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Cytocompatible polyion complex gel of poly(Pro-Hyp-Gly) for simultaneous rat bone marrow stromal cell encapsulation

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Abstract

Polyion complex (PIC) gel of poly(Pro-Hyp-Gly) was successfully fabricated by simply mixing polyanion and polycation derivatives of poly(Pro-Hyp-Gly), a collagen-like polypeptide. The polyanion, succinylated poly(Pro-Hyp-Gly), and the polycation, arginylated poly(Pro-Hyp-Gly), contain carboxy (pK_a = 5.2) and guanidinium (pK_a = 12.4) groups, respectively. Mixing the polyanion and the polycation at physiological pH (pH = 7.4) resulted in PIC gel. The hydrogel formation was optimum at an equimolar ratio of carboxy to guanidinium groups, suggesting that ionic interaction is the main determinant for the hydrogel formation. The hydrogel was successfully used for simultaneous rat bone marrow stromal cell encapsulation. The encapsulated cells survived and proliferated within the hydrogel. In addition, the cells exhibited different morphology in the hydrogel compared with cells cultured on a tissue culture dish as a two-dimensional (2D) control. At day one, a round morphology and homogeneous single cell distribution were observed in the hydrogel. In contrast, the cells spread and formed a fibroblast-like morphology on the 2D control. After three days, the cells in the hydrogel maintained their morphology and some of them formed multicellular aggregates, which is similar to cell morphology in an *in vivo* microenvironment. These results suggest that the PIC gel of poly(Pro-

Hyp-Gly) can serve as a cytocompatible three-dimensional scaffold for stem cell encapsulation, supporting their viability, proliferation, and *in vivo*-like behavior.

Keywords

3D scaffold

collagen-like polypeptide

cytocompatible

polyion complex gel

poly(Pro-Hyp-Gly)

rat bone marrow stromal cells

simultaneous cell encapsulation

1. Introduction

Encapsulating stem cells in three-dimensional (3D) scaffolds has gained interest in tissue regeneration because of their advantages over two-dimensional (2D) culture in providing more natural microenvironments for cell growth and differentiation [1,2]. Hydrogels are attractive 3D scaffolds because of their structural and functional similarities to cell microenvironments [3,4]. Their hydrated polymeric networks allow diffusion of nutrients, oxygen, and waste during embedded cell culture [3–5]. Importantly, hydrogels can encapsulate cells simultaneously during their formation. Simultaneous cell encapsulation with scaffold fabrication offers homogeneous cell distribution, which enables homogeneous tissue reconstruction [6–8].

Chemical crosslinking strategies are usually used to fabricate hydrogels for stem cell encapsulation [3,4]. However, these strategies utilize chemical crosslinking reagents, which may adversely affect the encapsulated cells [4,9,10]. Physical crosslinking strategies offer great promise for encapsulating cells because they can be simply fabricated in the absence of any chemical crosslinking reagents [4,11–14]. Self-assembled collagen type I hydrogel have been widely used for stem cell encapsulation [15]. However, the collagen needs to be dissolved in acidic solution which may damage the encapsulated cells. In addition, collagen has a possibility to transfer pathogenic substances, such as prion, to patients [16]. Physically-crosslinked hydrogels made from alginate and calcium ion (Ca^{2+}) have been widely used to encapsulate a range of cells, such as human mesenchymal stem cells (hMSCs) [17], calf adrenal chromaffin cells [18], and neuronal stem cells [19]. However, the number of Ca^{2+}-alginate bonds in the hydrogels decrease with time because of Ca^{2+} release. It has been reported that Ca^{2+} released from the hydrogels upregulates the expression of inflammatory cytokines and chemokines when the hydrogels were injected subcutaneously into mice [20].

Polyion complex (PIC) gel formed by ionic bonding between high molecular weight polyanions and polycations offers a better stability. Few studies have reported the use of PIC gel as 3D scaffolds for simultaneous stem cell encapsulation [11–13]. PIC gel of hyaluronic acid (HA) and block copolymers of poly(2-aminoethyl methacrylate) and poly(ethylene glycol) (PEG) have been used for simultaneous encapsulation of hMSCs [12]. However, the hydrogel disintegrated within 15 min in the presence of phosphate-buffered saline (PBS), indicating low stability of the hydrogel at physiological conditions [12]. PIC of alginate and poly-L-lysine has been used for immunoprotective encapsulation of pancreatic islet cells [21]. However, poly-L-lysine may induce inflammatory responses through its necrosis-inducing abilities [22,23]. In addition, alginate has several drawbacks, such as limitation in supply, variability in different batches, and the risk of contamination with pathogenic substances [24]. Therefore, development of PIC gels that show high stability in a physiological environment, can be produced with consistent physicochemical characteristics, and are free from pathogenic contamination is important for designing 3D scaffolds for tissue regeneration.

