Published in final edited form as:

Polym Chem. 2015 August 21; 6(31): 5683–5692. doi:10.1039/C5PY00282F.

Multifunctional and Spatially Controlled Bioconjugation to Melt Coextruded Nanofibers

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Abstract

Polymeric fibers have drawn recent interest for uses in biomedical technologies that span drug delivery, regenerative medicine, and wound-healing patches, amongst others. We have recently reported a new class of fibrous biomaterials fabricated using coextrusion and a photochemical modification procedure to introduce functional groups onto the fibers. In this report, we extend our methodology to control surface modification density, describe methods to synthesize multifunctional fibers, and provide methods to spatially control functional group modification. Several different functional fibers are reported for bioconjugation, including propargyl, alkene, alkoxyamine, and ketone modified fibers. The modification scheme allows for control over surface density and provides a handle for downstream functionalization with appropriate bioconjugation chemistries. Through the use of multiple orthogonal chemistries, fiber chemistry could be differentially controlled to append multiple modifications. Spatial control on the fiber surface was also realized, leading to reverse gradients of small molecule dyes. One application is demonstrated for pH-responsive drug delivery of an anti-cancer therapeutics. Finally, the introduction of orthogonal chemical modifications onto these fibers allowed for modification with multiple cell-responsive peptides providing a substrate for osteoblast differentiation.

Introduction

Functional polymeric nanofibers have seen increasing interest in biomedical applications, particularly for use in regenerative medicine.^{1,2} Nanofibers present high-surface area to volume ratios, allowing for dense presentation of biochemical cues, often times leading to improved cellular adhesion, proliferation and differentiation.³ Ideally, these nanofibers can provide directional cues based on the macroscopic alignment of the fibers.^{4,5} When polymeric fibers are fabricated into non-woven materials, they provide the additional advantage of high porosity, allowing for influx of oxygen and nutrients into damaged tissues and efflux of waste products. Likewise, polymeric fibers are effective drug delivery materials owing to the low diffusion distances of drug-loaded fibers.^{2,6} Polymeric nanofibers remain promising for biomedical applications, but several hurdles remain. The most popular way to fabricate fibers of nanoscale dimensions is through the use of

electrospinning. Even when used industrially, the throughput is relatively low, with a maximum production output of hundreds of grams per hour.⁷ Electrospinning is a solvent-based process, where high dielectric and high boiling point solvents are typically required to produce uniform fibers. This can lead to solvent contamination that is detrimental to cell viability and differentiation.^{8–10} In this manuscript, we expand upon our previous reports using melt coextruded nanofibers as a biomaterial platform.^{11,12} The process is solvent free, uses polymers that have been extensively used in FDA approved products, and has the potential to access throughputs three orders of magnitude higher than electrospinning.¹³

A key component of biologically functional scaffolds is the need to introduce chemical or biochemical cues that allow for cellular adhesion, proliferation, and differentiation. In biological systems, however, a single functional cue on a scaffold does not recapitulate the natural biological environment. More often, multiple cues are needed to direct cell biology.^{14–16} Additionally, chemical cues are arranged spatially in natural systems, making it imperative that haptotactic and chemotactic gradients can be realized in synthetic scaffolds.^{17–19} A number of examples of synthesized telechelic polymers have been used to fabricate chemically functionalized fibers after processing.^{20–22} A recent example from Becker et al has demonstrated multi-component chemical functionalization of telechelic polymers synthesized from orthogonally functionalized monomers.²³ These electrospun fibers were functionalized post-processing to yield chemically complex fibers with multiple functionalities in the scaffold. However, no spatial control is reported in any of these seminal examples. Herein, we report upon a photochemical modification strategy of coextruded polymeric fibers to introduce multiple chemical functionalities in a spatially controlled manner. The photochemistry has been optimized to modulate the surface density of functional groups. Several types of ‘click’ functional groups have also been incorporated into extruded polymeric fibers, leading to functionalization with multiple small molecules and ultimately peptides for biological response.

Materials and Methods

Materials

Doxorubicin-HCl (>99 %) was purchased from TSZ Chem BIOTANG Inc. AzideFluor 488 (HPLC), 5-bromovaleric acid, and levulinic acid were purchased from Sigma Aldrich. Rink Amide MBHA resin LL (100–200 mesh, 0.52 meq) was purchased from Novabiochem. Aminooxy-5(6)-TAMRA was purchased from Biotium, Inc. MC3T3-E1 Subclone 4 (ATCC® CRL-2593™) was purchased from ATCC. Fmoc-protected amino acids and O-(1H-6-Chlorobenzotriazole-1-yl)-1, 1, 3, 3-tetramethyluronium hexafluorophosphate (HCTU) were obtained from Peptides International.

Instruments

Multilayer coextrusion was performed using the CLiPS two-component coextrusion system with 12 multipliers. Scanning electron microscopy (SEM) was performed using a JEOL SEM under an emission voltage of 20kV. Proton nuclear magnetic resonance (¹H NMR) and proton-decoupled carbon (¹³C NMR) spectra were recorded on a Varian Inova 600 MHz NMR spectrometer in deuterated solvents. Chemical shifts are reported in parts per million

(ppm, δ) relative to residual protio solvent (CDCl_3 , δ 7.26). A Thermo Finnigan LCQ Advantage LC/MS (ESI) was used to confirm the molecular weight of synthesized benzophenones. A high-intensity UV lamp (Bluepoint 4 Ecocure from Honle UV America Inc.) was used for surface modification of the poly(ϵ -caprolactone) (PCL) fibers with functionalized benzophenones using a 320–390 nm filter. ATR-FTIR was conducted on a Digilab FTS 7000 spectrometer, with a UMA 600 microscope. Surface analysis of materials was investigated on a PHI Versaprobe 5000 Scanning X-Ray Photoelectron Spectrometer (XPS) with an Al K α X-ray source (1486.6 eV photons) from the Swagelok center at Case Western Reserve University. Each spectrum was collected over a $100 \times 1400 \mu\text{m}$ sample area. The molecular weight of CYGFGG was measured on a Bruker AUTOFLEX III MALDI-TOF/TOF mass spectrometer using α -Cyano-4-hydroxycinnamic acid (CHCA) as a matrix. The molecular weight of azido-GRGDSP was confirmed by ESI-MS. Fluorescent images of dual dye-modified PCL were scanned on a Maestro imaging system from PerkinElmer. The drug release study and the alkaline phosphatase assay were monitored using a Biotech Synergy HT microplate reader.

