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Poly(ethylene glycol) hydrogels conjugated with a collagenase-sensitive fluorogenic substrate to visualize collagenase activity during three-dimensional cell migration

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Abstract

We have developed collagenase-sensitive hydrogels by incorporating a collagenase-sensitive fluorogenic substrate (CS-FS) within the backbone of a polyethylene glycol (PEG) copolymer to visualize collagenase activity during three-dimensional cell migration. CS-FS was synthesized by conjugating Bodipy dyes to a peptide with collagenase-sensitive sequence, Leu-Gly-Pro-Ala (LGPA), and the products were grafted into the collagenase-sensitive PEG hydrogels. CS-FS both in solution and hydrogels had an increase in the fluorescence intensity after proteolytic degradation by collagenase, but not by non-targeted proteases nor in the absence of an enzyme. Fibroblasts inside the hydrogels conjugated with CS-FS spread and extended lamellipodia in three dimensions over several days, and their pericellular collagenase-mediated proteolysis of the hydrogel was visualized via confocal microscopy. A matrix metalloproteinase inhibitor, served as a negative control, significantly reduced the degradation rate of CS-FS by collagenase and prevented cell migration and cell-mediated collagenase activity inside these hydrogels. In summary, we have fabricated collagenase-sensitive hydrogels incorporated with CS-FS and successfully visualized the collagenase activity during three-dimensional cell migration.

Keywords: Hydrogel; Polyethylene oxide; Fluorescence; Peptide

1. Introduction

Cell migration plays a key role in biological events such as in morphogenesis, wound healing, tumor metastasis and inflammation [1]. Cellular migratory behavior is closely regulated by two intertwined cellular functions: binding of the cell surface adhesion receptors and degradation of the extracellular matrix (ECM) by proteases [2,3]. Proteolytic activity during cell migration aids cell detachment and creates migratory pathways by degrading and remodeling ECM [4]. Then the cell surface adhesion receptors bind to the ECM domains, and the cell body is pulled forward.

Classes of proteinases known to be involved in cell migration are cysteine proteinases, serine proteinases, and matrix metalloproteinases (MMPs) [5]. Considered a major determinant of cell migration, MMPs are a family of extracellular zinc dependent neutral endoproteinases capable of degrading ECM proteins such as collagen, laminin and fibronectin [6].

Recently, molecular imaging of MMP activity by various fluorogenic substrates has been utilized to delineate the role and regulation of proteolysis by MMPs [7,8]. For this purpose, protein-based fluorogenic substrates such as dye-quenched bovine serum albumin (DQ-BSA) and dye-quenched collagen (DQ-collagen) have been used. These proteins utilize the concept of self-quenching, a phenomenon that occurs when certain fluorophores such as fluorescein and Bodipy dyes are in close proximity to each other. Fluorescence of Bodipy dyes bound to these proteins is highly self-quenched due to several fluorescent dye molecules closely conjugated to each protein. As in fluorescence resonance energy transfer (FRET), intramolecular self-quenching is inversely related to the distance

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between the fluorophores; after degradation of BSA or collagen by proteases, Bodipy dyes are separated in distance, effectively abolishing the self-quenching effect, and the degraded fragments generate strong fluorescence upon excitation. Petty et al. used gelatin gels mixed with the DQ-BSA substrate and dihydrotetramethylrosamine to visualize proteolytic activity during neutrophil locomotion over a period of several minutes. Migrating neutrophils on gelatin gel showed alternating fluorescent pattern of proteolytic and oxidative function [9]. Sameni et al. also used DQ-BSA substrate mixed into gelatin to image proteolysis by living breast cancer cells. They demonstrated that depending on the type of living human breast cancer cells, different mode of proteolysis is utilized. BT20 and BT549 breast cancer cells degraded DQ-BSA substrates pericellularly and intracellularly, respectively [10]. In addition, DQ-collagen type IV was used to investigate pericellular and intracellular proteolysis of tumor cells and analyze the contribution of cathepsin B activity on capillary formation by endothelial cells [11,12]. However, these fluorogenic substrates are proteins that can be degraded by a wide range of enzymes. Thus, while fluorescent products indicate the presence of active proteases, these substrates lack the specificity to delineate the identities and relative activities of each protease secreted by the cells. Additionally, diffusion of the degraded substrates over time not only resulted in blurry fluorescence images but also limited their usage to relatively short term studies up to 48 h [7,10].

