

Injectable *in situ* crosslinkable hyaluronan-polyvinyl phosphonic acid hydrogels for bone engineering

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ABSTRACT

A novel injectable hydrogel that was synthesized by *in situ* crosslinking of hyaluronan and polyvinyl phosphonic acid was proposed in this study. Fourier transform infrared spectrum (FT-IR) analysis, scanning electron microscope (SEM), pH measurement, and biodegradation test were used to confirm its characteristics. The results permitted to prove successful crosslinking, observe the inner morphology of hydrogel and pore sizes distribution, and determine the decomposition of hydrogel components during incubation time. Result of pH measurement showed that the pH scale of hydrogel decreased when volume of PVPA increased. As a consequence, it affected the cytotoxicity value, cell proliferation, and cell growth behaviors of each hydrogel. Optical microscope observation showed that chondroblasts cell proliferated well on HA-PVPA hydrogel. Therefore, these results suggest that the new injectable hydrogel is appropriate for bone/cartilage regeneration applications.

Keywords: Hyaluronan; PVPA; Injectable Hydrogel; Mesenchymal Stem Cells; Chondroblast Cells

1. INTRODUCTION

Hydrogels are three-dimensional hydrophilic polymeric networks consisting of interconnected pores, which absorb and retain water/human liquids [1,2]. Injectable *in situ* crosslinkable hydrogels have been widely applied in biomedical investigations, such as drug delivery [3], stem cell delivery [4] as anti-adhesive agent [5], encapsulating protein [6] due to their highly hydrophilic and non-toxicity characteristics [1,7]. For bone tissue engi-

neering, injectable *in situ* crosslinking hydrogels were widely used due to their capability of carrying osteoconductivity and osteoinductivity such as VEGF protein [6], receptors and stem cells [8], calcium phosphate [9] leading to accelerate bone formation and enhance the bone healing process [10].

Specially, injectable hydrogels are the favorite biomaterials for cartilage regeneration [11] and vocal fold lamina propria regeneration [12] because of three main reasons. First, injectable method is convenient in comparison with conventional operations since it reduces sore after treatment. Second, an injectable biomaterial could be formed into any complex shapes and then subsequently crosslinked to conform to the required dimensions at the site of injury. Third, the crosslinkable hydrogel would adhere to a tissue during the gel formation.

Hyaluronan is a non-sulfated glycosaminoglycan (GAG) and glucuronic acid, and is a major constituent of the extracellular matrix and organizes the extracellular matrix. Hyaluronan has been developed for bone augmentation [13], or cartilage [13-17] applications. Specially, injectable hyaluronan hydrogel has been widely used for knee osteoarthritis, knee, intra articular injection [18] and hip treatments [19].

A phosphonate-containing polymer, poly(vinyl phosphonic acid) (PVPA) is hypothesized to mimic the action of bisphosphonates, a group of drugs used in the treatment of osteoporosis. The close proximity of P-C groups within the structure is speculated to mimic the P-C-P backbone found in bisphosphonates which act by inducing osteoclast apoptosis leading to an increase in bone formation [20]. Phosphonic acid containing polymers has also been described to improve mineralisation in a dose-dependent manner, therefore, promoting osteoblast maturation. Moreover, Polyvinylphosphonic acid (PVPA) which shares a similar structural formula to

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poly(alkenoic acids) and has a quality similar to that of glass polyalkenoates [21], is used as an additive hardening ingredient in dental cements. Besides, PVPA has been shown to be beneficial due to its similarity to the phosphate groups of the natural bone hydroxyapatite [22], which can assist in the bone-bonding between the restorative material and the native bone tissue [23,24].

So far, there has been no report about injectable *in situ* crosslinkable hydrogel HA-PVPA. Therefore, the purpose of this investigation is to synthesize and analyze the characteristics and properties of a novel injectable *in situ* crosslinkable hydrogel HA-PVPA for bone regenerative applications. HA-PVPA hydrogel composes of a natural GAG of HA and osteoconductivity of PVPA. Hydrogel was formed by covalent crosslinking between -OH groups of hyaluronan and -P-OH groups of PVPA which were detected by appearing -CH₂-O-P-O-CH₂- stretching peaks from FT-IR analysis. By increasing the volume of PVPA, the pore sizes of hydrogel increased but the biocompatibility of hydrogel decreased through MTT assay results. The mesenchymal stem cell and chondroblast cell proliferated remarkably on hydrogel are fundamental evidences in order to approve HA-PVPA hydrogel as a novel biomaterials for bone regenerative applications.