Poly(Pro-Hyp-Gly), a collagen-like polypeptide, is a synthetic polymer containing Pro-Hyp-Gly sequences, which are commonly found in triple-helical domain of collagen [25]. The poly(Pro-Hyp-Gly) can be synthesized with high reproducibility in its physical and chemical properties and it is free from pathogenic contamination [25,26]. Tissue regeneration using poly(Pro-Hyp-Gly) sponges showed that epithelialization of full-thickness wounds on rabbit's ear pad were significantly promoted in the presence of the poly(Pro-Hyp-Gly) sponges in comparison with Terudermis® (Olympus Terumo Biomaterials, Tokyo, Japan), a heat-crosslinked bovine atelocollagen [27]. In addition, the sponge has been shown to degrade at the same rate as Terudermis® when embedded subcutaneously into the dorsal area of rats [27]. Therefore, poly(Pro-Hyp-Gly) is an attractive material for fabricating 3D scaffolds for tissue regeneration. An ideal 3D scaffold can be developed by further modification of the poly(Pro-Hyp-Gly).

Recently, Kusumastuti et al. fabricated PIC gel by simply mixing a polyanion, succinylated poly(Pro-Hyp-Gly) (Suc-poly(Pro-Hyp-Gly)), and a polycation, chitosan. The hydrogel was successfully used to encapsulate rat bone marrow stromal cells (rBMSCs) simultaneously during its formation [11]. The cells in the hydrogel proliferated during seven days of the culture period. However, at day one, the number of viable cells decreased drastically because of the low pH of the chitosan solution [11].

In this study, poly(Pro-Hyp-Gly) was modified with a succinyl group and arginine methyl ester to obtain a polyanion, Suc-poly(Pro-Hyp-Gly), and a polycation, Arg-poly(Pro-Hyp-Gly), respectively. At physiological pH (pH = 7.4), the carboxy group of Suc-poly(Pro-Hyp-Gly) (pK_a = 5.2) and the guanidinium group of Arg-poly(Pro-Hyp-Gly) (pK_a = 12.4) form negative and positive charges, respectively, allowing simultaneous cell encapsulation with PIC gel formation while maintaining the

viability of the encapsulated cells. To investigate the capability of the PIC gel to serve as a 3D scaffold for stem cell encapsulation, rBMSCs were simultaneously encapsulated into the PIC gel during its formation. Viability and morphology of the encapsulated cells were then assessed. We assumed that the PIC gel of poly(Pro-Hyp-Gly) would be suitable as a 3D scaffold for tissue regeneration because of its nontoxicity, ease of fabrication, and cytocompatibility.

2. Materials and methods

2.1. Materials

Pro-Hyp-Gly, 1-hydroxybenzotriazole (HOBt), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC·HCl), and *N*-hydroxysuccinimide (HOSu) were purchased from the Peptide Institute (Osaka, Japan). *N,N*-Diisopropylethylamine (DIPEA) was purchased from Applied Biosystems (Carlsbad, CA, USA). L-Arginine methyl ester dihydrochloride was purchased from Sigma-Aldrich (St Louis, MO, USA). Succinic anhydride was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and recrystallized from 2-propanol prior to use. Other reagents were purchased from Wako Pure Chemical Industries Ltd. The amino acids used in this study are all in the L-form.

2.2. Synthesis of poly(Pro-Hyp-Gly), Suc-poly(Pro-Hyp-Gly), and Arg-poly(Pro-Hyp-Gly)

Poly(Pro-Hyp-Gly) was synthesized according to the previous report [25]. Briefly, Pro-Hyp-Gly (0.7 mmol) and HOBt (0.14 mmol) were dissolved in 4 mL phosphate buffer (PB, 10 mM, pH 7.4) and mixed with EDC·HCl (3.5 mmol). The mixture was stirred at 400 rpm for 2 h at 0 °C and then for 46 h at 20 °C. The reaction was terminated by addition of 8 mL of Dulbecco's PBS (pH 7.4). After homogenization using a Waring blender (Waring Products Division, New Hartford, CT, USA), the mixture was stirred for 24 h at room temperature and then dialyzed against Milli-Q water (Merck Millipore, Billerica, MA, USA) for eight days at 4 °C using a dialysis membrane (molecular weight cut-off = 14,000 Da, UC2032100; EIDIA Co., Ltd., Tokyo, Japan) to remove any residual reagents.

