Biomaterials and Biomechanics in Bioengineering, *Vol. 2, No. 1 (2015) 23-32*
(Formerly, Biomaterials and Biomedical Engineering)
DOI: http://dx.doi.org/10.12989/bme.2015.2.1.023

Injectable TGF-beta 3-conjugated hyaluronic acid hydrogel for cartilage regeneration

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(Received January 3, 2015, Revised February 11, 2015, Accepted February 12, 2015)

Abstract. Facile immobilization of growth factors in hyaluronic acid (HA) hydrogels using dual enzymes is reported in the paper. The hydrogels were formed by using horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2) and transforming growth factor-β3 (TGF-β3) was covalently conjugated on the hydrogels in situ using tyrosinase (Ty) without any modifications. For the preparation of hydrogels, HA was grafted with poly(ethylene glycol) (PEG), which was modified with a tyrosine. The gelation times of the HA hydrogels were ranging from 415 to 17 s and the storage moduli was dependent on the concentration of H_2O_2 and Ty (470-1600 Pa). A native TGF-β3 (200 ng/mL) was readily encapsulated in the HA hydrogels and 17% of the TGF-β3 was released over 1 month at the Ty concentration of 0.5 KU/mL, while the release was faster when 0.3 KU/mL of Ty was used for the encapsulation (27%). It can be suggested that the growth factors resident in the hydrogels for a long period of time may lead cells proliferating and differentiating, whereas the growth factors that are initially released from the hydrogels can induce the ingrowth of cells into the matrices. Therefore, the dual enzymatic methods as facile gel forming and loading of various native growth factors or therapeutic proteins could be highly promising for tissue regenerative medicines.

Keywords: biocompatible polymer; biomaterials; biomedical engineering; cartilage; hydrogels

1. Introduction

Tissue engineering research is one of the emerging biomedical fields which has focused on developments of viable biomaterial substitutes for the restoration and maintenance of the function of human tissues (Peppas and Langer 2004). Hydrogels, water-swollen polymer networks are widely used as scaffolds in tissue engineering since their distinctive features make them excellent physicochemical biomimetics of natural extracellular matrix (ECM) (Lavik and Langer 2004). The molecular architecture of hydrophilic 3D polymer networks can result in tissue-like viscoelastic material, diffusive transport, and interstitial flow characteristics. However, the synthetic hydrogels

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 ISSN: 2288-3738 (Print), 2288-3746 (Online)

made from either synthetic or natural sources are severely limited by a lack of microvascularization within engineered constructs and cell-conductivities, meaning that cells do not fully adhere, proliferate and differentiate in the matrices (Babensee *et al.* 1998).

Current efforts in tissue engineering attempt to address this problem by enhancing angiogenesis and eventual tissue production by introducing single or multi-growth factors (Leslie-Barbick *et al*. 2009). Growth factors are mediators that act locally or systemically to modulate cell functions including cell differentiation, migration, adhesion, and gene expression. A wide range of approaches have been attempted to introduce growth factors into the hydrogels. In the previous reports, direct loading, electrostatic interaction, covalent binding, and the use of carriers are the main strategies to prepare the hydrogels containing growth factors (Zisch *et al.* 2003). For regeneration of natural tissues, it is very important not only that the growth factors should be slowly released in the early stage for cell migration into the hydrogels, but also that long-term maintenance of effective concentration of the growth factors in the matrices have to guide cells to adhere and proliferate on the polymer networks. Therefore, the immobilization of growth factors on the hydrogels is favored to maintain the activity of growth factors until degradation of the hydrogels and replacement with natural tissues.

