



# One-Pot Preparation of Alpha-Chymotrypsin Degradable Hydrogel Micropatterns for Controlled Drug Release

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## Abstract

Fluorouracil (5-FU) has been the most widely used chemotherapy agent since its clinical introduction in 1957, and it continues to form the basis of treatment for various cancers. However, due to the side effects of an antimetabolite, a strategy fulfilling stringent requirements placed on a 5-FU delivery system requires controlled and extended release of 5-FU in a localized manner. Here, an in-situ gel-forming method for the preparation of micropatterned,  $\alpha$ -chymotrypsin-degradable hydrogel (PHcd) for controlled release of 5-FU is introduced. More specifically, methacrylated hyaluronic acid (HA) and polyethylene glycol diacrylate (PEGDA), known for their excellent moisture retention capacity, are chosen for skin therapy. They are crosslinked with CYKC peptide through a thiol-ene click reaction. The synthesis of the CYKC peptide and its cleavage by  $\alpha$ -chymotrypsin were confirmed using high-performance liquid chromatography (HPLC). Additionally, the in vitro release behavior was accurately monitored using the micropatterning method, demonstrating the stable immobilization and successful sustained release of 5-FU upon the addition of  $\alpha$ -chymotrypsin in micropatterned PHcd hydrogel. Consequently, this micropatterned PHcd hydrogel can be considered a promising scaffold for localized and sustained delivery of cytotoxic drugs for skin cancer treatment.

**Keywords** 5-FU · Enzyme-degradable hydrogel · Drug delivery · Controlled release · Micropatterned hydrogel

## Introduction

Fluorouracil (5-FU) is a widely used hydrophilic anticancer drug employed in the treatment of breast, stomach, pancreatic, and colorectal cancers [1–4]. Its synergistic effects

with other anticancer drugs, such as oxaliplatin and irinotecan, have established it as a key agent in combating various malignant tumors [5–8]. Despite its broad-spectrum anti-tumor activity, 5-FU, known as an antimetabolite, can cause dermatitis, myelosuppression, diarrhea, vomiting, nausea, and gastrointestinal issues [9–11]. Consequently, systemic administration often results in undesirable outcomes like high-dosage requirements, poor bioavailability, and side effects [12, 13]. In response to these challenges associated with systemic administration, numerous therapeutic approaches have been explored to discover more efficient methods, including combination therapy, nano-formulation, and localized delivery, aiming to target the desired amount of drugs specifically to tumor sites [14–18].

In cancer resection scenarios, where the quantity of remaining cancer cells is minimal, effective removal can often be achieved by delivering a precise amount of drug [19–21]. For various skin-related lesions like malignant melanoma and breast cancer, which typically do not necessitate biopsies, 5-FU stands as a more suitable topical treatment compared to cryotherapy due to its higher success rate in long-term lesion removal from the treated area

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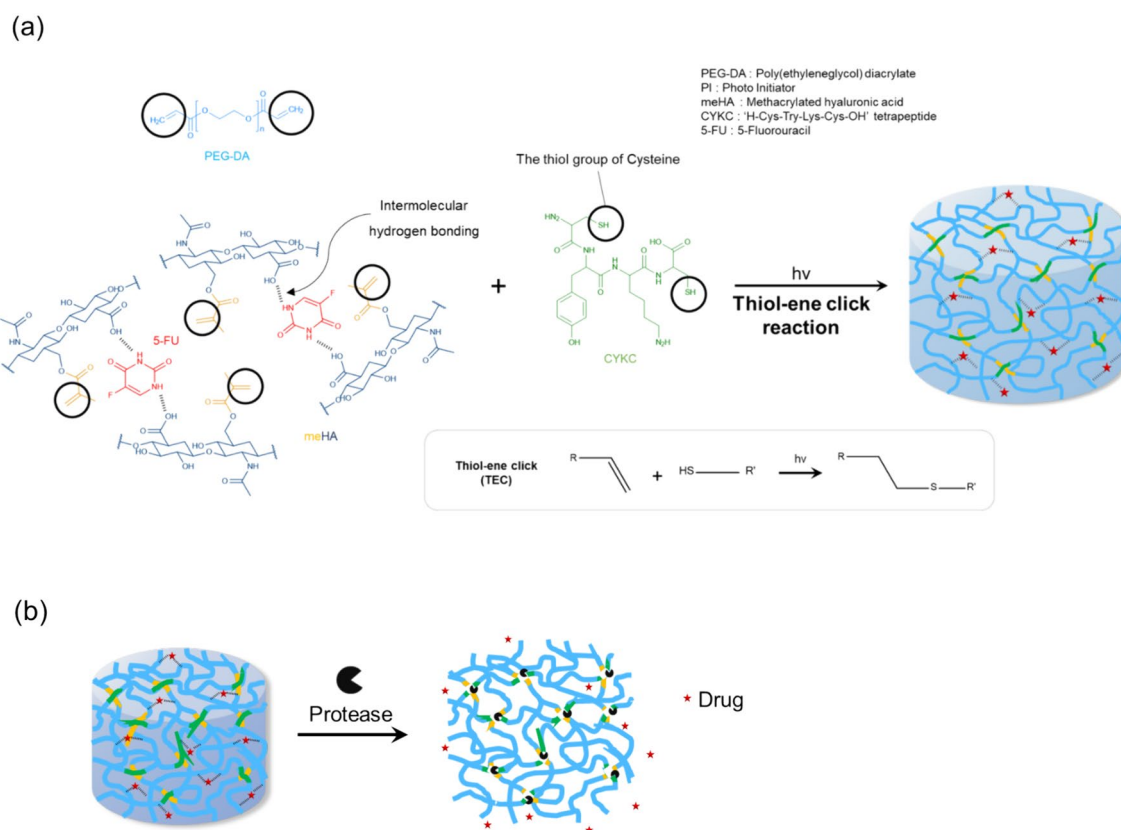
[22]. To enhance the adjuvant therapy of 5-FU, several researchers have reported on a biopolymer patch loaded with chemotherapeutic drugs, enabling continuous administration in small doses [14, 23–25].

