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Chitosan-Lysozyme Conjugates for Enzyme-Triggered Hydrogel Degradation in Tissue Engineering Applications

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Abstract

Tuning hydrogel degradation enables effective and successful tissue regeneration by modulating cellular behaviors and matrix formation. In this work, we develop a novel degradable hydrogel scaffold based on a unique enzyme-substrate complex by photocrosslinking. Chitosan and lysozyme are chemically modified with methacrylate moieties to be tethered in hydrogels, and in the presence of riboflavin initiator, these hydrogels are cured by blue light irradiation. The incorporation of lysozyme to chitosan hydrogels accelerates the degradation rate of the crosslinked hydrogels in a dose dependent manner, as evidenced by an increase in pore size and interconnectivity through cryogenic scanning electron microscopy (cryo-SEM) over time. Those noncytotoxic materials significantly enhance cellular proliferation and migration, which contribute to osteogenic differentiation of encapsulated mesenchymal stem cells *in vitro* and bone formation in mouse calvarial defects. These findings suggest a promising strategy to modulate the degradation behavior of hydrogels for use in tissue engineering.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Cytotoxicity and osteogenic effect of lysozyme. Relative cell growth of MeLy210. Water content, swelling ratio, and theoretical mesh size of hydrogels. *In vivo* cell infiltration images.

Keywords

hydrogel; chitosan; lysozyme; degradation; osteogenesis

1. Introduction

Tissue-engineered hydrogels mimicking native extracellular matrix (ECM) microenvironments offer a spatiotemporally heterogenic and dynamic nature^{1–3}. The nature of hydrogels can direct the fate of the encapsulated cells such as migration, infiltration, proliferation, differentiation, and tissue rearrangement^{4–6}. Cell migration and infiltration are favored in porous structure which can provide enough space for cell movement as well as ECM depositions guiding differentiation pathways^{7–9}.

The porous architecture of hydrogels can be obtained by modulating hydrogels degradation via various mechanisms. Hydrolytic degradation originated from water-susceptible functional groups may encounter high initial mass loss which makes it difficult to interact with the encapsulated cells¹⁰. Photoactive degradation triggered by photolabile groups is challenging to apply in tissue engineering due to a need of additional light activation¹¹. Protease-mediated degradation mimics local enzymatic breakdowns such as matrix metalloproteinases (MMP) regulation in tissue remodeling of natural ECM¹². However, MMP-mediated degradation is limited to the localized cells where the MMP concentration is high enough to cleave MMP-sensitive bonds, as well as hard to control MMP production level correlated with cell types, numbers, and metabolic activities¹³. Therefore, incorporation of exogenous hydrogel-specific enzymes that can control degradation kinetics regardless of cell encapsulation is an appealing strategy¹⁴.

Chitosan is a well-proven polysaccharide with high biocompatibility and has numerous primary amines that can be conjugated with diverse functional groups such as the photocrosslinkable methacrylate group^{6,15}. Chitosan hydrogels have been used in various tissue engineering applications including bone, cartilage, and neural regeneration^{16,17}. Lysozyme, a well distinguished endo-carbohydrase, ubiquitously presents in saliva, tears, or other body secretions¹⁸ and cleaves 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine of a chitosan backbone. Moreover, lysozyme is capable of destructing bacterial cell walls that contain peptidoglycan¹⁹. It also exhibits a stable three-dimensional structure and maintains stability at various pH (5–9), suitable to utilize in tissue remodeling environments¹⁹. Lysozyme is widely applied in chitosan scaffolds or films to modulate its material characteristics since *in vivo*, chitosan is mainly degraded by lysozyme targeting the acetylated part, and the final product of degradation, glucosamine, is nontoxic to cells^{20–23}.

Therefore, lysozyme incorporated chitosan hydrogels can potentially induce porous structure by causing degradation.

We have previously developed a visible blue light-crosslinkable hydrogel system using methacrylated glycol chitosan (MeGC) and riboflavin, an aqueous initiator from natural vitamins¹⁵. Enhanced proliferation and osteogenic or chondrogenic differentiation of mesenchymal stem cells (MSCs) encapsulated in MeGC hydrogel were demonstrated^{6,15,24,25}. However, it had relatively slow degradation profile which may interfere with tissue regeneration.

