In coronary artery bypass grafting (CABG), blood flow can be directed around occluded coronary arteries using autologous tissue such as saphenous veins. However,

![Image of vascular grafting](image_url)

**Figure 1.1.** The Vascular diseases can be caused by various ways such as deposition of fatty plaques, damaged intima, or accumulation of cholesterol. It leads to CAD, heart attach, and stroke. (www.cornerstone-msc.net/nham/index.cfm?menuid=13).
many patients do not have suitable donor tissue due to peripheral vascular disease or prior surgery (Burke et al., 1996, Huynh et al., 1999, Nikalson, 1999).

Synthetic materials have been developed for blood vessel substitutes but have experienced significant limitations. ePTFE (expanded polytetrafluoroethylene), Dacron (PET, polyethylene teraphthalate), and microporous polyurethanes have been successfully used as large diameter (ID > 6mm) vascular substitutes (Edward et al., 1998, Nerem et al., 1998, Geary et al., 1993). However, they are not suitable for small diameter applications such as CABG due to rapid occlusion caused by thrombosis and intimal hyperplasia (Burke et al., 1996, Clowes, 1993, Geary et al., 1993, Thompson et al., 1994).

Figure 1.2. Synthetic ePTFE vascular grafts. Synthetic materials have been successfully used for large diameter vascular grafts but have experienced significant limitations for small diameter vascular grafts. (www.bhecorp.com/gore.htm).
Vascular grafts in low blood flow positions are more susceptible to failure of patency than those under high blood flow because of thrombogenicity and anastomotic intimal hyperplasia (Seifalian et al., 2002). Geary et al. studied a time-course analysis of smooth muscle cell proliferation and intimal thickening in porous ePTFE grafts. High blood flow inhibited intimal thickening but a thick intima developed when grafts were placed in normal flow. Intimal thickening is caused by increased smooth muscle cell proliferation and extracellular matrix production (Geary et al., 1993). Compliance mismatch between grafts and host vascular tissues also leads to intimal hyperplasia near anastomotic sites (Seifalian et al., 2002). Especially, Dacron and ePTFE grafts are rigid compared to elastic artery (Seifalian et al., 2002). Thus, polyurethane vascular grafts have been developed due to their elastic properties (How and Clarke, 1984, Lyman et al., 1978, Zhang et al., 1994b) and reduced neointimal formation compared to ePTFE (Jeschke et al., 1999). Introduction of microporous structures into the vascular grafts has also shown to improve compliance (Doi et al., 1996, Matsuda and Nakayama, 1996).

Another important factor in graft failure is the lack of endothelial cells on the surface of grafts because endothelial cells synthesize thromboresistant molecules, increase resistance to bacterial infection, and reduce smooth muscle cell proliferation (Burke et al., 1996, Herring et al., 1978, Holt et al., 1994, Kobayashi et al., 1992).
1.2. Endothelialization

1.2.1. Endothelialization on synthetic vascular grafts

Blood vessels are composed of three layers: the intimal endothelial layer, medial smooth muscle cells, and an outer connective tissue layer called the adventitia. The inner endothelial cell layer, the intima, prevents thrombosis and regulates smooth muscle cell tone and proliferative activity (Nikalson, 1999). Endothelial seeding on artificial vascular grafts has been demonstrated to decrease thrombogenicity and increase patency (Graham et al., 1980, Herring et al., 1978, Kobayashi et al., 1992, Thompson et al., 1994). Graham

![Diagram of artery and vein](https://www.gpcsd28.ab.ca/stm/how_veins_work.htm)

**Figure 1.3.** The structure of artery and vein. Blood vessels consist of intimal endothelial cell layer, medial smooth muscle cell layer, and outer connective tissue layer. The endothelial cell layer prevents thrombosis and regulates vascular smooth muscle cell tone. (www.gpcsd28.ab.ca/stm/how_veins_work.htm).
et al. seeded autologous endothelial cells on Dacron grafts and implanted then into dogs. Endothelial cells covered approximately 70% of the graft surfaces after two weeks. Endothelialization of inner surfaces was confirmed via immunofluorescence (Graham et al., 1980). However, though these results are promising, 30% un-endothelialized surface leads to longer term clinical complications. Knitted Dacron grafts were seeded with autologous endothelial cells before implantation into dogs, and the seeded grafts were more thromboresistant than unseeded grafts (Herring et al., 1978). The luminal surfaces of PTFE grafts were coated with an endothelial cell monolayer and implanted into the carotid artery of rats. The patency rate at one month was ~86 % in the endothelial cell coated grafts compared to ~20 % in the unseeded controls (Kobayashi et al., 1992). However, incomplete endothelial coverage in early stage of implantation on exposure to shear stress of blood stream generally lead to failure of patency, especially for small diameter vascular grafts (Holt et al., 1994). In a canine model, ~30 % of the endothelial cells was detached from the PTFE grafts after 30 minutes of arterial flow and only ~17 % of endothelial cells remained after 24 hours (Rosenman et al., 1985). Thus, the success of patency was dependent on the retention and attachment of endothelial cells on the graft surfaces under physiological conditions.
1.2.2. *Surface modification to enhance endothelialization*

Surface modifications to increase adhesion and thus retention of endothelial cells on artificial prostheses have been tried with plasma treatment (Dekker et al., 1991, Tseng and Edelman, 1998), adhesive peptides (Holt et al., 1994), and growth factors (Doi and Matsuda, 1997). Highly hydrophobic surfaces of ePTFE grafts have been shown to restrict endothelial cell attachment. However, modifications of ePTFE grafts with nitrogen-containing plasma have been shown to promote endothelial cell attachment (Dekker et al., 1991). Tseng and Edelman immobilized nitrogen-containing functional groups via amide and amine plasma treatment of the surfaces of ePTFE grafts. The plasma-modified ePTFE surfaces showed increased hydrophilicity, and enhanced endothelial cell lining under constant and pulsatile flow conditions (Tseng and Edelman, 1998). The surfaces of Dacron vascular grafts have also been modified with polyethylene oxide (PEO) and Gly-Arg-Glu-Asp-Val-Tyr (GREDVY) peptides. This peptide modification increased endothelial cell binding (Holt et al., 1994). Basic fibroblast growth factor (bFGF) has also been used to enhance endothelial cell proliferation. Microporous polyurethane grafts that were coated with a solution of photoreactive gelatin, bFGF, and heparin enhanced proliferation of endothelial cells (Doi and Matsuda, 1997). However, bFGF is able to stimulate endothelial cell growth and proliferation (Lindner et
al., 1990), but it also acts as a potent mitogen for smooth muscle cells, potentially causing intimal hyperplasia (Lindner et al., 1991).

1.3. Bioactive biomaterials

1.3.1. Cell adhesive peptides

The integrin family is a class of well-known cell adhesion receptors that are heterodimers consisting of an α-subunit and β-subunit. Integrin receptors mediate both cell-cell and cell-substrate adhesion by recognizing specific peptide sequences in adhesion proteins (Yamada, 1991). These peptides include the Arg-Gly-Asp (RGD)
sequence of fibronectin, fibrinogen, collagen and von Willebrand factor (Yamada, 1991, Graf et al., 1987), and the Arg-Glu-Asp-Val (REDV) sequence found in fibronectin (Massia and Hubbell, 1991, Hubbell, 1995, Yamada, 1991). Nonintegrin receptors for laminin are also present on endothelial cells and bind to the Tyr-Ile-Gly-Ser-Arg (YIGSR) sequence of laminin (Graf et al., 1987, Grant et al., 1990, Massia et al., 1993). Cell adhesion on biomaterials is mediated by cell surface receptors and cell adhesion proteins adsorbed to the material surfaces (Hubbell et al., 1992). Adhesion proteins such as fibronectin and vitronectin are found in blood plasma and many biological fluids. Laminin, von Willebrand factor, vitronectin, fibronectin, and collagen are also present in the extracellular matrix (Hubbell, 1995). Since biochemical interactions between ligands and receptors at the cell surfaces influence cellular behavior (Yamada, 1991), introduction of these ligands into synthetic biomaterials allow us to develop biomimetic materials.
<table>
<thead>
<tr>
<th>Proteins</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibronectin</td>
<td>RGDS, LDV, REDV</td>
</tr>
<tr>
<td>Vitronectin</td>
<td>RGDV</td>
</tr>
<tr>
<td>Laminin A</td>
<td>LRGDN, IKVAV</td>
</tr>
<tr>
<td>Laminin B1</td>
<td>YIGSR, PDSGR</td>
</tr>
<tr>
<td>Laminin B2</td>
<td>RNIAEIIKDA</td>
</tr>
<tr>
<td>Collagen I</td>
<td>RGDT, DGEA</td>
</tr>
<tr>
<td>Thrombospondin</td>
<td>RGD, VTXG</td>
</tr>
</tbody>
</table>

**Table 1.1.** Adhesive peptide sequences in extracellular matrix (Hubbell, 1995).

1.3.2. *Surface modification of biomaterials*

Modifications of biomaterials with adhesive peptide sequences have been explored by number of groups. For example, the RGD peptide has been covalently grafted to the surfaces of PET, PTFE (Massia and Hubbell, 1991) and a copolymer of poly(ethylene glycol) and acrylic acid (PEG-co-AA) (Drumheller et al., 1994). Adhesion of fibroblast cells or adhesion and spreading of endothelial cells increased on these RGD peptide-modified surfaces. Incorporation of the RGD peptide into PEG hydrogels promoted spreading of fibroblast cells (Hern and Hubbell, 1998). In addition,
biodegradable polymers such as block copolymers of biotinylated poly(ethylene glycol) (PEG) with poly(lactic acid) (PLA) (Cannizzaro et al., 1998) and poly(lactic acid-co-lysine) (Barrera et al., 1993, Barrera et al., 1995, Cook et al., 1997) have been also modified with RGD peptides. Endothelial cells on these polymers showed enhanced spreading. Polyurethanes covalently grafted with RGD peptide also supported endothelial cell attachment and spreading (Barrera et al., 1993, Barrera et al., 1995, Cook et al., 1997, Lin et al., 1992, Lin et al., 1994).

Figure 1.5. Surface modifications of biomaterials with cell adhesive peptide sequences. Ligand-receptor binding affects cellular behaviors such as proliferation, migration, and extracellular matrix production. Thus, it allows us to design biomimic materials.
YIGSR peptides have been also used to modify biomaterials to promote functions of endothelial cells. Kubota and Mizoguchi reported that YIGSR peptide site of laminin was necessary for endothelial cell differentiation and capillary formation (Kubota and Mizoguchi, 1993). Massia and Hubbell modified glycophashe glass, PET, and PTFE by immobilizing YIGSR peptide. The attachment and spreading of human umbilical vein endothelial cells (HUVECs) on substrates modified with YIGSR increased (Massia and Hubbell, 1991). Hubbell et al. also reported that HUVEC on YIGSR immobilized surface showed resistance to detachment at 20 dyne/cm² shear stress for 8 hours (Hubbell et al., 1992). Proliferation and spreading of BAECs also increased on glass modified with covalently-bound YIGSR (Dee et al., 1994). In addition, the combination of YIGSR peptide and fibroblast growth factor supports an increase in proliferation and population motility of BAEC (Dee et al., 1995). Importantly, YIGSR immobilized on glycophashe glass or PEG modified PET supported enhanced adhesion and spreading of HUVEC but did not support platelet attachment (Hubbell et al., 1991). Thrombosis is one of the major reasons of vascular graft failure, so it is vital that biomaterials used in this application should be highly resistant to platelet adhesion. RGD is known to interact with platelet integrin glycoprotein IIb/IIIa (Andrieux et al., 1991, Mazur et al., 1994), so while RGD may enhance endothelial cell adhesion, it may ultimately decrease graft performance.
1.3.3. **Surface peptide concentration**

The surface concentration of adhesive peptides is another important factor affecting cellular functions. Massia and Hubbell reported that morphologies of fibroblast cells were varied with surface peptide concentrations. They suggested that minimal peptide spacing of 440 nm for spreading and 140 nm for focal contact formation were required (Massia and Hubbell, 1991). The speed of cell migration depends on the strength of interaction between a cell and its substratum (DiMilla et al., 1993, Palecek et al., 1997). A maximal cell migration can generally be obtained at an intermediate surface ligand concentration. Cell attachment and extracellular matrix production are also affected by peptide density. Smooth muscle cell attachment increased at higher peptide densities but extracellular matrix production decreased (Mann et al., 1999). Migration and proliferation of smooth muscle cells were also lower at higher surface concentration of RGDS peptides (Mann and West, 2002). These results indicate that cellular functions of endothelial cells could be controlled by changing surface concentrations of peptides.

1.4. **Polyurethane and polyurethaneurea**

1.4.1. **Chemistry of polyurethane and polyurethaneurea**

Polyurethane (PU) and polyurethaneurea (PUU) have been widely used for
biomedical applications due to their excellent mechanical properties and relatively good biocompatibility. These applications include vascular prostheses (Huang et al., 1992, Zhang et al., 1994a), artificial hearts (Burke et al., 1996, Wu et al., 1999), catheters (Lamba et al., 1998), and artificial tracheae (Yanagi et al., 1994). The performance of polyurethanes changes depending on their chemical composition. The two most used polyols are polyesters and polyethers. Polyurethanes synthesized with polyether, such as polytetramethylene oxide (PTMO), have shown comparable mechanical properties yet better hydrolytic stability than polyester-based polyurethanes (Lamba et al., 1998). Silver et al. reported that the MW of soft segments affected hemocompatibility, the degree of phase separation, crystallinity, and surface hydrophobicity (Silver et al., 1994).

The most commonly used isocyanates in polyurethane synthesis are toluene diisocyanate (TDI) and methylene diphenyl diisocyanate (MDI). MDI has a high reactivity and is crystallizable. Polyurethanes based on MDI generally show better physical properties than those based on TDI (Lamba et al., 1998). Hergenrother et al. studied the effect of hard segment chemistry on biostability. Various polyurethanes were synthesized on a 3:2:1 molar ratio of methylene diphenyl diisocyanate (MDI) or methylene dicyclohexane diisocyanate (H_{12}MDI), polytetramethylene oxide (PTMO), and butanediol (BD) or ethylene diamine (ED). Polymers based on MDI showed more
stability related to surface cracking and MW change than polymers based on H$_{12}$MDI, a non-crystallizable hard segment (Hergenrother et al., 1994). The symmetry of isocyanate affects the crystallization of the hard segment domains, increasing degree of phase separation (Lamba et al., 1998). The chain extenders also affect the physical properties of polyurethane by increasing the molecular weight (MW) of hard segments and by facilitating phase separation. Diamine chain extenders increase phase separation by forming stronger urea linkages but cause lower solubility in organic solvent (Hergenrother et al., 1994, Garrett et al., 2000, Lamba et al., 1998, Sung et al., 1980). Therefore, it is very important to understand phase separation because degree of phase separation is influenced by composition, crystallization, and segment length and contributes to solid properties of polyurethanes (Lamba et al., 1998).

1.4.2. Phase Separation of Polyurethane and polyurethaneurea

As described above, Polyurethane (PU) and polyurethaneurea (PUU) are composed of a soft segment, a low glass transition temperature (Tg) polyether, and a hard segment, a high Tg diisocyanate, extended with a diol (PU) or a diamine (PUU). Hard segment domains serve as a crosslinking sites by dispersion in soft segment domains and allow materials to possess high elastic moduli (Blackwell and Gardner, 1979, Blackwell

![Micro phase separation of hard segments](image1.png) ![AFM image of hard segment domains](image2.png)

**Figure 1.6.** Phase separation of polyurethane and polyurethanuera. (a) micro phase separation of hard segments of polyurethane (www.azom.com/details.asp?articleid =218), (b) AFM tapping mode phase image of polyurethanuera (chapter 3).

Recently, PUU synthesized from a diamine as a chain extender instead of a diol has been attractive because PUU showed better phase separation compared to PU (Garrett et al., 2000, Sung et al., 1980). Sung et al. studied the effect of the urea linkage in the hard segment on phase separation. The phase separation of PUU extended with ethylenediamine (ED) was improved dramatically compared to PU extended with
butanediol (BD). These results could be explained; first, two urea groups linked with one carbonyl group in a hard segment provide an additional stronger hydrogen bond and a stronger three-dimensional structure. Second, PUU showed a much lower Tg of the soft segment and a much higher Tg of the hard segment (Sung et al., 1980). Garrett et al. synthesized polyurethaneurea with MDI, PTMO, and ED or diamine mixture of ED and 1,4-diaminocyclo hexane, and studied microdomain morphology using small angle X-ray scattering (SAXS). Introduction of second diamine resulted in reduced phase separation due to disruption of hard segment hydrogen bonding (Garrett et al., 2000). In addition, polyurethaneureas extended with ED showed more thromboresistance than polyurethanes extended with BD (Takahara et al., 1985).

1.4.3. Modification of Polyurethanes and polyurethaneureas

Even though most polyurethanes and polyurethaneureas have shown excellent physical properties and relatively good biocompatibility, they are generally thrombogenic on exposure to blood. Several surface modification strategies have been evaluated to improve blood compatibility. One approach is the introduction of ionic groups into polyurethanes. Surface modifications with propyl sulphonate showed lower platelet deposition in a canine ex vivo shunt model, though a large amount of fibrinogen had
adsorbed to the materials by the end of the study (Silver et al., 1994). The anticoagulant heparin has also been widely used to modify the surface of blood-contacting biomaterials. Park et al. immobilized heparin on polyurethaneurea by using hydrophilic PEO spacers. PEO chains enhanced the bioactivity of heparin as well as decreased platelet adhesion and protein adsorption (Park et al., 1991). Thrombomodulin (TM), a thrombin-binding protein, was also applied for the modification of biomaterials. Recombinant human TM was immobilized to poly(ether urethaneurea) (PEUU), improving antithrombogenic activity (Kishida et al., 1994).

In other ways, functional groups were incorporated into the polyurethane backbone to achieve desired properties. A series of polyurethanes were synthesized from amine-terminated polydimethylsiloxane oligomers and extended with hexane diisocyanate to give urea linkage. These silicon-urea polyurethanes showed lower platelet adhesion and fibrinogen deposition compared to a polyetherurethane (Hergenrother et al., 1994). Glycerophosphonylcholine (GPS) was incorporated as a chain extender in PTMO based polyurethanes. These materials showed decreased bacterial adhesion and neutrophil adhesion and were thus expected to result in lower inflammatory responses (Yung and Cooper, 1998, Baumgartner et al., 1997).

Immobilization of bioactive adhesive peptides has been also investigated to
promote endothelialization on the surface of polyurethanes. RGDS peptides were covalently bound to carboxylated polyurethanes via amide linkages. The amount of grafted peptides was determined by amino acid analysis with approximately 120 μmol of peptide per gram of polymer. GRGDSY grafted to polyurethane surfaces showed increased HUVEC attachment and spreading (Lin et al., 1992). Again, though, this peptide may increase graft thrombosis.

1.4.4. Polyurethane and polyurethaneurea vascular grafts

Vascular grafts require appropriate biocompatibility and physical properties close to natural arterial tissues (How and Clarke, 1984). PU and PUU have been used for vascular grafts due to its biocompatibility and elastic properties. For clinical patency, microporous structure is required to induce anastomotic tissue ingrowth into grafts and reduce compliance mismatching (Lamba et al., 1998, Doi and Matsuda, 1997, Ballyk et al., 1998). Porous polyurethanes have been fabricated with several methods. Lyman et al. developed a technique that precipitates a solution of polyether urethaneurea (PEUU) onto a mandrel by exchanging solvent (DMF) and nonsolvent (water). 23 grafts (4 mm id) out of 49 were patent in femoral arteries of dogs at 18 months (Lyman et al., 1978). Annis et al. fabricated polyetherurethane elastomer (10 mm id) by a process of electrostatic
spinning. Polymer solution was ejected from a syringe into electrostatic field. Fibers drawn from the solution were collected on the rotating mandel to form an elastic tube with pores of 10 μm diameter found on the surfaces. The grafts implanted in the thoracic aorta of pigs were patent at 12 months and cellular ingrowth through the pores was observed (Annis et al., 1978). In other approaches polyurethanes in liquid phase were extruded through fine orifices, stretched and wrapped on a rotating mandrel. When it reached the mandrel, fiber to fiber bonding occurred and it produced a stable, helically wound porous tube. When they were implanted in the aorta of dogs, patency was observed at 3 month in both small (4 mm) and large (10 mm) diameter grafts (Wilson et al., 1983, Hess et al., 1983, Leidner et al., 1983). Hess et al. developed a fibrous polyurethane microvascular prosthesis of 1.5 mm inner diameter by spraying liquid directly onto rotating rods, and implanted in the rat aorta (Hess et al., 1983). Grafts were lined with neointima consisting of several layers of smooth muscle cells and endothelium by the third week.