Various tumor-microenvironment-responsive hydrogel patches have been designed to modulate the plasticity of cancer in flat and scaly areas. Hyaluronic acid (HA), an anionic polysaccharide consisting of  $\beta$ -1,4-linked D-glucuronic acid and *N*-acetyl-D-glucosamine disaccharide units, is a component of the extracellular matrix, displaying excellent biocompatibility [26–29]. Furthermore, HA's exceptional hydrophilicity promotes tissue and dermal regeneration. With diverse functional groups and a high negative charge, various anionic and synthetic forms of HA, such as methacrylated hyaluronic acid (meHA) and thiolated hyaluronic acid (HA-SH), facilitate convenient click reactions to prepare multifunctional and adjustable hydrogels [30–32]. Additionally, polyethylene glycol (PEG)-based materials are widely used in scaffolds for drug release and tissue engineering. PEG plays a critical role in preventing protein uptake and fouling, thereby reducing antigenicity, immunogenicity, and systemic

toxicity, while also extending the half-life of drugs in the plasma [33–35].

Stimuli-responsive hydrogels are promising biomaterials capable of changing their properties in response to various environmental stimuli, including temperature, pH, light, and biochemical reagents [36–39]. Many researchers have reported these hydrogels for their potential applications in drug delivery, tissue engineering, and other biomedical fields. Specifically, protease-degradable hydrogels can be used for wound healing and cancer treatment due to their ability to respond to enzymatic activity in the body, facilitating controlled drug release induced by biodegradation [40].

In this study, we developed a straightforward method to create ultrathin, micropatterned hydrogel patterns of PEGDA, and meHA (designated as PHcd) on solid substrates (Fig. 1). These films were designed with adjustable crosslinking densities and biochemical cues facilitating cell attachment and controlled release of 5-FU. Methacrylated hyaluronic acid was conjugated with the CYKC peptide using a 'clickable' photochemical thiol-ene addition. This adaptable addition reaction allowed for one-pot fabrication using a single photo-crosslinking reaction, effectively



**Fig. 1** Crosslinking of a protease-degradable hydrogel and subsequent drug release following the hydrogel degradation. **a** Methacrylated hyaluronic acid and PEG-DA are crosslinked with the CYKC peptide using a clickable photochemical thiol-ene addition

in a single-batch fabrication process. 5-FU is entrapped within the hydrophilic polymer networks. **b**  $\alpha$ -Chymotrypsin degrades between the CY-KC sequences in the peptide crosslinker, leading to drug release

entrapping 5-FU within the hydrophilic polymer networks. To create an enzyme-responsive hydrogel system for controlled 5-FU release, we employed  $\alpha$ -chymotrypsin to degrade the peptide chain located between the CY-KC sequence. This combination of materials presents an efficient delivery system capable of meeting sustainable and intermittent requirements for hydrophilic and cytotoxic drug delivery, particularly targeting skin care.

## Experimental

### Materials and Methods

Fmoc-amino acids, *N,N,N',N'*-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt), filtered reaction tubes (Libra tube®) were purchased from BeadTech Inc. (Ansan, Korea). H-Cys(Trt)-2-ClTrt resin (loading level: 0.7 mmol/g) was purchased from Gyros Protein Technologies (Uppsala, Sweden). Dichloromethane (DCM), methanol (MeOH), *N,N'*-dimethylformamide (DMF), piperidine, *N,N'*-diisopropylethylamine (DIPEA), triisopropylsilane (TIPS), trifluoroacetic acid (TFA), 70% Ethanol (EtOH), dimethyl sulfoxide (DMSO), pyridine, *n*-butanol, and phenol were purchased from Daejung Chemical & Metals Co., Ltd. (Siheung, Korea). Potassium cyanide (KCN) was purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). 3,6-Dioxa-1,8-octanedithiol (DODT) was purchased from TCI Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Diethyl ether was purchased from Carlo Erba Reagents, (Milan, Italy). Dialysis tubing 6–8 kDa was purchased from Spectrum Chemical Mfg. Corp., (Gardena, CA, US). Ninhydrin, deuterium oxide (D<sub>2</sub>O), 3-(trimethoxysilyl)propyl methacrylate (TMSPM), (3-aminopropyl)trimethoxysilane (APTMS), poly(ethylene glycol) diacrylate (PEGDA: average  $M_n = 700$ ), 2-hydroxy-2-methylpropiophenone (Darocur 1173; photoinitiator), chloroform, 5-fluorouracil (5-FU), methacrylic anhydride (MA), sodium hydroxide (NaOH), sodium chloride (NaCl),  $\alpha$ -chymotrypsin from bovine pancreas ( $\alpha$ -chymotrypsin: molecular weight = 25 kDa), thiazolyl blue tetrazolium bromide (MTT solution), calcein-AM, and ethidium homodimer (EthD-1) were purchased from Sigma-Aldrich (St. Louis, MO, US); hyaluronic acid (CN Lab: 10kDA HA); microscope glass slides (76 mm  $\times$  26 mm) were purchased from Heinz Herenz (Hamburg, German), respectively. Microscope cover glass slides were purchased from Duran (Mainz, German). A-431 human epidermoid carcinoma cells were purchased from Korea Cell Line Bank (Seoul, Korea). RPMI 1640 with stable glutamine (RPMI 1610), fetal bovine serum USA (FBS), penicillin–streptomycin solution 100X (PS), and Dulbecco's phosphate buffered saline (DPBS) were purchased from BLOWEST (Nuailé,