Experimental

Coextrusion of PCL fibers

A multilayered film was extruded by a multiplication coextrusion process to fabricate polymeric fibers. PCL (CAPA 6800 pellets, MW=80 kg/mol) were coextruded with poly(ethylene oxide) (PEO) to produce a PCL/PEO composite tape. In order to match the rheology of PCL and PEO melts during the extrusion, two grades of PEO (Dow POLYOX N80 (MW = 200 kg/mol) and POLYOX N10 (MW = 100 kg/mol)) with a weight ratio of 30:70 were pre-blended using a Haake Rheodrive 5000 twin screw extruder. The viscosities of the obtained PEO blend and PCL melt match at the extrusion temperature, 200 °C. Ten vertical multipliers and two horizontal multipliers were used throughout the extrusion line to generate a 256×4 matrix architecture that contains 128×4 PCL domains embedded in PEO. The chill roll speed was 40 rpm and the dimensions of the exit die are $0.5'' \times 0.02''$. PEO was removed by securing the ends of the composite tape and stirring in water at room temperature for 24 hours, to yield aligned PCL fiber bundles. The PCL fibers were washed with methanol 3 $\times$ and vacuum dried overnight.

Synthesis of functionalized benzophenones

Propargyl benzophenone (1) was synthesized following a previous procedure.¹¹

Propenyl benzophenone (2). 4-hydroxylbenzophenone (1 g, 5 mmol) and anhydrous potassium carbonate (1.38 g, 10 mmol) were stirred in a round bottom flask in acetone (30 mL) for 6 hours at room temperature to deprotonate the hydroxyl group. After 6 hours, allyl bromide (1.85 g, 15 mmol) was added and the reaction mixture was refluxed for 24 hours. The crude material was concentrated under reduced pressure and re-dissolved in CH_2Cl_2 . The organic layer was washed with water (3 $\times$) and brine (3 $\times$) and dried over Na_2SO_4 . The residual solvent was evaporated under reduced pressure and the crude material was recrystallized in n-hexane (80% yield). ^1H NMR (600 MHz, chloroform-*d*) δ 7.83 (d, 2H), 7.75 (d, 2H), 7.56 (t, 1H), 7.47 (t, 2H), 6.98 (d, 2H), 6.0(m, 1H), 5.46 (d, 1H), 5.34(d, 1H),

4.65(d, 2H) ppm. ^{13}C NMR (600 MHz, chloroform-*d*) δ 195.7, 162.4, 138.5, 132.7, 132.1, 130.5, 129.9, 128.4, 118.4, 114.5, 69.1 ppm. ESI-MS (*m/z*, rel%) 239.3 ($[\text{M}+\text{H}]^+$, 100 %), calculated for $\text{C}_{16}\text{H}_{14}\text{O}_2$.

2-(4-benzoylphenoxy)ethanol

4-hydroxylbenzophenone (2 g, 10 mmol) and anhydrous potassium carbonate (2.76 g, 20 mmol) were stirred in a round bottom flask in N,N-Dimethylformamide (DMF). 2-bromoethanol (1.07 mL, 15 mmol) and potassium iodide (KI) (0.8 g, 4.8 mmol) were added, and the reaction mixture was refluxed for 24 hours at 65 °C. After the reaction, the mixture was cooled to room temperature and then filtered. The yellow filtrate was slowly precipitated in 700 mL of deionized water in an ice bath. A white powder was collected by centrifuge at 10,000 rpm for 10 min. (80% yield) ^1H NMR (600 MHz, chloroform-*d*) δ 7.80 (d, 2H), 7.72 (d, 2H), 7.55 (t, 1H), 7.45 (t, 2H), 4.15 (t, 2H), 3.98 (t, 2H), 2.6 (s, 1H) ppm. ^{13}C NMR (600 MHz, chloroform-*d*) δ 195.8, 162.5, 138.4, 132.8, 132.2, 130.7, 129.9, 128.4, 114.3, 69.6, 61.5 ppm. ESI-MS (*m/z*, rel%) 243.4 ($[\text{M}+\text{H}]^+$, 100%), 281.3 ($[\text{M}+\text{K}]^+$, 13%) calculated for $\text{C}_{15}\text{H}_{14}\text{O}_3$.

Aminoxy benzophenone (3). 2-(4-benzoylphenoxy)ethanol (1g, 4 mmol), N-hydroxyphthalimide (3.26 g, 20 mmol), and triphenylphosphine (PPh_3) (5.3 g, 20 mmol) were dissolved in dichloromethane (DCM) in a round bottom flask at 0°C. Diisopropyl azodicarboxylate (3.94 mL, 20 mmol) in DCM (10 mL) was added dropwise to the solution and was stirred for 24 h. The reaction mixture was flushed over a silica plug and evaporated under reduced pressure. The dried reaction mixture was re-dissolved in acetonitrile (40 mL). Hydrazine monohydrate was added and the solution was stirred for two hours. After concentrating the reaction, 15 mL of DCM was added and the mixture was filtered over a plug of celite under vacuum. The product was purified via silica flash chromatography (n-hexane/ethylacetate, 1:2). (47% yield) ^1H NMR (600 MHz, chloroform-*d*) δ 7.83 (d, 2H), 7.74 (d, 2H), 7.56 (t, 1H), 7.47 (t, 2H), 5.56 (s, 2H), 4.26 (t, 2H), 4.06 (t, 2H) ppm. ^{13}C NMR (600 MHz, chloroform-*d*) δ 195.8, 162.8, 138.5, 132.8, 132.1, 131.5, 129.9, 128.4, 72.7, 66.9 ppm. ESI-MS (*m/z*, rel%) 258.4 ($[\text{M}+\text{H}]^+$, 13%) 279.5 ($[\text{M}+\text{K}]^+$, 100%), calculated for $\text{C}_{15}\text{H}_{14}\text{NO}_3$.