2. MATERIALS AND METHODS

2.1. Materials

Polyvinyl phosphonic acid (PVPA, Mw = 20,000, 30% solution) was purchased from Polysciences, Inc. (Warrington, PA, USA). Hyaluronan from *Streptococcus equisimilis* sp. purchased from Fluka (St. Louis, MO, USA). 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT formazan) purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS), phosphate buffered saline (PBS) were purchased from Hyclone (Waltham, MA, USA).

2.2. Synthesis of Hyaluronan-PVPA Hydrogel

0.5 wt% hyaluronan was dissolved in PBS solutions and gently stirred at room temperature to dissolve completely and became transparent solution. Yet pH scale of hyaluronan (HA) was recorded. Then, 1 ml hyaluronan solutions were taken by cylinder and poured into the glass vial. The formation of hydrogels was determined by injecting PVPA solution in various volumes (10, 20, 30, 40 and 50 μ l) which labeled HP-10, HP-20, HP-30, HP-40 and HP-50. The blend components were mixed by vortex; when the liquid level in the glass vial stopped moving, the gelation time of each sample was recorded. The pH scale of hydrogels (HP-10 - HP-50) were immediately measured after gelation using pH a meter (PHS-3BW).

2.3. Characterization of HA-PVPA Hydrogel

2.3.1. Scanning Electron Microscope

In order to measure the hydrogel structure, hydrogels were then frozen at -20°C and lyophilized. Next, the samples were mounted on an aluminum holder. Then, the holder was coated by platinum. The morphologic structure of hydrogels was observed using scanning electron microscope (SM-65F, JEOL, Japan).

2.3.2. FT-IR Analyze

The IR spectra of HA (a), HA-PVPA (b) and PVPA (c) were characterized by attenuated reflectance Fourier transform spectroscopy (Spectrum GX, PerkinElmer, USA). The infrared spectra of the samples were measured over a wavelength range of 4000 - 500 cm⁻¹. All spectra were taken in the spectral range by the accumulation of 64 scans with a resolution of 4 cm⁻¹.

2.3.3. Mechanical Properties

Tensile strength of the hydrogels was performed on a Universal Testing Machine (Unitech TM, R&B, Korea). Hydrogels (n = 4) were prepared in Teflon mold with uniform ribbon shapes and then placed on a metal plate, where the mechanical characterization was achieved by applying tensile test loads to specimens prepared at a crosshead speed of 1 mm/min.

2.3.4. Biodegradable Evaluation

Hydrogels were degraded for up to 30 days at 37°C with 0, 1, and 10 U/mL bovine testes hyaluronidase (Sigma) in PBS as described in previous study [25].

2.4. In Vitro Studies

2.4.1. Cell Maintenance

Fibroblast cells (ATCC, USA), chondroblast cells (ATCC, USA) and mesenchymal stem cells (ATCC, USA) were subcultured and changed media every 2 days. Media were supplemented with 10% (v/v) FBS (fetal bovine serum) and 1% PS (penicillin/streptomycin (antibiotics)). Cells were dissociated with trypsin-EDTA (GIBCO) centrifuged and resuspended in medium for using [26].

2.4.2. MTT Assay

The cytotoxicity of the hydrogels was evaluated by an extraction test according to ISO 10993-5 Standard [27]. The hydrogels were immersed in a culture medium (RPMI) at an extraction ratio of 0.1 g/ml and incubated in a humidified atmosphere with 5% CO₂, for 24 h, at 37°C. Fibroblast cells, seeded in 96-well culture plates at 1000 cells/well in 100 RPMI, were incubated with extraction fluid for 72 h, and then 20 μ l of filtered MTT Solution (0.1 mg/mL in PBS) was added. After incubation at 37°C for 3.5 h, the medium was removed from the

well and 150 μl of DMSO (dimethyl sulfoxide) was added to dissolve any insoluble formazan crystals. The absorbance was measured at 560 nm using an ELISA reader (Turner Biosystems CE, Promega Corporation, USA) and the cell viability was calculated as the percentage relative to the untreated control cells [26].

