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# High Viability of Cells Encapsulated in Degradable Poly(carboxybetaine) Hydrogels

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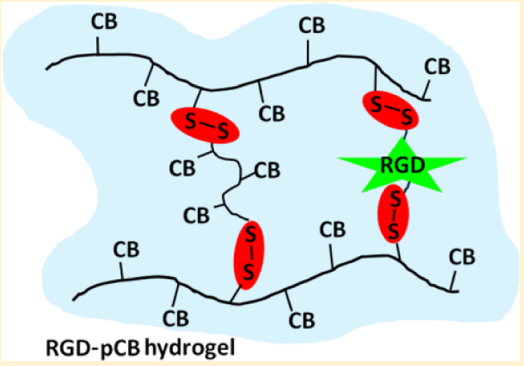
Supporting Information

**ABSTRACT:** In this study, we report a degradable poly(carboxybetaine) (pCB) hydrogel, produced via a thiol−disulfide exchange reaction for cell encapsulation. A pCB dithiol was synthesized as a cross-linker and reacted with a pyridyl dithiol-containing CB copolymer to form a hydrogel. We evaluated the biocompatibility of the pCB-based hydrogel via encapsulation of three cell types, including NIH3T3 fibroblasts, MG63 osteoblast-like cells, and HepG2 hepatocarcinoma cells. Up to 90% of cells retained their viability in the pCB hydrogel even at low cell-seeding densities under serum-free conditions after a 9-day culture. Results are compared with a degradable poly(ethylene glycol) methacrylate (PEGMA) hydrogel, which showed very low cell viability under serum-free condition after a 3-day culture. We incorporated an RGD peptide into the CB hydrogel using a cysteine-terminated cross-linker, which was shown to promote cell proliferation.




## 1. INTRODUCTION

Zwitterionic materials have received increasing attention due to their importance in recent years. Recently, a series of water-soluble zwitterionic compounds, such as carboxybetaine (CB) monomers and cross-linkers, has been developed.[1] Surfaces grafted with CB-based polymers have been demonstrated to be ultralow fouling, which is defined as less than 5 ng/cm$^2$ adsorbed proteins.[2] These surfaces are highly resistant to nonspecific protein adsorption, even from undiluted blood plasma and serum, and prohibit long-term bacterial colonization for up to 10 days.[1] The ultralow-fouling properties of zwitterionic materials result from high hydration around their opposing charges via electrostatically induced hydration and the high energy barrier required to remove hydration layers.[3] Due to the high hydration and ultralow-fouling properties of zwitterionic materials, they have found a variety of biomedical applications. For example, proteins conjugated to a CB polymer are able to maintain their stability without sacrificing binding affinity,[4] and CB polymer-based drug delivery carriers with encapsulated drugs are very stable.[5] CB-based hydrogels prepared from a CB monomer and a CB cross-linker have several advantages:[6,7] very low protein adsorption and cell adhesion, functionalizability, and excellent mechanical properties. Hydrogels with chemical and mechanical gradients have also been developed.[8] Recently, we demonstrated that cells that were directly encapsulated in biodegradable and RGD-functionalized CB hydrogels displayed a tissue-like morphology after 4-week culture.[9]

Three-dimensional (3D) scaffolds in tissue engineering serve as a temporary support for cell accommodation and growth.[10,11] Hydrogels have been attractive candidates for tissue-engineering scaffolds due to their tissue-like elasticity and high water content, mimicking the interstitial tissue environment. Poly(ethylene glycol) (PEG)-based hydrogels have been the most widely used synthetic materials for cell encapsulation[10−13] because they provide a highly swollen environment similar to native tissues and possess a low level of nonspecific protein adsorption.[13] Direct cell encapsulation during hydrogel formation in situ is a popular strategy, but a minimally invasive operation is required.[11] The requirements for such a strategy include the nontoxicity of hydrogel materials, including their precursors and final products, and an innocuous process of cell encapsulation.[10] One popular method is using diacrylate- or dimethacrylate-containing molecules that are cross-linked via a free radical polymerization to make hydrogels.[12] The toxic photoinitiator or radiation treatment can cause high cellular damage during in situ cell encapsulation. In order to reduce toxicity and free radical damage, macromolecules with various end group functionalities, such as thiols, as used in this work, are versatile for hydrogel formation.[13,14]

In this study, we prepared a degradable pCB hydrogel as a cell encapsulation material via a thiol−disulfide exchange reaction (Figure 1). The disulfide-cross-linked hydrogel, which is cleavable chemically by biological reductants such as glutathione (GSH) or cysteine, is of particular interest as an implantable scaffold due to its potential degradability. We