Levulinic benzophenone (4)

4-hydroxyl benzophenone (1 g, 5 mmol) and levulinic acid (2.3 mL, 25 mmol) were dissolved in 50 mL of DCM and cooled in an ice bath. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (3.88 g, 25 mmol) was slowly added in the solution and then 4-(dimethylamino) pyridine (DMAP) (0.62 g, 5 mmol) was added. The reaction was stirred for 48 hours. The final solution was flushed over a short silica plug with DCM. The organic layer was washed with DI water, dried over sodium sulfate and concentrated under reduced pressure. (82% yield) ^1H NMR (600 MHz, chloroform-*d*) δ 7.83 (d, 2H), 7.77 (d, 2H), 7.58 (t, 1H), 7.47 (t, 2H), 7.21 (d, 2H), 2.87 (t, 2H), 2.85 (t, 2H), 2.23 (s, 3H) ppm. ^{13}C NMR (600 MHz, chloroform-*d*) δ 206.5, 195.8, 171.2, 154.1, 137.7, 135.3, 132.7, 131.9, 130.2, 128.5, 121.7, 38.1, 30.0, 28.4 ppm. ESI-MS (*m/z*, rel%) 279.5 ($[\text{M}+\text{Na}]^+$, 100%), calculated for $\text{C}_{18}\text{H}_{16}\text{O}_4$.

Photochemistry

A functionalized benzophenone, was dissolved in methanol (MeOH) (10 mg/ml). Extruded PCL fiber bundles were cut to 3 cm in length. Each sample was soaked in the solution for 5 mins and then air-dried at room temperature. The dried samples were placed on slide glass and irradiated using a UV source with a 320–390 nm filter for 5, 10 and 20 mins respectively (n=3). The UV intensity was 33.5 mW/cm². Remaining reagents were removed by washing in MeOH overnight and the samples were vacuum dried.

Copper-catalyzed azide-alkyne cycloaddition (CuAAC) chemistry

After immobilizing **1** onto the PCL fiber bundles, each sample (n=3) was immersed in an aqueous solution of AzideFluor 488 (AF₄₈₈) (2 mM, 100 μ L) or azido-GRGDSP (4 mM, 50 μ L). A premixed solution of CuSO₄ (10 μ L, 50 μ M) and THPTA (50 μ L, 50 μ M) was then added, and finally a fresh solution of sodium ascorbate (100 μ L, 100 mM). The samples were incubated for 2 hours at room temperature. The samples were washed sequentially in DMSO and ultrapure water. The samples were vacuum dried for 24 hours. The total concentration of AF₄₈₈ attached onto the PCL fiber bundle was measured by dissolving the AF₄₈₈-PCL fibers in DCM and quantified by UVVis absorbance at 501 nm. This value was compared to the BET surface area of the fibers to determine surface coverage density. To confirm RGD modification, the atomic chemical composition of RGD-PCL fibers was analyzed by XPS.

Oxime chemistry

Photochemical conjugation of either **3** or **4** was accomplished as described above. Either sample was placed in a 1.5 mL Eppendorf tube with 1.0 mL of PBS (pH 5.3), 15 μ L of aniline (1mM in DMSO) and aminooxy-5(6)-TAMRA dye or doxorubicin hydrochloride (DOX), (2 mM, 100 μ L) and the solution was gently mixed. Reactions proceeded for 4 hours at 37 °C. After the reaction, the excess dye or doxorubicin was removed by sequential DMSO and water washing. The samples were vacuum dried for 24 hours.

Thiol-ene chemistry

Photochemical conjugation of **2** was carried out as described above to yield propenyl functionalized fibers. The alkene fibers were characterized by ATR FT-IR (Figure S3). The PCL fiber bundles were immersed in a scintillation vial with 2.5 mL DI water and the synthesized SH-PEG-FITC or CYGFGG peptide (1mM, 200 μ L) were added. The samples were irradiated with UV light for 60 sec (33.5 mW/cm²). After conjugation, the samples were washed in DMSO and water to remove excess SH-PEG-FITC (or CYGFGG) and vacuum dried.

Dual dye conjugation

Photomasks were printed as 1 $\times$ 3 cm linear gradient images on transparency film (3M™ PP2410/100) with a commercialized inkjet printer (Epson, WF 3540). To apply the gradient photomask, 3 cm of a PCL fiber bundle derived from a single tape was soaked in a solution of **1** (10 mg/mL in MeOH) for 5 mins and then air-dried at room temperature. The samples were covered with the gradient photomask and irradiated for 20 minutes (right to left). After

irradiation, the samples were washed in methanol overnight to remove excess **1**, and solvent was evaporated under reduced pressure overnight. The same procedure was carried out for **4** by covering the opposite face (left to right) with the gradient photomask. Click chemistry was performed with AF₄₈₈ as described above. After washing AF₄₈₈ in DMSO and water sequentially to remove non-reacted AF₄₈₈, samples were vacuum dried. Aminooxy-5(6)-TAMRA was then conjugated onto the same fiber bundle as described in the oxime chemistry.