As mentioned above, a high level of endothelialization on the graft surfaces is very important, especially for clinical patency of small diameter vascular grafts. In order to promote rapid endothelialization, microporous structures have been combined with additional modifications. Doi and Matsuda developed small caliber (1.5 mm)
microporous polyurethane grafts using an excimer laser technique and incorporated growth factors into the grafts. Growth factors and microporous structures were expected to enhance endothelial cell proliferation and migration from the anastomoses. Grafts were coated with a solution of photoreactive gelation, basic fibroblast factor (bFGF), and heparin, then photocured by exposure to UV light. Grafts were implanted into the rats for 4 weeks (Doi and Matsuda, 1997). Grafts were patent and transmural ingrowth of tissues including smooth muscle cells and fibroblasts were observed. It indicated that incorporation of bFGF promoted neoarterial regeneration. However, the mitogenic effect of bFGF on smooth muscle cells could lead to intimal hyperplasia (Lindner et al., 1991, Geary et al., 1993). In contrast, vascular endothelial growth factor (VEGF) has shown specific mitogen to endothelial cells (Guo et al., 1995) and promoted endothelial cell migration (Visweswaran et al., 1997). Incorporation of VEGF into the grafts enhanced ingrowth of both anastomotic and transmural tissues as well as capillaries into the grafts (Masuda et al., 1997).
1.5. Conclusions

Polyurethaneureas (PUU) have been an attractive material for vascular grafts, but like other synthetic materials their general thrombogenicity on exposure to blood requires additional modifications. Several modifications of polyurethaneureas have been tried but they have not been enough to solve the problems. Endothelialization on PUU might be a good strategy to improve graft patency, especially for small diameter vascular grafts. Endothelial cell behaviors have been altered by the surface concentration of YIGSR peptide sequences in chapter 2. Based on these results, the bioactive PUU has been synthesized by incorporating the peptide sequences into polymer backbone as a chain extender in chapter 3. Furthermore, in chapter 4, PEG and peptide-modified PUU has been developed to simultaneously enhance endothelialization and improve thromboresistance. This bulk modification allows us to fabricate microporous scaffold in chapter 5, and this scaffold has been used as a carrier of vascular endothelial growth factor (VEGF) in chapter 6. In addition, nitric oxide releasing PU has been developed by incorporating NO donor into the polymer backbone in chapter 7.
Chapter 2. Surface modification with YIGSR peptide to alter endothelialization

2.1. Introduction

Cell adhesion on biomaterials is mediated by cell surface receptors and cell adhesion proteins adsorbed to the material surfaces. Thus, modification of biomaterials with adhesive peptides has been shown to promote cell adhesion and spreading (Lin et al., 1992, Lin et al., 1994, Hern and Hubbell, 1998, Dee et al., 1998). In addition, the surface peptide concentration has been shown to affect cellular functions; Maximal cell migration is generally obtained at intermediate surface ligand concentrations (Palecek et al., 1997, DiMilla et al., 1993). Mann and West reported that migration and proliferation of smooth muscle cells were lower at higher surface concentration of RGD peptides (Mann and West, 2002). Cell morphology (Massia and Hubbell, 1991), attachment, and extracellular matrix production (Mann et al., 1999) have been shown to vary with surface peptide concentration for a number of different cell types.

Massia and Hubbell suggested that minimal RGD peptide spacing of 440 nm for spreading and 140 nm for contact formation were required on glass substrates. They found focal contacts of spreading human foreskin fibroblasts at 10 and 100 fmol/cm²
(Massia and Hubbell, 1991). Endothelial cell spreading was improved at 13 pmol/cm² of RGD-modified poly(lactic acid-co-lysine) (Cook et al., 1997). Adhesion of endothelial cell was increased more than 160% at 30 - 40 pmol/cm² of RGDS-modified poly(ester-urethane)ureas compared to tissue-culture polystyrene (Guian et al., 2002). Hubbell et al. found that cellular attachment was affected by peptide surface concentration, spacer length, and particular peptide immobilized (Hubbell et al., 1991). Therefore, the optimal surface concentrations of the peptides might be dependent on cell type, peptides, and substrates. The chapter 2 focused on investigating if surface modification with YIGSR peptides can enhance endothelialization without increasing thrombosis. YIGSR peptides were covalently immobilized to aminophase surfaces. Responses of endothelial cells were studied at various YIGSR surface concentrations.
2.2. Materials and Methods

2.2.1. Immobilization of YIGSR peptides to aminophase glass surfaces

The peptide YIGSR (Sigma Chemical Co., St. Louis, MO) was used for this study. First, the amine terminus of the YIGSR peptide was acetylated by reaction with an equimolar amount of acetic anhydride, lyophilized, and stored in $N,N$-dimethylformamide (DMF). Aminophase glass slides were made by reacting with 3-aminopropyltriethoxysilane (APTS) (Aldrich Chemical Co., Milwaukee, WI) in dry acetone at 37 °C overnight. The slides were then rinsed in acetone and sonicated in deionized (DI) water. The acetylated YIGSR peptides were coupled to the aminophase glass slides; the aminophase glass slides were placed in DMF containing 1.2 g/ml 1-ethyl-3-(3-diethylaminopropyl)carbodiimide (EDAC; Sigma Chemical Co., St. Louis, MO), 1.6% (v/v) N-ethylmorpholine (Sigma Chemical Co., St. Louis, MO), and 2.5 – 20 nmol/ml acetylated YIGSR peptide. After 4 hours incubation at 37 °C, the slides were rinsed in DMF and DI water, and sonicated in 4 M urea and 1 M NaCl for 10 minutes, respectively. After rinsing with DI water and drying at room temperature, average amine concentrations on the aminophase glass slides and YIGSR peptide concentrations on the YIGSR-modified slides were evaluated using the ninhydrin assay. Leucine solutions were used as standards at concentration ranging from 12.5 μM to 100 μM. The average amine
concentration on the aminophase glass slides was approximately 1.53 nmol/cm², and the YIGSR peptide concentrations on the YIGSR-modified slides were determined to be 0.23, 0.34, 0.61, and 1.53 nmol/cm². The slides were sterilized under UV light overnight prior to use.

\[
\begin{align*}
\text{H}_3\text{C} & \text{C} \text{O} \\
\text{H}_3\text{C} & \text{C} \text{O} \\
\text{NH}_2 & \text{YIGSR} \\
\text{Acetic anhydride} & \rightarrow \text{CH}_3\text{CONH}_2\text{YIGSR} \\
\text{Acetylated YIGSR} & \\
\end{align*}
\]

Coupled to aminophase glass

**Figure 2.1.** Immobilization of YIGSR peptide to aminophase glass surface. Acetylated YIGSR peptide was coupled to aminophase surface at various concentrations.
2.2.2. Cell maintenance

Bovine aortic endothelial cells (BAEC; Clonetics, San Diego, CA) were used for this study. Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma Chemical Co., St. Louis, MO) was prepared with 10% fetal bovine serum (FBS; BioWhitaker, Walkersville, MD), 2 mM L-glutamine, 1 unit/ml penicillin, and 100 mg/L streptomycin (GPS; Sigma). Endothelial basal medium (EBM; Sigma Chemical Co., St. Louis, MO) was prepared with endothelial medium supplement (Sigma Chemical Co., St. Louis, MO) that contained fetal bovine serum, basic fibroblast growth factor, heparin, epithelial growth factor, and hydrocortisone. BAECs were maintained on mixture of EBM and DMEM (25/75 %) at 37 °C in a 5% CO₂ environment.

2.2.3. Attachment and spreading of endothelial cells

To evaluate attachment of endothelial cells, cell suspensions were prepared and seeded at 17,000 cells/cm² in eight well FlexiPerms (Sigma Chemical Co., St. Louis, MO) attached to the modified slides. Cells were cultured at 37 °C in a 5% CO₂ environment. After 4, 8, or 24 hours of incubation, non-adherent cells were removed by rinsing and adherent cells were treated with trypsin and counted using a Coulter Counter (Multisizer 3, Beckman Coulter). To assess spreading of endothelial cells, after 4, 8, or 24
hours of incubation, non-adherent cells were removed by rinsing and fresh medium was added. Cells were observed by phase contrast microscopy (Zeiss Axiovert 135), and digital image processing (Scion Image) was used to determine cell areas.

2.2.4. **Strength of adhesion of endothelial cells**

To evaluate the strength of adhesion, cell suspensions were prepared and seeded at a concentration of 30,000 cells/cm² in eight well FlexiPerms on the modified slides. After 24 hours incubation at 37 °C in a 5% CO₂ environment, each slide was placed in a 50 ml centrifuge tube that was filled with culture medium. Cells on YIGSR-modified and aminophase glass surfaces were exposed to 2100 xg force via centrifugation for 15 min. The cells remaining on slides were counted using a Coulter counter and compared to those on stationary control slides.

2.2.5. **Proliferation of endothelial cells**

To evaluate proliferation, eight well rectangular FlexiPerms were attached to YIGSR-modified slides and aminophase glass slides. A cell suspension was prepared and seeded at a concentration of 17,000 cells/cm² in the eight wells on each slide. After 48 hours incubation at 37 °C in a 5% CO₂ environment, proliferating cell nuclear antigen-
horse radish peroxidase (PCNA-HRP; DAKO, Carpinteria, CA) conjugate was used for immunohistochemical staining and followed by treatment with AEC chromagen (DAKO, Carpinteria, CA). Counter staining was performed with Mayer's hematoxylin. PCNA-HRP conjugate stains cells in the S-phase of mitosis. Proliferating cells appear red and non-proliferating cells appear blue. Two wells of the eight were used as positive and negative controls.

2.2.6. Migration of endothelial cells

To assess migration, a fence-style assay was utilized. Cell suspensions were prepared and seeded at a concentration of 57,000 cells/cm² in three wells of eight well FlexiPerms on each slide. When cells reached confluence, the original boundaries were recorded, and the FlexiPerm strips were removed. After 24 hours incubation at 37 °C in a 5% CO₂ environment, the cells that migrated over the original straight boundary were counted using a phase contrast microscope.

2.2.7. Extracellular matrix production of endothelial cells

To evaluate extracellular matrix (ECM) production, eight well FlexiPerms were attached to the YIGSR-modified slides and aminophase glass slides. Cell suspensions
were prepared with 5 μg/ml ascorbic acid in the culture medium and seeded at a concentration of 17,000 cells/cm². Four of the wells on each slide were cultured with 1 μCi/ml ³H-glycine to measure ECM production. The other four wells were cultured in the absence of ³H-glycine and used to count adherent cells on the surfaces. After 48 hours of incubation at 37 °C in a 5% CO₂ environment, adherent cell numbers were determined using a Coulter Counter. Cells in the remaining wells were rinsed with PBS and DI water, and incubated with a lysis solution (25 mM ammonium hydroxide), and then the remaining ECM was rinsed with 70% ethanol. The ECM was sequentially digested with 200 μg/ml trypsin, 58 U/ml elastase, 76 U/ml collagenase, and 1N NaOH. ECM production was determined by measuring the amount of radioactivity in the glycoprotein, elastin, and collagen fractions using scintillation counting.

2.2.8. Platelet adhesion

Glass slides were coated with a solution of 2.5 mg/ml collagen I in 3% glacial acetic acid in DI water for 45 minutes in a humidified environment at room temperature to provide a highly thrombogenic reference material. Blood was obtained from a healthy volunteer with 10 U/ml heparin. Mepacrine (10 μM) solution was added to fluorescently labeled platelets. Collagen I (positive control), aminophase glass, and YIGSR modified
surfaces were incubated with mepacrine labeled whole blood at 37 °C for 20 minutes and rinsed with PBS. The number of adherent platelets per field of view (200x) was counted using a fluorescent microscope (Zeiss Axiovert 135, Thornwood, NY).

2.2.9. Statistic analysis

Data were compared with two-tailed, unpaired t-tests. $P$-values less than 0.05 were considered to be significant. Data are presented as mean ± standard deviation.
2.3. Results and Discussion

In this chapter, the YIGSR peptide was covalently bound onto a glass surface at varying concentrations, and its effects on the responses of endothelial cells were examined. The effect of YIGSR surface concentration on attachment of endothelial cells is shown in Figure 2.2. Attachment of endothelial cells was significantly higher on YIGSR-modified surfaces than on the aminophase glass. After 4 and 8 hours of incubation, improved cell attachment over the entire range of YIGSR concentrations was found, but after 24 hours of incubation, there was increased attachment only at concentrations above 0.61 nmol/cm².

Figure 2.2. Effect of YIGSR surface concentration on endothelial cell attachment. * $P < 0.05$, # $P < 0.01$, compared to aminophase. Empty column;aminophase, vertical column; YIGSR(0.23 nmol/cm²), horizontal column;YIGSR(0.34 nmol/cm²), diagonal column;YIGSR (0.61 nmol/cm²), solid column;YIGSR (1.53 nmol/cm²).
Figure 2.3 shows the effect of YIGSR surface concentration on spreading of endothelial cells. These results were similar to those for the endothelial cell attachment. Surface areas of endothelial cells were significantly greater on YIGSR-modified surfaces than on the aminophase glass over the entire range of YIGSR concentrations. After four

![Graph showing cell area over time](image)

**Figure 2.3.** Effect of YIGSR surface concentration on cell spreading. *P < 0.05, # P < 0.01, compared to aminophase. Empty column; aminophase, vertical column; YIGSR(0.23 nmol/cm²), horizontal column; YIGSR(0.34 nmol/cm²), diagonal column; YIGSR (0.61 nmol/cm²), solid column; YIGSR (1.53 nmol/cm²).
hours of incubation, most cells on aminophase surface were round in shape. However, on surfaces with a YIGSR concentration of 1.53 nmol/cm$^2$, over 90% of cells were spread.

Although many researches have tried to improve endothelial cell retention on synthetic vascular grafts, endothelial cells generally detach under physiological flow conditions (Rosenman et al., 1985). Even though modifications of synthetic grafts such as plasma treated ePTFE (Tseng and Edelman, 1998) and immobilization of REDV peptide on the Dacron (Holt et al., 1994) showed increased endothelial cell coverage, better results are still required. Therefore, the effect of YIGSR surface concentration on the strength of endothelial cell adhesion was evaluated and shown in Figure 2.4. With increasing YIGSR concentration, strength of adhesion also increased. Even at the low YIGSR concentrations, about 85% endothelial cells remain after exposure to shear stress (20 dyne/cm$^2$) for 15 minutes. However, improved adhesion alone may not be sufficient to obtain endothelialization of vascular prostheses and clinical success. Cell proliferation, migration, and extracellular matrix production are also required.
Figure 2.4. Effect of YIGSR surface concentration on strength of endothelial cell adhesion. *P < 0.05, compared to aminophase glass not modified with YIGSR peptides.

The effect of YIGSR surface concentration on endothelial cell proliferation is shown in Figure 2.5. Proliferation of endothelial cells was reduced at YIGSR surface concentrations above 0.61 nmol/cm². There was no significant difference at the lower YIGSR surface concentrations. This result indicates that high YIGSR surface concentrations may impair the proliferation of endothelial cells.
Figure 2.5. Effect of YIGSR surface concentration on proliferation of endothelial cell. *$P < 0.05$, # $P < 0.01$, compared to aminophase glass not modified with YIGSR peptides.

Cell migration is mediated by adhesion receptors such as integrins that link the cells to extracellular matrix ligands to transmit forces and signals necessary for locomotion. Maximal cell migration is known to occur at an appropriate adhesion between cells and substratum (DiMilla et al., 1993, Palecek et al., 1997). With poor adhesion, cells can not form enough stable adhesion to generate migration. However, very strong adhesion might also prevent migration because cells are unable to disrupt cellular adhesion sites. Therefore, appropriate ligand concentrations may allow cells to
form stable bonds with the substratum and give rise to maximal migration. Figure 2.6 shows the results of the effect of YIGSR surface concentration on migration of endothelial cells. The number of cells that had migrated over the original boundary was increased at 0.23 nmol/cm² and 0.34 nmol/cm², and decreased above 1.53 nmol/cm² as compared to the number of cells on amoniphase glass surface.

![Bar chart showing the effect of YIGSR concentration on cell migration.](chart.png)

**Figure 2.6.** Effect of YIGSR surface concentration on migration of endothelial cell. *P* < 0.05, # P < 0.01, compared to amoniphase glass not modified with YIGSR peptides.
ECM is composed of collagens, proteoglycans, glycoproteins, and elastic fibers (Ross, 1998). A interaction between cells and ECM is able to affect cell proliferation, adhesion, and migration. In addition, ECM also provides supporting materials to cells and tissues. Thus, ECM production is very important for the maintenance of cells and tissues. The results of the matrix production assay are shown in Figure 2.7. Matrix production increased at 0.23 nmol/cm$^2$ and 0.34 nmol/cm$^2$ and decreased at 1.53 nmol/cm$^2$.

**Figure 2.7.** Effect of YIGSR surface concentration on production of ECM proteins by endothelial cell. *$P < 0.05$, # $P < 0.01$, compared to aminophase glass not modified with YIGSR peptides.
The effect of YIGSR surface concentration on platelet adhesion is shown in Figure 2.8. Platelet adhesion on the YIGSR-modified and the aminophase glass surfaces were dramatically lower than on collagen I, the positive control. Additionally, the number of adherent platelets on YIGSR modified surface was significantly lower than on aminophase glass surface, indicating that this surface modification can selectively

**Figure 2.8.** Effect of YIGSR surface concentration on platelet adhesion. *P < 0.05, # P < 0.01, compared to aminophase glass not modified with YIGSR peptides.
enhance endothelial cell adhesion and growth. This is essential for vascular graft applications, where thrombosis is a common complication.
2.4. Conclusions

In chapter 2, YIGSR peptides were covalently grafted to aminophase glass surfaces at various concentrations. Strength of adhesion was increased with increasing YIGSR concentration, but proliferation was decreased at high concentrations. Cell migration and extracellular matrix production were increased at low peptide concentrations but decreased at high concentrations. Platelet adhesion on YIGSR modified-surfaces were less than on the aminophase glass surfaces. Therefore, we can identify optimal peptide concentrations to enhance endothelialization without increasing thrombosis. The results optimized in this chapter were used in the design of bioactive polyurethaneureas in chapter 3.
Chapter 3. Development of a YIGSR peptide-modified polyurethaneurea to enhance endothelialization

3.1. Introduction

Polyurethane block copolymers have been widely used for biomedical applications due to their excellent mechanical properties and relatively good biocompatibility. These applications include vascular prostheses (Huang et al., 1992, Zhang et al., 1994), artificial hearts (Burke et al., 1996, Wu et al., 1999), catheters (Lamba et al., 1998), and artificial tracheae (Yanagi et al., 1994). However, like other synthetic materials, they are generally thrombogenic on exposure to blood. Several surface modification strategies have been evaluated to improve blood compatibility such as introduction of ionic groups (Silver et al., 1994), heparin with hydrophilic PEO spacers (Park et al., 1991), and thrombomodulin (Kishida et al., 1994). Nevertheless, these modifications have not been enough to completely solve the problem.

Adhesive peptide sequences have been extensively used for modification of biomaterials to enhance endothelial cell adhesion. RGD peptides have been covalently grafted to carboxylated polyurethanes via amide bonds, resulting in improved endothelial cell attachment and spreading (Lin et al., 1992, Lin et al., 1994). Biodegradable
poly(ester-urethane)ureas (PEUUs) was synthesized from polycaprolactone and 1,4-diisocyanatobutane and extended with lysine ethyl ester or putrescine (Guian et al., 2002). RGDS peptide was coupled to the polymer surface with radio frequency glow discharge. Human endothelial cells on RGDS-modified surface showed increased cell attachment compared to both unmodified surface and polystyrene tissue culture plate. However, thrombosis is one of the major causes of vascular graft failure. RGD is known to interact with platelet integrin glycoprotein IIb/IIIa (Andrieux et al., 1991, Mazur et al., 1994), so while RGD may enhance endothelial cell adhesion, it may ultimately decrease graft performance.

The laminin-derived peptide YIGSR has also been used to modify biomaterials to promote endothelialization. Enhanced attachment, spreading, proliferation, and migration of endothelial cells on vascular graft materials are essential to obtain successful endothelialization. Massia and Hubbell modified glycopase glass, PET, and PTFE with immobilized YIGSR peptide and found that attachment and spreading of human umbilical vein endothelial cells (HUVECs) on substrates modified with YIGSR increased (Massia and Hubbell, 1991). Hubbell et al. also reported that HUVECs on YIGSR immobilized surface showed resistance to detachment at 20 dyne/cm² shear stress for 8 hours (Hubbell et al., 1992). Importantly, YIGSR immobilized on glass or PEG-modified
PET supported enhanced adhesion and spreading of HUVECs but did not support platelet attachment (Hubbell et al., 1991).

