The effect of Simvastatin Acid Loaded Photocurable Hyaluronic Acid Hydrogel for Bone Regeneration *in vitro* and *in vivo*

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Introduction: Therapeutic repair of skeletal tissues has received significant interest by the scientific community. Advances in reconstructive orthopaedic surgery, such as total hip replacement, spinal fusion and plastic surgery, resulting from progress in surgical procedures and the increase in an ageing population, have lead to a demand for bone grafts that exceeds the current available supply. Therefore, the loss of bone tissue due to congenital defects, trauma and following tumor resection represents a major clinical problem [South-Paul JE 2001, Percival M 1999]. Bone regeneration has been an important issue for millions of patients worldwide who have suffered from bone disease or defect, such as osteosacoma, osteoporosis, bone fracture, and etc. Regeneration of bone tissues is among the most promising area of biological repair that has the potential of providing a broad spectrum of potential clinical applications. Recently, bone tissue engineering, an interdisciplinary field at the intersection of engineering, biology and medicine, has emerged as one of approaches to develop biological bone substitute that restore, maintain or improve bone tissue function.

To date, there has been significant progress in developing tissue engineered constructs using various scaffolds and cells. For instance, in bone tissue engineering, many suitable materials have been generated, including synthetic hydroxyapatite (HAp)/tricalcium phosphates (TCPs) and polyglycolic and polylactic acids. Transplants of hydroxyapatite ceramics supplemented with periosteal cells or bone marrow cells appear to be effective for forming bone-like tissue [Brook IM 1991, Mankani MH 2004, Bianco P 2001]. In addition, it was reported that the tissue engineered bone constructs, which composed of bone marrow derived stem cells and 3D HAp ceramics, were implanted into the original patients demonstrating bone healing potential without any side effects [Morishita T 2006]. De Girolamo et at evaluated the effect of various clinically available biomaterials, such as hydroxyapatite, cancellous human bone fragments, deproteinized bovine bone granules and titanium, on stem cell adhesion, proliferation and osteogenic differentiation. In this research, stem cells were induced to differentiate into osteogenic lineage on monolayer culture, and then loaded on various scaffold, showing no significant difference in their adhesion and proliferation activity, but much more mineralized matrix could be observed in the culture on human bone fragments and deproteinized bovine bone granule [De Girolamo L 2008]. Other commonly used biomaterials, such as synthetic polylactic acid or poly L-lactic acid hybridized with natural collagen, have been used as porous scaffold to provide suitable environment for osteogenic cells growth and function [Hutmacher DW 2000, Suh H 2000, Suh H 2001], and some of polymers could be combined with hydrogels, bioglass, or ceramics [Endres M 2003, Blaker JJ 2005, Chen OZ 2006]. Recently, those biomaterials have been used in conjugation with bone inducing factors. For instance, it was reported that bone morphogenic protein (BMP)-inoculated 3D scaffold, composed of poly L-lactic-glycolic acid (PLGA) and hydroxyapatite, was developed as a stem cell-derived osteoblasts delivery vehicle for generating bone like tissue in vivo, reporting successful bone tissue formation by stem cell-derived osteoblasts in subcutaneous site of implanted immunodeficient mouse [Kim S 2008].

To dates, in addition to synthetic biomaterials in bone tissue engineering, natural biomaterials have been used as a cell carrier or a 3D scaffold due to their biological activity and good interaction with host cells in implantation. Among various natural biomaterials, hyaluronic acid (HA) has been in attention as cell carrier or 3D matrix for desired tissue formation in the field of tissue engineering due to its very low immunogenicity and various biological activities [Bot PT 2008, Collier JH 2000, Solchaga LA 1999]. HA is an extremely negative charged and heavily hydrated glycosaminoglycan present in almost all extracellular matrix in the body, and it has been well known that it affects cell attachment and migration [Lesley J 1993]. However, in spite of favorable biological activities of HA, for the application to tissue engineering, the development of HA with more durable mechanical strength might be needed for easy handling and resistance enough to allow 3D host cell ingrowth in implantation. Therefore, the goal of this study was to create a HA-based biomaterial that has inherent biological properties which specifically trigger bone growth and regeneration. For this purpose, we have developed

photocurable HA based hydrogels for bone regeneration, and loaded simvastatin, which known to be effective on osteogenic differentiation [Song C 2003], in hydrogels as a bone inducing factor in order to accelerate bone ingrowth. The mechanical property of hydrogel and simvastatin release activity was controlled by crosslinking degree and the initianl loading amount of simvastatin, and the cytotoxicity and osteogenic inducing activity was evaluated. Finally, the in vivo bone forming activity was evaluated by implanting the developed biomaterial into white New Zealand Rabbit.