Drug release study

1.5 mL of MES buffer (100 mM, pH 4.5) was added to each well of a 24 well-plate. PCL fibers decorated with Doxorubicin (PCL-DOX) were placed in the each of the wells on the 24-well plate. The well plate was immediately placed in a Plate Reader at 37 °C for 48 hours. The release kinetics of DOX from the PCL-DOX was observed by monitoring the UV-Vis absorbance at 490 nm every 10 minutes, corresponding to the maximum absorption of DOX. After 48 hours, the remaining fibers were dissolved in 2 ml of PBS titrated to pH 11 to determine if any Doxorubicin remained on the PCL fibers. The UV spectrum showed no absorbance at 490 nm.

Solid Phase Peptide Synthesis

The N₃-GRGDSP and CYGFGG were synthesized using solid phase peptide synthesis in a Torviq 25 mL filtered syringe specifically designed for peptide synthesis. Peptide synthesis started from 0.5 g of Rink Amide MBHA resin (0.52 mmol/gram) and this resin was pre-swelled in 15 mL of DCM for 10 minutes and washed in DMF 3 times. 20% 4-methyl piperidine solution in DMF is added to the resin (15 mL), this is performed twice for 5 minutes and 20 minutes. A Kaiser test was performed immediately after to confirm Fmoc deprotection. After a positive Kaiser test, the amino acid (0.78 mmol, 3 equiv), HCTU (323 mg, 3 equiv) and diisopropylethyl amine (DIPEA) (272 μ L, 6 equiv) were dissolved in a minimal amount of DMF. This solution was added to the resin at room temperature and allowed to react for 1 hour on a rotary shaker. Following the coupling, the resin was again washed with DMF and DCM for 5 mins, 3 times each, and a qualitative Kaiser test was performed to ensure coupling. This synthetic process was repeated until the full peptide sequence was completed. After synthesis of the full RGD peptide (GRGDSP), the N-terminus was conjugated with 5- Bromovaleric acid– as described above. After washing 3 times with DMF (10 mL) and DCM (10 mL), sodium azide (170 mmol, 10 equiv) was added in 20 mL of DMF in order to convert the alkyl bromide to an azide. The reaction was allowed to react overnight at room temperature. Subsequently, the resin was washed with DMF to remove excess sodium azide and the resin was dried completely in n-hexane. To cleave the peptide, a solution of TFA/H₂O (95/5, v/v) (10 mL) was incubated with the resin for 4 hours at room temperature and the resin was removed by filtration. The cleaved peptide solution was precipitated in cold ether, centrifuged (10,000 rpm for 10 minutes at 4 °C), and decanted to yield a white pellet. The final crude peptide was dried under vacuum to yield a white powder. The crude N₃-GRGDSP or CYGFGG peptides was purified by reverse phase-HPLC. Collected fractions were pooled and lyophilized to generate a white powder. The desired peptide product, Azido-GRGDSP, was confirmed by ESI-mass spectra

($[M+H]^+ = 712$ Da). CYGFGG peptide was confirmed via MALDI-TOF (positive ion mode) using α -cyano-4-hydroxycinnamic acid (CHCA) as a matrix ($[M] = 601$ Da).

Dual peptide conjugation on PCL fibers

Equal volumes of **1** (10 mg/ml in MeOH) and **2** (10 mg/ml in MeOH) were mixed homogeneously. The PCL fiber bundles were soaked in the mixed solution of **1** and **2** for 5 mins, air-dried, and then UV irradiated for 20 min. After UV irradiation, samples were washed in MeOH and then air-dried completely. In order to conjugate the N₃- GRGDSP peptide (RGD), click chemistry was performed first following the aforementioned method. After click chemistry, the fibers were washed in water overnight and then vacuum dried. The dried fiber bundle was immersed in a solution of CYGFGG (OGP) peptide (1.5 mL, 3mM) and 500 μ L of DI water and mixed gently. To perform thiol-ene chemistry, UV irradiation proceeded for 60s (33.5 mW/cm²). The peptide conjugated fiber bundle was washed in deionized water overnight and vacuum dried. To investigate the dual peptide conjugation on the same fiber bundle with two different chemistries, surface analysis of the fibers was performed with XPS.

Cell culture

MC3T3-E1 Subclone 4 cells were cultured in Alpha Minimum Essential Medium (MEM) with ribonucleosides, deoxyribonucleosides, 2 mM L-glutamine and 1 mM sodium pyruvate, but without ascorbic acid. To make complete growth medium, 10% newborn calf serum (NCS) and 1 % penicillin were included in the MEM medium. Cells were incubated in 75 cm² cell culture flasks at 37 °C, in a 95% air and 5% CO₂ environment. At 80–90% confluency, the cells were detached with PBS/EDTA for 10 minutes at 37 °C. The detached cells were collected by centrifugation at 800 g for 5 minutes. 2.9×10^4 cells were placed on each samples (RGD-PCL, OGP-PCL and RGD/OGP-PCL, n=3) in a 12-well cell culture plate and cultured at 37 °C, 5 % CO₂ in a humid environment for 72 hours. The cell culture medium was refreshed every day (The cells placed on the scaffolds were passaged 3 $\times$).

Alkaline phosphatase (ALP) activity assay

After 72 hours, cells on the peptide conjugated fibers (RGD-PCL, OGP-PCL and Dual peptide-PCL (RGD and OGP-PCL) were washed with Dulbecco's phosphate buffered saline (DPBS) 3 times. 400 μ L of 0.1M Triton X buffer for cell lysis was added and cells were detached using a cell scraper. The cell lysis buffer was placed in an Eppendorf tube and centrifuged at 10,000g for 5 mins. 100 μ L of the supernatant for each replicate was placed in a 24-well plate and 500 μ L phosphatase assay buffer was added. Baseline samples were recorded without enzyme. Finally, 500 μ L of *p*-nitrophenol phosphate substrate was added to each well with incubation at 37 °C for 30 min. Samples measured via UV-Vis spectroscopy at 405 nm in a microplate reader.

