3D Printing with Peptide-Polymer Conjugates for Single-Step Fabrication of Spatially Functionalized Scaffolds

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3D Printing with Peptide-Polymer Conjugates for Single-Step Fabrication of Spatially Functionalized Scaffolds

by

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A Thesis
Presented to the Graduate and Research Committee of Lehigh University in candidacy for the degree of Master of Science in Materials Science and Engineering

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Abstract

Each year, millions of people worldwide suffer from organ failure or tissue loss due to injury, disease, or congenital malformation. With a progressively aging population and limited supply of donor tissues and organs, there is increasing demand for therapies to regenerate or replace tissues long-term. Tissue engineering has emerged as a promising approach to restore, maintain, or improve damaged tissues or whole organs by providing lab-grown replacements or promoting tissue regeneration. Biodegradable polymer-based scaffolds are widely used in tissue engineering strategies to provide support during early stages of regeneration and are commonly functionalized with various chemical groups or bioactive cues to promote desired cellular behavior. However, these functionalized scaffolds are often modified post-fabrication, which can lead to undesired changes and homogeneously distributed chemistries that fail to mimic the spatial biochemical organization found in native tissues. To address these challenges, surface functionalization can be achieved by 3D printing with pre-functionalized biodegradable polymers, such as peptide-modified polymer conjugates, to control the spatial deposition of preferred chemistries. In this work, peptide-PCL conjugates were synthesized with the canonical cell adhesion peptide motif RGDS or its negative control RGES and 3D printed into scaffolds displaying one or both peptides. The peptides were also modified with bioorthogonal groups, biotin and azide, to visualize peptide concentration and location by labeling with complementary fluorophores. Peptide concentration on the scaffold surface increased with increasing peptide-PCL conjugate concentration added to the ink prior to 3D printing, and scaffolds printed with the highest RGDS(biotin)-PCL concentrations showed a significant increase in NIH3T3 fibroblast adhesion and spreading. To demonstrate spatial control of peptide functionalization, multiple printer heads were used to print both peptide-PCL conjugates into the same construct in alternating patterns. Cells preferentially attached and spread on RGDS(biotin)-PCL fibers compared to RGES(azide)-PCL fibers, illustrating how spatial functionalization can be used to influence local cell behavior within a single biomaterial. This presents a versatile platform to generate multifunctional biomaterials that can mimic the biochemical organization found in native tissues to support functional regeneration.
1. Introduction

In the United States, approximately 20 patients in need of organ transplants die each day while waiting for a suitable donor. The current demands for transplant organs and tissues is far outpacing the supply, and projections indicate that this gap will continue to widen. Therefore, there is increasing demand for therapies to regenerate or replace organs and tissues long-term. Tissue engineering has shown tremendous promise in creating biological alternatives for harvested tissues and organs. Tissue engineering strategies generally involve the combination of biomaterials, cells, and biologically active factors to induce tissue formation. The underlying concept of tissue engineering involves cells isolated from a patient, expanded in cell culture, and seeded onto an engineered construct. The resulting tissue-engineered construct is then implanted back into the same patient to function as the replacement tissue. In this approach, an artificial extracellular matrix (ECM) or scaffold is needed to accommodate cells and guide their proliferation and differentiation in three dimensions. However, despite significant progress, tissue engineered constructs remain functionally inferior to their native counterparts because they fail to mimic the complex compositions that imparts tissue function.

Biological tissues are multiscale materials with unique organization from the microscopic level of cells surrounded by their ECM to the macroscopic level of functional tissues and organs. This hierarchical structure combines multiple components in specific arrangements to perform functions that could not be achieved by each component alone or with homogeneous configurations. This results in biochemical and biophysical gradients critical for tissue function, as observed in numerous tissues including those of the musculoskeletal, cardiovascular, and neural systems. For example, articular cartilage is a musculoskeletal tissue that covers the ends of long bones and is necessary to facilitate low-friction movement while distributing mechanical loads. This tissue exhibits depth-dependent gradients in ECM composition, structure, and cell type, leading to anisotropic properties that are critical for biomechanical function. These complex tissues provide a guiding inspiration for the design of next-generation biomaterials. A central challenge in this field is to develop fabrication methods for biomaterials that can mimic the
spatial compositions seen in native tissues and ultimately guide ECM organization in vivo to achieve functional tissue replacements.\textsuperscript{19,20}

To address these challenges, we have developed a strategy that involves functionalizing biodegradable polymers before scaffold fabrication.\textsuperscript{21} This platform enables control over surface concentration and location within a single construct without significantly affecting the scaffold topography or architecture. Specifically, this technique has been optimized for 3D printing to generate scaffolds with user-defined architectures and biochemical organization within a continuous biomaterial. The overall objective of this research is to demonstrate the ability to control the organization of different biochemical cues within a single scaffold to guide local cell behavior.

1.1. Biodegradable polymers in tissue engineering

Biodegradable polymers are commonly used as scaffolds in tissue engineering because they provide mechanical support during the early stages of regeneration and degrade over time to allow for cell infiltration and matrix formation.\textsuperscript{22} Biodegradable polymers used in tissue engineering can either be natural or synthetic depending on their intended uses. Natural polymers, such as collagen, starch, chitin and chitosan, are considered as the first biodegradable biomaterials used clinically\textsuperscript{23} and have been used to repair skin,\textsuperscript{24,25} cartilage,\textsuperscript{26,27} and bone\textsuperscript{28–30} tissues. They are advantageous because they have bioactive properties that allow them to enhance cell performance in biological systems. However, they often exhibit insufficient mechanical properties, limiting their ability to be used for a wide array of tissue engineering applications.\textsuperscript{31} Synthetic biodegradable polymers, such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(lactic-co-glycolide) (PLGA), and poly(ε-caprolactone) (PCL), offer several key advantages compared to natural polymers. They can be reproducibly manufactured with a wide range of mechanical properties and degradation kinetics to enable the production of scaffolds with properties tailored for a particular application.\textsuperscript{32} For example, PCL scaffolds with different properties have been investigated for the regeneration of tissues ranging from blood vessels\textsuperscript{33} to bone\textsuperscript{34} by tailoring polymer molecular weight and fabrication techniques to suit desired applications.

While synthetic polymers are biologically inert, they can be functionalized with various chemical groups or bioactive cues\textsuperscript{35,36} to promote desired cellular behavior by encouraging cell adhesion and differentiation,\textsuperscript{37–39} binding specific biomolecules,\textsuperscript{40} or mimicking ECM components\textsuperscript{41}. Techniques for
scaffold functionalization typically include one or more post-fabrication processing steps. For example, scaffolds can be modified by physisorption or covalent linking of biomolecules after the scaffold has been formed, which requires chemical modification of the surface via aminolysis, hydrolysis or chemical grafting in order to generate functional groups necessary to react with a given biomolecule. These steps can lead to unwanted side reactions that negatively impact biomolecule activity and presentation as well as undesirable changes to scaffold topography and morphology. In addition, post-fabrication functionalization often produces biomaterials with homogeneously distributed chemistries, which fail to mimic the spatial biochemical gradients found in native tissues. Therefore, there is a persistent need to design and develop new biomaterials that address challenges with post-fabrication functionalization.

1.2. Peptide-polymer conjugates

To address challenges associated with post-fabrication functionalization, peptide-modified polymer conjugates can be used to manufacture scaffolds functionalized in a single step, without the need for post-fabrication modification. Peptides are short chains of amino acid sequences. They can be derived from active domains of ECM proteins and designed to mimic biochemical processes. Therefore, this versatile strategy can be tailored to target desired cellular responses by simply changing the peptide sequence. In addition, peptides can be synthesized easily using solid phase synthesis techniques. These shorter sequences are more stable than native proteins and can therefore be subjected to scaffold fabrication processes without denaturation.