In this study, a collgenase-sensitive peptide, Leu-Gly-Pro-Ala (LGPA), with cleavage site between leucine and glycine, was incorporated into a polymer material to render the scaffold material biodegradable. We have previously reported the development of collagenase-sensitive PEG hydrogels incorporating the LGPA sequence into the hydrogel backbone, which was susceptible only to collagenases [13,14]. Due to the increased specificity shown by this peptide compared to larger protein substrates, we sought to modify the LGPA sequence with multiple fluorescent dyes to generate self-quenched fluorogenic materials. In addition to the increased proteolytic specificity, the peptide-based fluorogenic substrates will have more easily controllable degradation properties. Moreover, simply by changing the amino acid sequence and the conjugated fluorophores, degradation specificity and fluorescence characteristics can be tuned to track many different proteases simultaneously. In this study, the GGCLGPACGK peptide was modified with Bodipy dyes to generate a self-quenched fluorogenic substrate. The peptide was further modified with acrylated PEG chains and covalently incorporated into hydrogels. This system allowed us to investigate the activity of cellular collagenases during cell migration. The results of this study suggest that by labeling proteolytically degradable peptides targeted toward different enzymes with different fluorophores, it should be possible to create materials where the activity of multiple proteases can be monitored simultaneously during cell migration.

2. Materials and methods

2.1. Synthesis of the degradable multi-block (GGGLGPAGGK-PEG) and PEG-RGDS conjugates

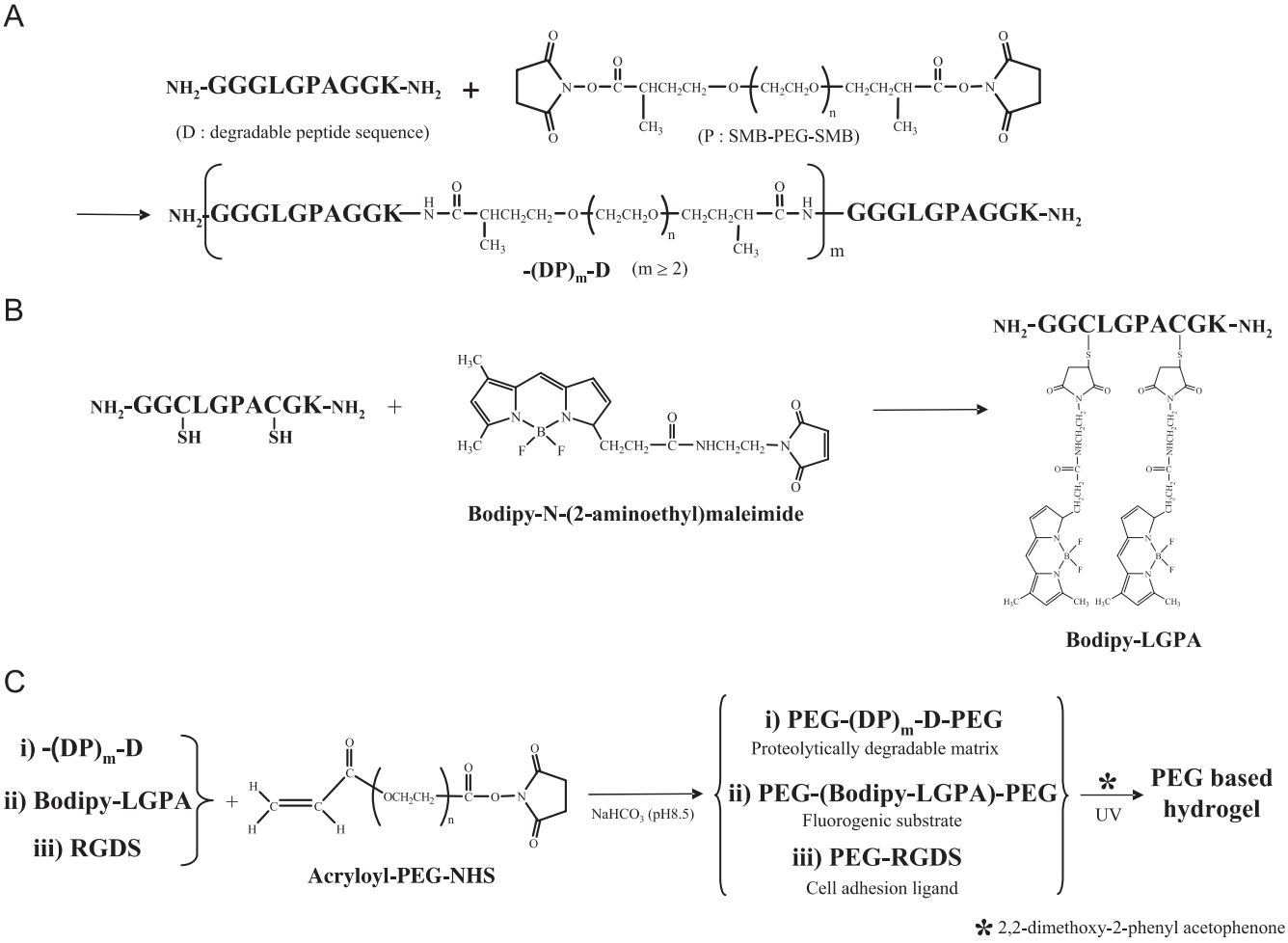
The collagenase-sensitive degradable peptide sequence GGGLGPAGGK was synthesized on an APEX 396 peptide synthesizer (Aapptec, Louisville, KY). This peptide has been shown to be degraded by collagenases [13,15]. Following purification, the reaction was completed in a stepwise manner in 50 mM sodium bicarbonate buffer solution (pH 8.5) at room temperature (Scheme 1A). First, the peptide was reacted with succinimidyl α-methylbutanoate-PEG-succinimidyl α-methylbutanoate (SMB-PEG-SMB, 3400Da; Nektar, Huntsville, AL) in a 2:1 (PEG:peptide) molar ratio, and then with a 2-fold excess of additional peptide. After the reaction, the product was dialyzed (MWCO 10,000; Spectrum Laboratories Inc., Rancho Dominguez, CA) to remove unreacted peptide and PEG moieties. The resulting product was reacted with acrylate-PEG-*N*-hydroxysuccinimide (acrylate-PEG-NHS, 3400Da; Nektar, Huntsville, AL) to introduce crosslinkable acrylate functional groups at both ends (Scheme 1C). The product (acrylate-PEG-(GGGLGPAGGK-PEG)$_n$-acrylate, $n \geqslant 3$) was dialyzed, lyophilized, and stored frozen under argon until use. The cell adhesive peptide RGDS (Arg-Gly-Asp-Ser, American Peptide, Sunnyvale, CA) was also reacted with acrylate-PEG-NHS in a 1:1 molar ratio as described above to give PEG-RGDS. The products were analyzed by ^{1}H-NMR (Advance 400, Bruker, Germany) and gel permeation chromatography (GPC; Polymer Laboratories, Amherst, MA) with UV/Vis and evaporative light scattering detectors.

