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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 have become increasingly attractive as an important form of scaffold in the fields of tissue engineering and biomaterials because of their high moisture, softness, elasticity, and good biocompatibility.[2] Hydrogels are especially suitable to mimic living organisms compared with other types of biomaterials.[3] Hydrogels are equivalent to the extracellular matrix (ECM) in terms of material characteristics. 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 for use in biomedicine and tissue engineering have attracted considerable attention owing to their rapid *in situ* formation of hydrogel under light irradiation.[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 photo-initiator, photo-cross-linked hydrogels can be formed upon exposure to visible or ultraviolet (UV) light. Therefore, photo-polymerisable hydrogels have 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 from a few seconds to several minutes at room temperature or physiological temperature; good solubility of polymer precursors, which makes it suitable for injection and *in situ* formation of cross-linked hydrogels.[12] With these unique advantages, photopolymerisable hydrogels have been widely applied in biomedical research. Photopolymerisable hydrogels have been prepared by physical cross-linking and chemical cross-linking methods.[13] The present photopolymerisable hydrogels are almost all 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, PEG–PLLA, *etc.*),[14] natural polymers (agarose, collagen, hyaluronic acid, gelatin, fibrin, *etc.*),[5,15] and hybrid polymer systems.[16]

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Synthetic polymer materials lack the biological motifs and must be carefully screened for potential cytotoxicity, and the complicated compositions of natural polymer materials make it 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 hydrogels 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 hydrogels 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, the hydrogel dissolved quickly in physiological environment due to the 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 polypeptides 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 multiple acrylate arms formed hydrogels in the presence of photoinitiator and UV light. The dynamic five-stranded bundles of the P domain were expected to provide paths for the 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-β-D-thiogalactoside (IPTG), ampicillin, kanamycin, calcein AM, and ethidium homodimer were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Restriction endonuclease BamHI, NheI, SpeI, 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 the Pcys segment and the PQE9P plasmid through DNA recombinant manipulation. The Pcys segment and the PQE9P plasmid were digested by BamHI to yield cohesive ends. The digested Pcys segment and PQE9P vector were ligated with T4 DNA ligase to construct the PQE9Pcys plasmid. The segment encoding RGD and containing NheI and SpeI restriction sites was also acquired by PCR. The digested RGD segment with NheI and SpeI was inserted into the NheI restriction site of PQE9Pcys to construct the PQE9RGDPcys plasmid. The sequences of PQE9Pcys and PQE9RGDPcys were verified at the DNA sequencing core facility of Sunny Institute at Shanghai. PQE9Pcys and PQE9RGDPcys plasmids were transformed into *E. coli* strain M15, respectively. Bacterial culture was grown at 37 °C in 1 L of 2× YT medium 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 (6000 *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 the 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 °C 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 °C, 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 in the dark. SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 12%) was used to monitor the degree of reaction. The excess PEGDA was removed by a 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 °C 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 the dark at room temperature for 4 h. The adipic acid-treated RGDP-PEG-acrylate was analyzed using 12% SDS-PAGE. The molar ratios 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 cross-links (chemical or physical) 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 each added to one of the hydrogel samples. Samples were shaken at room temperature for 10 h to examine whether the hydrogel dissolved or not. The solubility of a 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 the 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 hydrogels were clipped with tweezers at different times 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 new 1.5 mL EP tubes. M1 was the weight of an empty EP tube, and M2 was the total weight of the EP tube and hydrogel. Four hundred microliters of PBS were added to the hydrogel and renewed every 12 h. After two days, PBS was removed, and the hydrogel and EP tube were weighed (M3) after quick blotting with filter paper. The swelling ratio (R) of the hydrogels was calculated using the following equation: $R = (M3 - M2)/(M2 - M1) \times 100\%$.[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 °C, and lyophilized for 3 days until their contained water was completely sublimed. 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