Electrospinning with peptide-PCL conjugates to form functionalized fibrous scaffolds with peptides displayed on the surface has been shown in literature. Peptide concentration on the surface was controlled by changing the concentration of peptide-PCL conjugate added to the electrospinning solution. Furthermore, this strategy enabled the formation of continuous constructs displaying multiple chemistries. Sequential electrospinning techniques resulted in continuous scaffolds with opposing gradients of biomolecule-binding peptides to guide the spatial organization of multiple biomolecules. Additionally, this platform was easily tailored for other applications by functionalizing the polymer with other peptide sequences or specific functional groups to initiate controlled radical polymerization. Displaying different functionalities in discrete regions within the same construct enabled control of local cell behavior for tendon repair or vascular grafts. Although this functionalization strategy produced scaffolds with
multiple biochemical gradients, sequential electrospinning offered limited spatial resolution needed for well-defined organization of biochemical cues and scaffold architecture. However, the proven versatility of peptide-polymer conjugates enables this strategy to be adapted for other fabrication techniques that offer more control of scaffold design and architecture.

1.3. 3D Printing

Additive manufacturing, or three-dimensional (3D) printing, has rapidly become a popular approach for creating biomimetic scaffolds for tissue engineering because of the ease and precision by which 3D scaffolds can be made.\textsuperscript{55-58} It offers highly controllable scaffold morphologies and greater spatial resolution compared to other techniques, including electrospinning, porogen-leaching, and directional freezing.\textsuperscript{59,60} Furthermore, multiple printer heads can be used to deposit different materials within the same construct, expanding the possibilities to mimic the complexity of native tissues.\textsuperscript{61-63} In general, 3D printing broadly involves the deposition of material in sequential layers to form a defined shape. There are several types of 3D printing, including laser sintering, stereolithography, heat-based extrusion, and direct-write techniques, which vary considerably in the types of materials used and the structural resolution that can be achieved (Figure 1.1).\textsuperscript{55,64-69} The most common methods involve melt-based techniques, which can cause polymer degradation due to the higher processing temperatures.

Solvent-cast 3D printing, a variation of direct-write 3D printing, was developed to create complex, 3D microstructures at room temperature with PLA.\textsuperscript{70,71} By varying polymer concentration, deposition speed, and pressure, this approach produced a variety of morphologies including layered orthogonal scaffolds, square and circular towers, and circular spirals.\textsuperscript{66,67} In addition, solvent-cast 3D printing can be executed with a larger range of polymer chemistries, including functionalized polymers or polymers that cannot be melt-extruded. This demonstrates its potential to produce complex 3D architectures, such as those found in native tissues, as well as expanding the range of biomaterials that can be used.
1.4. Experimental approach and objectives

Our approach combines solvent-cast 3D printing with the peptide-polymer conjugate strategy for unprecedented control of both surface peptide concentration and spatial organization during a single print, without requiring post-fabrication modification (Figure 1.2). Similar to electrospinning, peptide-PCL conjugates were co-dissolved with unmodified, high molecular weight PCL in a volatile solvent prior to 3D printing. After printing, the solvent evaporates, and the peptides are present on the surface of 3D-printed fibers, producing peptide-functionalized scaffolds. This novel approach allows for simultaneous, high-resolution control of scaffold architecture and spatial arrangement of biochemical cues.

The main objective of this research is to demonstrate how the combination and spatial organization of different biochemical cues can be used to guide local cell behavior. Our goals are to: (1) demonstrate control of peptide concentration on the scaffold surface by changing peptide-PCL ink prior to fabrication; (2) demonstrate that we can 3D print different peptide-PCL conjugates in one construct; (3) show that cells respond to specific peptide chemistry and preferentially adhere to scaffolds and fibers displaying a canonical cell adhesion sequence. To achieve this, we designed and synthesized two different peptide-PCL conjugates, which included the well-known amino acid sequence RGDS to promote cell adhesion or its negative control RGES (Figure 1.2B and 1.2C). Scaffolds were 3D-printed with these two different peptide-PCL conjugates and characterized using a variety of spectroscopic and microscopy techniques. Biorthogonal reaction pairs were included in the conjugate design to specifically label and determine the
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This work introduces a versatile technique that utilizes peptide-polymer conjugates as modular building blocks that can be spatially arranged during scaffold fabrication to produce continuous heterogenous constructs to guide organized tissue formation. The long-term impact of this work is the fabrication of functional tissue constructs that mimic the complex structures and hierarchal organization seen in native tissues.

Figure 1.2. Schematic illustration of solvent-cast 3D printing with inks containing both unmodified, high molecular weight poly(caprolactone) (PCL) and peptide-PCL conjugate in a volatile solvent hexafluoroisopropanol (HFIP). As ink is deposited and solvent evaporates, the surface of the resulting 3D-printed scaffold is functionalized with peptides in a single fabrication step. Chemical structures of the (B) biotinylated RGDS peptide-PCL conjugate (RGDS(biotin)-PCL) with amino acid sequence CYGGGRGDSK(biotin) and (C) azide-modified RGES peptide-PCL conjugate (RGES(azide)-PCL) with amino acid sequence CYGGGREGSK(azide) used to print peptide-functionalized scaffolds.21
2. Materials and Methods

2.1. Materials

All fluorenylmethyloxycarbonyl chloride (Fmoc)-protected amino acids were purchased from AAPPTec (Louisville, KY, USA) except for Fmoc-Lys(N\textsubscript{3})-OH, which was purchased from Chem-Impex (Wood Dale, IL, USA). Piperidine, O-benzotriazole-N,N,N',N'-tetramethyluronium-hexafluoro-phosphate (HBTU), Fmoc-Rink-amide 4-methylbenzhydryalmine (MBHA) resin, and diisopropylethylamine (DIEA) were also purchased from AAPPTec. Diethyl ether (DEE), trifluoroacetic acid (TFA), acetonitrile (ACN), N,N-dimethyl formamide (DMF), dichloromethane (DCM), dimethyl sulfoxide (DMSO), anhydrous N-methyl pyrrolidine (NMP), deuterated dimethyl sulfoxide (DMSO-d\textsubscript{6}), diisopropylcarboimide (DIC), sodium hydroxide (NaOH), and isopropyl alcohol (IPA) were purchased from VWR (Radnor, PA, USA). Trisopropylsilane (TIS), biotin, poly(caprolactone) (PCL) (M\textsubscript{w} 14,000 Da), deuterated dichloromethane (CD\textsubscript{2}Cl\textsubscript{2}), bovine serum albumin (BSA), polyoxyethylenesorbitan monolaurate (TWEEN 20), streptavidin-conjugated fluorescein isothiocyanate (FITC), streptavidin-gold from streptomyces avidinii (10 nm colloidal gold), dibenzocyclooctyne-cyanine3 (DBCO-Cy3), Triton-X, N-acetyl cysteine, papain, ethylenediaminetetraacetic acid (EDTA), Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), and Oxyma were purchased from MilliporeSigma (Burlington, MA, USA). Dithiothreitol (DTT) was purchased from AGTC bioproducts (Alachua, FL, USA). Ninhydrin test kit was purchased from Anaspec (Fremont, CA, USA), while p-maleimidophenylisocyanate (PMPI) was purchased from Chem-Impex. PCL (M\textsubscript{w} 80,000 Da) was generously provided by Polysciences, Inc. (Warrington, PA, USA). 1,1,1,3,3,3,-Hexafluoro-2-propanol (HFIP) was purchased from Oakwood Chemical (Estill, SC, USA), and phosphate buffer saline (PBS) tablets were purchased from AMRESCO (Solon, OH, USA). All cell culture reagents were purchased from ThermoFisher Scientific (Waltham, MA, USA) and all cell culture plasticware were from VWR, unless otherwise stated. Trypsin ethylenediaminetetraacetic acid (EDTA) was purchased from ATCC (Manassas, VA, USA). Antibiotic/antimycotic was purchased from Caisson Labs (Smithfield, UT, USA), while calf thymus DNA (CTDNA) was purchased from VWR. Quan-iTTM Picogreen® dsDNA Assay Kit and rhodamine-phalloidin were purchased from ThermoFisher Scientific. Paraformaldehyde (PFA) was purchased from Alfa Aesar (Haverhill, MA, USA) and Hoescht 33258 was purchased from MilliporeSigma.
2.2. Peptide synthesis and purification

CYGGGRGDSK(biotin) (RGDS(biotin)) and CYGGGRGESK(azide) (RGES(azide)) peptides were synthesized at a 1 mM scale using standard Fmoc solid phase peptide synthesis (SPPS) techniques using a CEM Liberty Blue automated microwave peptide synthesizer (CEM Corporation, Matthews, NC, USA). For all amino acids added on the automated microwave peptide synthesizer, coupling reactions were activated using 3 molar equivalents each of DIC and Oxyma. Both peptides were synthesized on Fmoc-Rink-amide 4-methylbenzhydrylamine (MBHA) resin (100-200 mesh, 0.67 mmol/g functionalization). For the RGDS(biotin), an Fmoc-Lys(Mtt)-OH was coupled manually using methods described below (see Biotinylation of RGDS(biotin)), and the remaining amino acids were coupled using the automated microwave peptide synthesizer. Peptides were cleaved from the resin in a solution of 95% (v/v) TFA, 2.5% (v/v) TIS, 2.5% (v/v) ultrapure water, and 2.5% (w/v) DTT for 3 hours. TFA was removed using rotary evaporation, and the product was precipitated in cold DEE. The precipitate was collected using centrifugation then allowed to dry overnight under vacuum.

