OSTEOGENIC PEPTIDE-FUNCTIONALIZED POLYMERIC MATERIALS FOR
BONE REGENERATION APPLICATIONS

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OSTEOGENIC PEPTIDE-FUNCTIONALIZED POLYMERIC MATERIALS FOR
BONE REGENERATION APPLICATIONS

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LIVE every moment, LAUGH everyday and LOVE beyond words. This dissertation is dedicated to my amazing family who taught me the power of these three simple words. Without their support, I would not have succeeded in completing this chapter of my life.
ABSTRACT

Recently, research has focused its attention on the synthesis of polymeric materials functionalized with bioactive peptides to overcome the limitations of both naturally occurring and synthetic polymers used in bone tissue regeneration applications. Poly(ester urea)s (PEUs) are a high modulus, non-toxic, amino acid-based class of polymers that have been thoroughly investigated by the Becker group. Both its tunable properties and ease of functionality makes this polymer ideal for tissue engineering applications. Phenylalanine-based PEUs (Poly(PHE)) can be easily functionalized with bioactive peptides for enhancement of its osteoinductive potential. OGP[10-14] is the active subunit of the naturally occurring tetradecapeptide that is known to upregulate proliferation, differentiation and matrix mineralization of osteoblast cell lines. In this dissertation, both OGP-tethered and OGP-crosslinked poly(PHE) materials were synthesized using efficient ‘click’ chemistry techniques. These materials were tested in vitro and in vivo to reveal the enhanced osteoinductive ability of poly(PHE) materials for tissue engineering applications. Osteogenic differentiation of human mesenchymal stem cells (hMSCs) was enhanced at both 2 and 4 weeks on OGP[10-14]-tethered poly(PHE)s both in vitro and in vivo. Crosslinking OGP[10-14] into the poly(PHE) resulted in an increase in toughness and a decrease in tensile modulus, suggesting mechanical properties can be tuned with peptide crosslinkers. Early osteogenic differentiation of mouse preosteoblast cells (MC3T3-E1) was also enhanced on these constructs.
Surface functionalization of peptides was also conducted in order to study the enhancement of osseointegration on TiO$_2$ as well as the synergistic effects of several osteogenic peptides on 2D gradient surfaces. Here, the surface functionalization of titanium oxide (TiO$_2$) substrates with catechol-bearing dendritic OGP[10-14] modular peptides is reported. Functionalized OGP[10-14] modular peptides were synthesized and non-covalently bound to the surface of TiO$_2$. Osteogenic differentiation of MC3T3-E1 cells was tested to result in enhanced expression of osteogenic genes compared to cells cultured without OGP-functionalization. The concentration-dependent synergistic effect of bone morphogenetic protein 2 (BMP-2) and the Arg-Gly-Asp (RGD) amino acid sequence was also studied on 2D gradient substrates. In nature, peptides do not exist and act as a single entity, therefore this study reveals a glimpse at what the future holds for studying concentration effects of multiple bioactive factors simultaneously for enhanced bone tissue regeneration.

Each of these studies reveals the importance of blending synthetic materials with naturally occurring peptides for optimal tissue engineering constructs. There is a clear need for polymeric constructs with both the mechanical and biological properties necessary for bone tissue engineering, and this dissertation provides a solid foundation for the future of bone regeneration biomaterials in the Becker group.
ACKNOWLEDGEMENTS

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>LIST OF TABLES</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>x</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LIST OF FIGURES</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>xii</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LIST OF SCHEMES</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>xxiii</td>
</tr>
</tbody>
</table>

## CHAPTER

### I. INTRODUCTION

1.1 Abstract ......................................................... 1
1.2 Introduction ................................................... 2
1.3 Bone Repair ..................................................... 3
1.4 Osteogenic growth peptide .................................... 10
1.5 OGP for tissue regeneration applications ..................... 18
1.6 Use of other osteogenic growth factors for bone regeneration applications ..... 24
1.7 Looking forward ................................................ 26
1.8 Acknowledgments ............................................... 27

### II. MATERIALS AND INSTRUMENTS

2.1 Materials ....................................................... 28
2.2 Instruments .................................................... 29

### III. OGP-FUNCTIONALIZED POLY(ESTER UREA)S FOR ENHANCING OSTEOINDUCTIVE POTENTIAL OF HUMAN MESENCHYMAL STEM CELLS

3.1 Abstract .......................................................... 33
3.2 Introduction .................................................... 34
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 Three main types of bone grafting methods are used: 1. autografts 2. allografts 3. synthetic bone grafts. The ratings of effectiveness for each grafting method in various areas of interest are shown in the compiled table. – inadequate, + adequate, ++ good, +++ very good. [Bone Grafting and Bone Grafting Substitutes. Finkemeier, C.G. J Bone and Joint Surg. 2002, Volume 84. Copyright 2002. The Journal of Bone and Joint Surgery, Inc.]</td>
<td>5</td>
</tr>
<tr>
<td>4.1 qRT-PCR forward and reverse SYBR green primers for RNA expression quantification</td>
<td>97</td>
</tr>
<tr>
<td>4.2 Thermal properties of poly(PHE) and crosslinked the crosslinked networks</td>
<td>98</td>
</tr>
<tr>
<td>5.1 SYBR Green primers used for RT-PCR. GAPDH was used as the endogenous control for comparing gene expression</td>
<td>136</td>
</tr>
<tr>
<td>5.2 The apparent disassociation constant ($K_d$), maximum adsorption ($B_{max}$) and enhancement parameter ($\beta$) of catechol-functionalized dendrons to TiO$_2$ surface</td>
<td>143</td>
</tr>
<tr>
<td>5.3 The immobilization of OGP-Cat and OGP-(Cat)$_4$ and their retention on TiO$_2$ substrates. Atomic Ratios of N/Ti for the TiO$_2$ surface, the surfaces after OGP-Cat and OGP-(Cat)$_4$ immobilization, and the OGP-Cat and OGP-(Cat)$_4$ bearing surfaces after incubation in HEPES buffer $^{a,b}$</td>
<td>151</td>
</tr>
</tbody>
</table>
Appendix 1 Substrates for bioactivity evaluation. The immobilization of OGP[10-14] on TiO$_2$ surface by immersion the substrates in the solution of OGP-PEG-(Cat)$_4$ at different concentration for overnight and their respective load amount calculated in theory and measured with XPS.
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 OGP is a naturally occurring 14-mer peptide with an active subunit from amino acids 10 to 14. The active domain is cleaved after dissociation from α2M binding protein, and drives mitogenic activity and differentiation of osteoblast cell lines. (Reprinted with permission from Ref 72. Copyright 2002 Wiley Periodicals Inc)</td>
<td>13</td>
</tr>
<tr>
<td>1.2 OGP signaling for proliferation starts with the endogenous release of OGP from the cell following bone ablation. The OGP binding protein, α2M non-covalently binds to OGP in serum to prevent proteolysis and to regulate a steady state concentration. OGP dissociates from α2M and is then proteolytically cleaved to generate the active OGP[10-14] subunit which binds to the G protein receptor to promote mitogenic activity of osteoblast cell lines via the MAPK pathway.</td>
<td>15</td>
</tr>
<tr>
<td>3.1 ¹H NMR of all three polymer synthesized and the characteristic peaks to show functionality.</td>
<td>41</td>
</tr>
<tr>
<td>3.2 ¹H NMR spectrum of OGP conjugated poly(1-PHE-6)-co-(1-TYR-6). Wide peaks inside red curves are characteristics of OGP peptide units. Peak a is the chemical shift of triazole hydrogens, and peak b arises from chemical shift of protons connected tyrosine units and triazole rings. (Solvents marked with asterisks).</td>
<td>51</td>
</tr>
<tr>
<td>3.3 Microcomputed tomography images obtained for poly(1-PHE-6) salt-leached scaffolds with both 100-250 μm (A and C) and 250-400 μm (B and D) pore sizes. (A-B) 2D reconstructed image of a z-direction slice through the scaffold. (C-D) 3D models of scaffolds.</td>
<td>52</td>
</tr>
<tr>
<td>3.4 Summary of cell viability on OGP-functionalized 3D porous scaffolds using the WST-1 assay. hMSCs are able to survive and proliferate every surface, indicating the polymers are non-toxic to the cells. * indicates p-value &lt;0.05 between day 1 and 3 within one scaffold group. ** indicates p-value &lt;0.05 between day 3 and 7 within one scaffold group.</td>
<td>53</td>
</tr>
</tbody>
</table>
3.5 Effect of pore size, functionalization with OGP, and crosslinking on bone differentiation gene expression for hMSCs after 2 and 4 weeks in vitro as observed using qRT-PCR, using (A) GAPDH as the endogenous reference gene for normalization of (B) Runx2, (C) BSP, and (D) OSC expression. SYBR Green primers were used for all qRT-PCR experiments. ** indicates p value <0.05 for 2 and 4w crosslinked and non-crosslinked OGP-functionalized scaffolds of the same pore size. *** indicates p value <0.05 for 2 and 4w 100-250 µm and 250-400 µm pore sizes of OGP-functionalized scaffolds. .......................................................... 58

3.6 Effect of pore size, functionalization with OGP, and crosslinking on ALP expression of hMSCs. * indicates p value <0.05 between the 2w OGP-functionalized scaffolds and controls. ** indicates p value <0.05 between 4w OGP-functionalized scaffolds and controls .......................................................................................................................... 59

3.7 Summary of RUNX2 and PPAR-γ expression of hMSCs after 2 and 4 weeks in vitro. Immunofluorescence staining for cell nuclei (blue), Runx2 (red), and PPAR-γ (green) and quantification of Runx2 and PPAR-γ immunofluorescence using Image J. * indicates p value <0.05 between the 2w OGP-functionalized scaffolds and controls. ** indicates p value <0.05 between 4w OGP-functionalized scaffolds and controls. Scale bar = 20µm. ........................................................................................................... 70

3.8 Summary of Col1A and BSP expression of hMSCs after 2 and 4 weeks in vitro. Cell nuclei (blue), Col1A (red), and BSP (green) and quantification of Col1A and BSP immunofluorescence using Image J. No significant difference was observed for Col1A and BSP expression for scaffolds. Scale bar = 20µm ................................................................. 71

3.9 Summary of osteocalcin (OSC) expression for hMSCs after 2 and 4 weeks in vitro. Immunofluorescence staining for cell nuclei (blue) and OSC (red) and quantification of immunofluorescence using Image J. * indicates p value <0.05 between 2w OGP-functionalized scaffolds and controls. ** indicates p value <0.05 between 4w OGP-functionalized scaffolds and controls. *** indicates p value <0.05 between 2w 100-250 µm and 250-400 µm pore sizes of OGP-functionalized scaffolds. Scale bar = 20 µm. ................................................................................................................ 72

3.10 Bright field histological images for calcium deposition using Alizarin Red after 2 and 4 weeks of culture in vitro hMSCs. A hematoxylin counter stain was conducted for all histological samples. The angle at which the samples were embedded and sliced for imaging influences the visualization of the size and shape of the pores. Black arrow: calcium deposition, yellow arrow: scaffold. Images were taken using a 10X objective. Scale bar = 100 µm. ................................................................................................. 73
3.11 Bright field histological images for collagen 1 deposition using H&E staining after 2 and 4 weeks of culture in vitro hMSCs. A hematoxylin counter stain was conducted for all histological samples. Images were taken using a 4x objective. Scale bar = 200 \( \mu m \). ................................................................. 74

3.12 Trichrome staining for 4 and 12 week in vivo poly(1-PHE-6) porous scaffolds with 100-250 \( \mu m \) and 250-400 \( \mu m \) pore sizes. We speculate that pore sizes are difficult to discern among sample sets because of enhanced tissue integration after only 2 weeks in vivo. In addition, the angle at which the samples were embedded and sliced for imaging influences the visualization of the size and shape of the pores. Images were taken using a 100X objective. Scale bar = 100 \( \mu m \)................................................................. 75

3.13 H&E staining of in vivo poly(1-PHE-6) scaffolds with 100-250 \( \mu m \) and 250-400 \( \mu m \) pore sizes after 4 and 12 weeks. We speculate that pore sizes are difficult to discern among sample sets because of enhanced tissue integration after only 2 weeks in vivo. In addition, the angle at which the samples were embedded and sliced for imaging influences the visualization of the size and shape of the pores. Black arrow: new bone, red arrow: scaffold. Images were taken using a 100x objective. Scale bar = 100 \( \mu m \)................................................................. 76

3.14 Alizarin histological images for in vivo rat studies for poly(1-PHE-6) with OGP functionalization and cross linking, and without. We speculate that pore sizes are difficult to discern among sample sets because of enhanced tissue integration after only 2 weeks in vivo. In addition, the angle at which the samples were embedded and sliced for imaging influences the visualization of the size and shape of the pores. Images were taken using a 10x objective. Scale bar = 100 \( \mu m \)................................................................. 77

4.1 AT-IR and Raman spectroscopy were used to verify the successful formation of the C-S bond during the thiol-ene ‘click’ reaction to form the crosslinked network. Poly(PHE)-100%crosslinked was used for this characterization so the C-S bond could be fully visualized. The appearance of the AT-IR peak at 663 cm\(^{-1}\) demonstrates the successful formation of the C-S bond. This stretching can be more easily detected using Raman spectroscopy, in which the appearance of the peak at 620 cm\(^{-1}\) is apparent................................................................. 99

4.2 Young’s modulus was measured for poly(PHE) and poly(PHE)-3%crosslinked. Prior to crosslinking, the tensile modulus of poly(PHE) 756.9 ± 30.9 MPa, whereas the poly(PHE)-3%crosslinked materials had a decreased tensile modulus of 10.2 ± 7.8 MPa. With such low crosslinking incorporation into the network, the hydrogen bonding of the PEU backbone is disrupted, causing an increase in elasticity and a decrease in stiffness. ........................................................................................................ 100
4.3 Swelling studies were performed on poly(PHE)-3%crosslinked and poly(PHE)-3%OGP in DMF over a period of 6 h. After 1 h, each polymer network was swollen to its maximum capacity of $0.671 \pm 0.038$ S% and $0.579 \pm 0.050$ S% for poly(PHE)-3%crosslinked and poly(PHE)-3%OGP.

4.4 Proliferation of MC3T3-E1 cells was tracked on days 1, 3 and 7 of in vitro culture. On day 1, the same cell density was calculated for each surface. By day 3, there is a significantly greater cell density on poly(PHE)-3%OGP compared to poly(PHE) and the glass control. This is an indication that even when OGP[10-14] is tethered at the N- and C-terminus, enhanced proliferation is observed. On day 7, the cell density on glass substrates is significantly greater than on poly(PHE)-3%OGP and poly(PHE). As cells progress from the proliferative to the differentiation phase of osteogenesis, proliferation ceases. The greater cell density on glass substrates may indicate that cells remain in the proliferative phase on these substrates, while they are progressing to the osteoprogenitor stage on poly(PHE)-3% and poly(PHE). Significant differences have a p value $\leq 0.05$.

4.5 Vinculin and actin filaments were stained using immunofluorescence on day 3 to observe cell focal adhesion and spreading. Strong adhesion and well-structured actin stress fibers were observed on all of the samples which is indicative of osteogenic differentiation. Scale bar for all images = 200 µm.

4.6 RNA expression is an accurate method of tracking gene expression during osteoblast differentiation. On day 7 of in vitro culture, MC3T3-E1 cells were tested for RNA expression of RUNX2 and BSP. RUNX2 is an early transcription factor, essential for osteogenic differentiation. As cells progress from the proliferative phase to the osteoprogenitor stage, cells begin to express BSP, which is vital for osteoblast cell adhesion and matrix maturation. RUNX2 expression is upregulated in MC3T3-E1 cells seeded on poly(PHE)-3%OGP, with 120% greater expression observed, respectively, compared to poly(PHE). These results indicate that there OGP[10-14] crosslinked into poly(PHE) networks is bioactive and capable of enhancing osteogenic differentiation of preosteoblast cell lines. * is used to denote significance between poly(PHE)-3%OGP and poly(PHE), RUNX2 gene expression with p $\leq 0.05$.

5.1 The adsorption of catechol-functionalized dendrons, OGP-Cat, OGP-(Cat)$_4$ and OGP-PEG-(Cat)$_4$ onto TiO$_2$ surface monitored by QCM-d. The experiment contains three processes: i) baseline in HEPES buffer; ii) adsorption of ligands; iii) buffer washing the adsorbed ligands, as indicated by the small peak due to the stop of flow. To reach similar level of frequency shift, tetravalent ligands OGP-(Cat)$_4$ and OGP-PEG-(Cat)$_4$ required solution at much lower concentrations compared to the monovalent ligand, OGP-Cat, indicating a stronger binding affinity. Signals from different overtones were close due to the rigidity of the adsorbed film and those of n= 7 were shown here.
5.2 The adsorption of OGP-Cat onto TiO$_2$ surface and its adsorption isotherm. (A) The adsorption of OGP-Cat onto TiO$_2$ surface at different concentrations was measured by QCM-d, while the concentration was increased sequentially ($c_1 = 0.068$ μmol/L, $c_2 = 0.34$ μmol/L, $c_3 = 1.8$ μmol/L, $c_4 = 7.3$ μmol/L, $c_5 = 13$ μmol/L, and $c_6 = 34$ μmol/L). At last the adsorbed layer was washed with 25 mM HEPES buffer. The flow rate was 0.150 mL/min. Three independent measurements were shown. (B) The dissociation constant of OGP-Cat was $5.7 \pm 0.2$ μM by fitting the adsorption isotherm with single-site specific binding model, as represented with the dashed line.

5.3 Tetravalent binding ligand OGP-(Cat)$_4$ (c= 1 μM) shows strong binding affinities to versatile metal and metal oxide surfaces due to coordination bond, including Fe$_2$O$_3$, Fe$_3$O$_4$, ZrO$_2$, TiO$_2$ and CeO$_2$ surfaces as measured by QCM-d.

5.4 XPS characterization confirmed the successful immobilization of OGP-(Cat)$_4$ on TiO$_2$ surface. (A) Survey scan of bare TiO$_2$, OGP-(Cat)$_4$, and OGP-(Cat)$_4$ immobilized TiO$_2$. The N1s signal comes from amide bonds in peptides. (B) 1min of Ar$^+$ plasma treatment to the OGP-(Cat)$_4$ immobilized TiO$_2$ surface removed the adsorbed OGP-(Cat)$_4$ layer. The N1s peaks are normalized to the highest intensity (O1s) for comparison of the signal to noise ratio. The signal changes in high resolution XPS spectra of O1s (C) and C1s (D) demonstrates the successful immobilization of OGP-(Cat)$_4$ on TiO$_2$ substrates. The multiple peaks were fitted with a Gaussian model. The atomic ratios of C2/C1, C3/C1, O2/O1, O3/O1 and C3/N of respective surfaces were calculated based on the integrated area of each peak.

5.5 The immobilization of modular peptides was viewed by labeling the peptide with fluorescein. Due to multivalent binding, the retention time of OGP-(Cat)$_4$ on TiO$_2$ in buffer pH=7.4 was longer than 2 weeks. (A)-(E) The immobilized FITC-labeled OGP-Cat on TiO$_2$ surfaces was observed under fluorescence microscope, and the mean intensity of fluorescence decreased after incubating the substrates in pH=7.4 25 mM HEPES buffer due to the diffusion of FITC-labeled OGP-Cat. TiO$_2$ substrates were incubated in the solution of 0.5 mM (A) FITC-labeled OGP-Cat and (B) FITC overnight, then thoroughly washed with water and dried with N$_2$. FITC-labeled OGP-Cat immobilized TiO$_2$ pattern on glass slides was observed under (C) bright field microscope and (D) fluorescence microscope. The scale bar is 50 μm. (E) Mean intensity of FITC-labeled OGP-Cat immobilized surfaces after incubation. The control sample was a TiO$_2$ substrate incubated in FITC solution overnight. (F)-(G) The immobilized OGP-(Cat)$_4$ was preserved on the surface for more than 2 weeks in buffer pH=7.4, in comparison, the diffusion of monovalent ligand OGP-Cat was detected after 12 hours. XPS spectra of N1s signals taken after incubation of (F) OGP-(Cat)$_4$ and (G) OGP-Cat immobilized on TiO$_2$ substrates in HEPES buffer (pH=7.4) for different time durations, respectively. The control is taken after
incubation of TiO$_2$ substrates in 25 mM HEPES buffer. To compare the signal to noise ratio, all spectra were normalized to the peak of highest intensity (O1s). ...... 149

5.6 Preferential adsorption of OGP-(Cat)$_4$ to the TiO$_2$ region of a partially TiO$_2$-coated glass slide observed with XPS. The green spots on the substrates are positions where XPS spectra were taken (three in SiO$_2$ region and three in TiO$_2$ region). The stronger signal of N1s in the TiO$_2$ region compared with that of the SiO$_2$ region indicates peptides preferentially adsorbed to the TiO$_2$ surface. The XPS signals were normalized with the strongest peak intensity (O1s). The atomic percentage of nitrogen in TiO$_2$ and SiO$_2$ region were 8 ± 1% and 1.7 ± 0.8%, respectively. ..................... 152

5.7 The adhesion of MC3T3-E1 cells were not significantly influenced by the immobilized OGP[10-14] peptides, while they proliferated faster on OGP-bearing surface with dose-dependency. After 24 hours, MC3T3-E1 cells were well spread on substrates with or without immobilized OGP[10-14] and formed focal adhesion contacts with the substrates, as indicating by the immunohistochemical staining of adherent cells on (A) OGP-99% and (B) bare TiO$_2$ substrates. Red corresponds to F-actin in cytoskeleton; green corresponds to vinculin in focal adhesion complex; and blue corresponds to the nuclei (scale bar is 50 μm). (C) The immobilized OGP-PEG-(Cat)$_4$ promoted the cell proliferation, and this effect was dose-dependent. Cell number on substrates after day 1 and day 3 were evaluated by PrestoBlue Assay. The error bar was calculated from three replicates. .................................................. 154

5.8 Bone sialoprotein (BSP) and osteocalcin (OCN), late markers of differentiation to osteoblast, were secreted by MC3T3-E1 cells on OGP-99% substrate, as demonstrated by the immunohistochemical staining after 2 weeks. (A) blue corresponds to the nuclei; (B) red corresponds to osteocalcin (OCN); (C) green corresponds to bone sialoprotein (BSP); and (D) is the merged image of the three channels (scale bar = 50 μm). Similar results were observed on OGP-50% and TiO$_2$ substrates. However, the cells on OGP-99% substrate expressed a much higher gene level of BSP and OCN, compare to cells on bare TiO$_2$, as shown in the (E) mRNA levels of transcription factor genes of BSP and OCN, in MC3T3-E1 cells measured by real-time PCR after cell culture for 18 days. Data represent relative expression to the level of the control (cells on TiO$_2$), set at 1, and mean value and standard deviation calculated from triplicates. ................................................................. 157

5.9 (A)-(B) The ALP activity and its mRNA level of MC3T3 cells on OGP-99% substrate was significantly higher compared to substrates with lower concentration or none. (A) ALP activity of MC3T3 cells cultured on OGP-99%, OGP-50%, and TiO2 substrates, respectively, on day 18. (B) mRNA levels of transcription factor gene of ALP, in MC3T3-E1 cells measured by real-time PCR after cell culture for 18 days. (C) Ca$^{2+}$ accumulation in the cell films quantified with ICP-OES and normalized with total amount of protein. The cell films on OGP-99% exhibited 2-fold higher
content of Ca2+ compared with those on other substrates. (D)-(F) Mineralization of MC3T3-E1 cells on substrates studied by Alizarin Red S. staining on day 14 and Ca2+ quantification by ICP-AES on day 18. Larger sized calcified nodules were observed on OGP-99%, indicating promoted mineralization results from the higher concentration of OGP[10-14]. Images of cell films on (D) OGP-99%, (E) OGP-50%, and (F) TiO2 substrates after Alizarin Red S. observed under bright field microscope. The mineralized osteoids, the spherulites with dark red color, ranging from 0.5 to 2 μm, were observed on all the three kinds of substrates. And only cell films on OGP-99% showed the dark mineralized chunks, ranging from 2 to 10 μm. .......................... 159

6.1 High resolution C1s peak fitting for (A) 100% coverer age of 4-Cl and (B) 100% coverage of 5-vinly silane surfaces. The spectrum shows the average result based on 10 identical samples, and the error bar was calculated as the............................................. 168

6.2 High resolution C1s signal tracking for the peptide immobilization process. (A) C1s peak before any reaction; (B) after the RGD covalent bonding; (C) after RGD and BMP-2 peptide immobilization; (D) the overlay of the C1s peak during the reactions. The peak strength increase is a result of the attachment of peptide, and was used to calculate the peptide surface coverage fraction according the previous equationEquation 6.8  BMP-2 surface coverage fraction equation taking into account the 125 carbons in the BMP-2 sequence. ................................................................. 169

6.3 (A) Schematic illustration of the fabrication of 2D orthogonal peptide concentration gradients. (a) Glass substrates were treated with UV-ozone. (b) The first component, 4-chlorobutylidimethylchlorosilane, was deposited using a gradient vapor deposition method. (c) The [-Cl] group was substituted with an azide functional group. (d) The substrate was rotated 90o, and the second gradient deposition process introduced vinyl functional groups in the [Y] direction. (e) Sequential thiol-ene addition and (f) strain promoted alkyne-azide cycloaddition (SPAAC) reactions were carried out to immobilize GRGDS and BMP-2 peptides, respectively. (B) The corresponding chemical reactions happened at each step. (C) The cysteine functionalized GRGDS peptide structure synthesized for the thiol-ene reaction. (D) BMP-2 peptide structure with 4-dibenzocyclooctynol (DIBO) end group for SPAAC reaction. ...................... 176

6.4 High-resolution XPS C1s signals were used to characterize the extent and efficiency of the peptide immobilization procedure. (A) The C1s peak area distribution before the thiol-ene reaction for GRGDS peptide (step (d) in Figure 1). (B) The C1s peak area increased at each point on the substrate after the GRGDS immobilization (step (e) in Figure 6.3). (C) The peak area increased further after the BMP-2 peptide was covalently attached (step (f) in Figure 6.3). ........................................................................ 177

6.5 The average value and standard deviation of GRGDS (A) and BMP-2 (B) peptide concentrations were plotted for the identical axis dimensions. The average
concentration values were based on three individually fabricated and characterized 2D substrates. For each 2D substrate, a total of 25 evenly distributed points (5×5) were measured. In total, 15 samples points were measured at each concentration. Error bars represent the standard deviation of mean values (n = 3).

6.6 The hMSC cell proliferation behavior on 1D only RGD (A) and BMP-2 (B) gradient substrates. The cell number was determined from 3 identical samples with images randomly picked (n = 3) at each concentration point utilizing a Click-iT EdU imaging kit. Error bars represent the standard deviation of mean values.

6.7 The hMSCs density distribution on the 2D GRGDS and BMP-2 peptide gradients. The columns on the left and below each 2D data set represent the cell density values on 1D GRGDS only concentration gradient surfaces and 1D BMP-2 only concentration gradient surfaces, respectively. (A) Cell density after 1 day in culture; (B) Cell density after 3 days in culture; (C) Cell density after 7 days in culture. The data reported represents cell density calculated from n=3 samples.

6.8 The hMSCs osteoblastic differentiation progression on the 2D gradient substrate was measured by Runx2 (red) and BSP (green) immunofluorescence imaging. The columns on the left and below each 2D data set represent the cell behavior on 1D GRGDS only and 1D BMP-2 only concentration gradient surfaces, respectively. From (A) day 7, (B) day 14 and (C) day 21 images, the Runx2 and BSP proteins showed spatially- and temporally-dependent expression behavior.

6.9 The quantitative RT-qPCR characterization for Runx2 (A1) (B1) and BSP (A2) (B2) gene expression levels at 14-day (A) and 21-day (B) time points. The single columns on the left and below each 2D data set represent the gene expression results on 1D GRGDS only and 1D BMP-2 only concentration gradient surfaces, respectively. The gene expression levels were normalized to 18S rRNA control at every sample point. In general, the gene expression patterns of the cells on the substrates complemented the protein secretion results by fluorescence shown in Figure 6. Statistics were performed to show significance in gene expression from 2 to 3 weeks and along the gradients. * indicates significance of p ≤ 0.05 from week 2 to week 3 in the same gradient position for both 1D and 2D gradients. ** indicates significant of p ≤ 0.05 at the same time point along the 1D and 2D gradients. The bolded box highlights the gradient position which is significantly different from every position marked with **.

Appendix 1. $^1$H NMR of 1-PHE-6.

Appendix 2. $^1$H NMR of 1-PHE-4.
Appendix 3. $^1$H NMR of 1-TYR-6. ................................................................. 239

Appendix 4. $^1$H NMR and composition calculation of poly(1-PHE-6)$_{0.99}$-co-(1-TYR-6)$_{0.01}$ using the provided equation. ................................................................. 240

Appendix 5. $^1$H NMR and composition calculation of Poly(1-PHE-6)$_{0.89}$-co-(1-TYR-6)$_{0.01}$-co-(1-PHE-4)$_{0.10}$ using the provided equation. ......................... 241

Appendix 6. ESI-MS of N$_3$-OGP[10-14]. ......................................................... 242

Appendix 7. $^{13}$C NMR of 1-PHE-6................................................................. 243

Appendix 8. ATR-IR of 1-PHE-6 monomer..................................................... 244

Appendix 9. ESI-MS of 1-PHE-6 monomer..................................................... 245

Appendix 10. $^1$H NMR of Boc-protected 1-PHE-3 monomer........................... 246

Appendix 11. ESI-MS of Boc-protected 1-PHE-3 monomer............................. 247

Appendix 12. $^1$H NMR of 1-PHE-3 monomer................................................ 248

Appendix 13. $^{13}$C NMR of 1-PHE-3 monomer.............................................. 249

Appendix 14. ATR-IR of 1-PHE-3 monomer................................................ 250

Appendix 15. $^1$H NMR of poly(1-PHE-6)$_{0.7}$-co-(1-PHE-3)$_{0.3}$ ...................... 251

Appendix 16. $^{13}$C NMR of poly(1-PHE-6)$_{0.7}$-co-(1-PHE-3)$_{0.3}$ ..................... 252

Appendix 17. ATR-IR of poly(1-PHE-6)$_{0.7}$-co-(1-PHE-3)$_{0.3}$ ....................... 253

Appendix 18. SEC trace of poly(1-PHE-6)$_{0.7}$-co-(1-PHE-3)$_{0.3}$ ................... 254

Appendix 19. DSC curve for poly(1-PHE-6)$_{0.7}$-co-(1-PHE-3)$_{0.3}$ .................. 255
Appendix 20. TGA curve for poly(1-PHE-6)_{0.7-co-(1-PHE-3)}_{0.3} ............................... 256

Appendix 21. Dogbone shape with dimensions used for tensile testing............................. 257

Appendix 22. MALDI-MS pre- and post-disulfide reduction of Cys-OGP[10-14]-Cys 258

Appendix 23. DSC curve of poly(PHE)-3%crosslinked...................................................... 259

Appendix 24. DSC curve of poly(PHE)-3%OGP................................................................. 260

Appendix 25. TGA trace of poly(PHE)-3%crosslinked..................................................... 261

Appendix 26. TGA trace of poly(PHE)-3%OGP................................................................. 262

Appendix 27. MALDI-ToF and ESI spectra of OGP[10-14] functionalized with catechol-bearing dendons................................................................. 263

Appendix 28. Adsorption isotherms of catechol-functionalized dendrons. Each dot with an error bar was calculated based on three independent measurements. The dashed line represents the fitting with single-site specific binding model. .......................................................................................................................................................................................... 264

Appendix 29. Tetravalent binding ligand OGP-(Cat)_{4} (c = 10 μM) weakly adsorbed onto SiO_{2}, Al_{2}O_{3} and hydroxyapatite (HA), and relatively strongly adsorbed onto Au measured by QCM-d.................................................................................................................. 265

Appendix 30. Thickness of TiO_{2} deposition on the top of glass slides measured with interferometric surface profiler. The red higher region represents the TiO_{2} deposition for 1h, while the blue lower region represents the glass slide covered with a silica wafer without any deposition............................................. 266

Appendix 31. XPS characterization of TiO_{2} deposition after RF sputtering coating for 1h. The obtained TiO_{2} shows the O/Ti ratio of 2, matching with the theoretical with the theoretical stoichiometry. Some carbon and fluorine contamination exists.......................................................................................................................................................................................... 267

Appendix 32. The surface roughness of TiO_{2} deposition measure by AFM (height image). The TiO_{2} layer was formed on the top of Si wafer after 1h deposition.. 268
Appendix 33. A Live-Dead assay was used to assess the viability (>98%) of MC3T3-E1 that were seeded on the surface of F-OGP-Cat immobilized TiO$_2$ substrates and cultured for 24 hours. Live cells are stained green and dead cells are stained red. The scale bar is 50 µm. ............................................................... 269

Appendix 34. XPS characterization of OGP-PEG-(Cat)$_4$ immobilized TiO$_2$ surface with 99% and 50% coverage of the maximum adsorption. N$_1$s signal that comes from the amide bond in peptides showed up in OGP-99% substrates. The XPS signals were normalized to the highest intensity (O$_1$s) for comparison of the signal to noise ratio. The nitrogen percentage of the total atomic amount was calculated based on measurements of three independent samples. ........................................................................................................ 271

Appendix 35. Immunohistochemical staining of bone sialoprotein (BSP) and osteocalcin (OCN) of MC3T3 cells on OGP-99% substrates after 2 weeks. OCN showed higher concentration in cytoplasm, while the distribution of BSP in cytoplasm and extracellular matrix is indistinguishable under fluorescence microscope. (A) Merged image of nuclei (blue) and OCN (red); (B) merged image of nuclei (blue) and BSP (green). ....................................................................................... 272
# LIST OF SCHEMES

<table>
<thead>
<tr>
<th>Scheme</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1 Synthesis of the bis-amine-toluenesulfonic acid salt monomers.</td>
<td>38</td>
</tr>
<tr>
<td>3.2 The three monomers synthesized for this study: (A) 1-PHE-6 (B) 1-PHE-4 with alkene functionality for crosslinking (C) 1-TYR-6 with alkyne functionality for peptide conjugation.</td>
<td>38</td>
</tr>
<tr>
<td>3.3 Generic interfacial polymerization reaction scheme.</td>
<td>39</td>
</tr>
<tr>
<td>3.4 Copper catalyzed azide-alkyne click reaction for the conjugation of N₃-OGP to poly(ester urea).</td>
<td>42</td>
</tr>
<tr>
<td>4.1 Synthesis of 1-PHE-3 monomer.</td>
<td>85</td>
</tr>
<tr>
<td>4.2 Interfacial polycondensation of 1-PHE-6 and 1-PHE-3.</td>
<td>86</td>
</tr>
<tr>
<td>4.3 Model reaction for photo-induced thiol-ene ‘click’ crosslinking reaction.</td>
<td>88</td>
</tr>
<tr>
<td>4.4 Cys-OGP[10-14]-Cys was synthesized via Fmoc-protected solid phase peptide synthesis.</td>
<td>90</td>
</tr>
<tr>
<td>4.5 Cys-OGP[10-14]-Cys was crosslinked into the poly(PHE) network via photoinitiated thiol-ene ‘click’ chemistry.</td>
<td>91</td>
</tr>
<tr>
<td>5.1 Synthesis of Fmoc-YGFGG-Resin.</td>
<td>122</td>
</tr>
<tr>
<td>5.2 Synthesis of Dendron-YGFGG-Resin.</td>
<td>123</td>
</tr>
</tbody>
</table>
5.3 Synthesis of Dendron-PEG-YGFGG-Resin. ................................................................. 124

5.4 Synthesis of FITC-labeled OGP-Cat. ............................................................................. 126

5.5 Molecular structures of OGP-(Cat)$_n$ and OGP-PEG-(Cat)$_n$; $n = 1, 2, 4$ .................. 137

5.6 The Fmoc-protected solid phase synthesis of OGP-PEG-(Cat)$_2$. ............................... 138
CHAPTER I

INTRODUCTION

Some of this work has been previously published as

Gina M. Policastro and Matthew L. Becker. WIREs Nanomedicine and Nanobiotechnology, 2015, doi: 10.1002/wnan.1376

1.1 Abstract

For nearly 2000 years, biomaterials have been used as damaged tissue implants. A field that started with wood and gold tissue replacements, has evolved into an advanced science that combines ideas of cellular biology, engineering and synthetic chemistry to produce bioresorbable materials capable of directing specific cell responses. With the overwhelming number of failed bone defect repairs every year, bone tissue engineering has become an important area of study. Both naturally occurring and synthetic polymeric materials have shown promising results for bone regeneration with their wide range of mechanical and degradation properties. Despite their favorable properties, these materials are limited by their lack the biological cues necessary for enhanced bone formation and osteogenic differentiation. For this reason, naturally occurring growth factors, such as osteogenic growth peptide (OGP), have been studied for use in bone tissue engineering constructs to elicit more efficient bone healing. OGP-functionalization of bioresorbable
polymers has been shown to enhance regeneration of bone and osteogenic differentiation of stem cells in defect models. Vast improvements on bone tissue engineering constructs have been made possible through the use of OGP as a functional bio-conjugate. As this field continues to expand, the hopes of overcoming the limitations of current bone defect repair treatment methods is becoming a reality.

1.2 Introduction

The use of biomaterials in the human body is an ancient practice with evidence of wood and metals such as gold being used nearly 2000 years ago. The discipline has advanced exponentially over the last half century with expanding knowledge in the subjects of biochemistry, systems biology, polymeric materials, tissue regeneration, and biological processes. Biomaterials can be described as any natural or synthetic material that comes in contact with biological systems for the replacement or repair of defect tissue. The field of biomaterials extends through three generational classes. The earliest biomaterials, such as gold and wooden implants, served to provide suitable mechanical properties and non-toxicity as a replacement tissue in the defect site. With advances in biology, synthetic bioactive materials with the capability of eliciting a controlled response of the physiological system emerged as the second generation of biomaterials. These included bioglass, ceramics, and synthetic composites. Third generation biomaterials include bio-inspired synthetic constructs that stimulate a molecular level response in the body. Tissue engineering constructs fall into this category of advanced biomaterials, with the ability to elicit directed cellular lineage commitment at the damaged tissue site. The interdisciplinary field of tissue engineering merges principles of engineering, synthetic
chemistry and life sciences to generate biological substitutes capable of regenerating healthy, functioning tissue in a defected site.\textsuperscript{6, 7}

With an overwhelming number of bone defects occurring around the world every year, a means to enhance bone regeneration and healing \textit{in vivo} is needed. Many synthetic and naturally occurring polymeric materials have been studied for efficacy toward bone tissue engineering applications.\textsuperscript{8} Although several classes of polymers have proven effective toward bone regeneration, they often lack the ability to specifically promote osteogenesis \textit{in vivo} and \textit{in vitro}. Osteogenic growth peptide (OGP) is a naturally occurring 14-mer peptide that was discovered in the 1990’s by Bab, et al.\textsuperscript{9, 10} Since its discovery, many studies have shown the ability of OGP to enhance proliferation and osteogenic differentiation in osteoblast cell lines, including mesenchymal stem cells (MSCs).\textsuperscript{11-16} OGP is efficiently synthetized in the lab using chemical approaches, resulting in an appropriate and easily accessible molecule for biomaterial functionalization for bone tissue engineering applications. Several methods of OGP delivery, from intravenous injection to a tethered moiety on a polymer substrate, have been studied for bone tissue engineering applications, yielding promising results.