To synthesize Suc-poly(Pro-Hyp-Gly), poly(Pro-Hyp-Gly) was mixed with a 50-fold molar excess of both succinic anhydride and DIPEA relative to the hydroxy group of Hyp residues of poly(Pro-Hyp-Gly) on ice for 2 h and then for 24 h at room temperature [11]. The obtained Suc-poly(Pro-Hyp-Gly) was dialyzed against 1 M NaCl for two days and then Milli-Q water for five days.

Arg-poly(Pro-Hyp-Gly) was synthesized by mixing Suc-poly(Pro-Hyp-Gly) with a 20-fold molar excess of both HOSu and EDC·HCl relative to the carboxy group of Suc-poly(Pro-Hyp-Gly) and stirred on ice. After 15 min, a 20-fold molar excess of arginine methyl ester and DIPEA were added to the mixture and stirred for 2 h. The mixture was then stirred overnight at room temperature and dialyzed against 1 M NaCl for two days and Milli-Q water for five days.

A schematic illustration of the syntheses of Suc-poly(Pro-Hyp-Gly) and Arg-poly(Pro-Hyp-Gly) is shown in Figure 1.

Figure 1. Schematic diagram of synthesis of Suc-poly(Pro-Hyp-Gly) and Arg-poly(Pro-Hyp-Gly).

*indicate L-isomers.

2.3. Characterization of poly(Pro-Hyp-Gly), Suc-poly(Pro-Hyp-Gly), and Arg-poly(Pro-Hyp-Gly)

Gel permeation chromatography (GPC) analysis of the polypeptides was carried out with an ÄKTA purifier system on a Superdex 200 HR 10/300 GL column (GE Healthcare Biosciences, Piscataway, NJ, USA). The elution buffer was PBS and flow rate was 0.5 mL/min at room temperature with the detection wavelength of 215 nm. The molecular weight of the polypeptides was calculated based on PEG standards (Waters, Milford, MA, USA).

Circular dichroism (CD) spectra of the polypeptides at a concentration of 0.25 mg/mL in Milli-Q water were recorded from 270 to 190 nm in a quartz cell of 0.1 cm optical path length on a J-820 spectropolarimeter (Jasco, Tokyo, Japan) at room temperature.

Fourier transform infrared (FTIR) spectra were recorded in the range 400–4000 cm^{-1} using a Spectrum One FTIR spectrometer (PerkinElmer, Wellesley, MA, USA) based on the KBr method with 16 scans and a resolution of 1 cm^{-1}.

^1H nuclear magnetic resonance (^1H NMR) spectra of the polypeptides were recorded on a JNM-ECX 500 spectrometer (JEOL, Tokyo, Japan). The concentration of the polypeptides was 4 mg/mL in deuterium oxide (D_2O; Cambridge Isotope Laboratories Inc., Andover, MA, USA) with tetramethylsilane (Cambridge Isotope Laboratories Inc.) as an internal reference. The degree of succinylation (DS_{COO^-}) and degree of arginylation (DS_{Arg^+}) were calculated as follows:

$$DS_{COO^-} = \frac{\text{Peak area of Hyp-C}_{\rgamma}H\text{of Suc- (Pro-Hyp-Gly) (a*)}}{\text{Sum of peak areas of Hyp-C}_{\rgamma}H\text{ of Pro-Hyp-Gly (a) and Suc- (Pro-Hyp-Gly) (a*)}}$$

$$DS_{Arg^+} = \frac{\text{Peak area of Arg-C}_{\rgamma}H\text{ (d)}}{\text{Sum of peak areas of Hyp-C}_{\rgamma}H\text{ of Pro-Hyp-Gly (a) and Suc- (Pro-Hyp-Gly) (a*)}} \times$$

To confirm the DS_{COO^-} and DS_{Arg^+}, and to determine the pK_a of the carboxy and guanidinium groups in the polypeptides, potentiometric titrations were conducted using a DL 58 titrator