Results and Discussion

Polymer Extrusion

The coextrusion of polymeric fibers with rectangular cross-sectional dimensions and their subsequent photochemical modification was recently reported.^{11,24} Briefly, our coextrusion

process starts with poly(ϵ -caprolactone) (PCL) and poly(ethylene oxide) (PEO) which are layered vertically in the extrusion line (Figure 1A). In step B, layers are split vertically and recombined horizontally to yield 2^n vertical layers, where n is the number of multipliers. After the original multiplication of vertical layers, a surface skin of PEO is layered on top of the extrudate (Figure 1C). Finally, the composite tape is split horizontally and recombined vertically prior to release from the exit dye (Figure 1D). The final composite is composed of a PCL fibrous matrix embedded within a PEO tape. The water-soluble PEO tape is then removed either through an aqueous wash or through a water jetting process, depending on the orientation of the fibers desired. The final fibers can be either microscopically aligned or entangled, contingent upon the washing procedure. For all fibers detailed within, the composite tape was affixed at either end and a slowly stirred water bath was used to provide aligned fibers of $0.72 \pm 0.21 \mu\text{m}$ cross-sectional dimensions. Due to the final fiber processing, only a single cross-sectional dimension can be seen as the water bath does not entangle the fibers.

Fiber Functionalization

The fiber modification scheme utilizes photochemistry in which a benzophenone derivative is irradiated with ultraviolet light and undergoes a radical insertion into the PCL backbone.^{25,26} The pendant functional group attached to the benzophenone is then available for secondary modification. The photochemical scheme was chosen for its simplicity and ability to spatially pattern molecules onto the fiber surface. Additionally, photochemistry provides a straightforward way to control surface density simply as a function of irradiation time. In our previous work, we used propargyl benzophenone at a fixed surface concentration to enable the copper-catalyzed azide-alkyne cycloaddition reaction.¹¹ We sought to better understand and manipulate the chemistry to enable control over surface density, implement several functional groups for bioconjugation, and pattern multiple species onto a single fiber scaffold.

Four benzophenone derivatives were synthesized starting from 4-hydroxybenzophenone, in which four orthogonal bioconjugation handles were realized; propargyl benzophenone (**1**) for traditional 'click' chemistry, an alkene (**2**) for thiol-ene chemistry, an alkoxyamine (**3**) for oxime and hydrazone ligations, and finally a ketone (**4**) for the inverse of the oxime or hydrazone ligation.

Initially, the impact of photoirradiation on each of the benzophenone derivatives was probed to determine surface coverage of the PCL fibers. Fiber were dip-coated in a concentrated solution of the indicated benzophenone derivatives and allowed to air dry. The coated fibers were then UV irradiated in three separate trials for varying times (5 min, 10 min, 20 minutes – 33.5 mW/cm^2). The maximum duration of irradiation was chosen because this UV fluence showed no detriment to the mechanical properties of the fibers.¹¹ The fibers were subsequently washed with DMSO and water, dried, and allowed to undergo the corresponding 'click' type reaction (Figure 2). To quantify functional groups that remained active for conjugation, we used an excess of fluorescent dye molecules to perform the corresponding conjugation reaction. In this case, we were primarily interested in the functional group availability rather than the total density of benzophenone deposited, as the

benzophenone will not be our active molecule for downstream applications. After dye conjugation, the fibers were extensively washed to remove unreacted components and then dissolved in a good solvent for PCL. Total dye modification is quantified via UV Vis absorption versus a standard curve and correlated to the surface area of the fibers, as determined by BET measurements (Table 1). This method allows us to determine the surface density of coverage expressed as total molecules per surface area. In the past, this strategy has worked well for us as a corollary to peptide modification owing to the fact that click-type reactions are of high efficiency and are extremely chemoselective.^{11,12,27}

The results of modification chemistry between propargyl benzophenone (**1**), levulinic benzophenone (**4**), and propenyl benzophenone (**2**), all exhibited increasing modification density with an increase in total fluence of UV irradiation (Table 1). Functionalized fibers were characterized by ATR-FTIR to determine functional group equivalence following irradiation. Unfortunately, for ketone modified fibers only the results of the oxime reaction could be characterized as the ketone peaks were overwhelmed by the carbonyl peak observed from PCL. Propargyl modified fibers were reacted via the ligand-accelerated CuAAC reaction with AzideFluor 488 (AF₄₈₈) and quantified with varying irradiation times.²⁸ Gratifyingly, we were able to manipulate surface modification density of our fibrous scaffolds by approximately an order of magnitude, by simply varying the irradiation time. This led to modification densities ranging from ~0.1 nmol/cm² to ~1 nmol/cm² (Figure 1). This trend continued for the ketone modified fiber, leading to approximately equivalent densities, when modified with an alkoxyamine TAMRA derivative. Lastly, we modified fibers with the alkene derivative (**2**) to measure the efficiency of thiol-ene reactions. Commercially available sources of thiol modified dyes are unavailable, thus a thiol modified fluorescein was synthesized from fluorescein isothiocyanate and a heterobifunctional PEG containing both thiol and amine functional groups (Figure S2). The thiolated FITC was incubated with alkene decorated fibers and UV irradiated to undergo the thiol-ene reaction (Figure S3).²⁹ This reaction resulted in approximately one half of the surface coverage relative to the alkyne and ketone modified fibers, however, with the same trend of increasing surface coverage with increased irradiation time. This discrepancy is likely a result of steric hindrance, as the PEGylated fluorescein is ~1.5 kDa, occluding accessibility to modification in accordance with the small molecule dyes.

