Korean J. Chem. Eng., 38(1), 98-103 (2021)
DOI: 10.1007/s11814-020-0672-5

pISSN: 0256-1115
eISSN: 1975-7220

Tyrosinase-mediated rapid and permanent chitosan/gelatin and chitosan/gelatin/nanohydroxyapatite hydrogel

Sujin Lim*, Dawoon Jeong*, Mi-Ran Ki, Seung Pil Pack**, and Yoo Seong Choi*,†**

*Department of Chemical Engineering and Applied Chemistry, Chungnam National University, Daejeon 34134, Korea
**Department of Biotechnology and Bioinformatics, Korea University, Sejong 30019, Korea
(*Received 8 July 2020 • Revised 26 August 2020 • Accepted 1 September 2020*)

Abstract−Chitosan/gelatin and chitosan/gelatin/nanohydroxyapatite hydrogels were rapidly and stably prepared without any crosslinking materials by using an engineered tyrosinase (mTyr-CNK) with high catalytic activity for tyrosine/DOPA-tethered polymeric biomaterials throughout a broad pH range. A dual-barrel syringe with one part containing chitosan/mTyr-CNK solution and the other containing gelatin solution with/without nanohydroxyapatite was successfully used to form homogeneous hydrogels at room temperature followed by 37 °C to simulate an in situ injection approach. The obtained hydrogels exhibited an average pore size greater than 150 μm and high swelling ratios with similar mechanical properties to other chemically crosslinked chitosan/gelatin hydrogels. The in vitro degradation properties and cellular viability suggested that the hydrogels could be used as biodegradable and biocompatible scaffolds for biomedical applications, such as space filling biomaterials and delivery vehicles for bioactive molecules and cells. These results demonstrated that mTyr-CNK-mediated hydrogels have remarkable promise as an injectable scaffold biomaterial.

Keywords: Chitosan, Gelatin, Nanohydroxyapatite, Hydrogel, Tyrosinase

INTRODUCTION

Artificial hydrogels are widely considered promising scaffolds for tissue engineering and drug delivery applications [1-3]. Their remarkable properties, such as minimal invasiveness, high swelling properties, and easy matching to irregular defects, potentially enable their use as three-dimensional cell culture scaffolds and controlled drug delivery materials. Moreover, biodegradable hydrogels composed of biocompatible natural biomolecules have been developed to minimize inflammation and immune responses resulting from the remaining scaffold materials [4,5]. In this respect, chitosan and gelatin are promising biomaterials for the preparation of injectable and biodegradable hydrogels based on their excellent biocompatibility and bioactivity. Chitosan has been widely used as a scaffold material due to its structural and compositional similarity to glycosaminoglycans (GAGs) in the extracellular matrix (ECM) and as a delivery vehicle because it is nontoxic, biodegradable and biocompatible [6]. However, its water insolubility at physiological pH needs to be addressed for efficient use it in practice. Although chitosan has been chemically modified by attaching functional groups to overcome this issue, the direct use of chitosan would be optimal to minimize potential changes in bioactivity, biodegradability and biocompatibility [6]. Gelatin obtained from the hydrolysis of collagen, as a component of the natural ECM, has been popularly used to prepare hydrogels. Gelatin is highly water soluble in physiological conditions and has superior biocompatibility and low immunogenicity; however, its extremely high degradation has hampered its practical use [7,8]. Accordingly, chitosan/gelatin composites have been prepared to compensate for the drawbacks of each biomolecule, and the mechanical and biological performance has been improved by various physical and chemical crosslinking methods [9-11].

We recently prepared a permanent and stable chitosan/gelatin hydrogel using an acid-tolerant tyrosinase (tyrosinase-CNK) as a nontoxic and naturally derived crosslinking biocatalyst [12]. The prepared hydrogel showed favorable mechanical properties for use as scaffold for soft tissue engineering, although the catalytic activity needed improvement for the preparation of fast and injectable hydrogels. Moreover, the demand for biomaterials related to bone regeneration has continuously increased. Hydrogels have potential advantages as bone repair biomaterials, as they provide a nutrient environment suitable for endogenous cell growth with mechanical strength mimicking that of the natural ECM and can be tailored to obtain the desired geometry for implantation or injection [2,13]. However, they still have some limitations, such as weak biomechanical properties and relatively fast biodegradation, for applications in bone tissue engineering. Organic-inorganic composite materials have emerged to improve these weak points for successful application in bone tissue regeneration, bone graft materials and drug delivery. Bioactive ceramics, such as bioactive glass and sintered hydroxyapatite, have been used as representative materials for the preparation of these organic-inorganic composites for biomedical applications [14,15]. In particular, hydroxyapatite (HA) is an inorganic component of natural bone that exhibits excellent biocompatibility with soft tissues, offering osteoconductive and osteoinductive property, good bonding ability, and slow degradation [16].