(Mettler-Toledo, Schwerzenbach, Switzerland). A solution containing 1 mL of Suc-poly(Pro-Hyp-Gly) or Arg-poly(Pro-Hyp-Gly), 5 mL of 0.02 M HCl, and 24 mL of Milli-Q water was titrated with 0.02 M NaOH. The volume of NaOH needed to titrate the Suc-poly(Pro-Hyp-Gly) or Arg-poly(Pro-Hyp-Gly) was determined by subtracting a blank titration curve from the sample titration curve. DS_{COO^-} and DS_{Arg^+} were calculated from the following equations:

$$\frac{DS_{COO_1^-}}{267.3\left(1 - DS_{COO_1^-}\right) + 389.3DS_{COO_1^-}} = \frac{\Delta V \times \text{Molarity of NaOH}}{\text{Weight of Suc- (Pro-Hyp-Gly)}} \quad (3)$$

$$\frac{DS_{COO_2^-}}{267.3\left(1 - DS_{COO_1^-}\right) + 389.3DS_{COO_2^-} + 573.8\left(DS_{COO_1^-} - DS_{COO_2^-}\right)} = \frac{\Delta V \times \text{Molarity of NaOH}}{\text{Weight of Arg- (Pro-Hyp-Gl}}$$

$$DS_{Arg^+} = DS_{COO_1^-} - DS_{COO_2^-} \quad (5)$$

where 267.3, 389.3, and 573.8 are average tripeptide unit molecular weights of Pro-Hyp-Gly, Suc--(Pro-Hyp-Gly), and Arg-(Pro-Hyp-Gly), respectively.

2.4. PIC gel formation

Suc-poly(Pro-Hyp-Gly) and Arg-poly(Pro-Hyp-Gly) solutions were concentrated by evaporation and dissolved in PBS by adding 10% of 10× concentrated PBS. PIC gels were fabricated by dropping the concentrated Suc-poly(Pro-Hyp-Gly) (16.7 mg/mL; DS_{COO^-} = 0.55) and Arg-poly(Pro-Hyp--Gly) (22.7 mg/mL; DS_{Arg^+} = 0.58) solutions on microscope glass coverslips at a molar ratio of carboxy to guanidinium groups of 1:2, 1:1, and 2:1. The microscope glass coverslips containing precursors were then placed in Petri dishes and incubated at 37 °C for 1 h. The obtained PIC gels were washed with PBS to remove unreacted polypeptides and weighed to obtain the weight of the hydrogels. The hydrogels were then washed with Milli-Q water and freeze-dried to obtain dried gels. The gelation and swelling ratio of the PIC gels were calculated using the following equations:

$$\text{Gelation}\ (\%) = \frac{\text{Weight of the PIC gel}}{\text{Total weight of precursor solutions}} \times 100\% \quad (6)$$

$$\text{Swelling ratio} = \frac{\text{Weight of wet gel} - \text{weight of dried gel}}{\text{Weight of dried gel}} \quad (7)$$

2.5. Encapsulation of rBMSCs into the PIC gel of poly(Pro-Hyp-Gly)

Bone marrow cells were obtained from the femora of a six-week-old female Wistar rat as described previously [28]. The rBMSCs obtained were suspended in α-minimum essential medium (α-MEM; Gibco Invitrogen Corp. Grand Island, NY, USA) containing 20% fetal calf serum (FCS; HyClone, Logan, UT, USA) and cultured in an 80 cm^2 tissue culture flask (153732; Nalge Nunc International, Roskilde, Denmark) at 37 °C under 5% CO_2. After three days, the attached cells were washed with PBS and treated with an aliquot of 0.02% ethylenediamine tetraacetic acid and 0.25% trypsin. After centrifugation at 1200 rpm for 5 min, the cells were suspended in 20% FCS/α-MEM. The rBMSC suspension was then prepared at a density of 5×10^6 cells/mL.

The concentrated Suc-poly(Pro-Hyp-Gly) (18.1 mg/mL; DS_{COO^-} = 0.58) and Arg-poly(Pro-Hyp-Gly) (21.6 mg/mL; DS_{Arg^+} = 0.52) solutions in PBS were sterilized using Millex-HP filters (Merck Millipore) with 0.45 μm pore size. One hundred microliters of the filter-sterilized Arg-poly(Pro-Hyp-Gly) (2.1 μmol) was dropped on a 24-well tissue culture plate (Nalge Nunc International). Ninety microliters of the filter-sterilized Suc-poly(Pro-Hyp-Gly) (2.1 μmol) was mixed homogeneously with rBMSCs (5×10^4 cells) and then dropped on the top of a droplet of Arg-poly(Pro-Hyp-Gly). The mixture was incubated at 37 °C under 5% CO_2. After one hour, the obtained PIC gels were washed with 1 mL of 20% FCS/α-MEM several times to remove unreacted polypeptides and free cells. The PIC gel was then cultured in 1 mL of 20% FCS/α-MEM at 37 °C under 5% CO_2 atmosphere for seven days.