2.4.3. Cell Proliferation

The cell proliferation on the hydrogels was assessed by the use of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assays and microscope observation using chondroblast cells and bone marrow mesenchymal stem cells. The cells were seeded in 24-well tissue culture plates coating with hydrogels. After being treated for 1, and 3 days, the culture plate moved to the microscope (Olympus, IX71, Japan) in order to observe cell proliferation before adding 20 μl of filtered MTT solution (5 mg/mL in PBS). After adding MTT solution, cells were incubated at 37°C for 3.5 h, and then the medium was removed from the well. Finally, 1 ml of DMSO was added to dissolve any insoluble formazan crystals. The absorbance was measured at 560 nm using an ELISA reader (Turner Biosystems CE, Promega Corporation, USA) after aliquot 200 μl from 1 ml purple solution of 24-well to 96-well. The absorbance value determined the cell viability on hydrogels.

3. RESULTS AND DISCUSSION

Injectable hydrogels are of great interest as components of drug delivery systems, cell encapsulation vehicles, and scaffolds for tissue engineering. Hydrogels offer several advantages over conventional solid implants such as their handling is easy, they effectively reduced scarring and healing periods, cause less pain to the patient, allow minimizing the surgery time, and provide good conformation to irregular defects. In this study, we developed an injectable hydrogel that supports cell proliferation and growth to permit *in vivo* engineering of new tissues. *In situ* crosslinking HA-PVPA hydrogel is formed by crosslinking the -OH group of HA and the P-OH group of PVPA as shown in **Figure 1**.

In order to verify that HA and PVPA crosslinked successfully, the FT-IR spectrum of PVPA, HA and HP hydrogels were analyzed (**Figure 2**).

The spectrum of hyaluronan HA, the functional groups were indicated by the peak of C-O-C stretching at 1035 cm^{-1} and the peaks of carboxyl groups at 1410 cm^{-1} and 1620 cm^{-1} [28]. In case of PVPA, the band at 1140 cm^{-1} can be attributed to the phosphoryl group (P=O) of PVPA. And, the absorption bands at 904 and 983 cm^{-1} are a symmetric and an asymmetric vibration of P-O of PVPA [24]. From the spectrum of the HA-PVPA hydrogel confirmed that HA and PVPA cross-linked by both ionic bonding and covalent bonding. The assign-

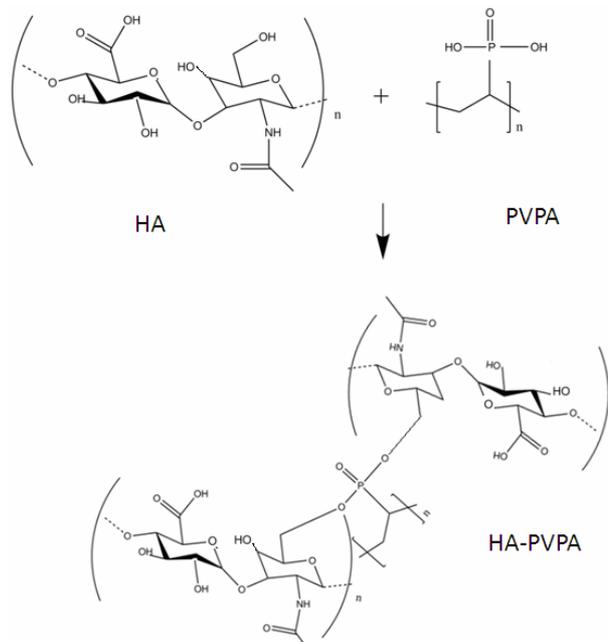


Figure 1. Schemes of the *in situ* crosslinking process of HA-PVPA hydrogels.