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 placed between two glass slides separated by a 1.1 mm thick spacer, followed by exposure to UV light (365 nm, 12.5 mW cm^{-2}) for 5 min to form hydrogels. The cover glass was removed carefully, and the hydrogel was transported to the parallel plate of a HR-2 discovery hybrid rheometer to perform the rheological test. A strain sweep test (0–10%) at an oscillatory frequency of 10 rad s^{-1} was 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, 1000 μm gap, 37 °C, 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 °C, 5% CO_2 in a 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 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 35 mm glass bottom culture dishes (MatTek) followed by exposure to long-wavelength UV light for 5 min. DMEM containing 1% penicillin–streptomycin antibodies and 10% fetal bovine serum was added to the surface of the hydrogels. Encapsulated fibroblasts were cultured at 37 °C, 5% CO_2 in a cell incubator for 26 h. The samples were stained with the calcein AM/ethidium homodimer for 20 min and examined with a 10× objective on an Olympus FV1000 confocal microscope. XYZ scanning mode was adopted for 2-D and 3-D images of cells in hydrogels.

2.13. Cytotoxicity

RGDP-PEG-acrylate$_{6k}$ (10% w/v) photo-cross-linked hydrogels were prepared in 96-well plates. NIH 3T3 fibroblasts were seeded on the hydrogel at 5000 cells per well and cultured at 37 °C and 5% CO_2 in a 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 pentamers was chosen to act as physical junctions in hydrogels. The polypeptide P as the major component of physical hydrogels has been thoroughly investigated.[24] Acryloyl groups are 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, nonimmunogenicity, and water-solubility is often used in tissue engineering applications. In order to prepare photo-cross-linkable macromers, one cysteine residue containing a free thiol was introduced at the C-terminus of the polypeptide by genetic engineering methods. Polypeptides (Pcys or RGDPcys) reacted with excess PEGDA through the Michael-type addition reaction to obtain the photo-cross-linkable macromers (P-PEG-acrylate or RGDP-PEG-acrylate) having PEG flanked by a photoreactive acrylate group and a self-assembling polypeptide P. It is known that a monomer with only one acrylate group usually forms a one-dimensional polymer, and the formation of 3-D polymeric networks require multiple branches or cross-linker. P-PEG-acrylate or RGDP-PEG-acrylate macromers containing only one acrylate group can form multi-branched macromers through physical assembly of polypeptide P. Under the conditions 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 polypeptides Pcys and RGDPcys are shown in Scheme 1. Because of the absence of toxic cross-linker and byproduct in the preparation of these physical hydrogels, they 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 structures of polypeptides 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 to 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]

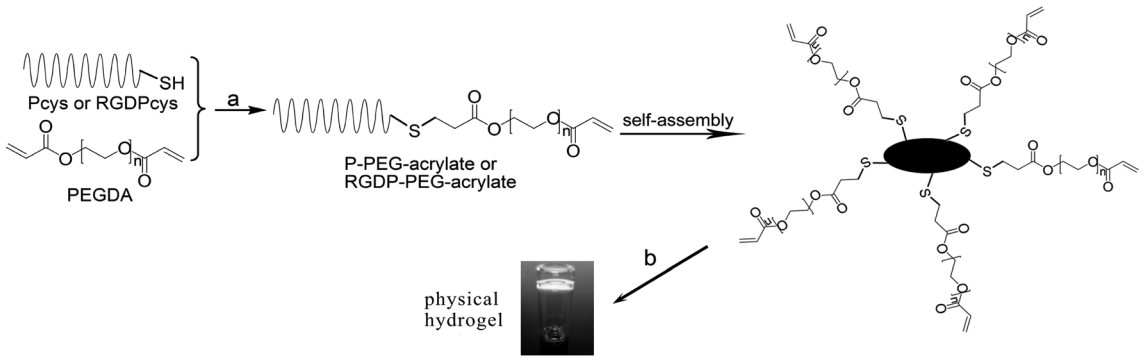
3.2. Covalent cross-linking of RGDP-PEG-acrylate

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, tetramers, trimers, dimers, 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 hydrogels in the presence of photoinitiator under 365 nm UV light.

3.3. Physical hydrogel or chemical hydrogel

To verify whether the photo-cross-linked hydrogel was a physical gel in nature, RGDP-PEG-acrylate hydrogels were immersed in 8.0 M urea or 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

(a)



(b)

Pcys : MRGS **[6H]**GSDAS **[P]**TSMGGC
RGDPcys: MRGS **[6H]**GSDASYAVTG**RGD**SPASSTS[P]TSMGGC
Abbreviations for peptide domains:
[6H]: HHHHHH
[P]: APQMLRELQETNAALQDVRELLRQQVKEITFLKNTVMESDAS

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.