In chapter 2, we identified optimal peptide concentrations to enhance endothelialization without increasing thrombosis. The goal of this chapter is to develop a bioactive peptide-modified polyurethaneurea to enhance endothelialization for small diameter vascular graft applications. A bioactive YIGSR peptide-modified polyurethaneurea was developed by incorporating GGGYIGSRGGGK peptide sequences into the polymer backbone as shown in Figure 3.1. The bioactive polyurethaneurea was characterized, and the enhancement of endothelialization was evaluated.

Figure 3.1. The distribution of peptide sequences incorporated into the hard segment domains in polyurethaneurea matrix.
3.2. Materials and Methods

3.2.1. Synthesis of polyurethaneurea (PUUPPD)

Prepolymer was synthesized by reacting methylene di(p-phenyl isocyanate) (MDI; Aldrich chemical Co., Milwaukee, WI) with poly(tetramethylene oxide) (PTMO; Aldrich chemical Co., Milwaukee, WI), and then extended with p-phenylene diamine (PPD; ACROS, New Jersy) (Lee and Ko, 1993) as shown in Figure 3.2. A 10% (w/v) solution of MDI (8 mmol, MW: 250) in 20 ml anhydrous N,N - dimethylformamide (DMF; Aldrich Chemical Co., Milwaukee, WI ) was prepared in a 100 ml three-neck round flask and stirred at room temperature. A 10% (w/v) solution of PTMO (4 mmol, MW: 1000) in 40 ml anhydrous DMF was added, and the mixture was heated to 75°C and held there for 2 hr under argon gas. The reactor was cooled to room temperature before PPD (4 mmol, MW:108) in 4 ml anhydrous DMF was added as a chain extender. The polymer solution was then incubated at 45°C for 2 hr under argon gas. The polymer solution was cooled to room temperature, precipitated in methanol, and dried under vacuum.
Step I

\[
\text{OCN-} \begin{array}{c}
\text{CH}_2 \\
\text{NCO}
\end{array} \quad + \quad \text{HO-CH}_2\text{CH}_2\text{CH}_2\text{O-}_{\text{n}}\text{H} \\
\text{4,4'-methylene di(p-phenyl isocyanate) (MDI)} \quad \text{poly(tetramethylene oxide) (PTMO)} \\
\text{OCN-prepolymer-NCO}
\]

Step II

\[
\text{OCN-prepolymer-NCO} \quad + \quad \text{H}_2\text{N-} \begin{array}{c}
\text{CH}_2 \\
\text{CH}_2 \\
\text{N}
\end{array} \quad \text{H}_2\text{N} \\
p\text{-phenylene diamine (PPD)}
\]

Soft Segment \quad Hard Segment \quad Soft Segment

(\text{PUUPPD})

**Figure 3.2.** Synthesis of polyurethaneurea (PUUPPD). Prepolymer was synthesized from MDI and PTMO and then extended with PPD.

### 3.2.2. Synthesis of bioactive YIGSR-modified polyurethaneurea (PUUYIGSR)

Prepolymer was synthesized by reacting MDI with PTMO as described above and extended with a combination of GGGYIGSRGGGK peptide (Sigma-Genosys, Woodlands, TX) and PPD (Figure 3.3). A 10% (w/v) solution of MDI (2.4 mmol) in 6 ml anhydrous DMF was prepared in 100 ml three-neck round flask and stirred at room temperature. A 10% (w/v) solution of PTMO (1.2 mmol) in 12 ml anhydrous DMF was
added, and the mixture was heated to 75°C and held there for 2 hr under argon gas. The reactor was cooled to room temperature before GGGYIGSRGGGK peptide (0.11 mmol) in 10 ml anhydrous DMF and PPD (1.1 mmol) in 10 ml anhydrous DMF were added as chain extenders. The polymer mixture was incubated at 45°C for 2 hr under argon gas.

The polymer solution was cooled to room temperature, precipitated in methanol, and dried under vacuum.

**Figure 3.3.** Synthesis of a peptide-modified polyurethaneurea (PUUYIGSR). Prepolymer was synthesized from MDI and PTMO and then extended with PPD and peptide.
3.2.3. *Polymer characterization*

PUUPPD and PUUYIGSR were characterized via $^1$H NMR using a 400 MHz NMR spectrometer (Advance 400, Bruker, Germany) in $N,N$-dimethylformamide-d$_7$ (DMF-d$_7$; Aldrich Chemical Co., Milwaukee, WI). Molecular weight distributions were obtained by GPC with UV and evaporative light scattering detectors (Polymer Laboratories, Amherst, MA). Samples for GPC analysis were dissolved in HPLC-grade DMF at a concentration of 1 mg/ml and run at 70°C through PLgel 5 μm Mixed-C columns (Polymer Laboratories, Amherst, MA) at a flow rate of 1 mL/min. Calibration was performed using polystyrene standards (PS; Polymer Laboratories, Amherst, MA), ranging in molecular weight from 5,000 – 96,400 Da. DSC thermagrams were obtained using a TA Instruments DSC 2920. Samples were cooled below -60°C and increased at 10 °C /min to 300°C. Helium was used for low temperature scans, and nitrogen was employed for high temperature scans. Fourier transform infrared (FT-IR) spectroscopy was performed using a Nicolet 500 spectrometer. The thin films of polyurethaneurea were prepared by mixing samples with KBr and pressed into pellets under vacuum. Sixteen scans were taken of each sample at a resolution of 4 cm$^{-1}$. 
3.2.4. Preparation and characterization of polyurethaneurea films

Polymers were dissolved in tetrahydrofuran (THF; 0.3 wt%) and sterilized using 0.2 μm pore size PTFE syringe filters (Whatman, NJ). Polymer films were prepared on glass coverslips (18 mm; Fisher Scientific, PA) by solvent casting at room temperature. Polymer films were held under vacuum for 48 hr to ensure removal of the solvent.

The equilibrium contact angles of DI water on PUUPPD and PUUYIGSR films were measured using a contact angle goniometer (CAM-Micro). Six measurements were taken to calculate average contact angles on the surface of each film.

ESCA analysis was performed using a Physical Electronics Model 5700 XPS instrument. Photo-emissions were produced through the use of a X-ray source (1486.6 eV) operated in the fixed retard ratio mode at a pass energy of 23.5 eV. Spectra were acquired over a 10 – 45 take off angle range. Charge neutralization was accomplished via bombardment with a low energy beam.

To evaluate the distribution of YIGSR peptide on the surface of the polyurethaneurea films, films were reacted with the fluorescent probe 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl; Molecular Probes, Eugene, OR). This fluorescent compound reacts with tyrosine residues on the peptides in the polymer. 250 mM NBD-Cl was prepared in DMSO. Polyurethaneurea films were placed in a well of a 12-well tissue
culture plate, 2 ml potassium phosphate buffer solution (pH 8.0) was added, and then 80 μl NBD-Cl solution was added. Samples were incubated at 37 °C for 2 hr. Unreacted NBD-Cl was rinsed with buffer solution over one week. The surfaces were observed using a Nikon E600 fluorescence microscope equipped with a Sony DXC-950P CCD camera. A Nikon multiband DAPI-FITIC-Texas red (excitation wavelengths: 385 - 415, 485 - 505, 555 - 585 nm, emission wavelengths: 450 – 470, 510 – 540, 590 – 650 nm) filter was used.

To investigate the distribution of hard and soft segment on the surfaces, the surfaces of polyurethaneurea films were imaged by AFM (NanoScope IIIa, Digital Instruments, Santa Barbara, CA). A 125 μm etched silicon cantilever was used. The radius of curvature of a silicon tip was 5 – 10 nm, and the resonance frequency was 300 kHz. Phase and height images were obtained using tapping mode under ambient conditions from $r_{sp} = 0.92$ to 0.80 ($r_{sp}$ = set point amplitude/free amplitude of oscillation). The data obtained were analyzed with Nanoscope IIIa controller and software.

Uniaxial mechanical testing was performed using an Instron model 5565 at a cross head speed of 25 mm/min with a 5 kN load cell. Polymers were dissolved in DMF (Aldrich Chemical Co., Milwaukee, WI) at 10 wt% and sterilized using 0.2 μm pore size PTFE syringe filters (Whatman, NJ). Polymer films were prepared in Teflon molds by
solvent casting at 60°C under vacuum for 48 hr. Test specimens were prepared according to ASTMD-638-VI. The tensile strength was calculated from the maximum load at break and the initial cross sectional area of the specimen. Sample thickness was measured using a digital caliper (Mitutoyo).

3.2.5. **Cell maintenance**

Bovine aortic endothelial cells (BAEC; Clonetics, San Diego, CA), passage 2 – 5, were used for this study. Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma Chemical Co., St. Louis, MO) was prepared with 10% fetal bovine serum (FBS; BioWhitaker, Walkersville, MD), 2 mM L-glutamine, 1 unit/ml penicillin, and 100 mg/l streptomycin (GPS; Sigma Chemical Co., St. Louis, MO). Endothelial basal medium (EBM; Sigma Chemical Co., St. Louis, MO) was prepared with 10% endothelial medium supplement (Sigma Chemical Co., St. Louis, MO) containing fetal bovine serum, basic fibroblast growth factor, heparin, epithelial growth factor, and hydrocortisone. BAECs were maintained on mixture of EBM and DMEM (25/75 %) at 37 °C in a 5% CO₂ environment.
3.2.6. Viability of endothelial cells on polyurethaneurea films

To evaluate viability of endothelial cells growing on polymer films, eight-well FlexiPerms (Sigma Chemical Co., St. Louis, MO) were attached to polyurethaneurea films to create culture wells on each surface. BAEC suspensions were prepared and seeded at a concentration of 17,000 cells/cm² on the films. Calcein AM and ethidium homodimer-1 (Live-Dead Assay Kit; Molecular Probes, Inc., Eugene, OR) were used to determine endothelial cell viability after 24 or 72 hr in culture. Calcein AM is converted to a green fluorescent product within live cells due to enzymatic activity, while ethidium homodimer-1, a red fluorescent compound, accumulates in dead cells due to increased membrane permeability.

To evaluate cytotoxicity of any leachable products, PUUPPD and PUUYIGSR films were placed in glass vials and dried under vacuum for 48 hr. Each film had a 6 cm² surface area. According to USP extraction ratio of synthetic polymer films with thickness less than 0.5 mm (Lee and Ko, 1993, USP, 2000, Shayne, 1997), 1 ml of HEPES-buffered saline (HBS, 10 mM, pH 7.4) solution was added to each vial. Samples were incubated at 37 °C. After 30 and 60 days of extraction, endothelial cells were seeded into 24-well tissue culture plate at 15,000 cells/cm². 24 hr after cell seeding, the extract solutions were sterilized using a 0.2 μm pore size syringe filter and added to the culture medium at 10,
15, or 25% (v/v). After 24 hr of incubation with the extracts, cell viability was evaluated by Calcein AM/ethidium homodimer staining as described above.

3.2.7. Adhesion and spreading of endothelial cells on polyurethaneurea films

To evaluate adhesion of endothelial cells, BAECs were seeded at a concentration of 17,000 cells/cm² in eight-well FlexiPerms attached to polyurethaneurea films. Cells were cultured at 37°C in a 5% CO₂ environment. After 4 or 24 hr of incubation, non-adherent cells were removed by rinsing, and adherent cells were removed with trypsin and counted using a Coulter Counter (Multisizer 3, Beckman Coulter). To evaluate endothelial cell spreading, after 4 or 24 hr of incubation, non-adherent cells were removed by rinsing, and fresh medium was added. Cells were observed by phase contrast microscopy (Zeiss Axiovert 135), and digital image processing (Scion Image) was used to determine cell areas.

Competitive inhibition of attachment and spreading of endothelial cells was also examined using soluble YIGSR peptides to ensure that increased BAEC adhesion and spreading was due to biospecific interactions with the YIGSR peptides that were incorporated into the polymer structure. Cells were seeded at a concentration of 17,000 cells/cm² in eight-well FlexiPerms attached to polyurethaneurea films and incubated with
soluble YIGSR peptide (Sigma Chemical Co., St. Louis, MO) at 0.01, 0.1, and 1 mM in EBM/DMEM (25%/75%) at 37 °C in a 5% CO₂ environment. After 4 hr incubation, attachment and spreading of cells were evaluated as described above.

3.2.8. Proliferation of endothelial cells on polyurethaneurea films

To evaluate endothelial cell proliferation, eight-well FlexiPerms were attached to polyurethaneurea films. Cells were seeded at a concentration of 17,000 cells/cm². After 48 hr incubation at 37 °C in a 5% CO₂ environment, immunohistochemical staining for proliferating cell nuclear antigen (PCNA) was employed. The PCNA antibody stains cells in the S-phase of mitosis. Cells were washed with PBS, fixed with 10% buffered formalin (Stephens Scientific, NJ) for 10 min, permeabilized with methanol (Sigma Chemical Co., St. Louis, MO) for 2 min, and incubated in 3% H₂O₂ (Fisher Scientifics, PA) for 5 min. Cells were incubated with mouse anti-human PCNA IgG (DAKO, Carpinteria, CA) for 1 hr, rinsed with PBS, and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (DAKO, Carpinteria, CA) for 40 min. Antibodies were diluted 1:100 in PBS containing 3% FBS, and incubations were performed at room temperature in a humidified chamber. After rinsing with PBS, cells were treated with aminoethylcarbazole chromagen (DAKO, Carpinteria, CA) for 10 min. Counter-staining was performed with Mayer's
hematoxylin (DAKO, Carpinteria, CA). Using this procedure, proliferating cells appear red and non-proliferating cells appear blue. Two wells were used as controls; one well with no primary antibody (anti-PCNA IgG), and one well with neither primary nor secondary antibody (HRP-conjugated anti-mouse IgG).

3.2.9. \textit{Migration of endothelial cells on polyurethaneurea films}

To assess migration, a fence-style assay was utilized. BAECs were seeded at a concentration of 40,000 cells/cm² in eight-well FlexiPerms attached to polyurethaneurea films. When cells reached confluence, the original boundaries were recorded, and the FlexiPerm strips were removed. After 24 hr of incubation at 37 °C in a 5% CO₂ environment, the cells that had migrated over the original boundary were counted using a phase contrast microscope (Zeiss Axiovert 135).

Endothelialization at an anastomotic site was also simulated in an \textit{in vitro} system (Figure 3.4). PUUPPD and PUUYIGSR were cast on glass, and then eight-well FlexiPerms were attached to the surfaces. Collagen I (Sigma Chemical Co., St. Louis, MO) was dissolved at 2.5 mg/ml in 3% glacial acetic acid. Surfaces coated with collagen I were prepared by adding 50 µl of the collagen I solution into each well and drying under vacuum. After washing the surfaces in PBS three times, endothelial cells were
seeded onto the surfaces at 34,000 cells/cm². After 24 hr the FlexiPerms were removed, the boundaries were marked, and fresh medium was added. After 48 hours incubation at 37 °C in a 5% CO₂ environment, the number of cells that had migrated across the boundary simulating an anastomotic site, from the collagen I-coated surface to the polyurethaneurea surface, were counted under a phase contrast microscope as described above.

![Diagram](image)

1) PUUPPD or PUUYIGSR was cast on the slides
2) collagen I was cast into one well of the FlexiPerm chamber
3) endothelial cells (EC)s were seeded on the collagen layer
4) when ECs become confluent, FlexiPerm was removed
5) ECs migrate across “anastomotic sites”

**Figure 3.4. In vitro** anastomotic site was simulated.

### 3.2.10. Extracellular matrix production by endothelial cells

To evaluate extracellular matrix (ECM) production, eight-well FlexiPerms were attached to the polyurethaneurea films. BAEC suspensions were prepared with 5 µg/ml ascorbic acid added to the culture medium and seeded at a concentration of 17,000
cells/cm². Four of the wells on each film were cultured with 1 μCi/ml ³H-glycine added to the medium to measure the ECM production via incorporation of the radioactive amino acid into newly synthesized ECM proteins. The other four wells were cultured in the absence of ³H-glycine and used for cell counting. After 48 hr of incubation at 37°C in a 5% CO₂ environment, cells cultured in the absence of ³H-glycine were trypsinized and counted on a Coulter Counter. Cells in the remaining wells were rinsed with PBS and DI water and then lysed in 25 mM ammonium hydroxide for 30 min. The remaining ECM was washed with 70% ethanol and air dried. In order to digest glycoprotein, elastin, and collagen, the ECM was sequentially exposed to 200 μg/mL trypsin for 4 hr, 58 U/ml elastase for 4 hr, and 76 U/ml collagenase for 8 hr at 37°C. Finally, the wells were incubated in 1 N NaOH for 1 hr at room temperature to remove any remaining proteins. All enzyme solutions were prepared in TEC buffer (25 mM Tris-HCl, 5 mM calcium chloride, pH 8). ECM production was determined by the amount of radioactivity in the glycoprotein (trypsin-sensitive), elastin (elastase-sensitive), and collagen (collagenase-sensitive) fractions using scintillation counting (Minaxiβ Tri-Carb 4000, Packard Instrument Co., Meridien, CT). NaOH fractions were also evaluated by scintillation counting to ensure that the ECM had been completely digested.
3.2.11. Effect of peptide incorporation on platelet adhesion

PUUPPD and PUUYIGSR films were cast on glass coverslips (18 mm; Fisher Scientific, PA) as described above. A solution of 2.5 mg/ml collagen I (Sigma Chemical Co., St. Louis, MO) solution was prepared in 3% glacial acetic acid. Glass coverslips were incubated with the collagen I solution for 45 minutes in a humidified environment at room temperature to provide a highly thrombogenic reference material. Blood was obtained from a healthy volunteer with 10 U/ml heparin (Sigma Chemical Co., St. Louis, MO). 10 μM mepacrine (Sigma Chemical Co., St. Louis, MO) was added in order to fluorescently label platelets. Collagen I (positive control), PUUPPD, and PUUYIGSR film surfaces were incubated with mepacrine-labeled whole blood at 37°C for 20 minutes and then rinsed with PBS. The number of adherent platelets per field of view (200x) was determined using a fluorescent microscope (Zeiss Axiovert 135, Thornwood, NY).

3.2.12. Statistic analysis

Data were compared with two-tailed, unpaired t-tests. P-values less than 0.05 were considered to be significant. Data are presented as mean ± standard deviation.
3.3. Results

3.3.1. Synthesis and characterization of polyurethaneurea

The NMR spectra of PUUPPD and PUUYIGSR were obtained, and the characteristic proton peaks of tyrosine (6.5 – 7.0 ppm) from the GGGYGSRGGGK sequence indicated the successful incorporation of the peptide sequence into the PUUYIGSR polymer. To ensure that the peptides were not just physically entangled or mixed in polymer matrix, the polymer was washed and filtered several times with methanol prior to NMR measurement. The peaks of prepolymer, PUUPPD, and PUUYIGSR were also assigned and characterized. Then the number of the protons was calculated from the intensity. The intensity was compared to theoretical values. The reactivity of the peptide into the polymer was nearly 100%. The peptide concentration of the polymer matrix was approximately 56 µmol per gram determined from NMR.

![NMR spectra](image)

**Figure 3.5.** $^1$H NMR spectra of PUUYIGSR. Tyrosine peak was identified in a.
The number-average molecular weight (Mn), the weight-average molecular weight (Mw), and the polydispersity index (PDI) were determined by GPC using polystyrene standards. The PUUPPD and PUUYIGSR polymers had similar molecular weights (PUUPPD: Mn = 40,001, Mw = 53,307, PDI = 1.33 and PUUYIGSR: Mn = 43,054, Mw = 54, 531, PDI = 1.27).

The surface atomic composition was determined using electron spectroscopy for chemical analysis (ESCA) with various take-off angles. Nitrogen (N) and oxygen (O) concentrations are related to the urea linkages of the hard segments and amide linkages of the peptide sequences. The higher level of nitrogen and oxygen detected on the PUUYIGSR surface indicates that the GGGYIGSRGGGK peptide sequences were successfully incorporated into the polymer backbone and present at the surface of the material.