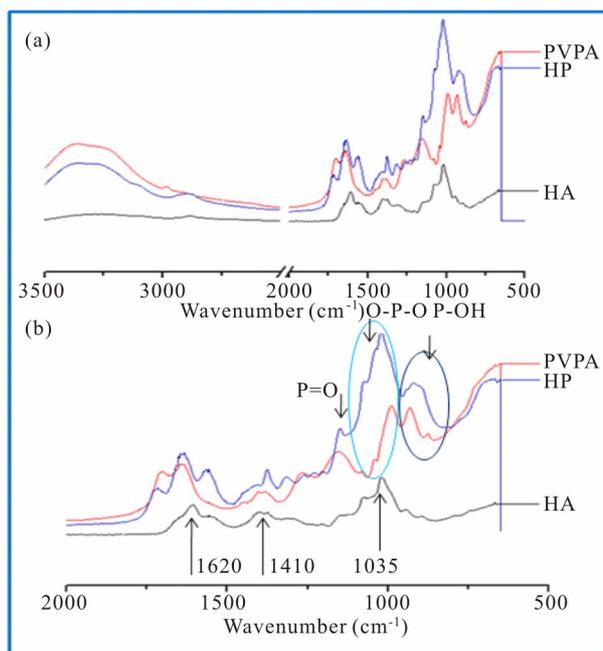


Figure 2. (a) FTIR spectra of original hyaluronan salt (HA), hyaluronan-polyvinyl phosphonic acid (PVPA) and hyaluronan-polyvinyl phosphonic acid hydrogels (HP); (b) Section of part A showing an enlargement of a section in the region 2000 - 500 cm^{-1} .

ments of some IR bands are as follows: 1210 cm^{-1} to the P=O stretching mode; 1168 cm^{-1} to the P-C stretching mode; 1000 - 1100 cm^{-1} to the predominantly uncomplexed -CH₂-O-P-O-CH₂- stretching mode [29]; 900 -

1000 cm^{-1} to the P-OH [7]. The proofs are that the absorbance of O-P-O stretching mode increased and the symmetric vibration of P-O at 904 cm^{-1} disappeared, and the ionic bonding between -NH- group of HA and -P-OH of PVPA resulted in the -PO₂-3 band at 1010 cm^{-1} increased [30].

The crosslinking between HA and PVPA was carried out at room temperature and aqueous solution as shown in **Figure 3**. **Figure 3(a)** shows the inverted vial in order to demonstrate hydrogel HP-10, 1 ml hyaluronan (0.5 wt%) and 10 μl PVPA, formation within 40 seconds. **Figure 3(b)** shows the hydrogel hanged on the needle to demonstrate hydrogel strength. Tensile stress and tensile strain of hydrogel increased directly proportionally to increase amount of PVPA. However, increase of stress is not remarkable in comparison with the increase of strain. For example, as the amount of PVPA increased from 10 - 50 μl , tensile stress increased from 10 - 25 (kPa) while tensile strain increased between 20% - 105% (**Table 1**). Gelation time decreases regularly and proportionally to amount of PVPA. For bone tissue regeneration, the scaffold with macroporous (over 50 nm) structure is recom-

mended for bone ingrowths and capillaries ingrowths [31,32]. Therefore, the pore sizes of hydrogels were confirmed by observing their morphology using scanning electronic microscope. Results of SEM observation showed that the pore sizes of PVPA-HA hydrogels are substantially larger than those prepared without the use of PVPA. Overall, all hydrogels meet the requirements of the pore sizes of bone substitute materials (larger than 50 nm). And, increasing of PVPA volume caused the pore sizes to increase (**Figure 4**). For example, SEM morphology of hyaluronan showed that hydrogel without PVPA composed of microporous and mesoporous (**Figure 4(a)**). When adding PVPA, the macroporous structure was obtained, and the pore sizes were larger as PVPA volume increased (**Figures 4(b)-(f)**). More specially, when adding 10 μl PVPA, the pore sizes were below 200 μm while adding 50 μl PVPA the pore sizes were larger than 200 μm . In previous report, it was found that the covalent crosslinking created a larger pore sizes than ionic crosslinking [7]. Therefore, the covalent crosslinking of -OH groups of hyaluronan and -P-OH groups of PVPA is probably the cause for the increase of the