1.3 Bone Repair

Damaged human tissue continues to pose a significant problem for surgeons across the globe. Nearly 6.2 million patients in the United States alone suffer from bone defects or injuries annually.\textsuperscript{17} Whether these injuries are a result of a traumatic event or congenital defect, there are several clinical repair options at the surgeon’s disposal in severe injuries and fractures. Many of the current treatment strategies result in additional complications
for the patient. Tissue engineering is a quickly expanding research area in the field of regenerative medicine, which seeks to overcome limitations of current medical treatment options by aiming to restore the form and function of the patient's damaged bone tissue.

1.3.1 Clinically practiced treatments for bone repair

Bone is a living tissue that is constantly being resorbed and rebuilt, therefore, the majority of small fractures and injuries are able to be healed using conventional therapies. However, when a patient suffers from severe defects or injuries, more aggressive treatments are necessary. Clinical repair options include the Ilizarov apparatus for patients born with limb length discrepancy, bone grafting, and amputation. Bone grafting is the most common form of treatment used in hospitals, with over 450,000 performed each year. Three options for bone grafting exist: (1) autografts (2) allografts (3) synthetic bone grafts. As shown in Table 1.1, there are several disadvantages and advantages to using each type of grafting material. Because autografts are bone tissue segments removed from the host patient’s iliac crest, they are considered to be the gold standard. Allografts consist of bone tissue that is removed from a cadaver or another patient. Both cancellous autografts and allografts show osteoinductive, osteoconductive, and osteogenic properties, however this spongy bone tissue, does not offer the immediate strength needed to support the repair of a load bearing bone. Cortical bone grafts can be used for whole bone segment replacement, however, there is a lack of tissue available for these types of bone grafting procedures. Synthetic bone grafts, including ceramics and bioglasses, have been considered for applications in bone repair because they are osteoconductive and have an unlimited availability. However, such materials often fail to reconstitute the bone due to their lack of osteoinductive properties and their inability to provide strength to the
defect site.\textsuperscript{19, 23} Several other limitations of grafting methods include risk of pathogen transfer, disease transmission, and the need for multiple surgeries.\textsuperscript{19, 32}

Table 1.1 Three main types of bone grafting methods are used: 1. autografts 2. allografts 3. synthetic bone grafts. The ratings of effectiveness for each grafting method in various areas of interest are shown in the compiled table. – inadequate, + adequate, ++ good, +++ very good. \[Bone Grafting and Bone Grafting Substitutes. Finkemeier, C.G. J Bone and Joint Surg. 2002, Volume 84. Copyright 2002. The Journal of Bone and Joint Surgery, Inc.\]

<table>
<thead>
<tr>
<th>Property</th>
<th>Autograft</th>
<th>Allograft</th>
<th>Synthetic</th>
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</thead>
<tbody>
<tr>
<td>Sources</td>
<td>Cancellous</td>
<td>Vas./Non-vas</td>
<td>Cortical</td>
</tr>
<tr>
<td></td>
<td>Iliac crest, distal tibia</td>
<td>fibula, ribs, iliac crest</td>
<td>cadaver</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pelvis, ribs, femur</td>
</tr>
<tr>
<td>Osteoinductive</td>
<td>++</td>
<td>+/-</td>
<td>++</td>
</tr>
<tr>
<td>Osteoconductive</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Osteogenesis</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Immediate Strength</td>
<td>-</td>
<td>+++</td>
<td>+/-</td>
</tr>
<tr>
<td>Best for...</td>
<td>&lt;5-6cm bone loss</td>
<td>&gt;12cm and &gt;5-6cm</td>
<td>large defect if lack of autologous tissue</td>
</tr>
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</table>

One of the greatest challenges facing orthopedic surgeons is the treatment of large bone defects. Defects that are deemed too large to heal during the lifetime of the patient are also known as critical size defects.\textsuperscript{22} When treated with bone grafting methods, delayed or non-unions most commonly result. Therefore, most patients suffering from defects of this magnitude, face amputation as their only option for treatment.\textsuperscript{33} As of 2008, there was an estimated 1.7 million people in the United States living with an amputated limb as a
result of a traumatic event, diabetes, vascular complications, cancer, and congenital defects.\textsuperscript{34}

The limitations of clinically practiced bone repair treatments span physiological, psychological, and emotional complications. To overcome these limitations, it is only fitting that scientists and physicians are looking toward regenerative medicine as a means of combatting bone tissue repair.

1.3.2 Tissue engineering as an alternative for clinical treatment options of bone repair

Tissue engineering (TE) attempts to combine the principles of biology and engineering to develop biomaterials with the ability to restore the form and function of the damaged human tissue.\textsuperscript{7} The basis for TE lies with the deep understanding of embryology and tissue formation. Current models of \textit{in vitro} bone TE applications are geared toward replicating the environmental cues necessary for healthy bone tissue generation during embryonic development.\textsuperscript{35} In the commonly employed “open system” approach for TE, autologous stem cells are implanted onto natural or synthetic polymer matrices or scaffolds, and implanted into the body at the defect site.\textsuperscript{7} During the healing process, the stem cells undergo an expansion process, and begin to differentiate into the desired tissue type, while the construct degrades, leaving behind newly regenerated, healthy, and functioning tissue. Using a TE approach for regenerating new bone, issues such as the lack of tissue sources, biological rejection, and failure to reconstitute the bone, will be resolved.
1.3.3 Bone tissue engineering

Using polymeric materials for bone tissue engineering applications has attracted increasing attention over the years. There are several requirements that must be met in order for a polymer-based scaffold to be an effective bone regeneration construct: 1) The polymeric scaffold must be non-toxic and biodegradable; 2) The mechanical properties of the polymeric scaffold should be similar to that of bone tissue; 3) The polymeric material must show osteoinductive properties to support the growth of healthy new bone at the defect site. These three requirements present challenges to those working in the synthesis and fabrication of bone repair constructs, however, promising results have been reported.

Bone marrow human mesenchymal stem cells (hMSCs) are the most widely studied cell line for bone repair. Because of their self-renewing and pluripotent properties, they are suitable for bone tissue engineering applications. Depending on chemical and mechanical cues received from their environment, hMSCs have the ability to differentiate into fat, cartilage, and bone. Driving hMSC osteogenic differentiation in vitro is well established in literature. Medium supplemented with dexamethasone, ascorbic acid, and \( \beta \)-glycerol phosphate, induces MSC osteogenic differentiation in vivo. Until recently, the natural cues resonating from the stem cell niche in vivo for driving osteogenesis were not well understood. The unique stem cell niche in bone consists of hematopoietic progenitors, MSCs, and both of their respective mature progenies, including osteoblasts, osteocytes, fibroblasts, blood cells, etc. \(^{37-39}\) In vitro studies in which MSCs were co-cultured with osteocytes and osteoblasts, revealed an enhanced osteogenic differentiation when compared to MSCs cultured in media with and without osteogenic supplements. \(^{38}\) These results are indicative of the necessity for the biochemical cues from the bone stem cell
niche support cells for osteogenesis and bone regeneration. It is unlikely that the supplements added to media for osteogenic differentiation are the same chemical cues present in the natural bone stem cell environment in vivo. The biochemical signals for enhanced osteogenesis that are released by osteoblasts and osteocytes may include growth factors and pre-osteogenic signaling molecules, such as fibroblast growth factor-2 (FGF-2) and nitric oxide (NO), respectively. With an enhanced knowledge of bone regeneration in vivo and the environment necessary for chemical signaling to MSCs, bone TE applications can be designed more effectively to better mimic the stem cell niche.

1.3.4 Polymeric materials for bone tissue engineering constructs

High modulus, degradable, non-toxic materials are needed for bone TE applications to overcome the limitations of clinically practiced methods of bone defect repair. The polymeric material itself must be able to support osteogenesis, and elicit no cytotoxicity for it to be considered for bone repair. Both naturally occurring and synthetic polymers have been studied for the fabrication of osteoinductive and osteoconductive scaffolds and matrices (Table 1.2). Although polymers of natural origin, including proteins, polysaccharides and microbial polyesters, have a low toxicity, are renewable and contain inherent biochemical signaling, their unsuitable mechanical properties, chemical stability and potential loss of biological properties during fabrication limit their use for bone TE. Synthetic polymers, such as poly-L-lactic acid (PLLA), polypropylene fumarate (PPF), poly(ε-caprolactone) (PCL) and poly(ester urea) (PEU) have shown a broad range of mechanical and degradation properties. In vivo studies have shown that while these materials are bioresorbable, the acidic degradation byproducts of most
biopolymers often results in an inflammatory response.\textsuperscript{54, 55} On the contrary, PEUs, have shown no \textit{in vivo} inflammatory response, which can be explained by the suspected acid-neutralizing urea byproducts released during degradation.\textsuperscript{53} While synthetic materials are advantageous because of their tunable properties, they generally lack inherent osteoinductive and osteoconductive ability for driving hMSC bone differentiation. Peptides such as osteogenic growth peptide, have been studied extensively, as both a soluble growth factor in culture medium, and as a chemically bound functional group on polymeric materials.

Table 1.2 Advantages and disadvantages of naturally occurring and synthetic polymeric materials used for bone tissue engineering. (Reprinted with permission from Puppi, D.; Chiellini, F.; Piras, A.M.; Chiellini, E. Polymeric materials for bone and cartilage repair. Prog Polym Sci. 2010, 35, 403-440. Copyright 2010 Elsevier).

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Natural Polymers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polysaccharides</td>
<td>Good biocompatibility, no immunogenic properties</td>
<td>Low mechanical strength, difficult to sterilize</td>
</tr>
<tr>
<td>Proteins</td>
<td>Good cell binding properties, slow degradability</td>
<td>Low mechanical stiffness, biocompatibility problems</td>
</tr>
<tr>
<td><strong>Synthetic Polymers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poly(L-lactic acid (PLLA))</td>
<td>Tunable mechanical and degradation properties</td>
<td>Difficult to functionalize, acidic degradation byproducts</td>
</tr>
<tr>
<td>Polypropylene fumarate (PPF)</td>
<td>Tunable mechanical and degradation properties, crosslinkable</td>
<td>Toxicity of crosslinking agents, acidic degradation byproducts</td>
</tr>
<tr>
<td>Poly(E-caprolactone) (PCL)</td>
<td>FDA approval for various applications</td>
<td>Very slow degradation rate, poor cell adhesion</td>
</tr>
<tr>
<td>Poly(ester urea) (PEU)</td>
<td>No acidic degradation byproducts, easily processed, amino-acid based</td>
<td>Lack biological cues without functionalization</td>
</tr>
</tbody>
</table>
1.4 Osteogenic growth peptide

Osteogenic growth peptide was first isolated in the early 1990s from culture medium conditioned with a newly formed rat bone resulting from an in vivo mechanical bone marrow ablation.\textsuperscript{10, 56} Chromatographic fractions were collected from the medium filtrate and tested in vitro for mitogenic activity. The mitogenic activity was attributed to the tetradecapeptide now known as osteogenic growth peptide (OGP).\textsuperscript{10, 56} OGP is identical to the C-terminus of the histone H4 (HH4) amino acid sequence 89-102 (H-Ala-Leu-Lys-Arg-Gln-Gly-Arg-Thr-Leu-Tyr-Gly-Phe-Gly-Gly-OH).\textsuperscript{10, 56} OGP is an endogenous peptide that is present in micromolar concentrations in human blood serum and has been found to enhance proliferation, differentiation, and matrix mineralization of osteoblast cell lines in vitro and in vivo.\textsuperscript{11, 13, 57-59} Histones are proteins housed in the chromatin structure of the nucleus and are characteristically linked to the replication of DNA.\textsuperscript{60-62} It is likely that when bone marrow ablation occurs, HH4 expression is enhanced due to increased cell cycle activity, therefore leading to an increased blood serum concentration of OGP. The amino acid sequence preceding OGP, includes a potential chymotrypsin cleavage site, which allows for post translational processing and OGP production.\textsuperscript{63} In blood serum, OGP exists as: 1) full length OGP bound to a binding protein, 2) free full length OGP, and 3) proteolytically cleaved free OGP[10-14]. Each form of OGP plays an important role in the regulation, protection, and activity of this osteogenic growth factor.
1.4.1 OGP-OGP binding protein complexes for regulation of OGP serum concentration

Cells originating in the bone marrow, such as hMSCs, serve as the precursors for osteoblasts and osteoclasts that are both necessary for the repair of healthy new bone.\textsuperscript{64-67} In both human and animal models, bone marrow injury has been linked to a localized osteogenic response that stimulates the bone formation at distal skeletal sites. This response has been attributed to the release and circulation of growth factors, such as OGP, by the regenerating bone marrow cells. Post ablation studies have shown that the concentration of OGP-OGP binding protein complex (OGPBPs) dramatically increases and is used to enhance new bone formation at the defect site.\textsuperscript{63} Fluctuations of OGP levels following bone injury, as well as the dose dependent response of OGP for the stimulation of osteogenesis, suggests the precise regulation of OGPBP is necessary for healing to occur.

For both human and rabbit marrow stromal cells, a relatively low concentration of OGP, $10^{-12}$ and $10^{-8}$ M, was shown to elicit accelerated proliferation \textit{in vitro}.\textsuperscript{58} This ~nanomolar dose dependency is one indicator that OGPBP may be necessary for circulation of OGP in serum. The major OGPBP is $\alpha_2$-Macroglobulin ($\alpha_2$M), which is a 720 kDa homotetramer that is found in the blood serum at high concentrations.\textsuperscript{68} Gavish, et. al. showed that in both its native and active form, $\alpha_2$M noncovalently binds to OGP, resulting in drastically different effects on mitogenic activity of osteoblast MC3T3 E1 cells.\textsuperscript{69} In its native form, $\alpha_2$M is hypothesized to deliver OGP to its target cells, allowing for enhanced mitogenic activity.\textsuperscript{69} However, OGP bound to activated $\alpha_2$M is thought to activate $\alpha_2$M-receptor/low density lipoprotein receptor-related protein complex ($\alpha_2$M-r/LRP) that inhibits enhanced mitogenic activity of osteoblast cells.\textsuperscript{69-71} OGPBP not only
function to maintain the “slow release” of OGP in a steady-state concentration in blood serum, but they also serve to protect the short 14-mer peptide from proteolysis and rapid clearance, which makes them necessary for OGP regulation in blood serum.

1.4.2 OGP enhances mitogenic activity of osteoblasts via the protein MAP kinase signaling cascade

Physiologically, OGP exists in high abundance in serum and serum-free culture medium of osteoblasts, primarily as an inactive form bound to OGPBPs. Other forms of endogenous OGP include full length, tetradecapeptide, and the C-terminal pentapeptide, OGP[10-14]. OGP in its full length form, consists of an accessory domain and a message domain (Figure 1.1). Within the accessory domain (amino acids 1-9) there is a mid-molecular sequence that allows the peptide to bind to α2M. Following release of α2M, OGP is proteolytically cleaved to form OGP[10-14]. Like full length OGP, OGP[10-14] plays a major role in the mitogenic activity of osteoblasts, however, it lacks to ability to bind to OGPBPs.

Structure-activity relationship studies have shown that OGP[10-14] is the minimal OGP-derived sequence necessary to drive proliferation of osteoblast cell lines, therefore it is considered to be the “active” form of OGP. Chen and colleagues studied various truncated analogs of OGP[10-14] to conclude that the C-terminal carboxylic group as well as the tyrosine and phenylalanine pharmacophores were essential for mitogenic activity of OGP. When the N-terminal tyrosine was replaced with alanine or phenylalanine, the relative mitogenic activity was between 12% and 41% compared to the OGP[10-14] reference peptide. Furthermore, any C-terminal modifications made to the peptide
resulted in less than 25% mitogenic activity compared to the reference OGP[10-14].\textsuperscript{74} The spatial proximity of the pharmacophores of tyrosine and phenylalanine play a crucial role in the recognition and activation of the OGP cell receptor for enhanced mitogenic activity.\textsuperscript{73-75}

Figure 1.1 OGP is a naturally occurring 14-mer peptide with an active subunit from amino acids 10 to 14. The active domain is cleaved after dissociation from α₂M binding protein, and drives mitogenic activity and differentiation of osteoblast cell lines. (Reprinted with permission from Ref 72. Copyright 2002 Wiley Periodicals Inc)

OGP[10-14] shares some structural similarities with naturally occurring opiate peptides, in which the phenolic ring of tyrosine and phenylalanine are crucial for cellular activity.\textsuperscript{75} Like several opiate peptides, OGP[10-14] initiates proliferation enhancement via activation of a G protein cell receptor.\textsuperscript{76} Binding of OGP[10-14] to this G protein leads to the downstream activation of mitogen-activated protein (MAP) kinase signaling pathway. Of the four subfamilies of MAP kinases, phosphorylation of the extracellular
signal-regulated kinases 1 and 2 (ERK1/2), has been linked to signaling of a cascade pathway for the regulation of osteoblast proliferation and runt-related transcription factor 2 (Runx2).\textsuperscript{77, 78} Similarly, OGP[10-14] mitogenic signaling involves the phosphorylation of ERK1/2, which elicits the downstream synthesis of mitogen-activated protein kinase-activated protein kinase-2 (Mapkapk2) mRNA and protein.\textsuperscript{79} The enhanced expression of Mapkapk2 links ERK1/2 activation to increased DNA synthesis and enhanced mitogenic activity of osteoblasts via activation of cAMP response-element binding protein (CREB) transcription factors.\textsuperscript{79} Figure 1.2 depicts the pathway for OGP signaling for enhanced proliferation in osteoblast cell lines.
OGP enhances osteoblast differentiation through the RhoA-ROCK cellular pathway.

Inducing bone formation is the fundamental strategy in bone tissue engineering for the treatment of defects as a result of traumatic events or congenital conditions, such as osteoporosis. Many *in vitro* and *in vivo* studies have revealed OGP's efficacy toward...
osteoblast differentiation of hMSCs and bone formation in animal defect models, respectively. However, until more recently the mechanism by which OGP works to promote bone formation through RhoA activation was undefined.

RhoA is a member of the guanidine triphosphatase (GTPase) family, which cycles between inactive guanidine diphosphate- (GDP-) bound and active GTP-bound forms. Through the downstream effector, Rho-associated protein kinase (ROCK), RhoA regulates the actin cytoskeleton and stress fiber formation. Several studies have suggested the effect of cell shape on proliferation and lineage commitment of MSCs, however, until 2004, these cell shape-mediated effects were loosely defined. The shape of each tissue cell type, plays a pivotal role in specialized cellular function and tissue generation through the Rho family GTPases.

The cells that make up various connective tissue vastly differ in phenotype, despite originating from the same pluripotent hMSCs. For example, adipose tissue cells are generally round, while osteoblasts that make up bone tissue are flat and elongated in shape. In 2004, Chen, et al. performed an in depth study on the link between morphology and lineage commitment, concluding that cell shape is a crucial cue necessary for regulating the adipogenic-osteogenic switch in hMSCs through the RhoA and ROCK activity. Previous studies have suggested that osteogenic differentiation of MSCs is accompanied by an increase in GTP-bound RhoA protein expression on the cell membrane. Cells that are well spread and elongated express greater RhoA and Rho-associated kinase (ROCK) activity, which promotes osteogenesis of hMSCs. The Rho family of GTPases, are known to play a role in cellular processes including proliferation, gene expression, cell polarity, and cytoskeleton dynamics. ROCK is the downstream
effector of RhoA involved in myosin-generated cytoskeleton tension.\textsuperscript{95} hMSC commitment is mediated by changes in cytoskeletal tension as a result of actomyosin activation. In the presence of ROCK inhibitor, Y-27632, hMSC expression of alkaline phosphatase (an osteogenic marker) was drastically decreased, while lipid production (an adipogenic marker) was enhanced.\textsuperscript{84} Interestingly, RhoA activation drives osteogenic differentiation in spread cells only, while ROCK activation drives osteogenic differentiation independent of cell shape.\textsuperscript{84} These findings suggest that mechanochemical cues as a result of cellular shape drive lineage commitment in hMSCs.

RhoA and ROCK activation was shown to be stimulated by both cell shape and soluble factors in media, to drive osteogenic differentiation.\textsuperscript{84} As a soluble growth factor in culture medium, OGP works in a similar manner to upregulate the activity of RhoA-ROCK signalling.\textsuperscript{96} A study conducted in 2011 revealed that the treatment of bone marrow MSCs with OGP increased RhoA activity.\textsuperscript{96} Downstream, increased activation of ROCK in OGP treated MSCs was detected via fluorescence staining.\textsuperscript{96} Downstream of RhoA-ROCK signaling, the phosphorylation of LIM domain kinase (LIMK) and myosin light chain (MLC) takes place.\textsuperscript{97, 98} This causes the inactivation of cofilin leading to enhanced actin polymerization and actomyosin contractions, respectively.\textsuperscript{97, 98} There is also evidence to suggest that OGP may enhance osteogenic lineage commitment of MSCs through the endothelial nitric oxide synthase (eNOS) pathway.\textsuperscript{16, 64, 99} Nitric oxide (NO) generated by eNOS stimulates bone morphogenic protein (BMP-2) levels and osteogenic differentiation of MSCs during bone remodelling.\textsuperscript{100-103} Several studies have shown that OGP administration to bone marrow MSCs \textit{in vitro} enhances
eNOS expression, suggesting it may play a pivotal role in OGP-mediated osteoblast differentiation of MSCs.\textsuperscript{16, 64}

1.5 OGP for tissue regeneration applications

Early studies, shortly after the discovery of OGP, revealed its ability to promote proliferation and differentiation of osteoblast cell lines.\textsuperscript{10, 58} Following bone marrow ablation, the circulating concentration of OGP increases, suggesting its essential role in the bone healing process.\textsuperscript{9, 63, 104} \textit{In vivo} and \textit{in vitro} administration of OGP increases bone mass and enhances alkaline phosphatase activity in osteogenic cell lines, respectively.\textsuperscript{13, 58} Preliminary studies suggested OGPs efficacy toward use in bone regeneration applications, and since it has been studied as a soluble growth factor in culture medium, as well as a functional moiety for enhanced osteoblast differentiation \textit{in vitro} and \textit{in vivo}.

In an effort to enhance the osteoinductive ability of polymeric materials, researchers have worked with an array for OGP-functionalized substrates to study enhanced osteogenic differentiation.\textsuperscript{14, 44, 105-107} Through both covalent and non-covalent bonding, surfaces and scaffolds can be functionalized with OGP to study the materials efficacy in bone tissue engineering applications.

While soluble OGP in growth medium and intravenously injected OGP have shown promising results for enhancing osteoblast formation, in most regenerative medicine applications, immobilization of OGP is more appropriate. This method of OGP presentation allows for retention of the peptide at the wound site for extended periods of time. Studying concentrations of immobilized synthetic OGP on gradient substrates allowed researchers to determine the optimal concentration necessary for enhanced
proliferation of MC3T3-E1 osteoblast cells. Via an efficient copper catalyzed click reaction, azo-functionalized OGP and the C-terminus fragment OGP[10-14] was reacted with pendant alkyne self-assembled monolayer (SAM) gradients to generate OGP-concentration gradient substrates.08 OGP[10-14] concentrations on the surface ranged from 0 pmol/cm\(^2\) to 140 pmol/cm\(^2\), as characterized by x-ray photoelectron spectroscopy (XPS).08 Cell culture studies revealed that both OGP and OGP[10-14] gradient substrates reduced the doubling time of MC3T3-E1 cells up to 40% compared to unstimulated values. Independent of peptide concentration, immobilized full length OGP and OGP[10-14] enhanced proliferation of mouse preosteoblasts compared to control samples in the early stages of proliferation, which is likely due to an induced paracrine effect.08 Unlike soluble OGP, which has a concentration dependence, these findings were unique to the immobilized form. This effect was found in the tethered full length sequence as well as the OGP[10-14] sequence. Perhaps when immobilized, the full length OGP is cleaved from the gradient surface and becomes a soluble factor, therefore, exposing cells to a homogeneous concentration of peptide.08 However, this is unlikely the case in regards to OGP[10-14] which lacks any specific recognition element. Despite the lack of concentration dependence, the use of immobilized OGP and OGP[10-14] on a SAM surface was shown to enhance proliferation of preosteoblast cells.

MSCs are the most widely studied cell line for tissue regeneration applications due to their pluripotent nature. Surface tethered OGP, both full length and the active subunit, had a similar effect on rabbit MSCs isolated from bone marrow. Compared to non-functionalized controls, OGP surfaces showed an enhanced proliferative effect by day 7, which was silenced after 14 days \textit{in vitro}.09 Observing a proliferative effect up to but not
past 7 days indicated that the MSCs were entering a differentiation phase prior to those on control samples. Furthermore, upregulation of osteogenic markers, such as Runx2, BMP2, and osteonectin (SPARC), was observed via quantitative real-time polymerase chain reaction (qRT-PCR), for MSCs cultured on OGP-tethered surfaces compared to controls at both 2 and 4 weeks in vitro culture. Overall, both studies discussed suggest the bioactivity and bioavailability of tethered OGP. Interestingly, only when OGP was tethered in such a manner that the C-terminus was exposed to the cells, did the proliferative and osteogenic differentiation enhancement occur.

When immobilized on polystyrene plates and SAM gradient surfaces, OGP and its active fragment acted similarly to soluble OGP in vitro. To translate these findings to biomaterial applications, OGP has been immobilized in polymer scaffolds and on titanium substrates to observe its effects on osteoblast cell lines. Titanium substrates are currently used in clinical practice as dental fixations, orthopedic pins and bone implants because of its high strength, minimal erosion tendencies and low density. Several methods to modify the surface of titanium implants with peptides have been conducted, to include both non-covalent interactions and covalent conjugation between the surface and peptide. Typically, non-covalent interactions require high doses of peptide and a low loading efficiency, while chemical bond formation induces a change in surface properties of the titanium surface. In an effort to overcome these limitations, we have generated a series of multivalent dendrons which result in efficient methods of peptide loading on a heterogeneous titanium surface.

Catechol-bearing modular OGP was synthesized and loaded onto TiO surfaces, yielding high loading efficiency and enhanced proliferation and differentiation of
MC3T3-E1 cells in vitro. Abundantly found in adhesive mussel proteins, 3,4-dihydroxyphenylalanine (DOPA) contains a functional catechol group, which is responsible for strong adhesion on multiple surfaces, including metal oxides, in wet conditions. The non-covalent interaction between catechol species and the d-orbitals of the metal oxide surfaces occurs through a coordination bond or hydrogen bonding. Dendritic modular OGP peptides were synthesized to contain two active components; 1) the DOPA surface binding moiety 2) the OGP[10-14] bioactive subunit to elicit cellular response. OGP modular peptides containing four DOPA groups (OGP-PEG-(Cat)₄) showed strong binding to TiO₂ surfaces with a low dissociation constant. Such strong binding, allowed for minimal amounts of OGP-PEG-(Cat)₄ needed for 99% coverage of the TiO₂ surface for MC3T3-E1 in vitro cell studies. Although proliferation on OGP-functionalized and non-functionalized TiO₂ surfaces yielded no statistical difference in proliferation up to 3 days, there was enhanced osteogenic differentiation on OGP-containing surfaces. By 18 days in culture, PCR results indicated that statistically greater amounts of bone sialoprotein (BSP) and osteocalcin (OCN) were expressed by the cells on OGP surfaces compared to TiO₂ surfaces.

Co-functionalization of OGP with fibronectin (FN) on TiO₂ surfaces revealed the promising synergistic effects of two bioactive molecules on osteogenic differentiation. As a multifunctional cell adhesive glycoprotein, FN plays a role in cell attachment through integrin receptor interactions. Simultaneous adsorption of OGP and FN on mineralized titanium substrates revealed a sustained peptide release profile, allowing for higher doses of peptide released from the substrate at later stages in rat MSCs (rMSCs) differentiation. Results of differentiation studies revealed enhanced proliferation, higher
ALP levels, and greater calcium deposition of rMSCs when compared to individually adsorbed substrates.\textsuperscript{105}

Along with titanium, hydroxyapatite (HA) is one of the most widely utilized implants for clinical bone repair because of both its compositional and structural similarity to natural bone.\textsuperscript{124, 125} Recently, HA-based materials were functionalized with modular peptides which contained both an HA-binding domain and a cell-responsive OGP motif.\textsuperscript{126} Compared to a non-osteogenic control, modular OGP-peptide functionalized HA displayed an upregulation of OCN, osteopontin (OPN) and Runx2 mRNA expression for rMSCs after two weeks \textit{in vitro}.\textsuperscript{126} Additionally, proliferation of rMSCs on the OGP-functionalized surfaces decreased within the first seven days of \textit{in vitro} culture, suggesting that these cells had entered the differentiation phase earlier than those on the non-osteogenic control surfaces.\textsuperscript{126, 127}

The use of titanium and HA implants is widely used in clinical orthopedics today, however the field of tissue engineering is progressing toward the use of biodegradable and tunable polymeric materials for the regeneration of damaged tissue. These third generation tissue engineering materials are designed to elicit a specific cellular response while mimicking the native tissue environment.\textsuperscript{2} Ideally, overtime the materials will degrade into innocuous byproducts that will be resorbed by the body as the new tissue is regenerated.\textsuperscript{2} As mentioned previously, polymers for bone tissue engineering applications include both naturally occurring and synthetic polymers. Bacterial cellulose is an unbranched polymer of β-1,4-linked glucopyranose residues that is extracellularly synthesized by the bacterium \textit{Acetobacter xylinum}.\textsuperscript{128} Its unique nanofibrous structure, high crystallinity, and high tensile strength make it a suitable candidate for bone
Recently, materials fabricated from bacterial cellulose have been shown to promote cell attachment and proliferation of human marrow osteoblast stem cells. Bacterial cellulose membranes functionalized with absorbed synthetic full length OGP promoted greater mineral deposition and ALP activity for rat calvarial bone cells compared to non-functionalized samples. When implanted into a critical size defect in the parietal bone of male Balb/c mice, bacterial cellulose-hydroxyapatite composites functionalized with OGP and OGP[10-14] upregulated Runx2 only three days post-operation. In addition, a high percentage of bone formation was seen for these membranes at 60 and 90 days post procedure, indicating enhanced bone formation.

OGP-functionalized phenylalanine-based PEU (poly(1-PHE-6)) scaffolds have also shown promising results for bone tissue engineering applications. While data suggested that Poly(1-PHE-6) salt leached scaffolds were capable of supporting bone regeneration in vitro and in vivo, this phenomenon was enhanced when functionalized with OGP[10-14] crosslinkers and tethers. With as little as 1% OGP[10-14] crosslinker incorporation, the tensile modulus of poly(1-PHE-6) thin films significantly increased from 3.05±0.24 GPa to 4.18±0.14 GPa. An enhanced stiffness using bioactive OGP[10-14] resulted in better osteoinduction in male Spraque-Dawley rats as indicated by 12 week post implantation mineralization histological staining. Poly(1-PHE-6) tethered with 10% OGP[10-14] revealed similar results. An intensive in vitro study with hMSCs suggested enhanced early and late bone differentiation markers, such as Runx2 and OCN, on OGP[10-14] functionalized salt leached scaffolds, compared to non-functionalized materials. In addition, greater matrix mineralization and ALP activity was observed via
histology and biochemical assays for both *in vivo* and *in vitro* OGP[10-14]-functionalized poly(1-PHE-6).

While functionalization of polymeric scaffolds and substrates is a popular research area for bone tissue engineering, the creative use of nanofibrous M13 bacteriophage as an ECM mimic for directed MSC osteogenic differentiation has been recently studied. Previous studies have revealed that this non-toxic, filamentous virus can be genetically engineered and used for drug delivery applications to treat various diseases.\(^\text{133-136}\) The development of an electrostatic self-assembly approach for coating M13 with specific peptides, allowed for the development of OGP-functionalized phage films for the directed osteoblast differentiation of MSCs.\(^\text{135}\) Both proliferation and early differentiation of MSCs was enhanced on OGP-phage films compared to wild type (WT) phage control films.\(^\text{137}\) After two weeks of *in vitro* culture in osteogenic media, greater expression of Runx2 was observed on films functionalized with OGP, indicating early enhancement of osteogenic differentiation.\(^\text{137}\) These results suggested that M13 genetically engineered phages can be incorporated into scaffold and substrates for proper functionalization to drive osteogenic differentiation of stem cells.\(^\text{137}\)

1.6 Use of other osteogenic growth factors for bone regeneration applications

The main focus of this dissertation research revolves around the use of OGP-functionalized materials for enhanced osteoinductivity of polymeric materials for healthy bone regeneration. In addition to OGP, several other peptides have gained attention for similar uses, including bone morphogenetic protein (BMP-2) and the Arg-Gly-Asp (RGD) amino acid sequence.
BMPs belong to the transforming growth factor β (TGF-β) superfamily and were first discovered in the early 1960’s. Nearly 20 BMPs have been identified, each with a specific role in osteogenic development through phosphorylation of serine/threonine kinase receptors on the cell surface and downstream activation of Smad proteins. From this large group of BMP proteins, a specific peptide fragment, BMP-2 (N-KIPKASSVPTELSAISTLYL-C), has been identified and shown to induce osteogenic differentiation of multipotent cell lines. Primarily, Runx2, the key osteogenic transcription factor, is a major target for BMP-2 downstream signaling, for the upregulation of osteogenic gene expression. BMP-2 is one of only two BMPs that have been approved for use by the Food and Drug Administration in the United States, and its use in tissue engineering applications is pivotal as research in this field progresses. Similar to OGP, BMP-2 has been studies as an injectable therapeutic treatment, loaded into drug delivery systems, and as a covalently bound growth factor to polymeric substrates for healthy bone regeneration and osteogenic differentiation of multipotent cell lines.

Along with BMP-2 and OGP, the RGD amino acid sequence is undoubtedly, the most widely employed bioactive molecule for tissue engineering applications. RGD is the same integrin binding motif that is found in extracellular matrix glycoproteins, such as fibronectin, vitronectin and osteopontin. Upon binding of RGD to cell surface integrin receptors, recruitment of specific proteins to form focal adhesion to the substrate occurs. Many studies have revealed that not only does this extracellular matrix binding cue trigger adhesion, but also leads to downstream regulation of lineage commitment and osteogenic differentiation. Optimal surface density of RGD immobilized on substrates has been shown to direct osteogenesis of multipotent cell lineages through the enhancement of actin
stress filament alignment and cellular spreading.\textsuperscript{151, 152, 160, 161} Upregulation of Runx2 and BSP were reported for osteogenic cell lineages when cultured in the presence of RGD.\textsuperscript{160-163}

1.7 Looking forward

When the first wooden implants were used in humans, no one could have imagined the advanced field of biomaterials that exists today. With each passing day, chemists, biologists and engineers, are working toward even more unimaginable goals. In just several decades, bone tissue regeneration expanded from an idea on paper, to a practiced technique. However, despite the vast improvement scientists have made in this field, there are still many questions to be answered.

Functionalization of polymeric materials with OGP has been shown to enhance osteogenic differentiation \textit{in vitro} and bone formation \textit{in vivo}.\textsuperscript{14, 105-107, 110} Several methods of functionalization were presented in this review, which portrayed promising results. It is important to note, however, that with each new technique of OGP-conjugation, there is a need to study the dosage of peptide delivered to the cells or damaged bone. Bone regeneration using OGP and OGP[10-14] is dose dependent, and studying the concentration of OGP delivered to damaged bone over a given amount of time with the varying functionalization techniques will be a huge step toward more efficient tissue engineering constructs.

In addition to dose dependence, extensive studies of the synergistic effects of OGP with various other peptides will be extremely useful for the future of bone regeneration. Within the bone marrow stem cell niche, many biological entities play a significant role in
bone healing.\textsuperscript{39} As the MSC niche becomes better understood, natural and synthetic polymers can be functionalized with multiple growth factors to even further enhance bone formation and osteogenic differentiation.

Many studies have already shown the efficacy of OGP, along with various other peptide sequences, including BMP-2 and RGD, toward new bone growth in tissue engineering constructs. By further expanding knowledge of the dose dependency and the synergistic effects of these growth factors, the field of tissue engineering can reach its full potential for bone regeneration. In a perfect world, there would be no limitations to clinical treatments of bone repair, and with the current knowledge of peptide-functionalized polymers, and the future questions answered, we will be closer to obtaining that goal than ever before.

In this chapter, tissue engineering for bone repair applications was discussed. Currently, surgeons have limited means of treating patients suffering from long bone defects. These clinical treatment methods are often met with limitations, including the need for multiple surgeries, pathogen transfer, and failure to reconstitute the bone. Fortunately, with advancements in the fields of synthetic chemistry and cellular biology, dreams of developing high modulus, bioresorbable and osteoinductive materials for efficient bone regeneration, is becoming a reality.

1.8 Acknowledgments

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CHAPTER II

MATERIALS AND INSTRUMENTS

2.1 Materials

All reagents were used as received without additional purification unless specified otherwise. Silica was purchased from Sorbent (Norcross, GA), with porosity 60 Å, size 200 x 400 mesh, surface area 450-550 m²/g, bulk density 0.5 g/mL, and pH 6.0-7.0. Preloaded C18-T columns for peptide purification were purchased from Phenomenex (Torrance, CA) (55 µm, 140 Å, sorbent mass 10 g) and were washed with 90 v% methanol and equilibrated with 0.1 v% TFA in H₂O before use. All fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids and preloaded Wang resins were purchased from Aapptec (Louisville, KY) and Advanced Chem Tech (Louisville, KY).

All organic solvents were purchased from Sigma Aldrich as ACS Grade and used as received without additional purification unless otherwise stated. Anhydrous dichloromethane (DCM) was purchased from Sigma Aldrich and used as received.

L-phenylalanine: Alfa Aesar, ≥99.0%
1,6-hexanediol: Sigma Aldrich, 99%
P-toluenesulfonic acid: Fisher Scientific, ≥99%
Triphosgene: Alfa Aesar, ≥98.0%
Sodium carbonate (NaCO₃): Fisher Scientific, ≥99.5%
Sodium bicarbonate (NaHCO₃): Sigma Aldrich, ≥99.7%
Boc-phenylalanine-OH (Boc-Phe-OH): Sigma Aldrich, ≥99.0%
3-allyloxy-1,2-propanediol: Sigma Aldrich, ≥99.0%
Hydrogen chloride solution (4M in dioxane, anhydrous): Sigma Aldrich
1,6-hexanedithiol: Sigma Aldrich, 96%
Hydroxybenzotriazole (HOBt): Fisher Scientific, ≥98%
$N\text{-}N'$-Diisopropylcarbodiimide (DIC): Sigma Aldrich, 99%
Phenol: Sigma Aldrich, ≥96.0%
Trifluoroacetic acid (TFA): Sigma Aldrich, 99%
Triisopropylsilyl (TIPS): Sigma Aldrich, ≥99%
2-hydroxy-4’-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959): Sigma Aldrich, 98%

All cell culture medium and supplements were purchased from Thermofisher (Life Technologies) (Waltham, MA) unless otherwise stated. Human mesenchymal stem cell (hMSC) cells and growth medium were purchased from Lonza (Walkersville, MD). MC3T3-E1 cells were purchased from ATCC (Manassas, VA). RNA isolation kits for qRT-PCR were purchased from Qiagen (Valencia, CA). All qRT-PCR reverse transcription reagents and PCR kits were purchased from Thermofisher (Applied Biosystems), unless otherwise stated.

2.2 Instruments

*Nuclear Magnetic Resonance (NMR)*: NMR spectra were obtained using a Varian NMRS 300 MHz spectrometer. Relaxation of $^1$H NMR was 1 s and scan numbers were
32. Relaxation of $^{13}$C NMR was 1 s and scans numbers were 128. Data analysis was conducted on MestReNova processing software. Chemical shifts were reported in parts per million (ppm, $\delta$) and referenced to the chemical shifts of the solvent ($^1$H NMR, DMSO-$d_6$ 2.50 ppm, $^{13}$C NMR, DMSO-$d_6$ 39.50 ppm).