Doxorubicin Conjugation and Release

As oxime chemistry is an important reaction for bioconjugation, alkoxyamine functionalized fibers, derived from **3**, were also fabricated. However, neither ketone nor aldehyde dyes are commercially available, making the inverse of the oxime reaction difficult to analyze. To further explore this reaction pathway, we chose to use a biologically relevant example that could be applied to cancer treatment. Doxorubicin (DOX), a commonly used anti-cancer therapeutic, can undergo site selective modification at the ketone position to form either an oxime or a hydrazone.^{27,30} These chemical linkages are reversible under acidic conditions, as would typically be found in tumor microenvironments.³¹ For DOX immobilization, 20 minutes of irradiation time was used to provide maximum alkoxyamine group density on the fiber surface. DOX conjugation was performed in PBS buffer in the presence of aniline as a catalyst. The fibers were noticeably red upon conjugation, as would be expected. Rather

than immediately quantifying DOX conjugation, we chose to harness the pH responsiveness of the oxime functional group. DOX-loaded fibers were immersed in MES buffer at a pH of 4.5. Release of DOX was quantified kinetically using the absorbance of the molecule at 490 nm (Figure 3).^{32,33} A rapid burst release is observed over the course of 30 minutes, resulting in ~60% DOX release. After the initial burst, the release slows and the overall release profile follows an exponential curve, as has been seen previously.³⁰ The total cumulative amount of DOX released was 0.93 ± 0.43 nmol/cm², which is consistent with other small molecule fiber modifications. Additionally, the buffer was adjusted to pH 11 to dissolve the fiber bundle and no residual DOX remained on the fiber scaffold. The results of this experiment indicate that coextruded fibers are a viable option for drug loading, and the oxime linkage provides stimuli-responsive release, which could lead to post-surgical treatment of cancerous tumors.

Spatial Control of Multiple Substrates

As our modification scheme relies on a photochemical reaction, the ability to perform spatial patterning based on exposure to UV intensity should be straightforward. Previously, we have demonstrated the ability to develop single substrate gradients that affect cell morphology in neural-derived cells.¹² In biology, multiple components usually act in concert to elicit a biological response. Several studies have explored surface patterning with multiple immobilized biologically active substrates and have found exciting conclusions where *in vitro* and *in vivo* responses have been magnified.³⁴ In this scheme, gradient modification with respect to two different chemistries is a relatively simple and expeditious task (Figure 4). First the fiber bundle is dip-coated in a concentrated solution of propargyl benzophenone (**1**) and allowed to air dry. A gradient photomask is then placed over the fiber bundle and the fibers are UV irradiated. The fibers are extensively washed and the same procedure is applied using the levulinic benzophenone (**2**), with the photomask aligned in the reverse orientation. The fibers then undergo sequential oxime chemistry with TAMRA and the CuAAC reaction with AF₄₈₈, to form gradient fibers with multiple components attached via two different types of chemistry. Fibers are imaged via a MAESTRO fluorescence imaging system in both the red and green channels, which correspond to TAMRA and AF₄₈₈, respectively (Figure 4B). Fluorescence images indicate two different gradients on the fiber substrate, with TAMRA increasing from right to left and the opposite orientation for AF₄₈₈. When the channels are overlaid, it is clear that multiple patterns can be expressed on the same fiber bundle. This also allows for the deposition of two different types of chemistry that can be readily controlled based solely on UV fluence.

Multiple Peptide Substrates

As a biologically relevant example of the fiber chemistry, we chose to deposit multiple peptide substrates and evaluate cellular response to two synergistic peptides. It has been shown that the RGD sequence and osteogenic growth peptide (OGP) act in synergy to cause pre-osteoblasts to differentiate into osteoblasts on polymeric substrates.^{35,36} The RGD sequence binds to integrin receptors, all wing for cellular adhesion and spreading,³⁷ while the OGP sequence, is able to cause differentiation into bone cells.³⁸ These two peptides, thus, allow for cellular attachment and differentiation, respectively. In principle, RGD by itself would only allow for adhesion and minimal differentiation, likewise, OGP would

allow for differentiation of cells but minimal adhesion. Both peptides should be necessary to see increased differentiation for adhered cells on the fibers. Fiber substrates were prepared by dip-coating the scaffolds in a mixture of propargyl benzophenone (**1**) and propenyl benzophenone (**2**), followed by air drying and UV irradiation. The fibers were then exhaustively modified using the CuAAC reaction to install N₃-GRGDSP. After removing the non-reacted N₃-GRGDSP and catalyst from CuAAC reaction, the alkenes were modified using the thiol-ene reaction with a cysteine modified OGP (CYGFGG). Control fibers were also studied, in which a single modification was made by either the CuAAC reaction or the thiol-ene reaction. In control substrates, only a single benzophenone was deposited on the fiber to eliminate cross-reaction between thiol-ene and thiol-yne reactivities. Fiber substrates were analyzed using X-ray photoelectron spectroscopy (XPS) to evaluate atoms that were unique to the peptide modified substrate, namely sulfur for OGP and nitrogen for both peptides. Neither of these peaks were present in unmodified or benzophenone modified PCL fibers.^{11,12} XPS analysis revealed a prominent sulfur (S2p) peak indicating attachment of OGP for the modification with a single peptide, as well as, an equivalent sulfur peak for the dually modified substrate. The nitrogen (N1s) peak was also analyzed to determine total peptide concentration on the surface. Gratifyingly, this peak was clearly visible with a single modification (~2%) and with the dual modification the total nitrogen content doubled relative to the single modification (~4%). This result is indicative of increased concentration with multiple chemical modifications, as would be expected with increased deposition of functional groups.