The crude peptides were dissolved in acidic mobile phase (95% (v/v) ultrapure water, 5% (v/v) spectroscopic grade ACN, 0.1% (v/v) TFA), sonicated until fully solubilized, and passed through a 0.45 µm filter before purification. Peptides were purified using reversed-phase preparative high-performance liquid chromatography (HPLC; Agilent 218 Prep HPLC, Agilent Technologies, Santa Clara, CA, USA) with a mobile phase gradient of 5%/95% ACN/water to 100% ACN with 0.1% TFA on an Agilent 5 Prep-C18 column (150 x 21.2 mm, 5 µm pore size, 100 Å particle size). Fractions were collected separately, rotary evaporated to remove excess ACN, and lyophilized with a Labconco Freezone freeze dryer (Labconco, Kansas City, MO, USA). The masses for each purified peptide were verified using electrospray ionization mass spectrometry (ESI-MS; Applied Biosystems 3200 Q Trap, Foster City, CA, USA). The purified peptides were also evaluated by HPLC with an Agilent 5 Prep-C18 analytical column (150 x 4.6 mm, 5 µm pore size, 100 Å particle size).

2.3. Biotinylation of RGDS(biotin)

For RGDS(biotin), Fmoc-Lys(Mtt)-OH was manually coupled to Fmoc-Rink-amide MBHA resin in a 100 mL synthesis vessel using standard Fmoc SPPS techniques. Manual SPPS was performed
using a 100 mL peptide synthesis vessel (Chemglass Life Sciences, Vineland, NJ, USA) with agitation on a wrist-action shaker (Burrell, Pittsburgh, PA, USA). The Fmoc protecting group on the resin was removed with 20% (v/v) piperidine in DMF followed by thorough washing with DMF and DCM. Fmoc-Lys(Mtt)-OH (4 molar equivalents) was activated with 3.95 molar equivalents of HBTU and 6 molar equivalents of diisopropylethylamine (DIEA) in DMF and added to the resin. After reacting for 2 hours, the resin was washed with DMF and DCM. A ninhydrin test was conducted at room temperature for 30 minutes to monitor the presence of free amines and confirm successful coupling of the Fmoc-Lys(Mtt)-OH. The Mtt protecting group was removed from the lysine side chain with a solution of 90% (v/v) DCM with 5% (v/v) TFA and 5% (v/v) TIS. This Mtt deprotection solution was added to resin in the synthesis vessel, which was placed on the wrist action shaker for 2 minutes, drained, and washed thoroughly with DCM. The solution turned bright yellow, indicating Mtt removal, and the deprotection and washing steps were repeated until the solution was clear. Biotin was dissolved at 2 molar equivalents with 1.98 molar equivalents of HBTU and 3 molar equivalents of DIEA in 50:50 DMF:DMSO. The biotin coupling solution was added to the resin and allowed to react for 3 hours before washing thoroughly with DCM and DMF. This coupling step was repeated before conducting a ninhydrin test at room temperature to verify successful coupling of biotin. The remaining amino acids were coupled using the automated microwave peptide synthesizer as described above.

2.4. Synthesis of peptide-PCL conjugates

Peptide-PCL conjugates were synthesized with RGDS(biotin) or RGES(azide) peptides using methods previously described. Briefly, PCL diol (Mw 14,000 Da) was dissolved at 80 mg/mL in anhydrous N-methyl pyrrolidone (NMP) under nitrogen atmosphere. P-maleimido phenyl isocyanate (PMPI) was dissolved in anhydrous NMP at 15 molar equivalents and added dropwise to the PCL solution while stirring under nitrogen. The reaction was continued overnight, and the resulting PCL-maleimide (PCL-mal) was precipitated in DEE to remove the excess PMPI. To conjugate the peptide, PCL-mal was reconstituted in anhydrous NMP under nitrogen. The peptides were separately dissolved in dimethyl sulfoxide (DMSO) at 8 molar equivalents and added dropwise to the PCL-mal solution while stirring under nitrogen and reacted overnight. The resulting peptide-PCL
conjugates were precipitated in cold DEE, washed with ultrapure water and 50 µM sodium hydroxide (NaOH) to remove excess peptide, and dried under vacuum overnight prior to analysis. Precipitation and washing steps were repeated as needed to remove excess peptide. Each synthesis step was confirmed by ¹H nuclear magnetic resonance (¹H NMR).

2.5. Solvent-cast 3D printing with peptide-PCL conjugates

PCL-based inks were created by dissolving unmodified PCL (Mₚ 80,000 Da) in 1,1,1,3,3,3,-hexafluoro-2-propanol (HFIP) at a concentration of 37% (w/v) with RGDS(biotin)-PCL or RGES(azide)-PCL added at concentrations of 1, 5, or 20 mg/mL. Inks without added peptide-PCL conjugates (PCL only) were used to create non-functionalized scaffold controls. All inks were 3D printed using a 3-axis EV Series automated dispensing system (Nordson EFD, Westlake, OH, USA) with a 32-gauge blunt-tip needle (100 µm inner diameter) to create 5 mm x 5 mm (width x length) cross-hatched orthogonal scaffolds with 160 µm programmed fiber spacings. The first layer was printed at 0.4 mm/s on a glass slide pretreated with hairspray at a z-height of 100 µm above the glass slide, while remaining layers were printed at 0.2 mm/s with 45 µm z-spacing between each subsequent layer. All scaffolds were printed with an applied pressure of 70 psi using a pressure multiplier connected to a compressed air line.

Scaffolds for scanning electron microscopy (SEM), fluorescence intensity measurements, and DNA quantification were printed as four-layer constructs (~160 µm thick). Spatially organized scaffolds presenting RGDS(biotin) and RGES(azide) peptides in alternating-fiber or alternating-layer patterns were fabricated as continuous, two-layer constructs (~80 µm thick) using two separate printer heads. Inks containing RGDS(biotin)-PCL or RGES(azide)-PCL were prepared as described above for single-peptide scaffolds and sequentially deposited in user-defined alternating-fiber or alternating-layer arrangements.

Scaffolds from each ink formulation were imaged using SEM (LEO 1550 SEM; Zeiss, Peabody, MA, USA) to evaluate fiber morphology and dimensions. The diameter of five fibers per scaffold was measured using Fiji⁷⁹, an image processing distribution package of the open-source program ImageJ⁸⁰.
2.6. **FTIR of peptide-functionalized scaffolds**

Surface functionalization of scaffolds was confirmed with Fourier transform infrared (FTIR) spectroscopy with a Perkin Elmer Spectrum 100 Spectrometer (PerkinElmer Inc., Waltham, MA, USA). Scaffolds samples (3 mm x 3 mm) with 90 µm programmed fiber spacings were printed using methods described above. FTIR spectra were taken of RGDS(biotin)-PCL and RGES(azide)-PCL scaffolds at concentrations of 0, 1, 5, or 20 mg/mL with scanning wavenumber range from 4000 cm\(^{-1}\) to 600 cm\(^{-1}\).

2.7. **SEM of peptide-functionalized scaffolds**

Samples were mounted on 12-mm aluminum stubs using carbon tape, then sputter-coated with iridium using a sputter coater (Electron Microscopy Sciences EMS575X, Hatfield, PA, USA). Samples were imaged using a scanning electron microscope (SEM) (LEO 1550 SEM; Zeiss, Peabody, MA, USA). Scaffolds were imaged using a secondary electron detector with an accelerating voltage of 5 kV.