The same number of rBMSCs without a PIC gel was cultured on another well of a 24-well tissue culture plate as a 2D control. The viable cells at days 1, 3, and 7 were quantified using water-soluble tetrazolium salt (WST-8-; Dojindo Molecular Technologies Inc. Kumamoto, Japan) according to the manufacturer's instructions. The optical density at 450 nm was measured using a SpectraFluor Plus microplate reader (Tecan, Männedorf, Switzerland). The morphology of the rBMSCs in the PIC gel was observed using a phase contrast microscope (Axiovert 100 M; Carl Zeiss, Oberkochen, Germany) and captured using an AxioCamHRc camera fixed to the microscope.

2.6. Statistical analysis

All statistical evaluations were performed using the one-way analysis of variance routine of KaleidaGraph 4.5 (Synergy Software, Reading, PA, USA) followed by Tukey's honest significant difference test. A value of $p < 0.05$ was accepted as statistically significant. All data are presented as mean ± standard deviation, with $n = 3$.

3. Results and discussion

3.1. Molecular weight of the polypeptides

In this study, poly(Pro-Hyp-Gly) as a precursor for preparing polyanion, Suc-poly(Pro-Hyp-Gly) and polycation, Arg-poly(Pro-Hyp-Gly), was synthesized by direct polycondensation of Pro-Hyp-Gly [25]. GPC profiles of the polypeptides showed peaks of molecular weight more than 120 kDa based on PEG standards (Figure 2(A) and (B)).

Figure 2. GPC profiles of Suc-poly(Pro-Hyp-Gly) (A) and Arg-poly(Pro-Hyp-Gly) (B). CD spectra (C) and R_{pn} value (D) of poly(Pro-Hyp-Gly), Suc-poly(Pro-Hyp-Gly), and Arg-poly(Pro-Hyp-Gly).

3.2. Triple-helical structure of the polypeptides

To observe the triple-helical structure of the poly(Pro-Hyp-Gly) after modification with succinyl group and arginine methyl ester, CD measurements were conducted. CD spectra of the polypeptides showed the appearance of a weak positive Cotton effect near 225 nm and a strong negative Cotton effect near 197 nm that are correlated to $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions, respectively, of the amide bond in the polypeptide backbone (Figure 2(C)). These results suggest that the polypeptides contain a collagen-like triple-helical structure [26,29].

The ratio of positive to negative Cotton peaks (R_{pn}) was used to estimate triple-helical content in the polypeptides [30]. The results showed that incorporation of succinyl group and arginine methyl ester into the poly(Pro-Hyp-Gly) significantly increased the triple-helical content of the poly(Pro-Hyp-Gly) (Figure 2(D)). The increase may be caused by the succinyl group and arginine methyl ester participating in the formation of water-mediated hydrogen bonding that can stabilize the triple-helical structure of the poly(Pro-Hyp-Gly). Crystal structure analysis of the triple-helical structure of a collagen-like peptide, $(Pro\text{-}Hyp\text{-}Gly)_3$-Ile-Thr-Gly-Ala-Arg-Gly-Leu-Ala-Gly-Pro-Hyp-Gly-$(Pro\text{-}Hyp\text{-}Gly)_3$, showed that the guanidinium group in the peptide can make intra- and interchain connections to the carbonyl backbone of the peptide through direct and/or water-mediated hydrogen bonding [31]. Another report showed that glutamic acid and lysine side chains in the triple-helical structure of a collagen-like peptide, $(Pro\text{-}Hyp\text{-}Gly)_4$-Glu-Lys-Gly-$(Pro\text{-}Hyp\text{-}Gly)_5$, form direct and/or water-mediated intra- and interchain hydrogen bonding with the carbonyls in the peptide backbone [32]. In addition, the electron-withdrawing effect of the succinyl group and arginine methyl ester may also stabilize the triple-helical structure of the polypeptides through the inductive effect. The inductive effect from electron-withdrawing groups constrains the puckering of the pyrrolidine ring and organizes the peptide backbone into conformations that are favorable for triple-helix conformation [33].

3.3. Chemical structure characterization of the polypeptides

To confirm the successful conjugation of the succinyl group and arginine methyl ester into the poly(Pro-Hyp-Gly), FTIR, ^{1}H NMR, and potentiometric titration analyses were conducted.