France). UV–Vis absorbance data were obtained with a UV–Vis spectrophotometer (UV-2550, Shimadzu, Japan) and microplate reader (SpectraMax i3x, Molecular Devices, CA, US). Ninhydrin color test (Kaiser test) was performed using a heat block (BioFree, Korea). High-performance liquid chromatography (HPLC) analysis data were obtained using Agilent 1260 Infinity system (CA, US). <sup>1</sup>H-Nuclear magnetic resonance spectroscopy (NMR) spectrum was acquired by a Bruker AvanceIII-HD500 spectrometer (Rheinstetten, Germany) operating at 500 MHz. O<sub>2</sub> plasma was performed using CUTE (Femto Science Co., Gyeonggi, Korea). Photo-polymerization was performed using Spot light source LC8 (L9588-01A UV illuminator, Hamamatsu, Japan) to generate 100 mW/cm<sup>2</sup> at 365 nm. Fluorescence-labeled cell images were obtained with a confocal laser scanning microscope (LSM700, Carl Zeiss AG, Jena, Germany).

### Preparation of CYKC Tetrapeptide

A solution of Fmoc-Lys(Boc)-OH (3.0 equiv.), HBTU (3.0 equiv.), and DIPEA (6.0 equiv.) in DMF (5 mL) was added to 500 mg of H-Cys(Trt)-2-ClTrt resin in a reaction tube and the suspension was shaken for 2 h at room temperature. The resin was then washed three times each with DMF, DCM, and methanol. The resin was treated with 20% (v/v) piperidine in DMF (5 mL) for 15 min to generate the dibenzofulvene–piperidine adduct that is quantified by UV–Vis spectrophotometer at 301 nm, and washed with DMF, DCM, and methanol (3 times each). The loading level was 0.92 mmol/g, calculated using the dibenzofulvene–piperidine adduct. For the synthesis of other amino acid sequences, the coupling and deprotection steps were repeated. In the coupling step, a solution of Fmoc-amino acid (3.0 equiv.) (sequentially, Tyr and Cys), HBTU (3.0 equiv.), HOBt (3.0 equiv.), and DIPEA (6.0 equiv.) in DMF (5 mL) was added to the H-Cys(Trt)-2-Cl Trt resin, and the suspension was shaken for 2 h at room temperature. The deprotection step involved the addition of a solution of 20% (v/v) piperidine in DMF (2  $\times$  5 mL) to the resin for 5 and 10 min, respectively. The resin was washed with DMF, DCM, and methanol (3 times each) at each step of coupling and deprotection. The completion of each amino acid coupling reaction used in the synthesis was confirmed using Kaiser's ninhydrin test. Kaiser's ninhydrin test was performed to verify the completion of deprotections and coupling reactions. 10–15 peptide beads were taken into a 1.7-mL microtube and heated at 110 °C for 5 min. Subsequently, 1–2 drops of each of the three reagents were added: (a) 1 mL of 10 mM KCN in 49 mL of pyridine, (b) a solution of 1.0 g of ninhydrin in 20 mL of *n*-butanol, and (c) a mixture of 40 g of phenol in 20 mL of *n*-butanol. Upon completion of the coupling reaction, the solution exhibited a yellow color, and the beads appeared colorless. If the coupling

reaction was incomplete, either the solution turned dark blue or the beads exhibited a dark blue color.

Once the peptide sequences were synthesized, a cleavage cocktail comprising TFA/DODT/TIPS/water (94:2.5:1:2.5, v/v/v/v; 5 mL) was added to dry resins for 1 h to cleave the peptides from the resin and remove the protecting groups from the side chains. The resulting solution was precipitated with cold diethyl ether, and the supernatant was decanted after centrifugation. This process was repeated two more times, and the collected peptides were dried *in vacuo*. The synthesized tetrapeptide was analyzed by HPLC and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS).