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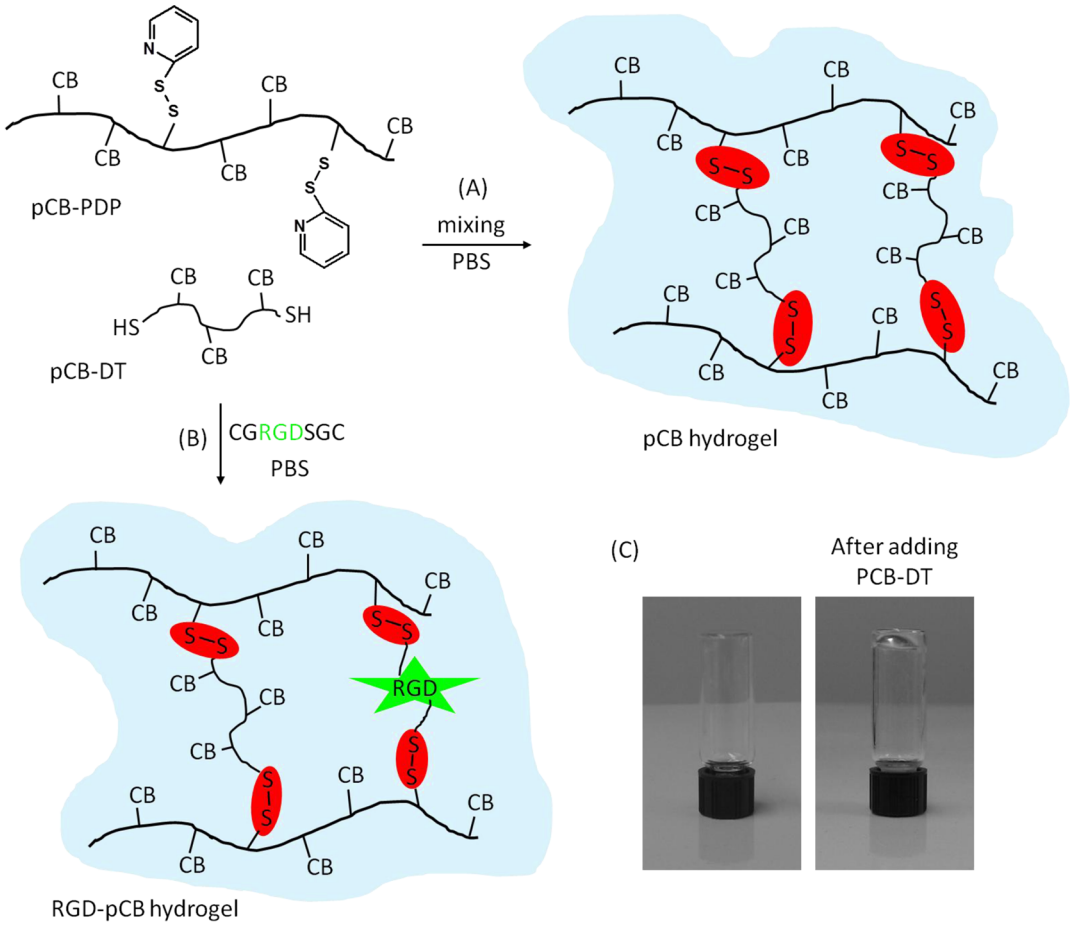


**Figure 1.** Illustration of the formation of pCB (a) and RGD-pCB (b) hydrogels by the thiol−disulfide exchange reaction. (c) pCB hydrogel was made from 7% (w/v) of pCB-PDP and 2% (w/v) of pCB-DT in PBS.

synthesized a pCB dithiol (pCB-DT) as a cross-linker; this reacts with a pyridyl dithiol-containing CB copolymer (pCB-PDP) to form a hydrogel. We evaluated the biocompatibility of the pCB-based hydrogel via several types of cell encapsulation. Finally, we incorporated a RGD peptide into the pCB hydrogel via a cysteine-terminated cross-linker and evaluated cell proliferation within the pCB hydrogel and RGD-pCB hydrogel.

## 2. EXPERIMENTAL SECTION

**2.1. Chemicals.** 2-Carboxy-*N*,*N*,-dimethyl-*N*-(2′-(methacryloyloxy)ethyl) ethanaminium inner salt (carboxybetaine methacrylate, CBMA) and (3-acryloylamino-propyl)-(2-carboxy-ethyl)-dimethylammonium(carboxybetaine acrylamide, CBAA) were synthesized using a previously published procedure.[15,16] 4-Cyano-4-(phenylcarbonothioylthio)pentanoic acid (CTP), diethylene glycol (DEG), *N*,*N*′-dicyclohexylcarbodiimide (DCC, 99%), 4-(dimethylamino)pyridine (DMAP), fluorescin diacetate (FDA), poly-(ethylene glycol) methacrylate (PEGMA, $M_n$ = 360), poly(ethylene glycol) dithiol (PEG-DT, $M_n$ = 3400), propidium iodide (PI), phosphate-buffered saline (PBS), triethylamine (TEA), dichloride methane (DCM), methanol, chloroform, ethyl ether, and dioxane were all purchased from Sigma-Aldrich. 2-Aminoethyl methacrylate hydrochloride (AEMA) and 4,4′-azobis(4-cyanovaleric acid) (V-501) were purchased from Acros. 2,2′-Azobis(4-methoxy-2.4-dimethyl valeronitrile) (V-70) was purchased from Wako Chemicals. *N* -Succinimidyl 3-(2-pyridyldithio)propionate (SPDP) was from ProteoChem. Cys-Gly-Arg-Asp-Ser-Gly-Cys (CGRGDSGC) peptide was purchased from Synthetic Biomolecules. The culture medium for L929 cells and MG63 cells contained Dulbecco's Modified Eagle Medium (DMEM) with 1% penicillin-streptomycin (PS). The culture medium for HepG2 cells consisted of DMEM, 1% nonessential amino acid, 1 mM sodium pyruvate, and 1% PS. All water used had been purified to 18.2 mΩ on a Millipore Simplicity water purification system.