†To whom correspondence should be addressed.
E-mail: biochoi@cnu.ac.kr

Crystalline nanohydroxyapatite (nHA) powders show improved sinterability, enhanced densification, and high bioactivity [17,18].

Here, we prepared stable chitosan/gelatin and chitosan/gelatin/nHA hydrogels using a tyrosinase engineered from tyrosinase-CNK, mTyr-CNK, without any additional crosslinking materials. mTyr-CNK exhibited high activity throughout a broad range of pH values and a distinguished monophenolase/diphenolase activity ratio (V_{max} mono/V_{max} di=3.83) with enhanced catalytic efficiency and stability at room temperature, but the stability above 37 °C markedly decreased (the half-life at 37 °C was approximately 25 min) [19]. The catalytic property is highly attractive in enzyme-mediated injectable hydrogel preparation because the enzyme can efficiently catalyze the homogeneous crosslinking of well-solubilized chitosan and gelatin at weak acidic pH values, and the instability in vivo could minimize unwanted adverse reactions after application. Aqueous precursor solutions of chitosan/mTyr-CNK and gelatin with/without nHA were prepared prior to gelation. The chitosan/gelatin and chitosan/gelatin/nHA hydrogels were prepared at room temperature upon mixing and cured at body temperature (37 °C). The morphological characteristics, swelling behavior, in vitro degradation properties, and cellular viability of the resulting hydrogels established their potential as an enzyme-catalyzed efficient biomaterial for bone tissue engineering applications.

EXPERIMENTAL

1. Preparation of Chitosan/Gelatin and Chitosan/Gelatin/nHA Hydrogels using mTyr-CNK

First, mTyr-CNK was produced in the previously described *E. coli* expression system [19]. Simply, *E. coli* BL21 (DE3) cells (Merck KGaA, Darmstadt, Germany) containing the plasmid pmTYR-CNK (for mTyr-CNK) were grown in Luria-Bertani (LB) medium with 50 μg ampicillin mL^{-1} (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C and 200 rpm. The inducer isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM final concentration; Sigma-Aldrich) was used for over-expression of mTyr-CNK at 20 °C and 200 rpm for 20 hr. The enzyme was purified from the soluble fraction of the cell lysate by Ni-nitrilotriacetic acid (Ni-NTA) (Qiagen, Germantown, MD, USA) affinity chromatography. The purity and concentration were assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the bicinchoninic acid (BCA) assay method (Sigma-Aldrich), respectively.

Then, chitosan/gelatin and chitosan/gelatin/nHA hydrogels were prepared using the mixtures of chitosan/mTyr-CNK and gelatin with/without nHA with different weight ratios of nHA and various concentrations of mTyr-CNK. Chitosan (from crab shells, with a 75-80% deacetylation degree and average molecular weight 500,000-700,000), gelatin (from porcine skin type A, bloom number ~175 and average molecular weight 40,000-50,000) and nHA (nanopowder, <200 nm particle size (BET), ≥97%, synthetic) were purchased from Sigma-Aldrich. Chitosan solution 2% (w/v) and gelatin solution (20%) (w/v) were prepared in 1% acetic acid solution and distilled water, respectively. To prepare the tyrosinase-mediated chitosan/gelatin and chitosan/gelatin/nHA hydrogels, mixtures (pH 4.0-4.5) of chitosan/mTyr-CNK and gelatin with/without nHA were injected into 1.5-mL microtubes with different weight ratios of nHA and various concentrations of mTyr-CNK. The mixture was placed at room temperature for 20 min and then soaked in a 37 °C water bath to form the gel. After gelation, the gels were washed with distilled water, immersed in 50% ethanol for 20 hr, and then dried in air before characteristic analysis.

2. Hydrogels Characterization

The gelation degree, rheological properties, morphology, swelling, in vitro degradation were analyzed by the same experimental procedure as previously [12]. Remarkably, scanning electron microscope with energy dispersive X-ray spectroscopy (SEM-EDS) (SNE-4500-EDS, SEC Co., Suwon, Korea) was additionally used to examine nHA deposits in the chitosan/gelatin/nHA hydrogels. Mixture without mTyr-CNK was also placed under the same conditions as a negative control. Swelling and in vitro degradation studies were carried out using phosphate-buffered saline (PBS) solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4; pH 7.4).

Compressive properties were tested using a rheometer (DHR-1, Ta Instruments, New Castle, DE, USA) at room temperature with a crosshead speed of 10 μm sec^{-1} using a cylindrical container with a diameter of 20 mm. Swollen hydrogel samples in PBS solution (pH 7.4) for 24 h were used for the measurement of compressive strength. All experiments were carried out on five samples.