### Preparation of Methacrylated Hyaluronic Acid

To methacrylate the hyaluronic acid (HA, 10 kDa), 200 mg of HA was dispersed in water to achieve a concentration of 1% (w/v), and DMF was added in a ratio of DMF to water (2/3, v/v). Methacrylic anhydride (MA) was added in a molar ratio of 3:1 of HA to MA. NaOH (1 N) was utilized to adjust the pH within a range of 8–10, and the solution was stirred overnight at 400 rpm. To reach a final DMF-to-water ratio of 1/10 (v/v), the solution was diluted with water, and NaCl (0.5 M) was added. The resulting solution was purified using a 7–11 kDa dialysis tubing cellulose membrane for 2 days. The purified solution was subsequently frozen at  $-80\text{ }^{\circ}\text{C}$  for 2 days and then lyophilized. For analysis of the synthesized methacrylated hyaluronic acid, 1 mg was dissolved in 1 mL of deuterium oxide ( $\text{D}_2\text{O}$ ) and subjected to  $^1\text{H}$  NMR spectroscopy to determine the degree of modification (MoD).

### Micropatterning of PHcd Hydrogel on the Glass

The glass slides were placed in an oxygen plasma for 6 min and then immersed in a solution containing 0.095% (v/v) APTMS and 0.005% (v/v) TMSPM in chloroform for 1 h. The glass slides, with introduced amino and acryl groups, were subsequently washed with DCM and dried. The hydrogel precursor solution was prepared by mixing 10% (w/v) solution of tetrapeptide (CYKC) in 50% (v/v) methanol and deionized (DI) water, 10% (w/v) solution of methacrylated hyaluronic acid (meHA) in DI water, 40% (v/v) solution of PEGDA in deionized (DI) water, 10% (v/v) solution of photoinitiator in DI water, and 100 mM of 5-FU in DI water, in a ratio of 3:3:2.5:1:0.5. The hydrogel precursor solution was dropped onto the amino and acryl-introduced glass and polymerized using UV exposure at 365 nm for 1.2 s with a designed photomask. The polymerized hydrogel was then rinsed with DI water and dried.

### Swelling Studies of PHcd Hydrogel

The hydrogel precursor solution (40  $\mu\text{L}$ ) with a final concentration of 10%, 15%, and 20% (v/v) PEGDA prepared in a mold (4 mm in diameter and 5 mm in height) were polymerized by UV exposure at 365 nm for 6.0 s from a distance of 3.0 cm. The hydrogels were dried up in a  $55\text{ }^{\circ}\text{C}$  incubator for 2 d, and then immersed in water at room temperature. At regular interval of time, PHcd hydrogels were transferred from the water, dried and then weighed. Swelling ratio at different times ( $H_t$ ) was calculated from the following equation:

$$H_t(\%) = \frac{m_t - m_0}{m_0} \times 100, \quad (1)$$

where  $m_0$  and  $m_t$  is the weight of the initial dry hydrogel and of the weight of the hydrogel at time  $t$ , respectively.

Another parameter, water content at different times ( $W_t$ ) in the hydrogel can be calculated from the following equation:

$$W_t(\%) = \frac{m_t - m_0}{m_t} \times 100, \quad (2)$$

where  $W_{\infty}$  is the water content when the equilibrium is reached.

### Cell Viability Test After Controlled Release of 5-FU by PHcd Hydrogel

A-431 cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin–streptomycin (PS) in humidified atmosphere containing  $37\text{ }^{\circ}\text{C}$  and 5%  $\text{CO}_2$ . A-431 cell suspension (500  $\mu\text{L}$ ) was seeded on the hydrogel-patterned glass at a density of 50,000–100,000 cells per well in a 24-well plate and incubated for 1 d. The cell culture medium was carefully discarded, and 500  $\mu\text{L}$  of  $\alpha$ -chymotrypsin (100  $\mu\text{g}/\text{mL}$ ) was added into the cells to degrade the PHcd hydrogel. After  $\alpha$ -chymotrypsin treatment, 40  $\mu\text{L}$  of MTT working solution was added into each well, and the plate was shaken for 20 min and incubated for 2 h at  $37\text{ }^{\circ}\text{C}$  in the dark. After incubation, the absorbance was determined at a wavelength of 490 nm using a microplate reader.

In addition, microscopic observation of cell viability test was performed after controlled release of 5-FU. After  $\alpha$ -chymotrypsin treatment, 2  $\mu\text{M}$  of calcein-AM and 4  $\mu\text{M}$  of EthD-1 working solution was added to cell and incubated for 30 min at room temperature. Following incubation, the cell suspension was transferred to the conical tube and centrifuged at 1000 rpm for 3 min. The supernatant was removed, and 200  $\mu\text{L}$  of DPBS was added to clean the remaining fluorescence. DPBS (10  $\mu\text{L}$ ) was added, and the cell suspension

was dropped onto the glass with a coverslip. Labeled cells were observed by a confocal laser scanning microscope at 488 nm and 555 nm.

