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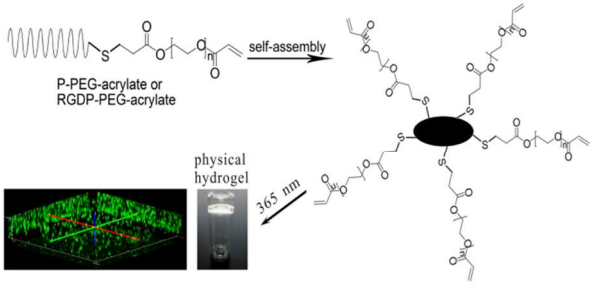
Graphical Abstract

A class physical hydrogel photo-cross-linked from the multi-arms photopolymerization monomers which based on the self-assembly of coiled-coil polypeptide P was developed.



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ARTICLE TYPE

Polypeptide-engineered physical hydrogels designed from the coiled-coil region of cartilage oligomeric matrix protein for three-dimensional cell culture

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Photo-cross-linkable physical hydrogels based on the coiled-coil region of the cartilage oligomeric matrix protein and polyethylene glycol diacrylate were designed and synthesized to mimic the natural extracellular matrix for three-dimensional cell culture. The engineered polypeptides (Pcys and RGDPcys) were modified with polyethylene glycol diacrylate to form photo-cross-linkable multifunctional macromers via the Michael-type addition reaction between the cysteine residues and acrylates. Gel formation was confirmed by rheological measurements. The swelling ratio and stability of 10% w/v RGDP-PEG-acrylate$_{6k}$ hydrogel were 38% and 15 days, respectively. Spreading and migration of encapsulated fibroblast cells were observed in these physical hydrogels, while round cells were observed in a covalent control hydrogel. In addition, rapid self-healing of these physical hydrogels can provide a flexible way to build tissue by self-assembly and bottom-up approach. The results demonstrate that such physical hydrogels are expected to have great potential applications in tissue engineering.

1. Introduction

Hydrogels are three-dimensional (3-D) polymeric networks that can absorb large amounts of water but remain insoluble due to the formation of chemical and/or physical cross-links between polymer chains.[1] During the last decade, hydrogels were increasingly attractive as an important form of scaffold in the field of tissue engineering and biomaterials because of their high moisture, softness, certain elasticity, and good biocompatibility.[2] Hydrogels are especially suitable to mimic the living organisms compared with other types of biomaterials.[3] Hydrogels are equal to the part of extracellular matrix (ECM) in terms of material characters. After absorbing water, the slippery effect reduces frictional irritation to the surrounding tissue.[4] Therefore, hydrogels have been used extensively in biomedicine and tissue engineering.[5,6]

Photo-cross-linkable hydrogels using in biomedicine and tissue engineering have attracted considerable attention owing to their rapid *in situ* formation of hydrogel under the irradiation of light.[7] Various photo-cross-linkable macromers, such as polyethylene glycol diacrylate (PEGDA),[8] polyethylene glycol dimethyl acrylate (PEGDMA),[7] polyethylene glycol divinyl sulphone (PEGVS),[9] and poly(propylene fumarate) (PPF),[10] have been synthesized and characterized. In the presence of photoinitiator, photo-cross-linked hydrogel can be formed upon exposure to visible or ultraviolet (UV) light. Therefore, photopolymerisable hydrogel has many promising properties: easily defined different sizes and shapes;[11] high photo-sensitivity against light irradiation; ignorable release of reaction heat in the process of polymerization;[10] fast hydrogel formation with a few seconds to several minutes at room temperature or physiological temperature; good solubility of polymer precursor, which makes it suitable for injection and *in situ* formation of cross-linked hydrogel;[12] With these unique advantages, photopolymerisable hydrogels have been widely applied in biomedical research. Photopolymerisable hydrogel have been prepared by physically cross-linked and chemically cross-linked methods.[13] The present photopoly-merisable hydrogels are almost chemically cross-linked hydrogels. However, the applications of chemically cross-linked hydrogels in 3-D cell culture are limited because the rate of hydrolysis or enzymolysis of chemical bonds is difficult to regulate.

Nowadays, physical hydrogels have attracted much attention as biomaterials to mimic the natural ECM.[14-16] Physical hydrogels include synthetic polymers (PNIPAAM, PEO-PPO-PEO, and PEG-PLLA, etc.),[14] natural polymers (agarose, collagen,

hyaluronic acid, gelatin, and fibrin, etc.),[5, 15] and hybrid polymer system.[16] Synthetic polymer materials lack the biological motifs and must be carefully screened for potential cytotoxicity, and the complicated compositions of natural polymer materials are difficult to obtain identical compositions.[17] As an alternative, hydrogels formed from genetically engineered polypeptides have been studied as scaffolds in tissue engineering applications.[18,19] Polypeptides are chains of various amino acids through peptide bonds on the basis of a certain order, which have excellent biocompatibility and controllable biodegradability. Compared with the synthetic materials, polypeptide-based hydrogel can better mimic the complex and dynamic natural ECM because proteins are major players in providing structural support, cell adhesion, and signal regulation in natural ECM.[20] Polypeptide-based hydrogel are expected to provide an effective way for settling the biocompatibility, functionality, and other crucial issues of biomedical materials. We have previously investigated a hydrophilic chain flanked by a terminal self-assembling leucine zipper domain and a terminal photoreactive acrylate group as photo-cross-linkable materials.[21] Although this system showed excellent biocompatibility and allowed reversible opening and closing of 3D cell migration paths, hydrogel dissolved quickly in physiological environment due to formation of intramolecular loops.

In this study, photo-cross-linkable physical hydrogels based on the coiled-coil region (named P) of the cartilage oligomeric matrix protein (COMP) and PEGDA were designed and synthesized. COMP is a noncollagenic glycoprotein present in cartilage, tendons, ligaments, and osteoblasts.[22] The engineered polypeptide Pcys and RGDPcys (each containing a C-terminal cysteine) were modified with PEGDA via the Michael-type addition reaction between the thiol and acrylate to form photo-cross-linkable macromers. The macromers with multi-arms of acrylate formed hydrogel in presence of photoinitiator and UV light. The dynamic five-stranded bundles of P domain were expected to provide the paths for spreading and migration of cells in the hydrogel. The photo-cross-linked hydrogels showed rapid self-healing characteristics. In addition, the cytotoxicity of these photo-cross-linked hydrogels was tested. These characteristics of physical hydrogels photo-cross-linked from self-assembled polypeptides will provide unique opportunities in tissue engineering.