To confirm the presence of RGDS(biotin)-PCL on the surface of the 3D printed fibers, scaffolds were labeled with streptavidin-conjugated gold nanoparticles and imaged using a FEI SCIOS Dual Beam Focused Ion Beam SEM (FEI, Hillsboro, OR, USA). Samples were incubated in a diluent phosphate buffered saline solution (PBS) containing 0.5% (w/v) bovine serum albumin (BSA) and 0.05% (v/v) polyoxyethylenesorbitan monolaurate (TWEEN 20) at room temperature for 30 minutes. Samples were then incubated in a streptavidin-gold solution (10 nm colloidal gold) at 1:10 in the diluent for 1 hour, washed twice with the diluent solution for 30 minutes and once with ultrapure H\(_2\)O for 30 minutes to remove any unbound streptavidin-gold nanoparticles before drying under vacuum overnight. Streptavidin-gold-labeled samples were mounted on 12-mm aluminum stubs using carbon tape then sputter-coated with carbon for approximately 30 seconds using a Denton Vacuum DV-502A sputter coater (Denton, Moorestown, NJ, USA). The samples were imaged with an accelerating voltage of 5 kV in backscattering mode.

To quantify the number of gold nanoparticles per scaffold, SEM images of five 4.14 µm x 2.74 µm sized areas on the surface of three different RGDS(biotin)-PCL scaffolds (N = 3 scaffolds per group) at each concentration were taken. The number of bound nanoparticles was manually counted.
2.8. Peptide presentation on 3D-printed scaffold

To visualize peptide functionalization and concentration on the scaffold surfaces, five scaffolds from five scaffolds of each group (PCL only; 1 mg/mL RGDS(biotin)-PCL; 5 mg/mL RGDS(biotin)-PCL; 20 mg/mL RGDS(biotin)-PCL; 1 mg/mL RGES(azide)-PCL; 5 mg/mL RGES(azide)-PCL; and 20 mg/mL RGES(azide)-PCL) were characterized. Each group was printed using two separate inks to ensure repeatability between ink batches. Printed constructs were fluorescently labeled after fabrication with streptavidin-fluorescein isothiocyanate (FITC) or dibenzocyclooctyne-Cyanine 3 (DBCO-Cy3), which specifically bind biotin and azide, respectively. RGDS(biotin)-PCL scaffolds were blocked with 0.5% bovine serum albumin (BSA) and 0.05% TWEEN in phosphate buffered saline (PBS) (blocking solution A) at room temperature for 20 minutes, washed with PBS, and incubated in 0.3 µM streptavidin-FITC in blocking solution A for 1 hour before washing thoroughly with PBS and ultrapure water. RGES(azide)-PCL scaffolds were blocked in a solution of 0.2% TWEEN and 0.2% Triton-X in PBS (blocking solution B) for 1 hour, washed with ultrapure water, incubated in 50 mM DBCO-Cy3 in PBS with 0.5% BSA for 30 minutes, and washed with PBS, blocking solution B, ultrapure water, and isopropyl alcohol. PCL only scaffolds were also labeled with streptavidin-FITC or DBCO-Cy3 following the same steps. Scaffolds were imaged with a Nikon C2+ laser scanning confocal fluorescence microscope (Nikon; Melville, New York, USA) using the appropriate excitation wavelengths and filters for FITC and Cy3. Alternating-fiber and alternating-layer scaffolds printed with both RGDS(biotin)-PCL and RGES(azide)-PCL were sequentially labeled with streptavidin-FITC then DBCO-Cy3 and imaged using methods described above.

To compare fluorescence across peptide-PCL conjugate concentrations, five individual peptide-functionalized fluorescently-labeled scaffolds printed using two different ink batches per scaffold group were imaged on a Nikon Eclipse Ts2R fluorescent microscope (Nikon; Melville, New York, USA) with same settings and exposure times maintained for all samples. To quantify fluorescence intensity, fluorescent images were converted to greyscale images and mean grey values were measured across the scaffold cross-sections using Fiji.
2.9. Cell seeding and culture

To prevent cell adhesion to cell culture plates, 24-well plates were coated with a silicone elastomer (Electron Microscopy Sciences) according to manufacturer’s instructions. The silicone-coated wells were sterilized in 70% (v/v) ethanol, rinsed with sterile water, and placed under UV light for 1 hour. For cell morphology experiments, alternating-fiber scaffolds were labeled with streptavidin-FITC and DBCO-Cy3 using methods described above before cell culture. All scaffolds were immersed in 70% (v/v) ethanol for 30 minutes before washing in sterile water three times and incubated in sterile PBS overnight. Prior to seeding, scaffolds were placed in the silicon-coated wells and immobilized with a 0.1 mm dissection pin through the center of the scaffold.

NIH3T3 fibroblasts were expanded in T75 culture flasks at a starting density of $1.3 \times 10^4$ cells/cm² using Dulbecco’s Modified Eagle’s Medium (DMEM)-GlutaMAX™ supplemented with 10% (v/v) calf serum (CS), 1% (v/v) antibiotic/antimycotic (anti/anti: containing amphotericin B, streptomycin, and penicillin), and 2% (v/v) sodium bicarbonate solution. The cells were expanded to 90% confluency and harvested (passage 18-24) using 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) in Hanks’ Balanced Salt Solution (HBSS). Harvested cells were suspended in media without CS from which $1.5 \times 10^5$ cells in 400 µL were added to each scaffold. Seeded scaffolds were cultured for 4 hours for DNA quantification and 24 hours for cell imaging and morphological analysis.

2.10. DNA Quantification

To compare differences in cell adhesion, DNA was quantified in three independent experiments, each with 3-5 scaffolds from the seven groups (N = 11 total scaffolds per group). After 4 hours, cell-seeded scaffolds were gently rinsed with PBS and immediately stored at -80°C until analysis. Samples were digested in a papain digest solution (0.01 M N-acetyl cysteine and 0.125 mg/mL papain in a phosphate buffer with 0.01M EDTA at pH 6.5) at 40°C for 24 hours followed by 60°C for 12 hours. DNA content was measured using Quant-iT™ PicoGreen® dsDNA Reagent according to manufacturer’s instructions. A standard curve was generated using calf thymus DNA to determine DNA content in experimental samples. Fluorescence intensity (excitation 485 nm, emission 535
nm) was quantified on a Tecan Infinite F200 fluorescence microplate plate reader (Tecan; Männedorf, Switzerland).

2.11. Cell staining and morphological analysis

To evaluate differences in cell morphology, cell spreading was quantified in an experiment with PCL only, 20 mg/mL RGDS(biotin)-PCL and 20 mg/mL RGES(azide)-PCL scaffold samples (N = 3 scaffolds per group). After 24 hours of culture, samples were fixed with 4% (w/v) paraformaldehyde (PFA) for 15 minutes, washed with PBS twice, and stored at 4°C. For cell staining, scaffolds were incubated for 10 minutes in 0.5% (v/v) Triton X-100 in PBS at room temperature to permeabilize attached cells, then washed in PBS and incubated in a 1:40 rhodamine-phalloidin solution in PBS for 30 minutes while protected from light to stain actin filaments. Next, cell nuclei were labeled by incubating samples in Hoescht 33258 at 1:500 dilution in PBS for 10 minutes. After washing with PBS and water, samples were placed on a glass slide with a coverslip and imaged on a Nikon C2+ laser scanning confocal fluorescence microscope using the appropriate filters. To quantify differences in cell spreading, five random regions of each scaffold were imaged and analyzed using Fiji. To evaluate changes in cell spreading in response to RGDS peptide functionalization, spreading was reported as a percentage of the total number of cells adhered to each sample. Parameters used to evaluate cell morphology on RGD-functionalized 2D structures, such as morphological score81 or shape factors82, were adapted to evaluate cell spreading on the 3D-printed fibers. Specifically, spread cells were defined as those with elongated morphologies with aspect ratio 2:1 or greater and/or cell areas greater than 400 µm², as measured using Fiji.