3. Cell Viability and Proliferation Assay

The mouse preosteoblast cell line MC3T3-E1 was maintained in complete α-MEM (89% α-MEM, 10% (v/v) fetal bovine serum, and 1% penicillin/streptomycin) (HyClone, GE Healthcare Life Science, Chicago, IL, USA) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. For the cell viability measurement, a total of 1×10^4 cells/well was placed both in complete α-MEM with 100 μg/mL mTyr-CNK and in complete α-MEM only. After 24 hr, the cell viability was quantitatively determined by 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) assay. Each MTT assay was performed in triplicate.

For the cell proliferation assay, the hydrogels were freeze-dried using a freeze drier (FDU-2200; EYELA, Tokyo, Japan) overnight, washed three times with PBS buffer (pH 7.4), and then immersed in complete α-MEM. A total of 4×10^4 cells/well was placed in the prepared hydrogels. The cell proliferation of attached cells was quantitatively determined by methyl tetrazole sulfate (MTS) assay (CellTiter 96®; Promega, Madison, WI, USA) after 1, 3, 7, and 10 days.

RESULTS

1. Preparation of Rapid and Injectable Chitosan/Gelatin and Chitosan/Gelatin/nHA Hydrogels by mTyr-CNK

First, a dual-barrel syringe with a plunger was prepared at room temperature with the chitosan/gelatin hydrogel components. One part of the syringe barrel was filled with chitosan and mTyr-CNK solution, and the other part was filled with gelatin solution because mTyr-CNK could convert only the tyrosine residues of gelatin into quinones via 3,4-dihydroxyphenylalanine (DOPA). Unwanted enzymatic reactions were not catalyzed by mTyr-CNK in the presence of chitosan alone, as expected (data not shown). Moreover, the gelatin from porcine skin (type A) had seven tyrosine residues per 1,000 total amino acid residues [20], and mTyr-CNK did not cata-

lyze the gelation in the gelatin-only solution due to the small number of tyrosine residues. Therefore, the gelation could be only conducted by a nonenzymatic reaction of the quinones of gelatin with chitosan to form covalently grafted gelatin/chitosan conjugates. Solutions of 5% (w/v) gelatin and 0.5% (w/v) chitosan were used to form the hydrogel catalyzed by mTyr-CNK based on previous similar experimental conditions for the in vitro gelation of chitosan and gelatin by tyrosinase-CNK [21]. As a result, the gelation of the mTyr-CNK-mediated hydrogel was stably and efficiently formed when more than 10 μg mTyr-CNK mL^{-1} was only applied (Fig. 1(a)), whereas more than 300 μg tyrosinase-CNK mL^{-1} was required for gelation, and mushroom tyrosinase did not catalyze gelation under the same conditions [12]. The rheological evolution results demonstrated that the in vitro gelation occurred rapidly after 65 sec at 20 °C (Fig. 1(b)), where the elastic modulus (G′) exceeded the viscous modulus (G″), meaning the solution behaves more like an elastic solid after the intersection point [22]. Because the gelatin solution itself undergoes physical gelation below 30 °C [23], the physical gelation of the chitosan/gelation solution also occurred after 170 sec at 20 °C (Fig. 1(b)). Physical gelation could influence rapid mTyr-CNK-mediated gelation; therefore, experiments were also conducted at 37 °C. Fig. 1(c) shows that the stable chitosan/gelatin gel was also rapidly formed at 37 °C after 120 sec in the presence of mTyr-CNK, whereas the elastic modulus (G′) did not exceed the viscous modulus (G″) in the solution without mTyr-CNK. Similarly, chitosan/gelatin/nHA hybrid hydrogels with 5% (w/v) gelatin/0.5% chitosan (w/v) and various weight ratios of nHA were also stably and rapidly formed by using a dual-barrel syringe with chitosan/mTyr-CNK and gelatin/nHA solutions.