Recently, enzyme-mediated *in situ* cross-linkable hydrogels that are capable of covalently immobilizing growth factors have received much attention in tissue engineering because of the less toxicity, high efficiency and the mild conditions. Park and his colleagues, one of the leading research groups of enzymatic cross-linking chemistry, have reported *in situ* formation of hydrogels based on tyramine conjugated Tetronic® *via* enzymatic oxidative reactions using a horseradish peroxidase (HRP) and a hydrogen peroxide (H_2O_2) (Park *et al.* 2010). The HRP is a hemoprotein that catalyzes the conjugation of phenol and aniline derivatives with decomposed H_2O_2 molecules (Babensee *et al.* 1998). A GRGDGGGGGY (RGD-Y) peptide was conjugated simultaneously in the presence of HRP and H_2O_2 promoting adhesion and spreading of osteoblasts. This research group has also reported the RGD immobilization onto TiO_2 substrate by using tyrosinase (Ty)-catalyzed oxidative reactions (Park and Park 2011). Tyrosinase is an enzyme that catalyzes the oxidation of phenol molecules into *o*-quinones immediately passing the intermediate state (*o*-dihydroxyphenol) in the presence of oxygen (Cooksey *et al.* 1997). Using the enzymatic immobilization chemistry, the phenol containing RGD peptide was rapidly immobilized within few minutes and the surface density of conjugated RGD ranged from 0.18 to 0.35 nmol/cm^2.

In this study, the enzyme-mediated crosslinkable hydrogels based on hyaluronic acid (HA) and poly(ethylene glycol) (PEG) were developed and a growth factor was immobilized *in situ* (Fig. 1). As one of the natural polymers (e.g., HA, collagen, alginate, and chitosan), HA have been used to offer biological interactions and functionality for healing of defects especially for cartilage. HA is known to play a key role in the retention of proteoglycans in the cartilaginous matrix. For the formation of the HA hydrogels, HA was grafted with tyramine modified PEG and crosslinked by HRP and H_2O_2 (Fig. 1(a)). Transforming growth factor-β3 (TGF-β3) that controls proliferation, cellular differentiation, and other functions in most cells was covalently immobilized on the hydrogels using Ty as a model growth factor (Fig. 1(b)). A native TGF-β3 could be incorporated *via* Ty-catalyzed oxidative reactions without any modifications. Amine groups of the growth factors were reacted with *o*-quinones transformed by Ty at the end point of the PEG chains. The chemical structures of the PEG conjugated HA was evaluated by ^{1}H NMR and UV spectroscopy. The gelation times and mechanical strength of the hydrogels were characterized using different concentrations of HRP and H_2O_2. In addition, the effect of the immobilization of TGF-β3 using Ty on the gel stiffness was investigated. Release kinetics of TGF-β3 simply mixed or conjugated with

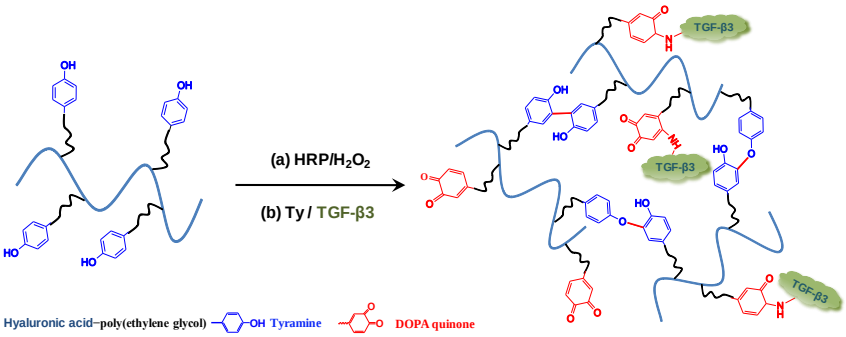

Fig. 1 Schematic gelation of the HAPT hydrogel and immobilization of TGF-*β*3 *via* enzyme-oxidative reaction within the HAPT hydrogel. HRP and H_2O_2 for the hydrogel formation (a), TGF-*β*3 and Ty for the growth factor immobilization reactions (b)

HA hydrogels was also evaluated.

2. Materials and methods

2.1 Materials

Hyaluronic acid (HA, MW=1,000 kDa) was obtained from Biorane. Tyramine (TA) was provided from ACROS. PEG (MW 4000 g/mol), 4-nitrophenyl chloroformate (PNC), 4-dimethylamino pyridine (DMAP), ethylene diamine (ED), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxy succinimide (NHS), MES, H_2O_2, HRP (type VI, 250-330 units/mg solid), albumin from bovine serum (BSA), Tween 20 and Ty from mushroom were purchased from Sigma-Aldrich. Triethylamine (TEA) was provided from KANTO CHEMICAL Corp. Recombinant Human TGF-*β*3 (TGF-*β*3) and Duoset® Human TGF-*β*3 were supplied from R&D systems. All other reagents were of analytical grade and used as received.