2.2. Synthesis of the PEG-GGC(Bodipy)LGPAC(Bodipy)GK-PEG (CS-FS-PEG) fluorogenic peptide substrate

The peptide GGCLGPACGGK was dissolved in *N,N*-dimethyl formamide (DMF) with a 5-fold molar excess of tris-(2-carboxyethyl)phosphine, hydrochloride (TCEP, Molecular Probes, Eugene, OR) and then reacted with a two-fold molar excess of Bodipy *N*-(2-aminoethyl)maleimide (Molecular Probes, Eugene, OR) in DMF for 2 h at room temperature (Scheme 1B). After the reaction, the sample was precipitated into diethyl ether and dried. The unbound Bodipy fluorophore and unreacted peptide were removed using a Sephadex G-25 fine chromatography column (Amersham Bioscience, Uppsala, Sweden) and dialysis (MWCO 1000). The acquired product was then conjugated to acrylate-PEG-NHS as described above (Scheme 1C). ^{1}H-NMR, FT-IR (660 plus, JASCO, Japan), reverse-phase HPLC (Varian Inc., Walnut Creek, CA) and GPC were used to analyze peptides and to confirm the conjugation of products.

2.3. Proteolytic degradation of collagenase-sensitive PEG hydrogels

One hundred microliter of crosslinkable multi-block (acrylate-PEG-(GGGLGPAGGK-PEG)$_n$-acrylate, $n \geqslant 3$) solution (0.1 g/mL) in 10 mM HEPES buffered saline (HBS, pH 7.4) was filtered (MILLEX GP filter, 0.22 μm PES membrane, Millpore Corporation Bedford, MA) and then mixed with 10 μL/mL of a photoinitiator solution (2,2-dimethoxy-2-phenyl acetophenone in *n*-vinylpyrrolidone, 300 mg/mL). The prepolymer solution was poured into a 96-well plate and photopolymerized under long-wavelength UV light (365 nm, 10 mW/cm^2). Resulting hydrogels were removed, weighed and then allowed to swell in HBS with 1 mM $CaCl_2$ and 0.2 mg/mL sodium azide at 37 °C for 24 h. Each hydrogel sample was then incubated at 37 °C with HBS, 0.2 mg/mL collagenase from *Clostridium histolyticum* (Sigma, St. Louis, MO), 0.2 mg/mL plasmin (Sigma) or 0.2 mg/mL proteinase K (Invitrogen, Carlsbad, CA). Degradation was



Scheme 1. Reaction of PEG modification (A), (C), fluorophore conjugation (B) and photopolymerization (C) for PEG hydrogel.

evaluated by monitoring changes in the wet weight of hydrogels over time. The enzyme solution was refreshed every 24 h.