**2.2. Synthesis of Poly(CB-co-PDPMA) (pCB-PDP).** *N*-(3-(2-(Pyridyldithio)propanamido)ethyl methacrylate (PDPMA) was synthesized as described in Scheme 1. 2-Aminoethyl methacrylate hydrochloride (AEMA) (26.5 mg, 0.160 mmol) was dissolved in 5 mL of dichloromethane. *N*-Succinimidyl 3-(2-pyridyldithio) propionate (SPDP) (50 mg, 0.16 mmol) and triethylamine (0.022 mL, 0.160 mmol) were added to the solution, which was stirred at room temperature for 60 min. The reaction was diluted with dichloromethane, silica gel (5 g) was added to the solution, and the solvent was removed under vacuum on the rotary evaporator. Solid sample was purified via silica gel automatic column chromatography using a gradient of dichloromethane:methanol (20:1 to 1:1). The pure product was obtained as a thick colorless oil (50.0 mg, 0.153 mmol). Yield: 96%. $^1$H NMR ($CDCl_3$, 300 MHz); $\delta$ 8.47 (d, 1H, $J$ = 5.1 Hz), 7.64 (m, 2H), 7.13 (m, 1H), 6.89 (bs, 1H), 6.10 (s, 1H), 5.58 (s, 1H), 4.29 (t, 2H, $J$ = 5.5 Hz), 3.63 (m, 2H), 3.09 (t, 2H, $J$ = 6.7 Hz), 2.64 (t, 2H, $J$ = 6.7 Hz), 1.93 (s, 3H).

pCB-PDP was synthesized by a reversible addition−fragmentation chain transfer (RAFT) polymerization according to the process briefly described as follows (Scheme 1). In a 25 mL ampule, CBMA monomer (1011 mg, 4.417 mmol), PDPMA monomer (60 mg, 0.184 mmol), CTP (5.14 mg, 0.0184 mmol), and V-501 (1.03 mg, 0.004 mmol) were dissolved in methanol (5.0 mL). The ampule contents were purged with nitrogen for 1 h, and then the ampule was placed in a preheated oil bath at 70 °C. The reaction was terminated after 24 h by cooling the reaction tube in an ice bath followed by exposure to air. Product was purified by dialysis against water and collected by lyophilization, and the product yield was 56%. pPEGMA-PDP was


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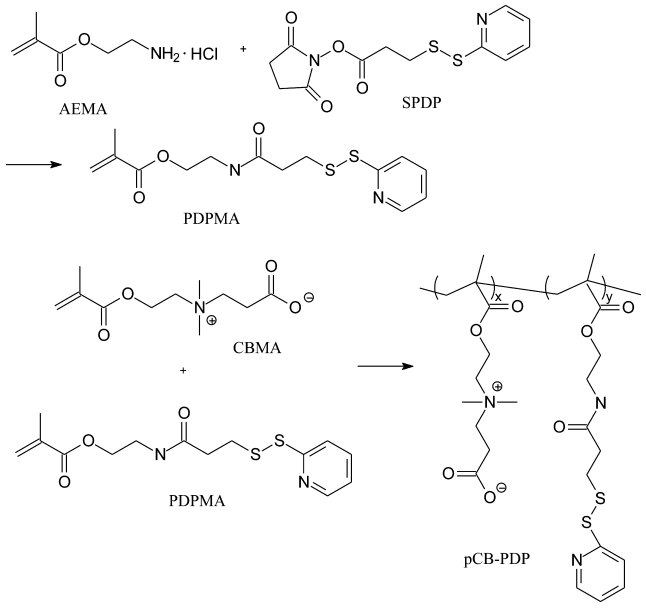
**Scheme 1. Synthetic Route of pCB-PDP**