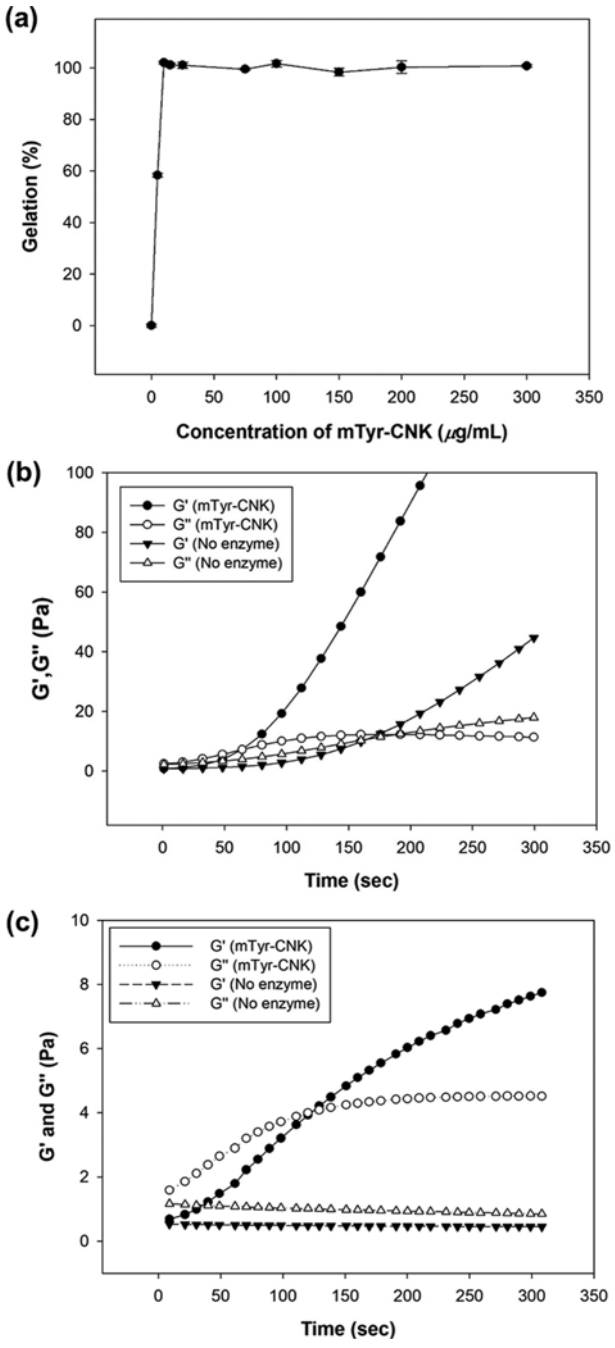

Fig. 1. Gel formation of chitosan (0.5% w/v)/gelatin (5% w/v) blends catalyzed by mTyr-CNK. (a) In vitro gelation degree with different concentrations of mTyr-CNK. (b) Rheological detection of gel formation in the absence (negative control) and presence of mTyr-CNK at 20 °C. (c) Rheological detection of gel formation in the absence (negative control) and presence of mTyr-CNK at 37 °C.

2. Morphological Analysis and Mechanical Properties of the Hydrogels

Fig. 2(a) shows the average pore diameter of the gels; the diameter for chitosan/gelatin gel with no nHA was approximately 164±6 μm, and the diameter gradually increased and then decreased with the addition of nHA, reaching a maximum at chitosan/gelatin/nHA gel containing 20% nHA (264±20 μm). A cross-section of the chitosan/gelatin/nHA gel showed that nHA nanoparticles were embedded into the gel surface (Fig. 2(b)). The compressive strength (290±9 kPa) of the prepared chitosan/gelatin gel without nHA gradually decreased and then increased with the addition of nHA, reaching a minimum at the chitosan/gelatin/nHA gel containing 20% nHA (202±4 kPa) (Fig. 2(c)). Interestingly, the addition of nHA into chitosan/gelatin did not enhance the compressive strength; however, compression caused only fracture in the chitosan/gelatin/nHA gels, and the shape was relatively well conserved, whereas compression crushed the chitosan/gelatin gel as a whole (Fig. 2(d)).

3. Swelling and In Vitro Degradation

Fig. 3(a) shows the water retention ability of the mTyr-CNK-mediated chitosan/gelatin and chitosan/gelatin/nHA hydrogels. The shape in the swelling was well conserved for all the gels, but the size was reversibly changed upon swelling and drying. The swelling degree of the chitosan/gelatin gel was approximately 1,135±37%, which was still high but lower than the previous results for the chitosan/gelatin gel catalyzed by tyrosinase-CNK [12]. The degradation gradually increased in vitro over time in PBS solution (pH 7.4, 37 °C), although less than 10% inconsistent weight loss occurred within 1 hr after the PBS application (Fig. 3(b)).

4. In Vitro Cytotoxicity of mTyr-CNK and Proliferation Assay in the Hydrogels

Because mTyr-CNK may have cytotoxic effects on cell growth, in vitro cell viability was investigated in the cell culture medium with mTyr-CNK. The cell viability was comparatively assessed in