2.4. Proteolytic degradation of CS-FS-PEG substrate in solution and hydrogels

For measuring the proteolytic action of CS-FS-PEG substrates, 50 μg/mL of the CS-FS-PEG was prepared in HBS with 1mM $CaCl_2$ and 0.2 mg/mL sodium azide. Throughout this study, 50 μg/mL of the CS-FS-PEG was used in the hydrogels since it was the minimum concentration necessary to detect cellular proteolytic activities using confocal microscopy as outlined later. HBS, collagenase, plasmin or proteinase K (all enzyme concentrations were 0.2 mg/mL) was added to the substrate solution, and then the fluorescence intensity of each sample was measured after incubation for 1 h at 37 °C using 495 ± 20 nm excitation and 528 ± 20 nm emission on a FLx 800 microplate fluorescence reader (Bio-Tek Instrument Inc. Winooski, VT). To inhibit collagenase activity, 100 μM of MMP inhibitor GM6001 (Chemicon International, Temecula, CA) was placed in the sample solution before adding collagenase solution. GM6001 is a synthetic polypeptide that specifically binds to the substrate binding sites of human MMP-1, -2, -3, -8 and -9, thereby inhibiting proteolytic activity [16,17]. For hydrogel assays, multi-block PEG-(GGGLGPA-GGK-PEG)$_n$-PEG hydrogels including 50 μg/mL of CS-FS-PEG were prepared by photopolymerization (Scheme 1C), allowed to swell for 24 h, and then increase in fluorescence intensity after exposure to the proteases was measured over 10 h as described previously. We have previously verified that the photopolymerization conditions do not alter Bodipy fluorescence [18].

2.5. Cell maintenance

Human dermal fibroblasts (HDFs; Clonetics, San Diego, CA) were maintained on Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS; Biowhittaker, Walkersville, MD), 2 mM L-glutamine, 500 U penicillin and 100 mg/mL streptomycin. Fibroblasts were incubated at 37 °C in 5% CO_2 environment. All experiments were conducted using fibroblasts at passages 3–6.

2.6. Preparation of hydrogels with encapsulated cells

The hydrogel precursor solution was prepared by dissolving multi-block PEG-(GGGLGPAGGK-PEG)$_n$-PEG (0.1 g/mL), PEG-RGDS (13.3 mg/mL) and CS-FS-PEG substrate (50 μg/mL) in DMEM. HDFs (300,000 cells/mL) were suspended in the resulting solution, followed by photopolymerization as described above. To inhibit collagenase activity of fibroblasts, 25 μM of MMP inhibitor GM6001 was incorporated into hydrogels during photopolymerization. In addition, in order to effectively investigate cell migration and proteolytic activity in a larger scale, we examined cell migration from cell clusters [19,20]. Briefly, fibroblasts in high concentration (1×10^5 cells) were clustered in fibrin clot prepared with 2 mg/mL of fibrinogen and 2 unit/mL of thrombin. The cell clusters were encapsulated in the hydrogels by placing the fibrin clots in the core of

the hydrogel during UV polymerization for 30 s. The hydrogels with individual HDFs or a cluster of HDFs were allowed to swell in DMEM for 24 h and incubated at 37 °C in a humidified atmosphere containing 5% CO_2, and cell migration was visualized as detailed below.

2.7. Confocal microscopy of cells within hydrogels

Differential interference contrast (DIC) and fluorescence images of cells within hydrogels were acquired with an LSM 510 META confocal microscope (Carl Zeiss Inc.; Oberkochen, Germany). The high degree of spectral overlap between the Bodipy fluorophore in CS-FS-PEG and fibroblast autofluorescence necessitated their separation by means other than physical bandpass filtering. This was achieved by spectral imaging with the META detector and linear unmixing [21]. At each time point, the "spectral fingerprints" of both CS-FS-PEG hydrogels without cells and cells in hydrogels without CS-FS-PEG were collected with the META detector in the range of measured emission wavelength (from 520 to 710 nm). The META detector was also used to collect the spectral emission of the hydrogel samples containing both cells and CS-FS-PEG. The spectral fingerprints of each fluorescent signal were used to linearly unmix the two signatures in the combined samples. Following successful unmixing, the two signals were visually distinguished by assigning pseudo-colors to the emission data: green for the Bodipy fluorescence from CS-FS-PEG and red for fibroblast autofluorescence. Thus, this unmixing technique and pseudo-color assignment allowed localization of fluorescence from each source within each image [18,21].

