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Calcium deposition in photocrosslinked poly(Pro-Hyp-Gly) hydrogels encapsulated rat bone marrow stromal cells

Short title: Calcium deposition in poly(Pro-Hyp-Gly) hydrogels

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Abstract

Reproducing the features of the extracellular matrix is important for fabricating three-dimensional (3D) scaffolds for tissue regeneration. A collagen-like polypeptide,

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poly(Pro-Hyp-Gly), is a promising material for 3D scaffolds because of its excellent physical properties, biocompatibility, and biodegradability. In this paper, we present a novel photocrosslinked poly(Pro-Hyp-Gly) hydrogel as a 3D scaffold for simultaneous rat bone marrow stromal cell (rBMSC) encapsulation. The hydrogels were fabricated using visible-light photocrosslinking at various concentrations of methacrylated poly(Pro-Hyp-Gly) (20–50 mg/mL) and irradiation times (3 or 5 min). The results show that the rBMSCs encapsulated in the hydrogels survived seven days of incubation. Calcium deposition on the encapsulated rBMSCs was assessed with SEM observation, Alizarin Red S and von Kossa staining. The most strongly stained area was observed in the hydrogel formed with 30 mg/mL of methacrylated poly(Pro-Hyp-Gly) with 5 min irradiation. These findings demonstrate that poly(Pro-Hyp-Gly) hydrogels support rBMSC viability and differentiation, as well as demonstrating the feasibility of using poly(Pro-Hyp-Gly) hydrogels as a cytocompatible, biodegradable 3D scaffold for tissue regeneration.

Keywords: collagen-like polypeptide; eosin Y; hydrogel; poly(Pro-Hyp-Gly); rBMSCs; calcium deposition; simultaneous encapsulation; photocrosslinking

1. Introduction

Tissue regeneration requires three-dimensional (3D) scaffolds that can support cell viability and differentiation (Chen *et al.*, 2002; Singh and Elisseff, 2010). To reproduce the features of extracellular matrices (ECMs), 3D scaffolds are usually fabricated with components of natural ECMs, such as animal-derived collagens (Cen *et al.*, 2008). Culturing cells in 3D scaffolds made from these components has significant effects on cell adhesion, morphology, and differentiation (Arakawa *et al.*, 2017; Singh and Elisseff, 2010). However, animal-derived collagens may contain pathogenic substances that could transfect humans (Cen *et al.*, 2008; Nemoto *et al.*, 1999) and they have low thermal stability (Leikina *et al.*,

2002). Furthermore, their properties are variable from batch to batch, which can result in inconsistent scaffold fabrication. Synthetic polymers, such as poly(ethylene glycol) (PEG) and its derivatives, are commonly used as alternative materials for fabricating 3D scaffolds, because they can be synthesized with high reproducibility of their physical and chemical properties (Nicodemus and Bryant, 2008; Ohya *et al.*, 2012). However, these polymers are not biodegradable, which limits their application in tissue regeneration (Kim *et al.*, 2010; Nicodemus and Bryant, 2008; Ohya *et al.*, 2012).

Poly(Pro-Hyp-Gly), a collagen-like polypeptide, is a synthetic polymer that has excellent physical properties, biodegradability, and biocompatibility (Kishimoto *et al.* 2005; Tanihara *et al.*, 2008). Compared with animal-derived collagens, poly(Pro-Hyp-Gly) is free from pathogenic substances and is highly stable up to 80 °C, and its hydroxy group can be easily tailored by specific modification (Kishimoto *et al.*, 2005; Kusumastuti *et al.*, 2017; Shibasaki *et al.*, 2011). Such properties are essential for producing reliable tissue regeneration.

Previously, we have demonstrated the use of a polyion complex (PIC) gel of poly(Pro-Hyp-Gly) and chitosan as a 3D scaffold for rat bone marrow stromal cell (rBMSC) encapsulation. The encapsulated rBMSCs survived and proliferated in the PIC gel during seven days of incubation (Kusumastuti *et al.*, 2017). However, the PIC gel is difficult to handle because of its low mechanical strength in physiological environments, which limits its usage for tissue regeneration.

Here, we report the fabrication of a chemically crosslinked hydrogel based on poly(Pro-Hyp-Gly) as a scaffolding material. The hydrogels were fabricated by visible-light photocrosslinking of methacrylated poly(Pro-Hyp-Gly). The properties of the hydrogels, such as gelation and swelling ratios, could be easily controlled by changing the concentrations of methacrylated poly(Pro-Hyp-Gly) and photoinitiator and the irradiation time. To evaluate the effect of the hydrogel properties on the encapsulated cells, we encapsulated rBMSCs into the

hydrogels at various concentrations of methacrylated poly(Pro-Hyp-Gly) (20–50 mg/mL) and irradiation times (3 or 5 min) and observed rBMSC viability and differentiation into osteogenic lineages.

2. Materials and methods

2.1 Materials

Pro-Hyp-Gly, 1-hydroxybenzotriazole (HOBt), and 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride (EDC·HCl) were purchased from the Peptide Institute (Osaka, Japan). *N,N*-Diisopropylethylamine (DIPEA) and dimethylformamide (DMF) were purchased from Applied Biosystems (Carlsbad, CA, USA). Methacrylic anhydride, eosin Y disodium salt, triethanolamine, and 1-vinyl-2 pyrrolidinone (NVP) were purchased from Sigma-Aldrich (St Louis, MO, USA). Other reagents were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

2.2 Synthesis and characterization of poly(Pro-Hyp-Gly) and methacrylated poly(Pro-Hyp-Gly)

Poly(Pro-Hyp-Gly) was synthesized in accordance with previous report, but with slight modifications (Shibasaki *et al.*, 2011). Briefly, Pro-Hyp-Gly (0.70 mmol) and HOBt (0.14 mmol) were dissolved in phosphate buffer (PB, 10 mM, pH 7.4) and mixed with EDC·HCl (1.04 mmol). The mixture was stirred at 400 rpm for 75 min at 4 °C and the temperature was then raised to 20 °C. The reaction was terminated by the addition of phosphate-buffered saline (Dulbecco's PBS; pH 7.4) and dialyzed against Milli-Q (Merck-Millipore, Billerica, MA, USA) water for five days at 4 °C using a dialysis membrane (molecular weight cut-off = 14000 Da, UC20-32-100; EIDIA Co., Ltd., Tokyo, Japan) to remove any residual reagents.

Methacrylated poly(Pro-Hyp-Gly) was synthesized by reacting the hydroxy group of

Hyp residues of poly(Pro-Hyp-Gly) with a 20-fold molar excess of both methacrylic anhydride and DIPEA (Figure 1a). Briefly, methacrylic anhydride (2.4 mmol) solution in DMF and DIPEA (2.4 mmol) was added to a poly(Pro-Hyp-Gly) (0.12 mmol) solution in PB. The mixture was stirred on ice for 1 h and then for 24 h at room temperature. After 24 h, an aliquot of PBS was added to the solution. The methacrylated poly(Pro-Hyp-Gly) obtained was purified by dialysis against Milli-Q water for seven days at 4 °C.