<table>
<thead>
<tr>
<th>Take-off angle</th>
<th>PUUPPD (%)</th>
<th>PUUYIGSR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>N</td>
</tr>
<tr>
<td>10°</td>
<td>83.61</td>
<td>1.06</td>
</tr>
<tr>
<td>45°</td>
<td>86.68</td>
<td>0.79</td>
</tr>
</tbody>
</table>

**Table 3.1.** Electron spectroscopy for chemical analysis (ESCA) measurements; atomic concentration of PUUPPD and PUUYIGSR films at variable take-off angles.
There was no significant difference in the FT-IR spectra between PUUPPD and PUUYIGSR. For both PUUPPD and PUUYIGSR, the hydrogen-bonded urea carbonyl peak occurred at 1640 cm\(^{-1}\). The urethane carbonyl peaks appeared at 1720 cm\(^{-1}\) for hydrogen bond and at 1740 cm\(^{-1}\) for free bond. The CH stretch peaks of the soft segment, PTMG, appeared at 2850 cm\(^{-1}\) and 2940 cm\(^{-1}\), and the hydrogen-bonded NH peak also appeared at 3310 cm\(^{-1}\) (Lee and Ko, 1993, Sung et al., 1980).

![Figure 3.6. FT-IR spectra of PUUYIGSR.](image)

The thermal behaviors of PUUPPD and PUUYIGSR were examined using DSC. The glass transition temperature (Tg) was determined to be -30 °C for PUUPPD and -45 °C for PUUYIGSR. The melting point (Tm) was approximately 260 °C for both polymers. Thus, the introduction of peptides into the polymer backbone lowered the glass
transition temperature of the soft segment, but did not affect the melting temperature of the hard segment.

![DSC thermogram of PUUYIGSR](image)

**Figure 3.7.** DSC thermogram of PUUYIGSR.

### 3.3.2. Characterization of polyurethaneurea film

The polymer films were fabricated using a solvent casting method on glass coverslips as described above. The bulk polymer characterization shows that GGGYIGSRGGGK peptide sequences were successfully incorporated into the polymer at a ratio of approximately 56 μmol per gram of polymer. Based on this, the surface peptide concentration of PUUYIGSR is approximately 0.2 nmol/cm². This concentration was calculated using the polymer density (1.25 g/cm³) and the thickness of the monolayer.
(20 nm). The thickness of the monolayer was obtained based on hard segment length (Blackwell and Gardner, 1979, Blackwell and Lee, 1983, Garrett et al., 2001). Water contact angle measurements showed that the contact angle on PUUYIGSR (68.8 ± 1.17) was approximately about 10° lower than the contact angle on PUUPPD (80.16 ± 1.72), indicating that the surface of PUUYIGSR is more hydrophilic and polar due to the incorporated peptides.

3.3.3. **Distribution of peptides and hard segment domains**

In order to study the peptide distribution on the surface of bioactive polyurethaneurea films, a fluorescent dye, NBD-Cl, capable of reacting with the tyrosine residues under aqueous conditions (Boccio et al., 1991), was employed. When reacted with NBD-Cl, PUUPPD showed no fluorescence; however, PUUYIGSR showed bright orange fluorescence evenly distributed over its entire surface area. This result suggests that peptide is uniformly distributed on the surface of the bioactive PUUYIGSR polymer.

The hard segment distribution on the surface was also examined using AFM. Figure 3.8. shows AFM tapping mode images from the surface of polyurethaneurea films at $r_{sp} = 0.88$. The $r_{sp}$ value is the ratio of set point amplitude and free amplitude of oscillation. At $r_{sp} = 0.88$, the very top layer of the surface is being detected (Garrett et al.,
The hard segment domains were exposed to the surface and appeared bright. The cylindrical and spherical hard segment domains were distributed in the soft segment matrix very randomly on the PUUPPD surface. However, on PUUYIGSR, most hard segment domains were cylindrical shape and arranged parallel to the plane of the surfaces. Even at low tapping force, $r_{sp} = 0.80$, the patterns of hard segment distribution in PUUPPD and PUUYIGSR were very similar to $r_{sp} = 0.88$.

**Figure 3.8.** AFM tapping mode phase images of the surfaces of polyurethaneurea at $r_{sp} = 0.88$. Phase images are 500 X 500 nm.

3.3.4. **Mechanical properties of polyurethaneureas**

The incorporation of the peptides into the polymer backbone did not significantly affect the tensile strength ($7.3 \pm 0.29$ MPa for PUUPPD and $7.6 \pm 0.71$ MPa for
However, the elastic modulus was decreased from $3.9 \pm 0.70$ MPa for PUUPPD to $0.9 \pm 0.07$ MPa for PUUYIGSR ($p < 0.005$), and elongation was increased from $123.2 \pm 18.6$ % for PUUPPD to $512 \pm 59.6$ % for PUUYIGSR ($p < 0.001$).

3.3.5. **BAEC viability and cytotoxicity of polyurethaneurea films**

BAECs were seeded and cultured on the polyurethaneurea films and also cultured in extract solutions from the films to evaluate cytotoxicity of any leachables. Over 95% of endothelial cells remained viable at 24 or 72 hr when cultured on PUUPPD and PUUYIGSR films. In addition, to evaluate cytotoxicity of any leachables under aqueous conditions, the polyurethaneurea films were incubated in HBS buffer solution for 30 or 60 days, and BAECs were then cultured with the extract solutions. Over 90% of BAECs were viable over the three different extract concentrations for both PUUPPD and PUUYIGSR.

3.3.6. **BAEC adhesion and spreading on polyurethaneurea films**

We examined whether the peptides incorporated into the polymer backbone could enhance BAEC adhesion and spreading. The attachment of BAECs on polymer films is shown in Figure 3.9. The number of adherent cells increased with increasing
incubation time. The number of adherent cells on PUUYIGSR was significantly higher than on PUUPPD after both 4 and 24 hours incubation (p < 0.01 for 4 hr and p < 0.05 for

Figure 3.9. Attachment and phase contrast micrographs of BAECs on polyurethaneurea films. (a) number of adherent cells after 4 and 24 hr incubation. Data represent the mean of three samples. * p < 0.05, # p < 0.01, compared to PUUPPD. Shaded columns represent PUUPPD and filled columns PUUYIGSR. Phase contrast micrographs of BAECs on (b) PUUPPD and (c) PUUYIGSR films after 4 hr incubation. Scale bar = 50 μm.
24 hr).

Spreading of endothelial cells on polyurethaneurea films was also investigated (Figure 3.10). Cell areas and the percent of spread cells increased with increasing incubation time for both surfaces. However, BAECs on PUUYIGSR showed significantly

![Graph A](image)

![Graph B](image)

**Figure 3.10.** Spreading of BAECs on polyurethaneurea films. (a) cell surface area, (b) percent of cells that were spread. Data represent the mean of thirty samples for cell surface areas and ten samples for percent of cell spreading. # $p < 0.01$, compared to PUUPPD. Shaded columns represent PUUPPD and filled columns PUUYIGSR.
greater cell surface area and percent of spreading cells than on PUUPPD after both 4 and 24 hours of culture ($p < 0.01$).

In order to ensure that the improved cell adhesion and spreading were due to biospecific interactions with YIGSR peptides in the bioactive polymer, competitive inhibition of endothelial cell attachment and spreading was investigated using soluble YIGSR peptides in the culture media. These can bind to cell surface receptors and block their involvement in cell adhesion. As shown in Figure 3.11 (a), the number of adherent cells on PUUYIGSR was significantly greater than on PUUPPD but the values were reduced in the presence of soluble YIGSR peptides over the entire ranges of the soluble peptide concentrations.

Cell surface area and the percent of cell spreading were also reduced over the entire range of soluble peptide concentrations (Figure 3.11 (b) and (c)). Thus, these results indicate that the improved cell adhesion and spreading were specifically mediated by YIGSR- sensitive cell adhesion receptors.
Figure 3.11. Competitive inhibition of attachment and spreading of BAECs by soluble YIGSR peptides. (a) competitive inhibition of attachment. Data represent the mean of four samples. * $p < 0.02$, compared to PUUPPD, # $p < 0.01$, compared to untreated PUUYIGSR. (b) cell surface area and (c) percent of cells that were spread. Data represent the mean of thirty samples for cell surface areas and ten samples for percent of cell spreading. * $p < 0.05$, compared to PUUPPD. # $p < 0.001$, compared to untreated PUUYIGSR.
3.3.7. *BAEC proliferation, migration, and ECM production on polyurethaneurea*

The effect of peptide incorporation on endothelial cell proliferation was examined using immunohistochemical staining with a PCNA-HRP conjugate (Figure 3.12). PCNA is present in cells in the S-phase of mitosis and indicates proliferative activity. The percent of PCNA-positive cells on PUUYIGSR was significantly greater than on PUUPPD after 48 hr of culture (78 ± 5% vs. 60 ± 4%, *p* < 0.001).

![Graph showing PCNA-positive cells percentage](image)

**Figure 3.12.** Proliferation of BAECs on polyurethaneurea films. *p* < 0.001 compared to PUUPPD.

A fence-style assay was used to assess endothelial cell migration on the polyurethaneurea films. The number of cells that had migrated over the original boundary was significantly greater on PUUYIGSR compared to that on PUUPPD (63 ± 8 vs. 37 ±
12, \( p < 0.05 \) as shown in Figure 3.13. In addition, endothelialization at an anastomotic site was simulated in an \textit{in vitro} system. It is very important for endothelial cells to migrate across the anastomotic site, from a natural ECM environment to the synthetic polymer material, to cover the surface of an implanted graft. A fence-style assay was also used, and the number of cells that had migrated over the original boundary from the collagen I-coated surface to the polyurethaneurea surface was determined using a phase contrast microscope. On PUUYIGSR, more cells migrated across anastomotic sites than on PUUPPD (216 ± 95 vs. 76 ± 29, \( p < 0.02 \)), as shown in Figure 3.14.

![Bar chart](image)

**Figure 3.13.** Migration of endothelial cells on polyurethaneurea films. *\( p < 0.001 \), compared to PUUPPD.
Figure 3.14. Migration of BAECs across simulated anastomotic sites; from collagen I surfaces to polyurethaneurea surfaces. Data represent the mean of six samples. * $p < 0.02$, compared to PUUPPD.

To evaluate extracellular matrix (ECM) production, cells were incubated in the presence of $^{3}H$-glycine on the polyurethaneurea films. Matrix production was determined by incorporation of $^{3}H$-glycine into glycoprotein, elastin, and collagen of ECM by using sequential enzyme digests (Figure 3.15). The ECM production by BAECs was significantly increased on PUUYIGSR compared to PUUPPD ($0.0075 \pm 0.0004 \text{ cpm/cell}$ vs. $0.0055 \pm 0.001 \text{ cpm/cell}$, $p<0.05$).
Figure 3.15. Extracellular matrix (ECM) production of endothelial cells on polyurethaneurea films. * $p < 0.05$, compared to PUUPPD.

3.3.8. Effect of peptide incorporation on platelet adhesion

Since some peptides such as RGD support adhesion of platelets (Mazur et al., 1994, Andrieux et al., 1991), the effect of the YIGSR peptide sequences incorporated in the polymer backbone on platelets adhesion was examined using mepacrine-labled whole blood (Figure 3.16). Platelets adhesion on PUUPPD (29.7 ± 7.1) and PUUYIGSR (28.6 ± 9.1) were dramatically lower than on collagen I (94.1 ± 13.1), the positive control. Additionally, there was no significant difference in the number of adherent platelet
between PUUPPD and PUUYIGSR, indicating that the incorporation of the YIGSR adhesion peptide did not enhance thrombogenicity.

![Graph showing number of adherent platelets for Collagen I, PUUPPD, and PUUYIGSR](image)

**Figure 3.16.** The effect of the YIGSR peptide in the polyurethaneurea structure on the platelet adhesion. Platelets were fluorescently labeled with mepacrine. Data represent the mean of ten samples. * $p < 0.001$, compared to Collagen I.
3.4. Discussion

Endothelialization of polyurethaneurea grafts is a possible strategy to improve graft patency, especially for small diameter vascular grafts. The laminin-derived peptide YIGSR has been used to improve endothelial cell adhesion and spreading (Jun and West, 2001, Hubbell et al., 1992, Dee et al., 1995) and thus may aid in graft endothelialization. We have developed bioactive polyurethaneurea by incorporation of the peptide sequence GGGYIGSRGGGK into the polymer backbone. This peptide contains two amine groups (one at the N-terminus and one on the lysine residue) to allow incorporation into the polymer backbone. Glycine was used as a spacer between the bioactive sequence and the synthetic polymer. Incorporation of peptides into the polymer structure is a very easy way to modify polyurethaneureas compared to surface modification because the peptide can be used as a chain extender during polymer synthesis. Additionally, bulk incorporation allows one to develop microporous materials that are uniformly cell adhesive for tissue engineering applications.

Successful incorporation of the peptide sequences into the polymer backbone was assessed by several polymer characterization techniques. The NMR spectra, ESCA, and contact angle results support the successful incorporation of the peptide sequence into the polymer backbone. Phase separation of polyurethaneurea was evaluated using
differential scanning calorimetry (DSC). Previously, the introduction of a second diamine was shown to decrease phase separation (Garrett et al., 2000). However, in this study, the peptide incorporation did not significantly affect phase separation according to DSC results. In addition, interesting results were found with respect to mechanical properties. The peptide incorporation made the polymer more elastic without decreasing its tensile properties. The improved elasticity may be helpful to transmit mechanical stimuli to the seeded endothelial cells and also improve compliance matching, hopefully reducing intimal hyperplasia at the anastomotic sites (Ballyk et al., 1998).

The distribution of the peptides on the surfaces of the PUUYIGSR films was investigated using fluorescent probes. Previously, YIGSR was immobilized to the glass surfaces, and dansyl chloride was reacted with the amine terminus of the peptides (Kouvroukoglou et al., 2000). They reported that the peptides were not uniformly distributed, and bright fluorescent clusters were found on the surfaces. In this study, bright orange fluorescence was found evenly on the entire surface area of the PUUYIGSR film, but no fluorescence was observed on PUUPPD. The distribution of the hard segment domains was also examined using AFM. The morphology of the phase-separated microdomains can be visualized using tapping mode AFM (Garrett et al., 2000). Cylindrical or spherical hard segment domains were visualized clearly on the tapping
mode images. In this study, we found that the second diamine (peptides) can affect the
distribution or the arrangement of hard segment domains on the surfaces: the hard
segment domains of PUUYIGSR were arranged parallel to the plane of the surface
compared to random distribution of those of PUUPPD.

We demonstrated that attachment, proliferation, migration, and extracellular
matrix production of endothelial cells were enhanced without increasing platelet adhesion
on PUUYIGSR. In addition, endothelialization at an anastomotic site was simulated in
an in vitro system. The greater number of cells had migrated across anastomotic sites on
PUUYIGSR compared to PUUPPD. To enhance endothelialization in vivo, it is very
important for endothelial cells to migrate across anastomotic sites, from the adjacent
vessel wall to the synthetic biomaterial, to cover the surfaces of implanted grafts.
3.5. Conclusions

A bioactive YIGSR peptide-modified polyurethaneurea (PUUYIGSR) has been successfully synthesized by incorporating the peptide GGGYIGSRGGGK into the polymer backbone. Cellular responses of endothelial cells on PUUYIGSR show that the incorporated peptides maintain their bioactivity, and may potentially improve vascular graft endothelialization. Platelet adhesion to this material (in the absence of adherent endothelium) was still moderately high and could lead to graft failure. Thus, a second generation of YIGSR-modified polyurethaneurea was synthesized with polyethylene glycol incorporated into the soft segment and GGGYIGSRGGGK incorporated into the hard segment.
Chapter 4. Modification of polyurethaneurea with PEG and YIGSR peptide to enhance endothelialization without platelet adhesion

4.1. Introduction

In chapter 3, we synthesized a bioactive polyurethaneurea by incorporating GGGYIGSRGGGK peptide sequences into the polymer backbone. Improved endothelial cell adhesion, proliferation, migration, and extracellular matrix production were observed. However, though the incorporation of the YIGSR peptide did not enhance platelet adhesion, platelet adhesion to the polyurethane urea was significant. This will likely limit its clinical impact. Thus, the focus of work presented in this chapter was the design of bioactive polyurethaneureas with the same positive endothelial cell interactions but with reduced affinity for platelets.

Polyethylene glycol (PEG) has been an attractive biomaterial due to its resistance to protein adsorption, platelet adhesion, and bacterial adhesion (Kim, 1996, Park et al., 1998, Wesslen et al., 1994, Deible et al., 1998b). These properties are believed to be caused by low interfacial surface energy, high chain motility, and molecular chain conformation as shown in Figure 4.1 (Park et al., 1998, Han et al., 1998). To improve biocompatibility, various efforts have been made to introduce PEG into biomaterials by
grafting (Wesslen et al., 1994, Lee and Matsuda, 1999, Park et al., 1999, Park et al., 1991), physical blending (Park and Bae, 2002), covalent attachment to the surface mediated by proteins (Deible et al., 1998a, Deible et al., 1998b), and synthesis of copolymers (Silver et al., 1994). Covalent incorporation of PEG into polyurethaneureas as at least a portion of the soft segment is desirable for long-term stability and three-dimensional fabrication of a non-thrombogenic biomaterial.

![Polyurethane Matrix](Diagram1.png)

**Figure 4.1.** The environment change leads to surface reorganization and minimize the interfacial free energy.

In this study, we developed a YIGSR peptide/PEG-modified polyurethaneurea by incorporation of GGYIGSRGGKK peptide sequences as a chain extender and PEG as a soft segment in the polymer backbone. The effects of the incorporated bioactive peptide sequences and hydrophilic PEG chain on adhesion of cells and platelets as well as bulk mechanical properties were evaluated.
4.2. Materials and Methods

4.2.1. Synthesis of polyurethaneurea (PUUPPD)

Prepolymer was synthesized by reacting methylene di(p-phenyl isocyanate) (MDI; Aldrich chemical Co., Milwaukee, WI) with poly(tetramethylene oxide) (PTMO; Aldrich chemical Co., Milwaukee, WI), and then extended with p-phenylene diamine (PPD; ACROS, New Jersey) (Jun and West, 2004). MDI was recrystallized in hexane, and PTMO was dried under vacuum for 48 hr. A 10% (w/v) solution of MDI (4 mmol, MW: 250 g/mol) in 10 ml anhydrous N,N-dimethylformamide (DMF; Aldrich Chemical Co., Milwaukee, WI) was prepared in a 100 ml three-neck round flask and stirred at room temperature. A 10% (w/v) solution of PTMO (2 mmol, MW: 2000 g/mol) in 30 ml anhydrous DMF was added, and the mixture was heated to 75°C and held there for 3 hr under argon. The reactor was cooled to room temperature before PPD (2 mmol, MW:108 g/mol) in 3 ml anhydrous DMF was added as a chain extender. The polymer solution was then incubated 45°C for 3 hr under argon gas. The polymer solution was cooled to room temperature, precipitated in methanol, and dried under vacuum.

4.2.2. Synthesis of PEG-modified polyurethaneurea (PUUPPD-PEG)

PTMO and polyethylene glycol (PEG, MW:4,600 g/mol; Aldrich chemical Co.,
Milwaukee, WI) were dried for 48 hr under vacuum and used as the soft segments (molar ratio of PTMO to PEG = 85/15). Polymer synthesis proceeded exactly as described above but using 85% PTMO/15% PEG mixture to form the soft segments.

![Chemical Structure]

**PUUPPD PEG**  
**PUUYIGSR PEG**

**Figure 4.2.** Synthesis of PEG and peptide modified polyurethaneurea. PEG was used as a soft segment and YIGSR peptide was incorporated into the hard segment.

4.2.3. *Synthesis of peptide/PEG-modified polyurethaneurea (PUUYIGSR-PEG)*

Prepolymer was synthesized by reacting MDI with PTMO/PEG mixture (85/15 molar ratio) as described above and extended with a combination of GGGYIGSRGGGK peptide (Sigma-Genosys, Woodlands, TX) and PPD. Prepolymer was synthesized using a 10% (w/v) solution of MDI (1.5 mmol), PTMO (0.64 mmol), and PEG (0.11 mmol) at 75°C for 3 hr under argon gas as described above. Next, chain extension was performed
using GGGYIGSRGGGK peptide (0.14 mmol) and PPD (0.61 mmol) in anhydrous DMF at 45°C for 3 hr under argon. The polymer solution was cooled to room temperature, precipitated in ethyl acetate, and dried under vacuum.

4.2.4. Polymer characterization

PUUPPD, PUUPPD-PEG, and PUUYIGSR-PEG were characterized via $^1$H NMR using a 400 MHz NMR spectrometer (Advance 400, Bruker, Germany) in $N,N$-dimethylformamide-d$_7$ (DMF-d$_7$; Aldrich Chemical Co., Milwaukee, WI).