*Attenuated Total Reflectance-Infrared (ATR-IR):* ATR-IR spectra were collected on a Shimadzu MIRacle 10 ATR-IR spectrometer from 600 to 4000 cm$^{-1}$. Resolution was 4 cm$^{-1}$ and scan numbers were 64 to 128. Data were plotted in Microsoft Excel.

*Thin-Layered Chromatographic Analysis (TLC):* TLC for specific organic compounds was conducted by spotting and developing samples on flexible silica gel plates (Sorbent, Norcross, GA) using customized solvents as eluents.

*Size Exclusion Chromatography (SEC):* SEC analysis was performed using a TOSOH EcoSec HLC 8320-GPC. Sample solutions were prepared at 5 mg/mL in 0.1 M LiBr solution in DMF as the eluent. The specified flow rate was 0.5 mL/min with an RI detector at 50 °C, with a polystyrene standard curve. Analysis was performed on EcoSec processing software and spectra were plotted in Microsoft Excel.

*Mass Spectra:* Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-ToF Spectroscopy) mass spectra were carried out on a Bruker Ultraflex-III ToF/ToF mass spectrometer (Bruker Daltonics, Inc, Billerica, MA) equipped with a Nd:YAG laser (355 nm). The instrument was calibrated using external polystyrene of PMMA standards as the molecular mass under consideration. Electrospray ionization (ESI) was performed using an HCT Ultra II quadrupole ion trap mass spectrometer (Bruker Daltonics, Billerica, MA) equipped with an electrospray ionization source. MALDI-ToF
and ESI were performed by Sahar Sallam in the Wesdimiotis group (Department of Polymer Science, University of Akron).

**Thermal Analysis:** All samples were dried under vacuum overnight prior to thermal testing. The degradation temperature ($T_d$) of polymers were determined by thermogravimetric analysis (TGA) (TA Instruments, Q500) from 0 to 600 °C at a scanning rate of 20 °C/min. Glass transition temperature ($T_g$) was measured using differential scanning calorimetry (DSC) (TA Instruments, Q2000) at a scanning rate of 10 °C/min over a temperature range of 0 to 200 °C. At the highest and lowest temperature, the machine was set to equilibrate for 5 min. All data for DSC were reported from the second heating cycle, with the average of three cycles used to determine $T_g$.

**Tensile Testing:** Tensile modulus (Young’s modulus, $E$) was determined using an Instron 5567 following ASTM D638. Dog bone shapes were stretched at a constant rate of 5 mm/min at ambient temperature. Data was plotted in Microsoft Excel as stress vs. strain %. Young’s modulus was determined in the linear region of the region of the graph for 0 to 5% strain.

**Fluorescence Microscopy Testing:** Fluorescence images were obtained using a IX81 Olympus Fluorescence Microscope with FITC, TRITC, and DAPI filters. Images were taken using CellSens and analyzed in Image J.

**Synergy MX Microplate Reader:** The microplate reader was utilized for measuring fluorescence and absorbance in biological assays. Specific protocols for the assay are listed in each section individually.

**Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR):** Total mRNA was extracted using the RNeasy Mini column kit (Qiagen). DNase digestion was performed
during RNA isolation using the Qaigen RNase free DNase set. Total RNA was quantified using a Take3 Multi Volume Plate and Synergy MX Microplate-Reader at 260 nm. Reverse transcription was performed using the Taqman Reverse Transcription Reagents kit (Life Technologies) in the 2720 Thermal Cycler (Applied Biosystems). Reverse transcription consisted of incubation at 25 ºC for 10 min, reverse transcription at 48 ºC for 30 min, and inactivation at 95 ºC for 5 min. qRT-PCR was performed on a 7500 Real Time PCR System (Applied Biosystems) using SYBR Green primers and reagents. For all genes, the thermal protocol consisted of reverse transcription at 50 ºC for 30 min, activation at 95 ºC for 15 min, and 40 amplification cycles of denaturing for 30 s at 95 ºC, annealing for 1.5 min at 58 ºC, and extension for 2 min at 72 ºC. Following amplification, a melt curve analysis was performed at 1°C increments from 50 ºC to 95 ºC to analyze the purity of the product generated. qRT-PCR results were used for relative quantification of the gene of interest compared to the reference gene (18S or GAPDH).
CHAPTER III

OGP-FUNCTIONALIZED PHENYLALANINE-BASED POLY(ESTER UREA) FOR ENHANCING OSTEOINDUCTIVE POTENTIAL OF HUMAN MESENCHYMAL STEM CELLS

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3.1 Abstract

Amino acid-based poly(ester urea)s (PEU) are high modulus, resorbable polymers with many potential uses, including the surgical repair of bone defects. In vitro and in vivo studies have previously shown that phenylalanine-based PEUs have non-toxic hydrolytic byproducts, and tunable degradation times. Phenylalanine PEUs (poly(1-PHE-6)) have been further modified by tethering osteogenic growth peptide (OGP) to tyrosine-based monomer subunits. These OGP-tethered PEUs have been fabricated into porous scaffolds and cultured in vitro to examine their effect on differentiation of human mesenchymal stem cells (hMSCs) toward the osteogenic lineage. The influence of tethered OGP on the hMSC proliferation and differentiation profile was measured using
immunohistochemistry, biochemistry and quantitative real time polymerase chain reaction (qRT-PCR). *In vitro* data indicated an enhanced expression of BSP by 130-160% for hMSCs on OGP-tethered scaffolds compared to controls. By 4 weeks, there was a significant drop (60-85% decrease) in BSP expression on OGP-functionalized scaffolds, which is characteristic of osteogenic differentiation. ALP and OSC expression was significantly enhanced for OGP-functionalized scaffolds by week 4, with values reaching 145% and 300% greater, respectively, compared to non-functionalized controls. *In vivo* subcutaneous implantation of poly(1-PHE-6) scaffolds revealed significant tissue-scaffold integration, as well as the promotion of both osteogenesis and angiogenesis.

3.2 Introduction

Strong, degradable polymers with osteoinductive potential are needed for regenerative applications in bone tissue engineering. Most polymeric materials that are being investigated for osteoconductive applications lack the mechanical properties necessary for supporting segmental bone defects. Intact cortical bone has a Young’s modulus of 17-30 GPa, depending on age and health of the patient. Non-filled, degradable polyesters, such as PLLA and PPF, have a reported Young’s moduli of 0.9-3.6 GPa, respectively. These synthetic polymers also often take years to fully resorb *in vivo*, with acidic degradation products causing inflammation.

Synthetic poly(ester urea) (PEUs) show promise in the engineering realm as they are amino acid-based and degradable with tunable mechanical properties and bioactivity. Phenylalanine-based PEU (poly(1-PHE-6)) was reported to have a Young’s modulus of 3.05±0.24 GPa. Previous studies have shown that peptide-based
cross linkers with poly(1-PHE-6) increases the Young’s modulus to 4.18±0.14 GPa in a concentration dependent manner, which exceeds most polymers under clinical investigation.\textsuperscript{169} \textit{In vitro} degradation studies show that PEUs degrade faster than PLLA, with a 5wt\% degradation after 112 days in physiologically relevant conditions.\textsuperscript{172} Furthermore, as PEU degrades, the acidic byproducts resulting from the labile ester bonds are buffered by the urea linkages at each repeat unit limiting inflammation from occurring at the implant site \textit{in vivo}.\textsuperscript{169}

A major challenge of tissue engineering results from the inherent inability of polymers to be osteoinductive. Significant effort has been focused on modifying polymers with peptides and growth factors to enhance their bioactivity and, specifically, osteoinductive behavior.\textsuperscript{17, 152, 173, 174} One such growth factor is osteogenic growth peptide (OGP), which is a naturally occurring 14-mer peptide with an active OGP[10-14] fragment that is cleaved \textit{in vivo} to directly regulate \textit{hMSC} osteoblast differentiation.\textsuperscript{96} OGP[10-14] has shown the ability to regulate proliferation, differentiation and ECM mineralization for osteoblast cell lineages, as both a soluble growth factor and a tethered peptide in concentration-dependent manner in previous literature.\textsuperscript{174-177}

Human mesenchymal stem cells (\textit{hMSC}s) are the most likely cell source for the repair process in an orthopedic injury. \textit{hMSC}s have been widely studied as a suitable option for cell-based tissue engineering treatments because of their self-renewing properties and differentiation potential.\textsuperscript{178-180} With proper cues from their environment, \textit{hMSC}s can be used to regenerate specific tissues such as bone. Precise functionalization of scaffolds with biomimetic molecules, such as OGP, can be used to better mimic the natural environment for osteogenic differentiation of \textit{hMSC}s. Previously, a subcutaneous
in vivo rat study using 1% OGP (by mass) as covalent crosslinkers for poly(1-PHE-6), showed mechanical enhancement in addition to a significant increase in bioactivity, including mineralization. Although the results were favorable, this is not a translation scalable approach, especially in large bone defect models. Therefore, in this study we maintained the covalent crosslinking through backbone alkene functionality, while using a functionalized tyrosine monomer for the post fabrication of scaffolds with OGP. In addition to crosslinking we also measured the influence of pore size on MSC differentiation and osteoconductive signaling. Scaffolds functionalized with OGP should be more osteoinductive, promoting the generation of osteoblasts from multipotent hMSCs. This study reveals enhanced hMSC osteogenic differentiation on poly(1-PHE-6) that have been modified using OGP, cross-linking, and pore sizes to generate a more osteoinductive construct that can be translated clinically.

3.3 Materials and Methods

All necessary purchasing and vendor information for materials and instrumentation will be listed in this section, followed by a complete experimental procedure.

3.3.1 Materials

All commercial reagents and solvents were purchased from Aldrich and Fisher Scientific and used without further purification unless otherwise specified. Chloroform, dichloromethane, and toluene were distilled from CaH₂ and stored under argon for use.
3.3.2 Characterization of chemical structure and thermal properties

Chemical structures were characterized with standard methods. NMR spectra were obtained using a Mercury 300 MHz NMR spectrometer. Deuterated solvents (TFA-d, or DMSO-d₆) were used based on the solubility of compounds. All chemical shifts are reported in ppm (δ), and referenced to the chemical shifts of residual solvent resonances (¹H NMR DMSO-d₆ 2.50, TFA-d 11.50; ¹³C NMR DMSO-d₆ 39.50, TFA-d 116.6 and 164.2). Infrared spectra were obtained from an Excalibur Series FT-IR spectrometer (DIGILAB, Randolph, MA) either by solution casting polymer films on KBr plates, or molding ground powder into film with KBr salt. The molecular mass of polymers was measured by size exclusion chromatography (TOSOH HLC-8320, 50 ºC, polystyrene standard, DMF with 0.01% LiBr as eluent, flow rate 0.8 mL/min). The thermal properties of PEUs were recorded using a TA Instruments DSC Q2000. The temperature ramp rate was 10ºC/min under nitrogen. The degradation temperatures (T_d) of the polymers were determined by thermogravimetric analysis (TA instruments, Q500 TGA) across a temperature range of 30 ºC to 500 ºC at a scanning rate of 20 ºC/min under nitrogen.

3.3.3 Monomer and polymer synthesis

The three monomers synthesized were prepared as a bis-amine-toluenesulfonic acid salt, following previous methods (Scheme 3.1).¹⁸¹,¹⁸² Scheme 3.2 (A, B, and C) shows the structure of the three monomers: 1-PHE-6, bis-L-phenylalanine and 1,6-hexanediol-based monomer; 1-PHE-4, bis-L-phenylalanine and cis-2-butene-1,4-diol-based monomer with alkene functionality for radical crosslinking; 1-TYR-6, tyrosine and 1,6-hexanediol-
based monomer with alkyne functionality for “click” peptide conjugation. The respective chemical structures were confirmed via $^1$H NMR (Appendix Figure 1-3).

Scheme 3.1 Synthesis of the bis-amine-toluenesulfonic acid salt monomers.

Scheme 3.2 The three monomers synthesized for this study: (A) 1-PHE-6 (B) 1-PHE-4 with alkene functionality for crosslinking (C) 1-TYR-6 with alkyne functionality for peptide conjugation.
The PEU polymers were synthesized using interfacial polycondensation as described in Scheme 3.3. The concentration of each monomer in the copolymers was controlled by the feed ratios in the copolymerization process. Four compositions of PEUs were prepared: poly(1-PHE-6), poly(1-PHE-6)-co-(1-PHE-4), poly(1-PHE-6)-co-(1-TYR-6), and poly(1-PHE-6)-co-(1-PHE-4)-co-(1-TYR-6). The content of 1-PHE-4 was 10% (by molar ratio) in both poly(1-PHE-6)-co-(1-PHE-4) and poly(1-PHE-6)-co-(1-PHE-4)-co-(1-TYR-6), and that of 1-TYR-6 was 1% in both poly(1-PHE-6)-co-(1-TYR-6) and poly(1-PHE-6)-co-(1-PHE-4)-co-(1-TYR-6). The chemical structures and compositions of the polymers were confirmed by $^1$H NMR spectroscopy and integration as shown in Figure 3.1 and Appendix Figure 4-5 respectively. The characteristic peaks of the alkene in 1-PHE-4 units are found at 5.6 ppm (i), and the methylene and alkyne peaks in the modified tyrosine units appear at 3.50 (k) and 4.75 ppm (j).

Scheme 3.3 Generic interfacial polymerization reaction scheme.

Poly(1-PHE-6): $^1$H NMR (DMSO-d6) δ =1.00-1.25 (b, 4H, -OCH2CH2CH2-), 1.30-1.55 (b, 4H, -OCH2CH2CH2-), 2.75-3.75 (m, 4H, -CH2-Ph) 3.95 (t, J=6.4, 4H, -OCH2CH2-), 4.25-4.50 (m, 2H, -NHCHCO-), 6.49 (d, 2H, -CONHCO), 7.00-7.40 (m, 10H, aromatic H); FT-IR (cm$^{-1}$): 3357, 3087, 3062, 3029, 2939, 2860, 1737, 1644, 1556, 1497, 1455, 1395, 1354, 1194, 1113, 1031, 744, 701. $M_n$=82 kDa, $M_w$=112 kDa. $T_g$=62 °C.

Poly(1-PHE-6)-co-(1-PHE-4): $^1$H NMR (DMSO-d6) δ =1.00-1.30 (b, 4H, -OCH2CH2CH2-), 1.30-1.55 (b, 4H, -OCH2CH2CH2-), 2.75-3.10 (m, 4H, -CH2Ph) 3.95 (t,
J=6.4, 4H, -OCH2CH2-, 4.25-4.50 (m, 2H, -NHCHCO-), 6.49 (d, 2H, -CONHCO), 7.00-7.40 (m, 10H, aromatic H); 4.60 (d, J=4.4, 4H of 1-PHE-4 units, -CH2CH=CHCH2-), 5.56 (t, J=4.1, 2H of 1-PHE-4 units, -CH2CH=CHCH2-). FTIR (cm-1) 3391, 3087, 3065, 3031, 1724, 1651, 1558, 1497, 1455, 1378, 1287, 1262, 1183, 1132, 1102, 1031, 894, 840, 736, 701, 686. Mn= 55 kDa, Mw=103 kDa. Tg= 58 °C.

Poly(1-PHE-6)-co-(1-TYR-6): 1H NMR (DMSO-d6) δ =1.00-1.25 (b, 4H, -OCH2CH2CH2-), 1.30-1.55 (b, 4H, -OCH2CH2CH2-), 2.75-3.75 (m, 4H, -CH2Ph) 3.95 (t, J=6.4, 4H, -OCH2CH2-), 4.25-4.50 (m, 2H, -NHCHCO-), 6.49 (d, 2H, -CONHCO), 7.00-7.40 (m, 10H, aromatic H), 3.55 (t, 2H, -OCH2C≡CH), 4.73 (d, 4H, -OCH2C≡CH) (Appendix Figure 4). FT-IR (cm-1): 3357, 3092, 3057, 3029, 2941, 2865, 1733, 1654, 1555, 1487, 1459, 1395, 1354, 1190, 1112, 1031, 745, 701. Mn= 90 kDa, Mw= 110kDa. Tg=60 °C.

Poly(1-PHE-6)-co-(1-PHE-4)-co-(1-TYR-6): 1H NMR (DMSO-d6) δ =1.00-1.30 (b, 4H, -OCH2CH2CH2-), 1.30-1.55 (b, 4H, -OCH2CH2CH2-), 2.75-3.10 (m, 4H, -CH2Ph) 3.95 (t, J=6.4, 4H, -OCH2CH2-), 4.25-4.50 (m, 2H, -NHCHCO-), 6.49 (d, 2H, -CONHCO), 7.00-7.40 (m, 10H, aromatic H); 4.6 (d, J=4.4, 4H of 1-PHE-4 units, -CH2CH=CHCH2-), 5.56 (t, J=4.1, 2H of 1-PHE-4 units, -CH2CH=CHCH2-), 3.55 (t, 2H, -OCH2C≡CH), 4.73 (d, 4H, -OCH2C≡CH) (Appendix Figure 5). FTIR (cm-1) 3367, 3087, 3060, 3039, 1732, 1649, 1551, 1495, 1461, 1379, 1287, 1261, 1185, 1133, 1101, 1035, 892, 841, 739, 701, 686. Mn= 75 kDa, Mw=133 kDa. Tg= 60 °C.
Synthesis of N\textsubscript{3}-OGP peptide

Azide-derivatized OGP (YGFGG) was synthesized using standard solid phase FMOC synthetic methods. The product was verified with Electrospray Ionization Mass Spectrometry (ESI-MS). \((N_3\text{-}OGP \ [M] = 638.4 \text{ (theoretical 638.5)})\) (Appendix Figure 6).

Conjugation of peptides to poly(ester urea)

\(N_3\text{-}OGP\) was tethered to alkyne functionalized PEUs, poly(1-PHE-6)-co-(1-TYR-6) and poly(1-PHE-6)-co-(1-PHE-4)-co-(1-TYR-6), via copper-catalyzed azide–alkyne cycloaddition (CuAAC) (Scheme 3.4). Briefly, PEU (5.00 g), \(N_3\text{-}OGP\) (0.20 g, 3 eq. to alkyne in PEU), and N, N, N, N', N''-pentamethyldiethylenetriamine (PMDETA) (20 µL)
were dissolved in 50 mL of DMF. After 2 freeze-pump-thaw cycles, CuBr (5 mg) was added into the reaction system with an argon purge, followed by two additional freeze-pump-thaw cycles. The reaction system was allowed to stir at 50 °C for 48 h. The polymer product was purified with a neutral alumina column and precipitated into excess cold methanol. After filtration, the polymer was dried in a vacuum oven at room temperature for 24 h (2.8 g, yield 56%).

Poly(1-PHE-6)-co-(1-TYR-6)-OGP: 1H NMR (DMSO-d6) δ=1.00-1.25 (b, 4H, -OCH₂CH₂CH₂-), 1.30-1.55 (b, 4H, -OCH₂CH₂CH₂-), 2.75-3.75 (m, 4H, -CH₂-Ph) 3.95 (t, J=6.4, 4H, -OCH₂CH₂-), 4.25-4.50 (m, 2H, -NHCHCO-), 6.49 (d, 2H, -CONHCO), 7.00-7.40 (m, 10H, aromatic H), 5.20-5.30 (broad, 2H, -OCH₂-triazole), 8.0-8.2 (b, 1H, H of triazole). FT-IR (cm⁻¹): 3360, 3085, 3057, 3031, 2943, 2869, 1735, 1654, 1551, 1490, 1458, 1395, 1350, 1195, 1109, 1036, 745, 701. Mₙ= 92kDa, Mₔ=118 kDa. Tₓ=60 °C.

Poly(1-PHE-6)-co-(1-PHE-4)-co-(1-TYR-6)-OGP: 1H NMR (DMSO-d6) δ=1.00-1.30 (b, 4H, -OCH₂CH₂CH₂-), 1.30-1.55 (b, 4H, -OCH₂CH₂CH₂-), 2.75-3.10 (m, 4H, -CH₂-Ph) 3.95 (t, J=6.4, 4H, -OCH₂CH₂-), 4.25-4.50 (m, 2H, -NHCHCO-), 6.49 (d, 2H, -
CONHCO), 7.00-7.40 (m, 10H, aromatic H); 4.6 (d, J=4.4, 4H of 1-PHE-4 units, -
CH₂CH=CHCH₂-,), 5.56 (t, J=4.1, 2H of 1-PHE-4 units, -CH₂CH=CHCH₂-), 5.20-5.30
(broad, 2H, -OCH₂-triazole), 8.0-8.2 (b, 1H, H of triazole). FTIR (cm\(^{-1}\)) 3367, 3082, 3060,
3039, 1740, 1649, 1557, 1498, 1461, 1379, 1297, 1260, 1182, 1139, 1111, 1039, 893, 847,
749, 701. \(M_n= 70\text{kDa}, M_w=96\text{kDa}. T_g= 65.\)

3.3.6 Fabrication of porous 3-D scaffolds

Polymers were processed into 3-D porous foams using a solvent-casting, particulate-leaching technique with NaCl as the porogen (100-250 µm or 250-400 µm salt crystals).\(^{183}\) Briefly, concentrated polymer solutions (60 mg/mL) in 1,1,1,3,3,3-
hexafluoro-2-propanol (HFIP) were cast into 12 cm crystallization dishes with a layer (0.5 cm) of sieved NaCl crystals. The samples were air dried for 48 h and then vacuum-dried for 24 h to remove any residual solvent. Polymer-salt composites were then soaked in deionized water (water change every 12 h) to leach out the salt. AgNO\(_3\) solution was used to check for the presence of NaCl until no white precipitation was found. The resulting porous foams were cut into round disks (1 cm) and stored at -20 °C.

For PEU containing the 1-PHE-4 unit scaffolds, azobisisobutyronitrile (AIBN) (10% as carbon-carbon double bonds in PEU) was dissolved in HFIP together with PEU. After the solution casting and solvent removal by nitrogen and vacuum dry stepwise, the resulting polymer-salt composites were placed in vacuum oven at 80 °C overnight. The subsequent steps were identical to those described previously. The porous scaffolds fabricated from poly(1-PHE-6), poly(1-PHE-6)-co-(1-TYR-6), and poly(1-PHE-6)-co-(1-
PHE-4)-co-(1-TYR-6) are referred to as poly(1-PHE-6), poly(1-PHE-6) 1% OGP, and poly(1-PHE-6) 10%-1% OGP, respectively.

3.3.7 Characterization of 3D porous scaffolds using micro-computed tomography (µCT)

Porous scaffolds were scanned using a Skyscan 1172 µCT porosity and pore size measurements, as well 3D pore interconnectedness observations. Scanning was conducted with no filter at 25 kV, 140 uA and 4W. All scans were taken with 5.9 um pixels and a small camera. Reconstructions of the 2D µCT scans were conducted using NRecon and analyzed using Ctan. Porosity calculations were conducted using Ctan by selecting a region of interest and manually thresholding the contrast so polymer was clearly visible in comparison to pores. A volumetric 3D porosity calculation was determined by measuring the porosity of every 2D reconstructed z-stacking slice. Pore interconnectedness was observed by generating a 3D model of the porous scaffolds in CTVox.

3.3.8 Human mesenchymal stem cell culture

Female hMSCs (Lonza, Walkersville, MD) were expanded following the manufacturer’s protocol using MSC growth medium (Lonza, Walkersville, MD) supplemented with 10 vol% FBS, 10 mL L-glutamine, 30 ug/mL gentamicin, and 15 ng/mL amphotericin. For proliferation and differentiation experiments, hMSCs (passage 5) were seeded on 3D porous scaffolds at ~2.5*10^5 cells/scaffold. hMSCs for proliferation studies were cultured in α-MEM (Invitrogen, Grand Island, NY) supplemented with 10 vol% FBS, 10^-7M dexamethasone, and 100 U/mL penicillin-streptomycin. Osteogenic differentiation media was used for all other experiments prepared from α-MEM media supplemented with
10 vol% FBS, 1.0*10^{-7} M dexamethasone, 100 U/mL penicillin-streptomycin, 50 µg/mL ascorbic acid, and 10 mM β-glycerol phosphate. All cells were cultured at 37 °C and 5% CO₂ for up to 4 weeks.

3.3.9 hMSC proliferation

Cell proliferation was measured using the WST-1 assay and protocol (Dojindo Molecular Technologies, Rockville, MD) on days 1, 3 and 7 of cell culture for poly(1-PHE-6) 1% OGP and poly(1-PHE-6) 10%-1% OGP of both 100-250 µm and 250-400 µm pore sizes. In brief, a serial dilution of cells was seeded in a 24 well plate at least 12 hours prior to the proliferation experiment. A working solution was prepared by mixing sterile water, WST-1 stock solution (22 mM) and 1-methoxy PMS stock solution (32 mM). The working solution was mixed with Tyrode’s HEPES Buffer and added to the samples. The samples were incubated for 3 hours at 37 °C and 5% CO₂. Endpoint absorbance was read on a Synergy Biomax plate reader at 450 nm. The absorbance reading for all samples and serial dilutions were normalized using the absorbance of the blank well. A new serial dilution was seeded before each time point, and the standard curve generated was used for the quantification of the cells.

3.3.10 Histological staining and immunohistochemistry (IHC)

All samples were fixed overnight in 3.7% paraformaldehyde (Sigma), then washed with 70% EtOH and 80% EtOH for 1 h each. Samples were washed in a 60 °C paraffin bath and embedded in paraffin blocks for sectioning. Blocks were removed from -20 °C, cut into 7 µm thick sections, mounted on glass slides, and dried for 2 days at 37 °C.
Samples were rehydrated through a series of washes: xylenes (2 x 2 min), 100% EtOH (1 x 2 min), 95% EtOH (1 x 2 min), 70% EtOH (1 x 2min), and deionized H2O (3 x 2 min).

Immunohistochemistry samples were incubated in 0.5% pepsin for 10 min at room temperature for antigen retrieval. Non-specific antibody binding was blocked by incubated in 10% donkey serum at room temperature for 1 h. Samples were incubated in the following primary antibodies overnight at 4°C: collagen type 1A (Col1A) (1:100), bone sialoprotein (BSP) (1:200), runt related transcription factor (Runx2) (1:100), osteocalcin (OSC) (1:100), and peroxisome proliferator-activated receptor gamma (PPAR-γ) (1:100).

Samples were then incubated in the appropriate secondary antibodies conjugated to Alexa Fluor 488 (Excitation 490 nm/Emission 525 nm) (PPAR-γ, BSP) and Alexa Fluor 546 (Excitation 556 nm/Emission 563 nm) (Runx2, Col1A, OSC). DAPI (Excitation 346nm/Emission 442) was used as the nuclear stain. Fluorescent images were obtained using an IX81 microscope (Olympus), and fluorescence was quantified using Image J. Individual cells were circled and the red, green, and blue fluorescence of the specified area was determined. Green and red fluorescence was divided by the total fluorescence to obtain a normalized value for comparison. The fluorescence of 50 cells was quantified for n=3 replicates of each scaffold type.

Hematoxylin and eosin Y (H&E), and alizarin staining were performed following rehydration. All histological samples were stained with Mayer’s Hematoxylin for 5 min and Scott’s Bluing reagent for 1 min. Samples were stained with eosin Y (Sigma) and alizarin (prepared fresh in 1X PBS, pH 4.2, Sigma) for 1 and 10 min, respectively. All histological samples were dehydrated in a series of washes; deionized H2O (3 x 2 min),
70% EtOH (1 x 2 min), 95% EtOH (1 x 2 min), 100% EtOH (1 x 2 min), and xylenes (2 x 2 min). Slides were imaged using an IX81 microscope (Olympus).

3.3.11 Biochemistry

Quantification alkaline phosphatase (ALP) and total DNA was done via biochemical assays. All samples were homogenized using a Tissue-Terror. Samples for ALP detection were processed according to the SensoLyte pNPP Alkaline Phosphatase Assay kit (Anaspec, Fremont, CA). Absorbance of ALP was measured at 405 nm. For normalization, total DNA was measured in samples using a Fluorescence DNA assay kit (Sigma, St. Louis, MO).

3.3.12 Quantitative real time reverse transcription polymerase chain reaction (qRT-PCR)

RNA isolation for qRT-PCR samples was conducted according to the RNeasy Mini kit instructions (Qaigen, Valencia, CA). The scaffolds were homogenized in 600 μL lysis buffer provided in the RNeasy Mini Kit using a tissue terror. The cell lysate was applied to the RNeasy Mini Column for total RNA isolation. DNase digestion was performed during RNA isolation using the Qaigen RNase free DNase set (Qaigen, Valencia, CA). Total RNA was quantified using a Take3 Multi-Volume Plate and a Synergy MX Microplate Reader (BioTek, Winooski, VT) at 260 nm. RNA was stored at -80 °C until used for reverse transcription into cDNA. The Taqman Reverse Transcription Reagents kit (Life Technologies, Grand Island, NY) was used following the manufacturer’s protocol for reverse transcription. The reverse transcription thermal protocol was conducted on a 2720 Thermal Cycler (Applied Biosystems) and consisted of incubation at 25 °C for 10
min, reverse transcription at 48 °C for 30 min, and inactivation at 95 °C for 5 min. cDNA was stored at -20 °C until PCR was performed.

The qRT-PCR was performed on a 7500 Real Time PCR System (Applied Biosystems) using SYBR Green Master Mix and SYBR Green primers. 100 µL reactions were prepared in PCR plates with 1X SYBR Green Master Mix, 209.4 nM forward primer, 209.4 nM reverse primer, and 10 ng of cDNA. The remaining volume of the reaction was filled with DNase, RNase free H₂O. For all genes, the thermal protocol consisted of reverse transcription at 50 °C for 30 min, activation at 95 °C for 15 min, and 40 amplification cycles of denaturing for 30 s at 95 °C, annealing for 1.5 min at 58 °C, and extension for 2 min at 72 °C. Following amplification, a melt curve analysis was performed at 1°C increments from 50 °C to 95 °C to analyze the purity of the product generated. qRT-PCR was used for the comparative quantification of Runx2, BSP, and OSC using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control. N=3 replicates were studied for each scaffold type, pipetting each replicate 3 times for the PCR reaction to eliminate pipet error.

3.3.13 Animal Surgeries

All animal protocols regarding the handling, care, maintenance and surgical procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Akron General Medical Center. A total of 16 male Sprague Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing > 250 grams were divided into groups containing 8 animals each per scaffold pore size, 100-250 µm and 250-400 µm. All animals were pre-anesthetized by a subcutaneous injection with 10-12 mg/kg of
butorphanol mixed with 0.04 mg/kg of atropine. Following inhalant anesthesia induction at 3% isofluorane in 100% oxygen, the animal was maintained at 1-1.5% isofluorane in 100% for the duration of the surgical procedures.

On the dorsum, four incisions were created with a sterile scalpel blade, two in the left and right lateral direction approximately 1 cm from the spine and approximately 2 cm apart. A subcutaneous pocket was tunneled using hemostats in the posterior-anterior direction. Thin plugs of polymers sterilized by ethylene oxide were inserted into each pocket and skin incisions were closed with Michel clips. Within the four subcutaneous spaces, each animal received: (1) poly(1-PHE-6); (2) poly(1-PHE-6)-co-(1-PHE-4); (3) poly(1-PHE-6)-co-(1-TYR-6)-OGP; (4) poly(1-PHE-6)-co-(1-PHE-4)-co-(1-TYR-6)-OGP. Positions 1-4 were randomized for each animal, while retaining diagonal distribution of control and test materials, to account for variability in positioning on the back. After 4 or 12 weeks post-surgical insertion, four animals from each group were euthanized and tissues containing the polymers were collected (2 cm x 2 cm), preserved in formaldehyde and prepared for histological evaluation.

3.3.14 In vivo histology analysis

Tissue sections (5 µm) were cut (Leica RM2235 micrometer) and stained by hematoxylin and eosin (Ventana ST5020 Automated Stainer, Hematoxylin 7211 and Eosin 71204) to show normal tissue architecture, Mallory’s Trichrome (Ventana NEXES Special Stains, Trichrome II Staining Kit 860-013) for collagen deposition and cellular infiltration, and Alizarin red to detect mineralization of calcium as evidence of bone cell activity. For Alizarin Red S staining, histological sections stained with 40 mM Alizarin Red S solution,
pH 4.2 at ambient temperature for 10 min, rinsed five times in distilled water and washed for 15 min in 1x PBS. Histological sections were counter-stained with hematoxylin, dehydrated with ethanol and rinsed in xylene before mounting with permamount. All slides were examined with an Olympus BX51 light microscope to identify the location of the polymer within the subcutaneous region and digital images were captured using the QImaging camera and software.

3.3.15 Statistics

All experiments were conducted with three replicates (n=3). A one-way analysis of variance (ANOVA) with Tukey post hoc analysis was conducted when applicable. A significance value of p≤0.05 was set for all statistical analysis. All quantitative data are presented as the average ± standard deviation.

3.4 Results

A summary of the results for all of the data collected for this chapter is provided in this section.

3.4.1 Conjugation of peptides to poly(ester urea)

The successful peptide conjugation was confirmed by the appearance of characteristic triazolium protons between 8.0 ppm and 8.3 ppm, as marked as peak “a” in Figure 3.2. Also the new peak between 5.0 and 5.2 ppm was attributed to protons of methylene group between tyrosine and triazole (peak “b” in Figure 3.3), which was located at 4.7 ppm prior to the “click” 1,3 Huisgen cycloaddition reaction. Broad peaks around 2.0
ppm and 3.5 ppm were characteristic proton resonances from peptides units, as marked inside the red lines in Figure 3.2.

Figure 3.2 $^1$H NMR spectrum of OGP conjugated poly(1-PHE-6)-co-(1-TYR-6). Wide peaks inside red curves are characteristics of OGP peptide units. Peak a is the chemical shift of triazole hydrogens, and peak b arises from chemical shift of protons connected tyrosine units and triazole rings. (Solvents marked with asterisks).

3.4.2 Characterization of 3D porous scaffolds using microcomputed tomography ($\mu$CT)

Data collected from $\mu$CT calculations and observations showed the successful fabrication of 3D porous scaffolds via salt leaching (Figure 3.3). Pore sizes were found in the ranges of 100-250 µm and 250-400 µm, for their respective salt crystal sizes. The percentages of porosity ranged from 85% to 90%, as calculated from the 3D reconstructed
models using manual threshold settings. When viewing the 3D models, the interconnectedness of the pores was easily observed, which facilitates cell migration through the entire scaffold construct.

![Poly(1-PHE-6) scaffolds](image)

Figure 3.3 Microcomputed tomography images obtained for poly(1-PHE-6) salt-leached scaffolds with both 100-250 μm (A and C) and 250-400 μm (B and D) pore sizes. (A-B) 2D reconstructed image of a z-direction slice through the scaffold. (C-D) 3D models of scaffolds.

3.4.3 hMSC proliferation

A low seeding density of 2.5*10^5 cells/scaffold was chosen for proliferation and differentiation for the promotion of osteoblast formation. hMSC proliferation was observed for day 1, 3 and 7 of cell culture using the WST-1 assay (Figure 3.4). Day 1 of
cell culture shows approximately $7.5 \times 10^4$ cells survives on each sample, which calculates to be a 30% seeding efficiency. For poly(1-PHE-6) 10%-1% OGP with 100-250 μm pore sizes, there is a significant decrease in cell number by day 3 of cell culture. By day 7, the cell density significantly increased for all four samples, with an average cell number of $\sim 2 \times 10^5$ cells/scaffold. All samples supported cell proliferation of hMSCs through day 7 of *in vitro* culture, indicating that poly(1-PHE-6) was non-toxic to the cells and supported cell growth.

Figure 3.4 Summary of cell viability on OGP-functionalized 3D porous scaffolds using the WST-1 assay. hMSCs are able to survive and proliferate every surface, indicating the polymers are non-toxic to the cells. * indicates p-value <0.05 between day 1 and 3 within one scaffold group. ** indicates p-value <0.05 between day 3 and 7 within one scaffold group.
3.4.4 Effect of cross linking and tethered-OGP on hMSC osteogenic differentiation on poly(1-PHE-6) porous scaffolds

Osteogenic differentiation of hMSCs on the six scaffold types poly(1-PHE-6), poly(1-PHE-6) 1% OGP and poly(1-PHE-6) 10%-1% OGP with 100-250 µm and 250-400 µm pore sizes) was characterized by tracking the expression of osteogenic markers and genes at 2 and 4 weeks of *in vitro* culture. These markers were observed and quantified using IHC, qRT-PCR and biochemistry.

For detection of Runx2, Col1A, BSP, and OSC proteins at 2 and 4 weeks *in vitro*, IHC staining was conducted. Runx2 was stained simultaneously with PPAR-γ for a comparison of hMSCs undergoing osteogenic and adipogenic differentiation, respectively (Figure 3.7). Similar to Runx2, PPAR-γ is a necessary transcription factor that regulates adipogenic differentiation.\(^{185,186}\) PPAR-γ expression was observed to be lower than Runx2 expression for all scaffold types, indicating that osteogenesis is more prevalent than adipogenesis. However, there was a clear difference in Runx2 expression relative to PPAR-γ expression when comparing experimental scaffolds to controls. Quantification (2 and 4 week) revealed that there is only ~1.2-1.3 times greater expression of Runx2 compared to PPAR-γ for poly(1-PHE-6) 100-250 µm and 250-400 µm scaffolds. poly(1-PHE-6) 1% OGP and poly(1-PHE-6) 10%-1% OGP (both pore sizes) show ~2-3.3 times greater Runx2 expression compared to PPAR-γ at 2 and 4 weeks. For poly(1-PHE-6) 10%-1% OGP with 100-250 µm pore sizes, there was significantly greater Runx2 protein compared to both poly(1-PHE-6) control scaffolds indicating that hMSCs reached the immature osteoprogenitor stage faster when OGP is present. Runx2 protein quantity remained constant (~1.6) and significantly greater than control scaffolds (~0.8-1.0) for
poly(1-PHE-6) 10%-1% OGP 100-250 µm from 2 to 4 weeks, suggesting that more hMSCs were progressing to the pre-osteoprogenitor stage in the presence of OGP and crosslinking. By 4 weeks, a significantly greater Runx2 expression of 1.63 was observed for poly(1-PHE-6) 1% OGP with 250-400 µm pores compared to the 2 week time point and the controls (1.0) at 4 weeks.

As hMSCs differentiate from progenitors to mature osteoblasts, expression of bone-associated genes, such as BSP, Col1A and OSC, is upregulated, fluctuates, or is reduced as a sign of ECM maturation. At both 2 and 4 weeks there was no difference in Col1A deposition or BSP protein expression detected using IHC (Figure 3.8). The persistent amount of BSP was attributed to the known fluctuations in BSP expression that occurs during hMSC osteoblast differentiation. BSP is expressed very early by osteoprogenitor cells, decreases in expression, and peaks again when mature bone-forming osteoblasts have been reached. By referring to Runx2 expression, it is apparent that the osteoprogenitor stage was reached at 2 weeks, suggesting that a peak in BSP expression was missed. All samples, regardless of OGP functionalization, pore size and crosslinking, showed fluorescence quantification of BSP between 0.7 and 1.0 with no significant difference. Similarly, Col1A fluorescence quantification was calculated to fall in the range from 0.6-1.3 with no significant difference for all samples. In previous literature, it has been shown that cells cultured in dexamethasone and β-glycerol phosphate produced mineralized bone nodules, as a result of an increase in Col1A and BSP mRNA expression. Regardless of sample type, the osteogenic media supplements had limited control over the effect of osteogenic gene expression, leading to BSP and Col1A expression for all samples. Col1A expression is detectable at the immature osteoprogenitor stage,
increases as these progenitor cells mature, and remains constant through the formation of a mature osteoblast. Figure 3.8 suggests that the hMSCs reached the progenitor cell stage, regardless of scaffold type, by 2 weeks. Col1A expression was found to remain constant, falling in the fluorescence value range of 0.6 to 1.3 with no significant difference, as the hMSCs progressed through osteogenic differentiation up to 4 weeks. The onset of Col1A deposition may have occurred prior to 2 weeks, resulting in the similarity among control and experimental samples.