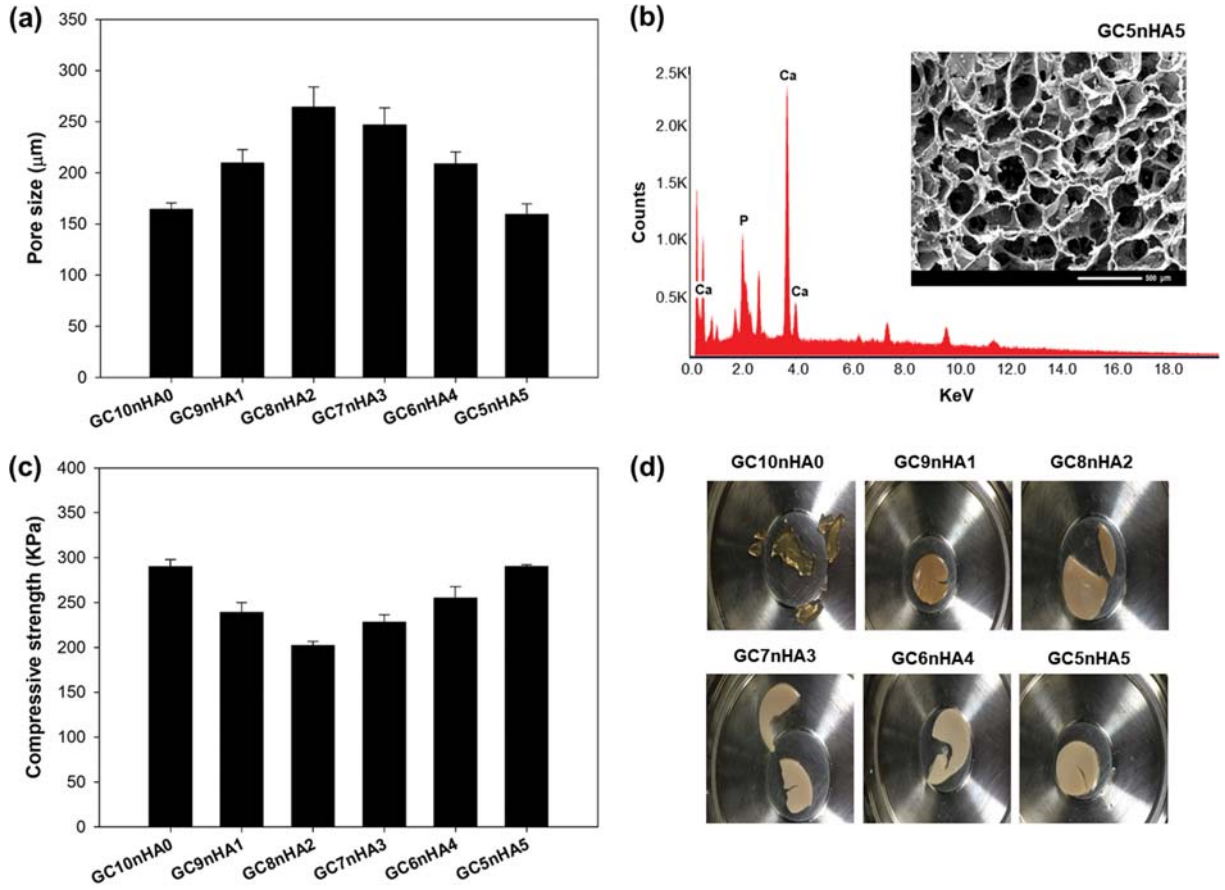

Fig. 2. Morphological analysis and compressive strength of mTyr-CNK-mediated hydrogels of chitosan (0.5% w/v)/gelatin (5% w/v) with various concentrations of nanohydroxyapatite (nHA). (a) Average pore size of the freeze-dried hydrogels. (b) Scanning electron microscopy (SEM) image with energy dispersive X-ray spectroscopy (EDS) for chitosan (0.5% w/v)/gelatin (5% w/v) with 50 wt% nHA. (c) Compressive strength of the hydrogels. (d) Photographs of the hydrogels after the compressive strength test. GC10nHA0, chitosan (0.5% w/v)/gelatin (5% w/v) with 0 wt% nHA; GC9nHA1: chitosan (0.5% w/v)/gelatin (5% w/v) with 10 wt% nHA; GC8nHA2, chitosan (0.5% w/v)/gelatin (5% w/v) with 20 wt% nHA; GC7nHA3, chitosan (0.5% w/v)/gelatin (5% w/v) with 30 wt% nHA; GC6nHA4, chitosan (0.5% w/v)/gelatin (5% w/v) with 40 wt% nHA; GC5nHA5, chitosan (0.5% w/v)/gelatin (5% w/v) with 50 wt% nHA.

MC3T3-E1 cells using complete α-MEM with 100 μg mTyr-CNK mL^{-1} and complete α-MEM only as a negative control, which was based on the applied enzyme amount for the preparation of chitosan/gelatin and chitosan/gelatin/nHA hydrogels. Fig. 4(a) shows that the viability in the medium with mTyr-CNK did not show a significant difference compared with the control ($p>0.2$).

The same amount of MC3T3-E1 cells was placed in the prepared hydrogels for the cell proliferation assay. Overall, the cell growth increased gradually with time on all the hydrogels, showing that they were non-toxic with cell growth (Fig. 4(b)). The total attached cell amount was higher in the chitosan/gelatin gel than in all the chitosan/gelatin/nHA gels after 24 hr. The hydrogel environment without nHA might be more favorable for initial cell attachment than that with nHA. However, the proliferation levels became similar when cell proliferation was investigated for seven days.