## Results and Discussion

### Preparation of Peptide and Acrylated Hyaluronic Acid

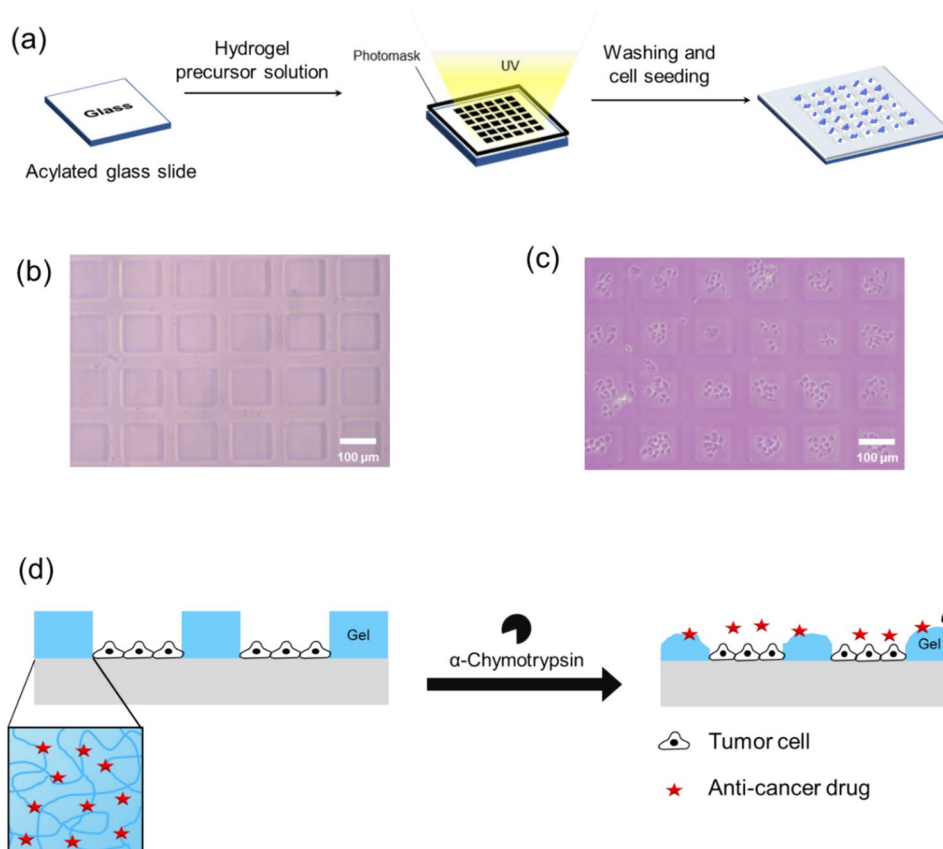
To prepare an enzyme-responsive hydrogel, we utilized the  $\alpha$ -chymotrypsin-cleavable tetrapeptide 'H-CYKC-OH (CYKC)', because  $\alpha$ -chymotrypsin recognizes and cleaves the amide bond of aromatic amino acids, including tyrosine, tryptophan, and phenylalanine [41]. Therefore, the CYKC tetrapeptide was synthesized using the solid-phase peptide synthesis (SPPS) method using H-Cys(Trt)-2-Cl Trt resin [42, 43] (Fig. S1) and characterized by HPLC and MALDI-TOF MS (Fig. S2). The crude HPLC spectrum showed 74.3% purity of peptide and the peptide was used after HPLC purification. MALDI-TOF MS exhibited the corresponding molecular weight of the peptide sequence. The degradation of the CYKC peptide by  $\alpha$ -chymotrypsin was analyzed using HPLC data. When the CYKC peptide was treated with 20  $\mu\text{g}/\text{mL}$  of  $\alpha$ -chymotrypsin for 30 min, 80% of the peptide was cleaved (Fig. S3a, b). When treated

with different concentrations of  $\alpha$ -chymotrypsin, the peptide was cleaved over 95% more than 30  $\mu\text{g}/\text{mL}$  of the protease concentration (Fig. S3c). Additionally, to incorporate hyaluronic acid in the PHcd hydrogel, the hyaluronic acid was chemically modified to introduce methacrylate. Figure S4 represents a  $^1\text{H}$  NMR spectrum of meHA, where the degree of methacrylate modification was calculated as 10.6% by integrating the peak of the vinyl singlets relative to the sugar ring of hyaluronic acid [44].

### Micropatterning of PHcd Hydrogel

Subsequently, the diacrylate group of PEGDA and the methacrylated hyaluronic acid reacted with the thiol group of cysteine in the CYKC tetrapeptide under UV exposure in the presence of a photoinitiator. Additionally, the anti-tumor agent 5-FU was embedded in the crosslinked PEGDA hydrogel through hydrogen bonding with the hydrophilic side chain of meHA. As depicted in Fig. 2, the hydrogel precursor solution was prepared by combining the following materials: tetrapeptide (CYKC), methacrylated hyaluronic acid (meHA), PEGDA, photoinitiator, and 5-FU. The mixture of hydrogel precursor solution was deposited onto glass surfaces functionalized with amino and acryl groups, and then polymerized using UV exposure with a designed photomask. The micropatterned PHcd hydrogel can be degraded

**Fig. 2** The drug delivery through the degradation of the hydrogel. **a** Fabrication of the PHcd hydrogel on an acrylated glass slide with photomask and immobilization of cells. **b** Bright-field image of patterned PHcd hydrogel on the glass (scale bar = 100  $\mu\text{m}$ ). **c** Bright-field image of cells after seeding for 24 h on the patterned PHcd (scale bar = 100  $\mu\text{m}$ ). Approximately 9.6 cells were seeded per well. **d** Schematic representation of 5-FU delivery to the cells by the  $\alpha$ -chymotrypsin-cleavable PHcd hydrogel



via the  $\alpha$ -chymotrypsin-induced cleavage reaction of CY/KC, enabling the sustained and localized release of 5-FU. The fluorescently labeled hydrogel micropattern was used to visualize the degradation of hydrogel upon the presence of protease. When the hydrogel labeled with rhodamine B isothiocyanate (RITC) was treated with 30  $\mu\text{g}/\text{mL}$  of  $\alpha$ -chymotrypsin, its fluorescent intensity decreased by 50% after 5 h compared to the hydrogel treated with PBS as a control experiment (Fig. 3). This result exhibits the protease triggered the degradation of the hydrogel.