Finally, we used the dual-modified substrates as cell seeding scaffolds for MC3T3 cells, a murine derived pre-osteoblast cell line known to differentiate in the presence of OGP.³⁹ In line with our hypothesis, MTT assays showed ~2-fold higher cell viability for the singly modified RGD substrate and the dually modified RGD/OGP substrate relative to the control OGP substrate (Figure S6). A common measure of bone differentiation is the upregulation of alkaline phosphatase (ALP), which is well-characterized in MC3T3 cells and portends mineralization of osteoblasts. Cells were seeded on all three substrates – the RGD fibers, OGP fibers and dual RGD-OGP fibers – and cultured for 72 hours in osteogenic growth media. Cells were subsequently harvested and lysed to release intracellular protein. The lysate was subjected to a commercial ALP assay, which relies on the cleavage of a *p*-nitrophenol substrate that is measured colorimetrically. The two negative controls, where single peptides were deposited, showed limited ALP activity, either due to lack of cellular adhesion or through lack of osteoinduction. Gratifyingly, the dual-modified substrate showed ~2.5-fold increase in relative ALP activity. This result indicates that the bioavailability of multiple peptides on our substrate was sufficient to induce both bone differentiation and cellular adhesion, providing a platform to pattern multiple biologically active substrates.

Conclusions

In this manuscript, the modification of melt coextruded nanofibers has been extended to include a diverse set of functional fibers for bioconjugation. Taking advantage of multiple ‘click’ types of chemistry allows for the covalent attachment of several different types of molecules. This strategy is especially useful if the CuAAC reaction may not be appropriate

or if orthogonal chemistry may be needed. Additionally, we report a simple method for surface modification that enables the manipulation of surface concentration based solely on UV fluence. The uniqueness of this approach is that simple chemical patterns can be established on fiber substrates and that multiple functional groups can be deposited on the fibers. This is advantageous as recapitulating biological systems must take a more globally informed outlook, where a large number of factors are involved. Such factors include haptotactic and chemotactic gradients, synergistic peptide and protein cues, surface topology of substrates, as well as the mechanical properties of the scaffold. With regard to the coextruded fiber scaffold, a number of these factors can be controlled including modulus,²⁴ fiber alignment,¹² and now the deposition of multiple chemical functionalities in a spatially controlled manner. By having control over all of these properties, application of these scaffolds will be pursued for a broad range of tissue engineered substrates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

J.K.P. acknowledges a Pathway to Independence award from the NIH (R00EB011530). J.K.P., S.E.K., and J.D.W. acknowledge the NSF Center for Layered Polymeric Systems (CLiPS) for financial support (DMR 0423914).

References

1. Barnes CP, Sell SA, Boland ED, Simpson DG, Bowlin GL. *Adv. Drug Deliv. Rev.* 2007; 59:1413–1433. [PubMed: 17916396]
2. Liu W, Thomopoulos S, Xia Y. *Adv Healthc Mater.* 2012; 1:10–25. [PubMed: 23184683]
3. Choi JS, Messersmith PB, Yoo HS. *Macromolecular Bioscience.* 2013
4. Chew SY, Mi R, Hoke A, Leong KW. *Adv. Funct. Mater.* 2007; 17:1288–1296. [PubMed: 18618021]
5. Li A, Hokugo A, Yalom A, Berns EJ, Stephanopoulos N, McClendon MT, Segovia LA, Spigelman I, Stupp SI, Jarrahy R. *Biomaterials.* 2014; 35:8780–8790. [PubMed: 25064803]
6. Sahoo S, Ang LT, Goh JCH, Toh S-L. *Journal of Biomedical Materials Research Part A.* 2010; 93A:1539–1550. [PubMed: 20014288]
7. Persano L, Camposeo A, Tekmen C, Pisignano D. *Macromolecular Materials and Engineering.* 2013; 298:504–520.
8. Nam J, Huang Y, Agarwal S, Lannutti J. *J. Appl. Polym. Sci.* 2008; 107:1547–1554.
9. Lannutti J, Reneker D, Ma T, Tomasko D, Farson D. *Materials Science and Engineering: C.* 2007; 27:504–509.
10. Fatih Canbolat M, Tang C, Bernacki SH, Pourdeyhi B, Khan S. *Macromol. Biosci.* 2011; 11:1346–1356. [PubMed: 21984502]
11. Kim S-E, Wang J, Jordan AM, Korley LTJ, Baer E, Pokorski JK. *ACS Macro Letters.* 2014; 3:585–589. [PubMed: 24977107]
12. Kim S-E, Harker EC, De Leon AC, Advincula RC, Pokorski JK. *Biomacromolecules.* 2015; 16:860–867. [PubMed: 25715836]
13. Repka MA, Shah S, Lu J, Maddineni S, Morott J, Patwardhan K, Mohammed NN. *Expert Opinion on Drug Delivery.* 2012; 9:105–125. [PubMed: 22145932]
14. Choi JS, Choi SH, Yoo HS. *J. Mater. Chem.* 2011; 21:5258–5267.
15. Kempen DHR, Lu L, Heijink A, Hefferan TE, Creemers LB, Maran A, Yaszemski MJ, Dhert WJA. *Biomaterials.* 2009; 30:2816–2825. [PubMed: 19232714]