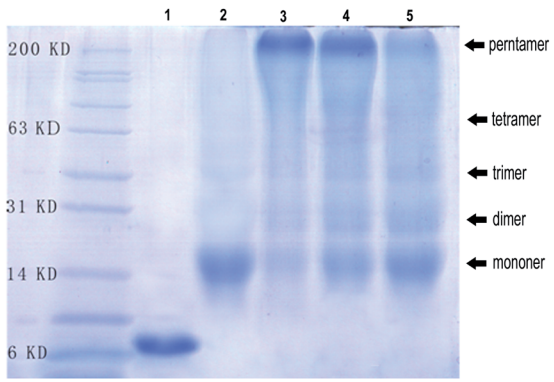
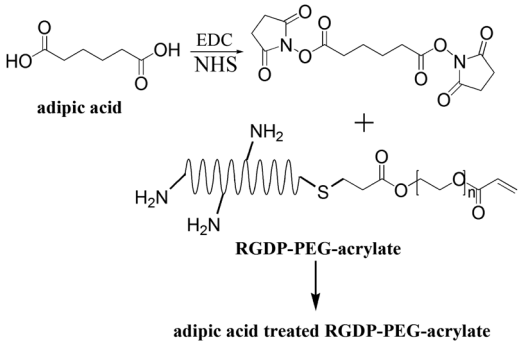


Fig. 1 SDS-PAGE of RGDP-PEG-acrylate and adipic acid-treated RGDP-PEG-acrylate. Lane 1, RGDPcys; 2, RGDP-PEG-acrylate$_{6k}$; 3–5, RGDP-PEG-acrylate$_{6k}$ treated with adipic acid at various molar ratios of adipic acid to RGDP-PEG-acrylate$_{6k}$ (lane 3, 10 : 1; lane 4, 5 : 1; lane 5, 2.5 : 1).



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

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.

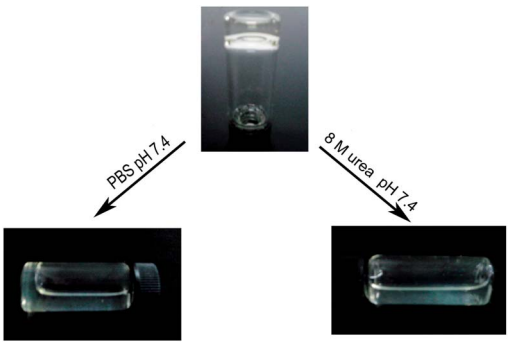


Fig. 2 Disassembly of photo-cross-linked RGDP-PEG-acrylate$_{6k}$ hydrogels (8% w/v) in 0.01M PBS (pH = 7.4) and 8 M urea (pH = 7.4) at 37 °C.

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. For clearer observations, one of the hydrogels was stained yellow with rhodamine 6G. We observed that the two hydrogels welded 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 possesses appropriate permeability for transport for oxygen, essential nutrients, and metabolic waste. Quick exchange of nutrients and metabolites is a prerequisite of biomaterials for tissue engineering applications. 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 the hydrogels.[24] To further determine the effect of concentration on healing, the self-healing times of hydrogels with various concentrations were investigated. The results show that the self-healing time becomes shorter with lower concentration. The

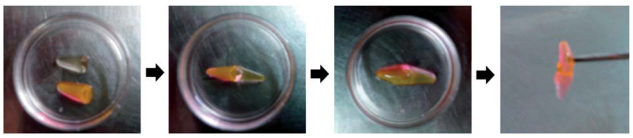


Fig. 3 Self-healing of photo-cross-linked RGDP-PEG-acrylate$_{6k}$ hydrogel (8% w/v) at 25 °C.

self-healing time depends strongly on the concentration of the hydrogel, demonstrating that the dynamic exchange rate of the self-assembled 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 damage and repair themselves as self-healing materials but also gain multi-responsive properties to environmental stimuli.[28] Thus, cells fixed in these hydrogels can be injected into the targeted positions for therapeutic and 3-D bioprinting applications.[29]