Molecular weight distributions were obtained by GPC with UV and evaporative light scattering detectors (Polymer Laboratories, Amherst, MA). Samples for GPC analysis were dissolved in HPLC-grade DMF at a concentration of 1 mg/ml and run at 70°C through PLgel 5 μm Mixed-C columns (Polymer Laboratories, Amherst, MA) at a flow rate of 1 mL/min. Calibration was performed using polystyrene standards (PS; Polymer Laboratories, Amherst, MA).

DSC thermograms were obtained using a differential scanning calorimeter (Pyris 1, Perkin Elmer, Wellesley, MA). Samples were cooled below -60°C and increased at 10°C/min to 300°C under nitrogen gas. Glass transition temperatures and melting temperatures were analyzed using Pyris software. Fourier transform infrared (FTIR)
spectroscopy was performed using a Nicolet 500 spectrometer. Thin films of polyurethaneurea were prepared by mixing samples with KBr and pressed into pellets under vacuum. Sixteen scans were taken of each sample at a resolution of 4 cm\(^{-1}\).

4.2.5. Preparation and characterization of polyurethaneurea films

Polymers were dissolved in tetrahydrofuran (THF; 0.3 wt\%) and sterilized using 0.2 μm pore size PTFE syringe filters (Whatman, NJ). Polymer films were prepared on glass coverslips (18 mm; Fisher Scientific, PA) by solvent casting at room temperature. Polymer films were held under vacuum for 48 hr to ensure removal of the solvent.

The equilibrium contact angles of DI water on PUUPPD, PUUPPD-PEG, and PUUYIGSR-PEG films were measured using a contact angle goniometer (CAM-Micro). Six measurements were taken to calculate average contact angles on the surface of each film.

ESCA analysis was performed using a Physical Electronics Model 5700 XPS instrument. Photo-emissions were produced through the use of a X-ray source (1486.6 eV) operated in the fixed retard ratio mode at a pass energy of 23.5 eV. Spectra were acquired over a 10 – 45 take off angle range. Charge neutralization was accomplished via bombardment with a low energy beam.
Uniaxial mechanical testing was performed using an Instron model 5565 at a cross head speed of 25 mm/min with a 5 kN load cell. Polymers were dissolved in DMF (Aldrich Chemical Co., Milwaukee, WI) at 10 wt% and sterilized using 0.2 µm pore size PTFE syringe filters (Whatman, NJ). Polymer films were prepared in Teflon molds by solvent casting at 60°C under vacuum for 48 hr. Test specimens were prepared according to ASTMD-638-VI. The tensile strength was calculated from the maximum load at break and the initial cross sectional area of the specimen. Sample thickness was measured using a digital caliper (Mitutoyo, Hauppauge, NY).

4.2.6. Cell maintenance

Bovine aortic endothelial cells (BAEC; Clonetics, San Diego, CA), passage 2 – 5, were used for this study. Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma Chemical Co., St. Louis, MO) was prepared with 10% fetal bovine serum (FBS; BioWhitaker, Walkersville, MD), 2 mM L-glutamine, 1 unit/ml penicillin, and 100 mg/ml streptomycin (GPS; Sigma Chemical Co., St. Louis, MO). Endothelial basal medium (EBM; Sigma Chemical Co., St. Louis, MO) was prepared with 10% endothelial medium supplement (Sigma Chemical Co., St. Louis, MO), which contained fetal bovine serum, basic fibroblast growth factor, heparin, epithelial growth factor, and hydrocortisone.
BAECs were maintained on mixture of EBM and DMEM (25/75 volume ratio) at 37°C in a 5% CO₂ environment.

4.2.7. Adhesion and spreading of endothelial cells on polyurethaneurea films

To evaluate adhesion of endothelial cells, BAECs were seeded at a concentration of 17,000 cells/cm² in eight-well FlexiPerm chambers (Sigma Chemical Co., St. Louis, MO) attached to polyurethaneurea films. Cells were cultured at 37°C in a 5% CO₂ environment. To evaluate endothelial cell spreading, after 4 hr of incubation, non-adherent cells were removed by rinsing, and fresh medium was added. Cells were observed by phase contrast microscopy (Zeiss Axiovert 135), and digital image processing (Scion Image) was used to determine cell areas. To evaluate cell attachment, the medium was removed by rinsing with PBS, and adherent cells were detached with trypsin and counted using a Coulter Counter (Multisizer 3, Beckman Coulter).

Competitive inhibition of attachment and spreading of endothelial cells was also examined using soluble YIGSR peptides to ensure that improved BAEC adhesion and spreading was due to biospecific interactions with the YIGSR peptides that were incorporated into the polymer structure. Cells were seeded at a concentration of 17,000 cells/cm² in eight-well FlexiPerm chambers attached to polyurethaneurea films and
incubated with soluble YIGSR peptide (Sigma Chemical Co., St. Louis, MO) at 0, 0.01, 0.1, and 1 mM in EBM/DMEM (25%/75%) at 37°C in a 5% CO₂ environment. After 4 hr incubation, attachment and spreading of cells were evaluated as described above.

### 4.2.8. Migration of endothelial cells on polyurethaneurea films

To assess migration, a fence-style assay was utilized. Polyurethaneurea films were placed in the 6-well tissue culture plates, and double-walled round Teflon molds (inner diameter: 6 mm, outer diameter: 17 mm) were placed on top of the films. BAECs were seeded at a concentration of 35,000 cells/cm² in the inner walls. After 24 hr, the original boundaries were recorded, and the inner walls were removed. Mitomycin C (Calbiochem, San Diego, CA) was added to the medium at 0.5 μg/mL to prevent cell proliferation. After 24 and 48 hr of incubation at 37°C in a 5% CO₂ environment, the cells that had migrated over the original boundary were observed using a phase contrast microscope (Zeiss Axiovert 135).

### 4.2.9. Adhesion of platelet on YIGSR peptide/PEG-modified polyurethaneurea

PUUPPD and PUUYIGSR films were cast on glass coverslips (18 mm; Fisher Scientific, PA) as described above. A solution of 2.5 mg/ml collagen I (Sigma Chemical
Co., St. Louis, MO) solution was prepared in 3% glacial acetic acid. Glass coverslips were incubated with the collagen I solution for 45 minutes in a humidified environment at room temperature to provide a highly thrombogenic reference material. Blood was obtained from a healthy volunteer with 10 U/ml heparin (Sigma Chemical Co., St. Louis, MO). 10 μM mepacrine (Sigma Chemical Co., St. Louis, MO) was added in order to fluorescently label platelets. Collagen I (positive control), PUUPPD, and PUUYIGSR film surfaces were incubated with mepacrine-labeled whole blood at 37°C for 20 minutes and then rinsed with PBS. The number of adherent platelets per field of view (200x) was determined using a fluorescent microscope (Zeiss Axiovert 135, Thornwood, NY).

4.2.10. Statistic analysis

Data were compared with two-tailed, unpaired t-tests. P-values less than 0.05 were considered to be significant. Data are presented as mean ± standard deviation.
4.3. Results

4.3.1. Synthesis and characterization of YIGSR peptide/PEG-modified polyurethaneurea

The NMR spectra of polyurethaneurea were obtained and characterized as described in chapter 3. The characteristic proton peaks of tyrosine (6.5 – 7.0 ppm) from the GGGYIGSRGGGK sequence were assigned, indicating the successful incorporation of the peptide sequence into the PUUYIGSR polymer. The peaks of prepolymer, PUUPPD-PEG, and PUUYIGSR-PEG were also assigned and characterized. Based on the number of protons calculated from peak intensities, the peptide concentration in the polymer was approximately 56 μmol/g. The surface peptide concentration of PUUYIGSR-PEG film was also estimated to be approximately 0.2 nmol/cm² using the

![NMR spectra](image)

**Figure 4.3.** $^1$H NMR spectra of PUUYIGSR-PEG. Tyrosine peaks were identified in a.

FT-IR spectra of the polyurethaneureas were also obtained and characterized. The incorporation of the PEG as a soft segment did not affect FT-IR spectra compared to PUUPPD in chapter 3. For both PUUPPD-PEG and PUUYIGSR-PEG, peaks for hard segments were observed at 1640 cm\(^{-1}\) (hydrogen-bonded urea carbonyl), at 1720 cm\(^{-1}\) (urethane carbonyl peaks for hydrogen bond), and at 1740 cm\(^{-1}\) (urethane carbonyl peaks for free bond). The CH stretch peaks of the soft segment, PTMO, appeared at 2850 cm\(^{-1}\) and 2940 cm\(^{-1}\), and the hydrogen-bonded NH peak also appeared at 3310 cm\(^{-1}\) (Jun and West, 2004, Lee and Ko, 1993, Sung et al., 1980).

![FTIR spectrum of PUUYIGSR-PEG](image)

**Figure 4.4.** FTIR spectra of PUUYIGSR-PEG
Thermal behaviors from DSC provide successful incorporation of PEG as a soft segment as shown in Figure 4.5. The glass transition temperature (Tg) of PUUPPD was observed at 9 °C. However, two distinct Tg were observed at 14 °C and 40 °C for PUUPPD-PEG and PUUYIGSR-PEG, likely caused by PTMO and PEG, respectively. The melting point (Tm) of PUUPPD and PUUPPD-PEG was determined about 180 °C, and the Tm of PUUYIGSR-PEG was approximately 165 °C.

Figure 4.5. DSC thermogram of PUUPPD, PUUPPD-PEG, and PUUYIGSR-PEG. A second Tg was found in PEG-modified polyurethaneurea.
The surface atomic concentration was determined using ESCA with various take-off angles. Nitrogen (N) and oxygen (O) concentrations are related to the urea linkages of the hard segments and amide linkages of the peptide sequences. The higher level of nitrogen and oxygen detected on the PUUYIGSR-PEG at 45° indicates that the GGGYIGSRGGGK peptide sequences were successfully incorporated into the polymer backbone and present at the surface of the material.

<table>
<thead>
<tr>
<th>Take-off angle</th>
<th>PUUPPD-PEG (%)</th>
<th>PUUYIGSR-PEG (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>N</td>
</tr>
<tr>
<td>10°</td>
<td>58.4</td>
<td>12.1</td>
</tr>
<tr>
<td>45°</td>
<td>75.1</td>
<td>0.4</td>
</tr>
</tbody>
</table>

**Table 4.1.** Electron spectroscopy for chemical analysis (ESCA) measurements; atomic concentration of PUUPPD-PEG and PUUYIGSR-PEG films at variable take-off angles.

The number-average molecular weight (Mn), the weight-average molecular weight (Mw), and the polydispersity index (PDI) were determined by GPC. PUUPPD-PEG and PUUYIGSR-PEG had similar molecular weights (PUUPPD: Mn = 113,489, Mw = 153,973, PDI = 1.36, PUUPPD-PEG: Mn = 92,645, Mw = 124,072, PDI = 1.34 and PUUYIGSR-PEG: Mn = 96,675, Mw = 116,103, PDI = 1.20).
Water contact angle measurement demonstrated that the contact angle dropped significantly on PEG-modified polyurethaneurea, PUUPPD-PEG (59.6 ± 1.7) and PUUYIGSR-PEG (50.6 ± 1.3), compared to PUUPPD (81.6 ± 1.1). The contact angle of PUUYIGSR-PEG was also significantly lower than that of PUUPPD-PEG. Thus, the combination of the incorporated peptides and PEG made the surface of PUUYIGSR-PEG more hydrophilic and polar.

4.3.2. Mechanical properties of polyurethaneurea

The incorporation of the PEG and peptide sequences into the polyurethaneurea backbone affected the mechanical properties. The mechanical properties of PUUPPD-PEG (elastic modulus: 0.44 ± 0.1 MPa, tensile strength: 3.1 ± 0.4 MPa, elongation: 370 ± 84.3 %) was significantly lower compared to PUUPPD (elastic modulus: 1.1 ± 0.2 MPa, tensile strength 27.2 ± 1.2 MPa, elongation: 2580 ± 544.7 %). However, the incorporation of the peptide sequences increased the mechanical properties of PUUYIGSR-PEG (elastic modulus: 0.21 ± 0.1 MPa, tensile strength 9.2 ± 1.8 MPa, elongation: 3532 ± 222.8 %).
4.3.3. Adhesion and spreading of BAECs on polyurethaneurea films

The effects of PEG and peptides incorporated into the polyurethaneurea on BAEC attachment and spreading were evaluated. BAECs were seeded on the PUUPPD, PUUPPD-PEG, and PUUYIGSR-PEG films, and adhesion and spreading were investigated after 4 hr. The number of adherent cells on PUUPPD-PEG was significantly lower than on PUUPPD ($p < 0.005$) as shown in Figure 4.6 (a). However, cell attachment increased dramatically on PUUYIGSR-PEG ($p < 0.001$). There was no statistical difference for endothelial cell attachment on PUUPPD versus PUUYIGSR-PEG. Similar results were also observed for cell area Figure 4.6 (b) and percent of cell spreading Figure 4.6 (c). Figure 4.7 also shows phase contrast micrographs of endothelial cells on PUUPPD-PEG and PUUYIGSR-PEG films. The incorporation of PEG decreased cell surface area and percent of cell spreading but the incorporation of the peptides promoted cell spreading; Few cells attached, and most of them were round shape and did not spread on PUUPPD-PEG. However, a greater number of cells attached and completely spread on PUUYIGSR-PEG.
Figure 4.6. Attachment and spreading of BAECs on polyurethaneurea films after 4 hr incubation. (a) number of adherent cells. * $p < 0.005$ compared to PUUPPD, # $p < 0.001$, compared to PUUPPD PEG. (b) cell surface area and (c) percent of cells that were spread. # $p < 0.001$ compared to PUUPPD, # $p < 0.001$ compared to PUUPPD-PEG.

Figure 4.7. Phase contrast micrographs of BAECs on (a) PUUPPD-PEG and (b) PUUYIGSR-PEG after 4 hr incubation. The length of the scale bar = 50 μm.
To ensure that improved cell adhesion and spreading were mediated by YIGSR specific cell adhesion receptors, competitive inhibition of endothelial cell attachment and spreading was studied using soluble YIGSR peptides in culture media. Fewer cells attached, and most attached cells were round in shape over the entire range of soluble peptide concentrations as shown in Figure 4.8.

![Micrographs](image)

**Figure 4.8.** Phase contrast micrographs of competitive inhibition of attachment and spreading of BAECs incubated with soluble YIGSR peptides at three different concentrations. (a) PUUYIGSR-PEG (b) + 0.01, (c) + 0.1, and (d) + 1 YIGSR. The length of the scale bar = 50 μm.

The adhesion of endothelial cells dramatically increased on PUUYIGSR-PEG compared to that on PUUPPD-PEG. However, adhesion of BAECs was reduced in the presence of soluble YIGSR peptides over the entire ranges of the soluble peptide
concentrations (0.01, 0.1, and 1 mM) as shown in Figure 4.9. These results suggest that adhesion of BAECs to this peptide-modified material is predominantly mediated by specific receptor-ligand interactions.

**Figure 4.9.** Competitive inhibition of attachment and spreading of BAECs by soluble YIGSR peptides after 4 hr incubation. BAECs were incubated with soluble YIGSR peptides at three different concentrations (0.01, 0.1, and 1 mM). (a) a number of adherent cells. * $p < 0.001$ compared to PUUPPD-PEG, # $p < 0.001$ compared to untreated PUUYIGSR-PEG. (b) cell surface areas and (c) cell spreading. * $p < 0.001$ compared to PUUPPD-PEG, # $p < 0.001$ compared to untreated PUUYIGSR-PEG.
4.3.4. Migration of BAECs on PUUYIGSR-PEG

Because cell adhesion was extremely low on PUUPPD-PEG, migration of BAECs was evaluated only on PUUYIGSR-PEG. As shown in Figure 4.10, by 24 and 48 hr after seeding, significant migration beyond the original boundary was observed. This suggests that these materials may be capable of supporting autologous endothelialization.

![Image](image.png)

Figure 4.10. Migration of BAECs on PUUYIGSR-PEG after (a) 24 hr and (b) 48 hr.

4.3.5. Adhesion of Platelets on YIGSR peptide/PEG-modified Polyurethaneurea

To generate a platelet-resistant material, we synthesized polyurethaneureas that included PEG as a potion of the soft segment domains. The adhesion of platelets on polyurethanurea films was evaluated using mepacrine-labeled whole blood (Figure 4.11 and 12). Adhesion of platelets on PUUPPD (124 ± 27) was lower than on Collagen I (310...
but still substantial. However, there was almost no adhesion of the platelets on PUUPPD-PEG (9 ± 4) or PUUYIGSR-PEG (7 ± 2). Thus, PUUYIGSR-PEG is a material that supports robust endothelial cell attachment and growth while being highly resistant to platelet adhesion and resultant complications.

**Figure 4.11.** The number of adherent platelets on the surfaces of collagen I, PUUPPD, PUUPPD-PEG, and PUUYIGSR-PEG. Data represent the mean of five samples. * p < 0.001 compared to Collagen I, # p < 0.001 compared to PUUPPD. Platelets were fluorescently labeled with mepacrine.
4.4. Discussion

NMR, GPC, DSC, ESCA, and contact angle measurements confirmed successful incorporation of the peptide sequences and PEG. It was noted from the DSC results that two distinct peaks (Tg) were observed in PEG-modified polyurethaneurea caused by PTMO and PEG. Higher molecular weight and crystallization of PEG might induce higher Tg. The peptide sequences didn't affect Tg but lowered Tm. These results were a little different from chapter 3; introduction of the peptide sequences lowered Tg without affecting Tm. In this study, the entanglement of the flexible PEG chain and the peptide sequences might affect thermal behavior; The high molecular weight PEG seems to prevent decrease of Tg but long peptide sequences also might restrict the crystallization of PEG.

The effect of PEG and the peptide sequences on the bulk properties of polymer has been clearly observed in mechanical properties. In spite of similar molecular weight, the incorporation of the peptide sequences resulted in enhanced mechanical properties that were dramatically decreased by the presence of the PEG chain. In a previous study, the incorporation of the peptide sequence increased elongation without affecting tensile strength. However, in this study the peptide sequences enhanced both tensile strength and elongation. This may be caused by molecular interactions in polymer chains, the degree
of distribution of hard segment domains in soft segment matrix, and restriction of motility of flexible PEG chain by long amino acid sequences. Thus, our study suggests the possibility that the bioactive peptide sequences are able to control not only cellular behaviors but also bulk mechanical properties.

The hydrophilicity of polymer surfaces was evaluated using contact angle measurement. The order of hydrophilicity was PUUPPD>PUUPPD-PEG>PUUYIGSR-PEG. The lower contact angle of PUUYIGSR-PEG compared to PUUPPD-PEG indicated that peptides were also exposed on the surface with PEG. Endothelial cell attachment and spreading were evaluated to determine if the peptide sequences maintain their bioactivity on the surface without interference by PEG. Few endothelial cells attached on PEG-modified surfaces and most didn’t spread. On the other hand a dramatic increase in cell attachment and spreading were observed on the peptide/PEG-modified surfaces. Competitive inhibition of cell attachment and spreading study confirmed that these results were mediated by specific YIGSR sensitive cell adhesion receptors.

The main reason to modify biomaterials using hydrophilic PEG is to prevent platelet adhesion, and the results depend on modification methods, ratio of hard and soft segments, and molecular weight as well as contents of PEG (Lee and Matsuda, 1999, Deible et al., 1998a, Deible et al., 1998b, Silver et al., 1994). In this study, 4600 g/mole
PEG was incorporated at a 15% molar ratio as a soft segment. As shown above, this amount was enough to form a PEG rich phase on the surface, preventing cell attachment. Platelet adhesion to the surface was also evaluated using mepacrine-labeled whole blood. There was no observable platelet adhesion on PEG- and PEG/peptide-modified polyurethaneurea surfaces in contrast to unmodified polyurethaneurea. This may be due to suppressed protein adsorption; adsorbed fibrinogen plays an important role in mediating platelet adhesion on surfaces (Andrieux et al., 1991, Mazur et al., 1994). These results also confirmed the YIGSR peptide is an appropriate peptide sequence for cardiovascular applications.
4.5. Conclusions

A bioactive peptide/PEG-modified polyurethanurea has been synthesized, and successful incorporation of the peptide sequences as a chain extender and PEG as a soft segment were confirmed. This material exhibited enhanced adhesion, migration, and growth of endothelial cells by a bioactive peptide sequences as well as suppressed platelet adhesion by hydrophilic PEG chains. Importantly, bioactive peptide sequences also supported improved mechanical properties. This material may be a good candidate for engineering small diameter vascular grafts.
Chapter 5. Endothelialization on a microporous bioactive YIGSR/PEG-modified polyurethaneurea

5.1. Introduction

Enhanced endothelialization and minimized compliance mismatching is essential for improving clinical patency of small diameter vascular grafts (Okoshi et al., 1996). Microporous structures can induce rapid transmural and anastomotic tissue ingrowth from surrounding tissue into the graft, thus resulting in enhanced endothelialization that improves thromboresistance (Lamba et al., 1998, Doi et al., 1996, Doi and Matsuda, 1997). The improved elasticity resulting from interconnected open micropores can also reduce compliance mismatching, decreasing intimal hyperplasia at the anastomotic sites (Ballyk et al., 1998, Doi et al., 1996).