2. Materials and Methods

2.1. Materials

Polyethylene glycol (PEG, molecular weight: 2 kDa, 6 kDa, 10 kDa) was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Acryloyl chloride was obtained from Aladdin Inc. (Shanghai, China). Photoinitiator 2-hydroxyl-1-(4-(hydroxyethoxy)phenyl)-2-methyl-1-propanone (Irgacure 2959) was a kind gift from Ciba Inc. (Tarrytown, NY). Tris(2-carboxyethyl)phosphine (TCEP), β–mercaptoethanol, isopropyl-b-D-thiogalactoside (IPTG), ampicillin, kanamycin, calcein AM, and ethidium homodimer were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Restriction endonuclease BamHI, NheI, Spe I, and T4 DNA ligase were obtained from New England Biolabs Inc. (Beijing, China). Ni-NTA separation column was purchased from Qiagen China (Shanghai) Co., Ltd. Tri-distilled water was used for all solutions.

2.2. Synthesis and purification of the polypeptide

PQE9P plasmid was a gift from Prof. David Tirrell at the California Institute of Technology Pasadena, CA. The gene encoding polypeptide Pcys was synthesized by the method of polymerase chain reaction (PCR) which used PQE9P plasmid as the template. The PQE9Pcys plasmid was constructed from Pcys segment and PQE9P plasmid through DNA recombinant manipulation. The Pcys segment and the PQE9P plasmid were digested by BamHI to yield cohesive ends. Digested Pcys segment and PQE9P vector were ligated with T4 DNA ligase to construct PQE9Pcys plasmid. The segment encoding RGD and containing NheI and SpeI restriction sites was also acquired by PCR. Digested RGD segment with NheI and SpeI was inserted into the NheI restriction site of PQE9Pcys to construct PQE9RGDPcys plasmid. The sequences of PQE9Pcys and PQE9RGDPcys were verified at the DNA sequencing core facility of Sunny Institute at Shanghai. PQE9Pcys and PQE9RGDPcys plasmid were transformed into *E. coli* strain M15, respectively. Bacterial culture was grown at 37 ºC in 1 L of 2xYT media supplemented with 50 mg L^{-1} of ampicillin and 25 mg L^{-1} of kanamycin. The culture was induced with 1 mM IPTG when the optical density at 600 nm reached 0.7-1.0. The culture was continued for an additional 4 h. Cells were harvested by centrifugation (6,000 g, 30 min) and lysed in 8 M urea (pH = 8.0). The cell lysate was centrifuged at 12,000 g for 30 min, and the supernatant was collected for purification. A 6×Histidine tag encoded in pQE9 vector allows the polypeptide to be purified by affinity chromatography on a Ni-NTA resin following the denaturing protocol given by Qiagen. The eluted fractions were dialyzed against sterile tri-distilled water for three days at room temperature, frozen, and lyophilized. The purified polypeptides were analyzed on a Bruker Reflex III reflectron MALDI-TOF mass spectrometer. Pcys (MS: 7050.3 Da, the theoretical calculation of molecular weight: 7053.8 Da), RGDPcys (MS: 8483.5 Da, the theoretical calculation of molecular weight: 8487.2 Da).

2.3. Synthesis of PEGDA

PEGDA was synthesized according to previously published methods.[8] Briefly, a solution of PEG in dichloromethane was reacted under argon with acryloyl chloride and triethylamine at an acryloyl chloride: OH molar ratio of 4:1. The product was precipitated in ice-cold diethyl ether, dried under vacuum, and stored at -20 ºC under the protection of argon. The final yields of the three products were more than 85%. High degree of

substitution (> 95%) was confirmed by ^{1}H NMR (Varian Unity spectrometer). ^{1}H NMR (300 MHz, $CDCl_3$, 25 ºC, TMS): δ = 6.2 (d, 2H), 6.0 (d, 2H), 5.7 (d, 2H), 4.1 (t, 4H), 3.4 ppm (m, 539H).

2.4. Preparation of polypeptide-PEGDA conjugates

Pcys or RGDPcys (27 μmol) was dissolved in 2.7 mL 8 M (pH = 8) urea buffer followed by addition of 300 μL TCEP (150 mM). The mixture was incubated at room temperature. After incubation for 1 h, 270 μmol PEGDA and 27 mL 8 M (pH = 8) urea were added. The pH of the mixture was adjusted to 8.0. The mixture was stirred at room temperature for 24 h under dark condition. SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 12%) was used to monitor the degree of reaction. The excess PEGDA was removed by Ni-NTA affinity column. The purified RGDP/P-PEG-acrylate was dialyzed against sterile tri-distilled water for 3 days, frozen, and lyophilized. The products were stored at -20 ºC under the protection of argon.

2.5. Preparation of covalently cross-linked RGDP-PEG-acrylate

To prepare a covalently cross-linked control hydrogel, the primary amines on the lysine and N-terminus of the polypeptide P were covalently coupled with adipic acid through 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC)/N-hydroxysuccinimide (NHS) chemistry. The mixture of 2 mM adipic acid, 20 mM EDC, and 60 mM NHS prepared in 100 mM MES (pH = 5.5) buffer was incubated at room temperature for 15 min. The pH of the solution was adjusted to 8.0, followed by addition of 0.2 mM RGDP-PEG-acrylate. The mixture was stirred in dark at room temperature for 4 h. The adipic-acid-treated RGDP-PEG-acrylate was analyzed using 12% SDS-PAGE. The molar ratio of 1:2.5 and 1:5 (RGDP-PEG-acrylate: adipic acid) were also covalently coupled according to the same method.