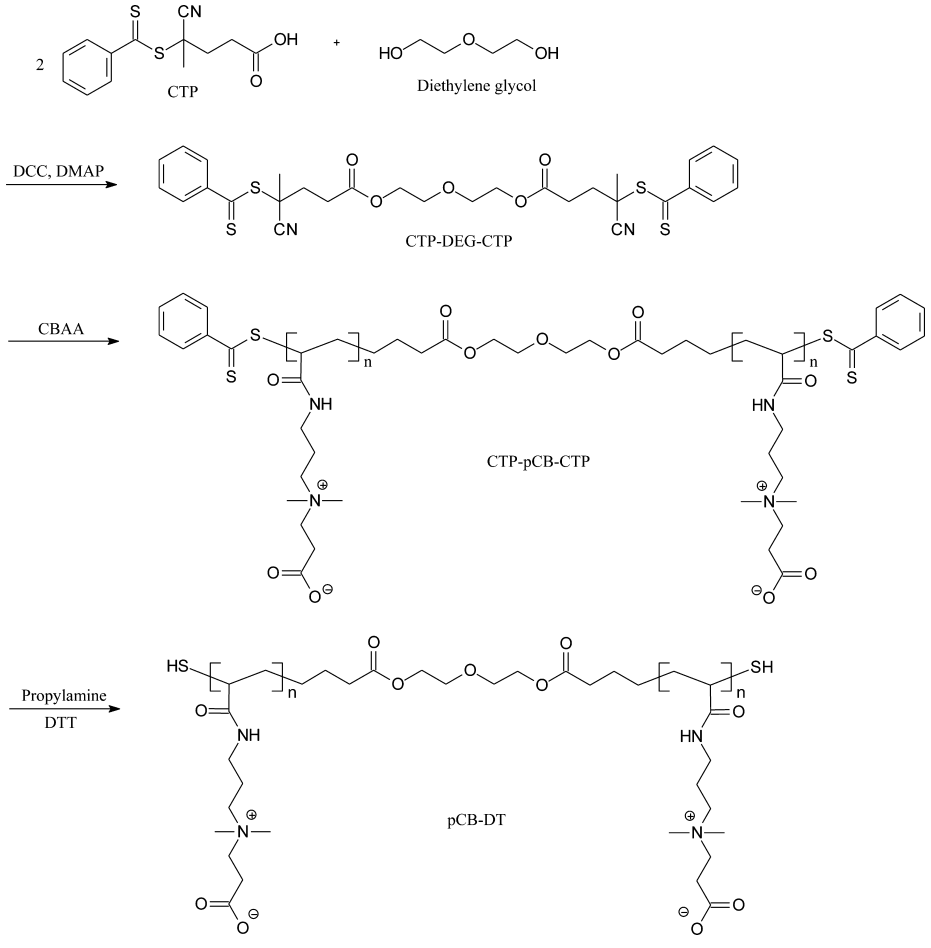



synthesized following the same procedure as illustrated in Scheme S1, Supporting Information.

**2.3. Synthesis of Thiol-Functionalized pCB (pCB-DT).** Difunctional chain transfer agent (CTP-DEG-CTP) was synthesized as described in Scheme 2. In a 50 mL one-neck round-bottom flask equipped with a magnetic stirring bar, CTP (2.02 g, 7.2 mmol), DEG (0.32 g, 3.0 mmol), and DMAP (0.73 g, 6.0 mmol) were dissolved in 10 mL of anhydrous chloroform. After the solution was homogenized by stirring, the flask was placed in an ice bath. DCC (3.09 g, 15.0 mmol) was then added in portions. After 4 h of stirring at 0 °C, the reaction mixture was allowed to warm to room temperature and stirred overnight. Precipitated dicyclohexylurea was removed by filtration to yield a clear red solution. After removal of the volatiles in vacuo, the 1.05 g of crude product was purified by column chromatography on silica gel with a mobile phase of ethyl acetate/hexane (1/3, v/v). Yield: 55.4%. $^1$H NMR ($CDCl_3$): $\delta$ 1.94 (s, −S−C(−CN, $-CH_2-$)−$\mathbf{CH_3}$); 2.41−2.78 (m, 8H, ≡C−$\mathbf{CH_2}$−$\mathbf{CH_2}$−(C═O)−); 3.75 (t, 4H, −(C═O)−O−$CH_2$−$\mathbf{CH_2}$−O−); 4.28 (t, 4H, −(C═O)−O−$\mathbf{CH_2}$−$CH_2$−O−); 7.42, 7.58, 7.94 (m, 10H, aromatic ring).

In a 25 mL ampule, CTP-DEG-CTP (0.189 g, 0.3 mmol) and CBAA monomer (1.93 g, 9.0 mmol) were dissolved in methanol (5.0 mL) and the solution pH was adjusted to 4.5. V-70 (42.0 mg, 0.15 mmol) dissolved in methanol (1.0 mL) was then added. The solution was stirred until all of the CTP-DEG-CTP was dissolved. The ampule contents were purged with nitrogen for ∼1 h, and then the ampule was placed in a preheated oil bath at 70 °C. The reaction was terminated after 8 h by cooling the reaction tube in an ice bath followed by exposure to air. The product (CTP-pCB-CTP) was purified by dialysis against water (pH 4−5) and isolated by lyophilization. The calculated average molecular weight ($M_n$) is 4500. Yield: 1.20 g, 56.6%. $^1$H NMR ($CD_3OD$): $\delta$ 1.92 (s, −S−C(−CN, $-CH_2-$)−$\mathbf{CH_3}$); 3.76 (t, −(C═O)−O−$CH_2$−$\mathbf{CH_2}$−O−); 4.30 (t, −(C═O)−O−$\mathbf{CH_2}$−$CH_2$−O−); 7.46−8.02 (m, aromatic ring); 1.36−1.76 (b, −(**CH**−$CH_2$)$_n$−); 1.88−2.08 (b, −(CH−$\mathbf{CH_2}$)$_n$− and −$CH_2$−$\mathbf{CH_2}$−$CH_2$−); 2.56−2.66 (t, −$\mathbf{CH_2}$−$COO^-$); 3.02−3.12 (b, $\mathbf{CH_3}$−$N^+$−$\mathbf{CH_3}$); 3.14−3.34 (b, −$\mathbf{CH_2}$−$CH_2$−$\mathbf{CH_2}$−); 3.50−3.59 (t, ≡$N^+$−$\mathbf{CH_2}$−$CH_2$−$COO^-$).