3.5. Swelling ratio and stability of hydrogels

The swelling ratios and stabilities of RGDP-PEG-acrylate$_{6k}$ hydrogels with different concentrations are shown in Fig. 4. The actual swelling ratio of a physical gel is larger than the experiment result because a part of the 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 ratios of 3% w/v and 11% w/v hydrogels are 19% and 38%, respectively. In addition, the swelling ratios of the hydrogels have a large variation at low concentrations and vary rarely at high concentrations. For instance, the swelling ratio increases by 17% when the concentration increases from 3% w/v to 5% w/v. In contrast, the swelling ratio increases by 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 hydrogels.

The biomaterials used in tissue engineering need the properties of high swelling ratio and excellent stability. Therefore, the stability of the photo-cross-linked hydrogels was also tested. As shown in Fig. 4b, the stability of the photo-cross-linked hydrogels 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 hydrogels based on leucine zipper A, hydrogels based on P domain showed significantly improved stability.[21] Hydrogels based on leucine zipper A readily form intramolecular loops because the 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 hydrogels reaches more than two months, suggesting that the stability of these photo-cross-linked physical hydrogels is mainly controlled by the rate of dissolution of the hydrogel. The

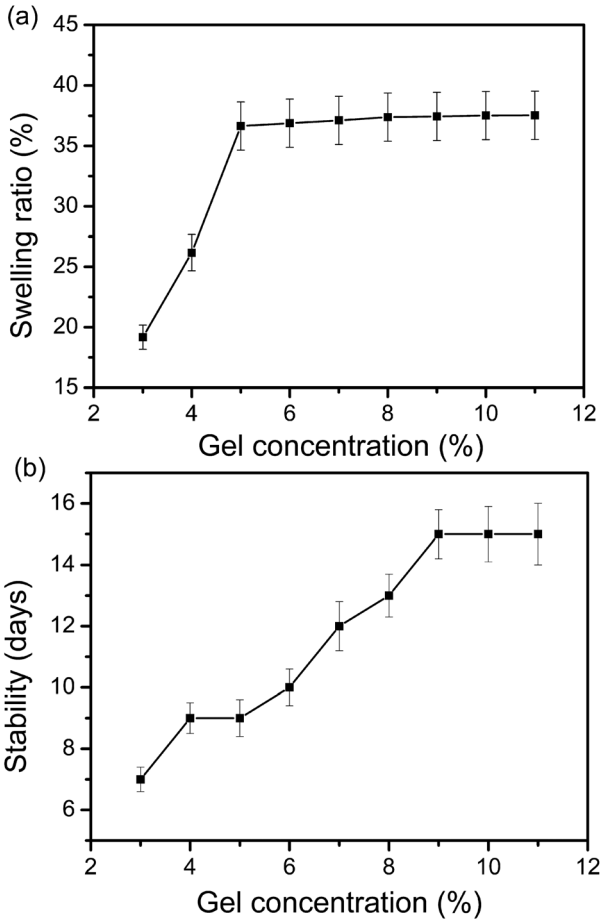


Fig. 4 Swelling ratio (a) and stability (b) of photo-cross-linked RGDP-PEG-acrylate$_{6k}$ hydrogels at 37 °C.

stability data of physical hydrogels are closely related to the volume of the gel and the volume of added PBS. As the stability of these physical hydrogels is mainly decided by the dissolving rate of gel, changing the concentration of gel will provide a convenient method to tune the stability of the 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$_{10k}$are not shown).

3.6. Hydrogel 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 those of 6% hydrogel (Fig. 5b). The mentioned pores have relatively large sizes with diameters of 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 cell growth and proliferation. The rapid permeation of rhodamine 6G (Fig. 3) further demonstrates large pores in the hydrogels. The

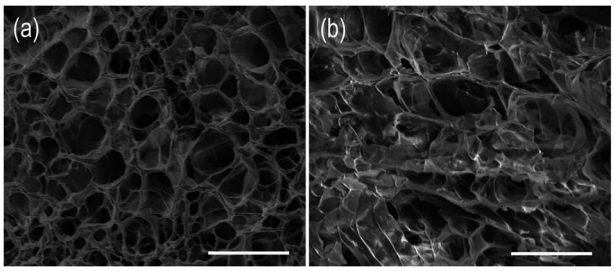


Fig. 5 SEM of photo-cross-linked RGDP-PEG-acrylate$_{6k}$ hydrogels: (a) 8% w/v; (b) 6% w/v. The scale bars are 40 μm.