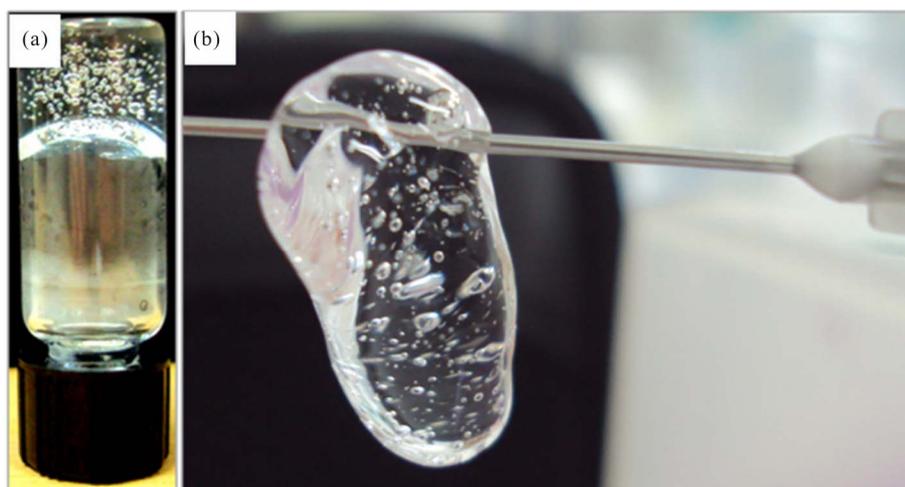


Figure 3. Photographs of the HA-PVPA hydrogels after mixing of HA and PVPA at 37 °C inside the reverse vial (a) and hang on a needle (b).

Table 1. Properties and characteristics of hydrogels.

	PVPA (ml)	Hyaluronan (%)	PH	Gelation time (seconds)	Pore size distribution (μm)	Tensile Strength	
						Strain (%)	Stress (kPa)
HA	0.00	1.0	6.8	-	<50	-	-
HP-10	0.01	1.0	5.8	40	50 - 150	20	10
HP-20	0.02	1.0	5.2	30	50 - 200	35	12
HP-30	0.03	1.0	4.9	25	50 - 250	58	17
HP-40	0.04	1.0	4.6	15	50 - 250	84	20
HP-50	0.05	1.0	4.2	10	50 - 300	105	25
PVPA	100	0.0	1.1	-	-	-	-

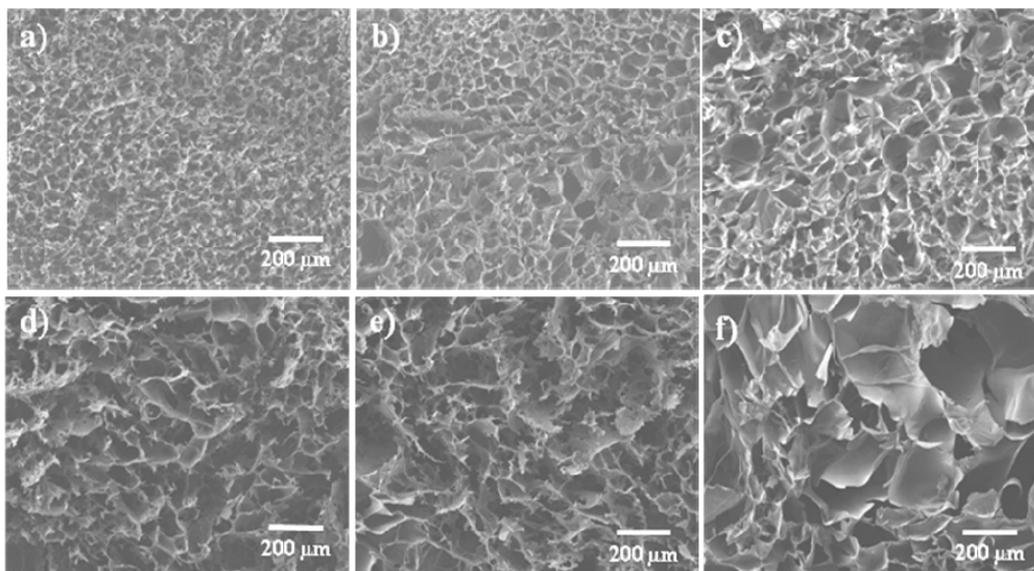


Figure 4. SEM morphology of HA-PVPA hydrogels at the range of PVPA volumes: (a) HP-0; (b) HP-10; (c) HP-20; (d) HP-30; (e) HP-40; (f) HP-50.

pore sizes of HP-50 in comparison with the pore sizes of HP-10.

