Biomaterials 31 (2010) 9266–9272

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

In situ thermal gelling polypeptide for chondrocytes 3D culture

Bo Gyu Choi [a,b], Min Hee Park [a], So-Hye Cho [c], Min Kyung Joo [a], Hye Jin Oh [a], Eun Hye Kim [a], Kwideok Park [b], Dong Keun Han [b,*], Byeongmoon Jeong [a,**]

[a] Department of Chemistry and Nano Science, Department of Bioinspired Science, BK21, Ewha Womans University, Seoul, 120-750, Republic of Korea
[b] Biomaterials Center, Korea Institute of Science and Technology, P.O. Box 131, Cheongryang, Seoul 130-650, Republic of Korea
[c] Nano-materials Center, Korea Institute of Science and Technology, P.O. Box 131, Cheongryang, Seoul 130-650, Republic of Korea

ARTICLE INFO

Article history:
Received 9 July 2010
Accepted 18 August 2010
Available online 22 September 2010

Keywords:
Secondary structure
Peptide
Thermally responsive material
Hydrogel
Chondrocyte
Cell culture

ABSTRACT

In the search for a cell-instructive or cell-interactive artificial extracellular matrix, synthetic hydrogels have been extensively investigated to apply three-dimensional (3D) cell culture and tissue engineering. Here, we are reporting a reverse thermal gelling L/DL-polyalanine block copolymer aqueous solution for chondrocyte 3D culture. The polymer aqueous solution undergoes sol-to-gel transition as the temperature increases, thus forming a 3D cell encapsulating scaffold *in situ* at 37 °C. In particular, the fraction of the β-sheet structure of the polyalanine dictated the population and thickness of fibrous nanostructure of the hydrogel, which in turn affected the proliferation and protein expression of the encapsulated chondrocytes. As an injectable tissue engineering system of chondrocytes, very promising results were confirmed for nude mice, using the current polypeptide aqueous solution. This paper not only provides important clues in designing an artificial extracellular matrix but also proves the significance of thermal gelling polypeptide as a minimally-invasive tissue engineering scaffold.

1. Introduction

The development of 3D environments mimicking the native extracellular matrix (ECM) has been the great challenge for the tissue engineering and regenerative medicine [1–5]. Compared with natural systems, synthetic hydrogels have been drawing attention due to their facile control of molecular parameters such as composition, degradability, and conformation of the hydrogel in a reproducible manner. However, they suffer from the limited methods of controlling biological functions due to the complexities of the biological events during differentiation and proliferation of cells. Since the pioneering comparative research on 3D versus traditional two-dimensional (2D) cell culture systems on human breast epithelial cells, neuronal cells, embryonic stem cells, and chondrocytes, a series of breakthrough works on 3D culture systems have been reported [6–9]. At the top of these breakthrough results come the study of the effect of stiffness of a hydrogel on the cell differentiation [10,11], the photodegradable hydrogel with an externally controllable biomechanical properties of the hydrogel [12], the cell-instructive hydrogels with modified functional groups [13], the self-assembled polypeptide hydrogel with specific nanostructures [14,15], and the hydrogel encapsulated microparticle [16]. They have contributed all together in improving the design principles for an artificial ECM for 3D cell culture.

Reverse thermal gelling polymer aqueous solutions undergoes sol-to-gel transition as the temperature increases. It can be utilized for the formation of drug or cell encapsulating 3D hydrogel matrix by mixing drugs or cells in a low viscous sol state, followed by increasing temperature [17–20]. Growth factors can be also simply incorporated, and by injecting the polymer aqueous solution, it can easily take the shape of the cavity or the disease site, avoiding the use of surgical procedures in certain cases. Therefore, the cell suspension in a reverse thermal gelling polymer aqueous solution has been proposed as a very promising system for minimally-invasive injectable tissue engineering.

Here, we are reporting the use of reverse thermal gelling polypeptides with well-defined secondary structures as promising platforms for the 3D culture of chondrocytes. Polyalanine–poloxamer–polyalanine (PA–PLX–PA) block copolymers, where L-alanine, DL-alanine, and their mixture (L/DL)-alanine were used in preparing the polyalanine to control the secondary structure as well as the resulting fibrous nanostructure of the polypeptide.

2. Materials and methods

2.1. Materials

Poly(propylene glycol)–poly(ethylene glycol)–poly-(propylene glycol) bis(2-aminopropyl ether) (PLX) (M_W = 600 Da) (Aldrich) was used as received. The

* Corresponding author. Tel.: +82 2 958 5282; fax: +82 2 958 5308.
** Corresponding author. Tel.: +82 2 3277 3411; fax: +82 2 3277 2384.
E-mail addresses: dkh@kist.re.kr (D.K. Han), bjeong@ewha.ac.kr (B. Jeong).