2.6. Hydrogel preparation and disassembly

Solutions containing various concentrations (3%-11% w/v) of P-PEG-acrylate or RGDP-PEG-acrylate and 0.2% photoinitiator Irgacure 2959 were prepared in PBS. The pH of each solution was adjusted to 7.4, and the solutions were exposed to UV light (365 nm, 12.5 mW cm^{-2}) for 5 min to form hydrogels. To determine which type of the cross-links (chemical or physical cross-link) were in hydrogels, 1 mL 8% (w/v) P-PEG-acrylate solution containing 0.2% 2959 was prepared in two transparent glass bottles. After hydrogel formation under UV, 8 M urea (pH 7.4) and 0.01 M PBS (pH 7.4) were added on the hydrogel, respectively. Samples were shaken at room temperature for 10 h to examine whether the hydrogel dissolved or not. The solubility of covalently cross-linked RGDP-PEG-acrylate hydrogel was also examined.

2.7. Self-healing of photo-cross-linked hydrogels

To test whether these photo-cross-linked hydrogels have the capability of self-healing, two P-PEG-acrylate hydrogels with same size were prepared. To observe conveniently, one of the hydrogel was soaked in PBS containing 0.01 M rhodamine 6G for 10 min. The hydrogels were brought into contact with each other without application of any external force. The hydrogel was clipped by tweezer in different period to determine whether two hydrogels have healed together.

2.8. Swelling ratio and stability of hydrogels

The P-PEG-acrylate hydrogels of different concentrations (3%-11% w/v) were prepared and transferred into a new 1.5 mL EP tube, respectively. M1 was the weight of empty EP tube, and M2 was the total weight of EP tube and hydrogel. Four hundred microliters of PBS was added on the hydrogel and renewed every 12 h. After two days, PBS was removed, and the hydrogel was weighed (M3) after quick blotting with filter paper. The swelling ratio (R) of the hydrogels was calculated using the following equation R = (M3-M2) / (M2-M1) × 100%.[23] The stability of hydrogels was defined as the time needed for the hydrogel to disappear. The experiments were performed in triplicate.

2.9. Scanning electron microscopy (SEM)

RGDP-PEG-acrylate$_{6k}$ hydrogels (6% and 8% w/v) were prepared, frozen overnight at -80 ºC, and lyophilized for 3 days until their containing water was completely sublimated. The lyophilized hydrogels were fractured carefully in liquid nitrogen. The fracture surfaces of the hydrogels were coated with gold for 30 s, and the interior morphology of the hydrogels was observed by a SEM (Nova NanoSEM450).

2.10. Rheological measurements

The solutions of P-PEG-acrylate$_{6k}$ (8% and 10% w/v) and adipic-acid-treated P-PEG-acrylate$_{6k}$ (8% w/v) containing 0.2% photoinitiator Irgacure 2959 were prepared. The pH of each solution was adjusted to 7.4, and the solution was added between two glass slides separated with 1.1 mm thick spacer, followed by being exposed under UV light (365 nm, 12.5 mW cm^{-2}) for 5 min to form hydrogel. The cover glass was removed carefully, and the hydrogel was transported to the parallel plate of HR-2 discovery hybird rheometer to perform the rheological test. A strain sweep test (0-10%) at an oscillatory frequency of 10 rad s^{-1} were performed to reveal the linear viscoelastic regime, followed by a frequency sweep test performed at a strain value in the linear regime. Correlation parameters: 20 mm parallel plate, 1,000 μm gap, 37 ºC, 1% strain, and 100-0.1 rad s^{-1} angular frequency. All measurements were repeated three times.

2.11. 2-D cell adhesion assay

To observe the adhesion of fibroblasts (NIH 3T3) on hydrogels photo-cross-linked from P-PEG-acrylate, RGDP-PEG-acrylate, and adipic-acid-treated RGDP-PEG-acrylate (all 10% w/v, 1.1 mm thick, prepared in pH 7.4 PBS), cells were seeded on the surface of each hydrogel in serum-free DMEM supplemented with penicillin-streptomycin (100 units mL^{-1}) at a density of 2.5 ×

10^5 cells cm^{-2} and allowed to adhere for 2 h at 37 ºC, 5% CO_2 in cell incubator. Hydrogels were washed with PBS three times. Each sample was stained with the calcein AM/ethidium homodimer for 20 min and examined with a 10× objective on an inverted fluorescent microscope (Olympus IX71, Japan) equipped with a cool color charge-coupled device (CCD) (Pixera Penguin 150CL, USA).

2.12. 3-D encapsulation of fibroblast

The solutions of RGDP-PEG-acrylate (8% w/v) and adipic-acid-treated RGDP-PEG-acrylate (8% w/v) were prepared respectively in 100 μL serum-free DMEM containing 0.2% Irgacure 2959 and supplemented with penicillin-streptomycin (100 units mL^{-1}). Fibroblasts (NIH 3T3, 2.5 × 10^4 cells) were dispersed in RGDP-PEG-acrylate and adipic-acid-treated RGDP-PEG-acrylate solutions and transferred to the 35 mm glass bottom culture dishes (MatTek) followed by exposure them to the long-wavelength UV light for 5 min. DMEM containing 1% penicillin-streptomycin antibodies and 10% fetal bovine serum were added to the surface of the hydrogels. Encapsulated fibroblasts were cultured at 37 ºC, 5% CO_2 in cell incubator. The samples were stained with the calcein AM/ethidium homodimer for 20 min and examined with a 10× objective on the Olympus FV1000 confocal microscopy. XYZ scanning mode was adopted for 2-D and 3-D images of cells in hydrogels.