The poly(Pro-Hyp-Gly) and methacrylated poly(Pro-Hyp-Gly) were analyzed by gel permeation chromatography (GPC) and circular dichroism (CD). The GPC analysis of the polypeptides at a concentration of 0.5 mg/mL in PBS was carried out using 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 the flow rate was 0.5 mL/min at room temperature with a detection wavelength of 215 nm. The molecular weight of the obtained polypeptides was calculated based on PEG standards (Waters, Milford, MA, USA). The CD spectrum of the poly(Pro-Hyp-Gly) at a concentration of 0.25 mg/mL in Milli-Q water was recorded from 190–270 nm in a quartz cell of 0.1 cm optical path length on a Jasco model J-820 spectropolarimeter (Jasco, Tokyo, Japan) at room temperature.

The conjugation of methacrylate groups into poly(Pro-Hyp-Gly) was confirmed using ¹H nuclear magnetic resonance (¹H-NMR; JNM-ECX 500, JEOL, Tokyo, Japan). Based on the ¹H-NMR spectrum of methacrylated poly(Pro-Hyp-Gly), the degree of methacrylation was calculated by comparing the peak area of vinyl protons of the methacrylate group with the sum of the peak areas of Pro-C_δH, Hyp-C_δH, and Gly-C_αH of Pro-Hyp-Gly.

2.3 Gelation and swelling ratios of photocrosslinked poly(Pro-Hyp-Gly) hydrogel

Methacrylated poly(Pro-Hyp-Gly) was dissolved in PBS containing 10–30 μM eosin Y, 10 mM triethanolamine, and 1 mM NVP. The final concentration of methacrylated

poly(Pro-Hyp-Gly) in the solution was 20–50 mg/mL. Photocrosslinked hydrogels were prepared by dropping 30 μ L of the solution on microscope glass coverslips separated by silicone rubber with circular holes punched out (height = 3 mm and diameter = 5 mm). The solution was photocrosslinked under visible light using a KL 1 500 LCD microscope illuminator (Carl Zeiss, Oberkochen, Germany) for 5 min. The hydrogels obtained were washed with PBS and weighed to obtain the weight of the hydrogel. The hydrogels were then washed with Milli-Q water for 30 min and freeze dried to obtain the dried gel. The dried gels were swollen in PBS at 37 °C for 24 h. Gelation (%) and swelling ratios were calculated, as defined by:

$$\text{Gelation ratio (\%)} = (\text{weight of the obtained hydrogel} / \text{total weight of precursor solution}) \times 100$$

$$\text{Swelling ratio} = (\text{weight of swollen gel} - \text{weight of dried gel}) / \text{weight of dried gel}$$

2.4 Cytotoxicity of methacrylated poly(Pro-Hyp-Gly), triethanolamine, and NVP

Bone-marrow cells were obtained from the femora of a six-week-old female Wistar rat. Animal experiments were conducted to comply with the requirements of the Nara Institute of Science and Technology Animal Experiment Committee. The cells were passed through a 40 μ m filter and washed three times with α -minimum essential medium (α -MEM; Gibco Invitrogen Corp., Grand Island, NY, USA). The washed cells were suspended in α -MEM containing 20% fetal calf serum (FCS; HyClone, Logan, UT, USA) and cultured in an 80 cm² tissue culture flask (153732; Nalge Nunc International, Roskilde, Denmark) at 37 °C under 5% CO₂. 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 1 200 rpm

for 5 min, the cells were suspended in 20% FCS/ α -MEM.

The rBMSC suspension obtained was cultured at a density of 1 250 cells/well in a 96-well tissue culture plate (167008; Nalge Nunc International) and incubated at 37 °C under 5% CO₂. After 24 h, filter-sterilized samples of methacrylated poly(Pro-Hyp-Gly), NVP, and triethanolamine solution in 20% FCS/ α -MEM were added to the wells and incubated for a further 24 h. The same number of rBMSCs without any addition of samples were cultured in a similar 96-well plate as a control. Viable cells were quantified with water-soluble tetrazolium salt (WST-8, Dojindo Molecular Technologies Inc., Kumamoto, Japan) according to the manufacturer's instructions. The optical density (OD) at 450 nm was measured using a SpectraFluor Plus microplate reader (Tecan, Männedorf, Switzerland). The relative cell viability was calculated, as defined by:

$$\text{Relative cell viability (\%)} = \frac{[(\text{OD}_{450} \text{ sample} - \text{OD}_{450} \text{ medium}) / (\text{OD}_{450} \text{ control} - \text{OD}_{450} \text{ medium})] \times 100}$$

2.5 Encapsulation of rBMSCs into photocrosslinked poly(Pro-Hyp-Gly) hydrogel

Twenty microliters of filter-sterilized solution containing 20–50 mg/mL of methacrylated poly(Pro-Hyp-Gly), 20 μ M eosin Y, 10 mM triethanolamine, and 5 mM NVP in 20% FCS/ α -MEM was homogeneously mixed with rBMSCs (2.5×10^4 cells). The mixture was dropped onto a 35 mm bacteriological petri dish (35-1008; Falcon-Becton Dickinson, New Jersey, USA) and irradiated under visible light for 5 min. The hydrogel obtained was washed immediately with 2 mL of 20% FCS/ α -MEM for several times, to minimize the cytotoxicity caused by the residual photocrosslinking components. The dish containing hydrogel was then cultured in 2 mL of 20% FCS/ α -MEM and incubated at 37 °C under 5% CO₂ atmosphere for seven days. Every two or three days, half of the medium was replaced with fresh medium. On

the day of the viability assay, hydrogels containing cells were transferred to a 24-well tissue culture plate. Viable cells were quantified using a WST-8 assay. Cell viability in the hydrogel was assessed using a Live-Dead staining kit (BioVision; 501-100, Milpitas, CA, USA) according to the manufacturer's instructions.

2.6 Osteogenic differentiation of rBMSCs in photocrosslinked poly(Pro-Hyp-Gly) hydrogel

Photocrosslinked poly(Pro-Hyp-Gly) hydrogels containing rBMSCs were fabricated as described in Section 2.5 and incubated in 20% FCS/ α -MEM at 37 °C under 5% CO₂. After 24 h, the medium was changed to an osteogenic medium, which was 20% FCS/ α -MEM supplemented with 10 nM dexamethasone, 100 μ M L-ascorbic acid-2-phosphate, and 10 mM β -glycerophosphate, and incubated at 37°C under 5% CO₂ atmosphere for 28 days. Every two or three days, half of the medium was replaced with fresh osteogenic medium.

At day 28, the cells in hydrogel were fixed with 4% formaldehyde solution in PBS for 2 h at 4 °C, washed with PBS, and treated twice with 20% sucrose in PBS for 4 h at 4 °C. The hydrogels were embedded into optimal cutting temperature compound (Sakura Finetek, Tokyo, Japan), frozen at -20 °C, and cut into 6 μ m slices using a Leica CM1100 cryotome (Leica Microsystems, Wetzlar, Germany). Calcium deposition on the slices was observed using a scanning electron microscope (SEM; Model S-4800, Hitachi, Tokyo, Japan) at an acceleration of 15 kV without gold coating, and Alizarin Red S and von Kossa staining.