0142-9612/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2010.08.067

numbers of propylene glycol and ethylene glycol units were 3.5 and 8.5, respectively for PLX with the molecular weight of 600. *N*-Carboxy anhydrides of L-alanine, *N*-carboxy anhydrides of DL-alanine (M&H Laboratory, Korea), cell counting kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan), and Live/Dead kit (Molecular Probes™, Invitrogen, Carsbad, CA, USA) were used as received. Toluene was dried over sodium before use. Matrigel™ (BD Biosciences, San Jose, CA, USA) was used as received.

2.2. Synthesis

The PA–PLX–PA (PII) was prepared by ring-opening polymerization of the *N*-carboxy anhydrides of alanine in the presence of PLX [21]. PLX (4.5 g, 7.5 mmol; M_W = 600 Da) was dissolved in toluene (200 mL), and the residual water was removed by azeotropic distillation to a final volume of about 10 mL. Anhydrous chloroform/*N,N*-dimethyl formamide (12 mL; 2/1 v/v), *N*-carboxy anhydrides of L-alanine (6.57 g, 57.13 mmol), and *N*-carboxy anhydrides of DL-alanine (4.38 g, 38.09 mmol) were added to the reaction mixtures. They were stirred at 40 °C for overnight. The polymer was purified by repeated dissolution in the chloroform, followed by precipitation into diethyl ether (three times). PI and PIII were similarly prepared by using *N*-carboxy anhydrides of L-alanine and *N*-carboxy anhydrides of DL-alanine, respectively.

2.3. ^{1}H NMR spectroscopy

^{1}H NMR spectra in CF_3COOD (500 MHz NMR spectrometer; Varian) were used to determine the composition of the polymer.

2.4. Gel permeation chromatography

The gel permeation chromatography system (Waters 515) with a refractive index detector (Waters 410) was used to obtain the molecular weights and molecular weight distributions of the polymers. *N,N*-Dimethyl formamide was used as an eluting solvent. The PEGs in a molecular weight range of 400–20,000 Da were used as the molecular weight standards. An OHPAK SB-803QH column (Shodex) was used.

2.5. Circular dichroism spectroscopy

Ellipticities of the PA–PLX–PA aqueous solutions (0.01 wt.%) were obtained by a circular dichroism instrument (J-810; JASCO) as a function of temperature.

2.6. IR spectroscopy

IR spectra (FTIR spectrophotometer FTS-800; Varian) of the polymer aqueous solutions (10.0 wt.% in D_2O) were investigated. In order to analyze the secondary structural information of polypeptide, deconvolution of the IR spectra was done in the amide I band. The deconvoluted spectra were fitted with Gaussian–Lorentzian sum function (20% Gaussian and 80% Lorentzian) using XPSPEAK software 4.1 (RCSMS Lab). The peak positions, number of components, and the assignment of the absorption bands of secondary structure were made as described in the reference [22].

2.7. Cryo-TEM

Cryo-TEM images at 37 °C of PA–PLX–PA aqueous solutions were obtained after equilibrating the solutions for 20 min at 37 °C. Vitrified specimens of polymer aqueous solutions were prepared on the 200 mesh copper grid coated with a perforated form film (Ted Pella). A small drop (10 μL) was applied to the grid and blotted with filter paper to form a thin liquid film of solution, which was immediately plunged into liquid ethane at −170 °C. The procedure was performed automatically in the Vitrobot. The vitrified specimens were studied on a FEI Tecnai G2 TEM, at 200 kV with a Gatan cryoholder maintained below −170 °C, and images were recorded on an Ultrascan 2 K × 2 K CCD camera. In the microscopes, images were recorded with the Digital Micrograph software package under low-dose conditions to minimize damage by the electron beam radiation.

2.8. Dynamic mechanical analysis

Changes in storage modulus of the polymer aqueous solutions were investigated by dynamic rheometry (Rheometer RS 1; Thermo Haake). The aqueous polymer solution was placed between parallel plates of 25 mm diameter and a gap of 0.5 mm. During the dynamic mechanical analysis, the samples were located inside a chamber with water soaked cotton to minimize water evaporation. The data were collected under a controlled stress (4.0 dyn/cm^2) and a frequency of 1.0 rad/s as a function of time at 3 °C and 37 °C.