Biodegradation is an important factor, which should be tested for a new material in order to observe its degradable behavior. In this investigation, the degradation of HP hydrogels was examined for 30 days as shown in **Figure 5**. Diagram shows that hyaluronan hydrogel degraded significantly over 95 wt% while HP hydrogels degraded only 20 wt% after 10 days in 37°C. However, degradation behavior was significantly different after incubating for 15 days, HP-10 and HP-20 degraded approximately 40 wt% and 30 wt%, respectively, and HP-30 to HP-50 did not change significantly after the 10th day. Between the 15th day and 30th day, the degradation ratios of HP hydrogels were unchanged except for HP-30, which degraded approximately 30 wt% after incubating 30 days. The discomponent behavior of hydrogels as shown in **Figure 5** were probably caused by the physical discomponent. In here, a mass of polymer was established by covalent cross-linking of HA and PVPA so that hydrogels composed of high PVPA volume sustained a longer degradable time due to a mount of crosslinking.

Cytotoxicity is a general test for any biomaterial to evaluate its biocompatibility. In this study, cytotoxicity of hydrogels was examined using MTT assay. MTT assay is a simple and easy method that widely used for examining the toxicity of biomaterial reported in our previous studies [26,31,33,34]. According to I ISO-109 93-5, biomaterials that have over 70% cell viability will be approved as biocompatible biomaterials. Therefore, HA-PVPA hydrogels with being added over 30 $\mu\text{l/ml}$ PVPA significantly toxic as shown in **Figure 6**. By contrast, hydrogels that were added less than 30 $\mu\text{l/ml}$ PVPA are

biocompatibility. For example, HP-10 and HP-20 hydrogel showed excellent biocompatible in comparison with other hydrogels which are significantly toxic. The toxicity occurred due to the pH scale of hydrogels. By increasing the amount of PVPA, the pH scale becomes acid leading to the change of the pH scale of the culture medium, which decreased cell viable on hydrogels.

Cell proliferation on hydrogels was assessed using MTT assay method because MTT molecular detect only cell live [35]. Therefore, MTT assay was employed to evaluate cell viability through optical density value. **Figure 7** shows a graph of cell growth behavior on hydrogels in comparison with the control (tissue culture plate) in a 2 days period. After 1 cultured day, cells that grew on HP-10 to HP-40 were not significantly different, approximately 0.65 O.D, but were significantly higher on HP-50. Overall, cell growth was significantly higher on the control sample than on hydrogels. After incubating for more than 2 days, on the 3rd day, cell proliferation remarkably changed on all samples. For example, the optical density of the control sample increased remarkably and significantly higher than that of the hydrogels. HP-10 hydrogel showed excellent cell proliferation from about 0.65 O.D. to about 1.6 O.D. The more PVPA volume increased, the more cell proliferation decreased. For example, cell proliferation on HP-20 was lower than on HP-10. Specially, the results showed that a half of cell was dead on HP-30, HP-40 and HP-50 after 3 days in cubation. By contrast, cell proliferated by twofold on HP-10 and HP-20 hydrogels after a 2 day period. Hyaluronan is well-known as an excellent natural material [4,17,36] but the pH scale of HP affected to the cell vi ability. It is possible to infer results from increasing the amount of