To evaluate differences in cell morphology on alternating-fiber dual peptide scaffolds, four independent experiments (N = 10 total scaffolds) were performed. Alternating-fiber scaffolds were labeled with streptavidin-FITC and DBCO-Cy3 prior to cell culture and minimally exposed to light. After 24 hours of culture, samples were incubated in rhodamine-phalloidin and Hoechst 33258 to label actin filaments and cell nuclei, respectively, using methods previously described. To quantify differences in cell adhesion and spreading on RGDS(biotin) and RGES(azide) peptide-functionalized fibers, five random regions of each scaffold were imaged on a Nikon C2+ laser scanning confocal microscope, and the fluorescent images were processed using Fiji. A sub-region
with dimensions of 480 µm x 480 µm was selected from each fluorescent image to ensure an equal representation of both RGDS(biotin) and RGES(azide) peptide-functionalized fibers. Cell attachment was quantified by manually counting cell nuclei and reporting the number of cells on RGDS(biotin)-PCL or RGES(azide)-PCL fibers as a percentage of the total number of cells within the imaged region. To account for differences in cell attachment and evaluate changes in cell spreading in response to the different peptides, spreading was reported as a percentage of the total number of cells adhered to each fiber type.

2.12. Statistical analysis

Welch analysis of variance (ANOVA) with Games-Howell post-hoc test was performed to compare mean fluorescence intensity of labeled scaffolds with varying concentration of peptides (N = 5 scaffolds). Data is presented as mean ± standard deviation. DNA quantification was normalized to PCL only scaffolds, and statistical comparisons between groups were performed by one-way ANOVA followed by Tukey post-hoc (N = 11 scaffolds). Data is presented as mean ± standard error. One-way ANOVA was performed to compare cell spreading behavior on PCL only, 20 mg/mL RGDS(biotin)-PCL, and 20 mg/mL RGES(azide)-PCL scaffolds samples. Independent samples t-tests, assuming equal variances, were performed to compare cell attachment and spreading on alternating-fiber scaffolds (N = 10 scaffolds). Data is presented as mean ± standard error. Values were considered to be significantly different when p-value was <0.05. Statistical analysis was carried out in SPSS (Version 25, IBM software, USA).
3. Results and Discussion

Results from this thesis has been accepted with minor revisions: Paula Camacho*, Hafiz Busari*, Kelly B. Seims, Peter Schwarzenberg. Hannah L. Dailey, and Lesley W. Chow. 3D printing with Peptide-Polymer Conjugates for Single-Step Fabrication of Spatially Functionalized Scaffolds. *Biomaterials Science (in revision), 2019. (*authors contributed equally)

3.1. Peptide and peptide-PCL conjugate synthesis

We utilized solvent-cast 3D printing with peptide–PCL conjugates with amino acid sequences CYGGGRGSDK(biotin) (RGDS(biotin)) and CYGGGRGESK(azide) (RGES(azide)) (Figure 3.1) to control local cell behaviour within a continuous scaffold. In addition to the specific sequences (RGDS and RGES), peptides used in this study were designed to include a glycine (G) spacer to enhance the display of the peptide on the scaffold surface and tyrosine (Y) residues for spectroscopic detection of peptides on the scaffold surface. Biotin and azide groups were included in the peptide design for specifically labeling each peptide on the 3D-printed scaffold surfaces. Each synthesis and purification step were confirmed via mass spectrometry and analytical–HPLC, validating that peptides were successfully synthesized and purified (Figures 3.2 and 3.3).

![Figure 3.1. Chemical structure of (A) RGDS(biotin) and (B) RGES(azide) peptides.](image)
Figure 3.2. (A) ESI-MS of purified RGDS(biotin) (MW 1224 g/mol) showing the [M/2 + H] ion and (B) corresponding analytical HPLC of RGDS(biotin).

Figure 3.3. (A) ESI-MS of purified RGES(azide) (MW 1038 g/mol) showing the [M/2 + H] ion and (B) corresponding analytical HPLC of RGES(azide).
Peptide-PCL conjugates were synthesized using methods previously described (Figure 3.4). Briefly, the terminal hydroxyl groups of PCL (Mw 14,000 Da) were modified with a heterobifunctional linker p-maleimidophenyl isocyanate (PMPI) to form a carbamate group and generate a maleimide-functionalized PCL (PCL-mal). The maleimide group on the PCL-mal then reacts with the cysteine on the N-terminus of the peptide via a Michael-type addition, therefore coupling the peptide to the PCL. This versatile strategy can therefore be tailored to desired applications by simply changing the bioactive sequence. Each synthesis step was confirmed by 1H NMR. PMPI and PCL-mal were dissolved in CD2Cl2, while conjugates were dissolved in DMSO-d6. Proton assignments of the PMPI and PCL-maleimide were based on published spectra and 1H-NMR showed the PMPI was successfully conjugated to the PCL to form PCL-mal (Figure 3.5). 1H-NMR also showed peptides were successfully conjugated to the PCL-mal to form each respective peptide-PCL conjugate (Figures 3.6 and 3.7).

![Diagram of synthesis scheme of peptide-PCL conjugates](image)

**Figure 3.4.** Synthesis scheme of peptide-PCL conjugates showing modification of PCL (1) with p-maleimidophenyl isocyanate (PMPI; 2) to form PCL-maleimide (3). The PCL-maleimide was reacted with the thiol group on the N-terminal cysteine of the peptides (4) to form peptide-PCL conjugates (5).
Figure 3.5. $^1$H NMR (400MHz, CD$_2$Cl$_2$, TMS) and corresponding chemical structures of p-maleimidophenyl isocyanate (PMPI; inset) with chemical shift assignments 6.84 (2H, s, maleimide vinyl, 1), 7.20 (2H, d, Ar-H ortho to isocyanate, 2'), 7.32 (2H, d, Ar-H ortho to maleimide, 2) and PCL-maleimide with chemical shift assignments 6.82 (4H, s, maleimide vinyl, 1), 7.24 (4H, d, Ar-H ortho to isocyanate, 2'), 7.51 (4H, d, Ar-H ortho to maleimide, 2), 4.02 (198H, m, -O-CH$_2$-), 2.27 (205H, m, -CO-CH$_2$-), 1.61 (440H, m, -CH$_2$), 1.36 (207H, m, -CH$_2$), 1.36 (207H, m, -CH$_2$), 1.36 (207H, m, -CH$_2$).
Figure 3.6. $^1$H NMR (400MHz, DMSO-d$_6$, TMS) and corresponding chemical structures of RGDS(biotin)-PCL (top) with chemical shift assignments 6.61 (4H, d, Ar-H, α), 3.93 (198H, m, -O-CH$_2$-, b'), 2.22 (207H, m, -CO-CH$_2$-, d, d'), 1.49 (436H, m, -CH$_2$-, e, g'), 1.24 (215H, m, -CH$_2$-, f, e, f, g) and RGDS(biotin) (bottom) with chemical shift assignment 6.62 (2H, m, Ar-H, α).
Figure 3.7. $^1$H NMR (400MHz, DMSO-d$_6$, TMS) and corresponding chemical structures of RGES(azide)-PCL (top) with chemical shift assignments 6.61 (4H, d, Ar-H, α), 3.94 (198H, m, -O-CH$_2$-, b'), 2.23 (210H, m, -CO-CH$_2$-, d, d'), 1.50 (421H, m, -CH$_2$-, e, g'), 1.25 (209H, m, -CH$_2$-, f, e, g) and RGES(azide) (bottom) with chemical shift assignment 6.61 (2H, m, Ar-H, α).

Peptide conjugation to PCL was also confirmed with Fourier Transform Infrared (FTIR) spectroscopy. FTIR spectra were taken with a scanning wavenumber range from 4000 to 600 cm$^{-1}$ and peaks were analyzed. The IR transmittance peaks at 3200 cm$^{-1}$ and 1630 cm$^{-1}$ indicating amide bonds were present in both RGDS(biotin)-PCL and RGES(azide)-PCL conjugates but not the
unmodified PCL (Figure 3.8). The RGES(azide)-PCL conjugate also showed a transmittance peak at 2100 cm\(^{-1}\), representing the azide group\(^8\) (Figure 3.9).

**Figure 3.8.** FTIR of unmodified PCL (black) and RGDS(biotin)-PCL (green) conjugate demonstrating successful conjugation of RGDS(biotin) peptide to PCL. Black boxes highlight the IR transmittance peaks at 3200 cm\(^{-1}\) and 1630 cm\(^{-1}\) that indicate the amide bonds associated with the peptide.