### Swelling Properties of PHcd Hydrogel

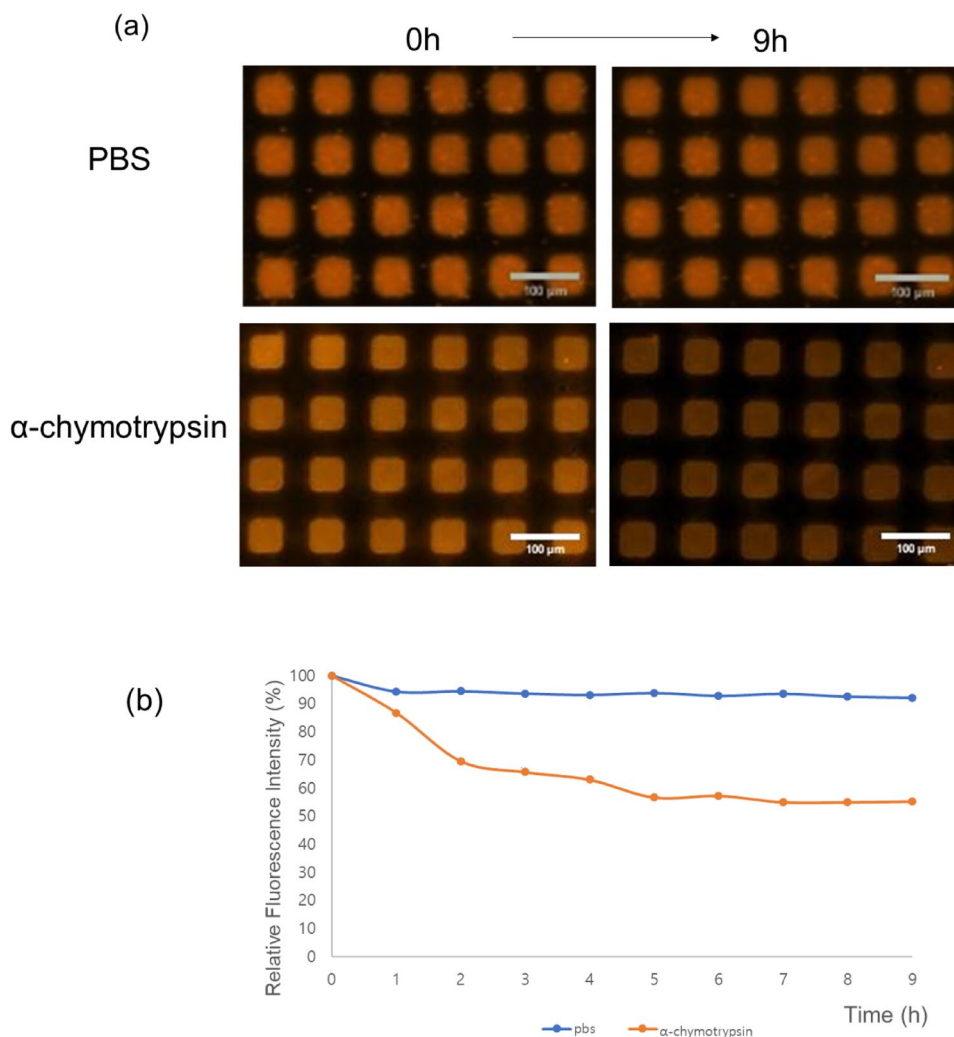
To investigate the volumetric changes in PHcd hydrogel due to the absorption of bound water, we conducted a swelling profile analysis of PHcd hydrogel at varying final concentrations of 10%, 15%, and 20% PEGDA without  $\alpha$ -chymotrypsin-induced gel degradation. The PHcd hydrogels crosslinked with 10%, 15%, and 20% PEGDA reached equilibrium at 120 min, 140 min, and 180 min, respectively.