2.9. Chondrocytes isolation and in vitro 3D culture

Chondrocytes were isolated from the knee articular cartilage of 4-week-old New Zealand white rabbits by collagenase digestion [23]. Isolated chondrocytes were monolayer cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin under 5% CO_2 atmospheres at 37 °C and then subcultured to passage 2. Harvested cells (passage 3, 2.0 × 10^5 cells) mixed with polymer aqueous solutions (0.25 mL) of PI (10.0 wt.%), PII (10.0 wt. %), PIII (17.0 wt. %), and Matrigel™ (as received) were incubated in 24-well culture plate at 37 °C for 30 min to form a cell encapsulating 3D matrix by the sol-to-gel transition of the system. DMEG (0.5 mL) containing 10% fetal bovine serum and 1% penicillin/streptomycin was added on the cell-encapsulated hydrogel under 5% CO_2 atmosphere at 37 °C and replaced every three days. Chondrocytes were also monolayer cultured in the plate without hydrogel for comparison.

2.10. Cell proliferation and viability

The proliferation of chondrocytes in hydrogels was assessed by CCK-8 methods (n = 3). CCK-8 solution (0.8 mL; 10% v/v in medium) was added to each well of the plate. After 2 h incubation, the absorbance value at 450 nm was measured with an ELISA reader (Model 550; Bio-Rad, Hercules, CA, USA), where the absorbance at 600 nm was used for baseline correction. Viability of chondrocytes in hydrogels was determined using Live/Dead kit. Briefly, samples were incubated at room temperature for 30 min in a solution of 4 μM ethidium homodimer-1 (EthD-1) and 2 μM calcein AM in PBS. Labeled cells were then viewed under a Nikon Eclipse E600 fluorescence microscope and images captured using Lucia software. Live cells were stained with calcein AM (green), whereas dead cells were stained with EthD-1 (red). For quantitative analysis, a total of 200 cells were counted from each sample over three randomly chosen areas and the live and dead cell counts were recorded.

2.11. sGAG contents

To determine the sGAG contents in chondrocytes encapsulated hydrogels, each sample was digested in papain solution. Then sGAG contents were measured using a dimethylmethylene blue (DMB) assay in 96 well plates. Chondroitin sulfate C was used as the standard.

2.12. Histology and immunohistochemistry

Samples were embedded in Tissue Tek® O.C.T. Compound (Sakura, Zoeterwoude, Netherlands). Cryosections with a 10 μm thickness were mounted on slides. sGAG was stained with alcian blue 8GX (Sigma) at pH 2.5 and counterstained with nuclear fast red (Sigma). For immunohistochemistry of type II collagen, the cryosections were incubated with monoclonal antirabbit type II collagen antibody (Calbiochem, San Diego, CA, USA). Subsequently, antibody staining was detected using the Histostain-Plus Kits (Invitrogen) according to the manufacturer's protocol.

2.13. RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

After 14th and 28th days of culture, the total RNA was extracted from the cell-encapsulated hydrogels using the TRI$_{ZOL}$™ reagent (Invitrogen), according to the manufacturer's protocol. The extracted RNA pellet was dissolved in nuclease-free water, and the RNA concentration was determined using a NanoDrop (ND-1000). After synthesize the cDNA from isolated RNA, PCR was performed using the Maxime PCR PreMix (iNtRON, Korea). The PCR amplifications were carried out for 30 cycles under the following conditions: 30 s at 95 °C for denaturation, 45 s for annealing at each temperature, and 30 s at 72 °C for extension. The PCR products were visualized by electrophoresis in a 1.0 wt.% agarose gel and visualized by SYBR green. The sequence of primers were as follows: for GAPDH (housekeeping gene, annealing temperature 57 °C): 5′-AGGTCATCCACGACCACTTC-3′ (forward), 5′-GTGAGTTTCCC-GTTCAGCTC-3′ (reverse); type I collagen (annealing temperature 57 °C): 5′-GGA-CCTCAAGATGTGCCACT-3′ (forward), 5′-CTTGGGGTTCTTGCTGATGT-3′ (reverse); type II collagen (annealing temperature 50 °C): 5′-GCACCCATGGACATTGGAGGG-3′ (forward), 5′-GACACGGAGTAGCACCATCG-3′ (reverse).

2.14. In vivo feasibility study

Chondrocytes (P3, 1 × 10^6) were mixed with 10.0 wt.% of PII (0.25 mL) in sol state and then subcutaneously injected at the back of the nude mice. The 3D matrix samples were collected to assay the amount of sGAG and the expression of collagens.

2.15. Animal procedure

All experimental procedures using animals were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Committee of Ewha Womans University.

2.16. Statistical analysis

Cell proliferation and sGAG content were statistically analyzed by a Student t-test ($p < 0.01$). At least triplicate experiments were carried out for statistical analysis.