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Encoding Cell-Instructive Cues to PEG-Based Hydrogels via Triple Helical Peptide Assembly

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Abstract

Effective synthetic tissue engineering scaffolds mimic the structure and composition of natural extracellular matrix (ECM) to promote optimal cellular adhesion, proliferation, and differentiation. Among many proteins of the ECM, collagen and fibronectin are known to play a key role in the scaffold's structural integrity as well as its ability to support cell adhesion. Here, we present photocrosslinked poly(ethylene glycol) diacrylate (PEGDA) hydrogels displaying collagen mimetic peptides (CMPs) that can be further conjugated to bioactive molecules *via* CMP-CMP triple helix association. Pre-formed PEGDA-CMP hydrogels can be encoded with varying concentration of cell-signaling CMP-RGD peptides similar to cell adhesive fibronectin decorating the collagen fibrous network by non-covalent binding. Furthermore, the triple helix mediated encoding allows facile generation of spatial gradients and patterns of cell-instructive cues across the cell scaffold that simulate distribution of insoluble factors in the natural ECM.

Introduction

Most mammalian cells grow within a scaffold composed of diverse structural and cell adhesive biomacromolecules that display tissue specific factors to control organization, proliferation and differentiation of cells.[1] This scaffold, broadly known as extracellular matrix (ECM), gives both mechanical and biochemical signals to the cells during tissue development that are both dynamic and spatio-temporal in nature. Many biochemical signals are soluble factors that freely permeate the ECM, but some cell-instructive signals are bound to the matrix scaffold. These insoluble factors are known to play a critical role in the development of complex tissues (e.g. vasculatures).[2,3]

As tissue engineers attempt to mimic this highly complex natural ECM structure, they often employ synthetic hydrogels as the starting scaffold material. The synthetic scaffolds consist of crosslinked hydrophilic macromolecules with viscoelastic properties similar to the natural ECM, and these scaffolds can be tailor designed to present cell-instructive cues that mediate cell-scaffold interactions.[4] Poly(ethylene glycol) (PEG) has been FDA approved for a number of biomedical uses, and PEG-based synthetic hydrogels are frequently used in tissue engineering research due to a wealth of information on their synthesis and physico-chemical

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†Electronic Supplementary Information (ESI) available: [MALDI-ToF mass spectra and CD melting curves of peptides, SEM and phase contrast micrographs of seeded cells]. See DOI: 10.1039/b000000x/

characteristics.[5] Because PEG is inert to cell interaction, it acts as a blank slate that accentuates cellular reaction to the bioactive cues conjugated to the scaffold.[6]

Previously, our lab showed that photocrosslinked PEG diacrylate (PEGDA) featuring collagen mimetic peptides (CMPs) can enhance the scaffold retention of collagens (and other ECM proteins) secreted by encapsulated chondrocytes.[7] Furthermore, this PEGDA-CMP scaffold promoted chondrogenic differentiation of encapsulated mesenchymal stem cells.[8] The improved bioactivity of the PEGDA-CMP scaffolds over PEGDA alone was attributed to CMP's ability to bind to cell-secreted collagens, which was first revealed by our research group.

Although CMPs were originally developed to model collagen's distinct triple helical structure, CMPs have since been shown to bind to natural collagen, presumably through CMP-collagen triple helical hybridization.[9] CMPs are short, synthetic peptides that feature collagen's prototypical (glycine-X-Y) repeating amino acid sequence in which X and Y positions are predominantly filled by proline and hydroxyproline, respectively.[10] The CMPs form thermally reversible triple helices in which three strands intertwine into a triple helix below the peptide's melting temperature (T_m) and melt into single strands above the T_m.[11] The CMP's ability to undergo such a specific folding process has motivated scientists to extend their studies beyond triple helix stability[12,13] to employing CMPs for diverse biomedical applications including natural scaffold functionalization,[14,15] macroscale peptide assembly,[16] and drug delivery.[17]

Taking advantage of their respective functionalities, researchers have begun using PEG-based hydrogels and CMPs together to generate synthetic tissue scaffolds. For example, physically crosslinked gels from CMP and starshape PEG complexes were reported to be a viable 3D scaffold for human mesenchymal stem cells.[18] Researchers have also created CMP platforms featuring direct cell-adhesive cues found in natural collagen such as GFOGER and GFPGER.[19–21] Since these adhesion peptides are active only in triple helical conformation, scientists have flanked this peptide with CMP sequences of high triple helical propensity.[22,23] These experiments demonstrated the compatibility of CMP-presenting scaffolds with cells that are either encapsulated or seeded onto them.

In our previous work, we demonstrated that PEG-CMP hydrogels featuring triple helix-mediated physical crosslinks can be further modified by strand-invasion and triple helix assembly of exogenously added CMPs.[24] These exogenous CMPs were able to compete with CMPs of the gel in triple helix assembly and bind to the hydrogel through heterotrimeric helix formation. Local CMP injection allowed modification of the gel in a spatially controlled manner, and particle tracking microrheology showed that exogenous CMPs can locally affect the mechanical properties of the gel by disrupting the triple helix crosslinks.

Here, we extend the exogenous CMP binding strategies to incorporate bioactive CMPs into a photocrosslinked PEGDA-CMP scaffold using the triple helical folding propensity of the peptides. The collagen triple helix is a unique multiplex protein structure rarely seen in other proteins. We present the use of CMP assembly as a non-covalent yet target-specific conjugation tool and demonstrate that CMP triple helix assembly can be a simple and robust tool for functionalization of scaffolds with cellular cues. While it is possible to use covalent grafting of cellular cues to promote cell-matrix interactions,[25] the CMP-mediated functionalization more closely mimics the physical binding interactions between structural and cell-signaling proteins in natural ECM.[26–28] We also demonstrate that the triple helix-mediated scaffold modification can create spatial patterns and gradients of cell-instructive

cues, which can help us understand the role insoluble factors play in cellular behaviors and complex tissue formation.

Experimental

Materials

Peptide synthesis reagents including Fmoc-amino acids, 2-(1*H*-benzo-triazole-1-yl)-1,1,3,3-tetra-methyluronium hexfluorophosphate (HBTU), *N,N*-diisopropylethylamine (DIPEA), N-methylpyrrolidone (NMP), and trifluoroacetic acid (TFA) were purchased from Advanced ChemTech (Louisville, KY). Rink-type Tentagel R RAM resin was purchased from Peptides International (Louisville, KY). Triisopropylsilane (TIS), piperidine, carboxyfluorescein (CF), rhodamine B, acrylic acid, and 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959) were obtained from Sigma-Aldrich (St. Louis, MO). Activating agents PyBOP, PyAOP, and HATU were obtained from EMD Chemicals (Philadelphia, PA). N-Fmoc-amido-dPEG$_4$-acid (dPEG) and polyethylene diacrylate (PEGDA; M.W. 3400 Da) were purchased from Advanced ChemTech and Glycosan BioSystems (Salt Lake City, UT), respectively. Round glass cover slips (15 mm diameter) were purchased from Electron Microscopy Sciences (Hatfield, PA). Fibroblasts (human dermal, neonatal) were obtained from American Type Culture Collection (Manassas, VA), and DMEM/F12+GlutaMAX media was obtained from Invitrogen. HUVECs and EGM-2 endothelial cell media were obtained from Lonza (Walkersville, MD). WST-1 proliferation assay kit was purchased from Roche Applied Science (Indianapolis, IN). For fixing & staining, 10% neutral buffered formalin was obtained from Sigma-Aldrich, tris-buffered saline (TBS) was obtained from Bio-Rad Laboratories (Hercules, CA), and Alexa-Fluor 488 phalloidin and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen and Roche Applied Science, respectively.

Peptide synthesis

Peptides were synthesized manually on Tentagel resin with conventional Fmoc mediated coupling chemistry using 4.5-fold molar excess of amino acids activated by HBTU and deprotected by treatment with piperidine. Coupling and deprotection steps were monitored with ninhydrin or chloranil tests. For Acrl-PEG-CMP synthesis, after completion of the CMP synthesis and Fmoc deprotection of the last Gly, the resin was reacted with Fmoc-dPEG spacer (4-fold molar excess) using PyBOP. The resin was treated with piperdine to remove the Fmoc from the dPEG spacer, and the N-terminus of the PEG-CMP conjugate was reacted with 5-fold molar excess of acrylic acid activated by PyBOP. Fluorescently labeled CMPs were prepared by reacting CMPs (on resin) with CF (6-fold excess) or rhodamine B (10-fold excess) using PyAOP and HATU, respectively. All peptide conjugates were cleaved from the resin by incubation with a cleavage cocktail (95% TFA, 2.5% TIS, 2.5% deionized water) for 3 h. The cleaved peptide solution was filtered from the resin and added dropwise to cold ethyl ether to precipitate crude CMP product. The peptides were then pelleted by centrifugation and dried under vacuum.