Porous scaffolds have been fabricated using various techniques such as solvent casting and salt leaching (Mikos et al., 1994), gas foaming (Harris et al., 1998), gas foaming and salt leaching (Yoon and Park, 2001), super critical CO\textsubscript{2} (Hile et al., 2000), excimer laser ablation (Doi et al., 1996, Matsuda and Nakayama, 1996), phase separation (Hu et al., 2002), and freeze-drying/salt leaching (Claase et al., 2003). Each method has its own advantages and disadvantages depending on the polymer system. For vascular
graft applications, the pore structure should be open and interconnected in order to induce cell migration through the scaffolds. The pore size also needs to be precisely controlled; large enough to guide cell ingrowth yet small enough to prevent blood from leaking through grafts. Higher porosity will improve tissue incorporation into the scaffolds but decrease mechanical properties. Thus, porosity must also be controlled to obtain appropriate strength and compliance (Doi et al., 1996, Matsuda and Nakayama, 1996, Lamba et al., 1998). One advantage to gas foaming and salt leaching methods is their ability to simultaneously control both pore size and porosity by changing parameters such as salt size, an amount of loading salts, and concentration of acid during the process. This method has shown to minimize the formation of closed pores and surface skin layers (Yoon and Park, 2001).

In addition to microporous structural design, the chemical properties of the biomaterial are also very important to enhancing endothelialization. The modification of biomaterial or scaffold with radio frequency glow discharge plasma (Tseng and Edelman, 1998), growth factors (Doi and Matsuda, 1997), or cell adhesive peptides sequences (Holt et al., 1994, Jun and West, 2004) can control the rate of tissue formation. However, the modification of the scaffold should promote endothelial cell proliferation or migration without changing the microporous structure (porosity) and biocompatibility (non-
In chapter 4, we developed a dually modified polyurethaneurea by incorporating endothelial cell adhesive YIGSR peptide sequences as chain extenders and non-thrombogenic PEG as a soft segment (PUUYIGSR-PEG) in the polymer backbone. Virtually no platelet adhesion was observed on PUUYIGSR-PEG, while endothelial cell adhesion, spreading, and migration were significantly greater on PUUYIGSR-PEG compared to PUUPPD-PEG. The bulk modification might be particularly useful for fabrication of porous scaffolds with cell adhesive characteristics throughout the pore structure. In this study, we have fabricated a bioactive microporous scaffold using a modified gas foaming and salt leaching method. The effects of bioactive peptide sequences and microporous structure on endothelialization have been explored.
5.2. Materials and Methods

5.2.1. Fabrication of microporous scaffolds using gas forming and salt leaching

PEG-containing polyurethane urea (PUUPPD-PEG) was synthesized by reacting 4,4’-methylene di(p-phenyl isocyanate) with poly(tetramethylene oxide)/PEG mixture (85/15 molar ratio) and extending with p-phenylene diamine as described chapter 4. A material also containing the YIGSR peptide (PUUYIGSR-PEG) was synthesized similarly except it was extended with a combination of GGGYIGSRRGGGK and PPD as described in chapter 4 (Jun and West, 2003).

Scaffolds were fabricated using gas foaming and salt leaching via incorporation of sodium bicarbonate as follows: PUUPPD-PEG and PUUYIGSR-PEG were dissolved in anhydrous N,N-dimethylformamide (DMF; Aldrich Chemical Co., Milwaukee, WI) at 10% (w/v) concentration, and DMF was partially evaporated at 60°C under vacuum. Sieved sodium bicarbonate salts (Sigma Chemical Co., St. Louis, MO; particle size of 100-200 µm, weight fraction = 90 wt%) were added to the polymer solutions at room temperature and mixed well. The paste of polymer and salts was placed in a Teflon mold (75x25x1 mm) and dried for 24 hr at room temperature. The polymer/salt films were immersed in 50% citric acid (Sigma Chemical Co., St. Louis, MO) for 30 min to induce gas foaming and then in DI water for 3 days to leach remaining salts. DI water was
changed every day, and samples were freezedried for 48 hr. Scaffolds were cut into disks using a cork borer (diameter = 7 mm, thickness = 1 mm, weight = 10-20 mg).

![Diagram](image)

**Figure 5.1.** Fabrication of microporous scaffolds using salt leaching and gas forming method. Sodium bicarbonate was used as a effervescent salt.

### 5.2.2. Characterization of scaffolds

The porosity of the scaffold was determined by mercury intrusion porosimetry (Autoscan-500, Quantachrome, Boynton Beach, FL). Scaffolds were loaded into the intrusion chamber for measurement. Mercury was intruded into samples at 500 psi, and the intruded mercury volume and pressure were recorded. Porosity was determined from the total intruded volume per unit mass (Mikos et al., 1994).

The microstructure of the scaffold was investigated using scanning electron
microscopy (SEM). Samples were sputter coated with gold for 30 sec at 100 mA using a sputter coater (Pelco Sputter Coater 91000, Ted Pella, Redding, CA) for a coating thickness of ~82 nm. The surface and cross-section of scaffolds were observed using SEM (JEOL, JSM-5300, Japan) operated at 30 kV.

Uniaxial mechanical testing was performed using an Instron model 5565 at a cross head speed of 25 mm/min with a 50 N load cell. Test specimens were prepared according to ASTM-D-638-VI. The tensile strength was calculated from the maximum load at break and the initial cross sectional area of the specimen. Sample thickness was measured using a digital caliper (Mitutoyo, Hauppauge, NY).

5.2.3. Cell maintenance

Bovine aortic endothelial cells (BAEC; Clonetics, San Diego, CA), passage 2 – 5, were used for this study. Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma Chemical Co., St. Louis, MO) was prepared with 10% fetal bovine serum (FBS; BioWhitaker, Walkersville, MD), 2 mM L-glutamine, 1 unit/ml penicillin, and 100 mg/l streptomycin (GPS; Sigma Chemical Co., St. Louis, MO). Endothelial basal medium (EBM; Sigma Chemical Co., St. Louis, MO) was prepared with 10% endothelial medium supplement (Sigma Chemical Co., St. Louis, MO) containing fetal bovine serum, basic
fibroblast growth factor, heparin, epithermal growth factor, and hydrocortisone. BAECs were maintained on mixture of EBM and DMEM (25/75 %) at 37 °C in a 5% CO₂ environment.

5.2.4. Seeding and culture of endothelial cells in the scaffolds

Scaffolds disks were immersed in 70% ethanol overnight prior to use. The scaffolds were then soaked in sterile PBS for 30 min three times, and in cell culture media for 15 min. For cell seeding, the scaffolds were placed in 6-well tissue culture plates, and excess medium was removed. 40 μL of cell suspension at 1×10⁶ cells/mL was added onto the top of the scaffold slowly and allowed to permeate through the matrix. The scaffold was turned over, and an additional 40 μL of the cell suspension was added to the opposite surface. 6-well plates containing cell seeded-scaffolds were maintained at 37°C in a 5% CO₂ environment for cell attachment. After 90 min, cell-seeded scaffolds were placed in 96-well plates, media was added, and constructs were incubated at 37°C in a 5% CO₂ environment. After three days of culture, each cell-seeded scaffold was placed in a well of a 24-well plate, washed three times with the sterile PBS, and fixed with 2.5% glutaraldehyde (Sigma Chemical Co., St. Louis, MO) at 4°C for overnight.

For evaluation by SEM, the cell-seeded scaffolds were dehydrated in a graded
series of ethanol/water solutions, and dried with tetramethylsilane on ice (TMS; Electron Microscopy Science, Fort Washington, PA). Samples were sputter coated, and morphology of cells in the scaffold was observed under SEM as described above.

For histological analysis, cell-seeded scaffolds were sectioned to 30 μm using a cryostat (HM505E; Microm, Walldorf, Germany). Sections were stained with hematoxylin and observed by light microscopy (Zeiss Axiovert 135).

5.2.5. Migration of cells through the scaffold matrix

The scaffolds were prepared as described above and placed in a 6-well plate, and 40 μL of cell suspension at 1x10^6 cells/mL was added onto only the top surface of the scaffold. The plates were then incubated at 37°C in a 5% CO₂ environment for 90 min to allow cell attachment. Each scaffold was placed into a transwell cell culture insert (6.4 mm diameter, 8 μm pore PET membrane: Becton Dickinson, Franklin Lakes, NJ) in 24-well companion plates (Becton Dickinson). Medium was added to the insert and the well, and then incubated at 37°C in a 5% CO₂ environment. After 7 days of culture, each scaffold was removed from the insert well, and each insert well was placed in another 24-well companion plate. The membrane of the insert well was washed with sterile PBS, and adherent cells on the top and bottom of the insert membrane were removed by
trypsinization and counted using a Coulter counter (Multisizer 3, Beckman Colulter).

Figure 5.2. Migration of endothelial cells through the scaffolds. Cell seeded scaffold was placed into a transwell cell culture insert. After 7 days of culture, a number of cells migrated through the scaffold were counted.

5.2.6. DNA and hydroxyproline measurement in scaffolds

Scaffolds were prepared as described above. The scaffolds were placed in 6-well plates, and 40 μL of cell suspension at 1x10^6 cells/mL was added onto the top and the bottom of the scaffold as described above. The cell seeded-scaffolds in the 6-well plates were placed at 37°C in a 5% CO₂ environment for cell attachment for 90 min and then transferred to 96-well plates. More media was added, and the cell-seeded scaffolds were incubated at 37°C in a 5% CO₂ environment. After three days of culture, cell-seeded
scaffolds were washed with the sterile PBS and cut into four pieces. Samples were digested with 0.1 N NaOH at 37°C overnight. The digested samples were neutralized with 0.1 N HCl. The digested samples and calf thymus DNA standards were diluted with TE buffer (200 mM Tris-HCl, 20 mM EDTA, pH 7.5) and combined with Hoechst 33258 (Molecular Probes, Eugene, OR), a fluorescent DNA binding dye. DNA content was determined by measuring fluorescence using a fluorometer (VersaFluor, BioRad Lab., Hercules, CA) with an excitation wavelength of 360 nm and emission wavelength of 460 nm, and compared to the calf thymus DNA standards (0 to 100 ng/mL).

For hydroxyproline measurement, the neutralized samples and hydroxyproline standards were combined with 4 N NaOH and autoclaved for 20 min at 247 °F to hydrolyze collagen. Samples and hydroxyproline standards (in DI water, 0 – 100 ng) were cooled to room temperature and neutralized with 4 N HCl. Then they were oxidized with chloramine T (ICN Biomedicals, Aurora, OH) and developed with p-dimethylbenzaldehyde (ICN Biomedicals, Aurora, OH). The production of hydroxyproline was determined by measuring absorption at 550 nm (Mann et al., 2001).
5.2.7. *Statistic analysis*

Data were compared with two-tailed, unpaired t-tests. *P*-values less than 0.05 were considered to be significant. Data are presented as mean ± standard deviation.
5.3. Results

5.3.1. Characterization of scaffolds

PUUPPD-PEG and PUUYIGSR-PEG scaffolds were successfully fabricated using gas foaming and salt leaching method. The microstructure of the scaffolds was characterized using mercury intrusion porosimetry and SEM. Both PUUPPD-PEG and PUUYIGSR-PEG scaffolds showed highly interconnected pore structures through the matrices with porosities of approximately 78% (PUUPPD-PEG: 78% ± 3.6 and...
PUUPPD-PEG: 77% ± 8.4) and pore sizes of 20 – 200 μm. The pores were highly open on both the surface and cross sections of the scaffolds (Figure 5.3).

![Image](c)

![Image](d)

**Figure 5.3.** SEM images of surface and cross section of the scaffolds. Highly interconnected pore structure was observed through the scaffold matrix. Length bar = 100 μm. (a) surface and (b) cross section of PUUPPD-PEG and (c) surface and (d) cross section of PUUYIGSR-PEG.

Uniaxial mechanical testing was performed to determine mechanical properties of
the scaffolds as shown in Figure 5.4. The PUUYIGSR-PEG scaffolds showed greater tensile strength and elongation compared to PUUPPD-PEG (tensile strength: 1.4 MPa ± 0.03 vs. 0.19 MPa ± 0.01, p< 0.01 and elongation: 796 MPa ± 122 vs. 129 MPa ± 2.08, p< 0.02). There was no significant difference in elastic modulus (PUUYIGSR-PEG: 0.33 MPa ± 0.1 and PUUPPD-PEG: 0.21 MPa ± 0.01).

![Graphs showing tensile strength and elongation](image)

**Figure 5.4.** Mechanical properties of the PUUPPD-PEG and PUUYIGSR-PEG scaffolds. (a) tensile strength (MPa), * p< 0.01 and (b) maximum elongation (%), * p< 0.02

### 5.3.2. Endothelial cell culture in scaffolds

Endothelial cells were seeded on the top and bottom of the scaffolds and cultured for three days. Cell attachment and morphology in the scaffolds were visualized using SEM as shown in Figure 5.5. On the PUUPPD-PEG scaffolds, few cells were found to
adhere throughout the scaffolds. Individual cells were distributed randomly on the scaffolds, and cell colonies were not found. However, confluent endothelial cell attachment and spreading were found throughout the PUUYIGSR-PEG scaffold. Endothelial cells created new monolayered-tissue along the interconnected scaffold network.

**PUUPPD-PEG**

**PUUYIGSR-PEG**

Figure 5.5. SEM images of endothelial cells cultured for 3 days in PUUPPD-PEG and PUUYIGSR-PEG scaffolds. Higher density cell colonies were found in PUUYIGSR-PEG scaffolds.
Histological analysis in Figure 5.6 also showed that few endothelial cells attach in PUUPPD-PEG scaffolds but higher cell densities were observed in PUUYIGSR-PEG scaffolds. Cells grew along the pores in uniform manner, and cell density close to surface was higher than that in center of the scaffold matrices.

Figure 5.6. Hematoxylin-stained endothelial cells on PUUPPD-PEG and PUUYIGSR-PEG scaffolds. Endothelial cells were cultured for 3 days. Higher cell density was observed throughout the PUUYIGSR-PEG scaffolds.
5.3.3. Migration of cells through the scaffold matrix

In order to study the interconnectivity of the scaffold matrix and bioactivity of the peptide sequences on the polymer matrix, the migration of the cells through the scaffolds were studied. Cells were seeded on the top of the scaffolds, and cell seeded-scaffolds were maintained in tissue culture plates for 90 min to ensure cell attachment on the scaffolds, then placed into the transwell insert. After 7 days of culture, scaffolds were removed. Then each insert well was transferred to another companion plates, and cell on the top and bottom of the membrane were counted. No cells were observed on the bottom of companion plates. As shown in Figure 5.7, the number of cells that had migrated through the PUUYIGSR-PEG matrices \( (4266.7 \pm 482.2) \) was significantly greater than for PUUPPD-PEG \( (1026.7 \pm 266.3, p<0.005) \).
Figure 5.7. Migration of endothelial cells through the PUUPPD-PEG and PUUYIGSR-PEG scaffold matrices after 7 days of culture. * p< 0.005.

5.3.4. DNA and hydroxyproline measurement in the scaffold

The effect of bioactive peptide sequence on endothelial cell proliferation and extracellular matrix production in the scaffolds were studied as shown in Figure 5.8. DNA concentration in PUUYIGSR-PEG matrices (4.41 ± 0.34 ng/scaffold) was significantly greater than that in PUUPPD-PEG (1.84 ± 0.26 ng/scaffold, p<0.001). Hydroxyproline production, a marker for collagen synthesis, in PUUYIGSR-PEG matrices (1.092 ± 0.323 ng/ng DNA) was also significantly greater than that in PUUPPD-
PEG (0.069 ± 0.062 ng/ng DNA, p<0.05). Higher DNA concentration in PUUYIGSR-PEG scaffolds indicates the greater numbers of cells, so higher hydroxyproline production in PUUYIGSR-PEG scaffold matrices could be expected. However, significantly greater hydroxyproline production per cell (ng/ng DNA) indicates that the bioactive peptide sequences might not only enhance cell adhesion but also facilitate extracellular matrix production.

**Figure 5.8.** DNA and Hydroxyproline production in PUUPPD-PEG and PUUYIGSR-PEG scaffold matrices after 3 days of culture. (a) DNA concentration (ng) per scaffold, * p< 0.001 and (b) hydroxyproline production (ng) per DNA (ng), * p< 0.05.
5.4. Discussion

The polyurethaneurea scaffolds fabricated by the gas foaming and salt leaching methods showed uniform distribution of open and interconnected pores throughout the matrix. In addition, there was no closed or nonporous skin on the external surface which was usually found in salt leaching or gas foaming method (Harris et al., 1998). Gas foaming and salt leaching method was also known to regulate porosity and mechanical properties by gas foaming reaction between acid and effervescent salts (Yoon and Park, 2001). In this study, sodium bicarbonate was chosen due to its superior stability compared to ammonium bicarbonate in polyurethaneurea systems. Thus, undesirable gas foaming at early processing stage was avoided, even in the peptide incorporated polyurethaneurea which has a low pH making it more susceptible to early gas foaming.

For vascular graft applications appropriate strength and compliance matching are very important (Lyman et al., 1978, Doi et al., 1996, Lamba et al., 1998). Porosity decreases mechanical properties but also promotes cellular invasion. The gas foaming and salt leaching method led to uniform distribution of micropores through the matrix, improving mechanical properties of the scaffolds. PUUYIGSR-PEG showed superior mechanical properties compared to PUUPPD-PEG. Since porosity and pore size were nearly identical for both scaffolds, the difference in mechanical properties may be
inherent to the material. In a previous study, we reported that the bioactive peptide sequences could affect not only cellular behavior but also bulk mechanical properties (Jun and West, 2003). Thus, the mechanical properties of PUUYIGSR-PEG may be influenced by both the molecular interactions in the polymer chain and the restriction of motility of the PEG chain by long amino acid peptide sequences and uniform distribution of micropores throughout the scaffold matrix.

One of the advantages of peptide incorporation into the polymer main chain was the ability to fabricate a bioactive scaffold easily without further modification. When modifying the surface of materials by traditional methods such as the grafting of adhesive peptides (Lin et al., 1992, Lin et al., 1994, Guian et al., 2002), it becomes difficult to gauge their bioactivity, since most grafted peptide sequences are embedded in the polymer during fabrication process. It is also challenging to modify scaffolds without changing the porous structure. The bioactivity of YIGSR peptide sequences incorporated was evaluated by assessing cell attachment, migration, and extracellular matrix production. Endothelial cells were seeded into the scaffolds and cultured. Cells adhered and spread through the PUUYIGSR-PEG matrix but very few cells adhered on PUUPPD-PEG matrix. Higher migration was also found in PUUYIGSR-PEG matrix compared to PUUPPD-PEG matrix. This indicates that incorporated peptide sequences maintain their
bioactivity and promote cell migration through the matrix. Thus, it can be also expected that this scaffold might promote endothelial ingrowth from the surrounding tissues and form a continuous monolayer on the surface of an implanted vascular graft.
5.5. Conclusions

A bioactive microporous scaffold has been fabricated using a gas forming and salt leaching method. This scaffold has an interconnected open pore structure with uniform distribution throughout the matrix which contributes to the material’s mechanical properties. The peptide sequences incorporated into the polymer main chain maintain their bioactivity, enhancing endothelialization. This bioactive scaffold might be an ideal candidate for small diameter vascular graft applications.
Chapter 6. Release of vascular endothelial growth factor from a microporous bioactive polyurethaneurea scaffold

6.1. Introduction

As described in earlier chapters, patency of small diameter vascular grafts (ID < 6 mm) might be achieved by rapid endothelialization (Okoshi et al., 1996, Masuda et al., 1997). Microporous grafts allow both transmural and anastomotic endothelial tissue ingrowth from surrounding tissues into the grafts, enhancing endothelialization (Lamba et al., 1998, Doi et al., 1996a, Doi and Matsuda, 1997). Thus, materials that support endothelial cell adhesion, migration, and growth with a microporous structure may offer the best opportunity for endothelialization of vascular grafts. In addition, angiogenesis is also required for vascular tissues integrated into the pores of grafts.