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 are less than those of 8% w/v hydrogel.

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 results of the storage modulus (G') and loss modulus (G'') are 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 4300 Pa, and the G' of 10% w/v hydrogel is 7200 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 hydrogels 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} are not shown). Changing the 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 crossover point in 0.1–100 rad s^{-1}. The result gives further evidence that RGDP-PEG-acrylate hydrogel is a physical gel, and adipic acid-treated RGDP-PEG-acrylate hydrogel is a chemical gel. In addition, with increasing the concentration of macromer, the crossover point shifts to the direction of low angular frequency, indicating that the higher concentration leads to stronger viscosity and better stability.

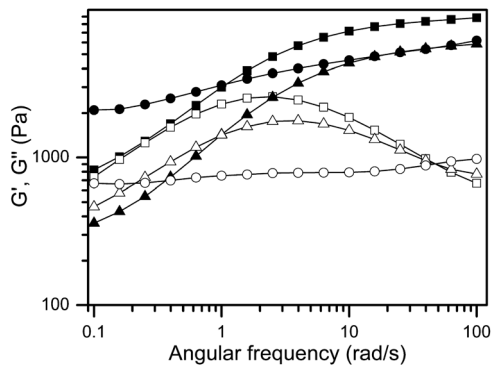


Fig. 6 Rheological oscillatory shear measurements of photo-cross-linked RGDP-PEG-acrylate$_{6K}$ hydrogels at various concentrations (10% w/v, squares; 8% w/v, triangles) and a covalently cross-linked control hydrogel at 8% w/v (circles). Storage modulus G': filled symbols; loss modulus G'': open symbols. 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 hydrogels, the investigation of cell adhesion and spreading on the surface of hydrogels (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 the 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] The RGD sequence could specifically combine with 11 kinds of integrins and effectively promote cell adhesion and spreading in biomaterials. To promote the adhesion and spreading of cells on the photo-cross-linked hydrogels, 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 is shown in 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 adipicacid-treated RGDP-PEG-acrylate hydrogels (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 effects on 2-D cell adhesion and 3-D cell growth and migration, has proven to be a useful strategy for

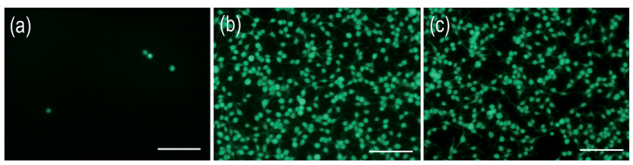


Fig. 7 Adhesion of NIH 3T3 fibroblasts on 10% w/v hydrogels of (a) P-PEG-acrylate$_{6k}$; (b) RGDP-PEG-acrylate$_{6k}$; and (c) adipic acid-treated RGDP-PEG-acrylate$_{6k}$. The scale bars are 200 μm.

understanding specific cell–materials interactions and the basic mechanism.[36–38] The precursor of photo-cross-linked hydrogels facilitated making hydrogels 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 numbers 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-based hydrogels such as collagen and elastin.

3.9. 3-D encapsulation of fibroblasts 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 cell growth and migration in the photo-cross-linkable hydrogels, 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 hydrogels are shown in Fig. 8. NIH 3T3 fibroblasts encapsulated in the RGDP-PEG-acrylate gel spread and migrated freely (Fig. 8a and g). NIH 3T3 fibroblast 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 hydrogels forms a dynamic construction through non-covalent self-assembly to provide the necessary paths for the spreading and migration of cells. Compared with untreated physical hydrogels, the adipic-acid-treated RGDP-PEG-acrylate control hydrogel is a chemical gel which would not provide fast spreading and migration conditions. Cell fast spreading and migration (26 h) indicate that cells can migrate and spread well through dynamic paths formed by the polypeptide P, and the dynamism of pentamers 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 cell migration across 3-D biomaterials has to deal with the physical obstruction posed by the matrix itself because the porosity of a 3-D matrix is significantly smaller than the average cell size.[17,41] Therefore, some strategies of cell migration within 3-D chemically cross-linked hydrogels 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 the hydrolysis rate of the whole materials is the same. Cell migration by the enzymolysis process only can be controlled in space through the action of the protease.[8] In the present study, we use dynamic polypeptide P in the absence of protease physical gel to provide the paths for cell migration which do not depend on spatio-temporal restriction. The cells in the proteolytically degradable hydrogels 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 hydrogels will not be affected because the assembly of coiled-coil polypeptide P is reversible.