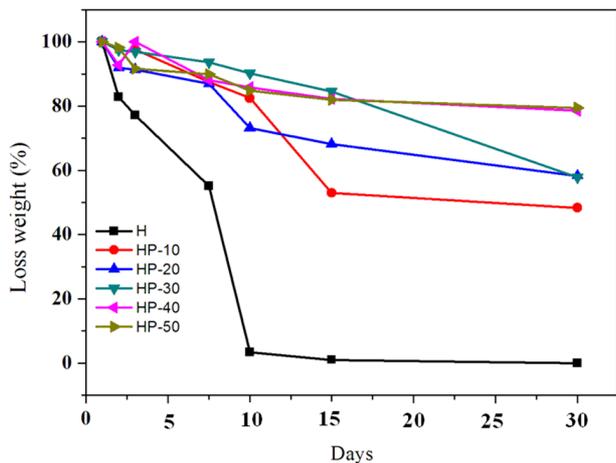


Figure 5. Biodegradable evaluation of HA-PVPA hydrogels during incubating in PBS solution.

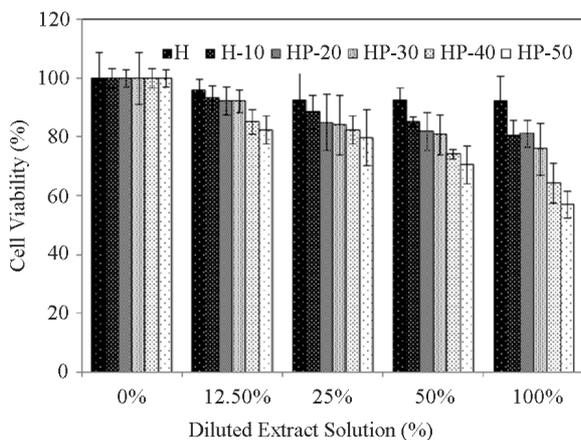


Figure 6. Cytotoxicity evaluation of hydrogels using MTT assay method.

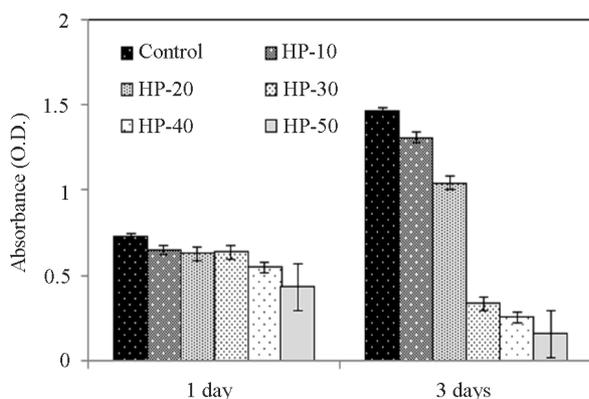


Figure 7. Optical images of mesenchymal stem cell proliferated on various hydrogels control and hydrogel sHP10-50 for 3 days.

PVPA.

Stem cells are sensitive even with vibration and under cold storage conditions [37]. Therefore, in case of

HA-PVPA injectable hydrogel sustain stem cells, it will probably proliferate and able to differentiate into other cell-lines [38]. In here, the cell viability of HA-PVPA injectable hydrogel was tested using rabbit bone marrow stem cells to evaluate interaction between hydrogel and stem cell [39]. In this study, rabbit stem cells were isolated as described in previous reports [40,41] and 106 cells were seeded in each well. To observe cell-hydrogel interaction after 3 days of culturing, the cell proliferation on each hydrogel in the cultured plate was inspected using the invert microscope. The optical images of mesenchymal stem cell (MSC) proliferated on hydrogel are shown in **Figure 8**. It shows that stem cells grew significantly on culture plate, higher than on hydrogels. Among hydrogels, HP-10 is the most compatible in comparison with control and the others HA-PVPA hydrogels. The results showed that almost HP hydrogels are not a favorite substrate for MSCs excepting hydrogel HP-10.

To apply HA-PVPA hydrogel for injecting and regenerating the cartilage damage, hydrogel was tested with chondroblast cell *in vitro*. **Figure 9** showed the proliferation of chondroblast cell on only HP-10 hydrogel. Cell growth behavior was observed for 60 minute and 1 day. **Figures 9(a), (b)** and **Figures 9(c), (d)** showed the morphology of chondroblasts cells proliferated on hydrogel HP-10 for the first 60 minutes and for 1 day, respectively. **Figures 9(a)** and **(b)** showed that chondroblasts cells were not spread at the early 60 min, then they spread well after 1 cultured day. Thus, HA-PVPA hydrogel is a potential injectable hydrogel for cartilage regeneration applications.