2.2 Synthesis of polymers

2.2.1 Synthesis of amino PEG−tyramine (H_2N−PEG−TA) conjugate

H_2N-PEG-TA conjugate was prepared by activation of terminal hydroxyl groups of PEG using excess of PNC followed by TA and ED conjugation (Fig. 2(a)) (Lih *et al*. 2012). In brief, PEG (20.00 g, 5.0 mmol) was dissolved in 150 mL of MC. DMAP (2.44 g, 20.0 mmol) and TEA (2.02 g, 20.0 mmol) were dissolved in 50 mL of MC and added to the PEG solution to activate the hydroxyl groups of the PEG. After 15 min, PNC (4.03 g, 20.0 mmol) which was dissolved in 50 mL of MC was dropped into the PEG solution and reacted with the activated PEG in N_2 atmosphere for 24 h under mild stirring. The product solution was filtered using aluminum oxide (STREM CHEMICALS) pad to remove the residual PNC salts and precipitated in cold diethyl ether. Subsequently, the PEG was isolated by filtration and dried under vacuum overnight resulting in a white powder.

The PNC conjugated PEG (10 g, 2.5 mmol) was dissolved in 40 mL DMF at 30℃ in the nitrogen atmosphere. TA (0.36 g, 2.7 mmol) was then dissolved in DMF and dropped onto PNC

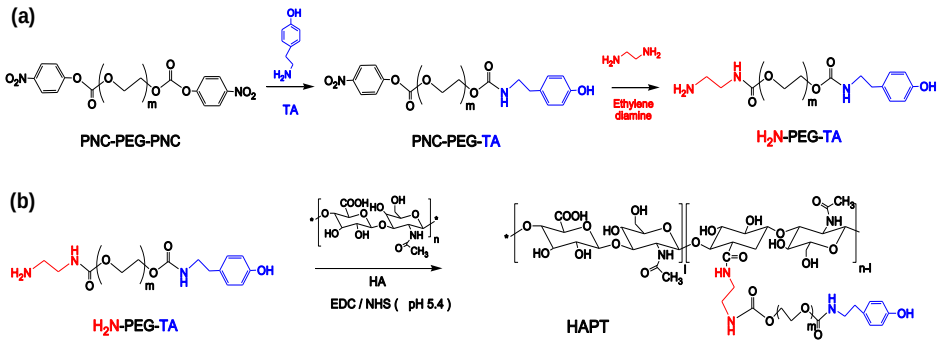

Fig. 2 Schematic representation for the synthesis of (a) H_2N-PEG-TA and (b) HAPT conjugates

conjugated PEG solution for the reaction for 6 h under mild stirring. This solution was mixed with ED (7.72 mL, 115.4 mmol) in DMF and further reacted for 4 h while maintaining the nitrogen atmosphere. TA conjugated amino PEG was filtered with aluminum oxide pad and precipitated in the cold ether. The chemical structure of the amino PEG-TA was assessed by ^{1}H NMR (VARIAN Bruker AMX-500 NMR spectrometer, 400 MHz).

2.2.2 Synthesis of hyaluronic acid−poly(ethylene glycol)−tyramine (HAPT) conjugate

HA was grafted with H_2N-PEG-TA conjugates using EDC/NHS chemistry (Fig. 2(b)). HA (0.25 g, 0.65 mmol) was dissolved in 300 mL of MES buffer solution (pH 5.4, 0.1 M) at 30℃. EDC (0.216 g, 1.13 mmol) and NHS (0.155 g, 1.35 mmol) in MES were applied to the HA solutions with a 30 min interval. H_2N-PEG-TA (4.5 g, 1.13 mmol) in 50 mL of MES was added to the mixture and stirred at R.T. for 24 h. After the reaction, the resulting solution was purified by dialysis (molecular weight cut off (MWCO) 12-14000 g/mol) for 2 days in the sodium chloride solution (4 L, 100 mM), subsequently in a co-solvent of distilled water (DI water) and ethanol (3:1) for 1 day and finally in DI water overnight. The purified solution was lyophilized for 3 days to obtain the HAPT polymer. The chemical structure of the HAPT conjugate was characterized by ^{1}H NMR and UV spectrophotometer (JASCO V-670).