For the Alizarin Red S and von Kossa staining, the slices were fixed on glass slides coated with Matsunami adhesive silane (Matsunami Glass, Tokyo, Japan), dried at room temperature, and washed three times with PBS. The fixed slices were incubated in 2% Alizarin Red S solution (pH 4.1–4.3) for 30 min at room temperature. The excess stain was removed by washing four times with Milli-Q water (Bianco *et al.*, 2006). Von Kossa staining

was conducted by incubating the fixed slices in 1% silver nitrate solution for 45 min under UV light at room temperature. After washing four times with Milli-Q water, the slices were then treated with 3% sodium thiosulfate for 5 min, washed four times with Milli-Q water (Bianco *et al.*, 2006), dried at room temperature, and observed with an optical microscope (Axioplan 2, Carl Zeiss). Quantification of the positively stained area in the hydrogel slices by Alizarin Red S and von Kossa was evaluated using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.7 Statistical analysis

All statistical evaluations were performed using the one-way analysis of variance routine of KaleidaGraph v. 4.5 (Synergy Software, Reading, PA, USA). Tukey's honest significant difference test was used to assess any differences between groups. A value of $p < 0.05$ was accepted as statistically significant. All data were expressed as mean \pm standard deviation, with $n = 3$.

3. Results and discussion

3.1 Characterization of poly(Pro-Hyp-Gly) and methacrylated poly(Pro-Hyp-Gly)

The peak molecular weights of poly(Pro-Hyp-Gly) and methacrylated poly(Pro-Hyp-Gly) measured by GPC analysis were about 12 and 9 kDa, respectively, based on the standard curve using PEG (Figure 1b). CD spectra of both polypeptides showed a positive Cotton effect near 225 nm and a negative Cotton effect near 197 nm, suggesting that the polypeptides contain a collagen-like triple-helical structure (Figure 1c).

The $^1\text{H-NMR}$ spectrum of methacrylated poly(Pro-Hyp-Gly) (Figure 2b) showed signals at 1.9, 5.8, and 6.2 ppm, which were attributed to a methyl proton and the two vinyl protons of the methacrylate group in the methacrylated poly(Pro-Hyp-Gly), respectively. The

spectrum also showed signals at 4.2 and 5.5 ppm that were assigned as Hyp-C_δH and Hyp-C_γH of methacrylated poly(Pro-Hyp-Gly), respectively, which appeared because of the electron-withdrawing effect of the ester group of methacrylated poly(Pro-Hyp-Gly), confirming that the methacrylate groups were conjugated successfully into the poly(Pro-Hyp-Gly). The degree of methacrylation of hydroxy groups was 42%, calculated from these peaks areas.

3.2 Effect of eosin Y, triethanolamine, NVP, methacrylated poly(Pro-Hyp-Gly) concentration, and irradiation time on hydrogel formation

Photocrosslinked poly(Pro-Hyp-Gly) hydrogel was fabricated by irradiating methacrylated poly(Pro-Hyp-Gly) with visible light in the presence of eosin Y as a photoinitiator, triethanolamine as a coinitiator, and NVP as a radical scavenger (Figure 3a). To optimize the concentration of the photocrosslinking components, we studied the effect of eosin Y and methacrylated poly(Pro-Hyp-Gly) concentrations and the irradiation time on the gelation and swelling ratios of the photocrosslinked hydrogels.

A gelation ratio shows the fraction of methacrylated poly(Pro-Hyp-Gly) that is involved in the crosslinking network compared with the amount of methacrylated poly(Pro-Hyp-Gly) before photocrosslinking. A higher gelation ratio represents a higher proportion of methacrylated poly(Pro-Hyp-Gly) involved in the hydrogel. Results from gelation and swelling experiments performed on hydrogels with different concentrations of eosin Y showed that changing the concentration of eosin Y in the range of 10–30 μM influenced the gelation and swelling ratios of the hydrogels significantly (Figures 3b, c). A hydrogel formed with 30 μM of eosin Y exhibited the highest gelation ratio ($82.5 \pm 8.2\%$). This suggests that a higher concentration of eosin Y will result in more radicals, leading to a hydrogel with a more crosslinked methacrylated poly(Pro-Hyp-Gly). However, for simultaneous cell encapsulation,

high numbers of radicals may have a cytotoxic effect on the cells; therefore, 20 μ M of eosin Y was chosen for further experiments.

The effects of the concentration of methacrylated poly(Pro-Hyp-Gly) and the irradiation time on the gelation of photocrosslinked hydrogels are shown in Figure 3d. At 5 min irradiation, the gelation ratio of the hydrogel increased as the methacrylated poly(Pro-Hyp-Gly) concentration increased ($p < 0.001$), and hydrogel formed with 50 mg/mL methacrylated poly(Pro-Hyp-Gly) showed the highest gelation ratio ($69.0 \pm 2.0\%$), followed by 40 mg/mL ($65.8 \pm 5.8\%$), 30 mg/mL ($53.4 \pm 2.2\%$) and 20 mg/mL ($44.6 \pm 2.9\%$). At 3 min irradiation, the gelation ratios of the hydrogels showed the same trend as those at 5 min, and there was no significant difference in the gelation ratios between hydrogels formed with 3 and 5 min irradiation at the same concentration of methacrylated poly(Pro-Hyp-Gly). However, there was no gel formation at 20 mg/mL of methacrylated poly(Pro-Hyp-Gly) and 3 min irradiation.

The swelling ratio of the hydrogels tends to decrease as the methacrylated poly(Pro-Hyp-Gly) concentration increases because of an increase in polymer network density (Figure 3e). At 5 min irradiation, the hydrogels showed a high swelling ratio in PBS, ranging from 19.5 ± 2.0 to 26.6 ± 1.0 , and the swelling ratio was significantly influenced by the methacrylated poly(Pro-Hyp-Gly) concentration ($p < 0.01$). The 3 min-irradiated hydrogels showed significantly lower swelling ratios than those of 5 min-irradiated hydrogels at the same concentration of methacrylated poly(Pro-Hyp-Gly). A longer irradiation time results in a hydrogel with a higher degree of crosslinking. However, a high degree of crosslinking may disturb the triple-helical structure of the hydrogel. As a result, a 3 min-irradiated hydrogel will have a higher triple-helical content than that of 5 min-irradiated hydrogels, and will therefore have reduced capacity to absorb water.

To study the effect of the photocrosslinking components on cell viability, rBMSCs were

incubated in medium containing either methacrylated poly(Pro-Hyp-Gly), triethanolamine, or NVP for 24 h. The results showed that NVP up to 5 mM had no effect on rBMSC viability (Figure 4a). The presence of 50 mM of triethanolamine in the medium was toxic to the rBMSCs, with only 31% of cells surviving under this condition (Figure 4b). At 10 mM of triethanolamine, rBMSCs showed high viability ($89.8 \pm 7.4\%$). About 43% of cells survived in the medium containing 20 mg/mL of methacrylated poly(Pro-Hyp-Gly) and there was no significant difference in cell viability between cells cultured in either 20 mg/mL or 30 mg/mL of methacrylated poly(Pro-Hyp-Gly) (Figure 4c). Based on gelation and swelling ratios and cytotoxicity, a setting of 20 μ M of eosin Y, 5 mM of NVP, and 10 mM of triethanolamine was chosen for further cell photoencapsulation experiments.