**Scheme 2. Synthetic Route of pCB-DT**





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Thiol-functionalized polymer (pCB-DT) was harvested by cleaving dithioester end groups in CTP-pCB-CTP. CTP-pCB-CTP (0.45 g, 0.1 mmol) and methanol (10 mL) were added to a 25 mL round-bottomed flask equipped with a magnetic stir bar. The mixture was stirred until a homogeneous solution was obtained. To this solution was added propylamine as a solution in methanol (2.0 M) (1.00 mL, 2.0 mmol). The resulting solution was stirred at room temperature for 5 h. Subsequently, the solution was loaded to a dialysis bag and dialyzed against water (pH 4–5) for 2 days. Crude product was isolated by lyophilization. Polymer was then dissolved in methanol (10 mL), and then DTT (0.154 g, 1.0 mmol) was added to the solution and allowed to stir at room temperature under $N_2$ atmosphere for 6 h. The resulting thiol-functionalized polymer (pCB-DT) was obtained as a white powder by precipitation into cooled ether, followed by drying under vacuum. Yield: 0.29 g, 74.4%. $^1$H NMR ($CD_3OD$): $\delta$ 1.90 (s, $-S-C(-CN, -CH_2-)-\mathbf{CH_3}$); 3.75 (t, $-(C{=}O)-O-CH_2-\mathbf{CH_2}-O-$); 4.28 (t, $-(C{=}O)-O-\mathbf{CH_2}-CH_2-O-$); 1.32–1.74 (b, $-(\mathbf{CH}-CH_2)_n-$); 1.81–2.07 (b, $-(CH-\mathbf{CH_2})_n-$ and $-CH_2-\mathbf{CH_2}-CH_2-$); 2.56–2.68 (t, $-\mathbf{CH_2}-COO^-$); 2.29–3.12 (b, $\mathbf{CH_3}-N^+-\mathbf{CH_3}$); 3.08–3.35 (b, $-\mathbf{CH_2}-CH_2-\mathbf{CH_2}-$); 3.51–3.60 (t, $\equiv N^+-\mathbf{CH_2}-CH_2-C-OO^-$).

**2.4. Preparation of Hydrogels.** Disulfide-cross-linked pCB hydrogels were prepared via thiol–disulfide exchange reaction with pCB-DT as a cross-linker (Figure 1A). The copolymer, pCB-PDP, was dissolved in PBS to make 3.5 and 7% (w/v) in a 100 $\mu$L solution, respectively. Three amounts, 0.5, 1, and 2 mg of pCB-DT, were added into the copolymer solution and allowed to gel. Gelation was observed by inverting the tubes until the gel solution stopped flowing. PEGMA hydrogels were prepared following the procedure as illustrated in Figure S1, Supporting Information.

**2.5. Cell Encapsulation in pCB and RGD-pCB Hydrogels.** Three cell types and three cell-packing densities were encapsulated in the pCB hydrogels and evaluated for cell viability. The three cell types studied were NIH3T3 fibroblasts, MG63 osteoblast-like cells, and HepG2 hepatocarcinoma cells; three cell-seeding densities, including $5 \times 10^4$, $2 \times 10^5$, and $1 \times 10^6$ cells/mL, were used. Cells were suspended in their respective medium and mixed with pCB-PDP and pCB-DT to allow gelation. Here, the final concentrations of pCB-PDP and pCB-DT in the cell suspension were 7% and 2% (w/v), respectively. Similarly, cells were also suspended in pPEGMA-PDP and PEG-DT for encapsulation as illustrated in Figure S1, Supporting Information. Hydrogels embedded with cells were incubated at 37 °C in 5% $CO_2$ and 100% relative humidity. After encapsulation, cells were immediately stained with 1 mg/mL FDA and 1 mg/mL PI for 30 min at 37 °C in the dark. Samples were washed with a large amount of PBS and then observed under a Nikon Eclipse TE2000-U fluorescence microscope. Cell viability was examined by Trypan Blue staining. The cell-laden hydrogels were immersed into a hydrogel-dissociation medium (cell medium contained with 2 mM L-cysteine) at 37 °C for 10 min. The cell suspension was then collected and stained with Trypan Blue. Dead cells showed a dark blue color under the microscope, while live cells showed a light color. Viability was calculated as the number of live cells/the number of total cells × 100(%).

A $5 \times 10^5$ cells/mL concentration of cell suspension was mixed with pCB-PDP and cross-linkers to evaluate cell proliferation. For preparation of RGD-pCB hydrogels, the cross-linkers contained equal molar pCB-DT and CGRGDSGC. Cell number was quantified via use of a cell-counting chamber. Cells were collected from dissociated hydrogels. After mixing with Trypan Blue, the cells were loaded onto a hemocytometer and then counted under a microscope. Cell numbers are normalized against the initial feed cell number.

## 3. RESULTS AND DISCUSSION

**3.1. Synthesis and Characterization of pCB-PDP and pCB-DT.** In order to make an overall zwitterionic hydrogel, we prepared a thiol-terminated zwitterionic cross-linker that can react with the pyridyl disulfide-contained polyzwitterion via a thiol–disulfide exchange reaction. Chemical compositions and molecular weights of the copolymers (pCB-PDP) are summarized in Table 1. The ratio of PDPMA to copolymer was estimated to be 3.5 mol % by $^1$H NMR according to the proton signals from the pyridyl disulfide at 7.0–8.5 ppm and the methyl group of the copolymer main chain at 2.5 ppm. The average molecular weight ($M_n$) is 65 000 as measured by gel permeation chromatography (GPC). Other ratios of PDPMA to CB were prepared to synthesize the copolymer; however, it was found that the copolymer was not dissolved in water when the ratio of PDPMA to copolymer was greater than 5 mol %. Therefore, 3.5 mol % of PDPMA in the copolymer was used in the study.

**Table 1. Synthesis of pCB-PDP**

| in feed (mole fraction) CB/PDPMA | composition (mole fraction)[a] CB/PDPMA | molecular weight (g/gmol) ($M_n$) | polydispersity ratio[b] |
|---|---|---|---|
| 96/4 | 96.5/3.5 | 65 000 | 1.35 |

[a]The composition of the copolymer was determined by $^1$H NMR. [b]The molecular weight and polydispersity ratio of the copolymer were determined by GPC.