3.3.1. Poly(Pro-Hyp-Gly)

FTIR spectra of the poly(Pro-Hyp-Gly) showed peaks at 1648 and 1552 cm^{-1} that were assigned as amide I and amide II of the poly(Pro-Hyp-Gly) backbone, respectively (Figure 3 (A)). The ^{1}H NMR spectrum of the poly(Pro-Hyp-Gly) showed peaks at 3.2 and 3.6 ppm, and 3.8 and 3.9 ppm that were assigned to Pro-$C_{\delta}H$ and Hyp-$C_{\delta}H$, respectively, in the triple-helical structure of poly(Pro-Hyp-Gly) (Figure 4 (A)) [34 ~~32~~]. The result suggests that the poly(Pro-Hyp-Gly) contains a collagen-like triple-helical structure supporting the CD result described previously [26].

Figure 3. FTIR spectra of poly(Pro-Hyp-Gly) (A), Suc-poly(Pro-Hyp-Gly) (B), and Arg-poly(Pro-Hyp-Gly) (C).

Figure 4. ^{1}H NMR spectra of poly(Pro-Hyp-Gly) (A), Suc-poly(Pro-Hyp-Gly) (B), and Arg-poly(Pro-Hyp-Gly) (C).
indicate proton signals in the triple-helical structure of poly(Pro Hyp-Gly). a* is a proton signal of Hyp-$C_{\gamma}H$ that was shifted downfield because of the electron-withdrawing effect of the succinyl group.

3.3.2. Suc-poly(Pro-Hyp-Gly)

The FTIR spectrum of Suc-poly(Pro-Hyp-Gly) showed a peak at 1735 cm^{-1}, which was assigned to the carbonyl stretching of the ester group of Suc-poly(Pro-Hyp-Gly) (Figure 3 (B)). The ^{1}H NMR spectrum of Suc-poly(Pro-Hyp-Gly) showed signals at 2.6 and 2.7 ppm, which were assigned to four methylene protons of a succinyl group of the Suc-poly(Pro-Hyp-Gly) (Figure 4 (B)). A signal at 5.5 ppm is assigned to the proton of Hyp-$C_{\gamma}H$ that was shifted downfield because of the electron-withdrawing effect of oxygen of the ester group of Suc-poly(Pro-Hyp-Gly). These results revealed that the succinyl group was successfully conjugated into the poly(Pro-Hyp-Gly) through an ester bond. From the ^{1}H NMR spectrum of Suc-poly(Pro-Hyp-Gly), the DS_{COO^-} was estimated to be 55%.

DS_{COO^-} of Suc-poly(Pro-Hyp-Gly) calculated from the potentiometric titration was 58% of the hydroxy group of Hyp residues of Suc-poly(Pro-Hyp-Gly), with a pK_a of approximately 5.2 (Figure 5 (A) and (C)).

Figure 5. Potentiometric titration curve of Suc-poly(Pro-Hyp-Gly) (A) and Arg-poly(Pro-Hyp-Gly) (B). Difference volume of titrant, 0.02 M NaOH, for blank and Suc-poly(Pro-Hyp-Gly) (C) and Arg-poly(Pro-Hyp-Gly) (D) titrations.

3.3.3. Arg-poly(Pro-Hyp-Gly)

The ^{1}H NMR spectrum of Arg-poly(Pro-Hyp-Gly) showed peaks at 1.7, 1.9, 3.3, 3.8, and 4.5 ppm that were assigned to Arg-C$_\gamma H$, Arg-C$_\beta H$, Arg-C$_\delta H$, methyl proton, and Arg-C$_\alpha H$ of arginine methyl ester, respectively (Figure 4(C)), indicating that arginine methyl ester was successfully conjugated into the poly(Pro-Hyp-Gly). Based on the ^{1}H NMR spectrum, about 100% of the succinyl group of Suc--poly(Pro-Hyp-Gly) has been conjugated with arginine methyl ester.

DS_{Arg^+} was also determined by measuring the remaining carboxy group that was not conjugated with arginine methyl ester. Based on the potentiometric titration curve (Figure 5(B) and (D)), DS_{Arg^+} was 52% of the hydroxy group of Hyp residues of poly(Pro-Hyp-Gly), indicating that about 95% of succinyl groups had been conjugated with arginine methyl ester through amide bond formation. The pK_a of the guanidinium group of the arginine methyl ester was estimated to be 12.4 (Figure S1, supplementary information).

3.4. PIC gels of poly(Pro-Hyp-Gly)

PIC gels of poly(Pro-Hyp-Gly) were fabricated by mixing the polyanion, Suc-poly(Pro-Hyp-Gly), and the polycation, Arg-poly(Pro-Hyp-Gly). To accomplish stable PIC gel formation, high molecular weight and high charge densities of the polypeptides are required [35]. However, polypeptides with higher charge densities (higher DS_{COO^-} and DS_{Arg^+}) may enhance their water solubility and decrease gel formation. Kramer et al. reported that increased negative surface charge of the protein correlates strongly with increased protein solubility [36]. In addition, polycations with high charge density tend to induce an inflammatory response of the encapsulated cells, which may cause cell necrosis [35]. Therefore, the polypeptides with about 50% of DS_{COO^-} and DS_{Arg^+} are thought to be appropriate for the PIC gel formation.