Materials & Methods: The methacrylated HA was prepared by reacting high molecular weight HA (1.43 × 10^6 Da, LG Life Sciences Co., Daejeon, Korea) with 2-aminoethyl methacrylate (AEMA, Sigma, St Louis, MO, USA). High molecular weight of HA (1 g) was dissolved in a buffer solution (0.5% w/v, pH 6.5) of 50 mM 2-morpholinoethanesulfonic acid (MES, Sigma, St Louis, MO, USA) with 0.5 M Sodium chloride (NaCl). N-hydroxysuccinimide (NHS, 0.53 g) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC, 1.75 g) (mole ratio of NHS : EDC = 1 : 2) were added to the mixture to activate the carboxylic groups of the HA. After 5 min, AEMA (20, 30, and 40 (w/w) % of HA) was added to the solution and the reaction was maintained at room temperature for 24 hr. The methacrylated HA was purified by dialysis membrane (MWCO 3500, Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) against DI water for 3 days, filtered, and lyophilized. To fabricate photocurable HA hydrogels, 0.1g of methacrylated HA was dissolved in 10ml volume of DI water with 0.05% w/v photoinitiator (Irgacure D-2959, Ciba Specialty Chemicals, Basel, Switzerland). The HA solutions were photocrosslinked with 365 nm UV light (CL-1000 UV-Crosslinker, UVP) at ×100 μJ/cm2 for 5 min to form the hydrogels.

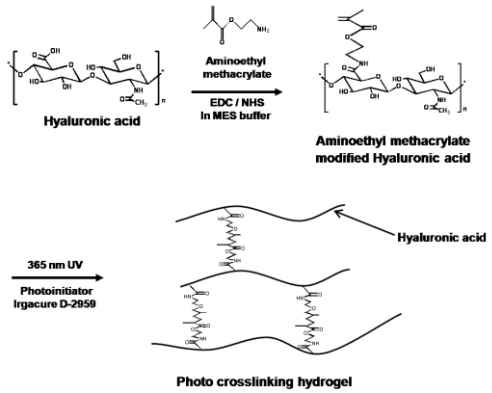

Figure 1. Schematic reactions of aminoethyl methacrylate (AEMA) modified HA.

The release kinetics of simvastatin acid of contraction of 0.1 mg (42 mM), 0.5 mg (210 mM), and 1.0 mg (420 mM) loaded photocurable HA hydrogel were investigated. Then, simvastatin acid loading hydrogel was immersed in 500 μL microcentrifuge tubes containing 1 ml phosphate-buffered saline (PBS, pH 7.4). These tubes were then incubated at 37°C with continuous agitation at 50 rpm. At predetermined time points of 1, 3, 6, 24 hr, 3, 7, and 14 day, the supernatant was harvested and the tubes were replenished with fresh PBS solution. The amount of simvastatin acid in the supernatants was determined using UV spectrophotometer (UV-1650PC, SHIMADZU, Japan). The absorbance of the samples was read at 240 nm wave length using a microplate reader (ELISA, Bio-Rad, Hercules, CA, USA). The amount of simvastatin acid was determined from a calibration curve based on known concentration of simvastatin acid. This experiment was performed in quintuplicate.

Results: HA based hydrogels were prepared via photopolymerization of pendent methacrylic ester, which schematic diagram is illustrated in figure 1. Prior to photopolymerization, HA was covalently reacted with different amounts of aminoethyl methacrylate, in order to present functional groups to HA molecule. The experimental methacrylation of the HA varied 20, 30, and 40 wt%, and was calculated on the basis of the concentration of AEMA added to the HA solution. The morphology and chemical modification of methacrylated HA with different extent was characterized by SEM, ATR-FTIR and ^{1}H NMR. As shown in Figure 2, the pore size of modified HA hydrogel in dry state decreased as the concentration of AEMA was increased.

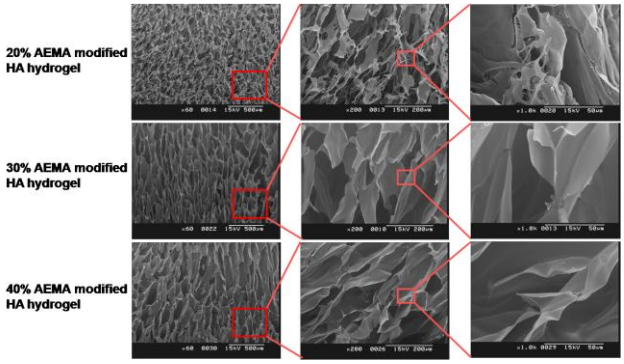

Figure 2. Cross-sectional scanning electron microscopy of 20%, 30%, 40% aminoethyl methacrylate modified HA hydrogels.