2.3 The gelation time and rheological measurements of HAPT hydrogels

HAPT hydrogel was prepared by using HRP and H_2O_2 in PBS (0.01 M, pH 7.4). The gelation time of the hydrogels was measured as a function of HRP concentration (from 0.001 to 0.016 mg/mL). For the experiments, the final concentration of H_2O_2 was fixed to 0.032 wt.%. HAPT polymer (0.015 g) was dissolved in both 500 μL of HRP solution (A solution) and 500 μL of H_2O_2 solution (B solution). The solutions were injected into 3 mL vials using a double syringe and measured the gelation time by the vial tilting method (Jin *et al.* 2007).

Rheological measurements of the HAPT hydrogels were carried out at 37℃ using an Advanced Rheometer GEM-150-050 (Bohlin Instruments, USA) in oscillatory mode. HRP (0.032 mg/mL) and H_2O_2 (0.025-0.125 wt.%) were separately dissolved in PBS (500 μL), and HAPT (0.015 g) was added to two individual solutions. For the measurements, 150 μL of the HRP solutions containing HAPT was firstly mounted on the plate of the instrument and subsequently added with the HAPT/H_2O_2 solution (150 μL). The elastic modulus (G') was recorded at a frequency of 0.1 Hz using the parallel plate geometry (plate diameter 20 mm, gap 1 mm, stress 0.1 Pa) for oscillatory

rheological measurements. To evaluate the effect of Ty and TGF-β3 on the G', a TGF-β3 solution (200 ng/mL) was added to the A solution (C solution). B and C solutions were mixed to form HAPT hydrogels and Ty (0-0.5 KU/mL) solutions were immediately added to the solution (B+C) in order to covalently immobilize TGF−β3.

2.4 In situ immobilization of TGF−β3 in HAPT hydrogels and release study

TGF-β3 was introduced in HAPT hydrogels using different concentrations of Ty. The hydrogels were formed by mixing B and C solutions, and Ty (0.3 or 0.5 KU/mL) solutions were immediately added to the solutions to immobilize TGF-β3 in the hydrogels. HAPT hydrogels, which are simply blended with free TGF-β3 were used as a control. For *in vitro* release of TGF-β3, the hydrogels were prepared in PBS in 10 mL vials and 1 mL of BSA solution (1 wt.%) was added as a release medium. The amount of released TGF-β3 was monitored by periodic withdrawals of samples (1 mL). The loading amount of TGF-β3 and concentrations of released TGF-β3 were determined by ELISA analysis (JASCO V-750, R&D Systems Duoset® kit) at 450 nm.

3. Results and discussion

3.1 Synthesis and characterization of H_2N−PEG−TA and HAPT conjugates

HAPT copolymers were prepared by conjugating H_2N-PEG-TA on the HA backbones. PNC-PEG-PNC was synthesized and coupled with ED and TA in a stepwise manner. ^{1}H NMR ($CDCl_3$): δ3.5-3.8 (ethylene group protons of PEG) and δ7.2 and 8.2 (aromatic proton of PNC). The PNC