OSC was expressed on all six samples, however OSC on poly(1-PHE-6) 1% OGP and poly(1-PHE-6) 10%-1% OGP scaffolds of both pore sizes was statistically greater with approximately 1.4 times more expression compared to controls (Figure 3.9). By 4 weeks, it is evident that more fluorescence signal was present on samples with OGP functionalization and crosslinking, regardless of pore size. The quantification of OSC expression is also shown in Figure 3.9. By 4 weeks in vitro OSC expression was significantly higher (~1.4 times greater) for samples containing only OGP (2.1-2.3 fold) and OGP with cross links (2.3 fold) than for control samples (1.6-1.7 fold). It is important to note that for poly(1-PHE-6) 1% and 10%-1% OGP with 250-400 µm pores, there was a consistent OSC gene expression from 2 to 4 weeks (~ 2.3 for both samples). This suggests that at 2 weeks, hMSCs may have reached the bone-forming osteoblast stage on these scaffolds. Furthermore, this is a good indication that OGP functionalization, cross linking, and a larger pore size may increase the rate of osteogenic differentiation for hMSCs.

It is important to note the auto fluorescence of the polymers in Figure 3.7, Figure 3.8 and Figure 3.9 was most likely due to the formaldehyde fixation process. Tyrosine molecules are known to be auto fluorescent. Additionally, the interaction of aldehydes
with amines and proteins leads to the generation of fluorescent molecules. As the PEUs contain peptide bonds, it is likely that formaldehyde fixation caused additional auto fluorescence of the polymer scaffolds.

To further quantitatively confirm the results from IHC, qRT-PCR was conducted for Runx2, BSP, and OSC, using GAPDH as an endogenous control (Figure 3.5). After 2 weeks of in vitro culture, a similar trend for Runx2 expression was observed; each scaffold showed similar expression of the essential osteoblast transcription factor (between 1.0 and 1.4 with no significant difference). After 4 weeks in culture, there was a 150% increase in Runx2 expression for poly(1-PHE-6) 1% OGP and 10%-1%OGP with 250-400 µm pores (2.5±0.8 and 2.3±0.9, respectively) when compared to the controls, which were normalized to 1.0. Using qRT-PCR, there was a noticeable difference in BSP expression between samples and from 2 to 4 weeks. At 2 weeks, BSP expression on poly(1-PHE-6) 1% OGP 100-250 µm, poly(1-PHE-6) 1% OGP 250-400 µm and poly(1-PHE-6) 10%-1% OGP 250-400 µm was 50%, 30% and 50% higher, respectively, compared to the controls. It is also important to notice that there was an 84% and 71% decrease in BSP expression from 2 to 4 weeks for poly(1-PHE-6) 1% OGP 100-250 µm pores and poly(1-PHE-6) 10%-1% OGP 250-400 µm pores, respectively. Although expression of OSC was similar for all scaffold types at 2 weeks, 4 week expression for poly(1-PHE-6) 1% OGP and 10%-1% OGP with 250-400 µm pores is 170% and 180% higher compared to controls. It appears that OGP functionalization, pore size, and crosslinking worked simultaneously to promote osteogenic differentiation on poly(1-PHE-6) scaffolds when cultured in osteogenic media.
Biochemical assays were conducted to quantitatively measure ALP expression at 2 and 4 weeks for the poly(1-PHE-6) scaffolds (Figure 3.6). ALP is an essential enzyme for mineralization that hydrolyzes the calcification inhibitor, inorganic pyrophosphate.\textsuperscript{192, 193} Expression of ALP was normalized to DNA for each scaffold. Poly(1-PHE-6) 10%-1% OGP with 100-250 µm and 250-400 µm pores showed 189 and 205 ng/mL/µg DNA, respectively, at 4 weeks. This was significantly greater than both controls which had a
calculated ALP quantification of 68 and 94 ng/mL/µg of DNA for poly(1-PHE-6) 100-250 µm and 250-400 µm pores, respectively.

Figure 3.6 Effect of pore size, functionalization with OGP, and crosslinking on ALP expression of hMSCs. * indicates p value <0.05 between the 2w OGP-functionalized scaffolds and controls. ** indicates p value <0.05 between 4w OGP-functionalized scaffolds and controls

3.4.5 Histological analysis of hMSC differentiation on poly(1-PHE-6) porous scaffolds

in vitro

As a final measure to observe differentiation of hMSCs, histological staining with alizarin red and eosin Y were conducted to observe calcium and collagen deposition, respectively, after 2 and 4 weeks (Figure 3.10 and Figure 3.11). Calcium deposition occurs during the final stage of hMSC osteogenic differentiation. Calcium and phosphate are crystalline salts that are regulated and deposited by cells into the organic matrix of bone,
in the form of hydroxyapatite. It is important to note that calcium deposition was greater at 2 and 4 weeks in all scaffolds functionalized with OGP, when compared to the poly(1-PHE-6) controls. There was no clear difference in hMSC calcium deposition between scaffolds with pore sizes ranging from 100-250 µm and 250-400 µm, nor was there a noticeable difference between scaffolds with and without 10% crosslinking. Calcium deposition remained constant from 2 to 4 weeks in all samples.

3.4.6 *In vivo* qualitative histological analysis

Trichome staining was conducted to qualitatively study the cellular response to poly(1-PHE-6) porous scaffolds *in vivo* (Figure 3.12). For scaffolds with 100-250 µm pore sizes, there was greater cell infiltration into the scaffold by 4 weeks for poly(1-PHE-6) 1% OGP and 10% cross linking-1% OGP. Also, samples containing OGP, showed greater collagen deposition (blue staining) within the scaffold at 4 weeks. From 4 to 12 weeks, a clear increase in cell infiltration and collagen deposition was noticeable. As evidenced by the dark red staining, an appreciable influx of blood vessels was observed for poly(1-PHE-6) 10% cross linking-1% OGP specimens. An enhanced blood supply provides nutritive support and permits cellular migration for new tissue growth within the polymer scaffold. Samples with larger pore sizes, 250-400 µm, showed less staining of erythrocytes but greater collagen deposition at 12 weeks, compared to small pore size samples, due to tissue remodeling that occurs in the later stages of tissue formation. It is also important to note that capsule formation around the scaffold did not increase in size from 4 to 12 weeks. The lack of fibrotic or granulomatous response confirms non-cytotoxicity of poly(1-PHE-6).
H&E staining (Figure 3.13) results showed a varying degree of cell infiltration for 100-250 µm pore size samples at 4 weeks, with more cell migration occurring in poly(1-PHE-6) with 1% OGP and 10% cross linking-1% OGP. A noticeable increase of cell infiltration from 4 to 12 weeks was seen, along with new bone formation in the extracellular matrix (pale pink staining). Samples with larger pore sizes, 250-400 µm, showed similar results, with greater cell infiltration and early bone formation on samples containing OGP. There was no clear difference between scaffolds with smaller or larger pore sizes.

Alizarin staining was conducted to observe mineralization of the cellular ECM at 4 and 12 weeks (Figure 3.14). Although there appeared to be no obvious difference for the varying pore sizes, samples containing OGP suggested faster matrix mineralization compared to non-OGP containing scaffolds as early as 4 weeks. Darker red staining was seen for poly(1-PHE-6) 10% cross linking, 1% OGP, and 10% cross linking-1% OGP, compared to poly(1-PHE-6) control samples.

3.5 Discussion

A discussion of the collected results is provided in this section to summarize the findings of this work.

3.5.1 hMSC proliferation

The first stage in bone regeneration is proliferation. During this time, hMSCs multiply while remaining pluripotent.\textsuperscript{190} Cell proliferation and lineage commitment is highly dependent on seeding density, with previous literature revealing that low seeding densities result in more hMSC osteogenic differentiation in osteogenic media.\textsuperscript{84, 195, 196}
Cells were seeded on the scaffolds with approximately 30% seeding efficiency, which was expected based on the method of seeding used. Proliferation data shows that regardless of sample type, hMSCs continue to proliferate after day 3 of culture. From day 1 to day 3, there is a significant decrease in cell number on scaffolds with 100-250 µm pore sizes. Although the seeding density was low, a lower seeding density may have been needed to see a difference between samples containing tethered-OGP and lacking tethered-OGP. Overall, the proliferation data suggests that a larger pore size is more conducive to hMSC proliferation in poly(1-PHE-6)-based scaffolds, and that the scaffolds are non-toxic to the hMSCs in vitro.

3.5.2 Effect of cross linking and tethered-OGP on hMSC osteogenic differentiation on poly(1-PHE-6) porous scaffolds

Following proliferation, hMSCs enter the early stages of differentiation, during which the extra-cellular matrix is deposited and matured. Early differentiation of hMSCs is marked by the expression of Runx2 and ALP. Runx2 is a necessary transcription factor for osteogenic differentiation, which regulates expression of later osteoblast-specific genes. As a crucial enzyme for ossification, detectable amounts of ALP are observed as hMSCs reach the mature osteoprogenitor stage. As hMSCs progress from immature to mature osteoprogenitor cells, expression of BSP and deposition of Col1a begins. BSP expression peaks during this phase of osteoblast formation, and therefore can be used as a detection method for rate of differentiation. The expression of the bone-specific protein OSC, and mineralization of calcium are terminal differentiation markers only observed when hMSCs have become mature osteoblasts.
IHC staining for PPAR-γ and Runx2 reveal that osteogenesis is more prevalent than adipogenesis on all samples at both 2 and 4 weeks. The comparison of adipogenic and osteogenic markers is essential as bone-marrow adipogenesis is unfavorable during the bone formation process. Adipogenesis of bone-forming mesenchymal stem cells is correlated to osteoporosis, which results in a loss of bone density and strength.\textsuperscript{202, 203} The ratio of osteogenic to adipogenic cells is much greater on scaffolds containing tethered-OGP at both 2 and 4 weeks, regardless of pore size, suggesting the presence of OGP is driving immature osteoprogenitor formation greater than non-functionalized controls. Samples containing OGP functionalization and cross linking showed a higher and constant expression of Runx2 at 2 and 4 weeks, suggesting a faster progression of osteogenesis compared to controls. When compared to qRT-PCR studies, there is a slight difference in results. Runx2 expression reveals a peak from week 2 to 4 for experimental scaffolds of 250-400 µm pore sizes. Genes are often expressed earlier than proteins, therefore, the characteristic peak in Runx2 expression is observed in qRT-PCR and not in IHC. Regardless, both data suggests that faster osteogenic differentiation is occurring in samples containing tethered-OGP and crosslinking.

As differentiation progresses, BSP, Col1a and OSC expression patterns are observed. Collagen 1 fibers make up nearly 90% of the organic matrix of bone, while the remaining 10% consists of non-collagenous proteins, such as BSP and OSC, which play an essential role in cell adhesion and matrix mineralization for the formation of healthy bone. BSP is a multifunctional protein that promotes cell adhesion and induces mineralization. BSP contains the amino acid sequence Arg-Gly-Asp (RGD), which is a cell attachment sequence.\textsuperscript{204} Previous studies have shown that BSP mRNA expression is induced by
glucocorticoids, such as dexamethasone, therefore, BSP expression was expected for all scaffold types because cells were cultured in osteogenic media. Even with osteogenic media inducing osteoblast formation, the characteristic drop in BSP expression was only observed on OGP-functionalized scaffolds using qRT-PCR. This drop in BSP expression suggests that samples with 1% tethered-OGP are inducing osteogenic proliferation faster than scaffolds without OGP functionalization. qRT-PCR reveals a significantly greater expression of BSP at 2 weeks for scaffolds with tethered OGP. This increased expression allows for better cell attachment to the polymer surface and therefore enhanced osteogenic differentiation. Cell attachment is essential for lineage commitment as revealed by Chen, et. al. When hMSCs are well attached and spread on a surface, they show a preference osteoblast formation over adipocyte formation. BSP protein also localizes at the sites where mineralization takes places, therefore greater expression of BSP at 2 weeks indicates that samples containing OGP will show mineralization quicker. Once again, a difference in BSP expression observed in IHC and qRT-PCR is observed, which can be attributed to the detection of protein and gene expression, respectively.

OSC is the terminal marker expressed when hMSCs have reached the bone-forming, mature osteoblast stage. In both IHC and qRT-PCR, a significant difference in OSC expression between poly(1-PHE-6) 1% OGP and 10%-1% OGP with pore sizes of 250-400 µm and control samples at 4 weeks in vitro culture. The data show that OGP-functionalization and the larger pore size aid in faster osteogenic differentiation of hMSCs on phenylalanine-based PEUs.

It is important to note that ascorbic acid is essential for bone formation in vitro, as it promotes collagen production and the expression of osteoblast-specific genes. In
this study, it was observed that genes specific to the osteoblast phenotype were expressed on all samples, showing that ascorbic acid aided in driving osteoblast differentiation regardless of OGP-functionalization. While the presence of OGP enhanced osteogenic differentiation, it was not the ultimate driving factor.

While OGP upregulates the osteogenic differentiation of hMSCs on poly(1-PHE-6) porous scaffolds, it was interesting to see the effect of pore size and cross linking on lineage commitment. Generally, mechanical strength decreases as porosity and pore size increases, therefore the void volume in bone tissue engineering scaffolds must be tuned to allow for proper osteogenic differentiation, as well as cell penetration. Previous studies have shown that larger pore sizes (200-400 µm) are necessary for bone differentiation to ensure cell penetration into the scaffold. Smaller pores (75-100 µm) have been shown to generate unmineralized osteoid tissue. Osteogenic differentiation occurred on all 6 samples, as indicated by expression of differentiation markers at both 2 and 4 weeks. The pore size difference may not have been large enough to see a true effect of pore size on osteogenic differentiation on poly(1-PHE-6) scaffolds. Finally, 10% covalent crosslinking was introduced to see the effect of stiffness on osteoblast formation. It appears that 10% crosslinking did not significantly enhance the differentiation of hMSCs. The cells responded similarly on uncrosslinked and crosslinked, OGP-functionalized scaffolds.

3.5.3 Histological analysis of hMSC differentiation on poly(1-PHE-6) porous scaffolds

In vitro.

Histological images for alizarin staining suggest that there is greater mineralization in tethered OGP-scaffolds compared to controls, however there is no clear difference for
scaffolds of different pore sizes. These results coincide with the previous quantitative results, suggesting that 10% crosslinking and the chosen pore size ranges do not change the mechanical properties of the scaffolds enough to induce a change in osteogenic differentiation. The increase in calcium deposition on OGP-functionalized scaffolds can be attributed to the increase in bone-specific genes expressed as detected by IHC and qRT-PCR.

3.5.4 In vivo qualitative histological analysis

Qualitative histological analysis reveals that there was no negative in vivo response elicited by the degradation of the poly(1-PHE-6) scaffolds. Although capsule formation is observed, the capsule size does not appear to increase from 4 to 12 weeks, suggesting that the poly(1-PHE-6) scaffolds do not generate inflammatory byproducts during degradation as previously shown. Fibrous capsule formation is routinely seen in vivo as a natural response to injury or implanted materials, and eventually decreases in size as time from implantation increases. In addition, for all scaffold types, little to no inflammatory cell nuclei are seen in implant-surrounding tissue (as indicated by black nuclei staining), which suggests there is minimal to no chronic inflammation occurring in vivo.

After 4 weeks, samples with OGP-functionalization show greater cell infiltration and collagen deposition, indicating that OGP incorporation enhances the ability for tissue-scaffold integration. For all scaffolds, there is clear increase in collagen and cell migration from 4 to 12 weeks, indicating maturation of the extracellular matrix. Along with collagen, blood vessel migration is integral for bone formation, as bone is a highly vascularized
Evidence of the influx of blood vessels and a collagen matrix are a good indication that the presence of OGP in poly(1-PHE-6) scaffolds promotes osteogenesis. Matrix maturation and mineralization are also observed in all scaffold types, however, it has occurred to a greater extent in scaffolds functionalized with OGP and cross linking.

3.6 Conclusions

The successful synthesis and fabrication of unfunctionalized and functionalized phenylalanine-based poly(ester urea) porous scaffolds is reported here. Scaffolds were fabricated with various pore size ranges, with and without tethered-OGP, and with and without chemical cross links. The PEU synthesis was confirmed using $^1$HNMR. The successful tethering of OGP on Poly(1-PHE-6)-co-(1-TYR-6) was also confirmed using $^1$HNMR to show the presence of the triazole group generated during the copper catalyzed click reaction. Microcomputed tomography revealed the successful fabrication of scaffolds with different pore size ranges, as well as interconnectedness or pores.

Tethered OGP, crosslinking and two pore sizes were tested to determine their individual effect on osteogenic differentiation of hMSCs on poly(1-PHE-6) poly(ester urea) scaffolds. The addition of OGP to poly(1-PHE-6) scaffolds resulted in faster differentiation, compared to the unfunctionalized poly(1-PHE-6) samples. Crosslinking and changes in pore size did not seem to change the stiffness of the scaffolds enough to cause a significant change in osteogenic differentiation on poly(1-PHE-6).

The active OGP fragment, OGP[10-14], has been previously shown to increase osteoblast formation in vitro on 2D substrates.\textsuperscript{177} It has been reported that OGP works by enhancing the expression of bone morphogenic protein 2 (BMP-2), which plays a critical role...
role in bone regeneration by upregulating the expression of bone-associated proteins such as ALP and OSC.\textsuperscript{139,174} The addition of OGP to poly(1-PHE-6) scaffolds resulted in faster differentiation, compared to the unfunctionalized poly(1-PHE-6) samples. Furthermore, samples with larger pore sizes allowed for greater osteogenic gene expression of hMSCs by 4 weeks compared to controls and smaller pore sizes. Previous studies have shown that larger pore sizes (200-400 µm) are necessary for bone differentiation to ensure cell penetration into the scaffold. Smaller pores (75-100 µm) have been shown to generate unmineralized osteoid tissue.\textsuperscript{205, 206, 217} Osteogenic differentiation occurred on all 6 samples, as indicated by expression of differentiation markers at both 2 and 4 weeks. The pore size difference may not have been large enough to see a true effect of pore size on osteogenic differentiation on poly(1-PHE-6) scaffolds. hMSCs also responded similarly on uncrosslinked and crosslinked, OGP-functionalized scaffolds, indicating that 10\% crosslinking is not enough to elicit a cellular response \textit{in vitro}. However, the increase in stiffness with 10\% crosslinking, much like 1\% crosslinking showed in previous work,\textsuperscript{169} should enhance ability of the scaffold to support the healing load-bearing bone in \textit{in vivo} defect models.

\textit{In vivo} data suggests that by 4 weeks, there is greater ability for subcutaneously implanted poly(1-PHE-6) scaffolds functionalized with OGP to promote ECM collagen deposition and matrix mineralization without any inflammatory response. Furthermore, the migration of blood vessels within the scaffold regions of the OGP-tethered samples was observed, revealing that these materials are not only osteogenic but also angiogenic. \textit{In vitro} and \textit{in vivo} data support the possibility for OGP-tethered poly(1-PHE-6) scaffolds to
be utilized in more advanced clinical bone tissue engineering models for the promotion of healthy, new bone formation.
Figure 3.7 Summary of RUNX2 and PPAR-γ expression of hMSCs after 2 and 4 weeks in vitro. Immunofluorescence staining for cell nuclei (blue), Runx2 (red), and PPAR-γ (green) and quantification of Runx2 and PPAR-γ immunofluorescence using Image J. * indicates p value <0.05 between the 2w OGP-functionalized scaffolds and controls. ** indicates p value <0.05 between 4w OGP-functionalized scaffolds and controls. Scale bar = 20µm.
Figure 3.8 Summary of Col1A and BSP expression of hMSCs after 2 and 4 weeks in vitro. Cell nuclei (blue), Col1A (red), and BSP (green) and quantification of Col1A and BSP immunofluorescence using Image J. No significant difference was observed for Col1A and BSP expression for scaffolds. Scale bar = 20µm
Figure 3.9 Summary of osteocalcin (OSC) expression for hMSCs after 2 and 4 weeks in vitro. Immunofluorescence staining for cell nuclei (blue) and OSC (red) and quantification of immunofluorescence using Image J. * indicates p value <0.05 between 2w OGP-functionalized scaffolds and controls. ** indicates p value <0.05 between 4w OGP-functionalized scaffolds and controls. *** indicates p value <0.05 between 2w 100-250 µm and 250-400 µm pore sizes of OGP-functionalized scaffolds. Scale bar = 20 µm.
Figure 3.10 Bright field histological images for calcium deposition using Alizarin Red after 2 and 4 weeks of culture in vitro hMSCs. A hematoxylin counter stain was conducted for all histological samples. The angle at which the samples were embedded and sliced for imaging influences the visualization of the size and shape of the pores. Black arrow: calcium deposition, yellow arrow: scaffold. Images were taken using a 10X objective. Scale bar = 100 µm.
Figure 3.11 Bright field histological images for collagen 1 deposition using H&E staining after 2 and 4 weeks of culture in vitro hMSCs. A hematoxylin counter stain was conducted for all histological samples. Images were taken using a 4x objective. Scale bar = 200 µm.
Trichrome staining for 4 and 12 week in vivo poly(1-PHE-6) porous scaffolds with 100-250 µm and 250-400 µm pore sizes. We speculate that pore sizes are difficult to discern among sample sets because of enhanced tissue integration after only 2 weeks in vivo. In addition, the angle at which the samples were embedded and sliced for imaging influences the visualization of the size and shape of the pores. Images were taken using a 100X objective. Scale bar = 100 µm
Figure 3.13 H&E staining of in vivo poly(1-PHE-6) scaffolds with 100-250 µm and 250-400 µm pore sizes after 4 and 12 weeks. We speculate that pore sizes are difficult to discern among sample sets because of enhanced tissue integration after only 2 weeks in vivo. In addition, the angle at which the samples were embedded and sliced for imaging influences the visualization of the size and shape of the pores. Black arrow: new bone, red arrow: scaffold. Images were taken using a 100x objective. Scale bar = 100 µm
Figure 3.14 Alizarin histological images for *in vivo* rat studies for poly(1-PHE-6) with OGP functionalization and cross linking, and without. We speculate that pore sizes are difficult to discern among sample sets because of enhanced tissue integration after only 2 weeks *in vivo*. In addition, the angle at which the samples were embedded and sliced for imaging influences the visualization of the size and shape of the pores. Images were taken using a 10x objective. Scale bar = 100 µm.
3.8 Acknowledgements

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CHAPTER IV

PHENYLALANINE-BASED POLY(ESTER UREA)S CROSSLINKED WITH OSTEOGENIC GROWTH PEPTIDE FOR BONE TISSUE ENGINEERING APPLICATIONS

4.1 Abstract

Functionalization of synthetic materials with naturally occurring growth factors has gained increasing attention in the field of tissue engineering in recent years. Although synthetic materials have tunable mechanical, physical, and chemical properties for multiple tissue regeneration applications, they lack the inherent biological cues necessary for driving specific lineage differentiation of cells. Through the use of osteogenic growth peptide (OGP)-based crosslinkers, both the mechanical properties and cellular response of preosteoblast cell lines on poly(PHE) materials can be tuned simultaneously. In this study, a pendant alkene-containing poly(PHE) copolymer was synthesized and crosslinked via an efficient thiol-ene ‘click’ reaction with cysteine-containing OGP (Cys-OGP[10-14] -Cys). With incorporation of 3% OGP crosslinking into the poly(PHE) network, the stiffness was decreased with an increase in toughness. Preliminary in vitro studies suggested that 3% OGP crosslinking upregulated proliferation and early osteogenic differentiation. By day 3, the cell density on OGP-crosslinked poly(PHE) (poly(PHE)-3%OGP) (4594 ± 352 cells/cm²) was significantly greater than the poly(PHE) (3460 ± 337 cells/cm²) and glass
controls (3823 ± 124 cells/cm²). Furthermore, Runx2 and BSP gene expression revealed a 120% and 130% enhancement, respectively, on poly(PHE)-3%OGP when compared to the poly(PHE) controls. Optimization of OGP crosslinking for enhanced mechanical properties is necessary for bone tissue engineering applications, however, this study reveals that even when OGP is tethered at both the N- and C-terminus, it remains bioactive and capable of upregulating osteogenic differentiation.

4.2 Introduction

Both natural and synthetic polymeric materials have been studied for use in bone tissue engineering applications, however as separate entities, they lack the mechanical and biological properties necessary for healthy bone regeneration. Proteins and polysaccharides, which are among the natural polymers studied for enhanced osteogenesis, have low toxicity and inherent biochemical signaling to induce healthy bone growth. However, unlike synthetic polymers, these materials lack the mechanical, thermal and chemical tunabilty and stability necessary for bone regeneration applications. Conversely, synthetic materials, including poly-L-lactic acid (PLLA) and polypropylene fumarate (PPF), have synthetic flexibility, which enables mechanical, physical and chemical properties to be tailored. Unfortunately, these materials lack the natural biochemical signaling for osteoinduction as well as cause chronic inflammation in vivo as a result of acid degradation byproducts.

Poly(ester urea)s (PEUs) are a class of degradable polymers that has been thoroughly investigated by the Becker group for biomedical applications. These amino-acid based materials possess synthetic flexibility, can be functionalized easily via click
chemistry reactions,\textsuperscript{181} and have shown tunable mechanical and degradation properties.\textsuperscript{228-230} More specifically, phenylalanine-based PEUs (Poly(1-PHE-6)) is a high modulus polymer (3.05 ± 0.24 GPa) with varying degradation rates, depending on diol chain length in the backbone of the monomer units.\textsuperscript{229-231} Furthermore, \textit{in vivo} toxicity studies have shown that after 12 weeks, subcutaneous implants of poly(1-PHE-6) have caused no chronic inflammation. While not definitive, we hypothesize that this is due to a self-neutralizing effect of the urea byproducts during degradation.\textsuperscript{231} The structural, mechanical and chemical properties of poly(1-PHE-6) make it a translationally relevant candidate for bone tissue regeneration applications, however, further functionalization with bioactive peptides is necessary to specifically drive osteoblast differentiation to enhance the extent and rate of bone regeneration.

Derivatization of synthetic polymers with bioactive peptides is an increasingly attractive strategy to induce specific cell responses.\textsuperscript{161, 232-234} Through incorporation of these bioactive motifs into polymeric networks, specific lineage commitment of cells can be enhanced for use in targeted tissue regeneration applications.\textsuperscript{151, 232-236} Osteogenic growth peptide (OGP) is a naturally occurring tetradecapeptide with an active subunit from amino acids 10 to 14 (OGP[10-14]) (\textit{N}-Tyr-Gly-Phe-Gly-Gly-C).\textsuperscript{237, 238} OGP[10-14] has been reported to upregulate proliferation, differentiation and matrix mineralization of preosteoblast cell lines \textit{in vitro}, which render it useful for polymer functionalization for bone regeneration applications.\textsuperscript{174, 233, 239, 240} It was recently demonstrated that tethering OGP[10-14] to tyrosine-like units in poly(1-PHE-6)-based copolymers, resulted in enhanced runt-related transcription factor (Runx2), bone sialoprotein (BSP) and
osteocalcin (OCN) expression of *human* mesenchymal stem cells (*hMSC*) at both 2 and 4 weeks of *in vitro* culture.232

In this study, both the mechanical and bioactive properties of poly(1-PHE-6)-based materials were altered, as a result of chemically crosslinking OGP[10-14] into the polymeric network. Crosslinking is a technique often used to increase the elastic modulus of materials by forming a more rigid network.241 The amount of crosslinking introduced to the network significantly changes the mechanical properties. Furthermore, this investigation uses the robust and non-toxic byproduct-forming thiol-ene ‘click’ reaction242-244 for the functionalization of poly(1-PHE-6) with OGP[10-14] crosslinkers.245 ‘Click’ chemistry is characterized by its use of mild reaction conditions, high yields and insensitivity to water or oxygen, making it an ideal choice for functionalization of biomaterials.246 This proves to be a fast and simple way of tuning the mechanical and osteoinductive properties of poly(1-PHE-6)-based materials. Herein, OGP[10-14] has been used to both enhance the osteoblast differentiation of MC3T3-E1 cells, and alter mechanical properties of poly(1-PHE-6), simultaneously, for the fabrication of a material for optimal bone tissue regeneration.

4.3 Materials and Methods

All necessary purchasing and vendor information for materials and instrumentation will be listed in this section, followed by a complete experimental procedure.
4.3.1 Materials

Commercial reagents and solvents were purchased from Aldrich (Milwaukee, WI) and Fisher Scientific (Pittsburgh, PA) and used without further purification unless otherwise specified. 1,6-hexanedithiol was transferred to a Schlenck ampule, degassed 3 times, and stored under N\textsubscript{2} to avoid oxidation. Chloroform was washed with deionized water 3 times, dried over CaCl\textsubscript{2} for 1 hour, and distilled from P\textsubscript{2}O\textsubscript{5} and stored over 4 Å molecular sieves. Fmoc-protected amino acids and resins for peptides synthesis were purchased from Aapptec (Louisville, KY) and Advanced Chem Tech (Louisville, KY). All biological supplements, medium, and assay reagents were purchased from ThermoFisher Life Technologies (Waltham, MA) unless otherwise stated.

4.3.2 Characterization of chemical structure and thermal properties

Proton \( ^1H \) and Carbon \( ^{13}C \) NMR spectra were obtained, using a Varian Mercury 300 MHz NMR spectrometer (Palo Alto, CA) to verify the structures of the desired compounds. Deuterated dimethylsulfoxide (DMSO-\textsubscript{d\textsubscript{6}}) was used as the solvent. All chemical shifts are reported in ppm (\( \delta \)), and referenced to the chemical shifts of residual solvent resonances \( ^1H \) NMR DMSO-\textsubscript{d\textsubscript{6}} 2.50 and \( ^{13}C \) NMR DMSO-\textsubscript{d\textsubscript{6}} 39.50). Infrared spectra were obtained from an DIGILAB Excalibur Series FT-IR spectrometer (Randolph, MA) by solution casting polymer films on KBr plates with chloroform. Characterization of the crosslinked materials was conducted using ATR-IR (Shimadzu, MIRacle 10 Single Reflection ATR Accessory) and Raman Spectroscopy (Horiba LabRam HR Micro Raman Spectrometer). The molecular mass and mass distribution of the polymers was measured by size exclusion chromatography (TOSOH, EcoSec HLC 8320-GPC, 50 °C, polystyrene
standard, 0.1 M LiBr in DMF as eluent, 5 mg/mL, flow rate 0.5 mL/min, RI detector). The glass transition temperatures ($T_g$) of the copolymers were recorded using a TA Instruments DSC Q2000. The temperature ramp rate was 10 °C/min under nitrogen. The degradation temperatures ($T_d$) of the polymers were determined by thermogravimetric analysis (TA instruments, Q500 TGA) across a temperature range of 0 °C to 600 °C at a scanning rate of 20 °C/min under nitrogen.

4.3.3 Monomer and copolymer synthesis

Two monomers were used in the synthesis of the desired copolymer. Monomer 1-PHE-6 was prepared following previously reported methods.$^{231, 232}$ Briefly, a bis-amine-toluenesulfonic acid salt was prepared by esterification of bis-L-phenylalanine and 1,6-hexanediol to generate 1-PHE-6. Purification was conducted via four recrystallizations from water.

The 1-PHE-3 monomer, containing a pendant allyl group for further functionalization (Scheme 4.1) was prepared using Boc-Phe-OH and 1,3-allyoxy-2-propanediol in anhydrous dichloromethane (1.8 M, N,N’-diisopropylcarbodiimide (DIC, 2.8 mole equivalents) and 4-(dimethylamino)pyridinium-4-toluene sulfonate (DPTS, 0.4 mole equivalents) overnight at room temperature under a N$_2$ atmosphere. The white precipitate was filtered and the yellow viscous liquid was dissolved in ethyl acetate and dried over Na$_2$SO$_4$. The concentrated product was purified using column chromatography to isolate the difunctional 1-PHE-3-Boc compound (yield = 84%) (4:1 hexanes: ethyl acetate, thin layer chromatography (TLC) 3:2 hexanes: ethyl acetate, $R_f$ = 0.62) (Appendix Figure 10-11). Removal of the Boc protecting groups was performed overnight in
anhydrous dioxane with the addition of 4 M HCl/dioxane under a N$_2$ atmosphere. Dioxane and HCl were removed under vacuum to yield 1-PHE-3 (white powder), which was washed in diethyl ether three times.

Scheme 4.1 Synthesis of 1-PHE-3 monomer.

1-PHE-6: $^1$H NMR (DMSO-d$_6$) $\delta =$ 1.03-1.12 (b, 4H, -OCH$_2$CH$_2$CH$_2$-), 1.32-1.46 (b, 4H, -OCH$_2$CH$_2$CH$_2$-), $\delta =$ 2.28 (s, 6H, CH$_3$-Ph-SO$_3$H), 2.96-3.20 (m, 4H, -CH$_2$-Ph), 4.02 (t, J=12.8, 6.4, 4H, -OCH$_2$CH$_2$-), 4.25-4.35 (t, J=13.8, 7.1, 2H, -NH$_2$CHCO-), 7.06-7.52 (m, 14H, aromatic), 8.39 (s, 4H, NH$_2$CH-) (Appendix Figure 1); $^{13}$C NMR (DMSO-d$_6$) $\delta =$ 20.84 (CH$_3$-Ph-SO$_3$H), 24.72 (-OCH$_2$CH$_2$CH$_2$-), 27.65 (-OCH$_2$CH$_2$CH$_2$-), 36.07 (-CH$_2$-Ph), 53.36 (-NH$_2$CHCO-), 65.48 (-OCH$_2$CH$_2$-), 125.38 (aromatic, -CH$_2$-Ph), 128.24-129.34 (aromatic, CH$_3$-Ph-SO$_3$H), 134.55 (CH$_3$-Ph-SO$_3$H), 138.14 (-aromatic, CH$_2$-Ph), 145.03 (aromatic, CH$_3$-Ph-SO$_3$H), 168.80 (-CH$_2$COO-) (Appendix Figure 7), ATR-IR characteristic peaks 1124, 1155, 1228, 1535, 1595, 1656, 1734, 2856 cm$^{-1}$ (Appendix Figure 8); ESI-MS [(M-TosOH)+Na]$^+$ = 398.38 g/mol (Appendix Figure 9); Yield = 86%.

1-PHE-3: $^1$H NMR (DMSO-d$_6$) $\delta =$ 3.00-3.27 (m, 4H, -CH$_2$Ph), 3.27-3.43 (m, 2H, -OCH$_2$CH=CH$_2$), 3.83-3.93 (d, J=4.8, 2H, -COOCH$_2$CH-), 3.97-4.08 (m, 1H, -COOCH$_2$CH-), 4.11-4.33 (m, 3H, -COOCH$_2$CH-, NH$_2$CHCO-), 4.95-5.32 (m, 2H, -OCH$_2$CH=CH$_2$), 5.74-5.91 (m, 1H, -OCH$_2$CH=CH$_2$), 7.17-7.40 (b, 10H, aromatic); 8.83
(b, 6H, \textsuperscript{+}NH\textsubscript{3}CH-) (Appendix Figure 12); \textsuperscript{13}C NMR (DMSO-d\textsubscript{6}) δ = 36.07 (-CH\textsubscript{2}-Ph), 53.36 (-NH\textsubscript{2}CHCO-), 67.04 (-OCH\textsubscript{2}CHO-), 67.25 (-CHCH\textsubscript{2}OCH\textsubscript{2}-), 67.31 (-CHCH\textsubscript{2}OCH\textsubscript{2}-), 72.15 (-OCH\textsubscript{2}CHO-), 117.24 (-CH\textsubscript{2}CH=CH\textsubscript{2}), 127.25-140.01 (aromatic, -CH\textsubscript{2}-Ph), 134.98 (-H\textsubscript{2}CH=CH\textsubscript{2}), 135.08 (-aromatic, CH\textsubscript{2}-Ph), 167.50 (-CHCOO-), 167.80 (-CH\textsubscript{2}COO-) (Appendix Figure 13); AT-IR characteristic peaks 1084, 1118, 1141, 1205, 1500, 1585, 1745, 2843 cm\textsuperscript{-1} (Appendix Figure 14). Yield = 66\%.

An interfacial polycondensation setup was employed for the polymerization of the poly((1-PHE-6)-co-(1-PHE-3)) copolymer (poly(PHE)). Scheme 4.2 shows the method of polymerization as described previously.\textsuperscript{231,232} The relative concentration of each monomer in the copolymer was controlled by the feed ratio in the copolymerization process, which was chosen as 0.70: 0.30 (1-PHE-6 : 1-PHE-3). The chemical structure and composition of the copolymer was confirmed by \textsuperscript{1}H NMR spectroscopy and ATR-IR. The characteristic peaks of the allyl peaks in 1-PHE-3 are seen at 5.09-5.24 ppm (l) and 5.81 (m).

Scheme 4.2 Interfacial polycondensation of 1-PHE-6 and 1-PHE-3.
Thiol-ene ‘click’ crosslinking of Poly(PHE) with 1,6-hexanediol (poly(PHE)-crosslinked) (Model crosslinking experiment)

A model reaction for the 3% crosslinked network using a thiol-ene ‘click’ reaction was conducted with 1,6-hexanediol (Scheme 4.3). Poly(PHE) was dissolved in DMF (0.02 M). Once dissolved, 1,6-hexanediol (1 mol/ene) and Irgacure 2959 (0.07 mol/ene) were added to the reaction flask and protected from ambient light. The reaction solution was degassed via three freeze-pump thaw cycles to remove O2. The solution was irradiated with 365 nm (8 W) at ambient temperature (Eiko F5T8 BLB) for 2 h without stirring. The
resulting gel product was purified by swelling and collapsing in DMF and water, respectively, 3 times. The poly(PHE)-crosslinked product was dried on the vacuum line prior to mechanical and swelling testing. Identical reaction conditions were employed for the synthesis of poly(PHE)-crosslinked with 100% of the pendant alkene bonds reacted (i.e. 30% crosslinking) for AT-IR and Raman spectroscopy. AT-IR characteristic peaks 663 cm$^{-1}$ (C-S stretching); Raman spectroscopy 620 cm$^{-1}$ (C-S stretching) (Figure 4.1)

![Scheme 4.3 Model reaction for photo-induced thiol-ene ‘click’ crosslinking reaction.](image)

4.3.5 Mechanical testing

Poly(PHE) and poly(PHE)-crosslinked were tensile tested following ASTM D638. In brief, samples were vacuum compression molded (TMP 35 ton vacuum molding press) into dog bone shapes (Appendix Figure 21) at 85 °C and 24 inHg, at 10, 15, 20, and 25 klbs for 2 minutes each, respectively. Tensile testing was performed on an Instron 5567 tensiometer. Samples were pulled at a rate of 5 mm/min at room temperature until they reached their breaking point. The Young’s modulus ($E$) was calculated from the stress-strain curve using Equation 4.1.
\[ E = \frac{\sigma(\varepsilon)}{\varepsilon} = \frac{F/A_0}{\Delta L/L} \]

Equation 4.1  Equation for calculated Young’s modulus \((E)\), where \(\sigma(\varepsilon)\) is the tensile stress (Pa), \(\varepsilon\) is the tensile strain, \(F\) is the force exerted on the object under tension (N), \(A_0\) is the original cross sectional area of the sample, \(\Delta L\) is the change in length (mm), and \(L\) is the original length of the sample (mm).