Angiogenesis, the development of new capillaries from existing blood vessels, is essential for treatment of certain vascular diseases and vascularization of constructs in tissue engineering (Chen and Mooney, 2003, Morbidelli et al., 2003) because cells more than hundreds micrometers from blood vessels suffer from limited nutrients and oxygen (Colton, 1995). Angiogenesis could be enhanced by controlled delivery of angiogenic growth factors such as basic fibroblast growth factor (bFGF) and vascular endothelial
growth factor (VEGF) (Belle et al., 1997, Hile et al., 2000, Lee et al., 2003). Growth factors are secreted by various cells and bind to their receptors on cell surfaces. This binding initiates signal transduction cascade through phosphorylation of secondary messengers in the target cell, regulate several cellular processes such as cell migration, differentiation, and proliferation (Chen and Mooney, 2003, Gooch et al., 1998).

![Diagram of VEGF receptors](http://www.rndsystems.com/asp/g_sitebuilder.asp?bodyId=230)

**Figure 6.1.** TVEGF functions through binding and activating VEGF-receptors. Receptors for VEGF are VEGF R1 (Flt-1), VEGF R2 (KDR), and VEGF R3 (Flt-1). Endothelial cells also express additional receptors such as Neuropilin-1 and -2. (http://www.rndsystems.com/asp/g_sitebuilder.asp?bodyId=230).

Polymeric scaffolds can be used as biomechanical supports for cell growth as well as local delivery vehicles for growth factors. Growth factors have been incorporated and
released from porous scaffolds (Hile et al., 2000, Sheridan et al., 2000, Murphy et al., 2000) or microspheres in alginate scaffolds (Perets et al., 2003). VEGF was incorporated into poly(lactide-co-glycolide) porous scaffolds with ~45% efficiency using a gas foaming/particulate leaching process. Sustained release was obtained after an initial burst release, and the VEGF maintained bioactivity over 12 days (Murphy et al., 2000). bFGF has also been released from microspheres incorporated in alginate scaffolds. The controlled delivery of bFGF stimulated vascularization after implantation on the mesenteric membrane in the rat peritoneum (Perets et al., 2003).

Vascular grafts have also been modified with growth factors to enhance endothelialization. Microporous polyurethane grafts were fabricated using excimer laser ablation techniques and coated with photoreactive gelatin, bFGF, and heparin (Doi et al., 1996b). Grafts were implanted in the aortas of rats for 4 weeks, and improved transmural tissue ingrowth was observed. However, bFGF is a potent mitogen for smooth muscle cells as well as endothelial cells, creating the potential for intimal hyperplasia (Lindner et al., 1991, Geary et al., 1993). In contrast, VEGF has been shown to be highly a specific mitogen for endothelial cells (Guo et al., 1995), and it also promotes endothelial cell migration (Visweswaran et al., 1997). VEGF and bFGF have been incorporated into alginate hydrogels and implanted subcutaneously in severe combined
immunodeficient (SCID) mice (Lee et al., 2003). Thickness of the granulation tissue layer and number of blood vessels increased with VEGF concentration. In addition, VEGF promoted higher blood vessel density compared to bFGF. In another study, VEGF and bFGF were also incorporated into microporous polyurethane grafts then implanted in aortas of rats (Masuda et al., 1997). VEGF enhanced both transmural capillary ingrowth and transanastomotic tissue ingrowth, whereas capillary ingrowth was not observed in bFGF-modified grafts. Therefore, incorporation of VEGF into small diameter vascular grafts might promote rapid anastomotic and transmural endothelialization without concern of intimal hyperplasia. In this chapter, additional effects of the controlled release of VEGF on rapid endothelialization have been explored.
6.2. Materials and Methods

6.2.1. Fabrication of microporous scaffolds using gas forming and salt leaching

PEG and YIGSR peptide modified-polyurethaneurea (PUUYIGSR-PEG) was synthesized by reacting 4,4'-methylene di(p-phenyl iso-cyanate) with poly(tetramethylene oxide)/PEG mixture (85/15 molar ratio) and extending with a combination of GGGYIGSRGGGK and p-phenylene diamine (PPD) as described in chapter 4 (Jun and West, 2003).

Scaffolds were fabricated using gas foaming and salt leaching via incorporation of sodium bicarbonate as described in chapter 5. Briefly, PUUYIGSR-PEG were dissolved in anhydrous N,N - dimethylformamide (DMF; Aldrich Chemical Co., Milwaukee, WI) at 10% (w/v) concentration, and DMF was partially evaporated at 60°C under vacuum. Sieved sodium bicarbonate salts (Sigma Chemical Co., St. Louis, MO; particle size of 100-200 μm, weight fraction = 90 wt%) were added to the polymer solutions at room temperature and mixed well. The paste of polymer and salts was placed in a Teflon mold (75x25x1 mm) and dried for 24 hr at room temperature. The polymer/salt films were immersed in 50% citric acid (Sigma Chemical Co., St. Louis, MO) for 30 min to induce gas foaming and then in DI water for 3 days to leach remaining salts. DI water was changed everyday, and samples were freeze-dried for 48 hr. Scaffolds
were cut into disks using a cork borer (diameter = 5.7 mm, thickness = 1 mm).

6.2.2. Cell maintenance

Bovine aortic endothelial cells (BAEC; Clonetics, San Diego, CA), passage 2 – 5, were used for this study. Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma Chemical Co., St. Louis, MO) was prepared with 10% fetal bovine serum (FBS; BioWhitaker, Walkersville, MD), 2 mM L-glutamine, 1 unit/ml penicillin, and 100 mg/l streptomycin (GPS; Sigma Chemical Co., St. Louis, MO). Endothelial basal medium (EBM; Sigma Chemical Co., St. Louis, MO) was prepared with 10% endothelial medium supplement (Sigma Chemical Co., St. Louis, MO) containing fetal bovine serum, basic fibroblast growth factor, heparin, epithelial growth factor, and hydrocortisone. BAECs were maintained on mixture of EBM and DMEM (25/75 %) at 37 °C in a 5% CO₂ environment.

6.2.3. Dose response of endothelial cells to VEGF

Recombinant human vascular endothelial growth factor (VEGF; Sigma Chemical Co., St. Louis, MO) was dissolved in PBS at 10 µg/1 mL with 1% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO). To evaluate the dose response of endothelial
cells to VEGF, endothelial cells were seeded at a concentration of 5,000 cells/cm² in six-well tissue culture plates. After 24 hr incubation at 37°C in a 5% CO₂ environment, medium was changed with fresh medium containing 0, 1, 5, 10, or 40 ng/mL VEGF. After 48 hr incubation at 37°C in a 5% CO₂ environment, medium was removed by rinsing with PBS, and adherent cells were detached with trypsin and counted using a Coulter Counter (Multisizer 3, Beckman Coulter).

6.2.4. In vitro release kinetics and bioactivity of released VEGF

Recombinant human vascular endothelial growth factor (VEGF; Sigma Chemical Co., St. Louis, MO) was dissolved in PBS at 10 µg/1 mL with 1% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO). Scaffold disks were immersed in 70% ethanol overnight prior to use. The scaffolds were then washed three times in sterile PBS and placed in 12-well FlexiPerm chambers (Sigma Chemical Co., St. Louis, MO). After removing excess PBS, 1 µg of VEGF in 100 µl PBS was evenly pipette-loaded into each scaffold disk. VEGF-loaded scaffolds were freeze-dried using lyophilizer. To evaluate VEGF release, each VEGF-loaded scaffold was placed in 1 mL PBS and incubated at 37°C.

To evaluate release kinetics and bioactivity of VEGF, the PBS was removed at
each time point and replaced with fresh PBS. 500 μl of each sample was analyzed using an ELISA for VEGF (Quantikine VEGF immunoassay kit, R&D systems, Minneapolis, MN). Another 500 μl of solution was sterilized using 0.22 μm pore size syringe filters (Millipore, Bedford, MA) and mixed with cell culture medium. Endothelial cells had been seeded at concentration of 5,000 cells/cm² in six-well tissue culture plate and incubated at 37°C in a 5% CO₂ environment. After 24 hr incubation, medium was replaced with VEGF-containing medium described above and incubated at 37°C in a 5% CO₂ environment. After 48 hr incubation, medium was removed by rinsing with PBS, and adherent cells were detached with trypsin and counted using a Coulter Counter (Multisizer 3, Beckman Coulter).

6.2.5. DNA and hydroxyproline measurement in scaffolds

Scaffolds disks were immersed in 70% ethanol overnight prior to use. The scaffolds were then soaked in sterile PBS for 30 min three times and placed in cell culture medium for 15 min. For cell seeding, the scaffolds were placed in 6-well tissue culture plates, and excess medium was removed. 40 μL of cell suspension at 2x10⁶ cells/mL was added onto the top of the scaffold slowly and allowed to permeate through the matrix. The scaffold was turned over, and an additional 40 μL of the cell suspension was added to
the opposite surface. 6-well plates containing cell seeded-scaffolds were maintained at 37°C in a 5% CO₂ environment for cell attachment. After 90 min, cell-seeded scaffolds were placed in 12-well FlexiPerm chamber (Sigma Chemical Co., St. Louis, MO) attached on glass slide, media was added, and constructs were incubated at 37°C in a 5% CO₂ environment.

After 3 days of culture, cell-seeded scaffolds were washed with sterile PBS and cut into 4 pieces. Samples were digested with 0.1 N NaOH at 37°C overnight. The digested samples were neutralized with 0.1 N HCl. The digested samples and calf thymus DNA standards were diluted with TE buffer (200 mM Tris-HCl, 20 mM EDTA, pH 7.5) and combined with Hoechst 33258 (Molecular Probes, Eugene, OR), a fluorescent DNA binding dye. DNA content was determined by measuring fluorescence using a fluorometer (VersaFluor, BioRad Lab., Hercules, CA) with an excitation wavelength of 360 nm and emission wavelength of 460 nm, and compared to the calf thymus DNA standards (0 to 100 ng/mL).

For hydroxyproline measurement, the neutralized samples and hydroxyproline standards were combined with 4 N NaOH and autoclaved for 20 min at 247 °F to hydrolyze collagen. Samples and hydroxyproline standards (in DI water, 0 – 100 ng) were cooled to room temperature and neutralized with 4 N HCl. Then they were oxidized
with chloramine T (ICN Biomedicals, Aurora, OH) and developed with p-
dimethylbenzaldehyde (ICN Biomedicals, Aurora, OH). The production of
hydroxyproline was determined by measuring absorption at 550 nm (Mann et al., 2001).

6.2.6. Evaluation of capillary ingrowth after subcutaneous implantation

To evaluate soft tissue response and blood vessel formation, VEGF-loaded
scaffolds were subcutaneously implanted into male Sprague-Dawley rats (375 - 400 g,
Charles River Lab., Houston, TX). After induction of anesthesia with isoflurane (Henry
Schein, Melville, NY), a longitudinal incision (1 cm) was made through the full thickness
of the dorsal skin, and a subcutaneous pocket was created. The VEGF- loaded scaffolds
were placed into the pocket followed by closure with polypropylene suture (1.0 metric,
Ethicon Inc., Somerville, NJ). After 14 days, the rats were euthanized via CO₂
asphyxiation, and the scaffolds with surrounding tissue were retrieved and immediately
frozen at -80°C.

For histological analysis, implants were sectioned to 30 µm using a cryostat
(HM505E; Microm, Walldorf, Germany). Sections were stained with hematoxylin and
observed by light microscopy (Zeiss Axiovert 135). For immunohistochemical analysis,
sections were stained with an antibody against CD31 (Serotec, Raleigh, NC), a marker
for endothelial cells (Kirkiles-Smith et al., 2000). Sections were incubated in cold acetone for 5 min, and endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol for 12 min. Sections were blocked with protein blocking solution (5% FBS in PBS) for 20 min and incubated with mouse anti-rat CD31 antibody (Serotec, Raleigh, NC; 1:50 dilution) at room temperature for 2 hr. After rinsing with PBS, sections were blocked with protein blocking solution for 15 min and incubated with HRP-conjugated anti-mouse IgG (Sigma Chemical Co., St. Louis, MO; 1:100 dilution) at room temperature for 1 hr. Incubations were performed in a humidified chamber at room temperature. 3,3’-diaminobenzidine (DAB, Vector Laboratories, Burlingame, CA) was used for detection of the HRP-conjugated secondary antibody.

6.2.7. Statistic analysis

Data were compared with two-tailed, unpaired t-tests. P-values less than 0.05 were considered to be significant. Data are presented as mean ± standard deviation.
6.3. Results

6.3.1. Dose response of endothelial cells to VEGF

To evaluate dose response of endothelial cells to VEGF, endothelial cells were seeded and allowed to attach for 24 hr in tissue culture plates. Medium containing known concentrations of VEGF (1, 5, 10, or 40 ng/mL) was added to endothelial cell cultures. Cells were incubated with VEGF for 48 hr and counted. At concentration of VEGF above 5 ng/mL, cell numbers were significantly increased (Figure 6.2.). There was no significant difference of cell proliferation between the 5, 10, and 40 ng/mL concentrations of VEGF.

![Graph showing dose response of endothelial cells to VEGF](image)

**Figure 6.2.** The dose response of endothelial cells to VEGF. Four known concentrations of VEGF were exposed to endothelial cells for 48 hr. * $p < 0.05$ compared to control.
6.3.2. *In vitro release kinetics and bioactivity of released VEGF*

The release profile of VEGF from the scaffolds revealed initial burst release over the first 24 hr (18.1 ± 5.3% based on initial load of VEGF in scaffold), followed by slower release up to 10 days (Figure 6.3). A negligible amount of release was found after 10 days. To investigate initial burst release kinetics, VEGF release study was repeated with time points only during the initial 24 hr. Burst release was observed over 6 hr.

![Graphs showing release profile of VEGF](image)

**Figure 6.3.** Release profile of VEGF from the scaffolds. Cumulative VEGF release (%) for 24 hr (a) and 14 days (c). VEGF release profiles at each time period for 24 hr (b) and 14 days (d).
In order to evaluate the bioactivity of VEGF released from the scaffolds, supernatant solution collected at each time period was added to endothelial cells seeded prior to 24 hr. Endothelial cells were incubated for another 48 hr and counted (Figure 6.4.).

![Graph showing increase of number of endothelial cells exposed to conditioned medium containing released VEGF at each time period for 48 hr. * p < 0.05 compared to corresponding control (normalized to 100%).](image)

**Figure 6.4.** Increase of number of endothelial cells exposed to conditioned medium containing released VEGF at each time period for 48 hr. * p < 0.05 compared to corresponding control (normalized to 100%).

Conditioned medium containing supernatant solutions collected at 1, 3, and 5 days (* p < 0.05) significantly stimulated proliferation of endothelial cells. In contrast, conditioned medium containing supernatant solutions after 5 days did not affect cell
proliferation. This result was in accordance with the VEGF release study, which the
amount of VEGF release was significantly decreased after 5 days. The bioactivity of the
released VEGF was evaluated to be about 94% for first 5 days.

<table>
<thead>
<tr>
<th>Time period (days)</th>
<th>0-1</th>
<th>2-3</th>
<th>4-5</th>
<th>6-7</th>
<th>8-10</th>
<th>11-14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. in cell culture (ng/mL)</td>
<td>45</td>
<td>4</td>
<td>1.2</td>
<td>0.1</td>
<td>0.7</td>
<td>0</td>
</tr>
<tr>
<td>Bioactivity (%)</td>
<td>94</td>
<td>95</td>
<td>93</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 6.1. Bioactivity of the released VEGF. There was no significant effect of VEGF on endothelialization after 5 days.

6.3.3. DNA and hydroxyproline measurement in the scaffold

To evaluate the effect of VEGF incorporated on endothelial cell proliferation and
extracellular matrix production in the scaffolds, DNA and hydroxyproline production was
measured after 3 days of culture (Figure 6.5.). DNA concentration in PUUYIGSR-PEG-
VEGF (35.5 ± 4.1 ng/scaffold) was significantly greater than that in PUUYIGSR-PEG
(13.9 ± 1.4 ng/scaffold, p < 0.01). Hydroxyproline production in PUUYIGSR-PEG-
VEGF matrices (37.6 ± 8.9 ng/scaffold) was also significantly greater than that in
PUUYIGSR-PEG (14.6 ± 3.1 ng/scaffold, p < 0.05).

Figure 6.5. DNA and hydroxyproline production of endothelial cells in PUUYIGSR-PEG and PUUYIGSR-VEGF scaffold after 3 days of culture. (a) DNA concentration (ng) per scaffold, * p < 0.01 and (b) hydroxyproline production (ng) per scaffold, * p < 0.05.

6.3.4. Evaluation of capillary ingrowth after subcutaneous implantation

VEGF-loaded scaffolds were retrieved after 14 days of subcutaneous implantation. Sections were stained with endothelial specific anti-CD31 antibody and counter stained with hematoxylin (Figure 6.6.). Host tissue completely penetrated the scaffold matrix. A large number of blood vessels were found in both interface region and inside of scaffold matrix. No significant inflammatory response was found on implanted scaffolds.
Figure 6.6. Histological sections of VEGF-loaded scaffolds implanted subcutaneously for 14 days. Stained with CD31 antibody and hematoxylin. Arrow indicates blood vessels.
6.4. Discussion

VEGF is a homodimeric glycoprotein of relative molecular mass 45,000 and has shown to stimulate proliferation and migration of endothelial cells (Guo et al., 1995, Visweswaran et al., 1997) without the risk of intimal hyperplasia (Belle et al., 1997). Incorporation of a growth factor such as VEGF into the scaffold might result in synergistic effects to promote the rate and level of endothelialization.

VEGF release profile demonstrated the initial burst release followed by sustained release. Bioactivity of VEGF released from the scaffolds was evaluated its ability to stimulate the proliferation of endothelial cells and correlated to the dose response of endothelial cells: minimum concentration of VEGF was required for inducing cell proliferation and saturated at specific concentration with no effect at higher concentrations as also reported by other groups (Kim and Valentini, 2002, Murphy et al., 2000, Lee et al., 2003). The efficiency of VEGF incorporation into the porous scaffolds using direct pipetting method was 20.5 ± 5.3%. This value was compatible with previous reports using same method (Kim and Valentini, 2002). Initial burst release in the first 24 hr might be also contributed by fast diffusion of dissolved VEGF through highly open interconnected pore structure since VEGF was physically adhered scaffold matrices.

Cells were seeded in VEGF-loaded PUUYIGSR-PEG scaffolds, and significantly
higher proliferation and extracellular matrix production were measured compared to pure PUUYIGSR-PEG scaffolds. It indicated additional effect of VEGF on endothelialization because PUUYIGSR-PEG showed enhanced endothelialization compared to PUUPPD-PEG in chapter 5.

In order to evaluate host tissue responses to the scaffolds as well as the ability of the scaffolds for local VEGF release to induce angiogenesis, an animal study was performed by implanting VEGF-loaded scaffolds subcutaneously into the rat. It was noted that surrounding tissues penetrated into the scaffolds with blood vessel formation by 14 days of implantation. It also indicated that VEGF-loaded scaffolds did not result in inflammatory or immune responses because host tissues could not penetrate through the scaffolds if inflammatory cells surrounded scaffolds.
6.5. Conclusions

Endothelial cell specific growth factor, VEGF, was incorporated into a bioactive polyurethaneurea scaffold and released. VEGF released from the scaffolds maintained its mitogenic effect on proliferation of endothelial cells \textit{in vitro} and induced angiogenesis \textit{in vivo}. These results seem to be contributed by the synergistic effect from microporous structure, bioactive peptide sequences, and local VEGF delivery.
Chapter 7. Nitric oxide-releasing polyurethanes

7.1. Introduction

Nitric oxide (NO) is an important signaling molecule that is involved in a wide range of biological processes including regulation of blood pressure, neurotransmission, and immune response as shown in Figure 7.1. (Morbidelli et al., 2003, Saavedra et al., 2000, Feldman et al., 1993).

Figure 7.1. Nitric oxide is an important molecule and involves in a wide range of physiological processes.
Nitric oxide is produced from L-arginine by nitric oxide synthase and has a very short half life (Morbidelli et al., 2003). Nitric oxide has been known to stimulate endothelial cell proliferation but reduce platelet aggregation and smooth muscle cell proliferation (Bohl and West, 2000, Morbidelli et al., 2003, Murohara et al., 1999, Pulfer et al., 1997). Thus, nitric oxide donors have been incorporated into biomedical polymers in order to reduce thrombosis and restenosis. For example, nitric oxide was released from cysteine modified-polyurethane (PU) and polyethylene terephthalate (PET) (Duan and Lewis, 2002), diazeniumdiolate NO-donor of PU and polyvinyl chloride (PVC) films (Mowery et al., 2000), polyethyleneimine microspheres (Pulfer et al., 1997), or PEG hydrogel (Bohl and West, 2000). In all cases, the released NO improved thromboresistance.