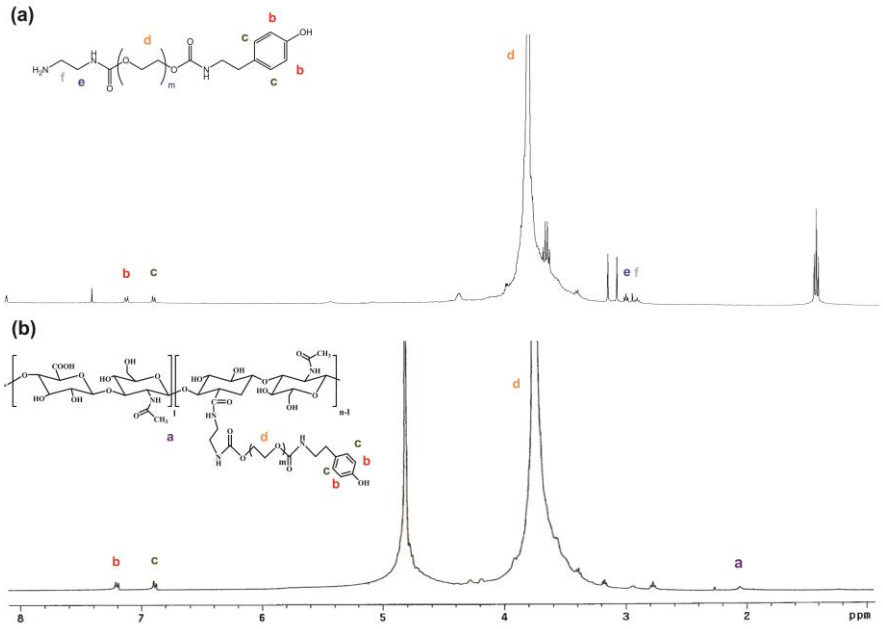

Fig. 3 ^{1}H NMR spectrum of H_2N-PEG-TA (a) and HAPT conjugates (b)

conjugation ratio against PEG was approximately 99% as previously reported (data not shown). The degree of TA conjugation with PEG was calculated by ^{1}H NMR spectroscopy and it turned out that 97% of PEG was functionalized with TA (Fig. 3a). ^{1}H NMR ($CDCl_3$): δ6.8 and 7.2 (aromatic protons of TA) and δ3.5-3.8 (ethylene group protons of PEG). ^{1}H NMR (D_2O): δ1.85-1.95 (the methyl resonance of acetamido moiety of the *N*-acetyl-D-glucosamine of HA), δ3.5-3.8 (ethylene group protons of PEG) and δ6.8 and 7.2 (aromatic protons of TA). The TA content in HAPT conjugates was also determined by UV measurements (275 nm) and the calculated TA content was about 280 μmol/g of HAPT.

3.2 Preparation and gelation time of in situ HAPT

The HAPT hydrogels containing growth factors were prepared by dual enzymatic reactions using HRP and Ty. Phenol groups in the HAPT copolymers were rapidly crosslinked by HRP/H_2O_2 and the residual phenol groups were transformed to *o*-quinine *via* Ty-mediated reactions forming covalent bonds with TGF-β3 by imine formation or Michael−type addition. The gel formations were achieved by simple blending of pre-fabricated polymer and enzyme solutions, and in general the *in situ* preparation of the hydrogels takes less than 10 min.

The gelation times of HAPT hydrogels were determined by the vial tilting method and they were shown to be controllable when different concentrations of the HRP were used. As shown in Fig. 4(a), the gelation times of the HAPT hydrogels were varied from 415 to 17 s as the HRP concentration increased from 0.001 to 0.016 mg/mL at the constant H_2O_2 concentration of 0.032 wt.%. This can be explained by the fact that the formation of the phenoxy radicals further accelerated during the gelation due to the high concentration of HRP. In Fig. 4(b), G's depending on different concentrations of H_2O_2 are represented. Below H_2O_2 concentration of 0.063 wt.%, it can be seen that the amount of H_2O_2 is not significantly influenced the G's. However, the elastic