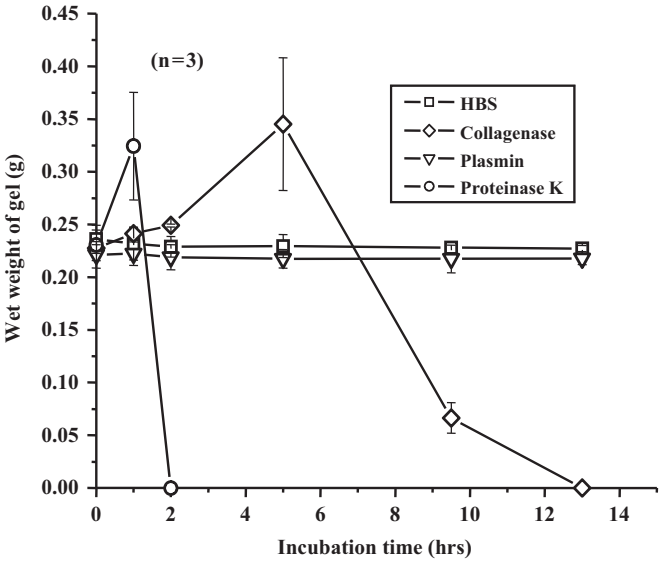


Fig. 1. Degradation profiles of GGGLGPAGGK-derivative PEG hydrogels in enzyme solution. Each hydrogel sample was swelled in HBS with 1 mM $CaCl_2$ and 0.2 mg/mL sodium azide at 37 °C for 24 h and then was incubated with 0.2 mg/mL enzyme solution at 37 °C: (□) HBS, (◇) collagenase, (▽) plasmin, (○) proteinase K.

3. Results

3.1. Characterization of PEG-modified peptide and fluorogenic substrate

^{1}H-NMR analysis demonstrated that acrylate-PEG was successfully conjugated to GGGLGPAGGK, GGCLGPAGCK, and RGDS. After purification, these PEG conjugated products showed the methylene protons of PEG as a triplet at 3.6–3.7 ppm as well as the acrylate protons at 6.0–6.5 ppm. The success of Bodipy and PEG conjugation was confirmed by FT-IR. The collagenase-sensitive fluorogenic substrate (CS-FS) conjugated to PEG (CS-FS-PEG) showed the characteristic peaks originated from Bodipy and PEG near 1700 and 2800 cm^{-1}, respectively. Furthermore, HPLC analysis showed that the peak of Bodipy conjugated peptide was located between hydrophilic peptide and hydrophobic Bodipy without unreacted residues, demonstrating the conjugation was successfully completed.

3.2. Proteolytic degradation of hydrogels

As shown previously, PEG diacrylate hydrogels did not degrade in enzymatic solution (data not shown) while incorporation of protease-specific peptide sequence renders the hydrogels degradable [13]. Degradation profiles of hydrogels derivatized with a peptide sequence GGGLGPAGGK were studied by measuring changes in the wet weight of hydrogels in enzyme solutions followed by equilibrium swelling in HBS for 24 h (Fig. 1). The incorporated sequence GGGLGPAGGK was selected as a collagenase-sensitive degradable peptide, which has a known cleavage site between leucine (L) and glycine (G) [15]. In the initial degradation phase of hydrogels, the wet weight increased as proteinases cleaved the peptides, loosening the hydrogel network and allowing more water to penetrate. Eventually, hydrogels were completely degraded as additional sequences were cleaved and degraded moieties diffused away from the hydrogel. Proteinase K served as a positive control because it is known to cleave most peptide sequences rapidly and non-specifically. As shown in Fig. 1, proteinase K showed much stronger proteolytic activity compared to collagenase and other enzymes, as evidenced by accelerated hydrogel degradation profile. Proteinase K (0.2 mg/mL) completely degraded hydrogels in 2 h whereas the same concentration of collagenase required 13 h. Meanwhile, hydrogels left in plasmin or HBS did not change in weight significantly throughout the course of the experiment as the LGPA sequence is neither sensitive to plasmin-mediated proteolysis, nor to simple hydrolysis.