Tissue is not a simple heap of cells, but an integrated complex with special functions formed by a certain regular arrangement of cells. The interactions between cells and well-organized arrangement of cells of different types are key issues

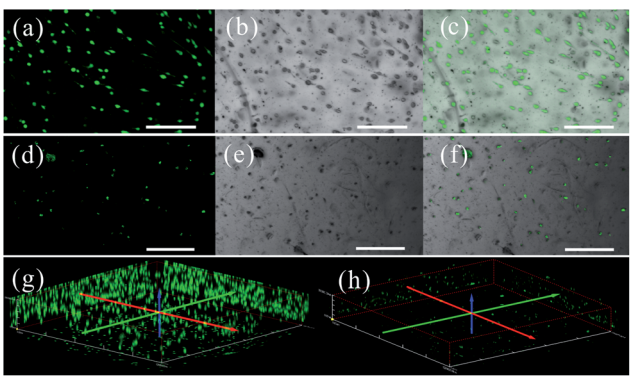


Fig. 8 Cell growth in 3-D photo-cross-linked hydrogels. Confocal fluorescence images of NIH 3T3 fibroblast encapsulation in 8% RGDP-PEG-acrylate$_{6k}$ hydrogel (a–c) and adipic acid-treated RGDP-PEG-acrylate$_{6k}$ (d–f). 3-D Confocal images of NIH 3T3 fibroblast encapsulation in 8% RGDP-PEG-acrylate$_{6k}$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

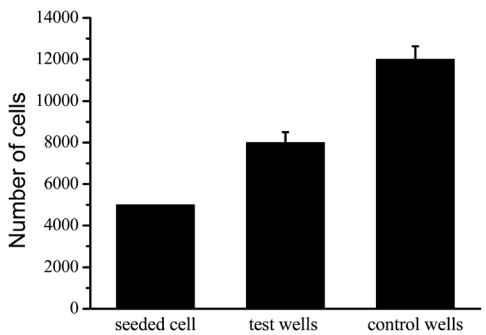


Fig. 9 Cytotoxicity of photo-cross-linked RGDP-PEG-acrylate$_{6k}$ hydrogels (8% w/v). NIH 3T3 fibroblasts were cultured on the surface of hydrogels for 48 h.

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 regulated. However, the rapid self-healing behavior of the photo-cross-linkable hydrogels containing the polypeptide P made it possible that different types of cells could be 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 way of self-assembly and bottom-up formation.[12,43]

Additionally, previous studies have shown that coiled-coil polypeptide P assembles into a pentameric cylinder-like and a 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 hydrogels is difficult work in tissue engineering, and the photo-cross-linked hydrogels containing the polypeptide P can provide an easy way to load hydrophobic drugs for controlled release.

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 hydrogels. Cells were seeded directly in 96-well cell culture plates as a control group. After 48 h of culture, only a few dead cells (live–dead assay) were observed. Although the number of cells in test wells was 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*.

4. Conclusions

Polypeptide-engineered physical hydrogels photo-cross-linked from multi-armed photopolymerization macromers 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 self-healing behavior. The physical bonds formed by self-assembly of the α-helical structure of the polypeptide allow reversible opening and closing of 3-D migration paths for spreading and migration of cells in these hydrogels. The self-healing characteristic of the photo-cross-linkable hydrogels enables these materials to conveniently build tissues through self-assembly and bottom-up construction. In addition, the main components of these physical hydrogels are biosynthetic polypeptides which are similar to natural tissue. Therefore, this kind of hydrogel is expected to have favorable prospects in tissue engineering as artificial scaffolds to mimic the natural ECM.

Acknowledgements

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