4.3.6 Synthesis of Cys-OGP[10-14]-Cys

Cysteine \(N\)- and \(C\)-terminated OGP[10-14] (CYGFGGC) was synthesized with standard solid phase FMOC synthetic methods (Scheme 4.4). In brief, the FMOC-Cys(trt)-OH preloaded Wang resin was swelled in DMF for 15 minutes. FMOC deprotection was completed using 10% piperidine in DMF for 15 min two times, followed by continuous washing of the resin with DMF and then DCM. Coupling of FMOC-Gly-OH was conducted for 2 h with DIC (10 equiv.) and hydroxybenzotriazole (HOBT, 10 equiv.) in DMF under a \(N_2\) atmosphere. Coupling was followed by continuous DMF and DCM washes under \(N_2\). This process was repeated for each successive amino acid. Following the \(N\)-terminal FMOC-Cys(trt)-OH coupling, the peptide was continuously washed with DMF and DCM, respectively, then dried under a \(N_2\) blanket for 1 h. Final deprotection and cleavage was conducted using an acidic cleavage cocktail (88 \(wt.\%\) trifluoroacetic acid (TFA), 5 \(wt.\%\) phenol, 5 \(wt.\%\) water and 2 \(wt.\%\) triisopropylsilyl (TIPS)) under \(N_2\) atmosphere for 3 h. The TFA solution was collected and concentrated under vacuum, precipitated into cold diethyl ether, and washed 3 times in cold diethyl ether (centrifugation at 4900 rpm for 5 min each).
MALDI-TOF mass spectrometry revealed disulfide bond formation as a result of oxidation, therefore a disulfide reduction was conducted (Appendix Figure 22). Cys-OGP[10-14]-Cys was dissolved in 0.05 M NaHCO3, pH 8.3 (1 mg / 10 mL) under a N2 blanket. Dithiothreitol (DTT) was added to the reaction peptide solution (0.05 M), and the reaction vessel was heated to 55°C under a N2 blanket with stirring. After 2 h, the pH was dropped to pH ~3 with concentrated HCl. The solution was loaded onto a C18 silica column (Phenomenex) for purification. Cys-OGP[10-14]-Cys, was eluted from the column with 90% MeOH, concentrated in vacuo, and lyophilized to remove water. The product was verified with MALDI-TOF mass spectrometry ([M] theoretical = 705.80 g/mol, [M + Na]^+ calculated = 728.14 g/mol) (Appendix Figure 22).

Scheme 4.4 Cys-OGP[10-14]-Cys was synthesized via Fmoc-protected solid phase peptide synthesis.
4.3.7 Thiol-ene ‘click’ crosslinking of Poly(1-PHE-6)-co-(1-PHE-3) with Cys-OGP[10-14]-Cys (poly(PHE)-3%OGP)

A 3% crosslinked network via a thiol-ene ‘click’ reaction was conducted using Cys-OGP[10-14]-Cys (Scheme 4.5) following the same procedure as described for poly(PHE)-crosslinked. Poly(1-PHE-6)0.70-co-(1-PHE-3)0.30 was dissolved in DMF (0.02 M). Once dissolved, Cys-OGP[10-14]-Cys (1 mol/ene) and Irgacure 2959 (0.07 mol/ene) were added to the reaction flask and protected from light. The reaction solution was freeze-pump thawed 3 times to remove O₂. The solution was irradiated with 365 nm at room temperature (Eiko F5T8 BLB 8W) for 2 h without stirring. The product was precipitated into H₂O and dried under vacuum.

Scheme 4.5 Cys-OGP[10-14]-Cys was crosslinked into the poly(PHE) network via photoinitiated thiol-ene ‘click’ chemistry.
4.3.8 Swelling studies for poly(PHE)-3%OGP

The initial mass ($M_{T=0}$) of the completely dried poly(PHE)-3%OGP samples (N=3) was recorded. Poly(PHE)-3%OGP was submerged in DMF and the mass of the swollen sample was recorded at each time point ($T = 1, 2, 4, 6, 10, 24$ h). Prior to recording the mass, the sample was allowed to dry on a paper towel for 1 min to remove all surface DMF. The swelling percentage ($%S$) was determined using Equation 4.2, where $M_T$ is the swollen mass at time $T$, and $M_{T0}$ is the dry initial mass. Samples were tested in triplicate.

$$%S = \frac{M_T - M_{T0}}{M_{T0}} \times 100$$

Equation 4.2 The swelling percentage of poly(PHE)-3%OGP and poly(PHE)-3%crosslinked was calculated over the course of 6 h.

4.3.9 Fabrication of spin coated slides for in vitro studies

Glass coverslips (18 mm diameter) (Fisher Scientific, Pittsburgh, PA) were washed with toluene, methanol, and toluene, then cleaned with UV ozone for 10 min, respectively. A solution of poly(PHE) (10 wt.% in DMF) was dissolved and filtered using a 5.0 µm and 1.2 µm filter, respectively. For poly(PHE)-3%OGP samples, Cys-OGP[10-14]-Cys (1 mol/ene) and Irgacure 2959 (0.07 mol/ene) were added to the filtered solution and protected from ambient light. Thin films (300 nm) were spin coated onto the glass slides using a Spin Processor (Model WS-400-6NPP/LITE, Laurell Technologies Corp.) at 2000 rpm for 2.5 min with an acceleration of 5. Following spin coating, poly(PHE)-3%OGP coated slides were protected from O₂ on the vacuum line, and irradiated for 5 min with 365 nm (8 W) UV light (Eiko F5T8 BLB) at ambient temperature. All thin films were annealed
in a vacuum oven at 60 °C for 6 h, EtO sterilized for 12 h (Anprolene Gas Sterilizer, AN 74i, Anderson Products, Inc.), and purged under vacuum for 74 h.

4.3.10 MC3T3-E1 cell culture

MC3T3-E1 mouse preosteoblast cells (bone/calvaria source, passage 17) (ATCC, Manassas, VA) were expanded and cultured in α-MEM medium supplemented with 10 vol% fetal bovine serum (FBS), 100 units/mL penicillin and 100 µg/mL streptomycin at 37 °C and 5% CO₂. The cells were subcultured every other day with 0.25% (w/v) trypsin and 0.5% (w/v) ethylenediaminetetraacetic acid tetrasodium salt (EDTA) solution.

4.3.11 PrestoBlue Metabolic Assay

MC3T3-E1 cells were seeded onto sterilized poly(PHE) and poly(PHE)-3%OGP thin films at 1600 cells/cm² (passage 19). Plain glass coverslips were seeded at identical concentrations as controls. Cells were cultured with α-MEM medium supplemented with 10 vol% fetal bovine serum (FBS), 100 units/mL penicillin and 100 µg/mL streptomycin at 37 °C and 5% CO₂. The PrestoBlue assay was performed on samples for days 1, 3 and 7 following the manufacturer’s protocol. In brief, a standard curve was prepared by seeding serial dilutions at known concentrations into a 12-well plate, 24 h before the experiment. Eight descending concentrations were seeded with a corresponding blank well. A 1:10 solution of PrestoBlue in culture medium was prepared and sterilized through a 0.22 µm syringe filter. Culture medium was aspirated from the samples and standard curve and 1 mL of PrestoBlue solution was added to each well. The samples were incubated at 37 °C and 5% CO₂ until the standard curve fluorescence could be fit to a linear
line. The samples’ fluorescence was read in the Plate Reader (Synergy Biomax) by excitation at 570 nm and emission at 615 nm. The blank absorbance value was subtracted from each reading, and the fluorescence of the samples was calculated using the standard curve generated.

4.3.12 Immunofluorescence staining of cytoskeletal actin and vinculin

All samples were pre-fixed in pre-warmed 0.8 mL cell culture medium and 1.2 mL 3.7% paraformaldehyde (PFA) in cytoskeletal (CS) buffer for 5 min at 37 °C on a dry block. Next, samples were fixed in 3.7% PFA in CS buffer for 5 min at 37 °C on a dry block, followed by three CS buffer washes. Permeabilization of the cells was performed by washing in Triton X-100 in CS buffer (0.5% v/v) for 10 min on the dry block at 37 °C. The samples were washed three times with CS buffer. Freshly made 0.1 wt.% NaBH₄ in CS buffer was added to the aspirated samples for 10 min at ambient temperature to quench any aldehyde fluorescence. The NaBH₄ solution was aspirated, and the samples were incubated in 5% donkey serum in CS buffer for 20 min at ambient temperature to block non-specific binding. The donkey serum solution was aspirated and the samples were incubated overnight at 4 °C in monoclonal vinculin primary antibody (mouse, v/v 1:200) (Sigma-Aldrich) in 1XPBS. After washing with CS buffer three times, the substrates were stained in rhodamine phalloidin (Excitation 540 nm/Emission 565 nm) (v/v 1:40) and Alexa Fluor 488 secondary antibody (Excitation 490 nm/Emission 525 nm) (mouse, v/v 1:200) in 1XPBS solution for 1 h at ambient temperature, protected from light. After washing with CS buffer three times, the nuclei were stained with DAPI dilactate in 1XPBS (Excitation 358 nm/Emission 461 nm) (6 µL/10 mL) for 20 min at room temperature, protected from
light. Samples were washed three times with 1XPBS and imaged using an IX81 Microscope (Olympus, Center Valley, PA) with a mercury bulb excitation and FITC (Excitation 467 nm/Emission 556 nm), TRITC (Excitation 532 nm/Emission 613 nm), and DAPI (Excitation 358 nm/Emission 461 nm) filters.

4.3.13 Quantitative real-time polymerase chain reaction (qRT-PCR)

On day 7 of in vitro culture, total RNA was isolated from the MC3T3-E1 cells using the RNeasy Mini Kit (Qiagen, Valencia, CA). In brief, the cells were scraped from the glass slides, homogenized using a tissue tearor, and lysed with 600 µL of the provided buffer solution. The cell lysate was applied to the RNeasy Mini column for total RNA isolation following the manufacturer’s protocol. DNase digestion was performed during RNA isolation using the Qiagen RNase free DNase set (Qiagen, Valencia, CA). Total RNA was quantified using a Take3 Multi Volume Plate and Synergy MX Microplate Reader (BioTek, Winooski, VT) at 260 nm. The 260 nm/280 nm ratio was used to determine purity of RNA with wells showing values of above 1.5 being used for qRT-PCR. RNA was stored at -80 °C until used for reverse transcription. The Taqman Reverse Transcription Reagents kit (Life Technologies, Grand Island, NY) was used for reverse transcription into cDNA, following the manufacturer’s protocol. The reverse transcription thermal protocol was conducted on a 2720 Thermal Cycler (Applied Biosystems) and consisted of incubation at 25 °C for 10 min, reverse transcription at 48 °C for 30 min, and inactivation at 95 °C for 5 min. cDNA was stored at -20 °C until qRT-PCR was performed.

The qRT-PCR was performed on a 7500 Real Time PCR System (Applied Biosystems) using SYBR Green Master Mix (Quanta Biosciences, Inc.) and SYBR Green
primers. In brief, 50 µL reactions were prepared in PCR plates with 2X SYBR Green Master Mix, 209.4 nM forward primer, 209.4 nM reverse primer (Table 4.1) and 20 ng of cDNA. The remaining reaction volume was filled with DNase, RNase free H₂O. For all genes, the thermal protocol consisted of reverse transcription at 50 °C for 30 min, activation at 95 °C for 15 min, and 40 amplification cycles of denaturing for 30 s at 95 °C, annealing for 1.5 min at 58 °C, and extension for 2 min at 72 °C. Following amplification, a melt curve analysis was performed at 1°C increments from 50 °C to 95 °C to analyze the purity of the product generated. qRT-PCR results were used for relative quantification of runt related transcription factor (Runx2) and bone sialoprotein (BSP) using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous reference gene. Relative expression of Runx2 and BSP was reported as a normalized gene expression value, 2^{\Delta\Delta C_t}, which was calculated using Equation 4.3. Here, C_{t-GoI} is the average threshold value for the gene of interest on poly(PHE)-3%OGP, C_{t-REF} is the average threshold value for GAPDH on poly(PHE)-3%OGP, C_{t-GoI} is the average threshold value for the gene of interest on the poly(PHE), C_{t-REF} is the average threshold value for GAPDH on the poly(PHE).
Table 4.1 qRT-PCR forward and reverse SYBR green primers for RNA expression quantification

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Sequence direction</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>mGAPDH-f</td>
<td>GAAGGGCTCATGACCACAG</td>
<td>Forward</td>
<td>SYBR Green</td>
</tr>
<tr>
<td>mGAPDH-r</td>
<td>GGGCCATCCACAGTCTTC</td>
<td>Reverse</td>
<td>SYBR Green</td>
</tr>
<tr>
<td>mRUNX2-f</td>
<td>ATGCCTCCGCTGTTATGAA</td>
<td>Forward</td>
<td>SYBR Green</td>
</tr>
<tr>
<td>mRUNX2-r</td>
<td>AGGTGAAACTCTTGCCTCGT</td>
<td>Reverse</td>
<td>SYBR Green</td>
</tr>
<tr>
<td>mBSP-f</td>
<td>GAGAAACAATCCGTGCGACT</td>
<td>Forward</td>
<td>SYBR Green</td>
</tr>
<tr>
<td>mBSP-r</td>
<td>GCTTTGATTCTTGCATGGAAAT</td>
<td>Reverse</td>
<td>SYBR Green</td>
</tr>
</tbody>
</table>

\[
\Delta C_{t-\text{Control}} = C_{t-\text{GOI}} - C_{t-\text{REF}} \\
\Delta C_{t-\text{Sample}} = C_{t-\text{GOI}} - C_{t-\text{REF}} \\
\Delta \Delta C_t = \Delta C_{t-\text{Sample}} - \Delta C_{t-\text{Control}} \\
\text{Fold difference} = 2^{-\Delta \Delta C_t}
\]

Equation 4.3 Fold difference was calculated for total RNA expression of Runx2 and BSP on day 7 using the provided equations.

4.3.14 Statistics

All experiments were conducted with three replicates (n=3). A Shapiro-Wilk test for normality of data was conducted prior to statistical analysis. A significance value greater than 0.05 indicated normality of data. A one-way analysis of variance (ANOVA) with Tukey post hoc analysis was conducted for proliferation data to compare each sample type at all three time points. A significance value of \( p \leq 0.05 \) with a 95% confidence interval was set for all statistical analysis. An independent student’s t-test was conducted for comparative qRT-PCR data between poly(PHE)-3%OGP and poly(PHE) with a
significance value of p ≤ 0.05 and a 95% confidence interval. All quantitative data are presented as the average ± standard deviation.

4.4 Results

A summary of the results for all of the data collected for this chapter is provided in this section.

4.4.1 Characterization of chemical structure and thermal properties

The complete characterization of poly(PHE), poly(PHE)-3%crosslinked, and poly(PHE)-3%OGP is shown in Table 4.2. Deviating from expected results, there is no statistical increase in $T_g$ when 3% crosslinking is added to poly(PHE). However, there is an increase in $T_d$ after 3% crosslinking has been incorporated into the network. AT-IR and Raman spectroscopy (Figure 4.1) for poly(PHE) compared to poly(PHE)-100%crosslinked, reveal the formation of the C-S peak after crosslinking at 663 cm$^{-1}$ and 620 cm$^{-1}$, respectively. Appendix 23-26 show the DSC and TGA traces for poly(PHE)-3%OGP and poly(PHE)-3%crosslinked.

Table 4.2 Thermal properties of poly(PHE) and crosslinked the crosslinked networks.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$M_n$ a (kDa)</th>
<th>$M_w$ a (kDa)</th>
<th>$D$</th>
<th>$T_d$ b (°C)</th>
<th>$T_g$ c (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(PHE)</td>
<td>26.5</td>
<td>39.0</td>
<td>1.47</td>
<td>264</td>
<td>60</td>
</tr>
<tr>
<td>Poly(PHE)-3%crosslinked</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>278</td>
<td>62</td>
</tr>
<tr>
<td>Poly(PHE)-3%OGP</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>278</td>
<td>60</td>
</tr>
</tbody>
</table>

a molecular weights determined at 50 °C, with a polystyrene standard, 0.1 M LiBr in DMF as the eluent, 5 mg/mL, flow rate 0.5 mL/min, RI detector
b $T_d$ determined at a ramp speed of 20 °C/min
c $T_g$ determined at a ramp speed of 10 °C/min with three cycles performed
Mechanical testing

Tensiometer testing was performed on vacuum compressed dog bone shaped tensile bars for both poly(PHE) and poly(PHE)-3%crosslinked to determine the effect of 3% crosslinking on Young’s modulus (Figure 4.2). Prior to crosslinking, the modulus of poly(PHE) was calculated to be 756.9 ± 30.9 MPa, with a yield point at 2.5 ± 0.7% strain.

Figure 4.1 AT-IR and Raman spectroscopy were used to verify the successful formation of the C-S bond during the thiol-ene ‘click’ reaction to form the crosslinked network. Poly(PHE)-100%crosslinked was used for this characterization so the C-S bond could be fully visualized. The appearance of the AT-IR peak at 663 cm\(^{-1}\) demonstrates the successful formation of the C-S bond. This stretching can be more easily detected using Raman spectroscopy, in which the appearance of the peak at 620 cm\(^{-1}\) is apparent.
After 3% crosslinking had been added, the Young’s modulus significantly decreased to 10.2 ± 7.8 MPa for poly(PHE)-3%crosslinked. However, the crosslinked material was reported to have a yield point at 158.3 ± 49.4 % strain. With the addition of just 3% crosslinking to poly(PHE), a decrease in stiffness and an increase in toughness resulted. Previous reports of hyperbranched phenylalanine-based PEUs revealed that as a higher percentage of branched monomers (up to 10%) were added to the polymer network, a decrease in Young’s moduli and an increase in toughness of the materials resulted. The data presented here are consistent with this trend.

Figure 4.2 Young’s modulus was measured for poly(PHE) and poly(PHE)-3%crosslinked. Prior to crosslinking, the tensile modulus of poly(PHE) 756.9 ± 30.9 MPa, whereas the poly(PHE)-3%crosslinked materials had a decreased tensile modulus of 10.2 ± 7.8 MPa. With such low crosslinking incorporation into the network, the hydrogen bonding of the PEU backbone is disrupted, causing an increase in elasticity and a decrease in stiffness.
4.4.3 Swelling studies of poly(PHE)-3%crosslinked and poly(PHE)-3%OGP

Swelling studies were performed on poly(PHE)-3%crosslinked and poly(PHE)-3%OGP samples, to prove crosslinking had been successfully accomplished. For both poly(PHE)-3%crosslinked and poly(PHE)-3%OGP the maximum swelling percentage was reached with $579 \pm 50 \text{ S\%}$ and $671 \pm 38 \text{ S\%}$, respectively (Figure 4.3). The swelling study conducted over a period of 6 hours without any significant change in swelling percentage.

Figure 4.3 Swelling studies were performed on poly(PHE)-3%crosslinked and poly(PHE)-3%OGP in DMF over a period of 6 h. After 1 h, each polymer network was swollen to its maximum capacity of $671 \pm 38 \text{ S\%}$ and $579 \pm 50 \text{ S\%}$ for poly(PHE)-3%crosslinked and poly(PHE)-3%OGP.
4.4.4 PrestoBlue proliferation assay of MC3T3-E1

Proliferation of MC3T3-E1 cells was tracked on days 1, 3 and 7 of \textit{in vitro} culture for detection of enhanced osteoblast proliferation in the presence of crosslinked OGP[10-14] (Figure 4.4). Day 1 proliferation revealed an efficient seeding process, as indicated by the equivalent cell density on each sample after 24 h of seeding. By day 3, there is a significantly greater cell density on poly(PHE)-3%OGP (4594 $\pm$ 352 cells/cm$^2$) samples compared to poly(PHE) (3460 $\pm$ 337 cells/cm$^2$) and glass controls (3823 $\pm$ 124 cells/cm$^2$). The poly(PHE) and glass samples are comparable in cell density by day 3. These results indicate that the 3% incorporation of OGP[10-14] crosslinker into the poly(PHE) network upregulates the proliferation of MC3T3-E1 cells as expected. By day 7, the cell number on each sample drastically increased to $5.73 \pm 0.52 \times 10^4$ cells/cm$^2$ and $5.65 \pm 0.79 \times 10^4$ cells/cm$^2$ on poly(PHE)-3%OGP and poly(PHE) respectively. These cell numbers are statistically lower than on glass controls ($7.23 \pm 0.17 \times 10^4$ cells/cm$^2$) with a p value of 0.001.
Figure 4.4 Proliferation of MC3T3-E1 cells was tracked on days 1, 3 and 7 of in vitro culture. On day 1, the same cell density was calculated for each surface. By day 3, there is a significantly greater cell density on poly(PHE)-3%OGP compared to poly(PHE) and the glass control. This is an indication that even when OGP[10-14] is tethered at the N- and C-terminus, enhanced proliferation is observed. On day 7, the cell density on glass substrates is significantly greater than on poly(PHE)-3%OGP and poly(PHE). As cells progress from the proliferative to the differentiation phase of osteogenesis, proliferation ceases. The greater cell density on glass substrates may indicate that cells remain in the proliferative phase on these substrates, while they are progressing to the osteoprogenitor stage on poly(PHE)-3% and poly(PHE). Significant differences have a p value ≤ 0.05.
4.4.5 Immunofluorescence staining of cytoskeletal actin and vinculin

Actin fibers and focal adhesion points were labeled with fluorescent markers via immunofluorescence on day 3 (Figure 4.5). Here, it is apparent that regardless of sample, the cells are well spread and show abundant adhesion to the material surface. MC3T3-E1 cells are a mouse preosteoblast cell line, therefore it is expected that the cells will have well defined actin filament structures, as seen in all of the images on each surface. Regardless of OGP[10-14] crosslinking into the poly(PHE) network, the cells are viable and well spread on the surface.

![Vinculin and Rhodamine Phalloidin Day 3](image)

Figure 4.5 Vinculin and actin filaments were stained using immunofluorescence on day 3 to observe cell focal adhesion and spreading. Strong adhesion and well-structured actin stress fibers were observed on all of the samples which is indicative of osteogenic differentiation. Scale bar for all images = 200 µm

4.4.6 Quantitative real-time polymerase chain reaction (qRT-PCR)

Relative gene expression of Runx2 and BSP compared to GAPDH was quantified on day 7 of in vitro culture to detect early and middle stage markers of osteoblast
differentiation. Runx2 is an essential transcription factor expressed by preosteoblast cells during the proliferative phase of differentiation. qRT-PCR was used to calculate the upregulation or downregulation of the RNA expression on poly(PHE)-3%OGP compared to the normalized value of gene expression on poly(PHE). On day 7, Runx2 expression on poly(PHE)-3%OGP was significantly upregulated 120% compared to the normalized poly(PHE) films (p = 0.03) (Figure 4.6). BSP expression is often used as a marker to track the osteogenic differentiation of cells. During the osteoprogenitor stage of differentiation, a spike in BSP expression is observed, indicating osteoblast lineage commitment and the progression of osteogenic differentiation. Comparative qRT-PCR for BSP on poly(PHE)-3%OGP and poly(PHE) surfaces shows no significant upregulation of expression on samples with crosslinked OGP[10-14] (Figure 4.6). qRT-PCR data suggests that crosslinked OGP[10-14] is bioactive and capable of upregulating osteoblast gene expression of preosteoblast cells \textit{in vitro} on PEU surfaces.
Figure 4.6 RNA expression is an accurate method of tracking gene expression during osteoblast differentiation. On day 7 of in vitro culture, MC3T3-E1 cells were tested for RNA expression of RUNX2 and BSP. RUNX2 is an early transcription factor, essential for osteogenic differentiation. As cells progress from the proliferative phase to the osteoprogenitor stage, cells begin to express BSP, which is vital for osteoblast cell adhesion and matrix maturation. RUNX2 expression is upregulated in MC3T3-E1 cells seeded on poly(PHE)-3%OGP, with 120% greater expression observed, respectively, compared to poly(PHE). These results indicate that there OGP[10-14] crosslinked into poly(PHE) networks is bioactive and capable of enhancing osteogenic differentiation of preosteoblast cell lines. * is used to denote significance between poly(PHE)-3%OGP and poly(PHE), RUNX2 gene expression with p ≤ 0.05.
4.5 Discussion

Incorporation of bioactive peptides into polymeric networks is a commonly studied area of research for tissue engineering applications. Various peptides have been tethered to polymer substrates and scaffolds, non-covalently incorporated into polymer composite materials, and added to medium as soluble growth factors for driving specific lineage differentiation on polymer surfaces. While these methods have been proven useful for tissue regeneration applications, room for improvement exists, particularly in the field of bone tissue engineering.

Bone tissue engineering requires polymeric materials that are high modulus, osteoinductive, and non-toxic. The Becker group has previously reported the use of phenylalanine-based PEUs for bone regeneration applications, as it is a stiff, biodegradable material with non-acidic degradation byproducts. PEUs are also easily functionalized using various methods of efficient ‘click’ reactions in order to enhance their osteoinductive capabilities. In earlier work, OGP was tethered to phenylalanine-based PEUs via an alkyne-azide ‘click’ reaction. As minimal as 1% incorporation of tethered OGP[10-14] resulted in enhanced bone differentiation of human mesenchymal stem cells. In an effort to further enhance the efficacy of these materials for bone regeneration applications, this work focuses on the crosslinking of OGP[10-14] into an alkene-functionalized phenylalanine-based PEU network via the well-established thiol-ene ‘click’ reaction to access tunable mechanical and osteoinductive properties.
4.5.1 Synthesis and characterization of poly(PHE), poly(PHE)-crosslinked and poly(PHE)-3%OGP

Poly(PHE) and poly(PHE)-3%OGP have been successfully synthesized and studied for alterations in thermal, mechanical, and biological characteristics. While incorporation of 3% crosslinking with both 1,6-hexanedithiol, as a model, and OGP[10-14] was difficult to detect using methods of spectroscopy (AT-IR and Raman), successful crosslinking was verified via swelling data collected over 6 h. Both the poly(PHE)-3%crosslinked and poly(PHE)-3%OGP polymers showed a swelling plateau around 1 h in DMF. Generally, swelling percentage is used for calculating the crosslink density of a polymer or hydrogel network\(^{254-256}\), however as this is a random step-growth polymerization, it is difficult to determine the chain length between crosslinks. For this reason, swelling was strictly used to prove crosslinking was successfully employed. The successful thiol-ene ‘click’ reaction was structurally characterized using the poly(PHE)-100%crosslinked. AT-IR and Raman spectroscopy reveal the formation of a C-S stretch\(^{257}\) for poly(PHE)-100%crosslinked. The broad peak from 2445-2611 cm\(^{-1}\) is seen in poly(PHE)-100%crosslinked as well, which may be indicative of free S-H bonds, therefore, it cannot be assumed that all of the available pendant alkene bonds underwent the photoinitiated thiol-ene ‘click’ reaction. Finally, the thermal properties (\(T_g\) and \(T_d\)) were determined for poly(PHE) and poly(PHE)-3%OGP. It is typically seen that low crosslinking densities of polymers will not increase \(T_g\) significantly,\(^{258}\) which was seen for 3% crosslinking of poly(PHE) with Cys-OGP[10-14]-Cys. Overall, the photoinitiated thiol-ene reaction was successfully utilized for the crosslinking of both 1,6-hexanedithiol and Cys-OGP[10-14]-Cys into the poly(PHE) network.
Poly(PHE)-3%crosslinked was employed as a model experiment for measuring the change in mechanical properties of poly(PHE). Thermal processing techniques were used for the fabrication of samples used for tensile testing, therefore poly(PHE)-3%OGP was not used for these studies to avoid peptide degradation during processing at high temperatures. Crosslinking of polymer chains is important for tailoring mechanical properties. Generally, high crosslink densities have been shown to increase the rigidity of polymer networks resulting in increased stiffness and decreased elongation. Here, it is observed that 3% crosslinking results in a lower Young’s modulus compared to the uncrosslinked, linear poly(PHE). These results coincide with the previously reported results of hyperbranched phenylalanine-based PEUs. Similar to what Yu, et. al, reported, the disruption in crystallinity as a result of crosslink formation between chains, decreases the rigidity of the polymer network. Phenylalanine-based PEUs make up a complex network that relies on strong hydrogen bonding between urea and carbonyl groups for high modulus and rigidity. It is probable that the 3% covalent crosslinking added to poly(PHE)-3%OGP upsets this physical network, resulting in a less stiff, and more elastic polymer. A similar trend was also observed for crosslinked poly(vinyl alcohol), in which the strong hydrogen bonded network was disrupted with low crosslink densities (2-10%), resulting in a lower modulus compared to the parent polymer. This is a trend typically seen for semicrystalline polymers.

4.5.2 MC3T3-E1 proliferation

The beginning of the cell differentiation timeline starts with a proliferative phase, during which cells are expanding on the culture surface. The proliferation of mouse
MC3T3-E1 preosteoblast cells was tracked on days 1, 3 and 7 using the PrestoBlue assay to determine metabolic activity at each time point. On day 1, each of the substrates shows equivalent cell densities, indicating seeding efficiency and precision. By day 3, there is a statistically greater cell density on poly(PHE)-3%OGP compared to poly(PHE) and glass controls. As both a soluble and tethered growth factor, OGP has been shown to enhance proliferation of preosteoblast cell lines in vitro.\textsuperscript{177, 233, 237} Day 3 results suggest that during this early proliferative phase of differentiation, OGP[10-14] that has been tethered at both the \textit{N}- and \textit{C}-terminus is still capable of enhancing cell proliferation of MC3T3-E1s. By day 7 of culture, the cell density of both poly(PHE)-3%OGP and poly(PHE) are statistically equivalent, however they are significantly lower than the glass controls. This may be an indication that the preosteoblast cells are progressing from the proliferative phase to the osteoprogenitor stage of differentiation on PEU-coated substrates, regardless of OGP[10-14] functionalization. The cells that have been cultured on the glass substrates appear to still be in the proliferative phase at day 7.

4.5.3 MC3T3-E1 adhesion and spreading

Lineage commitment of cells has been correlated to chemical, physical and biological signaling from the cell’s environment. Many studies have shown that both adhesion and cell spreading to a surface regulates lineage commitment.\textsuperscript{160, 262, 263} It has been reported that well adhered, flattened and spread cells predominantly undergo osteogenic differentiation, whereas round, unspread cells tend to follow the pathway toward adipogenic differentiation.\textsuperscript{262} In this respect, the substrate with which a cell comes in contact plays a major role in the chosen differentiation pathway. Vinculin and
rhodamine phalloidin staining revealed that the cells are well spread and adhered to each surface that was tested. It was not expected that the cell morphology would differ from poly(PHE) to poly(PHE)-3%OGP samples. The OGP[10-14] peptide does not contain the well-known Arg-Gly-Asp (RGD) adhesive amino acid sequence,\textsuperscript{163, 264, 265} therefore OGP[10-14] would not be expected to yield enhanced cell adhesion and spreading. Cells adhere to surfaces via membrane spanning integrins, which bind specific motifs in the extracellular matrix with proteins in the cell.\textsuperscript{160} Vinculin is a protein that is recruited to form focal adhesions when this binding phenomenon occurs between a cell and its environment.\textsuperscript{155} The images clearly show that on all surfaces the cells are well adhered through vinculin focal adhesions. Furthermore, it is evident that the cells are well spread, as indicated by the formation of long actin stress fibers. Previous literature has shown that this well-organized actin fiber formation is observed for cells undergoing osteogenic differentiation.\textsuperscript{154, 262, 266} MC3T3-E1 cells are a mouse preosteoblast cell line, therefore it is expected that spreading results would not differ between samples. From this data, we can conclude that the cells are strongly adhered to the surface of the poly(PHE) and poly(PHE)-3%OGP and that OGP[10-14] does not enhance spreading and adhesion on the polymer surface.

4.5.4 qRT-PCR

During the proliferative phase of osteogenic differentiation, cells begin to express Runx2, which is a multifunctional transcription factor necessary for regulating osteoblast formation.\textsuperscript{177, 237, 267-269} Binding of OGP[10-14] to the G protein cell receptor leads to downstream activation of the mitogen-activated protein (MAP) kinase signaling pathway.
The activation of the MAP kinase pathway aids in the regulation of osteoblast Runx2 RNA expression. By day 7, there is significantly greater Runx2 expression on poly(PHE)-3%OGP compared to poly(PHE), indicating the progression of cells through early differentiation of osteogenesis. As cells continue through the osteoprogenitor stage of differentiation, a spike in BSP expression should be observed. BSP is an acidic phosphoprotein that is expressed in the organic matrix of mineralized tissue. This RGD-containing protein promotes cell adhesion and induces mineralization of osteoblasts. By day 7, there appears to be an upregulation of BSP RNA expression detected on poly(PHE)-3%OGP compared to poly(PHE), however, statistics reveal no significant difference. BSP is generally expressed during the osteoprogenitor stage, and by day 7, the MC3T3s are just starting to enter this phase of differentiation, therefore a statistical difference in BSP expression could possibly be observed at a later time point. As cells progress through the timeline of osteogenesis, an upregulation in Runx2 is observed, followed by an increase in BSP expression. It remains justifiable that by day 7, the MC3T3-E1 cells are undergoing this osteogenic progression on poly(PHE)-3%OGP samples. It appears that the use of phenylalanine-based PEU networks with OGP[10-14] crosslinkers enhances the differentiation of preosteoblast cells in vitro.

4.6 Conclusion

Much improvement has been made in the field of tissue engineering over the past decade, particularly for bone regeneration. While synthetic polymeric materials can be fine-tuned for particular biomaterial applications, they lack the natural bioactive signals necessary to drive specific lineage differentiation for optimal tissue regeneration. To
overcome these limitations, researchers have studied the functionalization effect of growth factors and peptides on polymeric structures to enhance cell lineage commitment. OGP[10-14] has attracted minimal attention for this application since its discovery in 1992 by Bab, et. al, however, it is known to upregulate proliferation, differentiation, and mineralization in preosteoblast cell lines. Functionalization of phenylalanine-based PEU networks with OGP[10-14] has been conducted previously in the Becker lab, to reveal its effect on hMSC osteogenic differentiation on peptide-tethered scaffolds. Here, OGP[10-14] was used as a crosslinker for poly(PHE) networks, in order to study the change in mechanical properties and osteoinductive ability of the functionalized polymer.

Mechanical properties of poly(PHE and poly(PHE)-3%crosslinked were determined. With only 3% incorporation of crosslinking, the stiffness of the high modulus poly(PHE) was decreased. Hydrogen bonding is a major contributor to providing stiffness to the poly(PHE) network, however, small incorporation of crosslinkers causes disorder in the hydrogen bonding network between chains and results in a more elastic polymer. It is expected that an increase in crosslink density would lead to an increase in Young’s modulus. Bone tissue engineering requires a high modulus material; therefore, the crosslink density would have to be optimized to ensure a comparable modulus to that of native bone tissue. Overall, it has been demonstrated that the thiol-ene ‘click’ reaction can be utilized as a means of generating peptide-crosslinked networks for tissue regeneration applications with the potential to achieve tunable mechanical properties.

MC3T3-E1 proliferation and differentiation studies up to 7 days in vitro were performed to briefly investigate the bioactivity of OGP[10-14] as a crosslinked peptide. Here, 3% incorporation of OGP[10-14] was added to the poly(PHE) network to test cell
activity on spin-coated samples. It has been demonstrated that while tethered at both the
$N$- and $C$-terminus in a 3% crosslinked network, OGP[10-14] remains bioactive and functions to upregulate proliferation and osteoblast differentiation of preosteoblast cells. To the best of our knowledge, this is the first report of synthetic polymeric networks being covalently crosslinked with growth factors via ‘click’ chemistry techniques for the enhancement of cellular response for osteoinductive applications.

The successful incorporation of OGP[10-14] as a crosslinked peptide into the poly(PHE) network suggests that this efficient functionalization method can be used for enhancement of mechanical properties and osteoinduction of polymeric networks for bone regeneration applications. Furthermore, this crosslinking strategy can be applied to synthetic materials across the board, as a means of incorporating bioactive peptides into networks for tissue engineering applications.
CHAPTER V

BIOACTIVE SURFACE MODIFICATION OF METAL OXIDES VIA CATECHOL-BEARING MODULAR PEPTIDES MULTIVALENT-BINDING SURFACE RETENTION AND PEPTIDE BIOACTIVITY

This work has been previously published as


5.1 Abstract

A series of multivalent dendrons containing a bioactive osteogenic growth peptide (OGP) domain and surface-binding catechol domains were obtained through solid phase synthesis and their binding affinity to hydroxyapatite, TiO₂, ZrO₂, CeO₂, Fe₃O₄ and gold was characterized using a quartz crystal microbalance with dissipation (QCM-d). Using the distinct difference in binding affinity of the bioconjugate to the metal oxides, TiO₂-coated glass slides were selectively patterned with bioactive peptides. Cell culture studies demonstrated the bioavailability of the OGP and that OGP remained on the surface for at least two weeks under in vitro cell culture conditions. Bone sialoprotein (BSP) and osteocalcin (OCN) markers were upregulated 3-fold and 60-fold, respectively, relative to controls at 21 days. Similarly, 3-fold more calcium was deposited using the OGP tethered dendron compared to TiO₂. These catechol-bearing dendrons provide a fast and efficient
method to functionalize a wide range of inorganic materials with bioactive peptides and have the potential to be used in coating orthopedic implants and fixation devices.

5.2 Introduction

Immobilization of bioactive peptides onto surfaces has been proven to be an effective avenue to improve cell attachment,\textsuperscript{272} influence proliferation,\textsuperscript{177} and direct differentiation in tissue engineering.\textsuperscript{249,250} Physical adsorption/encapsulation and chemical conjugation have both been applied to derivatize tissue engineering scaffolds with bioactive peptides.\textsuperscript{236,273-276} Most of these methods were developed for polymeric materials, while the surface decoration of inorganic surfaces has received less attention, due to the lack of diversity in presenting functional groups for highly efficient chemical reactions.\textsuperscript{277-279} However, many inorganic materials are useful in the medical applications field. For instance, titanium and zirconia are widely used in prosthetic devices and dental implants;\textsuperscript{280,281} cerium oxide nanoparticles are potent antioxidants in therapeutics;\textsuperscript{282,283} and iron oxide magnetic nanoparticles are used to enhance the magnetic resonance imaging contrast in disease diagnostics.\textsuperscript{284} Thus the development of efficient and convenient methods to immobilize bioactive peptides onto the surface of metal oxide materials (TiO\textsubscript{2}, ZrO\textsubscript{2}, CeO\textsubscript{2}, Fe\textsubscript{3}O\textsubscript{4} and so on) will not only influence the cell behavior locally but will also contribute to the improvement of diagnostic and therapeutic techniques in the clinic.\textsuperscript{285}

Titanium is the most widely used material in bone implants and dental fixations due to its low density, high strength and high resistance to erosion. In physiological conditions, the oxide passivation layer of 2-20 nm TiO\textsubscript{2} is quickly formed on titanium implants.\textsuperscript{286} Several methods have been developed to decorate titanium implants with bioactive
peptides/proteins. Modifications can be achieved through physical interactions, such as protein-encapsulated coating, erosion and subject protein adsorption, and peptide-grafted polycation adsorption. However, the diffusion of loaded bioactive components may require high doses, and lead to low drug efficiency, and other adverse reactions. Chemical conjugation by generating reactive functional groups using electrochemical anodization, acid-etching, and oxidation have been utilized to covalently conjugate the bioactive moieties onto the titanium implant surface, which requires complicated procedures and changes the surface properties during the fabrication.