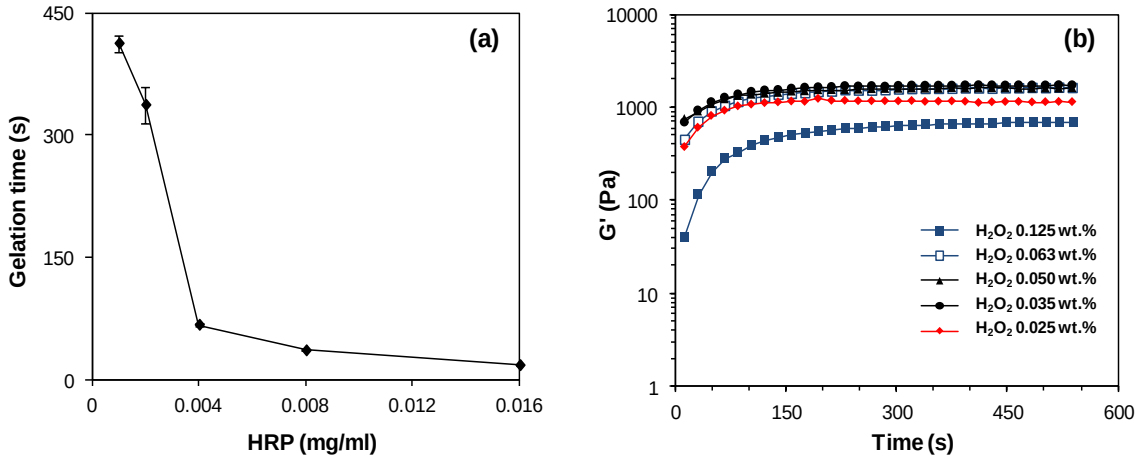

Fig. 4 Gelation times depending on the HRP concentrations (a) and elastic moduli as a function of H_2O_2 concentrations (b) for the HAPT hydrogels. For the (a) measurements, 0.025 wt.% of H_2O_2 was used. The constant concentration of HRP (0.008 mg/mL) was applied for the rheology studies (b). Error bars represent the standard deviations of mean values (n=5)

modulus lowered due to the relatively high concentration of H_2O_2 possibly by the inactivation of HRP enzymes, which has been reported in the literature (Jin *et al.* 2007). It is recommended that the H_2O_2 should be minimally used since the presence of excess H_2O_2 can also cause the cytotoxicity (>0.25 wt.%) (Jonas and Riley 1992).

3.3 Rheological measurements

The elastic properties of the HAPT hydrogels treated with Ty were compared with the non-treated hydrogels. Fig. 5(a) represented the elastic moduli of the HAPT hydrogels formed with 0.008 mg/mL of HRP and different concentrations of Ty. Mechanical strength of the HAPT hydrogels gradually decreased by increasing the concentration of Ty. The decrease in the modulus is because Ty reacted with more phenol groups, which was participating in the conjugation of TGF-β3 rather than internal crosslinking of HAPT. The G' values of HAPT hydrogels were reduced from 900 to 200 Pa as more Ty was used (0.1-0.5 KU/mL). Interestingly, the effect of Ty on the reduction of the elastic modulus became less significant when 0.05 wt.% of H_2O_2 was used (Fig. 5(b)). The reduction of the G' values was 500 Pa, while it was 800 Pa at the H_2O_2 concentration of 0.025 wt.%. This could be related with the differences in the gel stiffness. The elastic modulus was slightly higher with 0.05 wt.% of H_2O_2 (1600 Pa) as compared to one with 0.025 wt.% (1300 Pa). We assume that this is because 0.025 wt.% of H_2O_2 was not enough to fully crosslink the hydrogels (Sakai *et al.* 2009). The relatively higher amount of H_2O_2 improved the crosslinking efficiency during the hydrogel formations, limiting conversion of phenol groups to *o*-quinones by Ty. These results demonstrated that the elastic modulus of the HAPT hydrogel could be varied by changing the crosslinking conditions or using different concentration of Ty. Generally, the matrix stiffness is a crucial factor for determining cell behaviors such as spreading, migration, proliferation, and differentiation in synthetic hydrogels (Discher *et al.* 2009).

3.4 In vitro release behavior of TGF−β3 from HAPT hydrogels

TGF-β3 was used as a model growth factor to study the release kinetics before and after the immobilization. Fig. 6 represents the release profiles of TGF-β3 from the HAPT hydrogels placed

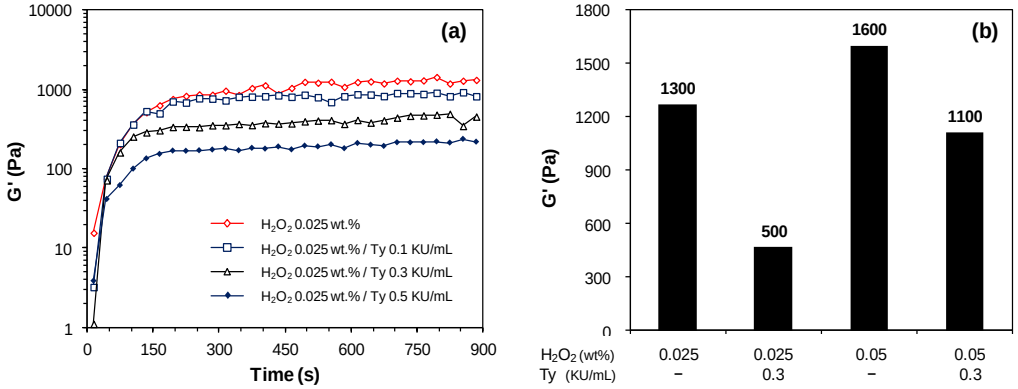

Fig. 5 The effects of Ty concentrations on the elastic modulus of the HAPT hydrogels (3 wt.%) (a) and comparison with untreated hydrogels (b)