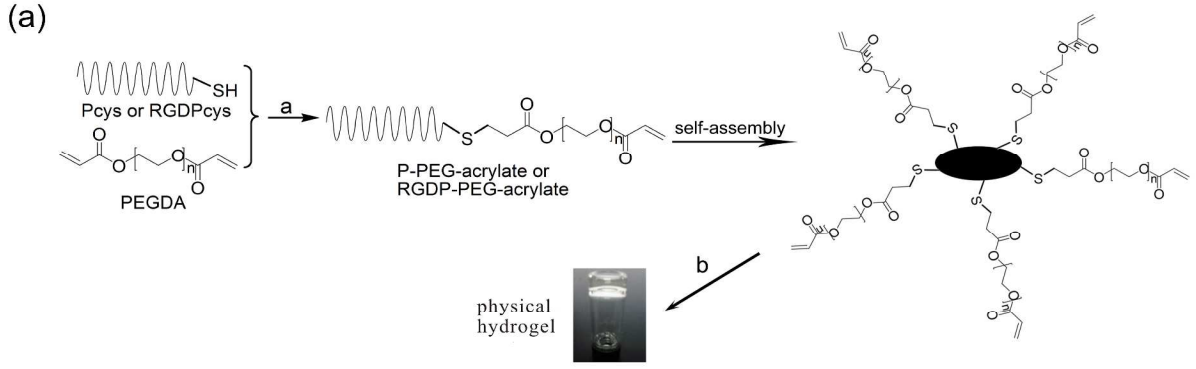
2.13. Cytotoxicity

RGD-PEG-acrylate$_{6K}$ (10% w/v) photo-cross-linked hydrogels were prepared in 96-well plate. NIH 3T3 fibroblasts were seeded on the hydrogel at 5000 cells per well and cultured at 37 ºC, 5% CO_2 in cell incubator for 48 h. Tests under all conditions were run in triplicate. Cells were stained with the calcein AM/ethidium homodimer for 20 min and examined on an inverted fluorescent microscope (Olympus IX71, Japan). Stained cells were trypsinized and counted using a hemocytometer.

3. Results and Discussion

3.1. Design of the hydrogel

To prepare photo-cross-linkable physical hydrogels, coiled-coil polypeptide P which can self-assemble into pentamer was chosen to act as physical junctions in hydrogels. The polypeptide P as the major component of physical hydrogel has been thoroughly investigated.[24] Acryloyl group is usually used to prepare the photo-cross-linked hydrogels because monomers with acryloyl groups can photo-cross-link in the presence of long-wavelength UV light and photoinitiator. PEG with high biocompatibility, nonimmunity, and water-solubility is often used in tissue engineering application. In order to prepare photo-cross-linkable macromer, one cysteine residue containing a free thiol was introduced at the C-terminus of polypeptide by genetic engineering method. Polypeptides (Pcys or RGDPcys) reacted with excessive PEGDA through the Michael-type addition reaction to obtain the photo-cross-linkable macromer (P-PEG-acrylate or RGDP-PEG-acrylate) having PEG flanked by a photoreaction acrylate group and a self-assembly polypeptide P. As known, monomer with only one acrylate group usually forms one dimensional polymer, and formation of 3-D polymeric networks require multi-branches or cross-linker. P-PEG-acrylate or RGD-PEG-acrylate macromer containing only one acrylate group can form multi-branched macromer through physical assembly of polypeptide P. Under the condition of long-wavelength UV light and photoinitiator, the multi-branched monomers cross-link and form physical hydrogels. The design and amino acid sequences of the polypeptide Pcys and RGDPcys are shown in Scheme 1. Because of the absence of



Scheme 1 (a): Illustration of the formation of photo-cross-linkable physical hydrogels from self-assembled multi-functional macromers. a: pH 8, 8 M urea buffer, 1.5 mM TCEP; b: 365 nm UV light, 5 min. (b): Amino acid sequences of Pcys and RGDPcys.

toxic cross-linker and byproduct in preparation of these physical hydrogel, these physical gels should be biocompatible. The P-PEG-acrylate or RGDP-PEG-acrylate macromer was verified by using 12% SDS-PAGE (Fig. 1). The conjugation reaction between each polypeptide and PEGDA was performed at a 1:10 molar ratio. Far-UV circular dichroism (CD) was employed to determine the secondary structure of polypeptide RGDPcys and RGDP-PEG-acrylate (Fig. S1). The result confirmed that modification of RGDPcys with PEGDA did not alter the coiled-coil structure of the polypeptide. Compared with traditional synthetic polymers, these physical hydrogels containing engineered polypeptide P as the major component have not only the advantages of the photo-cross-linkable hydrogel, but also similar components with natural ECM. Sequences of interest, such as binding domains and enzyme cleavage sites can be incorporated into engineered polypeptides through the flexibility of recombinant DNA technology.[25] In addition, in order to promote adhesion and spreading of cells in the hydrogel, a RGD cell-binding domain was successfully incorporated into the N-terminus of polypeptide P.[26] The dynamic polypeptide P in these physical hydrogels is expected to provide paths for cell spreading and migration.[24, 27]

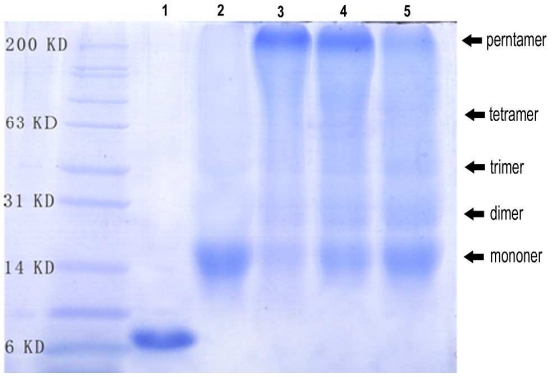
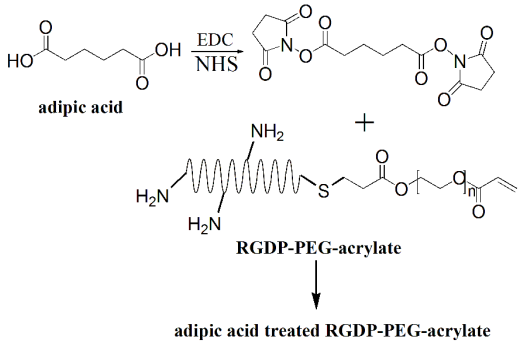


Fig. 1 SDS-PAGE of RGDP-PEG-acrylate and adipic-acid-treated RGDP-PEG-acrylate. Lane 1, RGDPcys; 2, RGDP-PEG-acrylate$_{6k}$; 3-5, RGDP-PEG-acrylate$_{6k}$ treated with adipic acid at various molar ratios of adipic acid to RGDP-PEG-acrylate$_{6k}$ (lane 3, 10:1; lane 4, 5:1; lane 5, 2.5:1).