Crude peptides were purified using reverse-phase high performance liquid chromatography (HPLC) on a Varian Polaris 210 series liquid chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a semiprep Vydac reverse-phase C18 column. Mobile phase gradients of deionized water (DI H_2O) and acetonitrile containing 0.1% TFA were passed through the column at a flow rate of 4 mL min^{-1}. Peptides were detected by UV absorption at 220 nm for Acrl-PEG-CMP or at 275 nm for CMP-RGD peptides. The elutions containing target peptides were collected, combined, and lyophilized. The purity of peptides was determined by matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass

spectrometry (Voyager DE-STR, Applied Biosystems, Foster City, CA) (Supplementary Information, Figure S1).

PEGDA-CMP hydrogel synthesis

PEGDA-CMP hydrogel precursor solutions were created from a mixture of 1 wt% Acrl-PEG-CMP, 9 wt% PEGDA, and 0.05 wt% Irgacure 2959 photoinitator dissolved in PBS. The gel precursor solution was heated at 80°C for 30 min to melt CMP triple helices into single strands. Each gel was prepared by first depositing 100 μL of hot precursor solution onto a glass cover slip followed by placing another cover slip on top of the solution to form a glass-solution-glass sandwich. These samples were then placed under a 365 nm UV lamp (McMaster-Carr, Atlanta, GA) for 10 min with ~5 mW cm^{-2} intensity. The resulting photocrosslinked gel was revealed by peeling off the top glass cover slip and the gel was transferred to a multiwell plate for further experiments as described below.

Circular dichroism study

To prepare samples for CD studies, gel precursor solution consisting of PEGDA (6.75 wt%), Acrl-PEG-CMP (0.75 wt%), and Irgacure 2959 (0.05 wt%) was dissolved in PBS. The sample was heated for 30 min at 80°C to melt CMPs into single strands, and 400 μL of this solution was pipetted into a CD cuvette. For CD measurements on uncrosslinked precursor solution, the sample was incubated in the cuvette at 4°C overnight to allow complete folding of triple helix. For CD measurements on photopolymerized gels, the hot precursor solution was exposed to UV light for 20 min, which led to formation of a self-supporting gel inside the cuvette. Longer exposure time ensured full photopolymerization of the gel which contained reduced amount of monomers to minimize background CD signal. This PEGDA-CMP gel was then incubated at 4°C overnight. For CD measurements on PEGDA-CMP gels photopolymerized under cold conditions, the precursor solution was first incubated at 4°C in the cuvette prior to UV light exposure for photocrosslinking. The CD spectra were recorded on a JASCO 710 spectrometer with JASCO PTC-348 WI temperature controller (Easton, MD). The CD thermal melting curves were generated by monitoring the triple helix CD ellipticity at 231–232 nm while heating the sample from 5°C to 90°C at a rate of 0.5°C min^{-1}. The T_m of CMPs was assigned to the minimum of the first derivative of ellipticity versus temperature curve.[29]

CMP-RGD binding and release study

After the photopolymerization, PEGDA-CMP gels were transferred to 24-well plates, covered with 500 μL PBS, and incubated at 37°C. CF-CMP-RGD solutions (761, 152, and 76 μM) in PBS were heated at 80°C for 30 min. After removing excess PBS coverage from the wells containing PEGDA-CMP gels, 500 μL of the 80°C CF-CMP-RGD solution was added to each well. The gels were incubated overnight at 37°C to allow CMP-scaffold triple helical hybridization. Gels were then washed with PBS (1 mL ×10) at room temperature to remove unbound CF-CMP-RGD. Subsequently, 500 μL of fresh PBS were added to the gels, and samples were returned to 37°C incubation. In the following days, for CF-CMP-RGD binding and release study, the PBS solution was removed from the well before each fluorescence measurement and replaced with fresh PBS solution. The amount of bound CF-CMP-RGD peptide was determined by measuring the CF absorbance at 493 nm with a Tecan Infinite 200 plate reader (Männedorf, Switzerland). The absorbance readings were converted into moles of peptide using a standard curve generated from absorbance values of a series of solutions with known CF-CMP-RGD concentration.

Cell culture

Human dermal neonatal fibroblasts were cultured in 1:1 DMEM/F12+GlutaMAX supplemented with 10% fetal bovine serum (FBS), amphotericin B, and gentamicin from Lonza (Walkersville, MD). HUVECs were cultured in a complete endothelial-cell growth medium (EGM-2). All cells were grown at 37°C with a fully humidified atmosphere and 5% CO_2. The growth media were changed every two days. For scaffold seeding experiments, passage 3–7 cells were harvested by treating the culture plate with 0.05% trypsin / 0.53 mM ethylenediaminetetraacetic acid (EDTA) in HBSS (Mediatech, Manassas, VA).

Cell seeding onto CMP-RGD modified PEGDA-CMP scaffolds

PEGDA-CMP precursor solutions were created under sterile conditions in the cell culture hood and photopolymerized using the previously described protocol. The synthesized gels were placed into a 24-well plate, covered with 500 μL PBS, and kept in 37°C incubator. Next, CMP-RGD solutions in PBS were heated at 80°C for 30 min. The PBS was removed from the wells containing the PEGDA-CMP gels and replaced with 500 μL of 80°C CMP-RGD peptides. The modified gels were then placed in the 37°C incubator overnight. The next day, CMP-RGD solutions were removed from the wells, and gels were washed with PBS to remove unbound peptides. Subsequently, fibroblasts were seeded onto scaffolds by adding 750 μL of media (without FBS) containing 50,000 cells mL^{-1} into the wells. All phase-contrast images of seeded cells were taken using an EVOS phase-contrast microscope (Advanced Microscopy Group, Botwell, WA)

Cell proliferation assays

Twenty four hours after fibroblast seeding, 50 μL of WST-1 reagent was added to each well and incubated for 2 h at 37°C. Afterwards, the formazan dye absorbance generated from metabolically-active cells was measured at 440 nm. Each modified scaffold condition was tested in triplicate, and two-tailed unpaired Student's t tests were conducted to determine the statistical significance of differences in cell proliferation.

Quantification of cell adhesion and morphology

Twenty four hours after seeding, fibroblasts were fixed with 200 μL of 10% neutral buffered formalin for 90 min. Fixed cells were then permeabilized and blocked with 200 μL TBS supplemented with 0.25% Triton X-100 and 5% donkey serum. The cells were then stained overnight with 200 μL of PBS solution containing DAPI (1 μg mL^{-1}) and phalloidin (0.165 μM). After washing the wells with TBS, fluorescent cell images were captured on a Nikon Eclipse TE2000-E (Nikon Instruments, Melville, NY). Each sample type was tested in triplicate, and fluorescent images from at least 5 regions were randomly taken from each scaffold. A customized pipeline from CellProfiler[30] software was used to analyze cell adhesion and morphology from each fluorescent image (1000×1000 pixels) corresponding to 0.41 mm^2 area. The mean of values from triplicate gels was plotted in a cell population vs area histogram for each condition.

Spatial modification of PEGDA-CMP with CF-CMP-RGD

For spatially controlled modification of PEGDA-CMP scaffolds with CF-CMP-RGD, 650 μL of 80°C CF-CMP-RGD solution (1 mM in PBS) was added to a dipping container. PEGDA-CMP gels were then partially submerged in the hot CF-CMP-RGD solution and incubated at 37° for 2 h to allow CF-CMP-RGD to bind to the exposed CMP in the gel. Gels were then removed from the dipping solutions and allowed to cool at room temperature for 30 min before transferring to a 24-well plate. Gels were covered with 500 μL of PBS and kept at room temperature overnight. The next day, gels were washed with PBS, a procedure

that mimics the cell seeding conditions, and CF-CMP-RGD absorbance at 493 nm was measured by scanning across the gel surface with a plate reader.