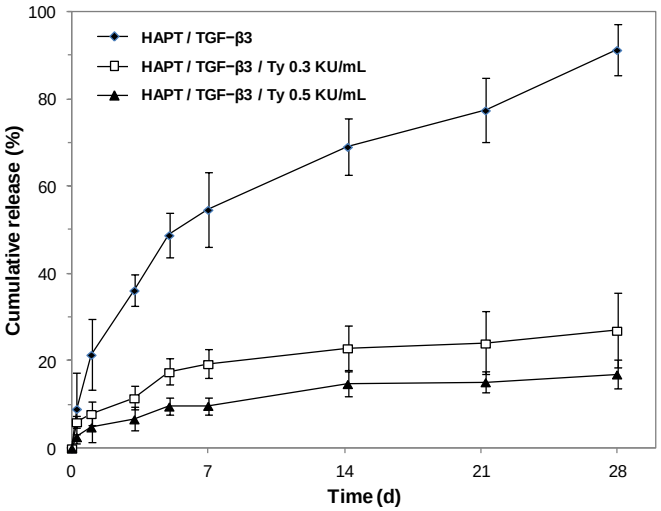

Fig. 6 *In vitro* release of TGF-*β*3 from HAPT hydrogels. Error bars represent the standard deviations of the mean values (*n*=4)

in vials with a PBS medium. A sustained release of TGF-*β*3 was observed for HAPT hydrogels physically loaded with TGF-*β*3 and complete release of the growth factor was accomplished after one month with a low initial burst. The release of growth factors from such matrix systems is governed by diffusion of the growth factors through the matrix, which can be influenced by the diffusion coefficient, solubility, pore size, and viscoelastic properties of the hydrogels. Similar shapes of release curves for TGF-*β*3 have been reported for hydrogels directly mixed with TGF-*β*3 (Park *et al.* 2010).

HAPT hydrogels conjugated with TGF-*β*3 released 27% of the TGF-*β*3 over 1 month (Ty 0.3 KU/mL), which is much lower than the release from the hydrogels mixed with TGF-*β*3. Obviously, this is related to the immobilization of TGF-*β*3 *via* covalent bonds. Phenol groups in the hydrogels were transformed to *o*-quinine by Ty-mediated reactions forming covalent bonds with amine groups of TGF-*β*3 (imine formation or Michael-type addition). At Ty concentration of 0.5 KU/mL, the release of TGF-*β*3 was further diminished for the same period of time (17%). Apparently, this is indicative that the immobilization of TGF-*β*3 was governed by Ty reactions and it was more efficient when higher concentration of Ty was used. In principle, the growth factors initially released from the hydrogels may induce the cell migration or attachment into the hydrogels and the growth factors immobilized in the hydrogels will lead cells proliferating and differentiating. It should be noted that the covalent bonding can cause relatively lower activity of the growth factors as compared to the native one, but this effect could be minimal due to the presence of PEG spacers in the hydrogels.

4. Conclusions

Enzyme-mediated *in situ* crosslinkable hydrogels based on HA and PEG were readily prepared by using HRP/H_2O_2 and a model growth factor (TGF-*β*3) was immobilized simultaneously in the

hydrogels. The elastic modulus of the hydrogels could be varied by changing the crosslinking conditions or using different concentration of Ty. It can be concluded that the HA/PEG hydrogels are capable of loading various native growth factors and therapeutic proteins less than 10 min and the dual enzymatic method is very a promising tool for tissue engineering applications. In our current program, the hydrogel will be further investigated the degradation behavior and the cell spreading/proliferation studies with varying the different mechanical strengths or loading of single or multi growth factors to visualize a feasibility of the hydrogels as a tissue regenerative medicine.

Acknowledgements

This work was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) grant funded by the Ministry of Science, ICT & Future Planning (NRF- 2012R1A2A2A06046885), and the Ministry of Education (NRF- 2009-0093826).

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