3.3 Cell encapsulation and viability in photocrosslinked poly(Pro-Hyp-Gly) hydrogels

The 3D microenvironment has been shown to play an important role in determining cell behavior, such as cell viability and proliferation (Edmondson *et al.*, 2014; Griffith and Swartz, 2006; Singh and Elisseff, 2010). WST-8 assays were performed to investigate the influence of hydrogel properties on cell viability. The results showed that the rBMSCs encapsulated in photocrosslinked hydrogels formed with 20–50 mg/mL of methacrylated poly(Pro-Hyp-Gly) survived during seven days of culturing (Figure 5a). The initial decrease in cell numbers in the hydrogels that appeared on day 1 compared with the initial cell seeding number was caused by the loss of free cells during hydrogel washing and it varied with the gelation ratio of the hydrogels: a lower gelation ratio resulted in fewer encapsulated cells. The rBMSCs in the hydrogels formed with 30–50 mg/mL of methacrylated poly(Pro-Hyp-Gly) did not exhibit any significant difference in cell proliferation throughout seven days of culturing. However, the rBMSCs proliferated in the hydrogels formed with 20 mg/mL of methacrylated poly(Pro-Hyp-Gly) ($p < 0.05$). The results may be caused by polymer network density. A

lower concentration of methacrylated poly(Pro-Hyp-Gly) results in a hydrogel with a lower polymer network density and a higher swelling ratio. These properties are crucial in determining diffusion of nutrients, waste, and oxygen through the polymer network, and they therefore influence cell viability and proliferation (Gasperini *et al.*, 2014; Park *et al.*, 2009). Park *et al.*, (2009) observed that hydrogels with high swelling ratio support rabbit marrow MSC proliferation. In addition, hydrogels with high polymer network density may act as a physical barrier preventing cell proliferation and migration (Bott *et al.*, 2010). Live-dead staining of the rBMSCs in the 20 mg/mL of methacrylated poly(Pro-Hyp-Gly) hydrogel revealed that most of the cells in the hydrogel are alive (Figure 5b), showing the biocompatibility of the hydrogels as 3D scaffolds for rBMSC encapsulation.

Phase-contrast microscope observations performed on the rBMSCs in the photocrosslinked poly(Pro-Hyp-Gly) hydrogels showed that the rBMSCs were distributed homogeneously in the hydrogels and had a spherical morphology (Figure 5b and 5c). These results are in agreement with Maia *et al.*, (2014), who observed that hMSCs in alginate hydrogels exhibited round shape morphology. This morphology is similar to cell morphology in their *in vivo* microenvironment (Edmondson *et al.*, 2014). After seven days of incubation, the rBMSCs in hydrogels with 30–50 mg/mL of methacrylated poly(Pro-Hyp-Gly) exhibited no difference in cell morphology compared with day 1 (Figure 5d). In contrast, some of the rBMSCs encapsulated in the 20 mg/mL methacrylated poly(Pro-Hyp-Gly) hydrogel exhibited an elongated and branched morphology. These results were in agreement with Engler *et al.* (2006), who observed that mesenchymal stem cells encapsulated in soft hydrogel exhibited an increasingly branched and filopodia-rich morphology, while they formed osteoblast-like morphology in rigid hydrogel. Similarly, Tan *et al.* (2014) showed that premyoblast C2C12 cells in soft and rigid transglutaminase crosslinked gelatin hydrogel (TG-gel) exhibited branched and round morphologies, respectively.

Our results support the view that the encapsulated cells could sense differences in hydrogel properties, such as matrix rigidity, and these differences influenced the cells' viability, proliferation, and morphology.

3.4 Calcium deposition in photocrosslinked poly(Pro-Hyp-Gly) hydrogels containing rBMSCs

To evaluate the ability of photocrosslinked poly(Pro-Hyp-Gly) hydrogels to support rBMSC differentiation into osteogenic lineages, the rBMSCs in hydrogels formed with 20–50 mg/mL of methacrylated poly(Pro-Hyp-Gly) were cultured in osteogenic medium for 28 days. The osteogenic differentiation of the rBMSCs in the hydrogels was observed by the deposition of calcium as a late marker for osteogenic differentiation. After 28 days of incubation, the hydrogels became opaque indicating calcium deposition in the hydrogels (Figure 6a). Phase-contrast microscope images of the rBMSCs in the hydrogels showed that bone nodule formation appeared in all hydrogels (Figure 6b), suggesting that the hydrogels supported the differentiation of the rBMSCs into osteogenic lineages.

For further confirmation of the calcium deposition, we observed SEM images of the hydrogels. These images, without gold coating, showed areas that were brighter than the surrounding area of the hydrogel because of the higher electron reflection by calcium atoms, which indicated calcium deposition in those areas (Figure 6c). The photocrosslinked poly(Pro-Hyp-Gly) hydrogel itself has a low electron reflection ratio, because it contains only of low-atomic-number elements (carbon, hydrogen, nitrogen, and oxygen), which results in darker areas than the calcium-containing areas.

Alizarin Red S and von Kossa staining was used for further confirmation of the calcium deposition in the hydrogels (Figures 7a, b). An area stained clearly red or dark brown was evidence for positive calcium deposition using Alizarin Red S or von Kossa staining,

respectively. The results revealed that high concentrations of methacrylated poly(Pro-Hyp-Gly) and 3 min-irradiated hydrogels showed less staining. Intense staining was observed in the hydrogel formed with a low concentration of methacrylated poly(Pro-Hyp-Gly) and 5 min irradiation. Quantification of the area stained by Alizarin Red S using ImageJ software showed that with 5 min irradiation, the hydrogel formed with 50 mg/mL of methacrylated poly(Pro-Hyp-Gly) exhibited the smallest stained area, while the hydrogel formed with 30 mg/mL of methacrylated poly(Pro-Hyp-Gly) had a significantly larger stained area (Figure 7c) ($p < 0.001$). Quantification of the areas stained by von Kossa showed similar results (Figure 7d) ($p < 0.05$).

These results suggest that hydrogels formed with 30 mg/mL of methacrylated poly(Pro-Hyp-Gly) with 5 min irradiation provided a suitable polymer network density for osteogenic differentiation of the encapsulated rBMSCs. An increase in cell differentiation may be caused by an increase in cell migration and aggregation (Figure 6b). In cell aggregates, cell-cell interaction and cell-matrix interaction are closely similar to cell behavior in *in vivo* microenvironments (Edmondson *et al.*, 2014); therefore, the microenvironment may increase cell differentiation and the expression of differentiation marker genes (Clause *et al.*, 2010; Deegan *et al.*, 2014; Tang *et al.*, 2010).