The presence of 3,4-dihydroxyphenylalanine (DOPA), which is found abundantly in mussel adhesive proteins, has been connected to the strong adhesion of mussels onto multiple surfaces in wet conditions. Catechol group is the functional group of DOPA, which is known to interact with titanium oxide surface through coordination bond or H-bond with pH sensitivity. Catechol is also crosslinked together under oxidative or basic conditions to form coating layers on surfaces. Thus it has been served as adhesive building blocks in the surface coating of variety of materials, including metal oxides, as well as organic polymers. Besides titanium oxide, the interaction of catechol with other metal oxides has also been studied, including iron oxide, chromium(III) oxide, manganese dioxide, aluminum oxide and zirconia. Anti-fouling ethylene glycol dendrons and glycocalyx layers have been successfully coated onto titanium oxide surfaces with catechol-functionalized oligomers as the surface-anchoring domain in the pioneered work. However, sequestering bioactive moieties, such as peptides that are known to direct cell behaviors, with catechol-bearing molecules on the surfaces of biomaterials has not been reported.
Modular peptides are conjugated molecules containing several different peptide sequences that are known to have specific bio-functionality. The Murphy group has successfully applied this molecular design in the modification of hydroxyapatite surfaces with osteoconductive peptides using a cost-effective dip-and-rinse procedure.\textsuperscript{310-312} In the modular peptides, there are two active components, the surface-binding peptide that sequesters the whole molecule on the hydroxyapatite surface and the bioactive subunit that influences the cell behavior. The loading concentration and retention time of the peptides on the surface are critical parameters that determine whether molecular signaling in the cell will be triggered. In many studies it was shown that the cell response to specific peptides is concentration-dependent.\textsuperscript{177, 313} However, in most applications, the concentrations that are required to trigger and sustain the cell response are less understood. Strong adsorption is the prerequisite to realize efficient immobilization with bioconjugate solutions at low concentration, and to retain the peptides on the surfaces over extended periods. Thus the key issue in the modification of surfaces with modular peptides is identifying the motifs that have strong binding affinity to the targeted surface and quantifying the binding affinity. Strategies and functional moieties that provide strong binding affinity are highly sought after.

Between two objects (\textit{eg.} molecules, proteins, surfaces of substrates and nanoparticles, cell membranes and so on), when there are more than one pair of ligand-receptor interaction binding simultaneously, a synergistic augment rises in binding affinity with an order of magnitude enhancement over the corresponding monovalent ligand.\textsuperscript{314, 315} This multivalent binding strategy has been used extensively in nature and synthetic molecules to enhance their respective binding affinity.\textsuperscript{316-320} Dendrimers are ideal...
platforms to construct multivalent binding ligands due to their abundant functional groups in the periphery region. Studies have shown that the molecular structure of the multivalent ligands, including binding valency, the flexible linkage units, molecular architecture and receptor density all play significant roles in the ultimate association constant of the multivalent ligand with its receptor. Models combining the macroscopic thermodynamics with microscopic probabilistic arguments based on the intramolecular reaction were employed to rationalize some experiment results. The optimization of molecular structures for the strongest binding is important in the understanding of multivalent binding process, as well as the design of strong adhesive motifs for drug targeting and sequestering purposes.

Osteogenic growth peptide (OGP) is an endogenous regulatory tetradecapeptide presents in mammalian serum with concentrations at the micromolar scale. Native or synthetic OGP regulates proliferation, alkaline phosphatase activity and matrix mineralization in studies of osteoblastic cell lines derived from human and other mammalian species. As its active portion, the carboxy-terminal pentapeptide, OGP[10-14] directs rat bone marrow mesenchymal stem cells to differentiate to osteoblasts. OGP or OGP[10-14]-functionalized biomaterials, including scaffolds for bone tissue engineering, gradient substrates, and peptide nanofibers have been prepared, and shown to promote both cell proliferation and osteogenic differentiation, in vitro and in vivo. Therefore, OGP[10-14] is a good candidate to be utilized in our attempts to enhance the osteoinductivity of conjugate-derivatized metal oxide implants.

In this paper, a series of modular peptides containing bioactive OGP[10-14], and a multivalent catechol-functionalized dendron were synthesized using solid phase synthesis.
The relationship between molecular structure, including binding valency (number of catechol groups in the molecule) and flexible linkage, and binding affinity was well elucidated to optimize the molecular structure for strong binding. The successful immobilization and retention time of peptides on the surface after a simple incubation-and-rinse procedure was studied using fluorescein-labeled peptides and X-ray photoelectron spectroscopy (XPS) analysis. The tetravalent ligand remained bioavailable on the surface in buffer at physiological pH for more than two weeks. The modular peptide exhibits strong interactions with several medically relevant materials (zirconia, cerium oxide, and iron oxide), demonstrating its usefulness in the surface modification of other biomedical devices. With the catechol-bearing modular peptides, selective surface modification was achieved on a patterned TiO$_2$-coated glass slide, which demonstrates a new method to fabricate surface with locally-restricted peptides. The immobilized OGP[10-14] was able to stimulate cell proliferation and promote osteogenic differentiation and mineralization for MC3T3-E1 cells cultured on the substrates.

5.3 Materials and Methods

All necessary purchasing and vendor information for materials and instrumentation will be listed in this section, followed by a complete experimental procedure.

5.3.1 Materials and equipment

Fmoc-protected amino acids were purchased from Novabiochem (San Diego, CA). Fmoc-NH-PEG6-Propionic acid was purchased from AAPPTec (Louisville, KY). Solvents were purchased from Sigma-Aldrich (St. Louis, MO). Unless otherwise stated, all solvents
used were reagent grade and all chemicals were used as supplied. The peptide synthesis was performed on a Liberty 1 peptide microwave synthesizer (CEM Cooperation, Matthews, NC). Reserved-phase high performance liquid chromatography (RP-HPLC) was performed on an Akta Purifier HPLC system by using a ZORBA 300SB-C18 column (5 μm, 9.4×250 mm). The HPLC-grade solvent was degassed before usage, with recipes as A: 0.1 % trifluoroacetic acid in H₂O and B: 0.085% trifluoroacetic acid in 95% acetonitrile and 5% H₂O. The flow rate was 4 mL/min with the pressure around 13 MPa. Fluorescence images were viewed on an IX81 Microscope (Olympus, Center Valley, PA). Quantification of the adsorption of catechol-bearing peptides onto surfaces was performed by a Q-sense E4 system (Bolin Scientific AB, Sweden). Electrospray ionization mass spectrometry (ESI-MS) spectra were recorded on a Waters Synapt HDMS quadrupole/time-of-flight (Q/ToF) instrument in positive mode. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectra were recorded on a Bruker Ultraflex III ToF/ToF mass spectrometer (Bruker Daltonics, Billerica, MA) equipped with Nd:YAG laser which emits at 355 nm. XPS measurements were performed on a Kratos AXIS Ultra DLD spectrometer (Manchester, U.K.) using silicon wafers or glass as substrates. A customized deposition system equipped with DC and RF magnetron sputtering sources was used to do the RF sputter coating. Thickness measurement was measured with NewView™ 7100 3D Optical Surface Profiler (Zygo, Middlefield, CT, USA). AFM images were achieved with a Veeco Nanoscope IIIA Atomic Force Microscope (Plainview, NY, USA). Absorbance or fluorescence was measured using a monochromator-based multi-mode microplate reader (Biotek, Winooski, VT). Cell culture media, immunohistochemical staining reagents, protein assays, Live/Dead assay,
PrestoBlue Assay, RNA extraction kit and cDNA reverse transcription kit was all purchased from Life technologies (Grand Island, NY). The concentration of calcium ions in the supernatant was measured with inductively coupled plasma optical emission spectrometry (ICP-OES) (Agilent Technologies 700 series, Santa Clara, CA, USA). Real time polymerase chain reaction (RT-PCR) was performed in the Applied Biosystems Real-Time PCR Instruments - 7500 Fast System (Life technologies, Grand Island, NY).

5.3.2 Fmoc-YGFGG-Resin

The synthesis was carried out with solid phase synthesis via microwave assistance in Liberty 1 peptide synthesizer. 0.25 mmol Fmoc-Gly-Wang Resin were added in the reaction vessel. The resin were first swelled in DMF for 15 minutes. And then the deprotection step, which was removing the Fmoc group to generate amine, was followed by adding 20 \( \nu\% \) piperidine in DMF with 0.1 M HOBT with microwave assistance programed by the Liberty 1 software. After thoroughly washing the resins, in the coupling step, 5 mL Fmoc-AA-OH (AA represents amino acid) solution (4 \( \text{equiv.} \), 0.2 M in DMF), the activator of HBTU (4 \( \text{equiv.} \), 2 mL of 0.5 M in DMF) and the activator base of DIPEA (8 \( \text{equiv.} \), 1 mL of 2 M in NMP) were added to couple the amino acid to the the N-termini of peptides on the resin with microwave assistance. The whole process was programed and carried out automatically by the peptide synthesizer. The obtained peptides on the resin were directly used for the next step (Scheme 5.1).

```
Fmoc-Gly\rightarrow \text{deprotection, } mw. \rightarrow Fmoc-Gly\rightarrow \text{coupling, } mw. \rightarrow Fmoc-Gly\rightarrow \text{deprotection, } mw. \rightarrow Fmoc-Gly\rightarrow \text{coupling, } mw. \rightarrow Fmoc-Gly
```

\( \text{Fmoc—Y—G—F—G—G—} \)

Scheme 5.1 Synthesis of Fmoc-YGFGG-Resin.
5.3.3 Dendron-YGFGG-Resin

The Lys-based dendron was conjugated to OGP[10-14] peptide by using Fmoc-Lys(Fmoc)-OH in the coupling step. Peptide 1 was used for the synthesis of OGP-(Cat)₂, and peptide 2 was used for the synthesis of OGP-(Cat)₄. The deprotection and coupling was carried out in the peptide synthesizer under standard conditions (Scheme 5.2). The obtained peptides on the resin were taken directly into the next step.

![Scheme 5.2 Synthesis of Dendron-YGFGG-Resin.](image)

5.3.4 Dendron-PEG-YGFGG-Resin

The hexaethylene glycol flexible linkage was conjugated by using Fmoc-NH-PEG6-Propionic acid in the coupling step. Then the Lys-based dendron was linked to the peptides. Peptide 3 was used for the synthesis of OGP-PEG-(Cat)₂, and peptide 4 was used for the synthesis of OGP-(Cat)₄. The deprotection and coupling was carried out in the peptide synthesizer under standard conditions (Scheme 5.3). The obtained peptides on the resin were taken directly into the next step.
The resins with peptides 1-4 were transferred into peptide reaction vessel, respectively. The resins were firstly swelled in DMF for 15 minutes. After aspiration, 20 mL DMF was added into the reaction vessel. With nitrogen bubbling the solution, acetonide-protected 3,4-dihydroxyhydrocinnamic acid (4 equiv. to each amine), HOBt (10 equiv. to each amine), and DIC (10 equiv. to each amine) were added sequentially. The reaction was carried out at ambient temperature with nitrogen bubbling for 4 hours. After aspiration, the resin was washed by DMF, DCM and MeOH, three times each for 2 minutes. Then the resins were immersed in 30 mL cleavage cocktail (trifluoroacetic acid 95%, triisopropylsilane 2.5%, H2O 2.5%, v/v) for 0.5 hour with nitrogen bubbling. The solution was collected and concentrated to ~ 3 mL with rotoevaporation. Peptides were precipitated in cold ether three times. Due to the poor solubility of products in 1×PBS buffer, the peptides were dissolved in mixed solvent of 1×PBS buffer and ethanol (v/v 1:1), followed by transferring into dialysis tube (MWCO 500 Da), and dialysis was conducted against dilute HCl solution (pH= 3~4). After dialysis, some product precipitated, therefore the
insoluble portion was dissolved with ethanol, combined with the solution inside the dialysis tube, and freeze dried. A gradient elution of RP-HPLC with linear change of 10%-80% B within 15 column volume. The catechol-bearing peptides were characterized with either ESI-MS or MALDI-ToF mass spectrometry, depending on the molecular weight. OGP-Cat: [M+H]+ m/z was calculated to be 792.4 and measured at 792.6. OGP-(Cat)2: [M+H]+ m/z was calculated to be 956.4 and measured at 956.5. OGP-(Cat)4: [M+Na]+ m/z was calculated to be 1562.7 and measured at 1562.7. OGP-PEG-Cat: [M+H]+ m/z was calculated to be 1127.5 and measured at 1127.6. OGP-PEG-(Cat)2: [M+Na]+ m/z was calculated to be 1313.6 and measured at 1313.7. OGP-PEG-(Cat)4: [M+Na]+ m/z was calculated to be 1897.9 and measured at 1898.3. The total yield calculated from the staring Fmoc-Gly-Wang Resin (0.25mmol) was: OGP-Cat: 25%, OGP-(Cat)2: 23%, OGP-(Cat)4: 12%, OGP-PEG-Cat: 20% OGP-PEG-(Cat)2: 15%, and OGP-PEG-(Cat)4: 9% (Error! Reference source not found.).

5.3.6 FITC-labeled OGP-Cat

To the solution of OGP-Cat (6 mg) and N,N-Diisopropylethylamine (DIPEA) (3 mg, 3 equiv.) in 0.2 mL anhydrous DMF, the solution of fluorescein isothiocyanate (FITC) (6 mg, ~2 equiv.) in 0.2 mL anhydrous DMF was added dropwise. The mixture was covered with aluminum foil and stirred at ambient temperature overnight. After the reaction, DMF was evaporated under vacuum, and the obtained orange solid was dissolved in 15 mL 25 mM HEPES buffer and filtered through a 0.22 μm filter to separate the overdosed insoluble FITC. The product was characterized with MALDI-ToF mass spectrometry. FITC-labeled OGP-Cat [M+H]+ m/z was calculated to be 1181.4 and found
to be 1181.6. The solution of FITC-labeled OGP-Cat (0.5 mM) was used directly for immobilization onto TiO$_2$ surface without further purification (Scheme 5.4).

5.3.7 Quartz crystal microbalance with dissipation (QCM-d) measurements

The AT cut sensors were purchased from Biolin Scientific AB (Sweden) and cleaned before use according to the protocol provided by the company. The sensor was excited at 5 MHz as its fundamental frequency. The frequency shift ($\Delta f$) and dissipation ($\Delta D$) were measured at 3$^{\text{rd}}$, 5$^{\text{th}}$, 7$^{\text{th}}$, 9$^{\text{th}}$, 11$^{\text{th}}$ and 13$^{\text{th}}$ overtones. 25 mM HEPES buffer (pH=7.40 at 25°C) was used as the flow medium. Sensors were mounted in the modules immediately after cleaning. HEPES buffer flowed above the sensors until a flat baseline was achieved at flow rate of 0.150 mL/min. Then solutions of catechol-bearing peptide in HEPES buffer were introduced and the flow continued until the adsorption reached its equilibrium state. If necessary, solutions at higher concentrations were introduced sequentially. Lastly, HEPES buffer was introduced again to wash the adsorbed layer. Three independent measurements were done simultaneously.

Scheme 5.4 Synthesis of FITC-labeled OGP-Cat.
5.3.8 Calculation of adsorbed area mass

The adsorbed area mass was proportional to the frequency shift ($\Delta f$) and calculated by the Sauerbrey Equation. Sauerbrey Equation is $\Delta m = -\frac{C}{n} \Delta f_n$, where $C$ is the mass sensitivity constant with the value of 17.7 ng Hz$^{-1}$ cm$^{-2}$ for 5 MHz fundamental frequency crystal, $n$ is the frequency overtone number, and $n=7$ was chose to calculate the adsorption area mass. As long as the adsorbed mass is small compared to the crystal, sufficiently thin and has limited viscoelastic coupling with the surrounding medium ($\Delta D < 1\times10^{-6}$ per 10 Hz), this relationship is valid. Adsorbed area mass measured from QCM-d includes water contained in the adhering layer. For adsorptions of OGP-(Cat)$_n$ and OGP-PEG-(Cat)$_n$ ($n=1,2,4$) onto substrates, $\Delta D$ were below $1\times10^{-6}$ per 10 Hz, and measurements from multiple overtones were close to each other, indicating adsorbed films were rigid, and the effect from content of water was slight.

5.3.9 Models used to fit the adsorption isotherm

The adsorption isotherms of OGP-(Cat)$_n$ and OGP-PEG-(Cat)$_n$ ($n=1,2,4$) binding to TiO$_2$ surface respectively were fit using a single-site specific binding model, $\Delta m = \frac{B_{max} \times C}{K_d + C}$, where $\Delta m$ is the amount of adsorbed analyte, $c$ is the concentration of the analyte solution, $B_{max}$ is the maximum adsorption of analyte on the surface, and $K_d$ is the apparent dissociation constant.

5.3.10 Sputter coating of TiO$_2$

Glass slides and silicon wafers were cleaned by sonication in 2% SDS solution for 30 minutes followed by thoroughly rinsing with water, dried with nitrogen and UV-ozone
treatment for 20 minutes. The TiO$_2$ target (99.99% pure, 2.00" diameter $\times$ 0.125" thickness) were purchased from Kurt J. Lesker (USA). The TiO$_2$ films were deposited by RF-magnetron sputtering using the following conditions at ambient temperature for 1h, power 75 W, 5 V$_{bias}$, deposition distance 5 cm, Ar 86 sccm, O$_2$ 9 sccm, and total pressure 42 mtorr ($p_{Ar}=34$ mtorr, $p_{o2}=8$ mtorr). For the fabrication of TiO$_2$ patterns on glass slides, 300 mesh Cu grids were placed on the top of glass.

5.3.11 Immobilization of OGP-(Cat)$_n$ and OGP-PEG-(Cat)$_n$ onto TiO$_2$-coated substrates

OGP-(Cat)$_n$ and OGP-PEG-(Cat)$_n$ ($n=1, 2, 4$) were dissolved in HEPES buffer at concentrations of 50 μmol/L. Substrates were immersed into the solution (800 μL for 20 mm $\times$ 20 mm glass slides, 500 μL for 5 mm $\times$ 5 mm silica wafer) and incubated at ambient temperature overnight. After that, the substrates were rinsed thoroughly with water to wash away the unbounded molecules, dried with nitrogen and subjected to further study.

5.3.12 X-ray photoelectron spectroscopy (XPS)

XPS measurements were performed on a Kratos AXIS Ultra DLD spectrometer. The X-ray source was monochromated Al K$_\alpha$, scanning over a binding-energy range of (0 to 700) eV with a dwell time of 100 ms. The analyzer pass energy was 110 eV for the survey spectra and 11 eV for the high-resolution C1s, N1s, and O1s scans. Each spectrum was collected over a 300 × 700 μm sample area.
5.3.13 Fluorescence intensity measurement

The florescence intensity was viewed with an inverted IX81 Microscope (Olympus, Center Valley, PA) with mercury bulb excitation and the appropriate filters. Displayed images were taken using identical settings, including exposure time (4.99 s), gain (10.04) and magnification (× 20). The mean intensity was calculated based on at least 10 randomly chosen sites observed under the same conditions.

5.3.14 Sterilization of substrates and OGP-PEG-(Cat)₄ solution

The TiO₂-coated glass slides were sterilized by washing with ethanol and UV irradiation for 30 minutes. The OGP-PEG-(Cat)₄ in HEPES buffer solution was sterilized by filtration through a 0.2 μm sterile syringe filter (EMD Millipore Millex).

5.3.15 Fabrication of substrates for cell studies

The immobilization of OGP-PEG-(Cat)₄ onto TiO₂ substrates is conveniently conducted by immersion of the substrates into the solution of OGP-PEG-(Cat)₄, followed by incubation overnight. Due to the strong binding affinity, the concentration of the OGP-PEG-(Cat)₄ solution is very low. By using \( C_1 = 100 \times K_d = 2.8 \mu\text{mol/L} \) and \( C_2 = K_d = 28 \text{nmol/L} \), OGP-PEG-(Cat)₄ modified TiO₂ substrates, OGP-99% and OGP-50%, were successfully prepare with 99% and 50% coverage of maximum adsorption, respectively, as shown in Appendix Table 1.
5.3.16 Cell Culture

MC3T3-E1 mouse preosteoblast (passage 17) were expanded and cultured in α-MEM media (Gibco, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 100 units/mL penicillin (Invitrogen), and 100 μg/mL streptomycin (Invitrogen) at 37°C in a 5% CO₂ humidified atmosphere. The cells were subcultured every 3 days in the presence of 0.25% (w/v) trypsin and 0.5% (w/v) ethylenediaminetetraacetic acid tetrasodium salt (EDTA) solution. Cells were seeded on substrates at 18 cells/mm² and fed every two days.

5.3.17 Viability assay

Viability was evaluated using a Live/Dead viability/cytotoxicity kit (Invitrogen, UK). Briefly, 5 μL of the 4 mM Calcein-AM stock solution and 10 μL of the 2 mM ethidium homodimer-1 (Etmd-1) stock solution were added to 10 mL of cell culture medium to prepare the Live/Dead staining solution. 1 mL of staining solution was added into each well in a 6-well plate, after aspiration of the old medium. The samples were incubated for 10 min in Live/Dead staining solution. The staining solution was removed and the samples were viewed under the IX81 fluorescence microscope (Olympus) with 494 nm (green, Calcein) and 528 nm (red, EthD-1) emission filters. For quantitative analysis a total of 250 cells were counted from each sample over 25 randomly chosen areas and the viable and non-viable cells counts were recorded.
5.3.18 Immunohistochemical staining of cytoskeletal actin and vinculin

All samples were pre-fixed in pre-warmed 0.8 mL cell culture media and 1.2 mL 3.7% paraformaldehyde (PFA) in CS buffer for 5 minutes on a dry block at 37 °C. After aspiration, samples were fixed in 3.7 % PFA solution at 37 °C for 5 minutes. After washing with 1×PBS 3 times, 1.5 mL of Triton X-100 in CS buffer (0.5% v/v) was added to each well to permeabilize the cells for 10 minutes on a dry block at 37°C. The substrates were washed 3 times with 1×PBS. Freshly made 0.1 wt% NaBH₄ in 1×PBS was then added for 10 minutes at r.t. to quench the aldehyde fluorescence, followed by aspiration and incubation in 5% donkey serum for 20 minutes at r.t. to block the non-specific binding. After aspiration, the substrates were incubated in vinculin primary antibody Mouse in 1×PBS (v/v 1:200) at 4°C overnight. After washing with 1% donkey serum 3 times, the substrates were stained in a solution of rhodamine phalloidin (v/v 1:40) and Alexa Flour 488 secondary antibody Mouse (v/v 1:200) for 1 hour at r.t., avoiding light. After washing with 1×PBS 3 times, the nuclei were stained with DAPI in 1×PBS (6 μL/ 10 mL) for 20 minutes at r.t. in the dark. After washing with 1×PBS 3 times to remove excess staining, the samples were mounted and viewed under an IX81 Microscope (Olympus, Center Valley, PA) with mercury bulb excitation and the filters of FITC, TRITC and DAPI.

5.3.19 Cell proliferation assay

Cell proliferation of MC3T3-E1 cells on OGP-99%, OGP-50% and TiO₂ substrates was evaluated by the PrestoBlue Assay (Life technologies, Grand Island, NY) following the provided protocol. The standard curve was prepared in duplicate by seeding cell suspensions at known concentrations into a 24-well plate at least 6 hours before the
experiment for full attachment. Nine descending cell concentration and one blank were included in the standard curve. The PrestoBlue solution was prepared by dilution with cell culture medium (v/v 1:9). After aspirating the old medium, 1.5 mL of PrestoBlue solution was added to each well, followed by incubation at 37°C in the incubator for 2-4 hours. A color change from blue to purple and to pink ultimately was observed during incubation. When the standard curve fluorescence could be fit to a linear line, the samples’ fluorescence was read. 100 μL solution was taken from each well in triplicate and placed in a 96-well plate. The change in cell viability was detected by fluorescence intensity (FI) in Plate Reader by excitement at 570 nm and emission at 615 nm. The standard curve was fit with a linear relationship by plotting FI vs Cell Number. The coefficient of determination (R²) was above 0.99. The cell number was calculated with the obtained equation.

5.3.20 Immunohistochemical staining of bone sialoprotein (BSP) and osteocalcin (OCN)

All samples were pre-fixed in pre-warmed 0.8 mL cell culture media and 1.2 mL 3.7% paraformaldehyde (PFA) in CS buffer for 5 minutes on a dry block at 37°C. After aspiration, samples were fixed in 3.7% PFA solution at 37°C for 5 minutes. After washing with 1×PBS 3 times, blocking buffer (10% normal donkey serum, 0.3% Triton X-100 in 1×PBS) was added into each well and incubated for 45 minutes at r.t. to block the non-specific binding. After aspiration, the substrates were incubated in bone sialoprotein (BSP) primary antibody Mouse (v/v 1:400) and osteocalcin (OCN) primary antibody Goat in 1×PBS (v/v 1:100) overnight at 4°C. After washing with 1% donkey serum 3 times, the substrates were stained by incubation in a solution of Alexa Flour 488 secondary antibody Mouse (v/v 1:200) and Alexa Flour 546 secondary antibody Goat (v/v 1:200) in 1×PBS for
1 hour at r.t. in the dark. After washing with 1% donkey serum 3 times, the nuclei were stained with DAPI in 1×PBS (6 μL / 10 mL) for 20 minutes at r.t. avoiding light. After washing with 1×PBS once, the samples were mounted and viewed under an IX81 Microscope (Olympus, Center Valley, PA) with mercury bulb excitation and the filters of FITC, TRITC and DAPI.

5.3.21 Alizarin Red S. staining

Samples were pre-fixed in pre-warmed 0.8 mL cell culture media and 1.2 mL 3.7% paraformaldehyde (PFA) in CS buffer for 5 minutes on a dry block at 37°C. After aspiration, samples were fixed in 3.7% PFA solution at 37°C for 5 minutes. Freshly made Alizarin Red S. solution (0.8 g in 40 mL dd H₂O, pH adjusted to 4.2), was added into substrates that are washed 3 times with dd H₂O to remove soluble calcium. After incubation at r.t. for 40 minutes, the Alizarin Red S. solution was carefully removed. The substrates was washed with dd H₂O 4 times, mounted and observed under bright field microscope.

5.3.22 Alkaline phosphatase (ALP) activity assay

ALP activity was measured by SensoLyte pNPP ALP Assay Kit (AnaSpec Inc, San Jose, CA, USA) following the provided protocol. The MC3T3-E1 cells on OGP-99%, OGP-50% and TiO₂ substrates were washed with 1× Assay Buffer twice. The cell film was peeled from the substrates and transferred into a 1.5 mL centrifuge tube, followed by addition of 0.5 mL lysis buffer (20 μL Triton X-100 in 10 mL 1× Assay Buffer). The cells were resuspended and incubated in the lysis buffer for 10 min at 2500 xg at 4°C. After centrifuge, the supernatant was collected for analysis. A standard curve was measured with
an ALP solution at concentrations of 0, 3.1, 6.2, 12.5, 25, 50, 100, 200 ng/mL. 50 μL of sample/standard solution and 50 μL pNPP solution was added into each well in a 96-well plate. The solution was mixed by gently shaking for 30 sec. After incubation for 30 min, the 96-well plate was shaken for 1 min before measuring the absorbance at 405 nm. Three replicates were measured for each sample. The standard curve was fitted with a linear relationship by plotting Ab. vs ALP concentration, with a coefficient of determination (R²) above 0.98. The ALP activity of cell lysate from each sample was calculated with obtained equation.

To normalize the ALP activity with total protein amount, the amount of total protein was quantified with a DC protein assay (Bio-Rad, Hercules, CA, USA). A standard curve was measure with BSA solution at amount of 0, 0.2, 0.5, 0.8, 1.1, 1.43 mg/mL with 1× ALP Assay Buffer as the dilution buffer. 5 μL of standards and samples were added into a 96-well plate. Then 25 μL of reagent A and 200 μL reagent B were added. The plate was shaken for 30 sec to well mix the solution, which was left to incubate for 15 min. The absorbance at 750 nm was read. The standard curve was fitted with a linear relationship by plotting Ad. vs BSA amount, with a coefficient of determination (R²) above 0.96. The total protein amount of cell lysate from each sample was calculated with obtained equation.

5.3.23 Calcium quantification

The MC3T3-E1 cells on OGP-99%, OGP-50% and TiO₂ substrates were washed with DPBS buffer (Mg²⁺, Ca²⁺ free) once. The cell film was peeled from the substrates and transferred into a 1.5 mL centrifuge tube, followed by addition of 300 μL dd H₂O. Three freeze-thaw cycles were carried out to destroy the cell membrane, followed by the addition
of 300 μL 1 M HCl. The samples were agitated at r.t. overnight. The concentration of calcium ions in the supernatant was measured with inductively coupled plasma optical emission spectrometry (ICP-OES) (Agilent Technologies 700 series, Santa Clara, CA, USA). The emission wavelength was set at 393.366 nm to quantify Ca$^{2+}$. A standard curve was measured with solutions of $c$(Ca$^{2+}$) equal to 0.125, 0.25, 0.5, 1, and 2 ppm. Triplicate measurements were carried out for each sample. The calcium amount of each sample was normalized with total protein amount, which was done in the same way as introduced in the ALP activity assay part.

5.3.24 Real time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated on day 18 with an RNA extraction and isolation kit (Applied Biosystems, Life Technologies), following the provided protocol, and was quantified by ultraviolet spectroscopy. The synthesis of complementary DNA (cDNA) was performed with a high-capacity cDNA reverse transcription kit with RNase inhibitor (Applied Biosystems, Life Technologies) using 800 ng total RNA as the template in a 100 μL reaction following the provided protocol. RT-PCR was performed with non-specific detection fluorescence, SYBR Green (Applied Biosystems, Life Technologies). For SYBR Green detection, 10 ng of cDNA product and 1× SYBR Green master mixture (Applied Biosystems, Life Technologies) were included in 50μL reaction mixture (209.4 nM each primer). The housekeeping gene used was glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All oligonucleotide primers (Table 5.1) were purchased from Applied Biosystems (Life Technologies). The ALP primer (alpl gene from Mouse) was also purchased from Applied Biosystems (Life Technologies). RT-PCR was performed in the
Applied Biosystems Real-Time PCR Instruments - 7500 Fast System (Life technologies, Grand Island, NY) with recommended programs by the supplier (Table 5.1).

Table 5.1 SYBR Green primers used for RT-PCR. GAPDH was used as the endogenous control for comparing gene expression.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Sequence direction</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>mGAPDH-L1</td>
<td>GACAGTCAGCCGCATCTT</td>
<td>Forward</td>
<td>SYBR Green</td>
</tr>
<tr>
<td>mGAPDH-R1</td>
<td>CCATGGTGCTGAGCGATGT</td>
<td>Reverse</td>
<td>SYBR Green</td>
</tr>
<tr>
<td>mBSP-L1</td>
<td>CCTGGCACAGGGTATACAGG</td>
<td>Forward</td>
<td>SYBR Green</td>
</tr>
<tr>
<td>mBSP-R1</td>
<td>CTGCTTCGCTTTCTTCGTTT</td>
<td>Reverse</td>
<td>SYBR Green</td>
</tr>
<tr>
<td>mOCN-L1</td>
<td>GTGCAGCCTTTGTGTCCAA</td>
<td>Forward</td>
<td>SYBR Green</td>
</tr>
<tr>
<td>mOCN-R1</td>
<td>GGCTCCCAGCCATTGAT</td>
<td>Reverse</td>
<td>SYBR Green</td>
</tr>
</tbody>
</table>

5.3.25 Statistics

All experiments were conducted at three replicates (n=3). All quantitative data are presented as the average ± standard deviation.

5.4 Results and Discussion

A summary of the results and a discussion for these findings is reported in this section of the chapter.

5.4.1 Synthesis and characterization of catechol-bearing modular peptides

Lysine-based dendrons were used as the platform to construct the catechol-bearing multivalent binding ligands with a bioactive peptide at the core as shown in Scheme 5.5. In the periphery of the dendron, tunable numbers of catechol (Cat) functional groups were attached with valency equal to 1, 2 or 4. In the focal point of the dendron, an
osteocomductive peptide, OGP[10-14], with the amino acid sequence YGFGG was linked. The two domains were connected with or without a hexaethylene glycol flexible linkage. We envision that the surface-binding domain will sequester the bioconjugate on the surface of metal oxide implants due to strong binding affinity, and the bioactive domain will interact with cells and influence their behavior in a concentration dependent manner.

Scheme 5.5 Molecular structures of OGP-(Cat)_n and OGP-PEG-(Cat)_n; n = 1, 2, 4

The synthesis of OGP-(Cat)_n and OGP-PEG-(Cat)_n (n=1, 2, 4.) was carried out using Fmoc based solid phase synthesis, and the synthesis of OGP-PEG-(Cat)_2 was shown
in Scheme 5.7 as an example. The last coupling step of acetonide-protected 3,4-dihydroxyhydrocinnamic acids with amines in the peptide chain terminus cannot tolerate microwave-assisted conditions, and the protection of catechol groups is essential for successful synthesis. No intervening purification was required, and synthetic process only took 6 hours in all. After RP-HPLC purification, the OGP[10-14] peptide-functionalized catechol-bearing dendrons were achieved with high purity with the yield of 9%-25% as shown in ESI or MALDI-ToF mass spectrometry (Appendix Figure 27).

Scheme 5.6 The Fmoc-protected solid phase synthesis of OGP-PEG-(Cat)$_2$.

5.4.2 Adsorption of TiO$_2$ measured by QCM-d

The adsorption processes of all molecules to TiO$_2$ surfaces at 25°C and pH 7.4 were monitored by QCM-d. Multivalent binding effects that enhanced the binding affinity were clear. In Figure 5.1, to obtain a similar level of adsorption, ~ 6Hz frequency shift, the
concentration of monovalent ligand, OGP-Cat, is 13 μM, while that of OGP-(Cat)₄ and OGP-PEG-(Cat)₄, is 0.32 and 0.16 μM, respectively due to the mass differences. A much smaller amount of sample (~80-fold less) is needed for the tetravalent ligand OGP-PEG-(Cat)₄ compared with the monovalent ligand OGP-Cat to achieve the same level (mass) of surface adsorption. Moreover, the tetravalent ligands remained on the TiO₂ surface under buffer washing, as no frequency shift was observed after switching the solution to HEPES buffer. This indicates that the tetravalent ligands are sequestered on the TiO₂ surface and are unlikely to diffuse away after being implanted into the body. Under similar conditions, the monovalent ligand was partially washed away. The adsorption kinetics were recorded by QCM-d, with regard to OGP-PEG-(Cat)₄, 2 hours are needed to reach the equilibrium state at a concentration of 0.16 μM.
To quantitatively compare the binding affinities of the multivalent binding ligands, the adsorption properties at several different concentrations were measured. The solutions at higher concentrations were switched to flow above the sensor, until the adsorption of previous solution at lower concentration reached the equilibrium state (the change in frequency shift is smaller than the signal fluctuation, 0.05 Hz/min), taking OGP-Cat as an example shown in Figure 5.2A. The corresponding frequency shift was calculated using

![Graph showing frequency shift versus time for different concentrations of OGP-Cat and OGP-Cat_4](image)

Figure 5.1 The adsorption of catechol-functionalized dendrons, OGP-Cat, OGP-(Cat)_4 and OGP-PEG-(Cat)_4 onto TiO_2 surface monitored by QCM-d. The experiment contains three processes: i) baseline in HEPES buffer; ii) adsorption of ligands; iii) buffer washing the adsorbed ligands, as indicated by the small peak due to the stop of flow. To reach similar level of frequency shift, tetravalent ligands OGP-(Cat)_4 and OGP-PEG-(Cat)_4 required solution at much lower concentrations compared to the monovalent ligand, OGP-Cat, indicating a stronger binding affinity. Signals from different overtones were close due to the rigidity of the adsorbed film and those of n= 7 were shown here.

5.4.3 Binding affinity and maximum adsorption

To quantitatively compare the binding affinities of the multivalent binding ligands, the adsorption properties at several different concentrations were measured. The solutions at higher concentrations were switched to flow above the sensor, until the adsorption of previous solution at lower concentration reached the equilibrium state (the change in frequency shift is smaller than the signal fluctuation, 0.05 Hz/min), taking OGP-Cat as an example shown in Figure 5.2A. The corresponding frequency shift was calculated using
the Sauerbrey Equation to get the adsorbed area mass. The adsorption isotherm of each molecule was drawn and fit with a single-site specific binding model to get the apparent disassociation constant ($K_d$) and maximum adsorption ($B_{max}$) (that of OGP-Cat as in Figure 5.2B and others in Appendix Figure 28) from the adsorbed area mass at the respective concentrations. The results are summarized in Table 5.2. The $K_d$ decreases as the valency changes from 1, to 2, and to 4. This clearly proved that multivalent binding ligands provide a stronger binding affinity. The enhancement parameters were calculated for the two series of molecules, with or without the PEG linkage. There is a 184-fold enhancement in binding affinity for OGP-(Cat)$_4$ with the $K_d$ of 31 ± 3 nM, when compared to OGP-Cat. Surprisingly, the PEG linkage also influences the binding affinity. When the valency equals 1 or 2, the molecules with PEG showed a 6-fold and 14-fold stronger binding compared with molecules without PEG. This is most likely because the PEG linkage serves as a spacer and weakens the effect of any intramolecular H-bond that may form between catechol groups and the OGP[10-14] peptide chain. From the quantification results of apparent $K_d$ and $B_{max}$, for the tetravalent ligand, the binding is strong enough to saturate and sequester the whole molecule on TiO$_2$ surface at very low bioconjugate concentrations. A solution of OGP-(Cat)$_4$ at 2.8 μM (100$K_d$) covers 99% of the binding sites on TiO$_2$ surfaces, at a OGP[10-14] concentration of 103 pmol/cm$^2$. The immobilization procedure simply involves immersing the TiO$_2$ surface in the solution for more than two hours.
Figure 5.2 The adsorption of OGP-Cat onto TiO$_2$ surface and its adsorption isotherm. (A) The adsorption of OGP-Cat onto TiO$_2$ surface at different concentrations was measured by QCM-d, while the concentration was increased sequentially ($c_1 = 0.068$ μmol/L, $c_2 = 0.34$ μmol/L, $c_3 = 1.8$ μmol/L, $c_4 = 7.3$ μmol/L, $c_5 = 13$ μmol/L, and $c_6 = 34$ μmol/L). At last the adsorbed layer was washed with 25 mM HEPES buffer. The flow rate was 0.150 mL/min. Three independent measurements were shown. (B) The disassociation constant of OGP-Cat was $5.7 \pm 0.2$ μM by fitting the adsorption isotherm with single-site specific binding model, as represented with the dashed line. The adsorbed area mass was calculated from the Sauerbrey Equation. Each dot with an error bar was calculated based on three independent measurements.
Table 5.2 The apparent dissociation constant ($K_d$), maximum adsorption ($B_{max}$) and enhancement parameter ($\beta$) of catechol-functionalized dendrons to TiO$_2$ surface.