3.3. Proteolytic degradation of CS-FS in solution and hydrogels

Fig. 2 shows the relative proteolytic degradation of CS-FS-PEG in solution using collagenase, plasmin and proteinase K. All fluorescence intensities were normalized to the fluorescence intensity of the untreated substrate in HBS. The fluorescence intensity of CS-FS-PEG substrate increased by approximately 3-fold in the presence of collagenase and proteinase K, but not in the same concentration of plasmin, illustrating that CS-FS-PEG has specificity toward collagenase (Fig. 2). PEG conjugation of

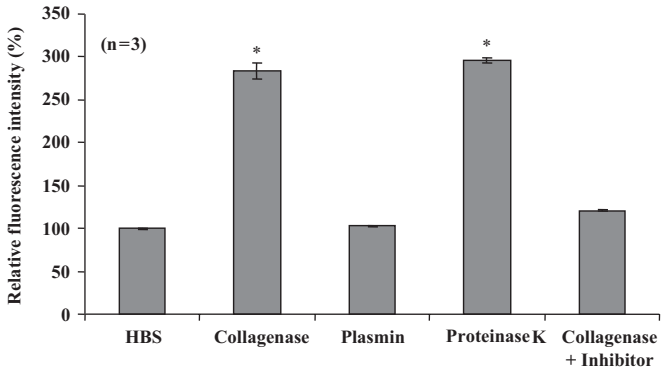


Fig. 2. The relative fluorescence intensity generated by proteolytic digestion of collagenase sensitive fluorogenic substrate (CS-FS-PEG) in solution. 50 μg/mL of the substrate was prepared in HBS with 1 mM $CaCl_2$ and 0.2 mg/mL sodium azide, and incubated with indicated enzymes at 0.2 mg/mL for 1 h at 37 °C. The fluorescence intensity of each sample was measured with 495 ± 20 nm excitation and 528 ± 20 nm emission. To inhibit collagenase activity, 100 μM of MMP inhibitor GM6001 was placed in the sample solution with collagenase. Data represent mean ± SD ($n = 3$). *$P < 0.01$, compared to the control (HBS).

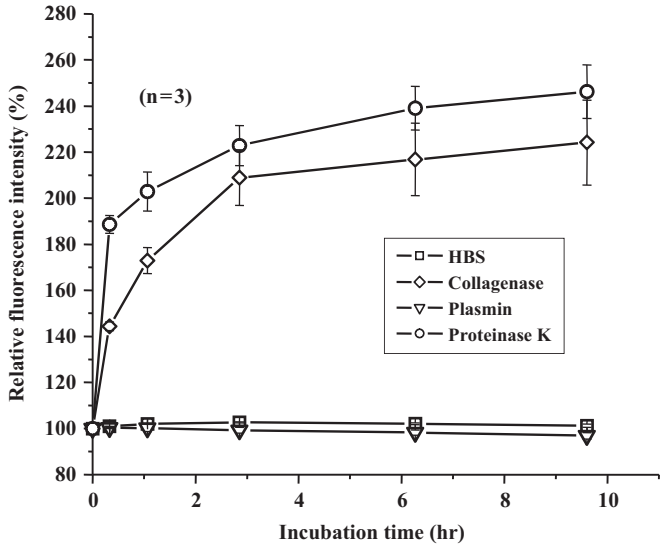


Fig. 3. The increase in fluorescence intensity with time by proteolytic digestion of degradable hydrogels containing the CS-FS-PEG substrate. Each hydrogel sample was incubated in enzyme solution (0.2 mg/mL) at 37 °C. The fluorescence intensity of each sample was measured over 10 h: (□) HBS, (◇) collagenase, (▽) plasmin, (○) proteinase K.

CS-FS did not affect degradation properties of the substrate as the identical experiment performed with CS-FS substrate without PEG conjugation resulted in the same results. The presence of an MMP inhibitor (100 μM of GM6001) significantly reduced the fluorescence intensity of CS-FS-PEG in collagenase solution, demonstrating that the collagenase indeed caused enhancement of fluorescence intensity.

Furthermore, we examined the fluorescence enhancement of CS-FS-PEG crosslinked into the backbone of hydrogels (Fig. 3). Hydrogels containing CS-FS-PEG and the degradable peptide sequence along the backbone linker (PEG-(GGGLGPAGGK-PEG)$_n$-PEG) demonstrated sensitivity to proteinase K and collagenase, but not to plasmin. Collagenase actively degraded the CS-FS-PEG substrate and hydrogel, enhancing fluorescence intensity of hydrogel as much as proteinase K (positive control). However, hydrogels incubated with plasmin did not show increase in fluorescence (Fig. 3). This result in combination with the hydrogel degradation tests (Fig. 1) demonstrates that the PEG hydrogels incorporated with the degradable peptide sequence and fluorogenic substrate are susceptible to collagenase-mediated degradation, generating fluorescence due to dequenching of the fluorophores.