At high concentrations of methacrylated poly(Pro-Hyp-Gly) hydrogels (40–50 mg/mL), cell migration and aggregation may be inhibited by high polymer network density. The low calcium deposition in 3 min-irradiated hydrogels may be caused by their low swelling ratio, which may reduce the ability of the hydrogels to facilitate transportation of nutrients, waste, and oxygen during cell culture (Gasperini *et al.*, 2014). Sahai *et al.*, (2013) confirmed that a low-oxygen environment reduced the capacity of adipose-derived mesenchymal stem cells to differentiate into osteoblasts.

The encapsulated cells could sense the difference in the hydrogel properties and then,

convert that information into morphological changes and lineage commitment (Engler *et al.*, 2006). In this study, we found that the differences in rBMSC morphology between 20 and 30 mg/mL of methacrylated poly(Pro-Hyp-Gly) hydrogels as a response to polymer network density may control the osteogenic differentiation of the encapsulated rBMSCs. The rBMSCs in 30 mg/mL methacrylated poly(Pro-Hyp-Gly) hydrogel exhibited a spherical morphology that is similar to that of osteoblastic cells in *in vivo* microenvironments; therefore, they produced more calcium as an osteogenic marker than that rBMSCs did in 20 mg/mL of methacrylated poly(Pro-Hyp-Gly) hydrogel. Similarly, Tan *et al.*, (2014) showed that premyoblast C2C12 cells in rigid TG-gel exhibited a round morphology and higher osteogenic marker expression than the cells in soft TG-gel.

Finally, these results show that by carefully controlling the photocrosslinking conditions for poly(Pro-Hyp-Gly) hydrogel fabrication, we successfully provided a cytocompatible 3D microenvironment for simultaneous rBMSC encapsulation. Importantly, our findings confirm that hydrogel properties, such as polymer network density and swelling ratio, are important factors in determining cell viability, morphology, and lineage commitment.

4. Conclusion

Photocrosslinked poly(Pro-Hyp-Gly) hydrogels were successfully fabricated by visible-light photocrosslinking, and the hydrogels' physical and chemical properties could easily be controlled by changing the concentrations of eosin Y and methacrylated poly(Pro-Hyp-Gly) and the irradiation time. An *in vitro* rBMSC encapsulation study showed that the photocrosslinked poly(Pro-Hyp-Gly) hydrogels were stable for long culture periods and supported the viability of encapsulated rBMSCs and their differentiation into osteogenic lineages. Taken together, these findings demonstrate the feasibility of fabricating a chemically

crosslinked poly(Pro-Hyp-Gly) hydrogel as a scaffolding material for tissue regeneration.

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Conflict of interest

The authors have no potential conflicts of interest to declare.

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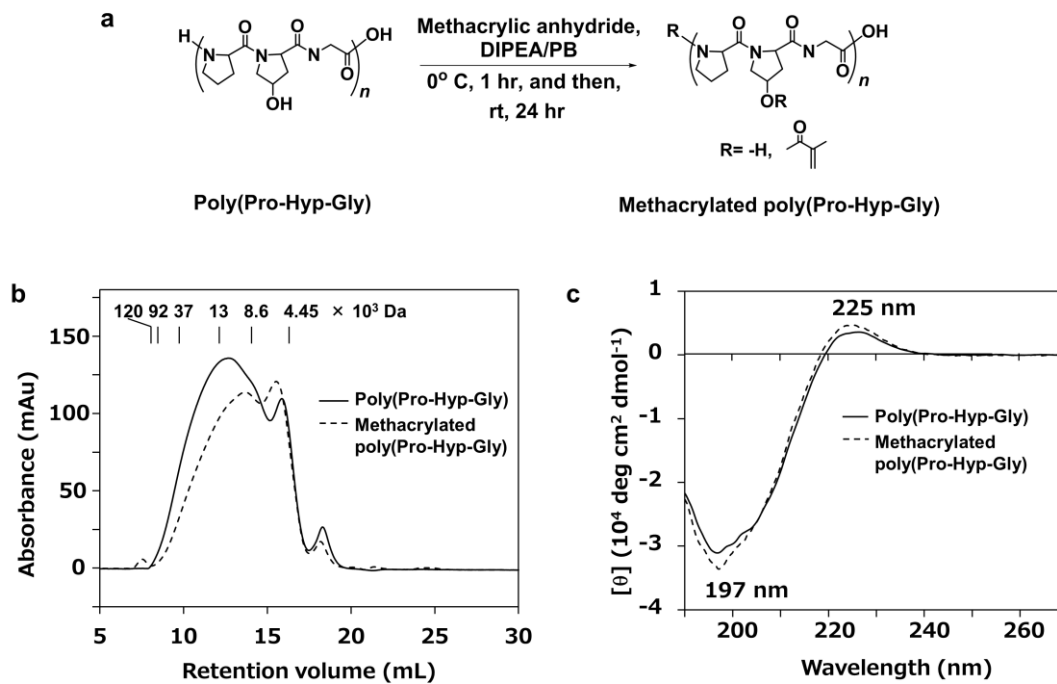


Figure 1. (a) Schematic diagram of the synthesis of methacrylated poly(Pro-Hyp-Gly). (b) GPC profiles and (c) CD spectra of poly(Pro-Hyp-Gly) and methacrylated poly(Pro-Hyp-Gly).

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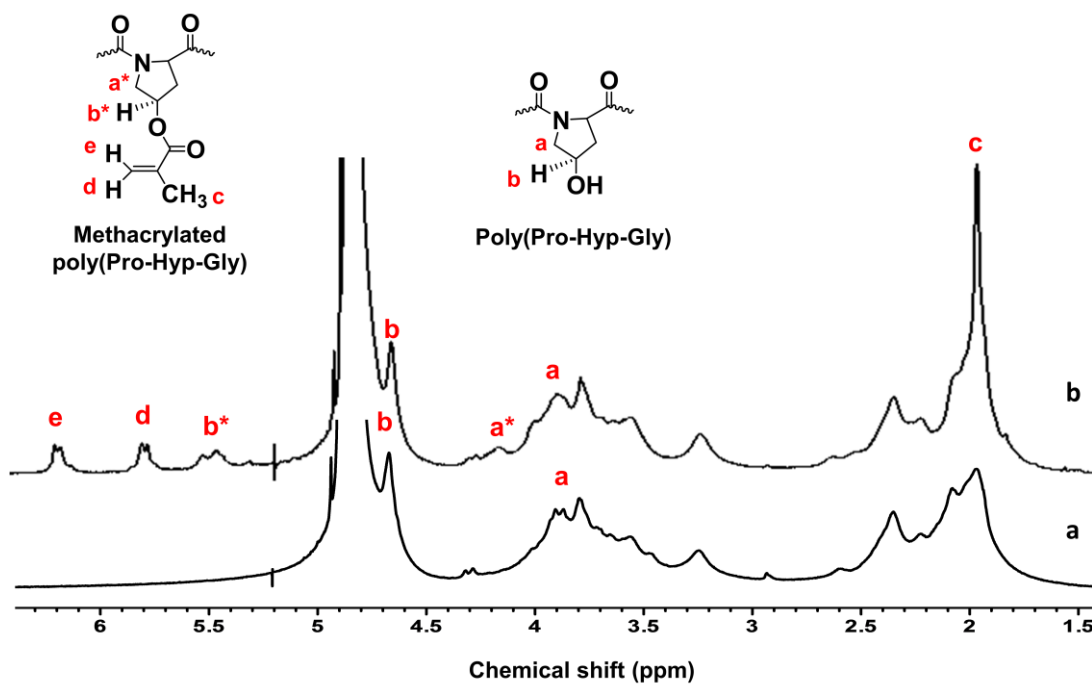


Figure 2. (a) $^1\text{H-NMR}$ spectra of poly(Pro-Hyp-Gly) and (b) methacrylated poly(Pro-Hyp-Gly). a^* and b^* are Hyp- C_δH and Hyp- C_γH , respectively, which are shifted downfield because of methacrylation of the hydroxy group of Hyp residues of poly(Pro-Hyp-Gly).