<table>
<thead>
<tr>
<th>Ligands to TiO$_2$</th>
<th>$K_d(\mu M)$</th>
<th>$B_{max}(\frac{ng}{cm^2})$</th>
<th>$\beta^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>OGP-Cat</td>
<td>5.7 ± 0.2</td>
<td>54 ± 4</td>
<td>--</td>
</tr>
<tr>
<td>OGP-PEG-Cat</td>
<td>1.0 ± 0.1</td>
<td>59 ± 2</td>
<td>--</td>
</tr>
<tr>
<td>OGP-(Cat)$_2$</td>
<td>1.1 ± 0.3</td>
<td>55 ± 3</td>
<td>5</td>
</tr>
<tr>
<td>OGP-PEG-(Cat)$_2$</td>
<td>0.08 ± 0.01</td>
<td>73 ± 4</td>
<td>12</td>
</tr>
<tr>
<td>OGP-(Cat)$_4$</td>
<td>0.031 ± 0.003</td>
<td>160 ± 6</td>
<td>184</td>
</tr>
<tr>
<td>OGP-PEG-(Cat)$_4$</td>
<td>0.028 ± 0.008</td>
<td>196 ± 23</td>
<td>36</td>
</tr>
</tbody>
</table>

$^a$ Adsorption isotherm was fit with single-site specific binding model, $\Delta m = \frac{B_{max} \times C}{K_d + C}$, where $\Delta m$ is the amount of adsorbed analyte, $c$ is the concentration of the analyte solution, $B_{max}$ is the maximum adsorption, and $K_d$ is the apparent dissociation constant.  

$^b$ Enhancement parameter is defined as the ratio of association constant of multivalent ligand to that of monovalent ligand, $\beta = \frac{K_{a,multi}}{K_{a,mono}}$, and it was calculated based on two sets of molecules with or without PEG linkage.

5.4.4 Binding ability of OGP-(Cat)$_4$ to versatile surfaces

The adsorption of tetravalent binding ligand OGP-(Cat)$_4$ to a wide range of materials was tested to identify the common features of materials that catechol-bearing ligands strongly bind to. As shown in Figure 5.3, OGP-(Cat)$_4$ showed the strongest binding to CeO$_2$, TiO$_2$ and ZrO$_2$, strong binding to iron oxide (Fe$_3$O$_4$ and Fe$_2$O$_3$), some adsorption to gold, and weak adsorption to SiO$_2$. This is because the coordination bond between catechol and metals with empty $d$-orbitals or $f$-orbitals provides a stronger interaction than Hydrogen bonding. Limited adsorption of OGP-(Cat)$_4$ with materials of compounds from main group elements, including SiO$_2$, Al$_2$O$_3$ and hydroxyapatite (HA), was confirmed even when applying a solution at 10-times higher concentration (Appendix Figure 29). Stronger
adsorption was observed for transition metal and transition metal oxide, which was attributed to coordination bonding. Despite the strong binding with TiO₂, OGP-(Cat)₄ showed similar strong binding affinity and persistence under buffer washing to other biomaterial-related surfaces, including zirconia, ZrO₂, a widely used material in prosthetic devices, cerium oxide, CeO₂, and iron oxide, Fe₃O₄. Therefore, we envision OGP-(Cat)₄ as highly useful for the functionalization of transition metal oxides.

Figure 5.3 Tetravalent binding ligand OGP-(Cat)₄ (c= 1 μM) shows strong binding affinities to versatile metal and metal oxide surfaces due to coordination bond, including Fe₂O₃, Fe₃O₄, ZrO₂, TiO₂ and CeO₂ surfaces as measured by QCM-d.

5.4.5 OGP-(Cat)ₙ on the TiO₂ surface

To directly prove the existence of OGP-(Cat)ₙ on TiO₂ surface, XPS and fluorescein labeling experiments were carried out. The TiO₂ layer was prepared by RF sputter coating on glass slides or silica wafers. The thickness of TiO₂ layer was measured to be around 36
nm with O/Ti ratio equaling to 2.0 (Appendix Figure 30-31). The surfaces roughness of deposited TiO$_2$ was measured by atomic force microscopy (AFM) with an RMS roughness around 1 nm (Appendix Figure 32).

Immobilization of peptides onto TiO$_2$ surface was conducted by immersing the TiO$_2$ substrates into the corresponding modular peptide solution and incubating overnight at ambient temperature. The successful immobilization of OGP-Cat and OGP-(Cat)$_4$ onto TiO$_2$-coated substrates were confirmed by XPS. Nitrogen is the element contained only in the modular peptides while not in bare TiO$_2$, as shown in the XPS survey scan of bare TiO$_2$ and OGP-(Cat)$_4$ in Figure 5.4A. Thus N1s signal at 400.3 eV corresponding to the amide in peptides can be used to prove the immobilization of OGP-(Cat)$_4$ onto TiO$_2$ surfaces (Figure 5.4A). The adsorbed OGP-(Cat)$_4$ layer was readily removed with Ar$^+$ plasma treatment for 1 min, indicating that the N1s indeed came from the very top adsorbed peptides layer (Figure 5.4B). It is noted that the N1s peaks in Figure 5.4B are normalized to the highest intensity (O1s) for comparison of the signal to noise ratio.

To quantify the increase in nitrogen due to adsorption of OGP-Cat and OGP-(Cat)$_4$, the nitrogen content (N1s) was normalized with Ti content (Ti2p), and compared with TiO$_2$ substrate after incubation in HEPES buffer overnight (TiO$_2$ as control), as show in Table 3. The N/Ti ratio increased from 0.03 (TiO$_2$ control) to around 0.4 (OGP-Cat: 0h incubation in buffer, OGP-(Cat)$_4$: 0 day incubation). The presence of modular peptide OGP-(Cat)$_4$ was further proven by the significant change of C1s and O1s signatures in high resolution XPS spectra before and after the immobilization. In Figure 4C and 4D, the C1s signals were fit with Gaussian model into three components based on their respective binding energy, including carbon of C-C bond (C1, 284.8 eV), of C-O bond (C2, 286.1 eV), and of
amide bond (C3, 287.8 eV). Similarly, the O1s signals were deconvoluted into three peaks: oxygen of Ti-O bond (O1, 530.2 eV), of C=O bond (O2, 531.5 eV), and of C-O bond (O3, 533.0 eV). The significant increase in the atomic ratios of C2/C1, C3/C1, O2/O1, and O3/O1 (table inserted in Figure 5.4 indicates the amide bonds and phenol rings contained in the OGP-(Cat)4. The C3/N ratio, from amide bond of OGP-(Cat)4, is 0.93, which is closed to the theoretical value of 1.

Fluorescein-labeled modular peptide FITC-labeled OGP-Cat was also synthesized to visualize the presence of the immobilized peptides on TiO2 surface using fluorescence microscopy. After immobilization of FITC-labeled OGP-Cat onto TiO2 surfaces, the fluorescence intensity was much stronger compared to the control sample, which was a TiO2-coated substrate incubated in a solution of FITC at an identical concentration, as shown in Figure 5.5A and Figure 5.5B. When a TiO2 pattern was present on the glass slides, it was observed that the TiO2 region showed a significantly stronger fluorescence signal due to the stronger binding affinity of FITC-labeled OGP-Cat to TiO2 compared with SiO2, Figure 5.5C and Figure 5.5D.
Figure 5.4 XPS characterization confirmed the successful immobilization of OGP-(Cat)$_4$ on TiO$_2$ surface. (A) Survey scan of bare TiO$_2$ surface, OGP-(Cat)$_4$, and OGP-(Cat)$_4$ immobilized TiO$_2$. The N1s signal comes from amide bonds in peptides. (B) 1min of Ar$^+$ plasma treatment to the OGP-(Cat)$_4$ immobilized TiO$_2$ surface removed the adsorbed OGP-(Cat)$_4$ layer. The N1s peaks are normalized to the highest intensity (O1s) for comparison of the signal to noise ratio. The signal changes in high resolution XPS spectra of O1s (C) and C1s (D) demonstrates the successful immobilization of OGP-(Cat)$_4$ on TiO$_2$ substrates. The multiple peaks were fitted with a Gaussian model. The atomic ratios of C2/C1, C3/C1, O2/O1, O3/O1 and C3/N of respective surfaces were calculated based on the integrated area of each peak.
Figure 5.5 The immobilization of modular peptides was viewed by labeling the peptide with fluorescein. Due to multivalent binding, the retention time of OGP-(Cat)$_4$ on TiO$_2$ in buffer pH=7.4 was longer than 2 weeks. (A)-(E) The immobilized FITC-labeled OGP-Cat on TiO$_2$ surfaces was observed under fluorescence microscope, and the mean intensity of fluorescence decreased after incubating the substrates in pH=7.4 25 mM HEPES buffer due to the diffusion of FITC-labeled OGP-Cat. TiO$_2$ substrates were incubated in the solution of 0.5 mM (A) FITC-labeled OGP-Cat and (B) FITC overnight, then thoroughly washed with water and dried with N$_2$. FITC-labeled OGP-Cat immobilized TiO$_2$ pattern on glass slides was observed under (C) bright field microscope and (D) fluorescence microscope. The scale bar is 50 μm. (E) Mean intensity of FITC-labeled OGP-Cat immobilized surfaces after incubation. The control sample was a TiO$_2$ substrate incubated in FITC solution overnight. (F)-(G) The immobilized OGP-(Cat)$_4$ was preserved on the surface for more than 2 weeks in buffer pH=7.4, in comparison, the diffusion of monovalent ligand OGP-Cat was detected after 12 hours. XPS spectra of N1s signals taken after incubation of (F) OGP-(Cat)$_4$ and (G) OGP-Cat immobilized on TiO$_2$ substrates in HEPES buffer (pH=7.4) for different time durations, respectively. The control is taken after incubation of TiO$_2$ substrates in 25 mM HEPES buffer. To compare the signal to noise ratio, all spectra were normalized to the peak of highest intensity (O1s).
5.4.6 Retention of OGP-(Cat)$_n$ on TiO$_2$ surface

To study the stability of sequestered modular peptides on targeting surfaces, the modular peptide immobilized TiO$_2$ substrates were immersed in 25 mM HEPES buffer (pH= 7.4 at 25°C) and incubated for different duration, respectively. The mean intensity of FITC-labeled OGP-Cat immobilized TiO$_2$ substrates after incubation was quantified to detect the diffusion of FITC-labeled OGP-Cat into surrounding solution, Figure 5.5E. After 3 days incubation, the intensity deceased by about a half of the original intensity coming from FITC-labeled OGP-Cat on surfaces, indicating the dissociation of monovalent ligand. Because the N1s signals were assigned to the modular peptides, the decrease of N1s corresponds to the dissociation of adsorbed peptides from TiO$_2$ surface. In Figure 5.5F, tetravalent ligand OGP-(Cat)$_4$ showed a longer retention time on the targeted surface, the decrease of N1s signal was not detected until after 14 days, while that of monovalent ligand OGP-Cat showed a reducing in N1s signal after 12 hours (Figure 5.5G). The atomic ratio of N/Ti were calculated, as shown in Table 5.3, the half life time of immobilized OGP-Cat and OGP-(Cat)$_4$ presenting on TiO$_2$ surface is around 36 hours and 14 days, respectively. The mismatch of retention time of monovalent ligands was attributed to the sensitivity difference of XPS and fluorescence microscopy. The tetravalent ligands OGP-(Cat)$_4$ are clearly present on the TiO$_2$ surface beyond 2 weeks, which is enough to trigger the cascade signaling reactions in adjacent cells.
Table 5.3 The immobilization of OGP-Cat and OGP-(Cat)$_n$ and their retention on TiO$_2$ substrates. Atomic Ratios of N/Ti for the TiO$_2$ surface, the surfaces after OGP-Cat and OGP-(Cat)$_n$ immobilization, and the OGP-Cat and OGP-(Cat)$_n$ bearing surfaces after incubation in HEPES buffer.$^{a,b}$

<table>
<thead>
<tr>
<th>Surface</th>
<th>TiO$_2$</th>
<th>OGP-Cat on TiO$_2$</th>
<th>OGP-(Cat)$_n$ on TiO$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer Incubation Time</td>
<td>0h (bare)</td>
<td>12h (control)</td>
<td>0h 12h 36h day 3 day 7 day 14 day</td>
</tr>
<tr>
<td>N/Ti</td>
<td>0.03</td>
<td>0.41 0.34 0.21</td>
<td>0.43 0.44 0.45 0.24</td>
</tr>
</tbody>
</table>

$^a$ Standard deviations are typically below 10% relative.
$^b$ 0h incubation in 25 mM HEPES buffer (pH = 7.41 at 25 °C) means surfaces just after TiO$_2$ coating or OGP-(Cat)$_n$ (n = 1,4) immobilization without incubation in buffer.

5.4.7 Preferential immobilization of OGP-(Cat)$_n$

We have shown that the catechol-bearing dendrons have strong binding to transition metal compounds due to coordination bond, while weak binding to materials when only H-bond or other weak non-covalent interactions exist. Thus immersing materials with both SiO$_2$ and TiO$_2$ present on the surface into the solution of OGP-(Cat)$_n$, the OGP-(Cat)$_n$ will preferentially adsorb onto TiO$_2$ region. This provides a method to preferentially functionalize selected regions on the surface, which is useful in the fabrication of surfaces with locally restricted functionality of peptides. It was demonstrated with a partially coated glass slides containing SiO$_2$ region in the middle and TiO$_2$ in the surrounding region. After incubating the slides in the solution of OGP-(Cat)$_n$ (c= 1 μM) overnight, the elements present on the surface in the respective regions was detected by XPS. As shown in Figure 5.6, the green spots are positions where X-ray was directed on the surface. The signals from SiO$_2$ and TiO$_2$ regions are quite different. The local existence of Si and Ti was confirmed in XPS. And notably, the TiO$_2$ region showed a much stronger signal in N1s,
which corresponds to the adsorbed OGP-(Cat)_4. The atomic percentage of nitrogen from N1s in TiO_2 and SiO_2 region were 8 ± 1% and 1.7 ± 0.8%, respectively.

Figure 5.6 Preferential adsorption of OGP-(Cat)_4 to the TiO_2 region of a partially TiO_2-coated glass slide observed with XPS. The green spots on the substrates are positions where XPS spectra were taken (three in SiO_2 region and three in TiO_2 region). The stronger signal of N1s in the TiO_2 region compared with that of the SiO_2 region indicates peptides preferentially adsorbed to the TiO_2 surface. The XPS signals were normalized with the strongest peak intensity (O1s). The atomic percentage of nitrogen in TiO_2 and SiO_2 region were 8 ± 1% and 1.7 ± 0.8%, respectively.
5.4.8 Cytotoxicity

MC3T3-E1 cells were seeded on the TiO$_2$ substrates with immobilized FITC-labeled OGP-Cat and cultured for 24 hours. Cell viability was studied (Appendix Figure 33). The dominant green fluorescence (viability > 98%) from live cells in the live/dead cell staining demonstrates the modular peptide bioconjugates are not toxic when tethered to the surface. The cells were well spread on the peptide-bearing surfaces, which is a consistent with an adherent proliferating cell population.

5.4.9 XPS of OGP[10-14] immobilized TiO$_2$-coated substrates

The loading amount was calculated based on the adsorption isotherm fitted with signal site specific model as shown in Table 5.2. The XPS characterization was applied to detect the immobilized peptides from the N1s signal, which is the element only contained in the amide bond in OGP-PEG-(Cat)$_4$. The nitrogen content normalized with total amount of elements on surface is 5.8 ± 0.3 for OGP-99% substrates, while that of OGP-50% are not distinguish from noise due to low content (Appendix Figure 34 and Appendix Table 1).

5.4.10 Effects of immobilized OGP[10-14] on cell adhesion and morphology

Because the capacity for cells to interact with growth factors is an important cell behavior, the MC3T3-E1 cells were fluorescently stained to visualize actin and vinculin proteins, to assess the organization of cytoskeleton and the spatial distribution of focal adhesion contacts, respectively. After 24 hours, the MC3T3-E1 cells were attached on both OGP-99% and bare TiO$_2$ substrates and the focal adhesion contacts between cells and
substrates formed, as shown in Figure 5.7A and B. There is no statistic difference in cell area and aspect ratio for the cell adhesion to OGP-99% and bare TiO\(_2\) substrates, because OGP peptides, immobilized or dissolved, show no effects in the adhesion of MC3T3-E1 cells as described in our previous work.\(^{177}\)

![Image](image_url)

Figure 5.7 The adhesion of MC3T3-E1 cells were not significantly influenced by the immobilized OGP[10-14] peptides, while they proliferated faster on OGP-bearing surface with dose-dependency. After 24 hours, MC3T3-E1 cells were well spread on substrates with or without immobilized OGP[10-14] and formed focal adhesion contacts with the substrates, as indicating by the immunohistochemical staining of adherent cells on (A) OGP-99% and (B) bare TiO\(_2\) substrates. Red corresponds to F-actin in cytoskeleton; green corresponds to vinculin in focal adhesion complex; and blue corresponds to the nuclei (scale bar is 50 \(\mu\)m). (C) The immobilized OGP-PEG-(Cat)\(_4\) promoted the cell proliferation, and this effect was dose-dependent. Cell number on substrates after day 1 and day 3 were evaluated by PrestoBlue Assay. The error bar was calculated from three replicates.
5.4.11 Effects of immobilized OGP[10-14] on cell proliferation

The OGP-99%, OGP-50% and TiO₂ substrates were seeded with preosteoblast MC3T3-E1 cells with cell density of 18 cell/mm². In the first 24 hours, the cells were mostly attaching to the surface and adjusting to the new environment, therefore, the cell number after 1 day for all three substrates was comparable. After 3 days, the MC3T3-E1 cells on OGP-99% substrates showed the highest cell number compared with the others, indicating that the OGP[10-14] peptide promoted a faster cell proliferation rate in a concentration dependent manner, as shown in Figure 5.7C.

5.4.12 Effects of immobilized OGP[10-14] on osteogenic differentiation

Bone sialoprotein (BSP) constitutes approximately 8% of all non-collagenous proteins found in bone, and is important in the nucleation process of hydroxyapatite formation.²⁴⁸ Osteocalcin (OCN) is expressed solely by the osteoblast, thus it is the most specific protein for osteoblast differentiation and mineralization.³³⁸ The fluorescent staining of BSP and OCN, the maker proteins of osteogenic differentiation, reveals that the MC3T3-E1 cells on the OGP-99%, OGP-50% and TiO₂ substrates secreted abundant amounts of BSP and OCN after 2 weeks, as indicated by the strong fluorescence of red (OCN) and green (BSP) on the substrates. In Figure 5.8, the immunohistochemical staining of cells on OGP-99% substrates is shown. Similar results were observed for OGP-50% and TiO₂ substrates. From the enlarged images, a difference in the distribution of OCN and BSP was observed. The amount of BSP in the cytoplasm and extracellular matrix (ECM) is similar (Appendix Figure 35), while the OCN showed a higher concentration in the cytoplasm (Appendix Figure 35). This is consistent with the fact that BSP is a component
in bone matrix, while OCN is secreted by osteoblasts to regulate the metabolic activities and bone-building process. Using RT-PCR, a quantitative comparison of the expressed total RNA level of BSP and OCN demonstrated a significant increase in expression of these osteogenic genes in cells on OGP-99% substrates (Figure 5.8). With enough OGP[10-14] present on the surface, the osteogenic differentiation of MC3T3-E1 cells was enhanced.

5.4.13 Effects of immobilized OGP[10-14] on ALP activity

Alkaline phosphatase (ALP) plays a critical role in the process of mineral formation in tissues such as bone, cartilage, and dentin. ALP activity is the most widely recognized biochemical marker for bone forming ability. A standard colorimetric assay was performed to quantify the ALP activity after culture for 18 days, and the values were normalized with total amount of protein to account for the difference of cell number content in samples. The immobilized OGP[10-14] at high concentrations exhibited an enhancement effect on the ALP activity (Figure 5.9). MC3T3-E1 cells on OGP-99% showed a 3-fold higher ALP level compared with those on OGP-50% and TiO₂. This up-regulation of ALP activity indicates the immobilized OGP[10-14] preserves its ability to stimulate the dephosphorylation, which is an essential activity involved in the mineralization process. The RNA expression level of ALP in cells on OGP-99% is also higher in comparison with those on OGP-50% and TiO₂ as indicated in RT-PCR (Figure 5.9B).
Figure 5.8 Bone sialoprotein (BSP) and osteocalcin (OCN), late markers of differentiation to osteoblast, were secreted by MC3T3-E1 cells on OGP-99% substrate, as demonstrated by the immunohistochemical staining after 2 weeks. (A) blue corresponds to the nuclei; (B) red corresponds to osteocalcin (OCN); (C) green corresponds to bone sialoprotein (BSP); and (D) is the merged image of the three channels (scale bar = 50 μm). Similar results were observed on OGP-50% and TiO₂ substrates. However, the cells on OGP-99% substrate expressed a much higher gene level of BSP and OCN, compare to cells on bare TiO₂, as shown in the (E) mRNA levels of transcription factor genes of BSP and OCN, in MC3T3-E1 cells measured by real-time PCR after cell culture for 18 days. Data represent relative expression to the level of the control (cells on TiO₂), set at 1, and mean value and standard deviation calculated from triplicates.
5.4.14 Effects of immobilized OGP[10-14] on mineralization

The appearance of calcium deposition is the phenotypic marker for the last stage of mature osteoblast. The extent of mineralized extracellular matrix (ECM) formed on OGP-99%, OGP-50% and TiO₂ substrates after 2 weeks was examined by staining with Alizarin Red S., a red dye that forms a complex with calcium depositions in ECM.³⁴ The cell films on all three substrates were positively stained red, indicating the MC3T3-E1 cells differentiated to osteoblast and secreted mineralized ECM. Under the microscope, the mineralized osteoids, spherulites with dark red color, ranging from 0.5 to 2 μm, were observed on all three substrates (Figure 5.9D, E and F). But only cell films on OGP-99% substrates showed the dark mineralized chunks, ranging from 2 to 10 μm, which is attributed to the higher content of calcium in the cell films on OGP-99% substrates. The calcium content was quantified with ICP-OES after 18 days culture in non-osteogenic medium. The result is consistent with that of Alizarin Red S. staining, as show in Figure 5.9C. The cell films on OGP-99% substrates exhibited more than two times higher concentration of Ca²⁺ normalized by total amount of protein to account for the difference in cell numbers. Therefore the immobilized OGP[10-14] on surfaces promotes the mineralization of osteoblasts.
Figure 5.9 (A)-(B) The ALP activity and its mRNA level of MC3T3 cells on OGP-99% substrate was significantly higher compared to substrates with lower concentration or none. (A) ALP activity of MC3T3 cells cultured on OGP-99%, OGP-50%, and TiO2 substrates, respectively, on day 18. (B) mRNA levels of transcription factor gene of ALP, in MC3T3-E1 cells measured by real-time PCR after cell culture for 18 days. (C) Ca2+ accumulation in the cell films quantified with ICP-OES and normalized with total amount of protein. The cell films on OGP-99% exhibited 2-fold higher content of Ca2+ compared with those on other substrates. (D)-(F) Mineralization of MC3T3-E1 cells on substrates studied by Alizarin Red S. staining on day 14 and Ca2+ quantification by ICP-AES on day 18. Larger sized calcified nodules were observed on OGP-99%, indicating promoted mineralization results from the higher concentration of OGP[10-14]. Images of cell films on (D) OGP-99%, (E) OGP-50%, and (F) TiO2 substrates after Alizarin Red S. observed under bright field microscope. The mineralized osteoids, the spherulites with dark red color, ranging from 0.5 to 2 μm, were observed on all the three kinds of substrates. And only cell films on OGP-99% showed the dark mineralized chunks, ranging from 2 to 10 μm.
5.5 Conclusion

Using straightforward synthesis, a series of multivalent catechol-bearing modular peptides were generated with the aim of providing a simple and efficient method to functionalize metal-oxide based orthopedic implants with bioactive peptides. With a multivalent binding strategy, tetravalent ligands were shown to persist on the metal oxide surfaces \emph{in vitro} beyond two weeks under near physiological conditions. The bioactivity of immobilized peptides was demonstrated in an \emph{in vitro} cell culture study. The tethered OGP[10-14] promoted the proliferation, osteogenic differentiation and mineralization of MC3T3-E1 cells. Considering their strong adhesion to versatile metal oxide surfaces, this modular peptide strategy is promising for the development of translational implants with improved bioactivity.

5.6 Acknowledgments

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CHAPTER VI

CONCENTRATION DEPENDENT HMSC DIFFERENTIATION ON ORTHOGONAL CONCENTRATION GRADIENTS OF GRGDS AND BMP-2 PEPTIDES

This work has been previously published as


6.1 Abstract

Self-assembled monolayer substrates containing tethered orthogonal concentration profiles of GRGDS (glycine/arginine/glycine/aspartic acid/serine) and BMP-2 (bone morphogenetic protein) peptides are shown to accelerate or decelerate, depending on the concentrations, the proliferation and osteoblastic differentiation of human mesenchymal stem cell (hMSC) populations in vitro without the use of osteogenic additives in culture medium. Concurrently, the single peptide gradient controls (GRGDS or BMP-2 only) induce significantly different proliferation and differentiation behavior from the orthogonal substrates. Bone sialoprotein (BSP) and Runt-related transcription factor 2 (Runx2) PCR data acquired from hMSC populations isolated by laser capture microdissection correspond spatially and temporally to protein marker data obtained from immunofluorescent imaging tracking of the differentiation process. Although genomic and protein data at high concentrations area GRGDS (71~83 pmol/cm²):BMP-2 (25 pmol/cm²) reveal an implicit
acceleration on the hMSC differentiation timeline relative to the individual peptide concentrations, most of the GRGDS and BMP-2 combinations displayed significant antagonistic behavior during the hMSC differentiation. These data highlight the utility of the orthogonal gradient approach to aid in identifying optimal concentration ranges of translationally relevant peptides and growth factors for targeting cell lineage commitment.

6.2 Introduction

Regenerative approaches to tissue engineering have been explored extensively over the last two decades, demonstrating stunning technical advances in pre-clinical applications. However, few comprehensive clinical trials have been carried out and the results have been somewhat limited. A thorough understanding of how growth factors and extracellular matrix (ECM) cues couple with materials to influence cell fate synergistically or antagonistically on the cellular and molecular levels is critical to provide a scientific foundation for more sophisticated tissue engineering designs and clinical regeneration applications. It is well understood that the molecular presentation and spatial distribution of growth factors can determine many important aspects of cell behavior, and threshold concentrations are often necessary to trigger cellular responses. Furthermore, it has been shown that cell responses occur in complex non-monotonic pathways, where signaling is a concerted, synergistic or antagonistic process. The quantitative evaluation of this concentration dependent synergistic or antagonistic effect is essential to gain a comprehensive understanding of growth factor-cell interaction for future tissue engineering constructs designed to control cell responses.
A recent study showed that the combination of a cell adhesion motif, arginine/glycine/aspartic acid, GRGDS, and BMP-2 (bone morphogenetic protein) peptides synergistically enhanced stem cell proliferation and osteoblast differentiation. Since normal cell function is dependent on surface anchorage, the introduction of the cell adhesion GRGDS peptide is used widely to aid cell localization and tissue development on synthetic substrates. BMP-2 peptide is pivotal for hMSC osteoblast differentiation and bone function development, and it is one of two BMP peptides that have been approved by the US Food and Drug Administration (FDA) for use in specific orthopedic applications. However, the complications and limitations surrounding the use of BMP-2 in the clinic implies the insufficiency of single growth factor delivery. The combination of BMP-2 with GRGDS peptide could potentially enhance osteoblast production and provide additional insight for more sophisticated tissue engineering design.

Herein, we employ orthogonal peptide concentration gradient substrates to study the influence of concentration and identify synergistic or antagonistic effects on cell differentiation in the presence of two growth factor peptides simultaneously. Significantly, these studies do not involve the use of dexamethasone or other osteogenic additives that are difficult to recapitulate in vivo. The cooperative interactions of GRGDS and BMP-2 peptides were quantified using orthogonal 2D (two-dimensional) GRGDS and BMP-2 concentration gradient substrates. The results from 2D experiments yield significant differences for hMSC proliferation and osteoblast differentiation from 1D (one-dimensional) GRGDS or BMP-2 peptide only. Studies revealed a dependency of enhanced or inhibited hMSC proliferation and osteogenic differentiation on GRGDS and BMP-2 concentration regimes. The results emphasize the importance of studying growth factor
combinations, with similar simultaneous stimulation properties, prior to their widespread use in tissue engineering applications.

6.3 Materials and Methods

All necessary purchasing and vendor information for materials and instrumentation will be listed in this section, followed by a complete experimental procedure.

6.3.1 Materials

4-chlorobutylidimethylchlorosilane and 5-hexenyldimethylchlorosilane, used for gradient deposition, were purchased from Gelest (Morrisville, PA). Fluorenylmethyloxycarbonyl (FMOC)-protected amino acids and reagents for peptide synthesis were purchased from EMD Millipore (Billerica, MA). All other chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. Two-dimensional (2D) gradient substrates were fabricated on glass coverslips (No 1; 25 mm×25 mm, Fisher Scientific) and used for cell culture after sterilization with a 24 h ethylene oxide cycle. For X-ray photoelectron spectroscopy (XPS) analysis, silicon wafers, (Si[100], one side polished, Silicon Quest International, were cut to 25 mm×25 mm) with identical glass substrate dimensions, were used as surrogates in the gradient fabrication process.

6.3.2 Orthogonal two-dimensional peptide gradient fabrication

Two-dimensional chemical gradient precursors were fabricated using a vapor deposition strategy\textsuperscript{360} with a few minor modifications. Briefly, substrates were pre-treated with UVO (Jelight Company Inc. Model No. 42A) for 20 min to generate a clean oxide
layer on substrate surfaces, followed by the deposition of 4-chlorobutylidimethylchlorosilane in the [X] direction using a “vacuum away” confined channel vapor deposition device.\textsuperscript{360, 361} Immediately following the chlorine gradient formation, an SN\textsubscript{2} substitution of the chlorine atom with NaN\textsubscript{3} in DMF was carried out with near quantitative conversion at 60 °C for 24 h. Subsequently, a chemical concentration gradient of 5-hexenyldimethylchlorosilane was deposited in the [Y] direction following a 90° rotation of the substrate. Following the formation of 2D orthogonal chemical gradients with [-Cl] and [vinyl] function in orthogonal directions, sequential thiol-ene and “click” reactions were carried out to immobilize the RGD and BMP-2 peptides on the gradient substrate. RGD or BMP-2 only 1D gradient samples were also prepared as controls using the same method, without the second peptide reaction. Samples were stored in vacuum desiccators at room temperature until used.

6.3.3 Functional peptide synthesis

The peptides were synthesized following standard solid phase FMOC chemistry without final cleavage. FMOC-protected PEG amino acid was synthesized and coupled to the respective bioactive peptide sequence as a spacer.\textsuperscript{362, 363} For thiol-functional RGD peptide (GRGDS), the amino acid cysteine was added to the N terminus using standard FMOC conditions. MALDI-ToF MS: RGD-thiol peptide [C\textsubscript{30}H\textsubscript{53}N\textsubscript{11}O\textsubscript{14}S, [M+H]\textsuperscript{+} m/z calculated 824.3, found 824.3; [M+2H]\textsuperscript{2+} m/z calculated 412.7, found 412.6. The 4-dibenzocyclooctynol (DIBO) end-functionalized BMP-2 peptide was synthesized using a resin-tethered BMP-2-PEG with a free amine at the N-terminus that was coupled to DIBO using a method described previously.\textsuperscript{364, 365} The cleavage reaction was carried out under
normal conditions, followed by trituration and purification. MALDI-ToF MS: BMP-2-DIBO peptide [C₁₂₄H₁₉₂N₂₄O₃₆], [M+H]^+ m/z calculated 2595.4, found 2595.0; [M+Na]^+ m/z calculated 2617.4, found 2617.0.

6.3.4 RGD immobilization by thiol-ene reaction

RGD-thiol peptide (5 mg/mL) was dissolved in H₂O/DMF (10:1 in volume) solution with Irgacure 2959 (2 mg/mL) as a photoinitiator. The substrate was immersed in the peptide solution and treated with UV (254 nm) for 1 h at room temperature. The substrate was washed with methanol, toluene and then methanol three times and blown dry under N₂ prior to characterization.

6.3.5 BMP-2 immobilization by strain-promoted alkyne-azide cycloaddition (SPAAC)

Following the thiol-ene reaction, the chemical gradient substrates with [N₃] was incubated in BMP-2-DIBO solution (5 mg/mL in DMF) at ambient temperature for 48 h. The substrate was washed with methanol, toluene and then methanol three times and blown dry under N₂.

6.3.6 X-ray photoelectron spectroscopy (XPS)

XPS measurements were performed on PHI VersaProbe II Scanning XPS Microprobe. High resolution C₁s signal was collected from binding energy 280 eV to 300 eV with 50 ms dwell time, analyzer pass energy 29.35 eV and 5 sweeps for every spectrum. The sample area was over 100 μm × 100 μm square. A total of 25 (5×5) evenly distributed sample points were characterized for one 2D gradient surface immediately after each
reaction, and the peak area values were calculated based on three individual identical gradient substrate measurements.

In order to ensure the consistency of XPS machine working conditions and minimize the systematical error, control samples were prepared freshly every time and tested in every XPS run. When there were unusual fluctuations for the control sample signals, the XPS results collected in the same butch would not be used. All the presented data were based on at least 3 identical samples measurements.

For the surface coverage calculation, a reference control sample with 100% coverage of silane was prepared by vapor deposition for 48 h at 50~60 °C. By comparing the control sample and gradient sample C_{1s} peak areas, the calculated ratio is the surface coverage fraction (Equation 6.1). According to the literature,^{366-369} fully covered self-assembled alkyl monolayer on silicon is 465 pmol/cm^2, therefore, the final surface concentration could be calculated using Equation 6.2.

\[
\text{surface coverage fraction} = \frac{\text{Peak area}_{\text{sample}}}{\text{Peak area}_{\text{control}}}
\]

Equation 6.1 The surface coverage fraction of the peptide was calculated using the ratios of the C_{1s} peaks for the sample and control samples.

\[
\text{surface concentration} = \text{surface coverage fraction} \times 465 \text{ pmol/cm}^2
\]

Equation 6.2 The surface concentration of RGD and BMP-2 were calculated using the surface coverage fraction value obtained from Equation 6.1.
In this study, two reference sample surfaces with 100% coverage of 4-chlorobutyltrimethylchlorosilane (4-Cl silane, containing 6 carbon atoms in one molecule) and 100% coverage of 5-hexenyldimethylchlorosilane (5-vinyl silane, containing 7 carbon atoms in one molecule), were prepared. Their high resolution C$_{1s}$ peaks were analyzed and averaged to give the C$_{1s}$ peak area value for 100% coverage of the surface with only one carbon atom (Figure 6.1). Ten reference samples were repeated for each silane surface to give precise results (Equation 6.3). It should be mentioned there that several other functional SAMs have been measured, and all the calculated C$_{1s}$ peak area one atom values were identical within the systematical error. The final peak area value for 100% coverage of silane surface with only one carbon atom under our specific XPS setting was 345

Figure 6.1 High resolution C$_{1s}$ peak fitting for (A) 100% coverage of 4-Cl and (B) 100% coverage of 5-vinyl silane surfaces. The spectrum shows the average result based on 10 identical samples, and the error bar was calculated as the

$$C_{1s} \text{ peak area}_{one \text{ atom}} = \frac{10 \times C_{1s} \text{ peak area}_{4-Cl}}{6} + \frac{10 \times C_{1s} \text{ peak area}_{5-vinyl}}{7}$$

$$= 345 \pm 18$$

Equation 6.3 The C$_{1s}$ peak area was calculated for one carbon atom using 10 samples for each reference surface type.
To quantify the peptide immobilization concentrations, high resolution C\textsubscript{1s} was collected along the 2D substrate for each sample point after every reaction step. Figure 6.6.2 illustrates one of the sample point’s C\textsubscript{1s} signal change during the fabrication process. The peak area increased from 2833 (before reaction) to 4739 after RGD, and further increased to 6030 after BMP-2 addition. When the peptide concentration was calculated using Equation 6.4 and Equation 6.5, the number of carbon atoms in each peptide sequence was considered. Specifically, RGD peptide contains 31 carbon atoms, and BMP-2 peptide contains 125 carbon atoms in the sequence.

\textit{RGD surface coverage fraction}

\[
\frac{C_{1s} \text{peak area}_{after \ RGD} - C_{1s} \text{peak area}_{before \ RGD}}{31 \times 345} = \frac{4739 - 2833}{31 \times 345} = 0.18
\]

Equation 6.4 RGD surface coverage fraction equation taking into account the 31 carbons in the GRGDS sequence.

\textit{BMP surface coverage fraction}

\[
\frac{C_{1s} \text{peak area}_{after \ BMP} - C_{1s} \text{peak area}_{before \ BMP}}{125 \times 345} = \frac{6030 - 4739}{125 \times 345} = 0.03
\]

Equation 6.5 BMP-2 surface coverage fraction equation taking into account the 125 carbons in the BMP-2 sequence.
During the high resolution analysis, the main peak binding energy of C\textsubscript{1s} was first calibrated to 285 eV, and the linear background was subtracted. The peaks from different carbons were assigned according to the structure, literature evidence, and an online database (http://srdata.nist.gov/xps/Default.aspx). The peak fitting was done automatically.

Figure 6.6.2 High resolution C\textsubscript{1s} signal tracking for the peptide immobilization process. (A) C\textsubscript{1s} peak before any reaction; (B) after the RGD covalent bonding; (C) after RGD and BMP-2 peptide immobilization; (D) the overlay of the C\textsubscript{1s} peak during the reactions. The peak strength increase is a result of the attachment of peptide, and was used to calculate the peptide surface coverage fraction according the previous equation.

During the high resolution analysis, the main peak binding energy of C\textsubscript{1s} was first calibrated to 285 eV, and the linear background was subtracted. The peaks from different carbons were assigned according to the structure, literature evidence, and an online database (http://srdata.nist.gov/xps/Default.aspx). The peak fitting was done automatically.
by CasaXPS software using Levenberg–Marquardt algorithm and the final total peak area was also analyzed by the software with the standard parameters setting.

6.3.7 hMSC cell culture

Male hMSCs (Lonza, Walkersville, MD) were expanded following the manufacturer’s protocol using Lonza MSC growth medium. For proliferation and differentiation experiments, hMSCs, passage 5, were seeded onto 25 mm x 25 mm gradient coverslips at a cell density of 80 cells/mm². Human MSCs were cultured in Lonza growth medium (supplemented with 10 vol% FBS, 10 mL L-glutamine, 30 µg/mL Gentamicin, and 15 ng/mL Amphotericin) without osteogenic additives to avoid driving the hMSC differentiation, in 37 °C incubators with 5% CO2 for up to 3 weeks.

6.3.8 hMSC proliferation

Cell proliferation was measured using a Click-iT EdU imaging kit (Invitrogen). The cells on the substrates were first fixed (3.7% formaldehyde in PBS for 15 min) and washed twice with 3% BSA in PBS. The cells were permeabilized in 0.5% triton X-100 in PBS at room temperature for 20 min and rinsed with 3% BSA in PBS solution twice. Click-iT reaction cocktails were prepared according to the manufacturer’s protocol, and added to each substrate (30 min incubation at room temperature, protected from light). The substrates were washed twice with 3% BSA in PBS and imaged with an IX81 microscope (Olympus). Days 1, 3 and 7 time points were studied. Five randomly selected images were taken at each concentration regime on the sample (n = 3) for statistical analysis. The cell number within each image was counted and the final cell density value was calculated and
averaged based on 15 images. The error bar represents the standard deviation of the mean (n = 3) for each concentration point.