The chemical modification was also characterized by ^{1}H NMR analysis after AEMA treatment of HA. The ^{1}H NMR spectra of AEMA treated HA with different concentration are shown in Figure 3. The ^{1}H NMR spectra of the AEMA treated HA also revealed the increase of peak indicating acrylate groups in a different position as the increase of AEMA concentration (Fig. 3b, c and d). In comparison, no peak was found in a and b position of HA structure (Fig. 3a). The performed ^{1}H NMR analysis proved the well-formed methacylation of HA.

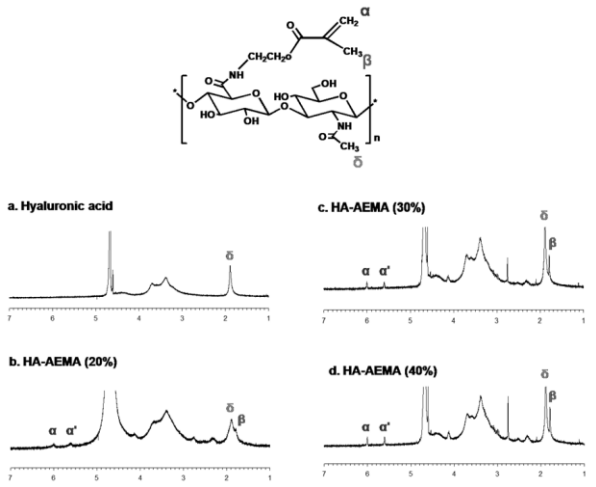

Figure 3. ^{1}H NMR spectra of 20%, 30%, 40% aminoethyl methacrylate modified HA.

The swelling test was done by measuring the size change of hydrogels after placing in DI water. Photocurable HA hydrogels were prepared with 20, 30, and 40 wt% AEMA treated HA. Swelling ratios of hydrogels in DI water are shown in Figure 4(b). As shown in Figure 4(a), the gradual increase of volume by swelling was detected until 2 weeks in 40 wt% AEMA treated HA hydrogel. In comparison, 20 and 30 wt% AEMA treated HA hydrogel showed rapid increase in their volume and reach at maximum state by 2 weeks (Fig. 4(b)). The changes of swelling ratio for the hydrogels measured over time might reflect changes in their physical and chemical structures.

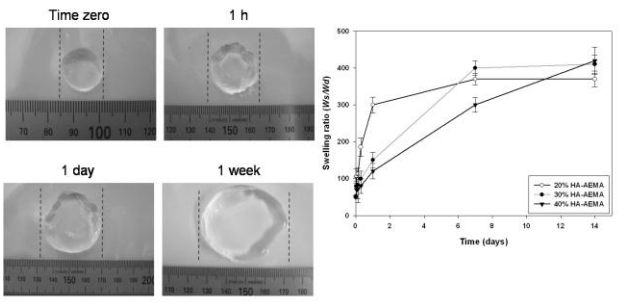

Figure 4. Morphology of 40 wt% AEMA grafted HA hydrogel after 1 hr, 1 day, and 1 week equilibrium in water (a). Swelling ratios of photocurable hydrogels (b).

After the development of photocurable HA hydorgel, we loaded simvastatin within hydrogels in order to present bone inducing activity. To determine the feasibility of AEMA treated HA hydrogel as a simvastatin delivery carrier, the release kinetics of simvastatin were examined using an UV spectrophotometer. Figure 5 showed the effect of different initial loading amount of simvastatin acid (0.1 mg, 0.5 mg and 1 mg) on release rate of AEMA treated HA hydrogels. When 0.5 and 1 mg of simvastatin was loaded, the rapid release at early time was detected with following sustained release after 1 day from AEMA treated HA hydrogel. Different from continuous release in 0.5 and 1 mg of simvastatin-loaded hydrogels, the sustained release was not observed in 0.1 mg of simvastatin-loaded hydrogel.