**Figure 3.9.** FTIR of unmodified PCL (black) and RGES(azide)-PCL (red) conjugate demonstrating successful conjugation of RGES(azide) peptide to PCL. Black boxes highlight the IR transmittance peaks at 3200 cm\(^{-1}\) and 1630 cm\(^{-1}\) that indicate the amide bonds associated with the peptide and the blue box shows the azide peak at 2100 cm\(^{-1}\) from the RGES(azide).

### 3.2. Fabrication of peptide functionalized 3D-printed scaffolds

Inks containing PCL with and without peptide-PCL conjugates were solvent-cast 3D printed into peptide-functionalized scaffolds, as shown schematically in Figure 1.2A. Processing parameters, such as needle gauge, print speed, print pressure, polymer concentration in the ink, and deposition height, are known to influence the final morphology of 3D-printed structures.\(^{66,67}\) In this study, printing parameters were optimized to produce consistent fiber diameters and cylindrical morphologies across each printed layer.
Printed fiber diameters were found to be approximately 38.5 µm ± 3.1 µm (N = 7 scaffolds per group) across all scaffold groups. As expected and observed by others, shrinkage occurred due to evaporation of the solvent after 3D printing, resulting in fibers with diameters smaller than the inner diameter of the printing needle (100 µm). Scaffold morphology and topography was unaffected by the addition of the peptide-PCL conjugates, even at the highest concentrations, as shown by SEM of PCL only, and peptide-PCL scaffolds (Figure 3.10). This suggests that peptide functionalization can be modified independently of scaffold morphology and differences in cell behavior can be attributed to changes in surface chemistry.

![Figure 3.10](image)

**Figure 3.10.** Representative scanning electron microscopy (SEM) images of scaffolds 3D printed with inks containing (A) PCL only or PCL with (B) 1 mg/mL RGDS(biotin)-PCL, (C) 5 mg/mL RGDS(biotin)-PCL, (D) 20 mg/mL RGDS(biotin)-PCL, (E) 1 mg/mL RGES(azide)-PCL, (F) 5 mg/mL RGES(azide)-PCL, (G) 20 mg/mL RGES(azide)-PCL with a higher magnification image shown in the inset. Scaffold morphology was not significantly affected by the addition of the peptide-PCL conjugates at any concentration.

### 3.3. Characterization of peptide-functionalized 3D-printed scaffold

FTIR verified the presence of peptides on the surface of peptide-functionalized scaffolds. IR transmittance peaks around 3200 cm⁻¹ and 1600 cm⁻¹, indicating peptide amide bonds, present only on peptide-functionalized scaffolds at concentrations of 1, 5, or 20 mg/mL and not on PCL only scaffolds. Additionally, an increase in IR peak intensity at 3200 cm⁻¹ and 1630 cm⁻¹ indicates amide
bonds corresponding to increasing peptide-PCL concentration in both RGDS(biotin)-PCL scaffolds and RGES(azide)-PCL scaffolds (Figures 3.11 and 3.12).

Figure 3.11. FTIR of scaffolds 3D-printed with PCL only or PCL with 1, 5, or 20 mg/mL RGDS(biotin)-PCL showed an increase in IR peak intensity at 3200 cm\(^{-1}\) and 1630 cm\(^{-1}\) indicating an increase in RGDS(biotin) peptide concentration on the scaffold surface that corresponded with increasing conjugate concentration in the ink.

Figure 3.12. FTIR of scaffolds 3D-printed with PCL only or PCL with 1, 5, or 20 mg/mL RGES(azide)-PCL showed an increase in IR peak intensity at 3200 cm\(^{-1}\) and 1630 cm\(^{-1}\) indicating an increase in RGES(azide) peptide concentration on the scaffold surface that corresponded with increasing conjugate concentration in the ink.

To enable direct and specific labeling of each peptide presented on the 3D-printed scaffolds, the peptide-PCL conjugate design included the bioorthogonal functional groups biotin and azide (Figures 1.2B and 1.2C). Each functional group only reacts with its complementary pair, allowing for highly selective reactions in the presence of other chemistries. Biotin specifically binds with streptavidin at femtomolar concentrations\(^\text{86}\) while azide and DBCO undergo a copper-free click reaction.\(^\text{87–89}\) Also, these reactions proceed specifically and efficiently under physiologically relevant conditions, exhibit fast kinetics, and are tolerant to aqueous environments,\(^\text{90–92}\) thus introducing the ability to localize biomolecules of interest within a scaffold before or during culture. For example, Ji et al. 3D printed polymers bearing click-ready pendant
groups to bind azide-heparin, which was used to bind and deliver bone morphogenetic protein-2 (BMP-2) to promote human mesenchymal stem cell (hMSC) osteogenic differentiation. Bioorthogonal chemistries offer several unique advantages, such as versatility with the choice of probe and applicability to many classes of biomolecules.

To verify the presence of RGDS(biotin), scaffolds 3D printed with 0, 1, 5 and 20 mg/mL RGDS(biotin)-PCL were labeled with streptavidin-conjugated gold nanoparticles (10 nm), coated with a thin layer of carbon and imaged by SEM. The gold nanoparticles appeared as white dots on the surface of 3D-printed fibers while imaging in backscattered electron mode. This contrast difference between the gold nanoparticles and 3D-printed polymer material arises from gold having a greater atomic mass than carbon, hydrogen, nitrogen, and sulfur. Using this visualization technique, gold nanoparticles were observed on the 1 mg/mL, 5 mg/mL and 20 mg/mL RGDS(biotin)-PCL scaffolds, but not on the 0 mg/mL scaffolds (Figure 3.13 A-D). This confirmed the presence of biotinylated peptides on the surface after 3D printing with ink containing the RGDS(biotin)-PCL conjugate. This result thus showed that the peptide introduced into solution prior to 3D printing was ultimately presented on the scaffold surface after fabrication, as demonstrated previously with electrospun scaffolds. Furthermore, the increase in concentration of RGDS(biotin)-PCL conjugate during ink preparation produced increases in gold-labeling on the surface. For quantitative analysis, we took images of five 4.14 µm sized areas on the surface of three different RGDS(biotin)-PCL scaffolds (N = 3) at each concentration and manually counted the amount of nanoparticles that were present. Quantitative analysis showed trends of increasing gold nanoparticles with increasing RGDS(biotin)-PCL concentration (Figure 3.14). However, there were no statistical significant differences among the groups due to the high variability of nanoparticles observed at higher concentrations of RGDS(biotin)-PCL. This high variability could stem from a combination of two factors, the first being the formation of peptide clusters instead of a uniform distribution of peptides across the scaffold surface. The second reason could be streptavidin’s ability to bind four biotin molecules at a time. Therefore, with more biotin molecules available, there is a higher likelihood that streptavidin binds to more of the biotinylated peptide therefore skewing the amount of gold nanoparticles observed. Although more investigation with this technique is needed, the correlation between the amount of gold nanoparticles and RGDS(biotin)-PCL concentration indicates control of surface peptide concentration in our 3D-printed constructs.
Figure 3.13. Representative FIB-SEM images in backscattering mode of scaffolds 3D printed with (A) PCL only or PCL with (B) 1 mg/mL RGDS(biotin)-PCL, (C) 5 mg/mL RGDS(biotin)-PCL or (D) 20 mg/mL RGDS(biotin)-PCL labeled with streptavidin gold nanoparticles (10 nm) diameter shows that increasing RGDS(biotin)-PCL conjugate concentrations corresponds with increases in the number of gold nanoparticles on the surface of the 3D-printed fibers illustrating the ability to fine-tune peptide density on the micron scale.
Quantitative analysis streptavidin gold nanoparticles shows that increasing RGDS(biotin)-PCL conjugate concentrations corresponds to increases in the number of gold nanoparticles on the surface of the 3D-printed fibers illustrating the ability to fine tune peptide density on the micron scale.