Studying morphology of cells on PEGDA-CMP hydrogel with CMP-RGD gradient

PEGDA-CMP gels were synthesized under sterile conditions in the cell culture hood. These gels were then partially submerged into dipping containers filled with 80°C CMP-RGD solutions (2.5 mM in PBS) and incubated at 37°C for 2 h. The gels were then removed from the dipping solution, allowed to cool to room temperature in the cell culture hood for 30 min, and transferred into a 24-well plate. Gels were covered with 500 μL PBS and stored at room temperature overnight. To prepare for cell seeding, gels were washed with fresh PBS to remove unbound CMP-RGD. Fibroblasts (50,000 cells mL^{-1} in 750 μL media without FBS) were added to the wells containing the modified PEGDA-CMP scaffolds. After 24 h, the cells were fixed, permeabilized, blocked, and stained using the previously described protocol. Fluorescent images were taken of the stained cells, capturing 9 or more images each from of the submerged, middle, and unsubmerged regions of the gels. These data were averaged to create histograms of cell frequency vs area for the corresponding three regions of the scaffolds featuring CMP-RGD gradient.

Creating multiple peptide gradients across PEGDA-CMP scaffolds

PEGDA-CMP scaffolds were photopolymerized from PEGDA, Acrl-PEG-CMP, and Irgacure 2959 precursor solutions in DI H_2O as described above. These gels were then partially submerged in 700 μL rhodamine-CMP-RGD (1 mM in DI H_2O) at 80°C and incubated for 1 h at 37°C. The rhodamine-CMP-RGD modified gels were removed and allowed to cool at room temperature for 30 min. A quenched CF-CMP-RGD solution was prepared by heating a stock CF-CMP-RGD solution (175 μL at 4 mM in DI H_2O) to 80°C and diluting it into room temperature DI H_2O (525 μL) to generate 700 μL of quenched single-stranded CF-CMP-RGD at 1 mM concentration. This quenched CF-CMP-RGD solution was pipetted into the dipping container. To create PEGDA-CMP gels with opposing peptide gradients, the gels were inserted into the quenched CF-CMP-RGD in the dipping container in the opposite gel orientation compared to the rhodamine-CMP-RGD exposure. To create gels with quadrants of peptide modification, the gels were inserted into CF-CMP-RGD at 90° rotation from the rhodamine-CMP-RGD dipping orientation. After 37°C incubation for 1 h, the multi-peptide treated gels were removed from CF-CMP-RGD solution and cooled to room temperature for 30 min. The gels were transferred to a 24-well plate with 500 μL DI H_2O and incubated overnight at room temperature. The gels were rinsed with DI H_2O, and the local CF-CMP-RGD absorbance was measured at 493 nm using a plate reader. For absorbance measurements of rhodamine B, the gel coverage solution was replaced with 500 μL of 0.1% acetic acid in DI H_2O, and the absorbance was read at 564 nm. The resulting data were presented as average values with the standard error of the mean (SEM).

Results and discussion

Design and Synthesis of Photocrosslinkable Collagen Mimetic Peptides and PEGDA-CMP Hydrogel

Photopolymerization of PEG diacrylate (PEGDA) to generate a covalently crosslinked biocompatible hydrogel is a well-established technique.[31] The photopolymerization, which involves photoinitiation followed by radical polymerization, occurs in minutes even under hot temperature (80°C). In order to incorporate CMP covalently into the PEGDA network, we designed polymerizable CMP in the form of acrylamide-PEG-CMP that can be copolymerized with PEGDA. Since the photopolymerization process is quick and can be performed at a moderately high temperature, the precursor solution can be heated prior to

the photopolymerization to ensure that the CMPs are incorporated into the PEGDA network in the form of melted single strands.

Despite previous success with $(POG)_7$, attempts to conjugate CMP of sequence G_3-$(POG)_8$ onto commercially available acrylate-PEG-NHS resulted in low yield. This could be explained by the lower reactivity of the long CMP [$(POG)_8$] compared to a shorter peptide sequence [$(POG)_7$], which was further exacerbated by the triple helical conformation that creates steric crowding at the N-terminus. Furthermore, dialysis or size exclusion column purification techniques were ineffective due to the size similarity between the acrylate-PEG-NHS and the CMP as well as triple helical associations of unreacted CMP with PEG-CMP complexes.

We chose to generate a new type of photocrosslinkable CMPs (Acrl-PEG-CMP) entirely by solid phase peptide synthesis (Scheme 1). The G_3-$(POG)_8$ peptide sequence was prepared on a solid support resin by manual peptide synthesis. Using Fmoc chemistry, we conjugated a short discrete PEG (dPEG) spacer at the N-terminus of the peptide to provide flexibility and hydrophilicity. The dPEG spacer gives flexibility to the CMP after it is photopolymerized into the PEGDA hydrogel but is short enough to mitigate triple helical association of neighboring CMPs in the photocrosslinked gel. Finally, acrylic acid was coupled to the peptide's N-terminus to provide the photopolymerizable vinyl group. MALDI-ToF mass spectrometry indicated pure product with no side products lacking in dPEG or acrylic acid groups (Supporting Information, Figure S1).

The high reaction yield was mainly a result of the peptide's enhanced reactivity as it resides on the solid support in single-stranded form without tertiary structure. In addition, unreacted reagents can be removed by washing the resin before Acrl-PEG-CMP cleavage, eliminating the need for yield-reducing dialysis purification.

PEGDA-CMP gels were created by first generating a precursor solution in PBS with 9 wt% PEGDA (3400 Da), 1 wt% Acrl-PEG-CMP, and 0.05% photoinitiator (Irgacure 2959). The precursor solution was heated to 80°C in order to melt CMPs into single strands, loaded between two glass cover slips, and exposed to UV-light (5 mW cm^{-2} for 10 min) to form a gel. The top cover slip was lifted to reveal the PEGDA-CMP gel for further experiments. This synthesis approach of co-photopolymerizing single-stranded Acrl-PEG-CMP with PEGDA allowed maximum preservation of CMP's single-stranded form in the hydrogel, which was critical for peptides to work as available binding partners for further modification of the hydrogel.

Study of Triple Helical Structure in PEGDA-CMP Hydrogel

To investigate the tertiary structure of CMPs in the photocrosslinked hydrogel, we measured the triple helical content using circular dichroism (CD). CD melting curve of PEGDA-CMP gel precursor solution (before UV photocrosslinking) showed a clear sigmoidal melting transition with T_m at 57°C (Figure 1). In the case of PEGDA-CMP gel photocrosslinked from 80°C precursor solution, CD melting curve showed an overall reduced intensity as well as a much broader melting transition. The CD melting curve of PEGDA-CMP gel photocrosslinked from 4°C precursor solution showed a partial recovery of CD signal towards the precursor solution.

The low CD signal for the gel photocrosslinked after 80°C incubation of precursor indicates that portions of the CMPs are conjugated to the PEGDA hydrogel network in a way that does not support triple helix assembly with other CMPs in the hydrogel. The shallow slope of this temperature-dependent CD melting curve mirrors the case of a mixture of triple helical CMPs and single-stranded peptides having non-triple helical polyproline-II structure

(Supporting Information, Figure S2). While the CD signal does show a gradual sigmoidal transition of triple helix melting, the signal's intensity and slope are tempered by the contribution from single-stranded CMPs in polyproline-II conformations whose CD signal (ellipticity at 231 nm) decreases linearly with rising temperature.[24]

The notion of a mixture of triple helical and single-stranded peptides in the PEGDA-CMP gel was further supported by studying the PEGDA-CMP samples photocrosslinked from cold (4°C) precursor solutions, which have full triple helical content at the start of gelation. The increase in CD signal and steeper sigmoidal curve suggest a higher triple helical content for these gels compared to those photopolymerized from 80°C precursors. This signal is still lower than that obtained from the precursor solution before the gelation. It seems that the heat generated during UV exposure may have melted some of the peptides during gelation. It is also possible that the polymerization/gelation processs forces imperfect triple helix registry that lower the triple helix thermal stability.

Triple helical hybridization between the CMPs in the photocrosslinked hydrogel and exogenously added CMPs can only occur if the two peptides are initially in single-stranded form. The results indicate that the CMPs are photocrosslinked into the PEGDA-CMP scaffold in single-stranded form, acting as open binding partners for bioactive CMPs that can be added to a pre-formed gel. We note that even triple helical CMPs in PEGDA-CMP can participate in binding with other CMPs if hot CMP solution is used since the hot temperature can melt the homotrimeric CMPs of the gel and allow CMP exchange for heterotrimeric triple helix formation.