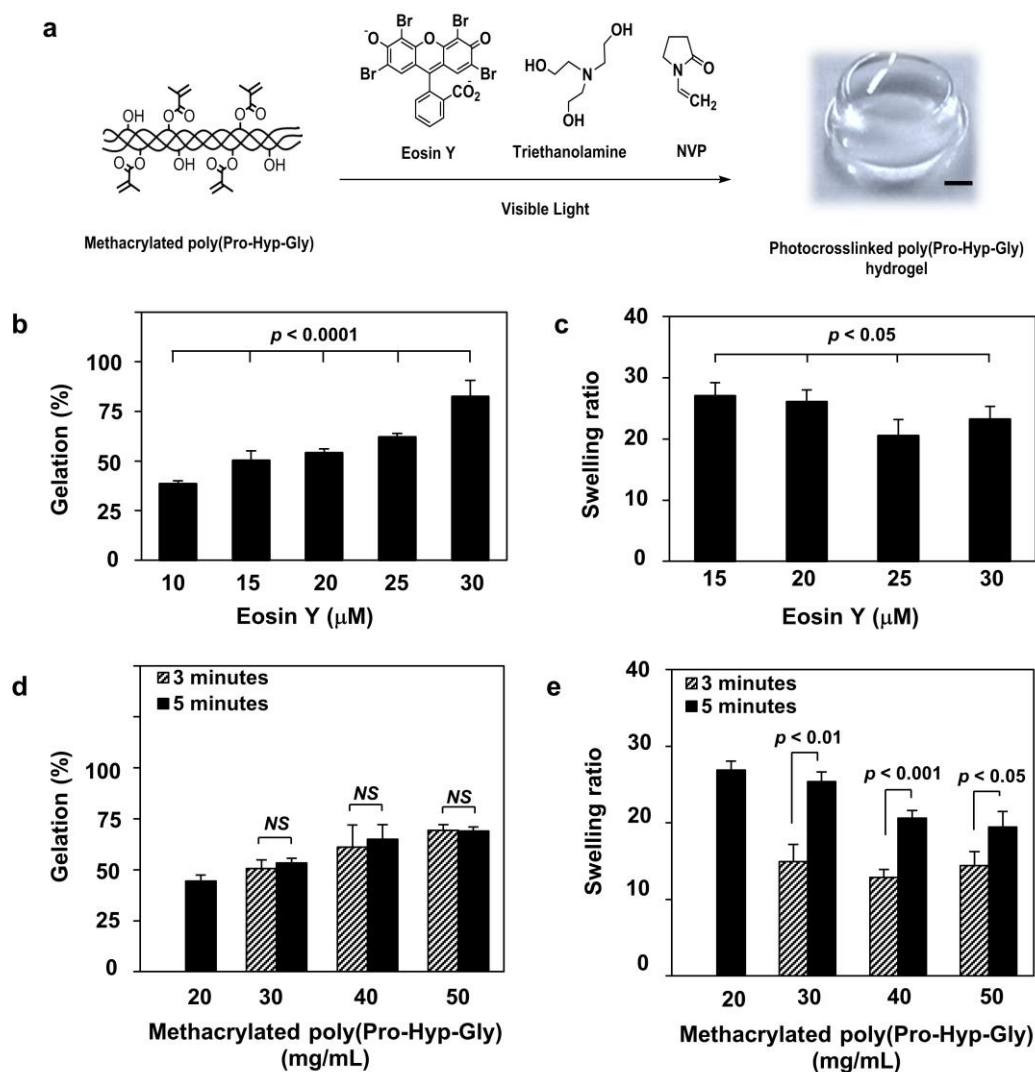


Figure 3. (a) Schematic diagram of the photocrosslinking of methacrylated poly(Pro-Hyp-Gly) with visible light in the presence of eosin Y, triethanolamine, and NVP. (b) Gelation and (c) swelling ratios of photocrosslinked poly(Pro-Hyp-Gly) hydrogels at different concentrations of eosin Y; the photocrosslinking system contained 30 mg/mL of methacrylated poly(Pro-Hyp-Gly), 10–30 μM eosin Y, 10 mM triethanolamine, and 1 mM NVP in PBS, and the irradiation time was 5 min. (d) Gelation and (e) swelling ratios of photocrosslinked poly(Pro-Hyp-Gly) hydrogels formed at different concentrations of methacrylated poly(Pro-Hyp-Gly) and irradiation times; the photocrosslinking system contained 20 μM eosin Y, 10 mM triethanolamine, and 1 mM NVP in PBS. The bar in an image of the obtained hydrogel represents 1 mm. NS = not significant.