3.2. Covalent cross-linking of RGDP-PEG-acrylate

To prepare negative control hydrogel containing covalently cross-linked polypeptide P, RGDP-PEG-acrylate was treated with adipic acid through EDC/NHS coupling chemistry (Scheme 2). The carboxylates of adipic acid reacted with NHS in the presence of EDC to synthesize activated NHS ester which subsequently reacted with the primary amines in the polypeptide portion of RGDP-PEG-acrylate. Different proportions of the coupling between NHS-activated ester of adipic acid and RGDP-PEG-acrylate macromer were investigated, and the efficiency of the coupling was analyzed with 12% SDS-PAGE. As shown in Fig. 1, five unequally intense bands of the treated RGDP-PEG-acrylate macromer indicated the presence of pentamers, tetramer, trimer, dimer, and monomer, which was consistent with previous reports.[21] With increasing the molar ratio of the NHS-activated ester of adipic acid and the RGDP-PEG-acrylate macromer (from 2.5 to 10), the ratio of pentamers increases, and the ratio of the monomer decreases. When the ratio of the NHS-activated ester of adipic acid and the RGDP-PEG-acrylate macromer was 10, almost no monomers were detected. Therefore, in this study, the ratio of 10 was chosen to prepare covalent cross-linking of RGDP-PEG-acrylate. The covalently cross-linked RGDP-PEG-acrylate macromer also formed hydrogel in the presence of photoinitiator under 365 nm UV.



Scheme 2 Preparation of adipic acid treated RGDP-PEG-acrylate.

3.3. Physical hydrogel or chemical hydrogel

To verify whether the photo-cross-linked hydrogel was physical gel in nature, RGDP-PEG-acrylate hydrogel was immersed in an 8.0 M urea and PBS, respectively. As expected, the hydrogel completely dissolved after 10 h shaking in the 8.0 M urea due to the disruption of the secondary structure of polypeptide P. In contrast, the hydrogel immersed in PBS retained its integrity (Fig. 2). The result indicates that these hydrogels are physical gels regarding the physical bonds formed by self-assembly of the polypeptide P as the junctions. The hydrogel formed from adipic-acid-treated RGDP-PEG-acrylate retained its integrity in 8 M urea over one week, further demonstrating that the untreated photo-cross-linked gels are physical but not chemical hydrogels.

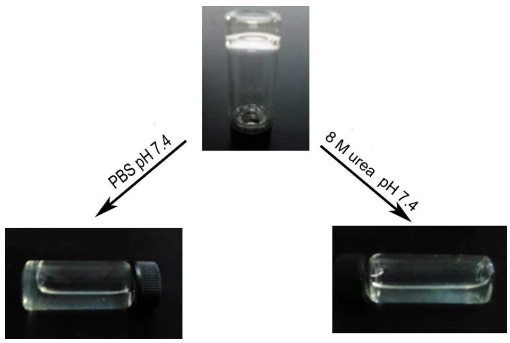


Fig. 2 Disassembly of photo-cross-linked RGDP-PEG-acrylate$_{6k}$ hydrogel (8% w/v) in 0.01M PBS (pH = 7.4) and 8 M urea (pH = 7.4) at 37 ºC.

3.4. Self-healing of hydrogels

To examine whether this photo-cross-linked hydrogel has the self-healing capability, two hydrogels were put together without any external force. In order to have a better observation, one of hydrogel was stained to yellow with rhodamine 6G. We observed

that two hydrogels weld rapidly after half an hour (Fig. 3), and the rhodamine 6G in the stained hydrogel diffused rapidly into the unstained hydrogel. The result indicates that this kind of hydrogel not only has the self-healing capability but also own proper permeability for transport for oxygen, essential nutrients, and metabolic waste. Quick exchange of nutrition and metabolites is prerequisite of biomaterials for tissue engineering application. The self-healing capability of the photo-cross-linked hydrogel may be due to the dynamic exchange of the polypeptide P at their interface of hydrogels.[24] To further determine the effect of concentration on healing, the self-healing time of hydrogel with various concentrations was investigated. The results show that the self-healing time becomes shorter with lower concentration. The self-healing time depends strongly on the concentration of hydrogel, demonstrating that the dynamic exchange rate of the self-assembly polypeptide P in the physical hydrogel relates to the concentration of macromer. As shown in Fig. 3, the healed hydrogel exhibits a certain mechanical strength. Hydrogels with reversible or dynamic physical bonds will not only manage external damages and repair themselves as self-healing materials but also gain multi-responsive properties to environmental stimuli.[28] Thus, the cells fixed in these hydrogels can be injected into the targeted positions for therapeutic and 3-D bioprinting applications.[29]

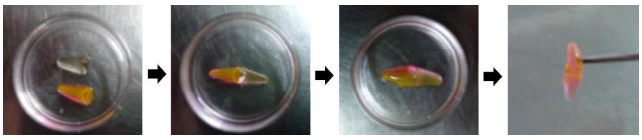


Fig. 3 Self-healing of photo-cross-linked RGDP-PEG-acrylate$_{6k}$ hydrogel (8% w/v) at 25 ºC.

3.5. Swelling ratio and stability of hydrogels

The swelling ratio and stability of RGDP-PEG-acrylate$_{6k}$ hydrogel with different concentrations are shown in Fig. 4. The actual swelling ratio of physical gel is larger than the experiment result because a part of hydrogel will dissolve in PBS during the experiment, especially at low concentration. As shown in Fig. 4a, the swelling ratio depends on the concentration of RGDP-PEG-acrylate$_{6k}$. For example, the swelling ratio of 3% w/v and 11% w/v hydrogel are 19% and 38%, respectively. In addition, the swelling ratio of the hydrogels is a large variation at low concentrations and varies rarely at high concentrations. For instance, the swelling ratio increases 17% when the concentration increases from 3% w/v to 5% w/v. In contrast, the swelling ratio increases only 1% when the concentration increases from 5% w/v to 11% w/v. This difference may be attributed to the poor stability of low concentration hydrogel.