Bioactive CMP-RGD Peptides and PEGDA-CMP Functionalization

For the bioactive CMP to be used for PEGDA-CMP gel functionalization, we synthesized bifunctional CMP-RGD with a sequence of $(POG)_8$-G_3-RGDSY that features a CMP sequence, $(POG)_8$, for triple helix-mediated binding to photopolymerized PEGDA-CMP and an RGDS sequence for cell adhesion. Tyrosine was added to the C-terminus for peptide quantification by UV-vis spectroscopy. The peptide was designed with the CMP sequence at the N-terminus so that after the triple helical hybridization to the PEGDA-CMP scaffold, the RGDS ligand is positioned away from the PEG backbone toward potential binding receptors (e.g. integrins on cell surface). These CMP-RGD peptides can be heated to melt into single-stranded state and then added to pre-formed PEGDA-CMP gels for triple helix-mediated conjugation. In this construct, the PEGDA-CMP system mimics the structural framework of the ECM while the CMP-RGD peptide imitates the adhesive proteins of the ECM (e.g. fibronectin) by physically binding to the scaffold and mediating cellular adhesion.

The RGDS cell adhesion sequence is one of many RGD-type sequences associated with integrin binding. Specific cell types produce the predominant cell adhesion integrins, such as $\alpha v\beta 3$, $\alpha 5\beta 1$ and $\alpha IIb\beta 3$, at different expression levels.[32] These different integrin types, in turn, exhibit varying affinities for different RGD sequences, e.g. RGDS, RGDVY, and RGDNP. For example, endothelial cells have shown enhanced attachment to $\alpha v\beta 3$ integrin selective RGD sequences, whereas fibroblasts preferably attach to $\alpha 5\beta 1$ integrin selective surface.[33] We chose to use an RGDS adhesion sequence because it shows intermediate affinity to both $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins and therefore provides some flexibility in the cell types to be used for adhesion studies.[34]

To investigate the CMP-RGD peptide binding to the PEGDA-CMP scaffold, the peptide's N-terminus was labeled with carboxyfluorescein (CF-CMP-RGD). CD studies showed that this peptide forms a stable triple helix with T_m = 57°C and was expected to form stable triple helices with photocrosslinked PEGDA-CMP at cell culture conditions (37°C). Hot CF-CMP-RGD solutions (80°C) were applied to photocrosslinked PEGDA-CMP gels, incubated

at 37°C overnight, and the gel was washed with PBS to remove unbound CMPs before measuring the fluorescence of the gels. The initial binding level was determined, and the cumulative release of peptides from the gels at 37°C was tracked for several days (Figure 2).

The cumulative release profile indicated sustained release behavior that correlated with the loading level. Adding more concentrated CF-CMP-RGD solution resulted in higher initial binding level. However, the percentage of added CF-CMP-RGD peptides that bound to the scaffold was decreased when the concentration of loading solution was increased. This may be due to the limited number of available Acrl-PEG-CMP binding partners in the gel. Additionally, concentrated CF-CMP-RGD peptide solutions have a greater tendency to form homotrimeric triple helices before hybridization with CMPs in the photocrosslinked gel. The initial binding assay from 761 μM CF-CMP-RGD solutions indicated 14.7 nmoles cm^{-2} of bound peptide, a density far exceeding the minimum RGD concentrations (picomole per cm^2) previously reported to promote cell adhesion *via* integrin-RGD interactions.[35,36] In contrast, peptide solutions added to PEGDA-only scaffolds did not exhibit significant binding because without the photocrosslinked CMPs in the scaffold, the bioactive peptides lack binding partners in the gel and are removed during washes.

Cell Seeding Experiments on CMP-RGD Modified PEGDA-CMP Scaffolds

To create bioactive PEGDA-CMP scaffolds, photopolymerized gels were covered with 80°C CMP-RGD peptide solutions, allowed to cool, and incubated overnight at 37°C. The modified gels were then washed with PBS to remove unbound CMP-RGD. Human dermal neonatal fibroblasts were seeded onto these scaffolds at 50,000 cells mL^{-1} in 750 μL of fetal bovine serum free media. Control samples included PEGDA-CMP gels modified with the CMP peptide lacking RGD sequence and PEGDA-only gels treated similarly with hot CMP-RGD solution.

Several hours after seeding, fibroblasts adhered and began to spread on CMP-RGD modified PEGDA-CMP scaffolds (Figure 3A and Supporting Information Figure S3). In contrast, primarily rounded cell morphology was seen on control scaffolds (Figure 3B and 3C). Cell culture experiments using human umbilical vein endothelial cells (HUVECs) showed similar results in which HUVECs only exhibited characteristic cobblestone morphology on CMP-RGD modified PEGDA-CMP scaffolds but not on control gels (Supporting Information, Figure S4). The bioactive PEGDA-CMP gels spurred fibroblast attachment and proliferation over several days and up through cell confluency (Supporting Information, Figure S5). However, cells on all control scaffolds remained rounded and began to clump together overtime as they underwent apoptosis.

The viability of cells on these scaffolds was quantified by a colorimetric WST-1 proliferation assay 24 h after cell seeding (Figure 4). Fibroblasts exhibited markedly increased proliferation on scaffolds with higher CMP-RGD concentrations. Control samples of unmodified PEGDA-CMP and PEGDA-CMP modified with CMPs without an RGD sequence retained the lowest cell viability. The PEGDA-only scaffold modified with CMP-RGD (761 μM) also showed significantly lower cell viability than the PEGDA-CMP modified with CMP-RGD (761 μM) ($p < 0.005$). These findings clearly indicate that cells require both the cell-adhesive capacity of the CMP-RGD ligand and the CMP-CMP hybridization partners that immobilize the RGD units to the PEGDA scaffold to mediate lasting cell adhesion and proliferation.

The cell morphology in response to CMP-RGD concentration was further quantified by image analysis. Fibroblasts cultured on PEGDA-CMP hydrogels were fixed and treated with DAPI and phalloidin for nuclei and F-actin staining, respectively. We took fluorescent images of these cells on scaffold surfaces and conducted image analysis to quantify the cell

coverage area using CellProfiler software. As shown in the cell area histograms of Figure 5, use of higher concentrations of CMP-RGD resulted in higher cell adhesion and average cell coverage area on the PEGDA-CMP scaffolds. In contrast, the control scaffolds showed fewer adhered cells and very low cell coverage area. This study demonstrates that the base PEGDA-CMP scaffold can be encoded with varying concentrations of cell-instructive cues to induce a range of cell spreading morphology.

Spatially-Controlled Modification of PEGDA-CMP Scaffolds

Natural ECMs often exhibit spatial gradients of biochemical and mechanical signals that allow continuous transition of one tissue type to another. Such gradients can be very helpful for engineering complex tissues but are difficult to reproduce in conventional polymeric scaffolds that do not possess specific sites for scaffold modification. We created a spatial gradient of CF-CMP-RGD in PEGDA-CMP gels and quantified it by mapping CF absorbance across the gel (Figure 6A). The gradient was achieved by partially dipping PEGDA-CMP gels into a hot CMP-RGD solution and allowing the peptide to diffuse into the gel and hybridize with CMPs of the PEGDA-CMP scaffold (Supporting Information, Figure S6). Fibroblasts were seeded onto these gels, and the cellular morphology across the scaffold was quantified using the CellProfiler image analysis as described above. The spatial transition of the cell morphology clearly mirrored the spatial gradient of the bioactive peptides on the gel (Supporting Information, Figure S7). Specifically, we saw highest number of adhered cells with well-spread morphology at the area of the scaffold with highest CF-CMP-RGD concentration. Moving from the submerged regions (high CF-CMP-RGD) towards the unsubmerged regions (low CF-CMP-RGD) of the scaffold, the fibroblast adhesion and spreading gradually decreased (Figure 6B). These results demonstrate that the CMP-based modification system can be used to develop biomaterials with spatial gradients of cell-instructive cues that continuously influence cell-scaffold interactions.

The CMP mediated gradient formation in PEGDA-CMP hydrogel can be further developed to produce spatial patterns and gradients of more than one component. We created PEGDA-CMP hydrogels modified with two opposing CMP gradients by dipping one side of the hydrogel in 80°C rhodamine-CMP-RGD solution followed by dipping the opposing side in a quenched CF-CMP-RGD solution. The second solution was quickly quenched from 80°C just prior to the hydrogel treatment to ensure that the CMPs that were already bound by prior treatment do not thermally melt and release from the hydrogel. Previously, we reported successful modification of collagen scaffold with quenched CMP solution by taking advantage of the slow the folding rate of the triple helix.[37,38] This procedure is readily applicable to modifying 3D scaffolds that contain live cells and heat sensitive bioactive molecules. Figure 7A shows the two opposing fluorescent CMP gradients in the PEGDA-CMP hydrogel across 12 mm distance. Using a similar approach of selectively exposing the hydrogel to CMP solutions, we also produced PEGDA-CMP hydrogels modified with the rhodamine tagged and CF tagged CMPs in quadrant patterns (Figure 7B).