4. CONCLUSION

We introduced a novel injectable hydrogel, composing of hyaluronan and polyvinyl phosphonic acid. This hydrogel seem to be a promising biomaterial because it is necessary, affective, and handy for bone applications. HA-PVPA hydrogels consisted of the upper 100 nm of pore sizes that recommended scaffold for bone regeneration. And, as PVPA volume increased, the mechanical strain (%) of hydrogels increased while mechanical stresses were not significantly different. HA and PVPA provided successfully *in situ* crosslinking through covalent bonding, which was determined by FT-IR spectrum. MTT assay confirmed that the pH scale decreased when the PVPA volume increased, resulting in decreasing the biocompatibility of hydrogels. Among hydrogels, HP-10 hydrogel is the best in term of biocompatibility. For example, the results of cell proliferation tests showed that the pH scale affected the sensitivity of bone marrow mesenchymal stem cells; but, HP-10 hydrogel showed biocompatible with MSCs. And, chondroblast cells proliferated very well on HP-10. Therefore, HP-10 hydrogel is proved to be appropriate for cartilage applications.

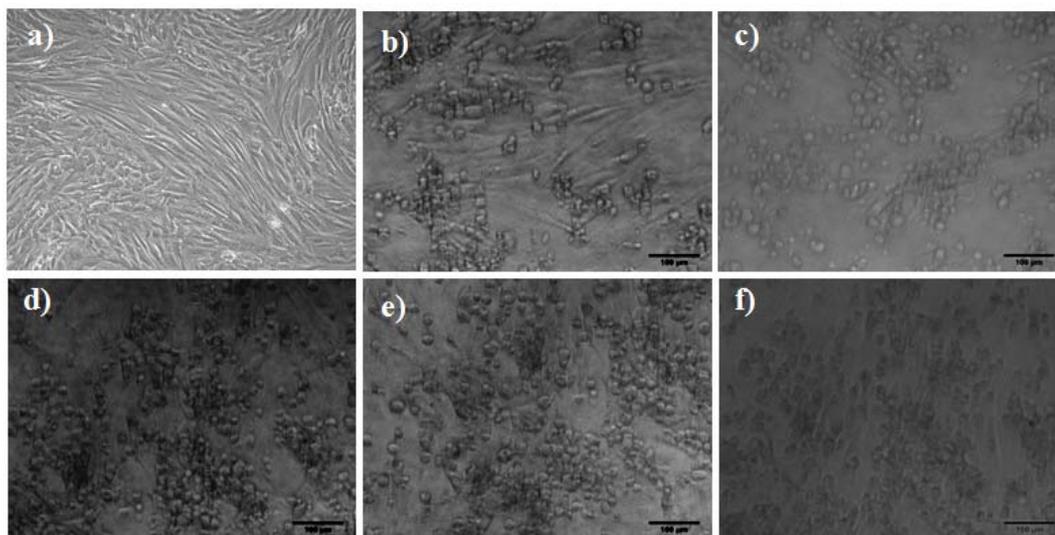


Figure 8. Optical images of mesenchymal stem cell proliferated on tissue culture plate (a) various hydrogels H10-50, (b)-(f) for 3 days.

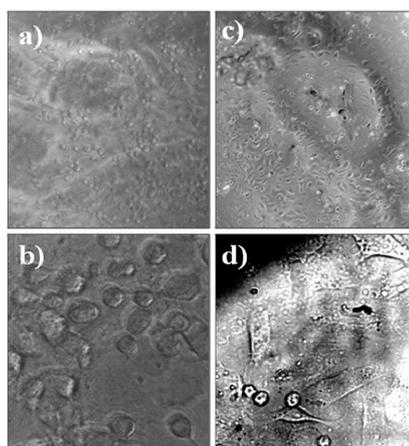


Figure 9. Optical images of chondroblasts cell proliferated on hydrogel HP-10 for 60 minutes (a), (b) and 1 day (c), (d) H10-50 (b)-(f) for 3 days.

5. ACKNOWLEDGEMENTS

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