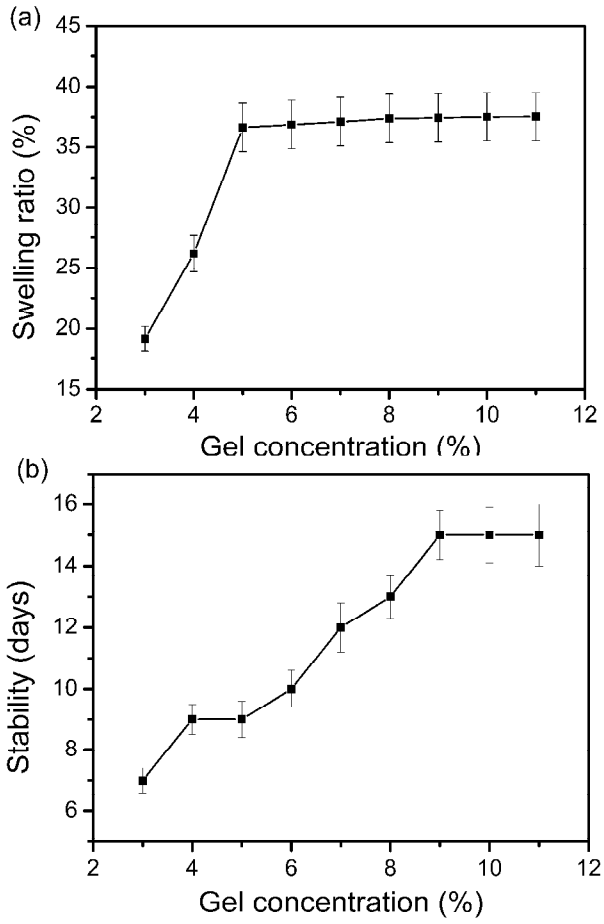


Fig. 4 Swelling ratio (a) and stability (b) of photo-cross-linked RGDP-PEG-acrylate$_{6k}$ hydrogels at 37 ºC.

The biomaterials used in tissue engineering need the capability of high swelling ratio and excellent stability. Therefore, the stability of the photo-cross-linked hydrogel was also tested. As shown in Fig. 4b, the stability of the photo-cross-linked hydrogel increases gradually from 7 days to 15 days with increasing the concentration until the concentration reaches 9% w/v. The stabilities are almost unchanged when the concentrations are above 9% w/v. Compared with our previous photo-cross-linked hyrogel based on leucine zipper A, hydrogel based on P domain showed significantly improved stability.[21] Hydrogel based on leucine zipper A forms readily intramolecular loops because leucine zipper domain adopts an antiparallel orientation.[24] The stability of these physical hydrogels can be regulated by the rate of dissolution of hydrogel, hydrolysis of ester bonds, and degradation of polypeptide. The stability of the covalently cross-linked RGDP-PEG-acrylate hydrogel achieves more than two months, suggesting that the stability of these photo-cross-linked physical hydrogels are mainly controlled by the rate of dissolution of hydrogel. The stability data of physical hydrogel are closely related to the volume of the gel and the volume of added PBS. As the stability of these physical hydrogel is mainly decided by the dissolving rate of gel, changing the concentration of gel will provide a convenient method to tune stability of gel. In

addition, the biodegradation products of polypeptide and PEGDA can be excluded through kidney without concentration in the human body.[30] The swelling ratio and stability of hydrogels were also tuned by altering the length of PEG (the swelling ratio and stability of the PEG_{2k} and PEG_{10k} not shown).

3.6. Hydrogels morphology

The interior of photo-cross-linked RGDP-PEG-acrylate$_{6k}$ hydrogels is shown in Fig. 5**.** It can be seen that hydrogels have a uniform distribution of interior pore size. The pore sizes of 8% hydrogel (Fig. 5a) are a little bit smaller than that of 6% (Fig. 5b). The mentioned pores have relatively large sizes with the diameter about 20 μm. The large pore sizes make these hydrogels act for the permeation of nutrients, exchange of oxygen and carbon dioxide, discharge of metabolites and so on, which can provide a comfortable environment for cells growth and proliferation. The rapid permeation of rhodamine 6 G (Fig. 3) further demonstrates large pores in the hydrogel. The result of 6% w/v hydrogel with larger pore size than 8% w/v is consistent with the results that the stability and mechanical strength of 6% w/v is less than 8% w/v.

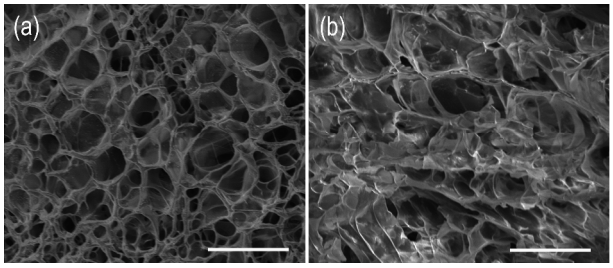


Fig. 5 SEM of photo-cross-linked RGDP-PEG-acrylate$_{6k}$ hydrogel. (a): 8% w/v; (b): 6% w/v. The scale bars are 40 μm.

3.7. Rheology of hydrogels

Hydrogel formation was further confirmed using small amplitude oscillatory shear experiments. For each hydrogel, a strain sweep test (0-10%) was performed at an oscillatory frequency of 10 rad s^{-1} first. It was revealed that a strain of 1% is in the linear viscoelastic range for all tested hydrogels. The linear viscoelastic behavior of hydrogels was characterized by oscillatory frequency sweep measurements. The result of the storage modulus (G') and loss modulus (G'') is shown in Fig. 6. With increasing the concentration of hydrogel, both G' and G'' increase gradually. The G' is larger than the G'' at high frequency. The G' of 8% w/v hydrogel is 4,300 Pa, and the G' of 10% w/v hydrogel is 7,200 Pa at an angular frequency of 10 rad s^{-1}. The G' of adipic-acid-treated hydrogel changes rarely compared with the untreated hydrogel at the same concentration. The G' and G'' of hydrogel can be tuned not only through the concentration of macromer, but also by altering the length of PEG (data of the G' and G'' of the PEG_{2k} and PEG_{10k} not shown). Changing stiffness of materials has proven to be a useful strategy for studying 2-D cell adhesion or 3-D cell growth.[31] The G' and G'' of no adipic-acid-treated RGDP-PEG-acrylate hydrogel can crossover (transition point of gel-liquid), while the G' and G'' of adipic-acid-treated RGDP-PEG-acrylate hydrogels have no cross point in 0.1-100 rad s^{-1}. The result gives the further evidence that RGDP-PEG-acrylate hydrogel is physical gel, and adipic-acid-treated RGDP-PEG-acrylate hydrogel is chemical gel. In addition, with increasing the concentration of macromer, the cross point shifts to the direction of low angular frequency, indicating that the higher concentration leads to stronger viscosity and better stability.