The triple helical hybridization interactions between CMP and the PEGDA-CMP provide a convenient way to encode spatially defined bioactive signals to the biologically inert PEGDA hydrogel. Therefore, in addition to serving as scaffolds for complex tissue engineering, the PEGDA-CMP system could be used for testing spatially defined library of matrix-bound cell signals to identify concentration/composition dependent activity of insoluble signals in the ECM which are critical for differentiation and tissue development of cells with therapeutic potential (e.g. stem cells).[39,40]

Conclusions

The collagen triple helix is a distinct protein structure that is rarely found in other non-collagenous proteins.[41] By taking advantage of the structural scarcity and the stability of the triple helix, we developed a highly specific, non-covalent conjugation tool based on triple helical hybridization. We synthesized PEDGA hydrogels displaying CMPs that serve as triple helix-mediated conjugation sites by copolymerization of PEGDA with Acrl-PEG-CMP. This PEGDA-CMP hydrogel can be readily encoded with CMP conjugates simply by the addition of a solution containing single-stranded CMP. With the use of CMP-RGD conjugates, we were able to create not only individual PEGDA-CMP hydrogels that vary in RGD peptide concentration but also those with spatially defined gradients and patterns, which mimic the distribution of matrix-associated cell signaling molecules in natural ECM. The morphology of seeded fibroblasts directly indicated the level of concentration and concentration gradients of CMP-RGD bound to the PEGDA-CMP hydrogel. Others have created cell adhesive gradients by photo-patterning or by using microfluidic device and gradient makers.[42–44] Although these methods may offer better control over the composition of the spatial gradients, they are either limited to 2D surfaces or require complicated fabrication processes. We believe that the CMP hybridization approach is a simple system that is conducive to producing multiple components of gradients. Since diffusion is used to create the gradient, it can be readily extended to fabricating 3D gradient systems. This is a new approach to encoding insoluble cellular signals to polymer scaffolds which could be useful for studying cell behaviors in response to spatially displayed signals and ultimately for complex tissue engineering.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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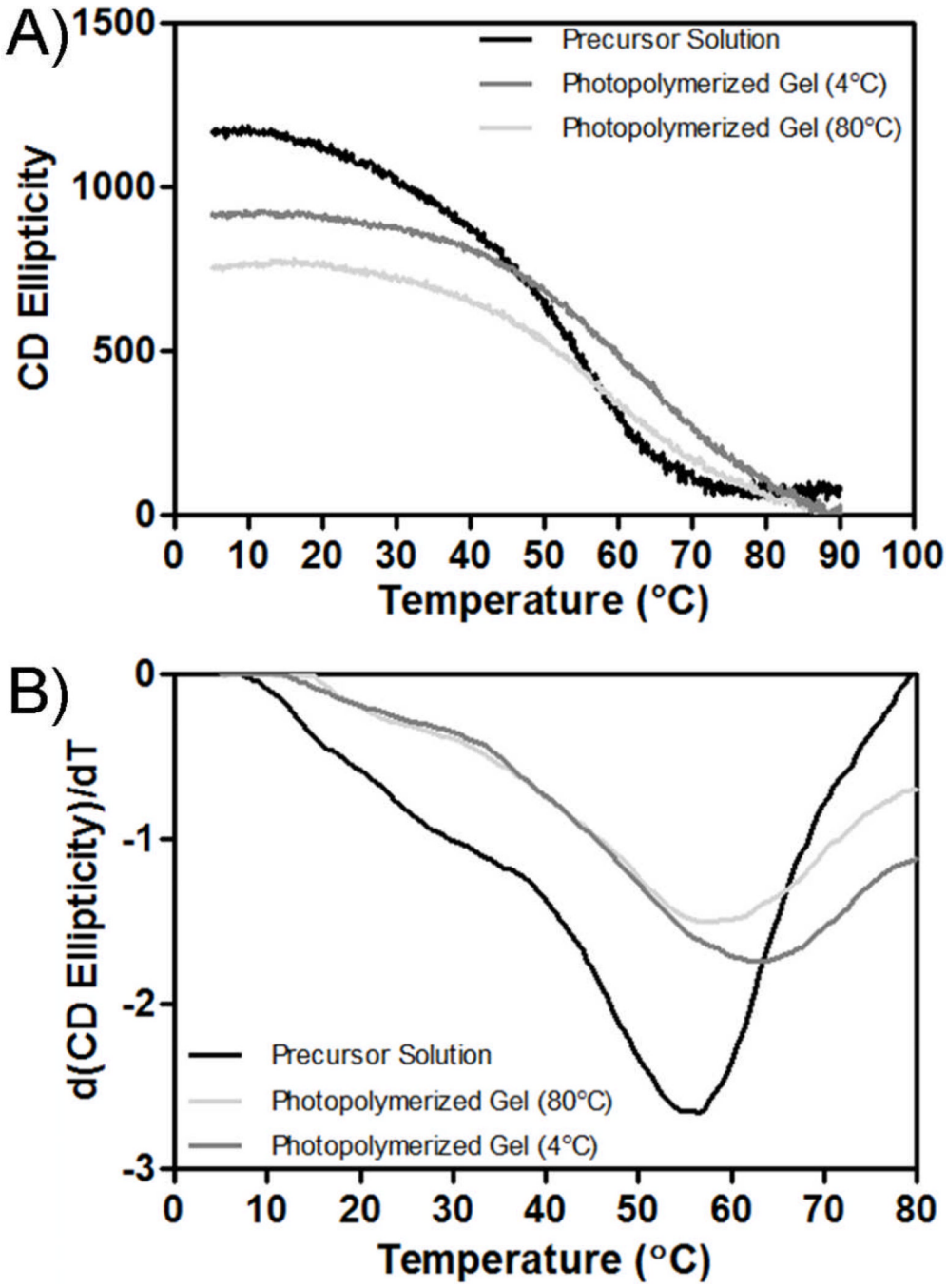


Fig. 1.

A) Circular dichroism melting curves of PEGDA-CMP precursor solution and PEGDA-CMP gel photopolymerized at 4°C and 80°C. B) First derivatives of corresponding CD melting curves.