To reproduce the pH and osmotic pressure of cell's physiological microenvironment, the Suc--poly(Pro-Hyp-Gly) and Arg-poly(Pro-Hyp-Gly) were dissolved in PBS. PIC gel was then fabricated by simply mixing the polyanion and polycation at physiological pH (pH = 7.4) and temperature (37 °C). At physiological pH, the carboxy group of Suc-poly(Pro-Hyp-Gly) (pK_a = 5.2) and the guanidinium group of Arg-poly(Pro-Hyp-Gly) (pK_a = 12.4) form carboxylate anion and guanidinium cation, respectively, enabling ionic interaction between the polyions. At physiological temperature, the polypeptide chain motion and flexibility are higher than that at room temperature because of a weakening interaction between the polypeptides chains in the triple-helical structure [29]. An increase in polypeptide chain flexibility increases the possibility of the polypeptides to interact with each other to form PIC gel. A schematic drawing for the PIC gel formation is shown in Figure 6(A). The hydrogel formation is initiated by macroscopic phase separation that is driven by diffusion process of a polyanion and a polycation, and resulted in the co-existence of dilute and rich phases of the polypeptides (Figure S2,

supplementary information) [37]. The dilute phase contains counter ions and unreacted polypeptides, while the rich phase contains PIC gel [37].

Figure 6. Schematic drawing of PIC gel formation (A). Gelation (B) and swelling ratio (C) of PIC gel of poly(Pro-Hyp-Gly). NS = not significant.

Notes: Scale bar represents 2 mm. ~~Gelation (B) and swelling ratio (C) of PIC gel of poly(Pro-Hyp-Gly). NS = not significant.~~

To estimate the degree of crosslinking of the obtained PIC gels, gelation and swelling ratio of the PIC gels in PBS were measured. A gelation shows the fractions of Suc-poly(Pro-Hyp-Gly) and Arg-poly(Pro-Hyp-Gly) that are included in the PIC gel compared with their initial amount before PIC gel formation. A higher gelation represents a higher amount of Suc-poly(Pro-Hyp-Gly) and Arg-poly(Pro--Hyp-Gly) in the PIC gel. The results showed that gelations of the PIC gels at molar ratios of carboxy to guanidinium groups of 1:2, 1:1, and 2:1 were 7.7 ± 1.6%, 19.9 ± 5.4%, and 9.2 ± 2.2%, respectively, and were significantly affected by the molar ratio ($p < 0.05$) (Figure 6(B)). The highest gelation ratio was obtained at 1:1 molar ratio of carboxy to guanidinium groups, suggesting that PIC gel formation is mainly caused by ionic interaction between the carboxy and guanidinium groups. These results are in agreement with our previous study, we observed that PIC gel formation between Suc-poly(Pro-Hyp-Gly) and chitosan was optimum at a 1:1 molar ratio between polyanion and polycation [11]. Another report also showed that PIC formation between poly(diallyldimethyl-ammonium chloride) and copolymers of acrylamide and sodium acrylate was optimum at a 1:1 molar ratio of the polyions [38].

The swelling ratio represents the amount of PBS that can be absorbed by the PIC gel. The swelling ratio is an indicator of the polymer network hydrophilicity and polymer network density [39]. The results showed that the PIC gels exhibited a high swelling ratio up to 22.8 ± 8.4 (Figure 6(C)). For stem cell encapsulation, hydrogels with a high swelling ratio are preferable. This property can control metabolic functionality of the encapsulated cells permitting better diffusion of nutrients, waste, and oxygen through the hydrogel networks [13,40]. Park et al. reported that cellular functions are enhanced when cells were encapsulated in hydrogels with a high swelling ratio [40]. Therefore, PIC gel formed with an equimolar concentration of carboxy to guanidinium groups, which showed the highest gelation and swelling ratio, was chosen for the cell encapsulation study.

3.5. Encapsulation of rBMSCs into the PIC gel of poly(Pro-Hyp-Gly)

To determine cytocompatibility of the PIC gel, rBMSCs were simultaneously encapsulated into the PIC gel. Morphology and viability of the cells in the hydrogel were compared with that of cells cultured on a tissue culture plate as a 2D control.