3.4. Confocal imaging of fibroblasts in CS-FS-PEG substrate hydrogels

Fibroblasts were encapsulated within the degradable PEG hydrogels including CS-FS-PEG substrate and RGDS-PEG, and cellular proteolytic activity was visualized. As we investigated the fluorescence image of the samples with confocal microscope, cellular autofluorescence interfered with fluorescence generated from the fluorogenic substrate. To differentiate extracellular proteolytic activity from intracellular autofluorescence, a META detector was used to collect the spectral fingerprint of CS-FS-PEG and fibroblasts as detailed in Section 2. Immediately after encapsulation, fibroblasts exhibited a round morphology (Fig. 4A), and green fluorescence near the cells was comparable to the background (Fig. 4B). After several days, the fibroblasts inside the hydrogel extended many processes, and bright fluorescence from CS-FS-PEG was observed at the surface of cells (Fig. 4C and D), suggesting that collagenases secreted from fibroblasts were causing localized substrate and hydrogel degradation. After 7 days (Fig. 4E and F), fibroblasts inside the hydrogels exhibited lamellipodia, and bright areas of fluorescence from CS-FS-PEG continued to be seen around all extended cell processes. On the other hand, fibroblasts incubated with 25 μM of MMP inhibitor GM6001 failed to extend lamellipodia, and green fluorescence of CS-FS-PEG near the cells was comparable to the background even to day 7 (Fig. 4G and H), suggesting that the MMP inhibitor inhibited collagenase-mediated cleavage of LGPA sequence in the hydrogel backbone and CS-FS-PEG substrate.

Fibroblast migration and proteolytic activity were investigated in a larger scale using dense cluster of cells embedded in the hydrogels. Sprouts of fibroblasts radiated out from the cell clusters, and migrated into the hydrogel materials, showing bright green fluorescence along the migratory pathway up to day 7 (Fig. 4I and J). The bright green fluorescence generated by CS-FS-PEG substrate in Fig. 4J was coincident with cellular migration pathways seen in Fig. 4I. There were distinct regions with fluorescence around cell sprouts from the bottom of the cell

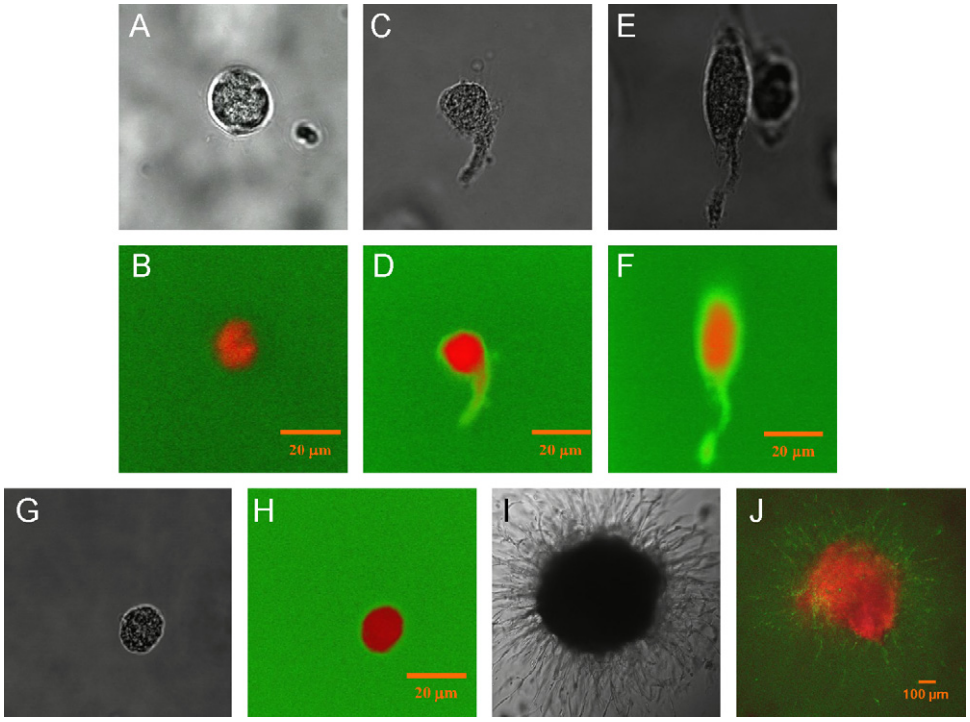


Fig. 4. The differential interference contrast (DIC) and confocal fluorescence images of fibroblasts inside degradable hydrogel containing CS-FS-PEG substrate. Fluorescence images were acquired with the META detector to collect spectral fingerprints of CS-FS-PEG and fibroblasts in the range from 520 to 710 nm. Fluorescence acquired from CS-FS-PEG substrate and autofluorescence in fibroblasts were pseudo-colored in green and red, respectively. (A), (C), (E), (G), (I) are DIC images, and (B), (D), (F), (H), (J) are optical sectioned images. DIC and fluorescence images are shown from day 0, 5, and 7 (A), (B), (C), (D), (E), (F), respectively. (G), (H) DIC and fluorescence images of fibroblasts in the presence of MMP inhibitor after 7 days. (I), (J) DIC and fluorescence images of migrating fibroblasts from cell cluster in the hydrogel after 7 days.

cluster up to the leading edge, indicating that fluorophores were indeed retained near the site of proteolytic activity rather than diffusing away into the matrix.