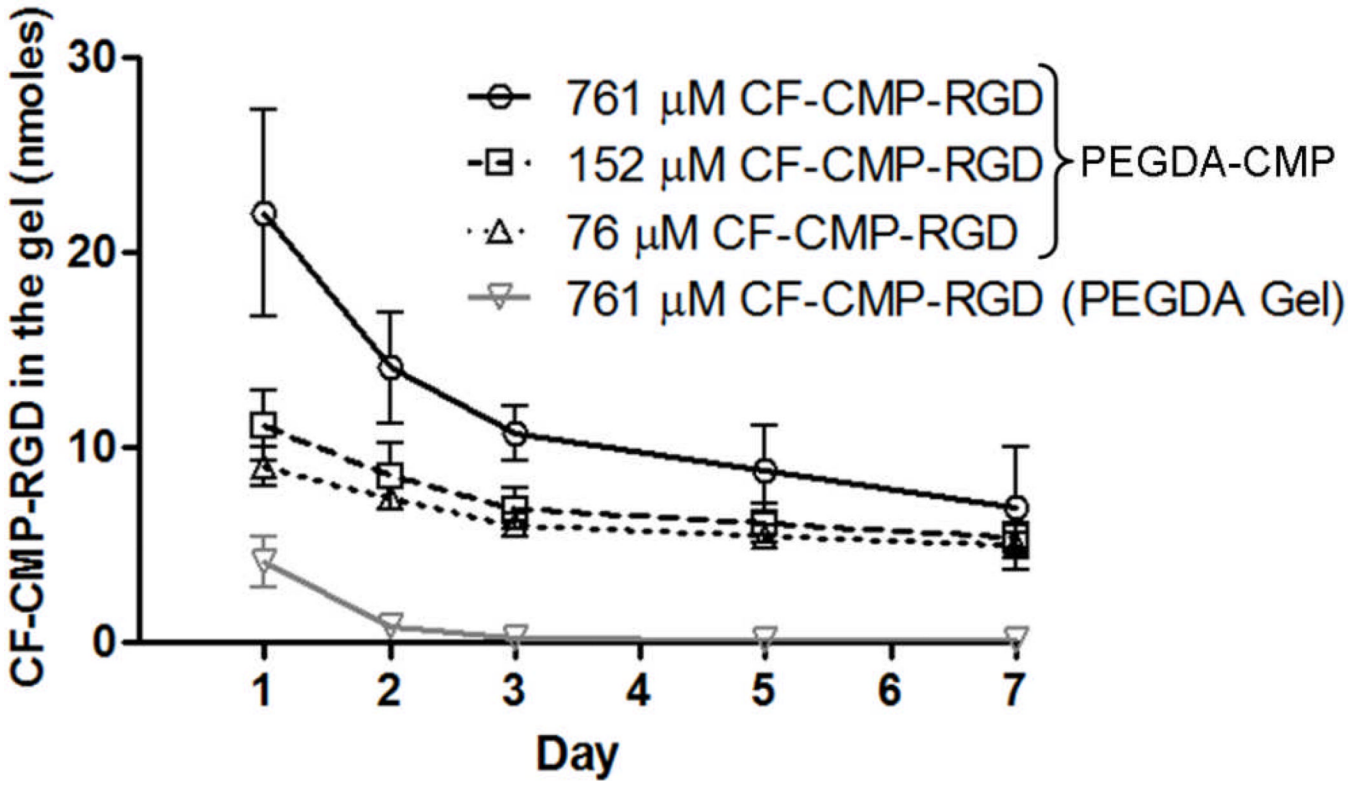


Fig. 2.

Cumulative release profiles of CF-CMP-RGD from PEGDA-CMP gels and from PEGDA-only gels. Single-stranded CF-CMP-RGD were added to gels at varying concentration and left at 37°C overnight to allow triple helix-mediated binding. The gels were washed with PBS to remove unbound peptides and their CF absorbance was monitored over the following days at 37°C. Data reported as mean ± SD.

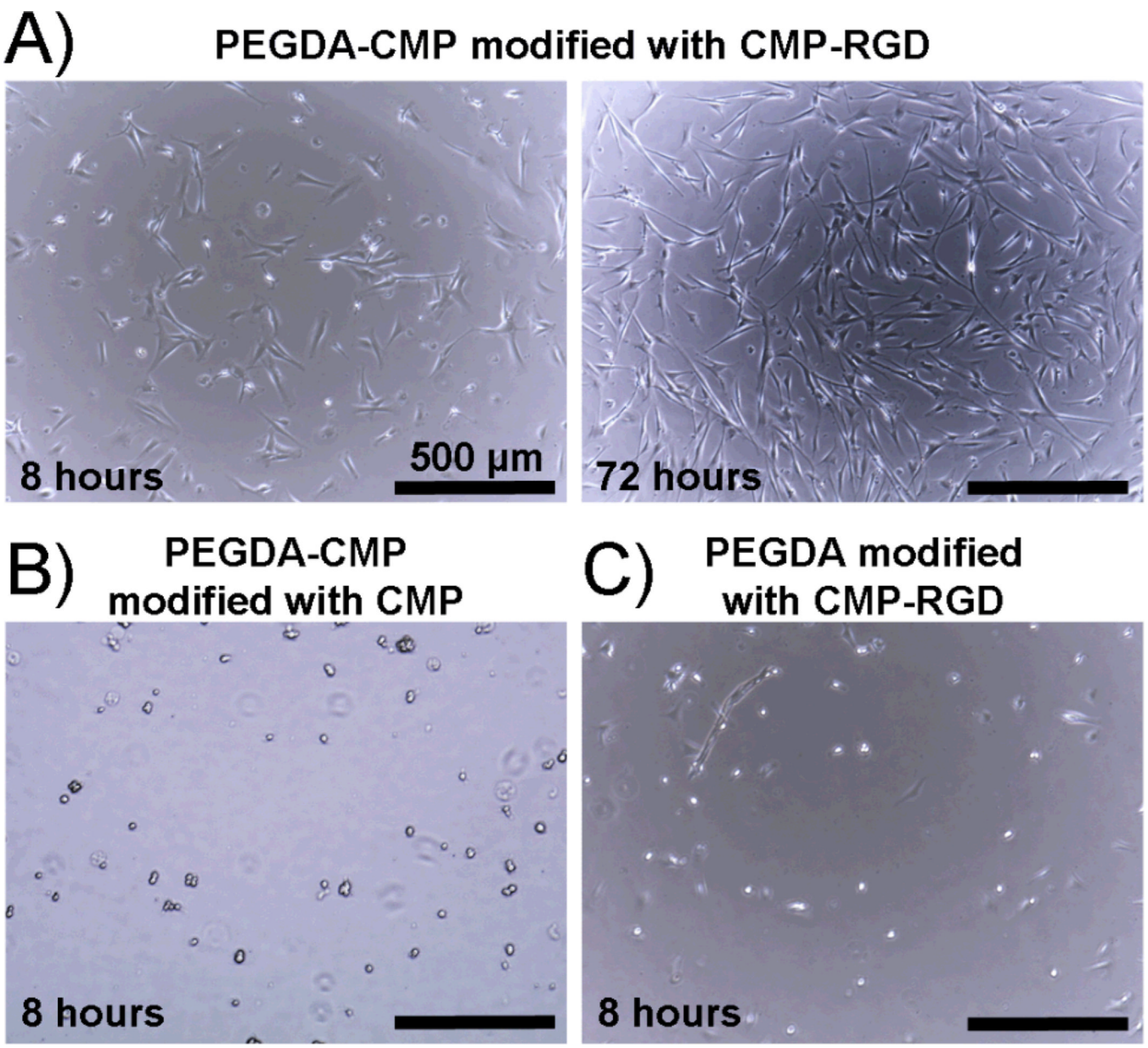


Fig. 3.
Phase contrast micrographs of human dermal neonatal fibroblasts seeded onto PEGDA-CMP scaffolds modified with CMP-RGD (A), PEGDA-CMP modified with CMP (B), and PEGDA modified with CMP-RGD (C).