DISCUSSION

mTyr-CNK, an engineered fragment of tyrosinase-CNK, had appropriate activity for the crosslinking of chitosan and gelatin throughout a broad range of temperature and pH. Moreover, the homology model structure of mTyr-CNK suggested that the entrance of the active site could be well opened by removing the C-terminal domain of tyrosinase-CNK, which was expected to minimize the steric hindrance of polymeric substrates such as chitosan and gelatin. The elimination of the C-terminal domain of tyrosinase-CNK enhanced the enzymatic crosslinking ability of polymeric chitosan/gelatin solution. Primarily, mTyr-CNK could be optimally applicable for the preparation of rapid and injectable chitosan/gelatin hydrogels. The activity of mTyr-CNK could be simply controlled only by temperature without any additional materials. The enzyme was very stable below 30 °C but can be efficiently and selectively inactivated at temperatures greater than 30 °C after in vivo gelation reaction minimizing unwanted side reactions [19]. In addition, the optimally rapid gelation time is an important parameter for characterizing the injectable properties. Excessively rapid gelation may cause difficulty in mixing precursor solutions and discontinuity in the formed gels; on the other hand, slow gelation may result in unwanted diffusion of the precursor solutions and gel formation with undesired shapes [24].

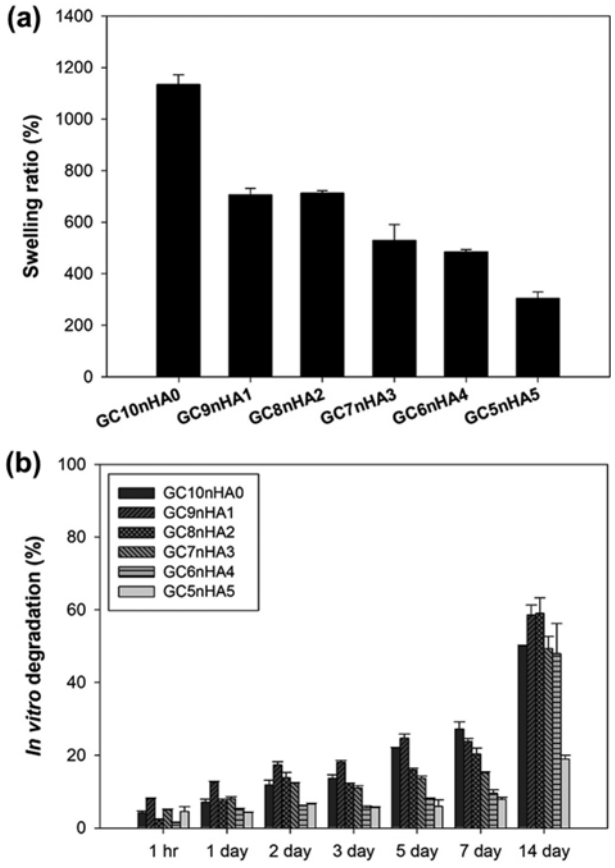

Fig. 3. Swelling ratio (a) and in vitro degradation (b) of chitosan (0.5% w/v)/gelatin (5% w/v) with various concentrations of nHA in phosphate-buffered saline (PBS). GC10nHA0, chitosan (0.5% w/v)/gelatin (5% w/v) with 0 wt% nHA; GC9nHA1: chitosan (0.5% w/v)/gelatin (5% w/v) with 10 wt% nHA; GC8nHA2, chitosan (0.5% w/v)/gelatin (5% w/v) with 20 wt% nHA; GC7nHA3, chitosan (0.5% w/v)/gelatin (5% w/v) with 30 wt% nHA; GC6nHA4, chitosan (0.5% w/v)/gelatin (5% w/v) with 40 wt% nHA; GC5nHA5, chitosan (0.5% w/v)/gelatin (5% w/v) with 50 wt% nHA.