6.3.9 hMSC differentiation investigation by Runx2 and BSP immunofluorescence

Cells on substrates were fixed (3.7% formaldehyde in PBS for 20 min) and permeabilized (0.1% triton X-100, 0.1% sodium citrate in PBS for 10 min) on days 7, 14 and 21. The cell-substrates were washed with 0.1% donkey serum in PBS for 30 min and then blocked with 10% donkey serum, 0.3% triton X-100 in PBS for 1h at ambient temperature. Incubation in the primary antibody solutions, Runx2 (rabbit polyclonal IgG, diluted 1:100 in PBS, Santa Cruz Biotechnology) and BSP (mouse IgG1, diluted 1:200 in PBS, Santa Cruz Biotechnology), was conducted at 4 °C overnight. The primary antibody solution was then aspirated the following day and subsequently the substrates were incubated in the secondary antibody solution with either anti-rabbit Alexa Fluor 546 (Invitrogen, diluted 1:400 in PBS) or anti-mouse Alexa Fluor 488 (Invitrogen, diluted 1:400 in PBS) for 1 h at room temperature. A nuclear fluorescent stain was conducted using a DAPI solution (6 µL/10 mL in PBS) for 20 min. The substrates were washed with 0.1% donkey serum in PBS for 1 h and rinsed with PBS buffer three times. The fluorescent-stained cells were imaged on the same day. Five images were taken for each concentration section on each substrate (n = 3) totaling 15 images for each concentration regime. The expression of Runx2 and BSP was qualitatively assessed.
6.3.10 RNA isolation by laser capture microdissection (LCM)

Using an ArcturusXT™ Laser Capture Microdissection System (Life Technologies), groups of cells were collected for quantitative gene expression studies at each peptide concentration point: 5 points (concentrations) for 1D peptide samples and 5×5 or 25 collection points for each of the 2D peptide gradients. Five substrates (n = 5) were used for each gene at each time point (14d and 21d). The cell-substrates were removed from the culture medium, immediately frozen in liquid nitrogen for 1 min and stored at -80 °C. Immediately before LCM, the cell-substrates were removed from -80 °C, fixed in 70% ethanol for 1 min, and subsequently dehydrated through graded ethanol of 95%, 100%, 100% for 1 min each, followed by xylene three times for 5 min each. The cell-substrate was air dried at room temperature for 1 min to allow xylene evaporation, and populations of cells were isolated by LCM from specific concentration regions. Immediately after capture, cells were extracted from the cap, total RNA was isolated and DNase-treated following the protocol provided with the PicoPure RNA Isolation Kit (Life Technologies, Catalog # KIT0202). The whole process was done as quickly as possible, and all reagents were kept on ice to minimize RNA degradation. The isolated RNA from each collection point was stored at -80 °C.

6.3.11 Real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Each sample RNA stored at -80 °C was reverse transcribed to cDNA using Taqman Reverse Transcription Reagents (Invitrogen, Life Technologies). The reverse transcription was performed on the 7500 Real Time PCR System (Applied Biosystems, Life Technologies) using the thermal protocol of 50 °C for 30 min followed by qPCR in a two-
step process. Subsequently, each well for qPCR contained 10 µL cDNA, SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies) and either Runx2 or BSP primer sequences designed previously. For all genes, the amplification protocol consisted of activation at 95 °C for 15 min, and 40 cycles of denaturation for 30 s at 95 °C, annealing for 1.5 min at 58 °C, and extension for 2 min at 72 °C. Following amplification, a melt curve analysis was performed at 1 °C increments from 50 °C to 95 °C to analyze the purity of the product generated. Standards were prepared for each gene and each PCR plate. 18S RNA gene was used as the endogenous reference. The relative gene expression levels of Runx2 or BSP were calculated utilizing the comparative $C_t$ methodology, $2^{-\Delta C_t}$, where $\Delta C_t = C_{t,\text{RUNX2 or BSP}} - C_{t,18S\text{rRNA}}$ for each sample point. Quantitative gene expression levels were measured from 5 identical samples at 14 days (n = 5) and 21 days (n = 5) for each concentration point (n = 5 or 25) and each gene (n = 3). Gene expression plots were generated in Microsoft Origin showing gene expression as a function of gradient concentration regime after normalization to 18S total RNA expression.

6.3.12 Statistical analysis

Proliferation and RT-qPCR data was conducted with 3 (n=3) and 5 replicates (n=5), respectively. Statistical analysis for gene expression along the gradient was completed using a one-way analysis of variance (ANOVA) with Tukey post hoc analysis. To determine significance at the same gradient position from 2 to 3 weeks, an independent t-test was used. A significance value of $p \leq 0.05$ was set for all statistical analysis.
6.4 Results

A summary of the results for all of the data collected for this chapter is provided in this section.

6.4.1 Orthogonal 2D gradient fabrication

Orthogonal (2D) GRGDS and BMP-2 peptide concentration gradients were fabricated using a sequential gradient vapor deposition method. After the initial chemical profiles were generated, sequential thiol-ene addition (GRGDS) and strain-promoted azide-alkyne cycloaddition (BMP-2) reactions were utilized to immobilize the GRGDS and BMP-2 peptides (Figure 6.3).

The 2D orthogonal chemical gradient fabrication methods have been described previously.\textsuperscript{360,371} Here, the application of simple chemical gradients has been extended to generate orthogonal peptide concentration gradients using sequential “click’ reactions. The fabrication process was monitored using high-resolution x-ray photoelectron spectroscopy (XPS) to calculate quantitatively the chemical profiles and peptide concentration (Figure 6.4).

The samples were carefully washed after each step, removing the potential non-covalent bonded peptides and contaminants, therefore the increase of $C_{1s}$ peak area after the click reaction results from the covalent bonding of the peptide on the surface. The peptide concentrations were calculated from the increased amplitude of $C_{1s}$ peak area for every sample spot respectively. The final spatially-dependent concentration distributions of GRGDS and BMP-2 peptides are plotted where the formation of orthogonal concentration gradient substrate for GRGDS and BMP-2 can be seen in Figure 6.5.
Figure 6.3  (A) Schematic illustration of the fabrication of 2D orthogonal peptide concentration gradients. (a) Glass substrates were treated with UV-ozone. (b) The first component, 4-chlorobutyldimethylchlorosilane, was deposited using a gradient vapor deposition method. (c) The [-Cl] group was substituted with an azide functional group. (d) The substrate was rotated 90°, and the second gradient deposition process introduced vinyl functional groups in the [Y] direction. (e) Sequential thiol-ene addition and (f) strain promoted alkyne-azole cycloaddition (SPAAC) reactions were carried out to immobilize GRGDS and BMP-2 peptides, respectively. (B) The corresponding chemical reactions happened at each step. (C) The cysteine functionalized GRGDS peptide structure synthesized for the thiol-ene reaction. (D) BMP-2 peptide structure with 4-dibenzocyclooctynol (DIBO) end group for SPAAC reaction.
The concentration gradient profiles decreased continuously in one direction (GRGDS in [Y] direction, BMP-2 in [X] direction) while remaining nearly identical in another direction, results which support the concept of orthogonal gradients. The final concentration values along the gradient were averaged across the identical substrate positions, and showed a 2D orthogonal gradient with GRGDS concentrations ranging from ~15 to 90 pmol/cm² and a BMP-2 concentration range from ~0 to 25 pmol/cm². All possible concentration value combinations of GRGDS and BMP-2 peptides in these ranges are presented on these 2D gradient substrates. More than 200 identical substrates were generated to facilitate the replicate experiments and multiple time points required in this study.

Figure 6.4 High-resolution XPS C₁s signals were used to characterize the extent and efficiency of the peptide immobilization procedure. (A) The C₁s peak area distribution before the thiol-ene reaction for GRGDS peptide (step (d) in Figure 1). (B) The C₁s peak area increased at each point on the substrate after the GRGDS immobilization (step (e) Figure 6.3) (C) The peak area increased further after the BMP-2 peptide was covalently attached (step (f) in Figure 6.3).
A quantitative evaluation of the synergistic or antagonistic effects of GRGDS and BMP-2 peptides on hMSC proliferation and differentiation was carried out using 2D orthogonal peptide concentration gradient substrates. The GRGDS only and BMP-2 only
one-dimensional (1D) gradient substrates were used as controls, under the same fabrication and cell culture conditions.

hMSC proliferation was quantified on days 1, 3 and 7 using cell counts. On the 1D GRGDS and BMP-2 control gradients, the cell density did not exhibit explicit concentration dependent behavior. There were no significant differences in the cell numbers over the entire concentration range for both the GRGDS and BMP-2 controls (Figure 6.6)

![Graph](image)

Figure 6.6 The hMSC cell proliferation behavior on 1D only RGD (A) and BMP-2 (B) gradient substrates. The cell number was determined from 3 identical samples with images randomly picked (n = 3) at each concentration point utilizing a Click-iT EdU imaging kit. Error bars represent the standard deviation of mean values.
Surprisingly the cell density distribution on the 2D GRGDS and BMP-2 orthogonal concentration gradients demonstrated strong spatio-temporal dependent behavior (Figure 6.7). Initially, the high GRGDS (71–83 pmol/cm²): low BMP-2 (0.5 pmol/cm²) concentration regime promoted the highest cell density with an average value of 6726 ± 2815 cells/cm². Regions toward the middle of the gradient (GRGDS 23-56 pmol/cm² and BMP-2 3.5-13 pmol/cm²) showed significantly lower cell density values with p ≤ 0.05. However, on day 3, the highest cell density was located in the low GRGDS (23 pmol/cm²): low BMP-2 (0.5 pmol/cm²) (15,029 ± 4858 cells/cm²) combination area and remained localized in this concentration regime section up to day 7. Statistics suggest that this gradient regime had statistically (p ≤ 0.05) higher cell density compared to the majority of GRGDS:BMP-2 concentration areas from GRGDS = 31-83 pmol/cm² and BMP-2 = 0.5-13 pmol/cm². Early time points showed that the high BMP-2 concentration tended to induce lower cell density regardless of GRGDS concentration, while from day 3 to day 7 the cell density in low GRGDS (23 pmol/cm²): low BMP-2 (0.5 pmol/cm²) area was similar to the low GRGDS (23 pmol/cm²): high BMP-2 (25 pmol/cm²) section, and the lowest cell density appeared in the high GRGDS: high BMP-2 corner gradually over time. Even though there was no evidence supporting the enhancement of BMP-2 peptide for hMSC proliferation in the 1D control samples, the colocalization of a low concentration of BMP-2 peptide with GRGDS did trigger different cell density distribution compared with the 1D control substrates. Through initial speculation, it is possible that the low GRGDS: low BMP-2 concentration regime delays the progression of hMSCs from the proliferative to the differentiation phase. The co-regulation of GRGDS and BMP-2 peptides to hMSC proliferation appears to have distinct effects than individual peptide environment.
Figure 6.7 The hMSCs density distribution on the 2D GRGDS and BMP-2 peptide gradients. The columns on the left and below each 2D data set represent the cell density values on 1D GRGDS only concentration gradient surfaces and 1D BMP-2 only concentration gradient surfaces, respectively. (A) Cell density after 1 day in culture; (B) Cell density after 3 days in culture; (C) Cell density after 7 days in culture. The data reported represents cell density calculated from n=3 samples.
6.4.3 *hMSC differentiation*

*hMSC differentiation* behavior was qualitatively and quantitatively assessed using Runx2 and BSP protein expression.\(^{372-374}\) Runx2 is a member of the Runt domain family and the essential master transcription factor during stem cell-specific osteoblast development and bone formation.\(^{375-379}\) Bone sialoprotein (BSP) is a major non-collagenous protein in bone extracellular matrix expressed by osteoprogenitor and mature osteoblasts for bone mineralization initiation.\(^{204, 248, 372}\) The expression is off set temporally with Runx2 rising and falling followed by BSP expression later in the commitment timeline.\(^{373}\) The secretion of BSP during differentiation implies the formation of mature osteoblasts and the onset of bone matrix formation.

Strong expression variation of Runx2 on day 7 and day 14 was not observed across the cell-substrates, which implied the onset of osteoblast differentiation on both 1D and 2D gradients at every concentration by day 7 (Figure 6.8). However, from day 14 to day 21, the lowest Runx2 expression was found in the high GRGDS: high BMP-2 concentration area. The elevated Runx2 expression gradually transitioned toward the low BMP-2 concentration sections.

BSP is expressed by osteoprogenitor and mature osteoblasts during osteoblast development, and expression level decreases after mature osteoblast formed. Its expression serves as an indicator for stem cell differentiation rate and progression, due to a peak in expression levels as cells advance from the osteoprogenitor to the mature osteoblast stage.\(^{190}\) BSP protein expression was observed as early as day 7, near the highest GRGDS (71~83 pmol/cm\(^2\)): highest BMP-2 (25 pmol/cm\(^2\)) concentration sections on the 2D gradient substrates showing the greatest expression level. This served as supporting
evidence for the enhanced acceleration of stem cell osteoblast differentiation with the combination of high GRGDS: high BMP-2 concentration. BSP signaling continuously increased from day 7 to day 14 on the 2D substrates, especially in the highest BMP-2 concentration regions (25 pmol/cm²) with GRGDS concentration from 31–83 pmol/cm². BSP immunofluorescent staining in the high concentration region reached its peak expression level on day 14. From day 14 to day 21, BSP expression dropped dramatically in the high concentration position, while it increased toward the relatively lower GRGDS and BMP-2 concentration regions indicating a slower progression of differentiation. The appearance of high level BSP expression on day 14 suggests the generation of mature osteoblasts and the onset deposition of bone extracellular matrix, which indicates faster differentiation compared with other concentrations. This confirmed the results that were obtained for Runx2 analysis, the highest GRGDS and BMP-2 concentration combination promoted the fastest hMSC osteoblast differentiation.

Surprisingly, on day 7, high levels of BSP expression on the 1D GRGDS only surfaces did not occur in the high RGD concentration regime on the 1D control gradients. By days 14 and day 21, the trend was reversed. Higher GRGDS peptide concentration promoted more BSP expression on a longer time scale. On the other hand, the spatial dependence of BSP on BMP-2 peptide was consistent with the 2D gradient trend, but with lower overall expression levels.
Figure 6.8 The hMSCs osteoblastic differentiation progression on the 2D gradient substrate was measured by Runx2 (red) and BSP (green) immunofluorescence imaging. The columns on the left and below each 2D data set represent the cell behavior on 1D GRGDS only and 1D BMP-2 only concentration gradient surfaces, respectively. From (A) day 7, (B) day 14 and (C) day 21 images, the Runx2 and BSP proteins showed spatially- and temporally-dependent expression behavior.
For quantitative characterization, total RNA gene expressions of Runx2 and BSP were measured by RT-qPCR at day 14 and day 21. Total RNA was isolated from specific concentration regimes by laser capture microdissection (LCM). The LCM enabled gene expression results were correlated with the immunofluorescence. In general, on 2D gradients, PCR measurements of Runx2 expression illustrated significantly greater levels from day 14 to day 21 for regions of the highest GRGDS concentration (71-83 pmol/cm²) in combination with low to mid BMP-2 concentrations (0.5-7.0 pmol/cm²). Significantly greater expression of Runx2 from 14 to 21 days is also observed in the lowest GRGDS concentration regime (23 pmol/cm²) with mid to high BMP-2 levels (7.0-25 pmol/cm²). At 2 weeks the Runx2 expression at moderately high GRGDS (31 pmol/cm²) and the highest BMP-2 (25 pmol/cm²) was significantly greater compared to almost all regions along the GRGDS gradient with low to mid BMP-2 concentrations (0.5-7.0 pmol/cm²). Overall, Runx2 expression trended with an increase across the 2D substrates along the diagonal direction x-axis, reaching the peak expression at GRGDS 31~56 pmol/cm²: BMP-2 13-25 pmol/cm² conditions on day 14. On day 21, statistical analysis revealed there was no significant difference in Runx2 expression along the 2D gradients, however, the general trend from week 2 to 3, shows significant increases in expression of this early osteogenic transcription factor. Quantitative data reveals a peak Runx2 expression in the 31-56 pmol/cm² GRGDS: 7.0-13 pmol/cm² concentrations regime on day 21. Combined with day 14, the overall trend reveals that at lower RGD concentrations (31 pmol/cm²): high BMP-2 concentrations (7.0-25 pmol.cm²), Runx2 expression is consistently greater. With no significant difference in expression in this regime the explanation that cells on the low GRGDS: high BMP-2 regime exhibited an accelerated osteoblast differentiation profile.
BSP gene expression patterns from day 14 to day 21 demonstrated an even clearer trend. At day 14, BSP levels appeared to have decreased from the high GRGDS: high BMP-2 concentration corner to low-low concentration region with the 31 pmol/cm² GRGDS concentration: 7.0 pmol/cm² BMP-s concentration regime showing a significantly lower expression compared to most regimes on the gradient. On day 21, the expression graphs show an elevated BSP expression in the low: low concentration regime, however, no significant difference was detected with statistical analysis. Although statistics did not reveal a difference in BSP expression along the gradient by day 21, significantly greater expression was seen from day 14 to 21 in regards to identical concentration regimes. It is important to note that generally, in mid to high BMP-2 concentration areas (7.0-25 pmol/cm²), the expression of BSP does not appear to significantly change from 2 weeks to 3 weeks. This data shows good correlation with Runx2 expression, suggesting that the low: high concentration levels of GRGDS:BMP-2 respectively, may drive osteogenic differentiation of hMSCs faster. All PCR quantification and statistical analysis can be seen in Figure 6.9. These quantitative PCR characterization findings correlate with the immunofluorescence study, indicating an accelerated hMSC osteoblast differentiation at highest concentration region.

Using BSP expression as the differentiation progress indicator, it is evident that the biological outcomes of using combinations of GRGDS and BMP-2 are not straightforward additions or subtractions of their individual functions. The RNA day 14 expression in the intermediate concentration section (GRGDS 23~83 pmol/cm²: BMP-2 0.5~13 pmol/cm²) was lower than GRGDS and BMP-2 only surfaces for BSP genes. Meanwhile, in the highest concentration region (BMP-2 25 pmol/cm²: GRGDS 23~83 pmol/cm²), the
expression level was slightly higher. However, even in the highest concentration region, the RNA accelerated expression level on 2D substrate is not qualified as explicit synergistic effects, as reported in some similar previous researches\textsuperscript{249, 359}. 
Figure 6.9 The quantitative RT-qPCR characterization for Runx2 (A1) (B1) and BSP (A2) (B2) gene expression levels at 14-day (A) and 21-day (B) time points. The single columns on the left and below each 2D data set represent the gene expression results on 1D GRGDS only and 1D BMP-2 only concentration gradient surfaces, respectively. The gene expression levels were normalized to 18S rRNA control at every sample point. In general, the gene expression patterns of the cells on the substrates complemented the protein secretion results by fluorescence shown in Figure 6. Statistics were performed to show significance in gene expression from 2 to 3 weeks and along the gradients. * indicates significance of $p \leq 0.05$ from week 2 to week 3 in the same gradient position for both 1D and 2D gradients. ** indicates significant of $p \leq 0.05$ at the same time point along the 1D and 2D gradients. The bolded box highlights the gradient position which is significantly different from every position marked with **,
6.5 Discussion

Results have shown that 2D orthogonal peptide concentration gradients provided a suitable and efficient platform to quantitatively evaluate the surface insoluble factor concentration dependence and multiple synergistic effects individually and simultaneously. The accuracy of the peptide density, which is critical to the future of well-defined material properties, was quantitatively and repeatedly characterized by high resolution XPS C\(_{1s}\) tracking. With gradient vapor deposition methods described in our previous work, individual chemical concentration gradients were formed by confined diffusion. Concentrations were decreased along the diffusion pathway, while remaining identical in the perpendicular direction.\(^{360}\) By sequential chemical deposition and “click” reactions in the \([X]\) and \([Y]\) axis, orthogonal peptide concentration gradients were achieved with well-defined concentration parameters Figure 6.5.

6.5.1 hMSC proliferation

In previous reports, researchers have shown that there is a dose-dependency of RGD on cell proliferation, however, the 1D gradient results seem to conflict with these well-established conclusions.\(^{272, 351, 352, 380}\) This inconsistency could arise from several factors, including different cell types and initial cell seeding densities used in each of the experimental designs. Lateral spacing of RGD has also been reported as a key player in cell adhesion to surfaces, suggesting that a critical spacing between RGD units is necessary for optimal cellular activity.\(^{381, 382}\) Here, only the peptide surface density is being controlled, however, lateral spacing is random, which may have led to similar cell densities at each gradient position. Moore, et. al, had reported an RGD dose-dependency on cell
proliferation, however, below a concentration of 70 pmol/cm\(^2\) there was no difference in cell density along the gradient, which is the concentration regime that was studied in this work.\(^{249}\)

Generally, BMP-2 peptide triggered lower cell density numbers relative to GRGDS peptide substrates, which was consistent with previous understandings regarding GRGDS promoting cell adhesion and proliferation, and BMP-2 peptide having limited influence on cell proliferation.\(^{383}\) Overall, the presence of a high BMP-2 concentration did not benefit hMSC early cell proliferation, and this limited influence weakened gradually until day 7. However, small amounts of BMP-2 peptide (0.5 pmol/cm\(^2\)) in combination with GRGDS triggered the highest cell density relative to GRGDS alone. It is also interesting to note that the cell proliferation did not depend on GRGDS or BMP-2 concentration in the studied range on 1D single peptide surface, while on 2D substrates it displayed concentration dependence. The highest cell density location changes from the high GRGDS section to low GRGDS concentration from day 1 to day 3, indicating that the cells in the high GRGDS regime are entering the differentiation phase.\(^{384}\) The adhesion function of GRGDS peptide explains the higher cell density localization on higher GRGDS concentration areas at day 1. The lower number shows that the localized cell population has slowed the proliferative phase and started the differentiation process by day 3. Another possible explanation of cell density data is that even though high GRGDS peptide dose can promote cell adhesion, the dose intrinsically did not support stem cell proliferation in early culture stages. The exact signaling and cell mechanism remains unclear and is under further investigation. Even though there was no evidence supporting the enhancement of BMP-2 peptide for hMSC proliferation in the 1D control samples, the colocalization of a low concentration of BMP-
2 peptide with GRGDS did trigger different cell density distribution compared with the 1D control substrates. Through initial speculation, it is possible that the low GRGDS: low BMP-2 concentration regime delays the progression of hMSCs from the proliferative to the differentiation phase. The co-regulation of GRGDS and BMP-2 peptides to hMSC proliferation appears to have distinct effects than individual peptide environment.

6.5.2 hMSC differentiation

As for hMSC osteoblast differentiation, high GRGDS concentration on the 1D gradient may inhibit BSP expression on day 7 while causing no significant variance in Runx2 protein expression and cell density. Similar Runx2 protein expression results were reported by Firth, et. al, with no significant change in Runx2 expression in relation to RGD dosage. The inhibitory influence on BSP decreased by day 14, showing higher BSP expression at the high GRGDS concentration position compared to the lower concentration sections. This observation demonstrated the temporal dependence of GRGDS peptide density influence on hMSC osteoblast development. It is well known that the GRGDS sequence promotes cell adhesion, and it has previously been reported that cell adhesion and spreading on a surface will promote osteogenic lineage commitment. These results coincide with the work of Chen, et. al, revealing that greater BSP expression correlates with higher concentration of the GRGDS cell adhesion peptide. hMSCs remain in the proliferative phase due to strong adhesion to the surface as a result of RGD functionalization, however, BSP is not expressed during this phase. As the cells progress into the osteoprogenitor stage, BSP expression is enhanced. High GRGDS concentration inhibited osteoblast formation at early time points, but later accelerated differentiation.
This inhibitory effect of GRGDS peptide was not demonstrated on GRGDS and BMP-2 combined 2D substrates. The highest BSP expression appeared in the high RGD (71~83 pmol/cm²): high BMP-2 (25 pmol/cm²) gradient corner on day 7, and throughout the entire GRGDS concentration range (23~83 pmol/cm²): high BMP-2 (25 pmol/cm²) section on day 14. Generally, the high GRGDS: high BMP-2 combination stimulated the fastest osteoblast differentiation during the entirety of the experiment, as indicated by the rise and fall in BSP expression by day 21. Meanwhile, the differentiation in the lower BMP-2 concentration region continued to progress; therefore Runx2 expression was at a continuously high level. This statement is further supported by BSP protein secretion patterns. The quantitative PCR analysis for the BSP gene was consistent with qualitative observations captured through immunofluorescence. Unlike BSP data, there is a difference in Runx2 expression across the gradient when comparing immunofluorescence and PCR data. Overall, from 7 to 14 days, there is a noticeable increase in Runx2 expression for both data sets, however, immunofluorescence suggests the highest Runx2 expression in the high RGD: low BMP-2 corner of the 2D gradient at 14 days. The Runx2 transcription factor is synthesized in the cytoplasm and transported to the nucleus. During immunofluorescence staining, if permeabilization was inefficient, only the newly synthesized Runx2, outside of the nucleus would have been stained, causing a discrepancy in the data.

In general, quantitative and qualitative gene expression characterization revealed that the outcomes of the GRGDS and BMP-2 combination were not the straightforward addition or subtraction of their individual functions. The enhancement or reduction effects
were spatio-temporal dependent, requiring specific analysis for confined conditions, and there is no explicit synergistic effect evidence in studied concentration range.

6.6 Conclusion

The synergistic mechanism of GRGDS and BMP-2 combination during hMSC osteoblast differentiation is extremely complex, considering the endless crosstalk between the activated intracellular pathways. The deciphering of this cooperative network on the cellular and molecular level remains urgent to provide insights about the cell-surface interactions for more sophisticated tissue engineering design. The acceleration of hMSC proliferation and osteoblast differentiation on 2D substrates possessing an orthogonal concentration gradient of GRGDS and BMP-2 peptides was shown to be concentration dependent. The results, obtained without the use of osteogenic additives, illustrated the concentration dependence and spatio-temporal features of the tethered peptides effects over the hMSC behavior that has not been quantitatively demonstrated previously. While the authors readily acknowledge that additional factors influence hMSC proliferation and osteoblast differentiation, the aforementioned data and controls required more than 300 identical substrates. In the future we envision that additional factors and combinations can be screened on these substrates, facilitating the acquisition of mechanistic understandings of cell-surface and cell-growth factor interactions. This strategy provides a novel platform for surface property identification and optimization, by rapidly surveying a wide array of chemical and structural variables. The approach is qualified as a solid model for investigations of surface chemical functions investigation in many other aspects.
6.7 Acknowledgements

The authors are grateful for financial support from the National Science Foundation (DMR-1105329).
CHAPTER VII

CONCLUSION

In the past several decades, polymeric materials for bone tissue engineering has become an increasingly studied field. Both naturally occurring and synthetic polymers have been investigated for this applications, however, as separate entities, they fall short of meeting all of the requirements necessary for regeneration of healthy bone tissue. In particular, these materials must be high modulus, resorbable, non-toxic, and osteoinductive, in order to reach full bone healing potential. While natural occurring polymers contain the inherent biochemical signaling necessary for driving bone formation, they often lack suitable mechanical properties and thermal stability. On the contrary, the mechanical, chemical, and thermal properties of synthetic materials can be tuned to meet the requirements for bone tissue engineering. Unfortunately, polymers do not inherently have osteoinductive ability and often degrade into acidic by products that cause chronic inflammation and failure of the implant. In more recent years, researchers have developed methods of combining the natural and synthetic materials, in order to meet all of the criterion for successful bone tissue regeneration.

Phenylalanine-based poly(ester urea)s are highly tunable synthetic materials that can be chemically functionalized for bone tissue engineering applications. In this dissertation, phenylalanine-based copolymers were both tethered and crosslinked with
OGP peptide in order to upregulate proliferation and differentiation of preosteoblast cell lines, including hMSCs and MC3T3s. OGP is a naturally occurring tetradecapeptide with an active domain from amino acids 10 to 14, which is known to enhancing preosteoblast proliferation, differentiation and matrix mineralization *in vitro*. Here, the successful synthesis of 1% OGP-tethered poly(1-PHE-6) was reported and tested both *in vitro* with hMSCs and *in vivo* with promising results for bone tissue engineering applications. Upregulated expression of early, middle and late markers of osteogenic differentiation were observed for OGP-tethered samples compared to non-tethered controls *in vitro*. In addition, two pore size ranges were studied for optimization of cell integration and bone tissue formation in the phenylalanine-based materials. Overall, larger pore sizes, 250-400 µm, were shown to have promote the greatest enhancement on OGP-tethered samples compared to the smaller pore sizes, 100-250 µm. Optimized cell adhesion and spreading, as well as matrix mineralization may be the leading factors that explain this upregulation of bone differentiation on larger pore-sized samples.

OGP-crosslinked phenylalanine-based poly(ester urea)s were also developed using an efficient thiol-ene crosslinking reaction. In an effort to ensure the OGP remained bioactive while tethered into the polymer network at both the *N*- and *C*- terminus, preliminary *in vitro* studies were conducted using mouse preosteoblast cells. These tests suggested that OGP-crosslinking at 3% has the capability to enhance proliferation and early osteogenic differentiation of MC3T3s. As previously mentioned, high modulus materials are necessary for bone regeneration. Crosslinking OGP into the poly(ester urea) network was meant to enhance not only osteogenic differentiation but was also employed for enhancement of mechanical properties. Here, 3% crosslinking of OGP into the network
resulted in a polymer with a decreased tensile modulus or stiffness, however, the overall
toughness of the network was increased. Although, these results were unfavorable for the
application, it is apparent that the mechanical properties of these materials can be tuned
using OGP crosslinkers. Future directions of this work includes tuning the mechanical
properties with varying degrees of crosslinking in order to enhance stiffness of the network
for bone tissue engineering applications. The tensile modulus will be tested on vacuum
compressed films. These polymers should then be fabricated into porous scaffolds via 3D
printing or salt leaching, for proper in vitro testing using multipotent hMSCs. The porous
scaffolds with varying crosslinking amounts should be testing for compression modulus to
gain sufficient information on the type of environment the cells will be exposed to during
the in vitro work. In addition to work with OGP crosslinkers, scrambled-OGP crosslinkers
should be synthesized to use as a negative control for the in vitro experiments. Controls
should also include soluble OGP and a PLLA standard material.

In this dissertation, OGP was not only used for functionalization of poly(ester
urea)s for enhanced osteogenesis, but it was also utilized for functionalized of TiO
surfaces for increased osseointegration. Titanium is one of the most widely used metals in
bone implants because of its low density, high strength and high resistance to erosion. To
improve the efficacy of titanium implants, greater osseointegration needs to be achieved.
Here, modular OGP peptides, bearing functional catechol groups were synthesized and
immobilized on TiO2 surfaces. In vitro results with MC3T3s showed greater osteogenic
gene expression on samples functionalized with OGP peptides, compared to non-
functionalized controls. Furthermore, sustained retention of OGP on these titanium
surfaces in physiological conditions was enhanced due to the strong interactions of the
catechol groups with the TiO$_2$, rendering this an efficient means of increasing osseointegration of metal implants in the body.

Finally, although OGP was the primary peptide studied in this dissertation, the use of other osteogenic peptides for bone tissue engineering applications was also investigated. Peptides do not exist in the body as separate entities and therefore, studying the synergistic osteogenic enhancement effects of both BMP-2 and RGD peptides is important for optimization of future tissue engineering constructs. BMP-2 and RGD were both chemically immobilized on the surface of glass slides via efficient click reactions. Extensive *in vitro* work, using hMSCs, was performed, in order to determine the synergistic effect of concentration of these peptides on osteogenic differentiation on 2D gradient substrates. It was observed that the interaction of these peptides with multipotent cells is no a simple addition of their individual concentrations along the gradient. Early and late time points indicate a shift in optimal osteogenic differentiation for the stem cells. Overall, this is an efficient means of studies the effect of multiple peptides for enhanced tissue engineering to more closely mimic what is occurring in the body.

This dissertation is a compilation of many studies that were used to show the impact that functionalized of polymeric materials with peptides has on osteogenic differentiation. While the results of each study were promising, there is still much work to be done in this field for optimal bone tissue formation in large defect areas. This work can also be extended to other areas of tissue engineering, with the hopes of using polymeric materials to their full potential for biomaterial applications.
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Appendix Figure 1 $^1$H NMR of 1-PHE-6.
Appendix Figure 2 $^1$H NMR of 1-PHE-4.
Appendix Figure 3 $^1$H NMR of 1-TYR-6.
Appendix Figure 4 $^1$H NMR and composition calculation of poly(1-PHE-6)$_{0.99}$-co-(1-TYR-6)$_{0.01}$ using the provided equation.
Appendix Figure 5 $^1$H NMR and composition calculation of Poly(1-PHE-6)0.89-co-(1-TYR-6)0.01-co-(1-PHE-4)0.10 using the provided equation.
Appendix Figure 6 ESI-MS of N3-OGP[10-14].
Appendix Figure 7 $^{13}$C NMR of 1-PHE-6.
Appendix Figure 8 ATR-IR of 1-PHE-6 monomer.

<table>
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<th>Wavenumber (cm⁻¹)</th>
<th>Assignment</th>
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<tr>
<td>2856</td>
<td>C-H stretch</td>
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<tr>
<td>1734</td>
<td>C=O stretch (ester)</td>
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<td>1656</td>
<td>C-C stretch (aromatic)</td>
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<tr>
<td>1595</td>
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<td>C=C stretch (aromatic)</td>
</tr>
<tr>
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<td>C-O stretch (ester)</td>
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<tr>
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<td>C-O stretch (ester)</td>
</tr>
<tr>
<td>1124</td>
<td>C-O stretch (ester)</td>
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<tr>
<td>1103</td>
<td>C-N stretch</td>
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</table>
Appendix Figure 9 ESI-MS of 1-PHE-6 monomer.
Appendix Figure 10 $^1$H NMR of Boc-protected 1-PHE-3 monomer.
Appendix Figure 11 ESI-MS of Boc-protected 1-PHE-3 monomer.
Appendix Figure 12 $^1$H NMR of 1-PHE-3 monomer.
Appendix Figure 13 $^{13}$C NMR of 1-PHE-3 monomer.
Appendix Figure 14 ATR-IR of 1-PHE-3 monomer.
Appendix Figure 15 $^1$H NMR of poly(1-PHE-6)$_{0.7}$-co-(1-PHE-3)$_{0.3}$
Appendix Figure 16 $^{13}$C NMR of poly(1-PHE-6)$_{0.7}$-$co$-(1-PHE-3)$_{0.3}$
Appendix Figure 17 ATR-IR of poly(1-PHE-6)_{0.7}-co-(1-PHE-3)_{0.3}

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<tr>
<td>3385</td>
<td>H-bonded N-H (urea)</td>
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<td>C=C stretch (aromatic)/C=C stretch (allyl)</td>
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<td>1546</td>
<td>C=C stretch (aromatic)</td>
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<td>N-H bending (urea)</td>
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<td>C-O stretch (ester)</td>
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<tr>
<td>1078</td>
<td>C-N stretch (urea)</td>
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</table>
Appendix Figure 18 SEC trace of poly(1-PHE-6)0.7-co-(1-PHE-3)0.3
Appendix Figure 19 DSC curve for poly(1-PHE-6)$_{0.7}$-$co$-(1-PHE-3)$_{0.3}$
Appendix Figure 20 TGA curve for poly(1-PHE-6)_{0.7}-co-(1-PHE-3)_{0.3}
Appendix Figure 21 Dogbone shape with dimensions used for tensile testing.
Appendix Figure 22 MALDI-MS pre- and post-disulfide reduction of Cys-OGP[10-14]-Cys
Appendix Figure 23 DSC curve of poly(PHE)-3% crosslinked.
Appendix Figure 24 DSC curve of poly(PHE)-3%OGP
Appendix Figure 25 TGA trace of poly(PHE)-3% crosslinked.

$T_g = 278 \, ^\circ C$
Appendix Figure 26 TGA race of poly(PHE)-3%OGP.
Appendix Figure 27 MALDI-ToF and ESI spectra of OGP[10-14] functionalized with catechol-bearing dendons.
Appendix Figure 28 Adsorption isotherms of catechol-functionalized dendrons. Each dot with an error bar was calculated based on three independent measurements. The dashed line represents the fitting with single-site specific binding model.
Appendix Figure 29 Tetravalent binding ligand OGP-(Cat)$_4$ (c = 10 μM) weakly adsorbed onto SiO$_2$, Al$_2$O$_3$ and hydroxyapatite (HA), and relatively strongly adsorbed onto Au measured by QCM-d.
Appendix Figure 30 Thickness of TiO$_2$ deposition on the top of glass slides measured with interferometric surface profiler. The red higher region represents the TiO$_2$ deposition for 1h, while the blue lower region represents the glass slide covered with a silica wafer without any deposition.
Appendix Figure 31 XPS characterization of TiO$_2$ deposition after RF sputtering coating for 1h. The obtained TiO$_2$ shows the O/Ti ratio of 2, matching with the theoretical stoichiometry. Some carbon and fluorine contamination exists.
Appendix Figure 32 The surface roughness of TiO$_2$ deposition measure by AFM (height image). The TiO$_2$ layer was formed on the top of Si wafer after 1h deposition.
Appendix Figure 33 A Live-Dead assay was used to assess the viability (>98%) of MC3T3-E1 that were seeded on the surface of F-OGP-Cat immobilized TiO₂ substrates and cultured for 24 hours. Live cells are stained green and dead cells are stained red. The scale bar is 50 μm.
Appendix Table 1 Substrates for bioactivity evaluation. The immobilization of OGP[10-14] on TiO$_2$ surface by immersion the substrates in the solution of OGP-PEG-(Cat)$_4$ at different concentration for overnight and their respective load amount calculated in theory and measured with XPS.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>c(OGP-PEG-(Cat)4) (µmol/L)</th>
<th>Immobilized OGP(10-14)a (pmol/cm$^2$)</th>
<th>Nitrogen content from XPS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OGP-99%</td>
<td>2.8</td>
<td>103</td>
<td>5.8 ± 0.3</td>
</tr>
<tr>
<td>OGP-50%</td>
<td>2.8×10-2</td>
<td>52</td>
<td>--</td>
</tr>
<tr>
<td>TiO2</td>
<td>0</td>
<td>0</td>
<td>--</td>
</tr>
</tbody>
</table>

Note: The load amount was calculated with the single site specific binding model, $\Delta m = \frac{B_{max} \times c}{K_d + c}$, where $\Delta m$ is the amount of adsorbed analyte, $c$ is the concentration of the analyte solution, $B_{max}$ is the maximum adsorption of analyte onto the surface, and $K_d$ is the apparent dissociation constant. For OGP-PEG-(Cat)$_4$, $K_d$ is $0.028 \pm 0.008$ µmol/L, and $B_{max}$ is $196 \pm 23$ ng/cm$^2$, as characterized in the previous work.
Appendix Figure 34 XPS characterization of OGP-PEG-(Cat)$_4$ immobilized TiO$_2$ surface with 99% and 50% coverage of the maximum adsorption. N1s signal that comes from the amide bond in peptides showed up in OGP-99% substrates. The XPS signals were normalized to the highest intensity (O1s) for comparison of the signal to noise ratio. The nitrogen percentage of the total atomic amount was calculated based on measurements of three independent samples.

<table>
<thead>
<tr>
<th></th>
<th>OGP-99%</th>
<th>OGP-50%</th>
<th>TiO$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1s (%)</td>
<td>5.8 ± 0.3</td>
<td>--</td>
<td>--</td>
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</table>
Appendix Figure 35 Immunohistochemical staining of bone sialoprotein (BSP) and osteocalcin (OCN) of MC3T3 cells on OGP-99% substrates after 2 weeks. OCN showed higher concentration in cytoplasm, while the distribution of BSP in cytoplasm and extracellular matrix is indistinguishable under fluorescence microscope. (A) Merged image of nuclei (blue) and OCN (red); (B) merged image of nuclei (blue) and BSP (green).