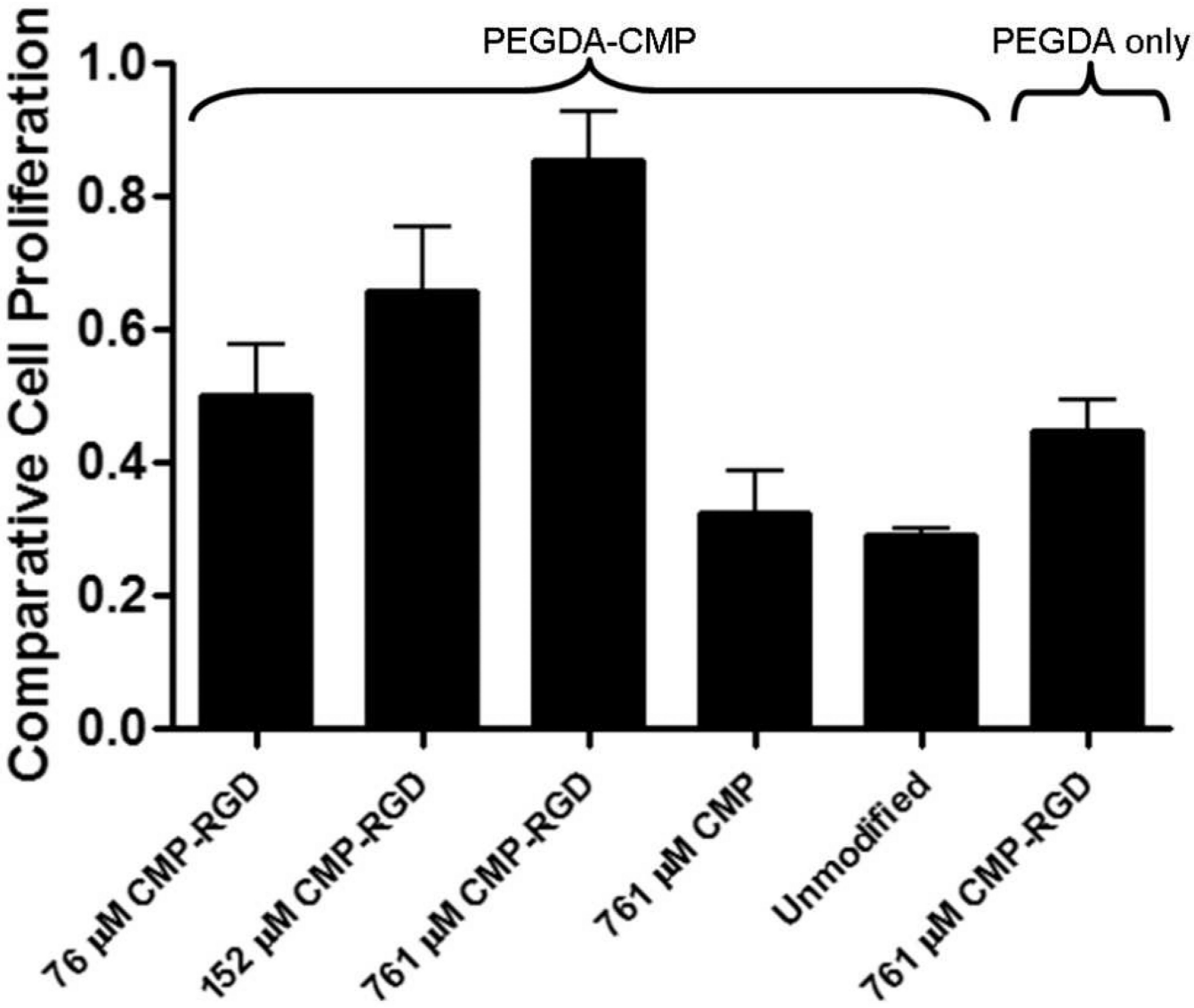


Fig. 4.

WST-1 proliferation assay (absorbance at 440 nm) of fibroblasts seeded on PEGDA-CMP modified with CMP-RGD and control hydrogels: PEGDA-CMP modified with CMP and PEGDA hydrogel (without CMP) modified with CMP-RGD. CMP-RGD modified PEGDA-CMP scaffolds showed higher cell proliferation compared to control samples (Student's *t* test, $p < 0.005$). Data reported as mean ± SD.

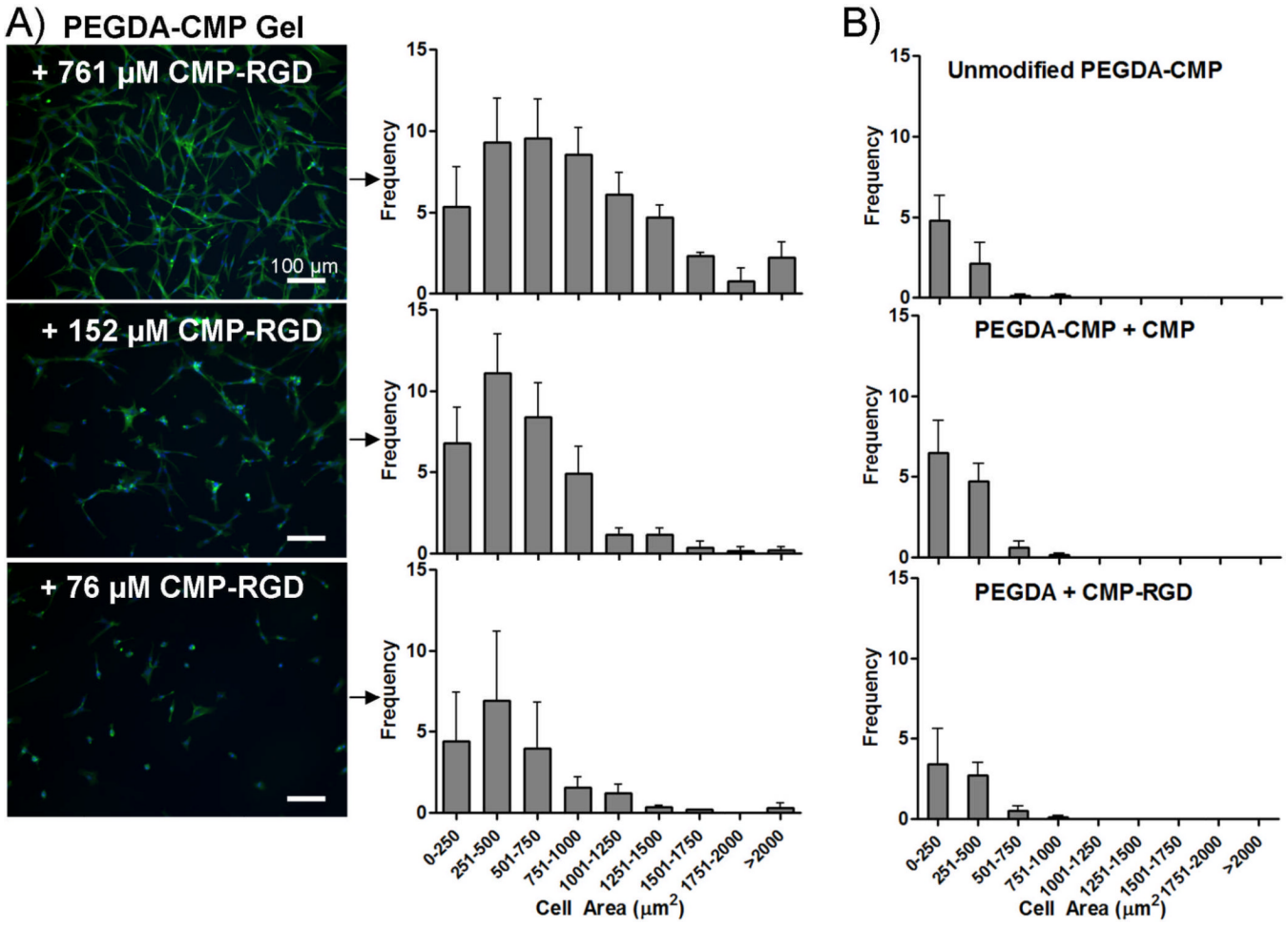


Fig. 5.

A) Fluorescence micrographs and corresponding cell area histograms of fibroblasts stained with DAPI (stains nucleus in blue) and phalloidin (stains actin in green). Cells exhibited enhanced adhesion and spread morphology on PEGDA-CMP scaffolds modified with increasing concentrations of CMP-RGD. B) Cell area histograms for cells seeded on control scaffolds showing reduced cell adhesion. Data reported as mean ± SD.

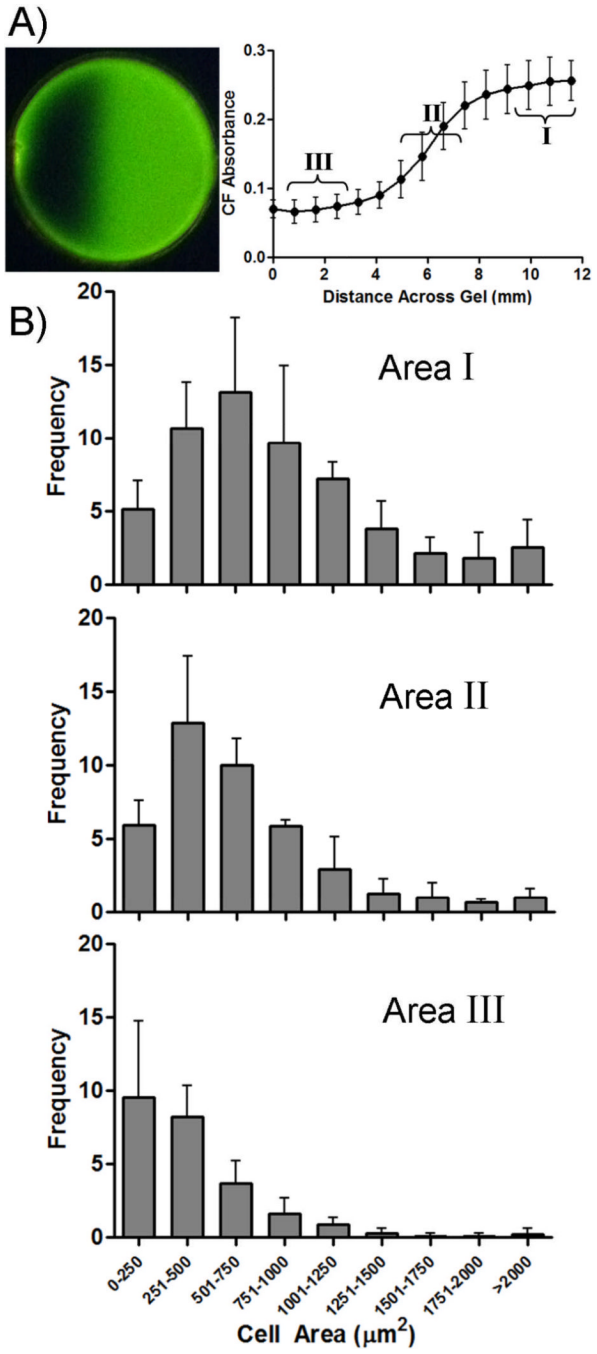


Fig. 6.