As an alternative to streptavidin gold nanoparticles, we exploited fluorophores conjugated to streptavidin groups to compare differences in surface peptide concentration and enable labeling of both peptides within the same 3D-printed scaffold. This technique also offers us the ability to characterize the RGES(azide)-PCL scaffolds, a method not afforded with gold nanoparticle characterization due to the difficulty of conjugating DBCO to gold nanoparticles. Scaffolds 3D printed with 0, 1, 5, or 20 mg/mL RGDS(biotin)-PCL or RGES(azide)-PCL were labeled with streptavidin-FITC or DBCO-Cy3, respectively, to confirm and visualize the presence of peptides. Although some fluorescence was detected on PCL only scaffolds due to autofluorescence of the material\textsuperscript{64}, fluorescence intensity was higher on peptide-functionalized scaffolds and demonstrated that peptide surface concentration corresponded directly with ink conjugate concentration (Figures 3.15A and 3.15B). Fluorescence intensity was statistically different between different concentrations of both RGDS(biotin)-PCL scaffolds (Welch's $F(3, 6.97) = 12.449$, $p = 0.003$) and RGES(azide)-PCL scaffolds (Welch's $F(3, 6.728) = 9.242$, $p = 0.009$) and increased with increasing conjugate concentration from 0 mg/mL to 1 mg/mL, 5 mg/mL, and 20 mg/mL. For RGDS(biotin)-
PCL scaffolds, fluorescence intensity from 0 mg/mL scaffolds was statistically different ($p < 0.05$) compared to 5 mg/mL and 20 mg/mL scaffolds. Differences between 1 mg/mL scaffolds and 5 mg/mL and 20 mg/mL RGDS(biotin)-PCL scaffolds were also statistically significant ($p < 0.05$) (Figure 3.15C). For RGES(azide)-PCL scaffolds, 20 mg/mL scaffolds had a significantly higher fluorescence intensity ($p < 0.05$) compared to the 0 mg/mL and 1 mg/mL RGES(azide)-PCL scaffolds (Figure 3.15D). This demonstrated that peptide concentration on the surface can be controlled by changing the concentration of peptide-PCL conjugates in the ink formulations prior to scaffold fabrication, without requiring post-fabrication modification steps.

**Figure 3.15.** Representative confocal fluorescence microscopy images of scaffolds 3D printed with PCL and (A) 0, 1, 5, and 20 mg/mL RGDS(biotin)-PCL (L to R) labeled with streptavidin-FITC (green) or (B) 0, 1, 5, and 20 mg/mL RGES(azide)-PCL (L to R) labeled with DBCO-Cy3 (red) (scale bar = 100 µm). (C) Quantification of mean fluorescence intensity of (C) 0, 1, 5, and 20 mg/mL RGDS(biotin)-PCL scaffolds showed that increasing conjugate concentrations corresponded with a statistically significant increase in mean fluorescence intensity from 0 mg/mL to 5 mg/mL and 20 mg/mL ($^*p < 0.05$) and from 1 mg/mL to 5 mg/mL and 20 mg/mL ($^#p < 0.05$) scaffolds. (D) Quantification of mean fluorescence intensity of RGES(azide)-PCL scaffolds 3D printed with conjugate concentrations of 0, 1, 5, and 20 mg/mL showed similar results. Increasing conjugate concentrations corresponded with statistically significant differences in
mean fluorescence intensity between 0 mg/mL and 20 mg/mL (*p < 0.05) and 1 mg/mL and 20 mg/mL (**p < 0.05). Data is presented as mean ± SD (N = 5 for all scaffold groups).^{21}

3.4. Optimizing peptide-PCL concentration to enhance cell adhesion and spreading

The canonical RGDS peptide motif has been extensively shown to promote integrin-mediated cell adhesion^{73–75}, while the single amino acid conversion of the aspartic acid (D) in RGDS to glutamic acid (E) in RGES serves as a negative control for the peptide’s cell adhesive properties^{52,77,78}. However, the surface density of RGDS has been shown to affect cell adhesion behavior on various biomaterials.^{77,95–97} For example, cell behavior assays conducted on polystyrene substrates with grafted RGDS peptides showed fibroblast adhesion and proliferation was highest on substrates with the maximum concentration of peptide and decreased as the density of peptide decreased.^{98}

Our first step was to compare cell behavior when cultured on 3D-printed scaffolds presenting different concentrations of each peptide. We seeded NIH3T3 mouse fibroblasts on RGDS(biotin)-PCL or RGES(azide)-PCL scaffolds with conjugate concentrations of 0, 1, 5, or 20 mg/mL under serum-free conditions and cultured for 4 hours to avoid confounding results due to proliferation.^{52} DNA was quantified using the PicoGreen Assay to compare the relative number of cells adhered to each scaffold. Normalized DNA (ng/ng) was significantly different between groups and functionalizing the scaffolds with 20 mg/mL RGDS(biotin)-PCL showed a significant increase in adhered cells compared to PCL only scaffolds (p < 0.05) (Figure 3.16). Furthermore, increasing the RGDS(biotin)-PCL concentration from 1 to 20 mg/mL also resulted in statistically significant differences (p < 0.05), indicating higher RGDS concentrations on the scaffold surface are required to enhance fibroblast adhesion. Comparisons with the negative control scaffolds demonstrated increased adhesion in the 20 mg/mL RGDS(biotin)-PCL scaffolds compared to 1 and 20 mg/mL RGES(azide)-PCL scaffolds. As expected, the amount of DNA detected on RGDS(biotin)-PCL scaffolds increased with higher concentration of the conjugate; however, the amount of DNA detected on RGES(azide)-PCL scaffolds remained stable with increasing RGES(azide)-PCL concentration and was not significantly different from the amount detected on PCL only scaffolds. This verified its use as a negative peptide-functionalized scaffold control for the RGDS(biotin)-PCL scaffolds. It is also interesting to note that there was no statistical significance between the 20
mg/mL RGDS(biotin)-PCL and 5 mg/mL RGES(azide)-PCL or 5 mg/mL RGDS(biotin)-PCL scaffolds. While this result can mostly likely be attributed to outliers in the case 5 mg/mL RGES(azide)-PCL scaffold group, there is still some investigation to be done in the case of the 5 mg/mL RGDS(biotin)-PCL scaffolds. However, the 20 mg/mL RGDS(biotin)-PCL and 20 mg/mL RGES(azide)-PCL scaffolds demonstrated the highest significant differences at the highest peptide concentrations, indicating cells can distinguish between these two peptide sequences.

These conjugate concentrations were therefore selected for subsequent experiments to investigate the spreading behavior of NIH3T3 cells on peptide-functionalized and PCL only scaffolds. We seeded NIH3T3 mouse fibroblasts on PCL only, 20 mg/mL RGDS(biotin)-PCL and 20 mg/mL RGES(azide)-PCL scaffolds for 24 hours to assess how the different scaffold surfaces supported cell spreading. In concurrence with the DNA quantification study, NIH3T3 fibroblasts seeded on 20 mg/mL RGDS(biotin)-PCL exhibited higher spread morphology when compared to 20 mg/mL RGES(azide)-PCL and PCL only scaffolds as demonstrated by confocal imaging of the scaffold surface (Figure 3.17A-C). Quantification of cell spreading showed that the 20 mg/mL RGDS(biotin)-PCL was statistically higher \( (p < 0.05) \) than the 20 mg/mL RGES(azide)-PCL and PCL only samples (Figure 3.17D). There was also no statistical difference between the 20 mg/mL RGES(azide)-PCL and PCL samples. Specifically, 56% of total cells adhered on the 20 mg/mL RGDS(biotin)-PCL surface were spread compared to 39.3% on the 20 mg/mL RGES(azide)-PCL scaffolds and 23.8% on the PCL only scaffolds. This further supports the use of these peptide concentrations to evaluate how the spatial organization of two distinct peptides influences spatial cell behavior within a single construct.
Figure 3.16. Normalized DNA quantification of NIH3T3 mouse fibroblasts cultured on 3D-printed scaffolds for 4 hours. Significant differences were found between 20 mg/mL RGDS(biotin)-PCL and 0 mg/mL (PCL only), 1 mg/mL RGDS(biotin)-PCL, 1 mg/mL RGES(azide)-PCL, and 20 mg/mL RGES(azide)-PCL scaffolds (*p < 0.05). Modifying conjugate concentration, which correlates with surface peptide concentration, and peptide sequence resulted in significant differences in cell adhesion. Data is presented as mean ± SE (N = 11 per scaffold group from three independent experiments).
3.5. Spatial organization of bioactive peptides in peptide-functionalized 3D-printed scaffolds