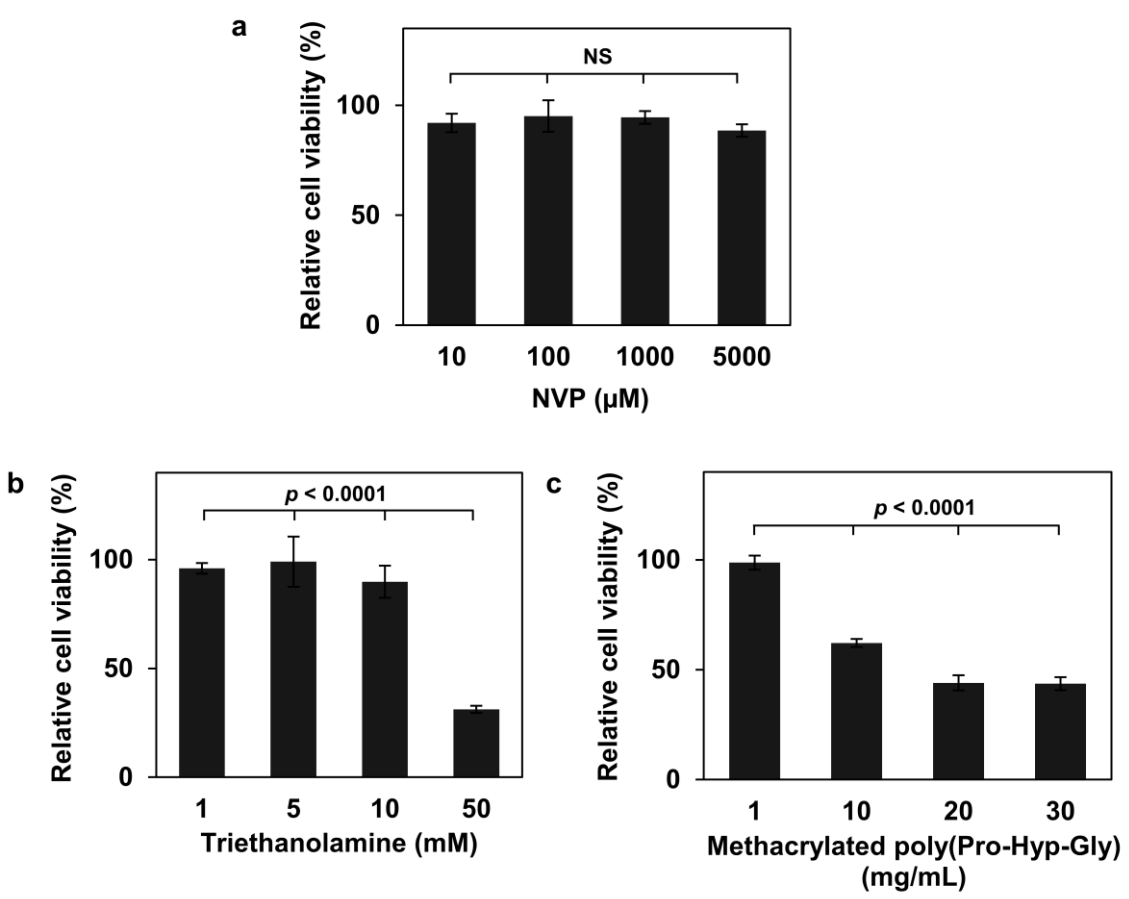


Figure 4. Relative cell viability of rBMSCs cultured in 20% FCS/ α -MEM containing (a) NVP, (b) triethanolamine, or (c) methacrylated poly(Pro-Hyp-Gly) for 24 h.

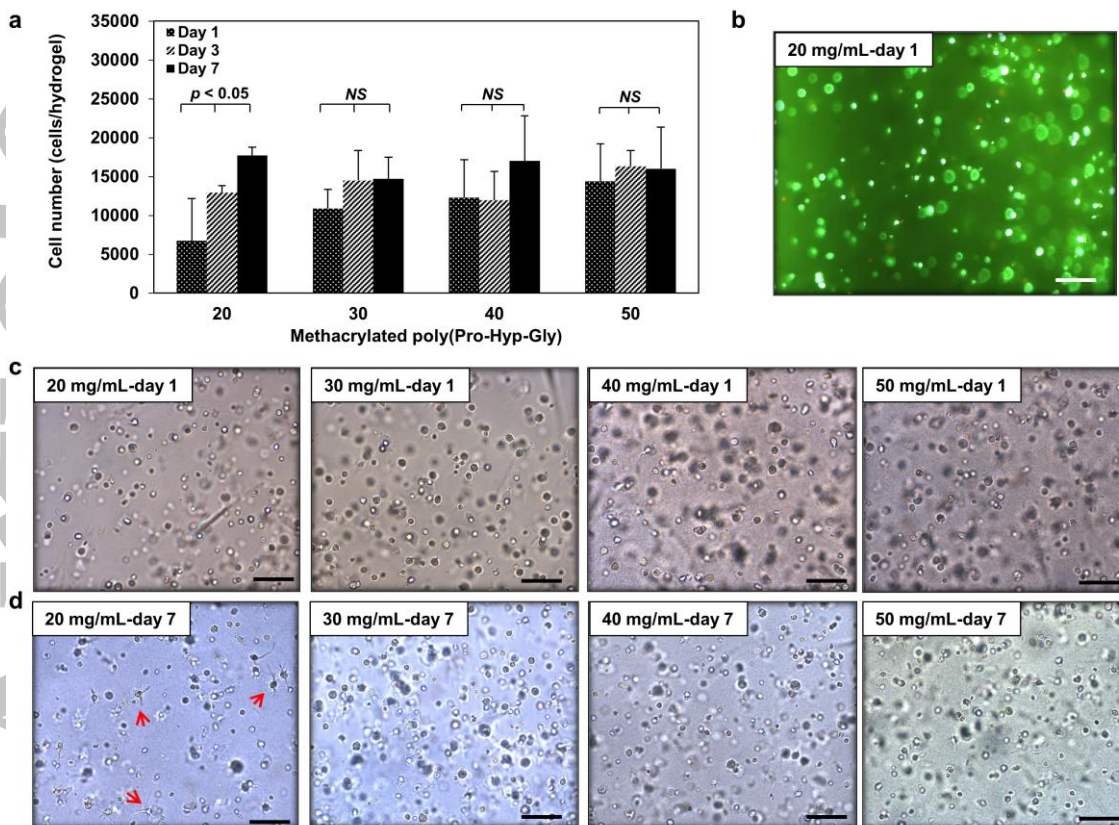


Figure 5. (a) Proliferation of rBMSCs in photocrosslinked poly(Pro-Hyp-Gly) hydrogels formed with 20–50 mg/mL of methacrylated poly(Pro-Hyp-Gly); the photocrosslinking system contained 20 μ M eosin Y, 10 mM triethanolamine, and 5 mM NVP in PBS, and the irradiation time was 5 min. Live-dead staining (b) and phase-contrast microscope images of the rBMSCs in the photocrosslinked poly(Pro-Hyp-Gly) hydrogels at day 1 (c) and day 7 (d). Red arrows indicate rBMSCs with branched morphology. The bars represent 100 μ m.