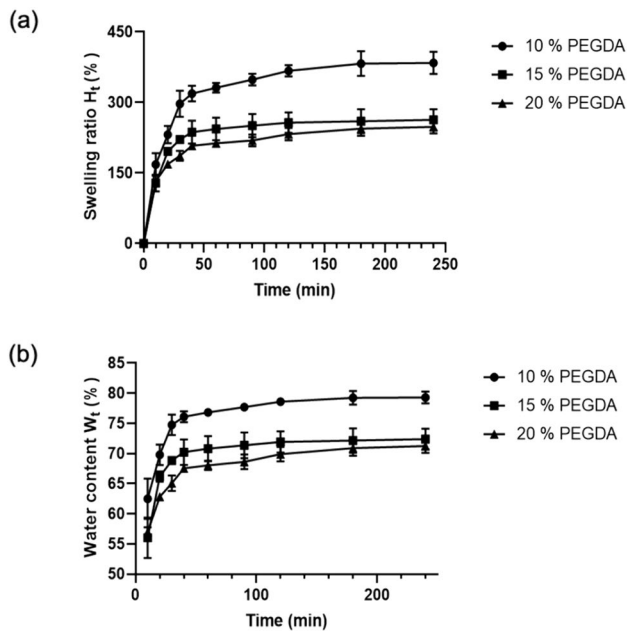
The swelling ratios of PHcd hydrogels crosslinked with 10%, 15%, and 20% PEGDA were approximately 370%, 275%, and 245%, respectively (Fig. 4a), while their water contents were 79%, 72%, and 71%, respectively (Fig. 4b). Notably, the PEGDA percentage within the PHcd hydrogel significantly affected the swelling ratios, particularly between 10 and 15% [45]. However, there were no substantial changes in water content observed among different PEGDA percentages after 40 min. Considering that even the lowest concentration of 10% PEGDA within the PHcd hydrogel exhibited stable swelling behavior after 100 min, similar to the behavior of PHcd hydrogels crosslinked with 15% or 20% PEGDA, the 10% PEGDA content was used to crosslink the PHcd hydrogel, aiming for higher water content in the hydrogel.

### Antitumor Activity of Micropatterned PHcd Hydrogel Containing 5-FU

The cell viability of A-431 cells on the PEGDA hydrogel according to the presence of 5-FU was compared

**Fig. 3** Degradation of fluorescently labeled PHcd hydrogel pattern. **a** Fluorescence images after 9-h treatment of PBS and  $\alpha$ -chymotrypsin, respectively (scale bar = 100  $\mu\text{m}$ ). **b** Due to the activity of  $\alpha$ -chymotrypsin, the fluorescence decreased by 40% after 5 h, whereas there was no significant change observed in the hydrogel incubated with PBS



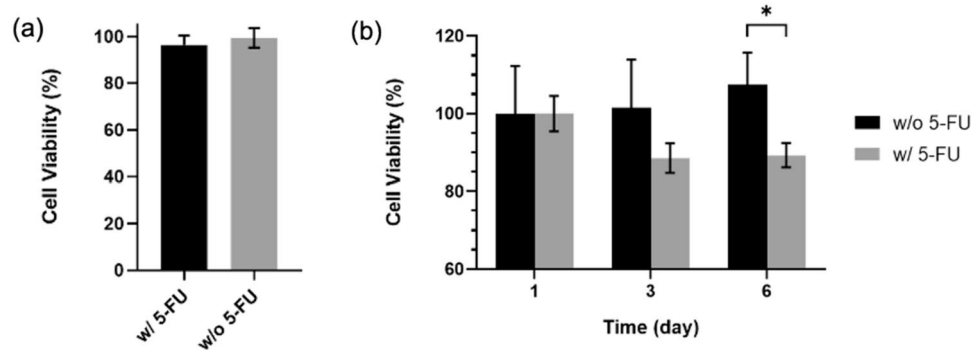


**Fig. 4** Swelling behavior of PHcd hydrogels based on PEGDA concentration. **a** Swelling ratio ( $H_t$ ) of PHcd hydrogels in water at various time intervals and room temperature. **b** Water content ( $W_t$ ) within PHcd hydrogel at different time points and room temperature, represented as mean  $\pm$  standard error

for 7 d by MTT assay. When the cells were treated with  $\alpha$ -chymotrypsin, the observed cell viability was 96.9%. This exhibited only a 2.3% difference compared to the PHcd hydrogel without 5-FU, which showed a viability of 99.2% (Fig. 5a). This result confirms that the hydrophilic PHcd hydrogel, primarily composed of PEGDA and hyaluronic acid, effectively adsorbed 5-FU between the polymer chains through hydrogen bonding with minimal loss. Next,

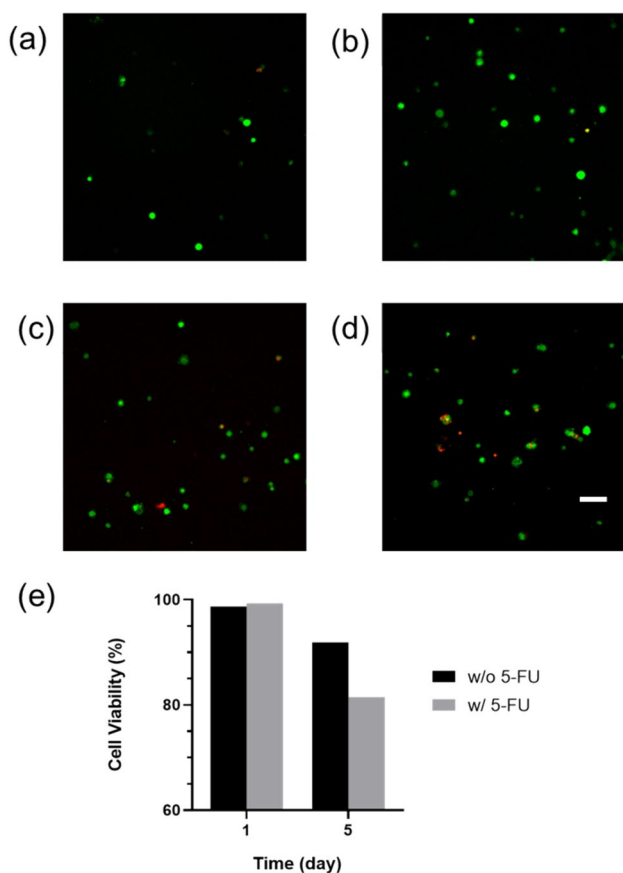
the cell viability of A-431 cells on the PHcd hydrogel was compared from day 1 to day 6 by MTT assay to evaluate the controlled release of 5-FU from PHcd hydrogel. The cell viability after adding  $\alpha$ -chymotrypsin for 1, 3, and 6 d to cells seeded into the hydrogel pattern without 5-FU was 92.2%, 108.0%, and 109.0%, respectively (Fig. 5b). In contrast, when using the 5-FU loaded PHcd hydrogel, the cell viability decreased to 91.4%, 92.4%, and 94.0% at 1, 3, and 6 d, respectively. Notably, the anticancer efficacy of the 5-FU loaded PHcd hydrogel was significantly evident on day 6 due to  $\alpha$ -chymotrypsin-induced gel degradation. These results indicate that the controlled release of 5-FU within the PHcd hydrogel sustained its efficacy for at least 6 d through  $\alpha$ -chymotrypsin-induced hydrogel degradation.