Herein, the thiol-terminated zwitterionic cross-linker was prepared through two steps. In step 1, the dithioester-terminated polymer (CTP-pCB-CTP) was prepared via RAFT polymerization using a specially designed difunctional chain transfer agent (CTA) (CTP-DEG-CTP). In step 2, the dithioester end groups were converted to thiols via a well-known aminolysis reaction.[17] The maximum UV absorbance at 318 nm is attributed to the CTP dithioester end groups on CTP-pCB-CTP. After the dithioester is converted to a thiol, the UV absorbance at 318 nm is significantly decreased (Figure 2).

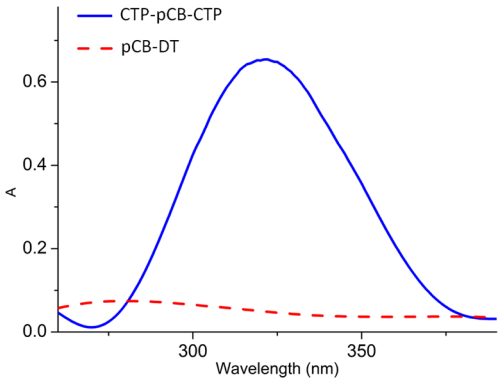


**Figure 2.** UV spectra of CTP-pCB-CTP and pCB-DT ($2 \times 10^{-4}$ mol/L) in methanol at room temperature. Dithioester end groups in CTP-pCBAA-CTP show a high signal at 318 nm. After being converted to thiols, the peak at 318 nm disappears.

This indicated that the reaction efficiency of converting dithioester to thiol is very high. For methacrylate polymer (i.e., pCBMA), when the thiol functionality generates through aminolysis of a CTP end group, there is a chance of forming a thiolactone structure via "backbiting" cyclization.[17] This cyclization results in the loss of thiol functionalities, leading to the consequent failure of hydrogel formation. Therefore, pCBAA (polycarboxybetaine acrylamide) was chosen to prepare the thiol-terminated polymeric cross-linker, pCB-DT.

**3.2. Preparation of Hydrogels.** pCB-based hydrogels were formed within a short period of time by simply mixing


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pCB-PDP copolymer and pCB-DT cross-linker in PBS or cell medium. The thiol group of pCB-DT was coupled to the pyridyl disulfide of pCB-PDP via a thiol−disulfide exchange reaction, releasing pyridine-2-thioe.[18] The existent disulfide-linked hydrogels can be degraded by a biological reducing agent, such as glutathione (GSH) or cysteine.[19] Here, various feed ratios of pCB-PDP to pCB-DT for making a hydrogel were tested. The results of CB hydrogel preparation with various copolymer (pCB-PDP)/cross-linker (pCB-DT) feed ratios are summarized in Table 2. The two concentrations of PCB-PDP were fixed at 3.5% and 7% (w/v) solutions, respectively. Three amounts, 0.5, 1, and 2 mg, corresponding to 0.5%, 1%, and 2% (w/v) of pCB-DT, were added into the copolymer solution, respectively. Before mixing with pCB-DT, the 3.5% and 7% (w/v) copolymer solutions were liquids of low viscosity. As 0.5, 1, or 2 mg of pCB-DT was added into the 3.5% copolymer solution, all conditions did not lead to a hydrogel. Therefore, we increased the copolymer concentration to 7% (w/v). Hydrogels were formed within 1 min after mixing with 1 and 2 mg of pCB-DT but not 0.5 mg of pCB-DT. The apparent gelation can be seen by inverting the tubes (Figure 1C). The 7% (w/v) of copolymer and 2% of cross-linker was chosen to make a hydrogel for cell encapsulation. For comparison, we also prepared a degradable PEGMA hydrogel following the same procedure via mixing of pPEGMA-PDP and PEG-DT (Scheme S1 and Figure S1, Supporting Information).

**Table 2. Preparation of pCB Hydrogels**

| copolymer concentration (pCB-PDP) (%, w/v) | cross-linker concentration (pCB-DT) (%, w/v) | gelation |
|---|---|---|
| 3.5 | 0.5 | no |
| 3.5 | 1 | no |
| 3.5 | 2 | no |
| 7 | 0.5 | no |
| 7 | 1 | yes |
| 7 | 2 | yes |

**3.3. Cell Encapsulation in Hydrogels.** To demonstrate the biocompatibility of the pCB materials and the process of gelation, three cell types, including NIH3T3 cells, MG63 cells, and HepG2 cells, and three cell-seeding densities, including $5 \times 10^4$, $2 \times 10^5$, and $10^6$ cells/mL, were encapsulated in the pCB hydrogels. pCB-PDP and pCB-DT were dissolved in cell suspensions and then formed cell encapsulation in the