4. Discussion

Among several fluorogenic substrates developed to investigate and visualize proteolytic activity in vitro and in vivo, DQ-BSA and DQ-collagen have been widely used due to their drastic changes in fluorescence intensity before and after proteolytic degradation. In many studies, DQ-BSA and DQ-collagen were non-covalently mixed into gelatin or collagen to investigate proteolytic activity [7,10]. However, simple mixing of the substrates is suboptimal because the fluorescent degradation products can diffuse out of the gels resulting in blurry fluorescence images. Furthermore, since DQ-BSA and DQ-collagen lack protease-specificity, it is difficult to decipher which protease is responsible for DQ-BSA or DQ-collagen degradation [9,10,18].

In previous studies, we developed a collagenase-sensitive biomimetic hydrogel system based on PEG to investigate some of the mechanisms of cell migration. This hydrogel system was fabricated by grafting an acrylate-PEG-modified cell adhesive peptide such as RGD and incorporating a collagenase degradable peptide sequence such as LGPA into the polymer backbone [14,22,23]. In this biomimetic system, the encapsulated cells degraded the PEG hydrogels and carried out normal cellular functions such as proliferation, migration, and ECM production to begin tissue formation [24,25].

The aim of the current study was to develop a novel PEG hydrogel system incorporated with a fluorogenic substrate that will allow us to monitor the proteolytic activity during cell migration. By conjugating Bodipy dyes and acrylated PEG chains to the GGCLGPACGK sequence, we have successfully developed a CS-FS-PEG that is specifically targeted to collagenase-mediated proteolysis but not to plasmin. Covalent incorporation of CS-FS-PEG into the backbone of the polymer prevented diffusion of the fluorophores and allowed detection of proteolytic activities for at least 7 days. The presence of the same degradable LGPA sequence in the hydrogel backbone as well as in the fluorogenic substrate allows investigation of the interaction between the proteolytic activity and cell migration simultaneously. As fibroblasts extended lamellipodia and migrated inside the hydrogels containing CS-FS-PEG, strong fluorescence was detected near the cells, presumably generated by collagenases secreted by the cells. In contrast, fibroblasts in the presence of an MMP inhibitor did not extend lamellipodia nor produce stronger pericellular fluorescence compared to the background, confirming that

MMPs indeed mediate degradation of the polymer backbone as well as the fluorogenic substrate.

Focal contacts are active site of integrin ligation with ECM proteins and assembly of cytoskeletons to form stress fibers. Since cellular spreading and migration are known to be tightly regulated by focal contacts, we expected to find distinct patches of fluorescence in the cell bodies corresponding to potential sites of focal contacts. Absence of such patches of fluorescence near cells bodies as in Fig. 4D and F does not preclude localized patterns of MMP activities. In order to further resolve the spatial and temporal patterns of MMP activity, we plan to investigate MMP activity in real time with time-lapse confocal microscopy with time intervals of hours rather than days.

Fibroblasts from highly concentrated cell clusters migrated much farther in the same hydrogel materials compared to the individual cells, allowing us to investigate cell migration and proteolytic activity much more effectively (Fig. 4I and J). Presumably, high concentration of cells in clusters would secrete much more collagenases to degrade the hydrogels and generate pores for cells to migrate through in a shorter time scale compared to the individual cells.

In addition, clear visualization of the fluorophores near the site of proteolytic activity up to 7 days as seen in Fig. 4A–J demonstrates that the fluorophores are indeed retained after degradation of the substrates. This result demonstrates that covalent modification of fluorophores into hydrogels is a superior mode of introducing fluorogenic substrates to the matrix. This is a great advancement from the currently available method in which fluorogenic proteins are physically mixed into the matrix, allowing visualization of proteolytic activity in a short time window limited by diffusion of the degraded fluorogenic products.

5. Conclusions

In summary, we have developed a PEG hydrogel system incorporated with CS-FS and used this system to visualize the collagenase activity during three-dimensional cell migration. Based on these results, we believe that this hydrogel system will be a useful tool to investigate the role of collagenases in proteolysis and cell migration in two and three dimensions. We will also be able to investigate proteolytic activities of proteases other than collagenases simply by altering the target peptide sequence. We can expand this idea further by modifying the current system with multiple fluorogenic substrates that have distinctive protease-specificity and emission spectra to probe the roles and interaction between numerous proteases in the course of cell migration.

Acknowledgments

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