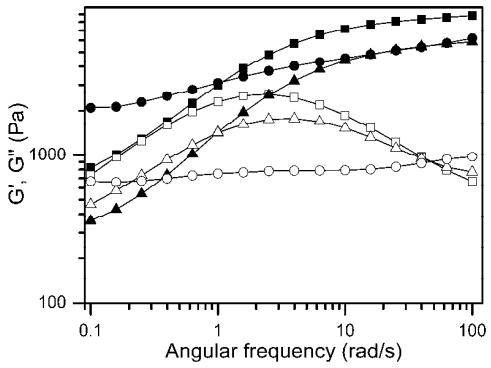


Fig. 6 Rheological oscillatory shear measurements of photo–cross-linked RGDP-PEG-acrylate$_{6K}$ hydrogels at various concentrations (10% w/v, squares; 8% w/v, triangles) and a covalently cross-linked control hydrogel at 8% w/v (circles). Storage modulus G': filled symbols; loss modulus G'': open symbols. Measurements were performed at 1% strain, pH 7.4, and 37 °C.

3.8. 2-D cell adhesion assay

To study 3-D cell culture in the photo-cross-linkable hydrogel, the investigation of cell adhesion and spreading on the surface of hydrogel (2-D cell culture) should be conducted firstly. 2-D cell culture is a powerful tool to study the basic mechanism of the force between cells and substrate. When the cells adhere to the substrate, the integrins relying on the cell membrane link to the ECM. The change of the connection between the integrins and ECM leads to the realignment of cytoskeleton, spreading, and migration of cells on the surface of substrate.[32-34] Previous studies have shown that the RGD sequences consisting of arginine, glycine, and aspartic acid exist in a variety of ECM.[35] RGD sequence could specifically combine with 11 kinds of integrins and effectively promote cells adhesion and spreading in biomaterials. To promote the adhesion and spreading of cells on the photo-cross-linked hydrogel, a RGD cell-binding domain was successfully incorporated into the N-terminus of polypeptide P. Cell adhesion on the hydrogels photo-cross-linked from P-PEG-acrylate, RGDP-PEG-acrylate, and adipic-acid-treated RGDP-PEG-acrylate are shown in the Fig. 7. Few NIH 3T3 cells adhered on the surface of P-PEG-acrylate hydrogel (Fig. 7a), while significant cell adhesion was observed on the surface of RGDP-PEG-acrylate (Fig. 7b) and adipic-acid-treated RGDP-PEG-acrylate hydrogel (Fig. 7c). These results suggest that the adhesion and spreading of cells on hydrogels might be mediated by the RGD sequence in the materials. In addition, the activity of RGD was not affected after treatment with adipic acid. Sequences of interest, such as binding domains and enzyme cleavage sites, can be incorporated into engineered polypeptide P because the

polypeptide P is biosynthesized by gene engineering. Biological functions of fused ligands can also be studied through 2-D cell culture.

The ability to control cell-binding ligand type and density, and thus to study their effect on 2-D cell adhesion and 3-D cell growth and migration, has proven to be a useful strategy for understanding specific cell-material interactions and the basic mechanism.[36-38] The precursor of photo-cross-linked hydrogel facilitated making hydrogel with similar mechanical properties and tuning the RGD ligand density. Because the polypeptide P is biosynthesized by gene engineering, the modular design of our engineered polypeptide facilitates the creation of identical P that differ only in the numbers of bioactive RGD ligand. By designing different number of cell-adhesive RGD ligands into each polypeptide P and by maintaining a constant polypeptide concentration with the engineered matrices we are able to tune the density of cell-adhesive RGD ligand in hydrogels with similar mechanical strength. This can provide a distinct advantage over performing such studies with natural protein-base hydrogels such as collagen and elastin.

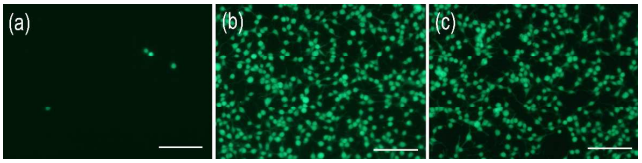


Fig. 7 Adhesion of NIH 3T3 fibroblasts on 10% w/v hydrogels of (a) P-PEG-acrylate$_{6k}$; (b) RGDP-PEG-acrylate$_{6k}$; and (c) adipic-acid-treated RGDP-PEG-acrylate$_{6k}$. The scale bars are 200 μm.

3.9. 3-D encapsulation of fibroblast in hydrogels

Building a 3-D complex that formed from cells and biomaterials is a technically challenging but key focus for studies of tissue engineering.[39] To investigate the situation and mechanism of cells growth and migration in the photo-cross-linkable hydrogel, NIH 3T3 fibroblasts were encapsulated into the photo-cross-linked hydrogels formed from RGDP-PEG-acrylate and adipic-acid-treated RGDP-PEG-acrylate (8% w/v, prepared in DMEM containing 10% fetal bovine serum). After 26 h culturing, the confocal images of encapsulated cells in the photo-cross-linked hydrogel are shown in Fig. 8. NIH 3T3 fibroblasts encapsulated in the RGDP-PEG-acrylate gel spread and migrate freely (Fig. 8a and g). NIH 3T3 fibroblasts spreading and migration occurred in multiple planes to the maximum imaging depth of approximately 200 μm (Fig. 8g), indicating that cell adhesion and spreading were not limited to the underlying culture dish. Previous studies have demonstrated that cells may capable of sensing on the underlying rigid substrate within several microns.[40] Meanwhile, cells encapsulated in the adipic-acid-treated RGDP-PEG-acrylate gel present a round shape (Fig. 8d and h). A possible reason for this is that the polypeptide P in RGDP-PEG-acrylate hydrogel form a dynamic construction through non-covalent self-assembly to provide the necessary paths for the spreading and migration of cells. Compared with untreated physical hydrogel, the adipic-acid-treated RGDP-PEG-acrylate control hydrogel is chemical gel which would not provide fast spreading and migration condition. Cell fast spreading and migration (26 h) indicates that cells can migrate and spread well through dynamic paths formed by the polypeptide P, and the dynamism of pentamer is continuous and fleet.