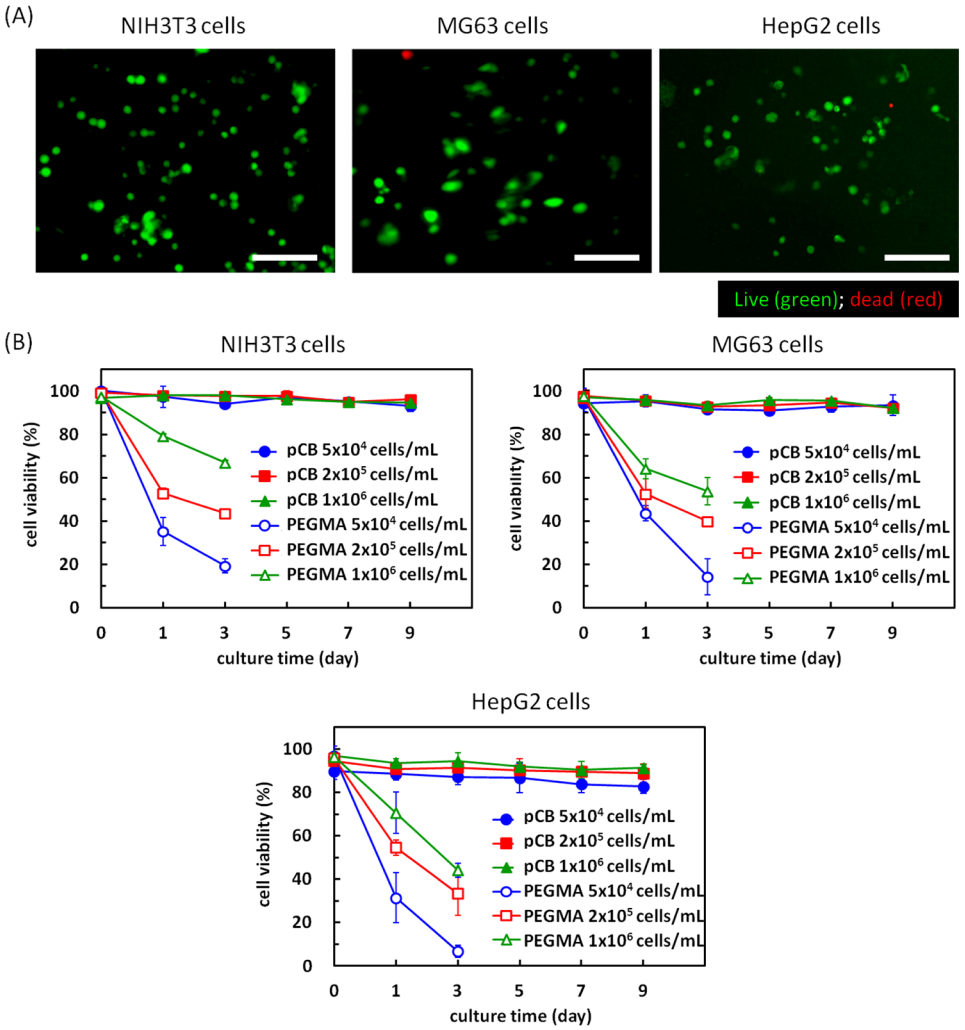


**Figure 3.** (A) Fluorescent images of NIH3T3, MG63, and HepG2 cells ($10^6$ cells/mL) encapsulated in pCB hydrogels. Viable cells were stained with Live/Dead staining. Green: live cells. Red: dead cells. Scale bar = 100 $\mu$m. (B) Viability of NIH3T3, MG63, and HepG2 cells encapsulated in pCB and PEGMA hydrogel was evaluated for different culture periods. Error bars represent standard deviation from three independent measurements.


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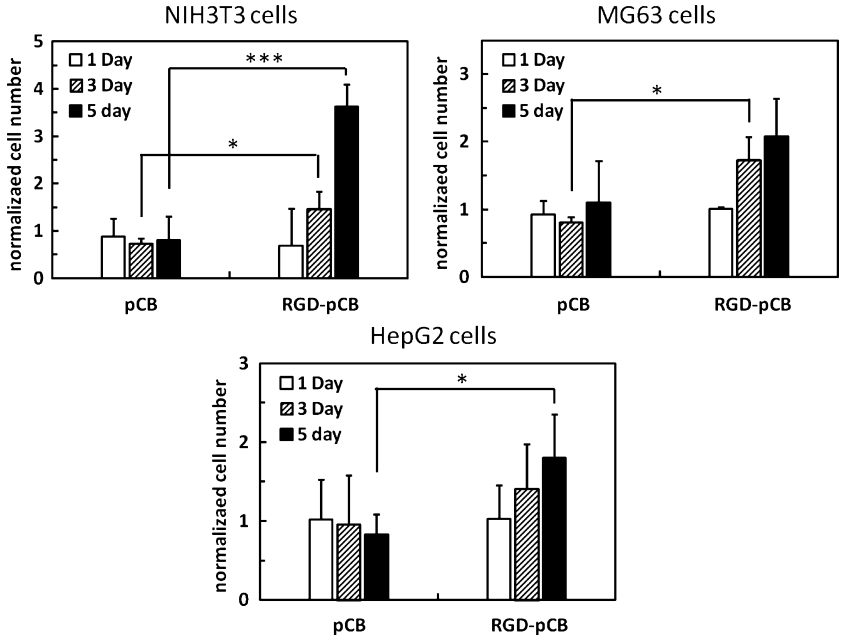


**Figure 4.** Cell proliferation in the 3D encapsulation of pCB and RGD-pCB hydrogels examined for 1-, 3-, and 5-day culture. Cell numbers are normalized against the initial feed cell number ($5 \times 10^5$ cells/mL). Error bars represent standard deviation from 4 independent measurements: (*) $p < 0.05$; (***) $p < 0.001$.

hydrogels. The viability of encapsulated cells in the hydrogels was observed under a fluorescent microscope by live/dead staining. Figure 3A shows that all three cell types are well distributed in the hydrogel, and the cells residing inside the hydrogels are fully encapsulated. The vast majority of the cells were viable within the hydrogels after gelation, and up to 95% of the cells were alive at the initial stage of cell culture (Figure 3B). The results confirmed that the thiol−disulfide exchange reaction is nontoxic for cells.