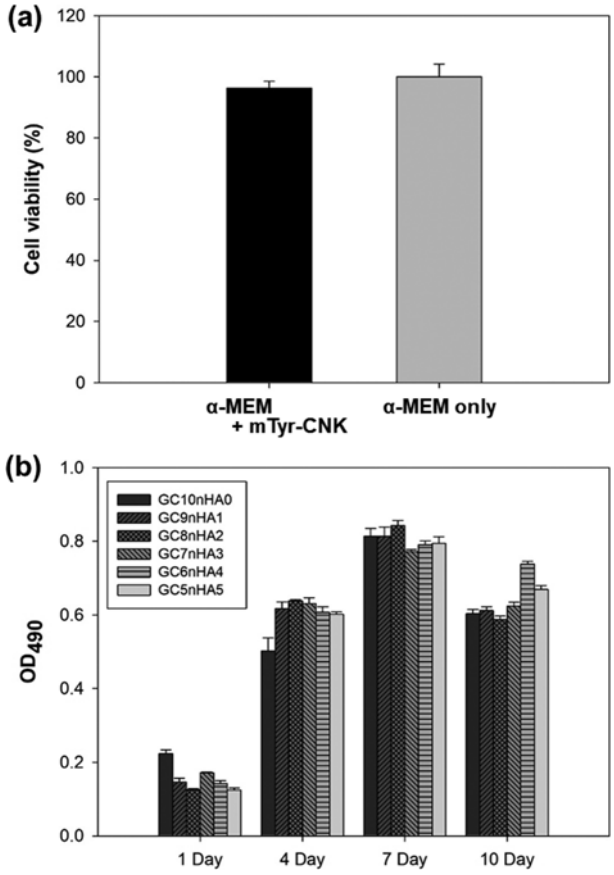

Fig. 4. (a) Cell viability of MC3T3-E1 in complete α-MEM with/without 100 μg/mL mTyr-CNK. (b) Cell proliferation assay of MC3T3-E1 in the mTyr-CNK-mediated hydrogels. GC10nHA0, chitosan (0.5% w/v)/gelatin (5% w/v) with 0 wt% nHA; GC9nHA1: chitosan (0.5% w/v)/gelatin (5% w/v) with 10 wt% nHA; GC8nHA2, chitosan (0.5% w/v)/gelatin (5% w/v) with 20 wt% nHA; GC7nHA3, chitosan (0.5% w/v)/gelatin (5% w/v) with 30 wt% nHA; GC6nHA4, chitosan (0.5% w/v)/gelatin (5% w/v) with 40 wt% nHA; GC5nHA5, chitosan (0.5% w/v)/gelatin (5% w/v) with 50 wt% nHA.

mTyr-CNK-mediated chitosan/gelatin/nHA gels showed relatively high average pore size (greater than 150 μm diameter), which could promote the permeation of nutrients, oxygen and waste and the internalization of cells into the gels, which requires above 80 μm pore size for endothelial cells and above 150 μm for vascular muscle cells and fibroblast cells [10,25]. Because mTyr-CNK catalyzes the formation of o-quinones from less than 1% tyrosine residues of gelatin and the embedded nHA nanoparticles could influence the crosslinking network formation, a relatively low-density crosslinking network between chitosan and gelatin might be formed, resulting in a relatively large pore size distribution. However, the compressive strength was highly improved compared with that of previous physical chitosan/gelatin gels and similar to that of other chemically crosslinked chitosan/gelatin gels. The mechanical properties depend on fabrication methods with different crosslinking mechanism even in the same materials, although different crosslinking density and pore size might influence the mechanical properties for the same polymerization conditions and component materials [26-28].

The swelling degree and in vitro degradation for the mTry-CNK-mediated hydrogels were investigated due to the importance for the sustained release of embedded biomolecules and cellular remodeling in hydrogel applications [10,29]. The swelling degree of approximately 1,135±37% gradually decreased with increasing nHA content in the chitosan/gelatin gels. The enhanced crosslinking ability of mTyr-CNK could increase the compressive strength, but the crosslinking and nHA content reduced the swelling ability. The overall in vitro degradation behavior was similar to that of previous tyrosinase-CNK-mediated chitosan/gelatin gels [12], and chitosan/gelatin/nHA hydrogels showed in vitro degradation behavior similar to that of chitosan/gelatin hydrogels, although increasing the amount of nHA appeared to somewhat reduce the in vitro degradation trend. Increased hydrophobic aggregation in the chitosan/gelatin/nHA gels may hamper the degradation of partially decomposed gel fragments. Finally, cell culture experiments demonstrated that the mTyr-CNK-mediated hydrogels are not cytotoxic and could

be usable as biocompatible hydrogels for a broad range of biomedical applications. Mouse preosteoblast MC3T3-E1 cells were used as a model because hydrogels with nHA may be more applicable in bone tissue engineering.

CONCLUSION

Biocompatible and stable chitosan/gelatin and chitosan/gelatin/nHA hydrogels were rapidly prepared without any additional cross-linking agents by using an engineered tyrosinase, mTyr-CNK, that had high catalytic activity for tyrosine/DOPA-tethered polymeric biomaterials. The obtained in situ injectable gels could be applicable for scaffolds for space filling agents, including nHA, delivery vehicles of bioactive molecules and cells, based on the pore size distribution and mechanical properties. These results suggest that the obtained hydrogels could be used in the design of in situ injectable and biocompatible hydrogels for various biomedical applications.