The ability to localize biomolecules or bioactive functional groups within 3D-printed scaffolds is critical for spatial control of cellular activities, such as cell migration, differentiation, and proliferation. In addition to providing control of peptide surface concentration, our 3D printing approach also introduces flexibility to tailor the spatial presentation of multiple peptides within a single construct. To illustrate this, we 3D printed scaffolds with both 20 mg/mL RGDS(biotin)-PCL and 20 mg/mL RGES(azide)-PCL inks in alternating-fiber or alternating-layer patterns (Figure 3.18). Labeling with both streptavidin-FITC and DBCO-Cy3 demonstrated how each peptide was localized to discrete locations within the same scaffold. Fluorescence microscopy revealed FITC fluorescence was only present on fibers or layers printed with inks containing RGDS(biotin)-PCL, while Cy3 fluorescence was only seen in fibers or layers printed with inks containing RGES(azide)-PCL. The corresponding bright-field images showed similar fiber morphologies and overall structure to previous scaffolds 3D printed with only one ink. This introduces fiber-level control of multi-peptide organization within a single construct.
3.6. Local cell behavior in spatially organized peptide-functionalized 3D-printed scaffolds

The specific peptides used in this study were selected to illustrate the potential of our platform in guiding local cell behavior within a single construct. Having demonstrated the ability to control multi-peptide organization within a continuous 3D-printed scaffold, we investigated how NIH3T3 fibroblasts responded to alternating-fiber scaffolds printed with both 20 mg/mL RGDS(biotin)-PCL and 20 mg/ml RGES(azide)-PCL inks in discrete regions (Figure 3.19). Integrin binding is highly sensitive to minor changes in the RGD sequence and affinity of RGE and scrambled peptides has been reported to be at least 2–4 orders of magnitude lower than RGD. Notably, both peptides have the same charge, which enabled us to investigate how cell attachment and spreading was affected by amino acid sequence rather than changes in surface properties (e.g., charge, hydrophilicity) due to peptide functionalization. When cultured on the scaffolds, cells preferentially attached and spread on RGDS(biotin)-PCL fibers compared to RGES(azide)-PCL fibers. Corresponding with results obtained with single-peptide scaffolds (Figures 3.16 and 3.17), the number of cells attached to RGDS(biotin)-PCL fibers was statistically higher ($p < 0.01$) compared to RGES(azide)-PCL fibers (Figure 3.19B). On average, 55.2%
cells attached to RGDS(biotin)-PCL fibers while 44.8% attached to RGES(azide)-PCL fibers. Furthermore, a higher proportion of the cells attached to RGDS(biotin)-functionalized fibers presented a spread morphology compared to cells on the RGES(azide)-functionalized fibers, which primarily maintained a spherical or ellipsoidal shape (Figure 3.19C). Specifically, 71% of total cells on RGDS(biotin)-functionalized fibers were spread compared to 54.7% of total cells on the RGES(azide)-functionalized fibers.

Similar behavior has been shown in other systems where RGDS was compared to a scrambled sequence or sequence with a single amino acid substitution. For example, Causa et al. compared NIH3T3 fibroblast response to PCL substrates functionalized with RGD or a scrambled control DGR. After 24 hours, cells were adhered and well-spread on RGD-functionalized PCL while cells on the scrambled peptide samples exhibit a rounded morphology that suggested insufficient adhesion to the substrate. Related to the current work, others have shown various cell types displaying enhanced spreading, focal contact formation, and cytoskeleton organization on RGD-presenting surfaces compared to RGE.

While the differences in cell adhesion and morphology obtained from the dual-peptide scaffolds were not as pronounced as with the single-peptide scaffolds, it should be noted that more dual-peptide scaffold samples were analyzed (N = 11 dual-peptide scaffolds compared to N = 3 single-peptide scaffolds). These larger sample sizes verify significance across groups. Additionally, quantification of cell adhesion and spreading for dual-peptide scaffolds excluded cells that were difficult to quantify. For example, cells that were located at the junction between RGDS(biotin)-PCL and RGES(azide)-PCL fibers were not counted. This limits observed differences in cell behavior between each type of peptide-functionalized fiber. However, our observations illustrate how cell behavior can be controlled on discrete fibers by organizing different peptides within the same scaffold. This introduces the ability to spatially present different biochemical cues to influence local cell-cell and cell-material interactions.
Figure 3.19. (A) Representative confocal fluorescence microscopy image of cells on 20 mg/mL RGDS(biotin)-PCL (green fibers) and 20 mg/mL RGES(azide)-PCL (red fibers) alternating-fiber scaffolds labeled with rhodamine-phalloidin (red) and Hoescht 33238 (blue) to stain for actin filaments and nuclei, respectively. Quantitative analysis of (B) cell attachment and (C) cell spreading at 24 hours that NIH3T3 mouse fibroblasts preferentially attach (*$p < 0.01$) to and spread (**$p < 0.05$) on RGDS-functionalized fibers compared to RGES-functionalized fibers when cultured on a single scaffold displaying both peptides. This demonstrates that scaffolds can be designed to control multi-peptide organization and guide local cell behavior within the same construct. Data presented as mean ± SE ($N = 10$ from four independent experiments).
4. Conclusion

This work demonstrates, for the first time, a single-step functionalization strategy using solvent-cast 3D printing with peptide-polymer conjugates to spatially control biochemical cues on printed scaffolds. The combination of this fabrication method with peptide-polymer conjugates represents a versatile platform that can be used to create controllably porous, biodegradable scaffolds with spatially organized surface chemistry at the single-fiber level. Varying peptide concentration presented on the surface was achieved by 3D printing with inks prepared with different peptide-PCL concentrations prior to scaffold fabrication. Multi-peptide organization was controlled by using multiple printer heads to sequentially deposit inks with peptide-PCL conjugates bearing different amino acid sequences in user-defined arrangements. The specific amino acid sequences used in this study, RGDS and RGES, were selected to illustrate the potential of this platform in guiding local cell behavior within a single scaffold environment. On single-peptide scaffolds, changing peptide concentration on the 3D-printed fiber surface influenced the degree of cell attachment. Furthermore, spatially presenting both peptide sequences within the same scaffold significantly affected local cell response at the fiber level, shown by enhanced cell attachment and spreading on fibers presenting RGDS(azide) compared to ones presenting RGES(biotin). This approach can be easily adapted for other applications by simply tailoring the peptide sequence or sequences for preferred cell behavior, both locally and globally within a scaffold. Printing with different peptide-PCL conjugate inks can be exploited to recreate the multi-component nature and organization of the ECM in different tissues.

Bioorthogonal groups can also be exploited for in situ modification of the surface chemistry over time in the presence of cells to mimic temporal changes that occur in the ECM during processes like development and remodeling. In addition, parameters such as needle gauge, print pressure, and print pattern can be modified to expand the range of possible scaffold architectures. Thus, both biochemical and structural organization can be tuned within a scaffold to match that of a native tissue of interest. The downstream potential of this approach is the ability to support functional regeneration by generating tissue-specific, multi-component biomaterials that mimic the complex biochemical, morphological, and mechanical properties of native tissues.
5. Future work

Future studies are focused on utilizing the biorthogonal chemistries to modify biochemical cues over time for temporal control of cellular behavior. The bioorthogonal groups present on the peptide-PCL conjugates developed in this work can be specifically tagged with their complementary chemistry in the presence of cells, allowing for temporal changes to the biochemical environment during culture. For example, RGES(azide)-PCL scaffolds can be reacted with an RGDS(DBCO) peptide at a later time to influence a different cellular behavior. In other words, cells localized by a cell adhesion peptide would therefore be exposed to this new biomolecule, changing the local biochemical environment. This will not only introduce strategies to create dynamic, multicomponent materials with controllable spatiotemporal compositions, but will also provide valuable insight to deepen our understanding about dynamic cell-ECM interactions and the structure-function relationships found in biological tissues.
6. Bibliography


7. Vita

Hafiz Busari completed his undergraduate studies at the University of Texas at Dallas (UTD) getting a B.S. in Biochemistry and a Psychology minor in 2015. While a UTD undergraduate, he was awarded with the Academic Scholars Award for his scholastic achievement in Biochemistry and graduated with honors. He participated in undergraduate research in Professor Jeremiah Gassensmith’s lab, working with functionalizing the bacteriophage Qbeta. Hafiz completed his Master’s at Lehigh University, graduating with an M.S. in Materials Science and Engineering in 2019. During his Master’s, he attended the 2018 Society for Biomaterials Conference (SFB) and gave a talk. He also attended and gave a talk at the 2019 Northeastern Bioengineering Conference (NEBEC).