Diazeniumdiolates spontaneously release NO in aqueous media at a rate dependent on pH (Bohl and West, 2000, Pulfer et al., 1997, Smith et al., 1996). Amine and diazeniumdiolate complex can be made by reacting NO with nucleophilic amines, and dissociation of NO then occurs at pH 7.4 at 37 °C as shown in Figure 7.2. (Saavedra et al., 2000, Smith et al., 1996).
\[ R_2NH + 2NO \quad \rightarrow \quad \begin{array}{c} \text{H} \\ \text{O}^- \end{array} \quad + \quad \begin{array}{c} \text{R}_2\text{N}--\text{N} \\ \text{N}--\text{O}^- \end{array} \]

Figure 7.2. The formation of diazeniumdiolates from amines and spontaneous release of NO.

In chapters 3 and 4, we have demonstrated that a bioactive polyurethaneurea could be synthesized by incorporating YIGSR peptide sequences into the main chain, and the incorporated peptide enhanced endothelialization. In this study, a NO releasing polyurethaneurea was synthesized by incorporating diazeniumdiolate-lysine peptide sequences into the polymer main chain in the similar manner. The NO release kinetics from diazeniumdiolate NO donor of polymer was studied, and the effects on platelet adhesion as well as endothelial and smooth muscle cell proliferation were also evaluated.
7.2. Materials and Methods

7.2.1. Polyurethane synthesis

A different synthesis scheme was utilized in these studies than in earlier work in this thesis because of the structure of the peptide used as the NO carrier. Prepolymer was synthesized by reacting methylene di(\(p\)-phenyl isocyanate) (MDI; Aldrich chemical Co., Milwaukee, WI) with poly(tetramethylene oxide) (PTMO; Aldrich chemical Co., Milwaukee, WI), and then extended with 1,4-butanediol (BD; Aldrich chemical Co., Milwaukee, WI) as shown in Figure 7.3. (Jun and West, 2003, Jun and West, 2004). A 10\% (w/v) solution of MDI (4 mmol, MW: 250) in 10 ml anhydrous \(N,N\)-dimethylformamide (DMF; Aldrich Chemical Co., Milwaukee, WI ) was prepared in a 100 ml three-neck round flask and stirred at room temperature. A 10\% (w/v) solution of PTMO (2 mmol, MW: 2000) in 20 ml anhydrous DMF was added, and the mixture was heated to 75\(^\circ\)C and held there for 3 hr under argon gas. The reactor was cooled to room temperature before BD (2 mmol, MW:90) in 2 ml anhydrous DMF was added as a chain extender. The polymer solution was then incubated at 45\(^\circ\)C for 3 hr under argon gas. The polymer solution was cooled to room temperature, precipitated in methanol, and dried under vacuum. This material is referred to as PUBD.
Step I

$$\text{OCN} - \text{CH}_2 - \text{NCO} + \text{HO-CH}_2\text{CH}_2\text{CH}_2\text{OH}_n$$

4,4'-methylene di(p-phenyl isocyanate) (MDI) poly(tetramethylene oxide) (PTMO)

OCN-prepolymer-NCO

Step II

$$\text{OCN-prepolymer-NCO} + \text{HO-CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$$

1,4-butandiol (BD)

Soft Segment Hard Segment Soft Segment
(PUBD)

Figure 7.3. Synthesis of polyurethane (PUBD). Prepolymer was synthesized from MDI and PTMO and then extended with BD.

7.2.2. Synthesis of lysine-modified polyurethane

SGGKKKKKGS (serine-glycine-glycine-lysine-lysine-lysine-glycine-glycine-serine) peptide was synthesized using standard fluorenlymethoxycarbonyl (fmoc) chemistry on an Applied Biosystems 431A peptide synthesizer (Foster, CA). The peptides were dissolved in DI water and reacted with NO gas at room temperature under argon gas in 100 ml round flask overnight. The extent of conversion of amine groups of lysine to diazeniumdiolates was measured using the ninhydrin assay (Bohl and West, 2000). The
peptides containing diazeniumdiolates, SGG[K(N(O)NO)]₄GGS, were freeze-dried and stored at -80 °C.

Prepolymer was synthesized by reacting MDI with PTMO as described above and extended with a combination of SGG[K(N(O)NO)]₄GGS peptide and BD (Figure 7.4) (Jun and West, 2003, Jun and West, 2004). A 10% (w/v) solution of MDI (1.5 mmol) in 5 ml anhydrous DMF was prepared in 100 ml three-neck round flask and stirred at room temperature. A 10% (w/v) solution of PTMO (0.75 mmol) in 15 ml anhydrous DMF was added, and the mixture was heated to 75 °C and held there for 3 hr under argon gas. The reactor was cooled to room temperature before SGG[K(N(O)NO)]₄GGS peptide (0.22 mmol) in 10 ml anhydrous DMF and BD (0.54 mmol) in 5 ml anhydrous DMF were added as chain extenders. The polymer mixture was incubated at 45 °C for 3 hr under argon gas. The polymer solution was cooled to room temperature, precipitated in ethyl acetate, and dried under vacuum. This material is referred to as PUBD-NO.
**Figure 7.4.** Synthesis of NO releasing polyurethane (PUBD-NO). Prepolymer was synthesized from MDI and PTMO and then extended with BD and peptide sequences containing diazeniumdiolates.

### 7.2.3. Polymer characterization

PUBD and PUBD-NO were characterized via \(^1\)H NMR using a 400 MHz NMR spectrometer (Advance 400, Bruker, Germany) in \(N,N\)-dimethylformamide-d\(_7\) (DMF-d\(_7\); Aldrich Chemical Co., Milwaukee, WI). Molecular weight distributions were obtained by GPC with UV and evaporative light scattering detectors (Polymer Laboratories, Amherst, MA). Samples for GPC analysis were dissolved in HPLC-grade DMF at a concentration
of 1 mg/ml and run at 70°C through PLgel 5 μm Mixed-C columns (Polymer Laboratories, Amherst, MA) at a flow rate of 1 ml/min. Calibration was performed using polystyrene standards (PS; Polymer Laboratories, Amherst, MA), ranging in molecular weight from 5,000 – 96,400 Da.

7.2.4. Preparation and characterization of NO-releasing PUBD-NO films

Polymers were dissolved in tetrahydrofuran (THF; 0.3 wt%) and sterilized using 0.2 μm pore size PTFE syringe filters (Whatman, NJ). Polymer films were prepared on glass coverslips (18 mm; Fisher Scientific, PA) by solvent casting at room temperature. Polymer films were held under vacuum for 48 hr to ensure removal of the solvent and sterilized under UV overnight.

Uniaxial mechanical testing was performed using an Instron model 5565 at a cross head speed of 25 mm/min with a 5 kN load cell. Polymers were dissolved in DMF (Aldrich Chemical Co., Milwaukee, WI) at 10 wt% and sterilized using 0.2 μm pore size PTFE syringe filters (Whatman, NJ). Polymer films were prepared in Teflon molds by solvent casting at 60°C under vacuum for 48 hr. Test specimens were prepared according to ASTMD-638-VI. The tensile strength was calculated from the maximum load at break and the initial cross sectional area of the specimen. Sample thickness was measured using
a digital caliper (Mitutoyo).

7.2.5. Cell maintenance

Bovine aortic endothelial cells (BAEC; Clonetics, San Diego, CA) and Sprague-Dawley rat smooth muscle cells (SDSMC; Cell Applications, SanDiego, CA), passages 2 – 5, were used for this study. For BAECs, Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma Chemical Co., St. Louis, MO) was prepared with 10% fetal bovine serum (FBS; BioWhitaker, Walkersville, MD), 2 mM L-glutamine, 1 unit/ml penicillin, and 100 mg/l streptomycin (GPS; Sigma Chemical Co., St. Louis, MO). Endothelial basal medium (EBM; Sigma Chemical Co., St. Louis, MO) was prepared with 10% endothelial medium supplement (Sigma Chemical Co., St. Louis, MO) containing fetal bovine serum, basic fibroblast growth factor, heparin, epithelial growth factor, and hydrocortisone. BAECs were maintained on mixture of EBM and DMEM (25/75 %) at 37°C in a 5% CO₂ environment. SDSMCs were maintained on Modified Eagle’s Medium (DMEM; Sigma Chemical Co., St. Louis, MO) prepared with 10% fetal bovine serum (FBS; BioWhitaker, Walkersville, MD), 2 mM L-glutamine, 1 unit/ml penicillin, and 100 mg/l streptomycin (GPS; Sigma Chemical Co., St. Louis, MO) at 37 °C in a 5% CO₂ environment.
7.2.6. **NO release**

PU-NO films were reacted with NO gas under argon at room temperature overnight. After rinsing the films with HEPES buffered saline (HBS) three times, the films were placed in HBS at 37°C at pH 7.4. Release of NO from the films was measured using the Griess assay (Scott-Burden et al., 1992, Bohl and West, 2000).

7.2.7. **Proliferation of BAEC and SDSMC**

PUBD and PUBD-NO films were prepared and sterilized as described above. PUBD-NO films were reacted with NO gas under argon at room temperature overnight and then rinsed with PBS three times. To evaluate BAEC and SDSMC proliferation, cells were seeded at a concentration of 17,000 cells/cm² in a 6-well cluster polystyrene plate (Corning Incorporated, Corning, NY). After 24 hr incubation at 37°C in a 5% CO₂ environment, PUBD and PUBD-NO films were placed into transwell inserts (24 mm diameter, 0.4 μm pore polycarbonate membrane: Corning Incorporated, Corning, NY) in 6-well plates. In an additional set of experiments, the films were incubated in HBS at 37°C for 48 hr before commencing the cell culture experiments. This was done to ensure that NO release in the post-burst phase was sufficient for bioactivity.

After 48 hr incubation at 37°C in a 5% CO₂ environment, immunohistochemical
staining for proliferating cell nuclear antigen (PCNA) was employed (Fields, 1997, Jun and West, 2003, Jun and West, 2004). The PCNA antibody stains cells in the S-phase of mitosis. Cells were washed with PBS, fixed with 10% buffered formalin (Stephens Scientific, NJ) for 10 min, permeabilized with methanol (Sigma Chemical Co., St. Louis, MO) for 2 min, and incubated in 3% \( \text{H}_2\text{O}_2 \) (Fisher Scientifics, PA) for 5 min. Cells were incubated with mouse anti-human PCNA IgG (DAKO, Carpinteria, CA) for 1 hr, rinsed with PBS, and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (DAKO, Carpinteria, CA) for 40 min. Antibodies were diluted 1:100 in PBS containing 3% FBS, and incubations were performed at room temperature in a humidified chamber. After rinsing with PBS, cells were treated with aminoethylcarbazole chromagen (DAKO, Carpinteria, CA) for 10 min. Counter-staining was performed with Mayer's hematoxylin (DAKO, Carpinteria, CA). Using this procedure, proliferating cells appear red and non-proliferating cells appear blue.

### 7.2.8. Platelet adhesion

PUBD and PUBD-NO films were prepared and sterilized as described above. PUBD-NO films were reacted with NO gas under argon at room temperature overnight and then rinsed with PBS three times. A solution of 2.5 mg/ml collagen 1 (Sigma
Chemical Co., St. Louis, MO) solution was prepared in 3% glacial acetic acid. Glass coverslips were incubated with the collagen I solution for 45 minutes in a humidified environment at room temperature to provide a highly thrombogenic reference material. Blood was obtained from a healthy volunteer with 10 U/ml heparin (Sigma Chemical Co., St. Louis, MO). 10 μM mepacrine (Sigma Chemical Co., St. Louis, MO) was added in order to fluorescently label platelets. Collagen I (positive control), PUBD, and PUBD-NO film surfaces were incubated with mepacrine-labeled whole blood at 37 °C for 20 minutes and then rinsed with PBS. The number of adherent platelets per field of view (200x) was determined using a fluorescent microscope (Zeiss Axiovert 135, Thornwood, NY). Again, additional sets of experiments were performed using films that had been pre-incubated in HBS at 37 °C for 48 hr.

7.2.9. Statistic analysis

Data were compared with two-tailed, unpaired t-tests. P-values less than 0.05 were considered to be significant. Data are presented as mean ± standard deviation.
7.3. Results

7.3.1. Synthesis and characterization of polyurethane

Conversion of amine groups on the side chains of lysines in the SGGKKKKGGS peptide to diazeniumdiolates was measured using ninhydrin assay, and 98% of amines were converted. PUUBD-NO was synthesized by incorporating the SGG[K(N(O)NO)]₆GGS sequence into the polymer backbone. The NMR spectra of PUBD and PUBD-NO were obtained, and the characteristic proton peaks of the SGGKKKKGGS sequence indicated the successful incorporation of the peptide sequence into the polymer (Figure 7.5). The peptide concentration of the polymer matrix was approximately 100 μmol/g (Fields, 1997, Jun and West, 2003, Jun and West, 2004).

![NMR spectrum](image)

**Figure 7.5.** $^1$H NMR spectra of PUBD-NO which was synthesized by incorporating lysine sequences. Lysine peaks were identified in a.
The number-average molecular weight (Mn), the weight-average molecular weight (Mw), and the polydispersity index (PDI) were determined by GPC using polystyrene standards. The PUBD and PUBD-NO polymers had similar molecular weights (PUBD: Mn = 54,701, Mw = 75,167, PDI = 1.37 and PUBD-NO: Mn = 50,323, Mw = 71,006, PDI = 1.41).

7.3.2. Mechanical properties of polyurethane

The incorporation of the peptides into the polymer backbone affected mechanical properties (Figure 7.6). The mechanical properties of PUBD-NO (elastic modulus: 1.2 ± 0.2 MPa, the tensile strength: 26.3 ± 4.2 MPa, elongation: 2239 ± 88 %) were significantly greater than those of PUBD (elastic modulus: 0.62 ± 0.1 MPa, the tensile strength: 10.8 ± 2.7 MPa, elongation: 1719 ± 279 %). The mechanical properties of PUBD-NO was comparable to commercial vascular grafts (Zhang et al., 1994, How and Clarke, 1984, Lyman et al., 1978, Edward et al., 1998).
Figure 7.6. The elastic modulus and the tensile strength of PUBD and PUBD-NO. *p < 0.01 compared to PUBD.

7.3.3. NO release kinetics

NO release from PUBD-NO is shown in Figure 7.7. NO was released over a 2 month period. Most of NO was rapidly released within 48 hr, and then more slowly released to 60 days. The initial burst of NO production might be caused by the spontaneous dissociation of NO on the surface of the film, and the sustained NO release after 48 hr might be explained by slow dissociation of NO from the film matrix.
Figure 7.7. Release profile of NO from PUBD-NO films at pH 7.4 in HBS. Each data point is the mean of three samples.

7.3.4. BAEC and SDSMC proliferation

The effect of NO release on BAEC and SDSMC proliferation was examined using immunohistochemical staining with a PCNA-HP conjugate (Figure 7.8 and 7.9). The percentage of PCNA-positive BAECs for PUBD-NO was significantly greater than for PUBD after 48 hr of culture (86.1 ± 4.5% vs. 58.4 ±10.9%, p < 0.001). However, the percent of PCNA-positive SDSMCs for PUBD-NO was significantly lower than for PUBD (3.7 ± 7.8% vs. 65.7 ± 5.8%, p < 0.001). In order to ensure that NO release in the 2-60 day time period is sufficient for bioactivity, PUBD-NO films were placed in HBS at 37 °C at pH 7.4 for 48 hr and cell proliferation was investigated again. Similar results
were found that the proliferation of BAEC by PUBD-NO was significantly greater (85.7 ± 1.1% vs. 42.9 ± 2.1%, p < 0.002) than by PUBD but the proliferation of SDSMC was significantly lower (5.5 ± 2.0% vs. 51.1 ± 3.1%, p < 0.002).

![Graph showing PCNA-positive Cells (%) for PUBD and PUBD-NO.](image)

**Figure 7.8.** Proliferation of BAEC for PUBD and PUBD-NO films. * p < 0.001 compared to PUBD. Proliferating cells appear red and non-proliferating cells appear blue.
Figure 7.9. Proliferation of SDSMC for PUBD and PUBD-NO films. * $p < 0.001$ compared to PUBD. Proliferating cells appear red and non-proliferating cells appear blue.

7.3.5. Platelet adhesion

Platelet adhesion on PUBD and PUBD-NO was examined using mepacrine labeled whole blood (Figure 7.10.). Platelet adhesion on PUBD (38.4 ± 7.9) was substantially lower than on collagen I (98.4 ± 5.2). However, platelet adhesion on PUBD-NO was dramatically lower, with almost no platelet adhesion (2.6 ± 0.5). Like cell proliferation study, bioactivity of released NO on platelet adhesion in the 2-60 day time period was evaluated after placing films in HBS at 37 °C at pH 7.4 for 48 hr. Similar results were found that platelet adhesion on PUBD-NO was significantly lower (9.6 ± 3.3, $p < 0.001$)
than PUBD (52.7 ± 8.8) and collagen I surface (127.2 ± 31.1).

**Figure 7.10.** The number of adherent platelets on collagen I, PUBD, and PUBD-NO. *p < 0.001 compared to collagen I, #p < 0.001 compared to PUBD.
7.4. Discussion

Previously we demonstrated that the cell adhesive peptide sequences was able to incorporated into the hard segment domains and maintained their bioactivity, enhancing endothelialization (Jun and West, 2003, Jun and West, 2004). In this study, NO generating polyurethane (PUBD-NO) was successfully synthesized by incorporating diazeniumdiolate-lysine peptide sequences into the polymer main chain. The active resistance to platelet adhesion and initial hyperplasia should improve performance as a small diameter graft. Ideally a future polyurethane graft material would contain PEG, YIGSR, and NO-donor groups.

Amine groups of the lysine sequences of polymer main chains were protected with NO molecules to avoid forming side reactions. 97% of the amines quantified by ninhydrin assay after polymerization indicates that NO molecules successfully protected amines of the lysine sequences during the polymerization. Since most NO gas released at high temperature during polymerization and under vacuum during film casting process, PUBD-NO films were reacted with NO gas overnight again, and then release kinetic was investigated. NO release profile revealed two-phase kinetics; an initial burst within 48 hours and slow sustained release over two months. It could be explained that the initial rapid NO might be produced by dissociation of diazeniumdiolate complexes on the
surface of the films followed by slow diffusion from embedded diazenium complexes inside of the film matrix since NO molecules are very small enough to diffuse across to cell membrane (Morbidelli et al., 2003, Saavedra et al., 2000).

NO has known to reduce platelet adhesion and smooth muscle proliferation (Bohl and West, 2000, Garg and Hassid, 1989, Groves et al., 1995) but stimulate endothelial cell proliferation (Murohara et al., 1999, Morbidelli et al., 2003). NO has been delivered from silicone rubbers (Zhang et al., 2002), polyurethane (Duan and Lewis, 2002), and microspheres incorporated into vascular grafts (Pulfer et al., 1997). However, the release profiles of NO molecules should be precisely controlled because a too high rate might cause cytotoxic response but too little may not be enough to reduce platelet adhesion or smooth muscle cell proliferation (Duan and Lewis, 2002, Smith et al., 1996). Recently, Bohl and West developed photopolymerizable hydrogels containing three NO donors (Bohl and West, 2000). NO release profiles could be controlled by changing the NO donor, and all three hydrogel NO donors reduced platelet adhesion and smooth muscle cell proliferation. This study is noteworthy because it might enable biomaterials to control local NO delivery precisely by employing appropriate NO donors.
7.5. Conclusions

NO generating polyurethane (PUBD-NO) has been successfully synthesized by incorporating diazeniumdiolate-peptide sequences into the polymer backbone. NO was successfully generated from the polymer and reduced platelet adhesion and smooth muscle cell proliferation but improved endothelial cell proliferation. This material might be useful for cardiovascular applications. For the future, PUBD-NO could be modified with cell adhesive peptide sequences, PEG, and NO donors and it might result in synergistic effects leading to fast and high level of endothelialization without platelet adhesion.
References


Cannizzaro, S. M., Padera, R. F., Langer, R., Rogers, R. A., Black, F. E., Daries, M. C.,


Cook, A. D., Hrkach, J. S., Gao, N. N., Johnson, I. M., Pajrani, U. B., Cannizzaro, S. M.

119.


Res.*, **41**, 252-256.