ACKNOWLEDGEMENTS

This work was supported by the Marine Biomaterials Research Center grant from Marine Biotechnology Program of the Korea Institute of Marine Science & Technology Promotion funded by the Ministry of Oceans and Fisheries, Korea and by the National Research Foundation of Korea (NRF), funded by the Ministry of Science, ICT & Future Planning (NRF- 2020R1I1A3072957).

REFERENCES

1. L. Fan, H. Yang, J. Yang, M. Peng and J. Hu, *Carbohyd. Polym.*, **146**, 427 (2016).
2. M. Liu, X. Zeng, C. Ma, H. Yi, Z. Ali, X. B. Mou, S. Li, Y. Deng and N. Y. He, *Bone Res.*, **5**, 17014 (2017).
3. J. Patterson, M. M. Martino and J. A. Hubbell, *Mater. Today*, **13**, 14 (2010).
4. Y. L. Li, J. Rodrigues and H. Tomas, *Chem. Soc. Rev.*, **41**, 2193 (2012).
5. R. LogithKumar, A. KeshavNarayan, S. Dhivya, A. Chawla, S. Saravanan and N. Selvamurugan, *Carbohyd. Polym.*, **151**, 172 (2016).
6. L. D. Zhu and K. M. Bratlie, *Biochem. Eng. J.*, **132**, 38 (2018).
7. K. Su and C. M. Wang, *Biotechnol. Lett.*, **37**, 2139 (2015).
8. Y. J. Yang, Y. S. Choi and H. J. Cha, *Biotechnol. J.*, **13**, 1800086 (2018).
9. L. Cui, J. F. Jia, Y. Guo, Y. Liu and P. Zhu, *Carbohyd. Polym.*, **99**, 31 (2014).
10. S. M. Saraiva, S. P. Miguel, M. P. Ribeiro, P. Coutinho and I. J. Correia, *RSC Adv.*, **5**, 63478 (2015).
11. M. Nieto-Suarez, M. A. Lopez-Quintela and M. Lazzari, *Carbohyd. Polym.*, **141**, 175 (2016).
12. Y. R. Choi, E. H. Kim, S. Lim and Y. S. Choi, *Biochem. Eng. J.*, **129**, 50 (2018).
13. X. Bai, M. Gao, S. Syed, J. Zhuang, X. Xu and X. Q. Zhang, *Bioact. Mater.*, **3**, 401 (2018).
14. M. Otsuka, *Biol. Pharm. Bull.*, **36**, 1653 (2013).
15. C. Ohtsuki, M. Kamitakahara and T. Miyazaki, *J. R. Soc. Interface.*, **6**, S349 (2009).
16. H. Zhou and J. Lee, *Acta Biomater.*, **7**, 2769 (2011).
17. R. Z. LeGeros, *Clin. Mater.*, **14**, 65 (1993).
18. S. I. Stupp and G. W. Ciegler, *J. Biomed. Mater. Res.*, **26**, 169 (1992).
19. H. Do, E. Kang, B. Yang, H. J. Cha and Y. S. Choi, *Sci. Rep.*, **7**, 17267 (2017).
20. R. N. Raja Mohd Hafidz, C. M. Yaakob, I. Amin and A. Noorfaizan, *Int. Food Res. J.*, **18**, 787 (2011).
21. E. H. Kim, S. Lim, T. E. Kim, I. O. Jeon and Y. S. Choi, *Biotechnol. Bioprocess Eng.*, **23**, 500 (2018).
22. C. Q. Yan and D. J. Pochan, *Chem. Soc. Rev.*, **39**, 3528 (2010).
23. L. Guo, R. H. Colby, C. P. Lusignan and A. M. Howe, *Macromolecules*, **36**, 10009 (2003).
24. S. F. Yan, T. T. Wang, X. Li, Y. H. Jian, K. X. Zhang, G. F. Li and J. B. Yin, *RSC Adv.*, **7**, 17005 (2017).
25. M. I. Gariboldi and S. M. Best, *Front Bioeng. Biotechnol.*, **3**, 151 (2015).
26. K. S. Anseth, C. N. Bowman and L. Brannon-Peppas, *Biomaterials*, **17**, 1647 (1996).
27. H. Jiankang, L. Dichen, L. Yaxiong, Y. Bo, Z. Hanxiang, L. Qin, L. Bingheng and L. Yi, *Acta Biomater.*, **5**, 453 (2009).
28. Y. Y. Pey, D. M. Guo, O. D. An, Z. Y. Xiao, S. R. Zhai and B. Zhai, *Korean J. Chem. Eng.*, **35**, 2384 (2018).
29. S. Jo, S. Park, Y. Oh, J. Hong, H. J. Kim, K. J. kim, K. K. Oh and S. H. Lee, *Biotechnol. Bioprocess. Eng.*, **24**, 145 (2019).