To further validate the anticancer effectiveness of the 5-FU loaded PHcd hydrogel, we employed confocal laser microscopy to observe live/dead cells and calculated the percentage of live cells during the controlled release of 5-FU from micropatterned PHcd hydrogel. The micropatterning method provided accuracy in the preclinical tumor model for assessing drug efficacy against cancer cells. A431 cells seeded into the hydrogel micropattern, with or without 5-FU, were stained with Calcein-AM/EthD-1 to distinguish live and dead cells (Fig. 6a–d). Upon the addition of  $\alpha$ -chymotrypsin to the PHcd hydrogel without 5-FU, the cell viability on day 5 was 91.9%. However, the 5-FU loaded PHcd hydrogel exhibited a significant reduction in cell viability (81.5%) after 5 days of  $\alpha$ -chymotrypsin addition (Fig. 6e). These results indicate that the PHcd hydrogel micropattern is capable of drug release in the presence of  $\alpha$ -chymotrypsin, acting as a trigger. This drug release platform can be used for the drug efficacy upon the controlled release.



**Fig. 5** Cell viability after controlled release of 5-FU from micropatterned PHcd hydrogel. **a** Viability of A-431 cells was determined using the MTT assay after treated with a solution of 10  $\mu$ M 5-FU for 1, 2, 4, and 7 days. Control experiment was performed without 5-FU treatment. **b** After 1, 3, and 6 days of exposure to  $\alpha$ -chymotrypsin, cell viability was measured using the MTT assay, with 5-FU released

in a controlled manner from PHcd hydrogel.  $\alpha$ -Chymotrypsin was introduced 24 h after cell seeding, and the MTT assay was performed on days 1, 3, and 6 post- $\alpha$ -chymotrypsin addition. Cells in the control group were seeded on patterned PHcd hydrogels without 5-FU. Results are presented as mean  $\pm$  standard error. \* $p$ -value was calculated using an unpaired  $t$  test ( $p < 0.05$ )



**Fig. 6** Confocal fluorescence images of A-431 cells were captured, depicting live cells stained green with calcein-AM and dead cells stained red with EthD-1. **a** A-431 cells were observed after exposure to  $\alpha$ -chymotrypsin for 24 h on the PHcd hydrogel without 5-FU. **b** A-431 cells were observed after exposure to  $\alpha$ -chymotrypsin for 24 h on the PHcd hydrogel containing 5-FU. **c** A-431 cells were observed after exposure to  $\alpha$ -chymotrypsin for 5 days on the PHcd hydrogel without 5-FU. **d** A-431 cells were observed after exposure to  $\alpha$ -chymotrypsin for 5 days on the PHcd hydrogel containing 5-FU (scale bar=50  $\mu$ m). **e** Cell viability following controlled release of 5-FU was assessed using confocal images

## Conclusion

To develop a controlled delivery system for the potent anticancer drug 5-FU, we introduced a novel and straightforward method to prepare micropatterned PHcd hydrogel. This hydrogel consists of methacrylated hyaluronic acid and polyethylene glycol diacrylate, susceptible to degradation by  $\alpha$ -chymotrypsin. The PHcd hydrogel, crosslinked by PEGDA, exhibits high water absorption and stable water retention capabilities. Its high water content prevents the rapid release of 5-FU through simple physical binding interactions. Cell viability assays also demonstrated that the  $\alpha$ -chymotrypsin-responsive PHcd hydrogel significantly enhances the controlled release of anticancer efficacy over a sustained period, particularly observed from

day 6 onwards. The micropatterned PHcd hydrogel allows for localized distribution within specific areas and is predominantly influenced by  $\alpha$ -chymotrypsin. This hydrogel exhibits substantial potential for broad applications in patch-type therapy for delivering hydrophilic drugs. Our next step involves broadening the scope of extended release applications by focusing on drug targeting and evaluating its efficacy.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s11814-024-00205-2>.

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**Data Availability** Data will be made available on request.

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