The migration of cells within a 3-D matrix is an important component of many cellular processes both *in vivo* and *in vitro*. Tissue formation during embryonic development, wound healing, and immune responses all require the orchestrated movement of cells in particular directions to specific locations. However, first of all cells migration across 3-D biomaterials have to deal with the physical obstruction pose by the matrix itself because the porosity of 3-D matrix is significantly smaller than the average cell size.[17,41] Therefore, some strategies of cells migration within 3-D chemically cross-linked hydrogel are to utilize the passive hydrolysis or enzymolysis of chemical bonds. However, the hydrolysis rate is hard to control in spatial and temporal synchrony with cellular infiltration and migration because hydrolysis rate of whole materials is same. Cells migration by the enzymolysis process only can be controlled in spatial through action of the protease.[8] In the present study, we use dynamic polypeptide P in the absence of proteases physical gel to provide the paths for cell migration which does not depend on spatio-temporal restriction. The cell in the proteolytically degradable hydrogel started to spread and migrate through the hydrogel matrix after three days,[8] while cell spreading and migration appeared after 26 h in RGDP-PEG-acrylate hydrogel. Therefore, the key advantage of these physical hydrogels over existing systems would be the fast migration. In addition, the mechanical strength of the polypeptide-based physical hydrogel will not be affected because the assembly of coiled-coil polypeptide P is reversible.

Tissue is not a simple heap up of cells, but an integrated complex with special functions formed by a certain regular arrangement of cells. The interaction between cells and well-organized arrangement of cells of different types are the key issue in the research of tissue engineering. For example, the vascular system is a well-organized arrangement of smooth muscle cells and endothelial cells.[42] Traditional biomaterials are only used to culture a type of cells, even if they are used to culture a variety of cells at the same time, the exact position of cells in the hydrogel is not be regulated. However, the rapid self-healing characteristic of the photo-cross-linkable hydrogel containing the polypeptide P made it possible that different types of cells are organized to form tissue. Various types of cells might be encapsulated into different shapes of microscale hydrogels (microgels), and the cell-laden microgels with different shapes will form a complex by the way of self-assembly and bottom-up.[12,43]

Additionally, previous studies have shown that coiled-coil polypeptide P assembles into a pentameric cylinder-like and hydrophobic core that is 7.3 nm long with a diameter of 0.2-0.6

nm, and it can specifically load some hydrophobic drugs, such as vitamin D_3, trans retinol (ATR), and curcumin (CCM).[22] Loading hydrophobic drugs in hydrogel is a difficult work in tissue engineering, and the photo-cross-linked hydrogel containing the polypeptide P can provide an easy way to load hydrophobic drugs for controlled release.

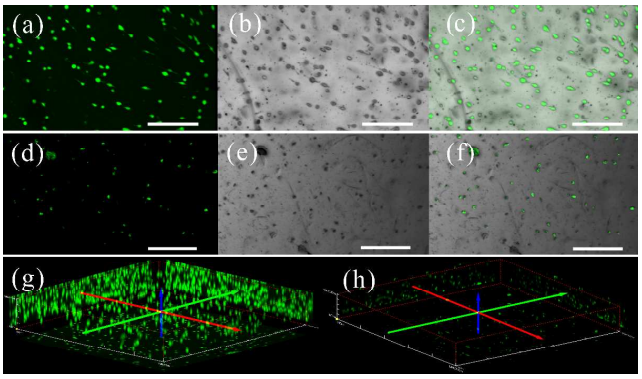


Fig. 8 Cell growth in 3-D photo-cross-linked hydrogels. Confocal fluorescence images of NIH 3T3 fibroblasts encapsulation in 8% RGDP-PEG-acrylate$_{6k}$ hydrogel (a)-(c) and adipic-acid-treated RGDP-PEG-acrylate$_{6k}$ (d)-(f). 3-D Confocal images of NIH 3T3 fibroblasts encapsulation in 8% RGDP-PEG-acrylate$_{6k}$ hydrogel (g), and adipic-acid-treated RGDP-PEG-acrylate$_{6k}$ hydrogel (h). Scale bar in (a)-(f) is 200 μm.

3.10. Cytotoxicity

To test the toxicity of the photo-cross-linked hydrogels to mammalian cells, NIH 3T3 fibroblasts were cultured on the surface of RGDP-PEG-acrylate hydrogel. Cells were seeded direct in 96-well cell culture plate as a control group. After 48 h of culture, only a few dead cells (live-dead assay) were observed. Although cells in test wells are less than those in the control wells, the amount of fibroblasts in test wells increased 1.6 times after 48 h of culture *in vitro* (Fig. 9). These results suggest that photo-cross-linked hydrogels containing P domains are not toxic to mammalian cells *in vitro*.

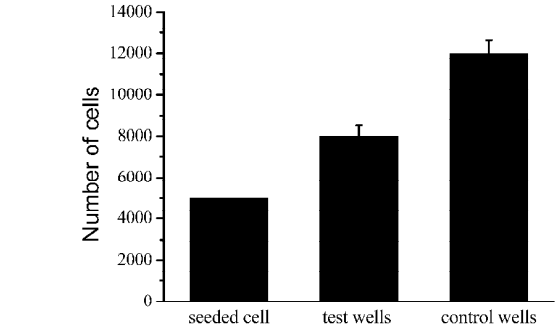


Fig. 9 Cytotoxicity of photo-cross-linked RGDP-PEG-acrylate$_{6k}$ hydrogel (8% w/v). NIH 3T3 fibroblasts were cultured on the surface of hydrogel for 48 h.

4. Conclusions

Polypeptide-engineered physical hydrogels photo-cross-linked from the multi-arms photopolymerization macromers which based on the self-assembly of coiled-coil polypeptide P were designed and synthesized. These hydrogels show high swelling ratio and stability. The dynamic pentamer structures formed by the polypeptide P in hydrogels endow the character of self-healing. The physical bonds formed by self-assembly of α-helical structure of polypeptide allow reversible opening and closing of 3D migration paths for spreading and migration of cells in these hydrogels. The self-healing characteristic of the photo-cross-linkable hydrogels gives these materials to conveniently build tissues through the way of self-assembly and bottom-up. In addition, the main components of these physical hydrogels are biosynthetic polypeptides which are similar to the natural tissue. Therefore, this kind of hydrogel is expected to have favorable perspectives in tissue engineering as artificial scaffolds to mimic the natural ECM.

Acknowledgments

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