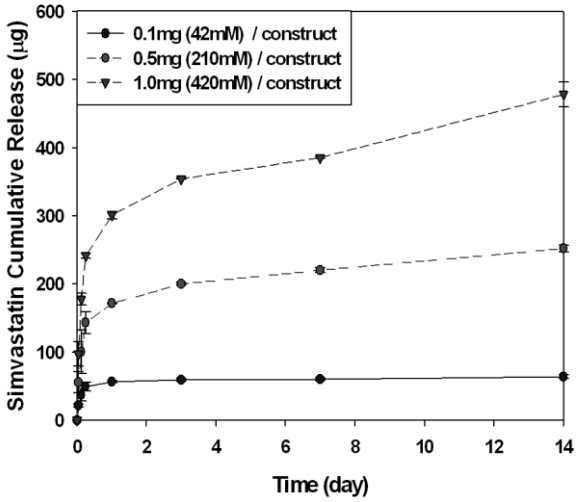

Figure 5. Release kinetics of simvastatin-loaded photocurable HA hydrogels.

The mineralization, indicating osteogenic differentiation, at early time was evaluated by Alizarin Red-S staining. For osteogenic differentiation, the ADSCs on simvastatin-loaded HA hydrogels were cultivated for 7 days in the presence of osteogenic supplements such as ascorbic acid, dexamethsone and □-glycerophosphate, and mineralization was evaluated at day 3, 5 and 7 of culture. As shown in Figure 6, mineralization was not detected in ADSC culture on hydrogels without simvastatin throughout whole culture period up to 7 days. In the other hands, ADSC culture on 0.1 and 1 mg of simvastatin-loaded HA hydrogels showed a statistically significant increase in mineralization from day 3 to 7.

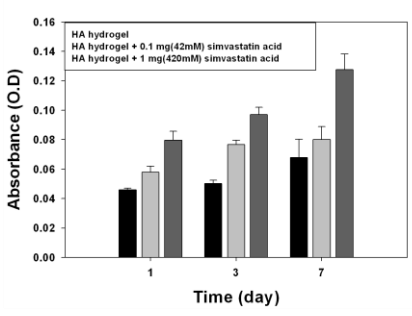

Figure 6. Mineralization of ADSCs culture on simvastatin-loaded photocurable HA hydrogels

The healing of defective bones was evaluated by soft X-ray radiography. On soft X-ray radiography, in the experimental group, those of which were terminated at 3, 5, 7 and 9 weeks after the formation of defective bones, showed the extended formation of trabeculation in the center and peripheral regions of the defective bone in comparison with in the controlled group. When comparing with the controlled group, in the experimental group, those of which were terminated 7 and 9 weeks after the formation of defective bone showed more dens trabeculations and advanced bony remodeling process on peripheral region at 7 weeks and complete coverage of bony defect area at 9 weeks (Fig. 7)

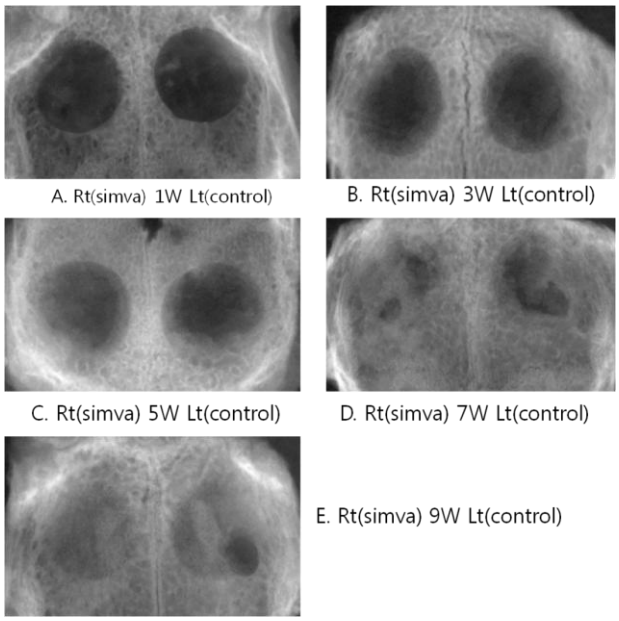

Figure 7. Soft x-ray radiographies at 1 week (A), 3 weeks (B), 5 weeks (C), 7 weeks (D), and 9 weeks (E) after implantation

Conclusion: Photocurable HA-hydrogel was confirmed to be successful carrier for sustained release of simvastatin acid in vitro. These results demonstrated that simvastatin acid was befound an important osteogenic differentiation factor, and increased simvastatin acid concentration promoted more osteogenic differentiation of ADSCs. In conclusion, simvastatin acid loaded HA-hydrogel would be valuable for tissue regeneration of bone.

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