The results showed that the rBMSCs were successfully encapsulated into the PIC gel, exhibited a round morphology, and were homogeneously distributed in the hydrogel (Figure 7(A)). A similar morphology was observed when breast adenocarcinoma cells were encapsulated in alginate hydrogels [41]. Another report showed that chondrocytes maintained their round shape morphology in chitosan hydrogels [42]. Cells in hydrogels exhibit a similar morphology to cells in an *in vivo* microenvironment. In contrast, the rBMSCs on the 2D control spread and exhibited a fibroblast-like morphology (Figure 7(B), (D), (F), and (H)). This morphology does not represent *in vivo* cell behavior because the cells have a more stretched morphology compared with that of cells in the hydrogels [1,42].

Figure 7. Phase contrast microscope images of rBMSCs in PIC gel and on tissue culture plate as 2D control. Scale bars represent 100 μm.

After three days of incubation, the rBMSCs in the PIC gel maintained their round morphology (Figure 7(C)). Interestingly, some of the cells started to form multicellular aggregates and the number of cell aggregates increased with longer incubation time (Figure 7(E) and (G)). Cells encapsulated in other types of physically crosslinked hydrogels have been reported to exhibit similar behavior; they formed aggregates in the hydrogel during incubation time [43,44]. hMSCs in a calcium alginate hydrogel formed aggregates because of cell migration that was promoted by hydrogel degradation [43]. Another report by Mie et al. showed that human lung adenocarcinoma epithelial A549 cells encapsulated in a self-assembled peptide hydrogel formed multicellular aggregates during seven days of incubation [44]. The ability of physically crosslinked hydrogels in promoting cell aggregation is presumably because of hydrogel degradation. Physically crosslinked hydrogels are reversible hydrogels and changes in environmental conditions, such as salt concentration, pH, and temperature, may lead to hydrogel degradation. For the PIC gel, in a physiological environment, ionic bonding between polyanion and polycation in the PIC gel may be interrupted by the screening effect of small ions in the cell culture medium [12]. This screening effect may decrease the amount of ionic bonding in the hydrogel, increase hydrogel mesh size, and allow cell migration, leading to the formation of cell aggregates. In the cell aggregates, cell–cell and cell–ECM interactions are similar to cell interactions in an *in vivo* cell microenvironment [1]. These results indicate that the PIC gel of poly(Pro-Hyp-Gly) can support *in vivo*-like cell behavior of rBMSCs.

Although there was slight degradation of the PIC gel during incubation in the cell culture medium, based on our observations, the hydrogel remains in the cell culture medium for more than two weeks of incubation, suggesting the stability of the hydrogel in a physiological environment.

Quantification of viable cells in the PIC gel was conducted using a WST-8 assay. The results demonstrated that the rBMSCs in the hydrogel were alive (Figure 8). An initial decrease in cell number

in the hydrogel at day one was caused by the loss of free cells during hydrogel washing and it is comparable with the gelation ratio of the hydrogel. After three and seven days of incubation, the number of viable cells in the hydrogels increased. These results suggest that the PIC gel of poly(Pro-Hyp-Gly) not only supports the formation of *in vivo*-like morphology but also supports the viability and proliferation of the encapsulated rBMSCs.

Figure 8. Quantification of viable cells in PIC gel and on 2D control.

Compared to our previous study where we used Suc-poly(Pro-Hyp-Gly) and chitosan to prepare PIC gel [11], PIC gel prepared in this study offers several advantages, such as (i) the PIC gel can be easily prepared by mixing the precursor at physiological pH and (ii) the PIC gel supported cell viability, proliferation, and the formation of *in vivo*-like morphology. These advantages showed that PIC gel made from poly(Pro-Hyp-Gly) derivatives is potential as a 3D scaffold for stem cell encapsulation.

4. Conclusion

We successfully fabricated cytocompatible PIC gel of poly(Pro-Hyp-Gly) by simply mixing polyanion, Suc-poly(Pro-Hyp-Gly), and polycation, Arg-poly(Pro-Hyp-Gly), at physiological pH and temperature. The PIC gel was successfully used for simultaneous rBMSC encapsulation and exhibited high stability in a physiological environment. The rBMSCs in the PIC gel were viable, proliferated, and formed multicellular aggregates. These results suggest that the PIC gel of poly(Pro-Hyp-Gly) is useful as a 3D scaffold for tissue regeneration supporting cell viability, proliferation, and *in vivo*-like morphology.

Disclosure statement

The authors have no potential conflicts of interest to declare.

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Supplemental data

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