A) Image of PEGDA-CMP gel partially treated with CF-CMP-RGD and CF absorbance profile across the gel. B) Cell area histograms (fibroblasts after 24 h culture) corresponding to the three areas defined in A demonstrating modulation of cell morphology across PEGDA-CMP scaffolds with RGD gradient formed by triple helical CMP association. Data reported as mean ± SD.

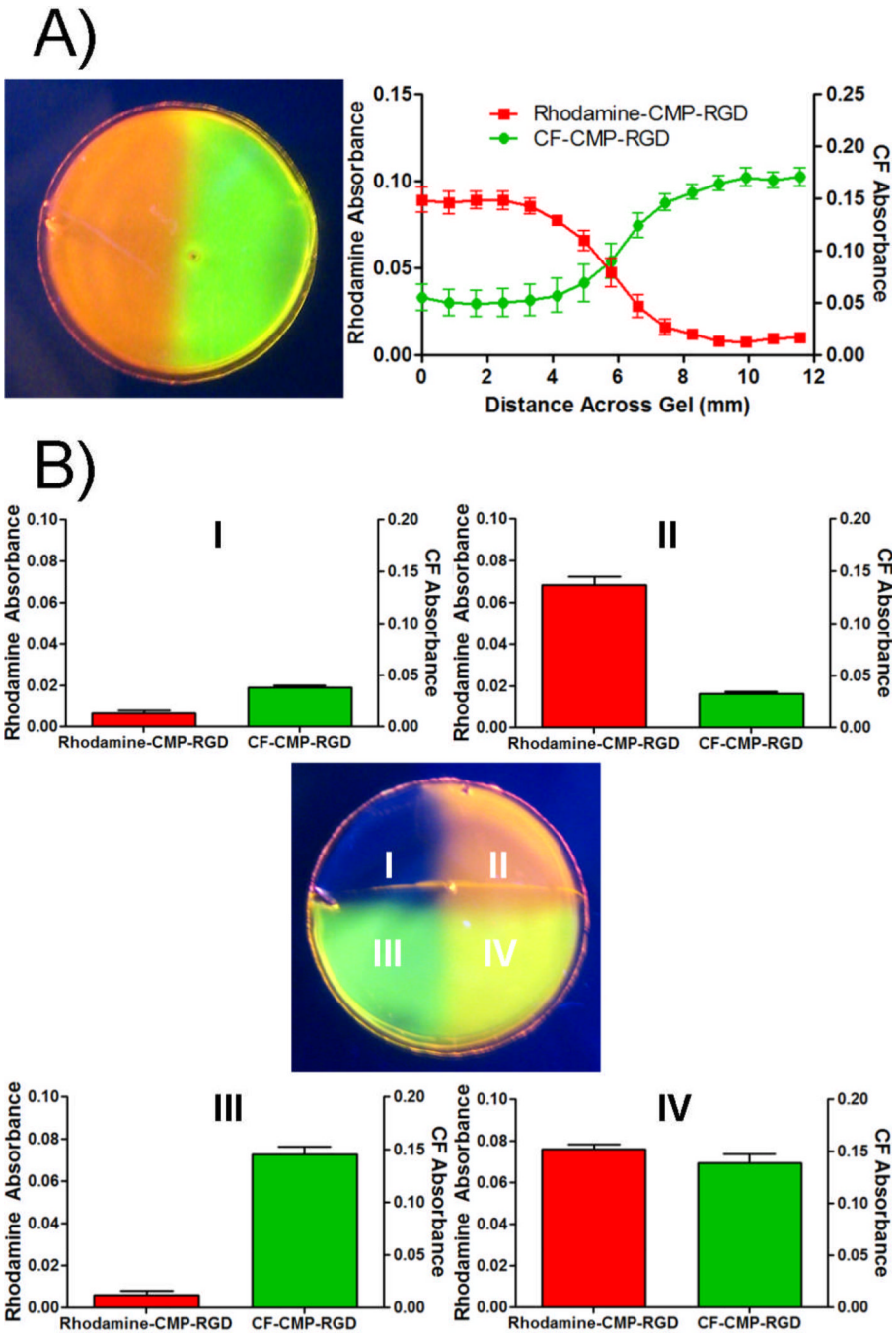
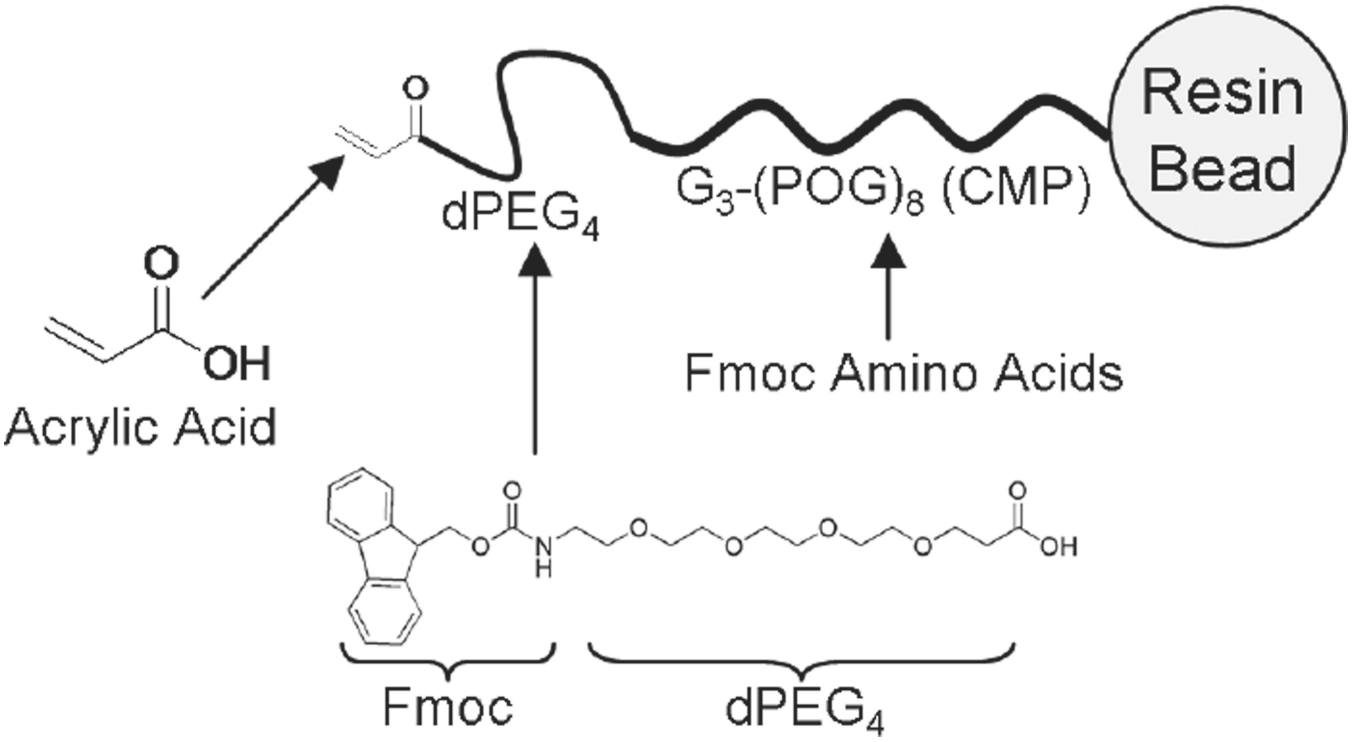


Fig. 7.

A) PEGDA-CMP gel modified with rhodamine (red) and CF (green) labeled CMP-RGD. Local absorbance measurements for rhodamine (564 nm) and CF (493 nm) labeled peptides show opposing gradients across the gel. B) PEGDA-CMP scaffolds modified into quadrant patterns with rhodamine and CF labeled peptides. Data reported as mean ± SEM ($n \geq 3$).



Scheme 1.
Design of photocrosslinkable Acrl-PEG-CMP peptide prepared by solid-phase peptide synthesis.