The average viability of the three cell types in the pCB hydrogels was around 90% under serum-free conditions up to 9-day culture (Figure 3B). The 9-day culture of NIH3T3 cells in the pCB hydrogels with an initial cell-seeding density of $5 \times 10^4$, $2 \times 10^5$, and $10^6$ cells/mL achieved 93.2%, 96.2%, and 94.5% viability, respectively. The viabilities of MG63 cells at 9-day culture were 93.3% ($5 \times 10^4$ cells/mL), 92.1% ($2 \times 10^5$ cells/mL), and 92.0% ($10^6$ cells/mL), respectively. The viabilities of HepG2 cells at 9-day culture were 82.7%, 88.9% ,and 91.4% with an initial cell-packing density of $5 \times 10^4$, $2 \times 10^5$, and $10^6$ cells/mL, respectively. It is worth mentioning that cells exhibited up to 90% survival even though a low cell-seeding density ($5 \times 10^4$ cells/mL) under serum-free conditions was used for cell encapsulation within the pCB hydrogels. We found that cell viability in the PEGMA hydrogel was better at high cell-seeding densities of $10^6$ cells/mL than at low cell-seeding densities of $5 \times 10^4$ cells/mL (Figure 3B). However, results for all cell types showed lower viability in the PEGMA hydrogel than in the pCB hydrogel with all initial cell-seeding densities. Generally, cell-seeding densities of $10^6$−$10^7$ cells/mL are used for cell encapsulation since cell−cell communication promotes cell survival.[20,21] It was reported previously that a minimum seeding density of $10^7$ cells/mL was necessary to maintain the survival of encapsulated $\beta$-cells in a PEG hydrogel. Bazou et al. reported that at concentrations greater than $10^6$ cells/mL HepG2 cells in alginate showed high viability.[21]

Contrasted with high cell-seeding densities, the viability of L929 cells with a low cell density ($1.8 \times 10^4$ cells/mL) was reported to be 92% for 8-day culture in a 2-methacryloyloxyethyl phosphorylcholine (MPC)-*co*-*n*-butyl methacrylate (BMA)-*co*-*p*-vinylphenylboronic acid (VPBA)/polyvinyl alcohol (PVA) (PMBV/PVA) hydrogel in the presence of serum in a microfluidic chip.[22] They attributed high cell viability to (a) the high gas and solution permeability and solute permeability of methacryloyloxyethyl phosphorylcholine (MPC)-based hydrogels and (b) the promoted mass transportation of flow when the cells were cultured in a microfluid chip.[22] However, cells cultured in medium in a 96-well microplate only maintained a 56.7% viability after 8 days and were entirely dead after 12 days.[22] These MPC-based hydrogels were composed of MPC copolymer (60% MPC unit, 30% MBA unit and 10% VPBA unit) and PVA. Here, we designed an overall zwitterionic hydrogel, pCB hydrogel, and demonstrated an outstanding viability in the pCB hydrogel without a fluid system, with low cell-seeding densities and under serum-free conditions. Cells were encapsulated in an overall zwitterionic hydrogel to better mimic three-dimensional matrices for cell growth.

**3.4. Cell Encapsulation in RGD-pCB Hydrogels.** After the three cell types were encapsulated in the pCB hydrogels, the number of cells did not obviously change throughout a 5-day culture (Figure 4). To improve cell−matrix interactions or enhance cell proliferation, it is highly desirable that the hydrogels have cell-specific adhesion. Here, we designed the RGD-decorated hydrogel to promote cell proliferation by adding a cystine-terminated RGD as a cross-linker to react with the copolymer (Figure 2B). We examined the proliferation of the three cell types in 3D encapsulation after 1-, 3-, and 5-day cultures. Figure 4 shows that the RGD-pCB hydrogel promoted proliferation of all cell types as compared to the pCB hydrogel. Particularly, both NIH3T3 and MG63 cells proliferated significantly in the RGD-pCB hydrogel after a 5-day culture. These results confirmed that incorporation of the RGD peptide


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into the pCB hydrogels promoted cell proliferation. This work suggests that it is possible to use pCB hydrogels to provide 3D aqueous environments for tissue regeneration.

The requirements of hydrogels to be used in biomedical applications include biocompatibility, biodegradability, and nonimmunogenicity.[13] Furthermore, resistance to nonspecific protein adsorption and cell adhesion is necessary for tissue scaffolds. In some applications such as islet encapsulation, nonfouling in immune protection and host reaction is even more critical.[23] pCB hydrogels provide a friendly environment for cells while also incorporating a specifically binding 3D environment for tissue engineering with incorporated functional peptides.

## 4. CONCLUSIONS

This study demonstrates the potential use of a zwitterionic hydrogel, pCB hydrogel, as a cell-encapsulating scaffold. The biodegradable pCB hydrogel was prepared by a thiol−disulfide exchange reaction with a pyridyl dithiol-containing CB copolymer (pCB-PDP) and a polyCB dithiol cross-linker (pCB-DT). The survival of cells encapsulated in the hydrogel was up to 90% after 9-day culture. Results show that cells maintained higher viability when encapsulated in the pCB hydrogels than the PEGMA hydrogels. Bioactive molecules can be easily incorporated into this pCB hydrogel system. As the hydrogel was incorporated with cysteine-terminated cross-linker, the RGD-pCB hydrogel enhanced cell proliferation in 3D hydrogel matrices.

## ■ ASSOCIATED CONTENT

### Supporting Information

Detailed description of pPEGMA-PDP synthesis and PEGMA hydrogel preparation. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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