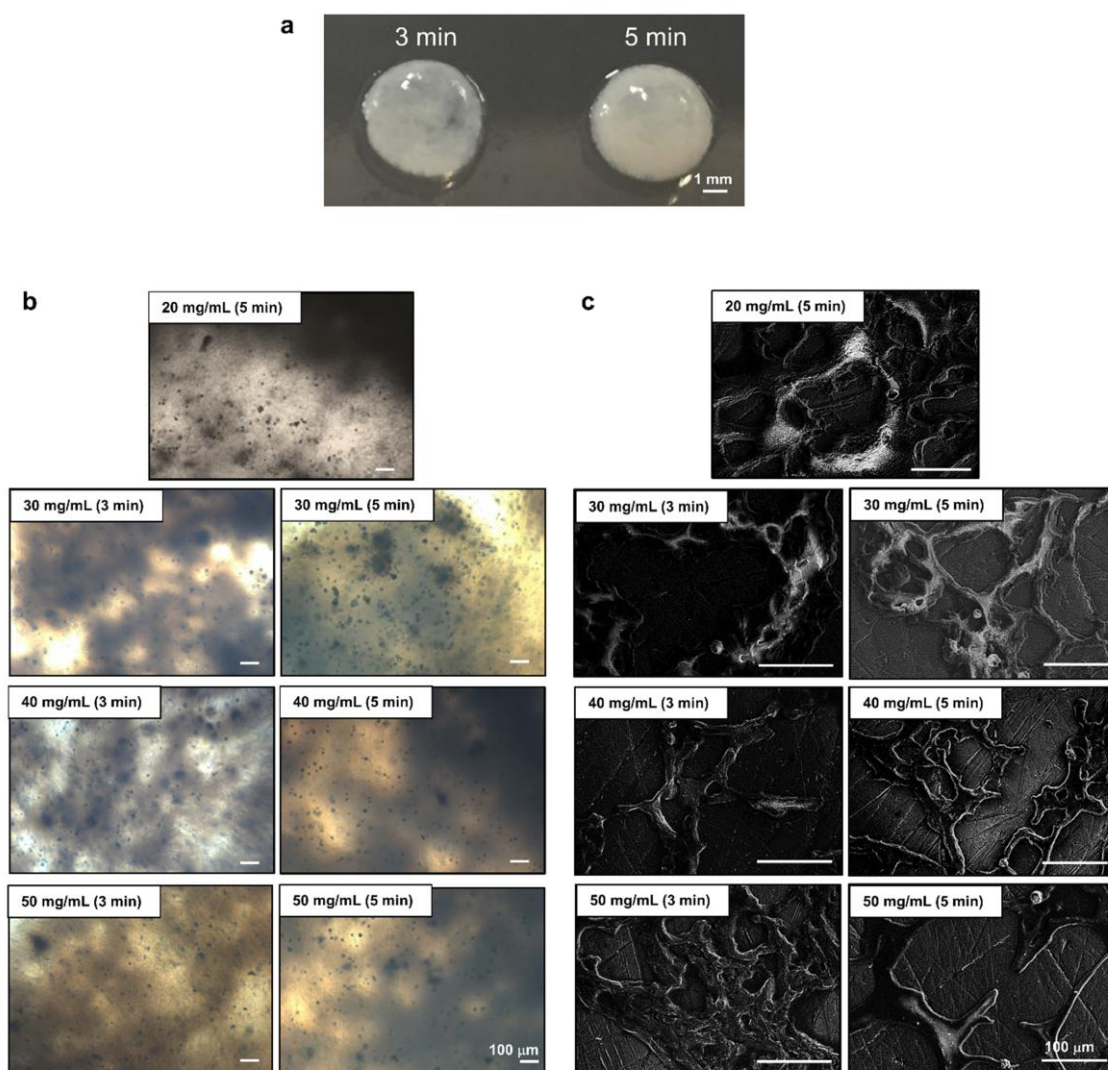


Figure 6. (a) Macroscopic appearance and (b) phase-contrast microscope images of rBMSCs in photocrosslinked poly(Pro-Hyp-Gly) hydrogels incubated with an osteogenic medium at day 28. (c) SEM images of 6 μm slices of photocrosslinked poly(Pro-Hyp-Gly) hydrogels containing rBMSCs.

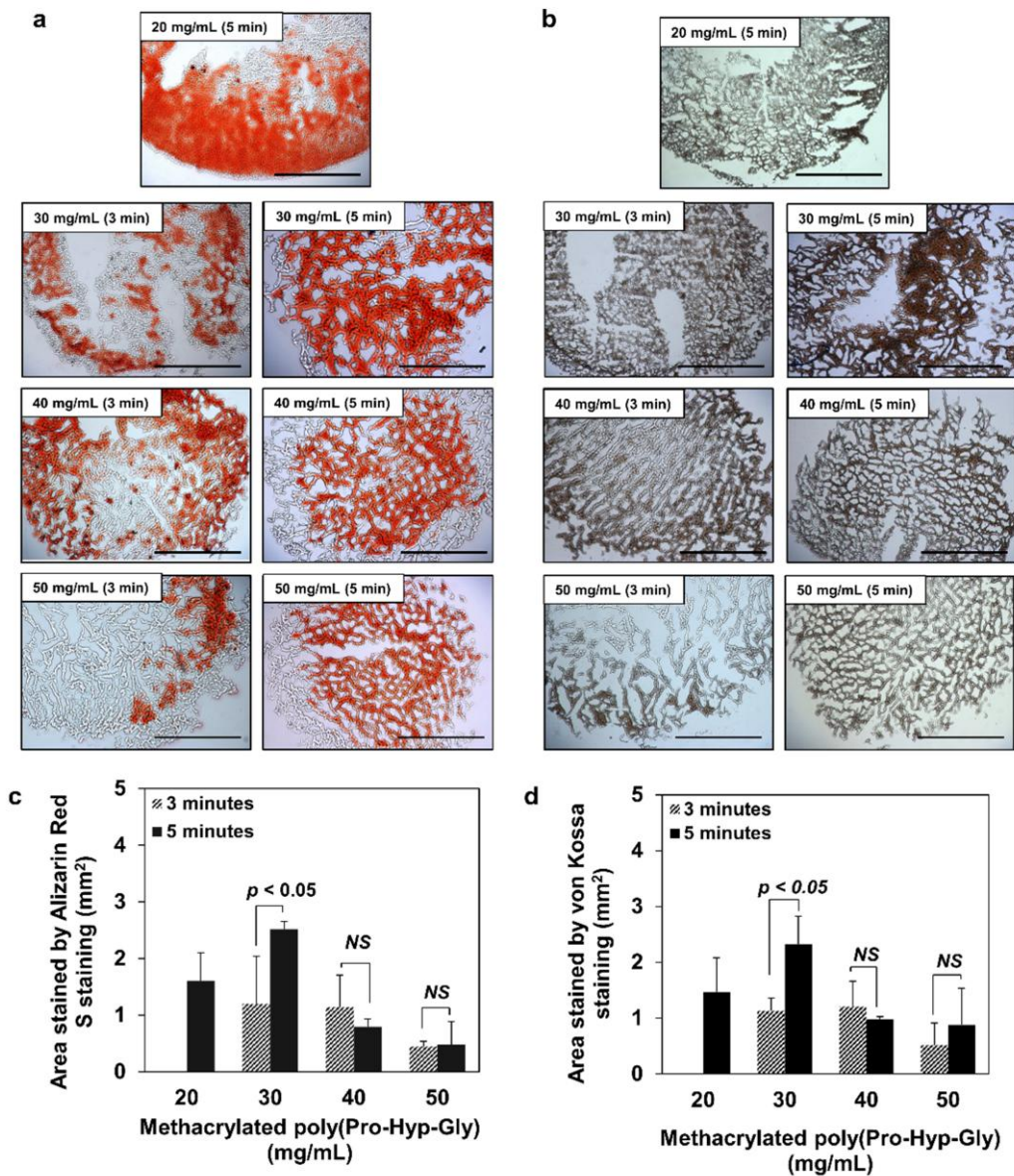


Figure 7. (a) Alizarin Red S and (b) von Kossa staining of 6 μm slices of photocrosslinked poly(Pro-Hyp-Gly) hydrogels containing rBMSCs after incubation with an osteogenic medium for 28 days. Areas of hydrogel slices that were stained by (c) Alizarin Red S and (d) von Kossa. The bars represent 1 mm.