16. Jin G, Prabhakaran MP, Kai D, Ramakrishna S. *European Journal of Pharmaceutics and Biopharmaceutics*. 2013; 85:689–698. [PubMed: 23791682]
17. Guarnieri D, De Capua A, Ventre M, Borzacchiello A, Pedone C, Marasco D, Ruvo M, Netti PA. *Acta Biomaterialia*. 2010; 6:2532–2539. [PubMed: 20051270]
18. Lagunas A, Comelles J, Martínez E, Prats-Alfonso E, Acosta GA, Albericio F, Samitier J. *Nanomedicine: Nanotechnology, Biology and Medicine*. 2012; 8:432–439.
19. Lamb BM, Luo W, Nagdas S, Yousaf MN. *ACS Appl. Mater. Interfaces*. 2014
20. Lancuški A, Fort S, Bossard F. *ACS Applied Materials & Interfaces*. 2012; 4:6499–6504. [PubMed: 23145558]
21. Zheng J, Liu K, Reneker DH, Becker ML. *Journal of the American Chemical Society*. 2012; 134:17274–17277. [PubMed: 23013452]
22. Zheng J, Xie S, Lin F, Hua G, Yu T, Reneker DH, Becker ML. *Polymer Chemistry*. 2013; 4:2215–2218.
23. Lin F, Zheng J, Yu J, Zhou J, Becker ML. *Biomacromolecules*. 2013; 14:2857–2865. [PubMed: 23795681]
24. Wang J, Langhe D, Ponting M, Wnek GE, Korley LTJ, Baer E. *Polymer*. 2014; 55:673–685.
25. Prucker O, Naumann CA, Rühle J, Knoll W, Frank CW. *J. Am. Chem. Soc.* 1999; 121:8766–8770.
26. Lancaster JR, Smilowitz R, Turro NJ, Koberstein JT. *Photochemistry and Photobiology*. 2014; 90:394–401. [PubMed: 24256104]
27. Pokorski JK, Breitenkamp K, Liepold LO, Qazi S, Finn MG. *Journal of the American Chemical Society*. 2011; 133:9242–9245. [PubMed: 21627118]
28. Hong V, Presolski S, Ma C, Finn MG. *Angewandte Chemie International Edition*. 2009; 48:9879–9883.
29. Slavin S, Khoshdel E, Haddleton DM. *Polymer Chemistry*. 2012; 3:1461.
30. Jin Y, Song L, Su Y, Zhu L, Pang Y, Qiu F, Tong G, Yan D, Zhu B, Zhu X. *Biomacromolecules*. 2011; 12:3460–3468. [PubMed: 21863891]
31. Estrella V, Chen T, Lloyd M, Wojtkowiak J, Cornnell HH, Ibrahim-Hashim A, Bailey K, Balagurunathan Y, Rothberg JM, Sloane BF, Johnson J, Gatenby RA, Gillies RJ. *Cancer Res*. 2013; 73:1524–1535. [PubMed: 23288510]
32. Missirlis D, Kawamura R, Tirelli N, Hubbell JA. *European Journal of Pharmaceutical Sciences*. 2006; 29:120–129. [PubMed: 16904301]
33. Karukstis KK, Thompson EH, Whiles JA, Rosenfeld RJ. *Biophys. Chem*. 1998; 73:249–263. [PubMed: 9700924]
34. Min HK, Oh SH, Lee JM, Im GI, Lee JH. *Acta Biomaterialia*. 2014; 10:1272–1279. [PubMed: 24370639]
35. He X, Ma J, Jabbari E. *Langmuir*. 2008; 24:12508–12516. [PubMed: 18837524]
36. Moore NM, Lin NJ, Gallant ND, Becker ML. *Biomaterials*. 2010; 31:1604–1611. [PubMed: 19932505]
37. Hersel U, Dahmen C, Kessler H. *Biomaterials*. 2003; 24:4385–4415. [PubMed: 12922151]
38. Bab I, Chorev M. *Biopolymers*. 2002; 66:33–48. [PubMed: 12228919]
39. Chen Y-C, Bab I, Mansur N, Namdar-Attar M, Gavish H, Vidson M, Muhlrud A, Shteyer A, Chorev M. *The Journal of Peptide Research*. 2000; 56:147–156. [PubMed: 11007271]

Figure 1.

Top: Schematic of coextrusion to produce fibers. Cartoon images (A–D) represent cross-sectional views within the extrusion line during multiplication steps. Bottom: Scanning electron micrograph of aligned fibers following PEO dissolution. Scale bar – 10 μm .

Figure 2. Fiber modification with AF₄₈₈. A) Digital images of fibers functionalized with AF₄₈₈ under varying UV irradiation times. B) UV-Vis spectra of AF₄₈₈ functionalized fibers following dissolution.

Figure 3. Oxime coupling of doxorubicin. A) Schematic for reversible oxime coupling of DOX. B) ATR FT-IR spectra of PCL (black) and PCL-Alkoxyamine (red). C) Doxorubicin kinetic release profile, measured using UV-Vis absorbance at 490 nm in pH 4.5 MES buffer in increments of 10 minutes.

Figure 4. Dual gradient modification results. A) Bifunctional gradient modification scheme. B) Fluorescent images of PCL bundles. Top: Red channel to visualize TAMRA, Middle: Green channel to visualize AF₄₈₈, Bottom: Overlaid green and red channels. Images were taken using a MAES RO fluorescence imaging system and fiber length is 3 cm.

Sample	Atomic component %				
	C 1s	N 1s	O 1s	S 2p	Cu 2p
RGD-PCL	83.9	2.0	14.1	-	-
OGP-PCL	79.9	2.8	17.7	0.4	-
Dual-PCL	84.4	4.2	11.1	0.4	-

Figure 5.
XPS spectra of peptide modified fiber scaffolds. Inset is an expansion of the sulfur (S2p) region of the XPS spectrum.

Figure 6. Relative ALP activity for peptide modified substrates. Dual indicates modification with both OGP and RGD.

Scheme 1.
Synthetic scheme for multiple functional fiber variants.

Table 1

Modification densities with varying UV irradiation time.

Irradiation Time (mins)	CuAAC (AzideFluor 488, nmol/cm²)	Oxime (TAMRA-ONH₂ 577, nmol/cm²)	Thiol-ene (SH-PEG-FITC, nmol/cm²)
5	0.17 ± 0.13	0.06 ± 0.04	0.05 ± 0.03
10	0.30 ± 0.10	0.25 ± 0.08	0.14 ± 0.03
20	0.91 ± 0.10	1.05 ± 0.35	0.50 ± 0.03