In this work, we report a novel hydrogel system with fine-tuned degradability based on a unique enzyme-substrate pair, lysozyme-chitosan. Lysozyme was modified with photo-crosslinkable moiety for stable conjugation in MeGC hydrogel. Degradation profiles and morphological changes of MeGC hydrogels were evaluated using cryogenic scanning electron microscopy (cryo-SEM). Cryo-SEM preserves native structures in a frozen hydrated state through rapid freezing and provides a direct imaging of samples in nanometer resolution. The degree of degradation with various lysozyme concentration was examined by analyzing the pore size change in the cryo-SEM micrographs. The effects of hydrogel degradation on cell proliferation, migration, and construct modulus were assessed by encapsulation or seeding of bone marrow stromal cells (BMSCs). We further investigated whether the hydrogel modification with lysozyme can promote osteogenesis of mesenchymal cells *in vitro* and bone regeneration in a mouse calvarial defect model.

2. Materials and Methods

2.1. Materials

Glycol chitosan (GC, B100 kDa), glycidyl methacrylate (GMA), lysozyme from chicken egg white, tween-20, *p*-nitrophenol phosphate, β -glycerophosphate, L-ascorbic acid, dexamethasone, riboflavin (RF), nitro blue tetrazolium (NBT), 5-bromo-4-chloro-3-indoxylphosphate, alizarin red S and ethylene diaminetetraacetic acid (EDTA) were supplied by Sigma-Aldrich (St. Louis, MO). Low glucose dulbecco's modified eagle's medium (DMEM), penicillin/streptomycin (P/S, 100 U mL⁻¹) and trypsin were purchased from Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was obtained by Mediatech Inc. (Manassas, VA). All solvents and products were used without further purification.

2.2. Preparation of Lysozyme Incorporated Hydrogel

Methacrylated glycol chitosan (MeGC) was prepared by following the previously developed method^{6,15}. Methacrylated lysozyme (MeLyz) was prepared by reaction of 200 mg mL⁻¹ lysozyme and GMA at a 1.1 molar ratio in distilled water (DW) for 18 h on a shaker at room temperature. After reaction, it was dialyzed in 3 kDa tubes against DW for 24 h, lyophilized and rehydrated in 1x phosphate buffered saline (PBS) at 200 mg mL⁻¹.

2.3. Bioactivity of Methacrylated Lysozyme

Vancomycin-resistant *Staphylococcus aureus* Mu50 (*S. aureus*, ATCC 700699) was used to test bioactivity of MeLyz²⁶. The bacterial culture was diluted to 5×10^7 colony-forming

units (CFU) mL⁻¹ with sterile Luria-Bertani (LB). Lysozyme solution was prepared as 0, 0.01, 0.1, 0.5, 1, and 5 mg mL⁻¹ with sterile LB. 500 µL of the bacterial solution was added to 500 µL of lysozyme solution and cultured for 5 h at 37 °C. The counts of bacteria were quantified by measuring the optical density of medium at 600 nm (OD₆₀₀) by UV/Vis spectrophotometer (Beckman Coulter). Based on the OD₆₀₀ value, bacterial survival rate was quantified and calculated as bacterial survival percentage.

2.4. Degradation Kinetics of Hydrogels

Degradation kinetics of hydrogels were measured by the dry weight change of hydrogels. The hydrogels with different MeLyz concentrations (0, 0.1, 1, and 10 mg mL⁻¹) were incubated in DW for two weeks at 37 °C. At day 0, 1, 7, and 14, hydrogels were collected ($n = 4$), and after lyophilization, the dry weight was weighed. The degradation of hydrogels was calculated by the following equation (1).

$$\text{Gel degradation (\%)} = \frac{W_0 - W_t}{W_t} \times 100 \quad (1)$$

where W_0 and W_t , refers to the dry weight of a hydrogel at day 0 and each time point.

2.5. Morphological Observations of Hydrogels

The morphology of hydrogels was observed with a Cryogenic-Scanning Electron Microscopy (Cryo-SEM, TESCAN Mira 3 filed emission SEM coupled with a Leica cryogenic system). Hydrogels were sandwiched between two freezer planchettes (Type “A”, Ted Pella, Inc.) with 100 µm depth. The total volume of the hydrogel should be enough (at least 5.6 µL) to fill the gap created by both planchettes. The hydrogel-loaded planchettes were vitrified in liquid ethane (~ -170 °C) for ~ 5 s and then transferred to a freeze fracture/sputtering coater (Leica ACE 600). The samples were freeze fractured and sublimated at -105 °C for 90 min to reveal the structure of hydrogel. The sublimation condition was optimized to observe the underlying surface feature of the hydrogel without distorting their native morphology. Following the sublimation, approximately 5 nm of gold layer was sputter-coated. The sample was imaged in the pre-cooled (~ -142 °C) TESCAN Mira 3 FE-SEM operated at 5 kV. An area less than 20 µm away from the planchette wall was imaged to minimize insufficient freezing. The size of pore created by hydrogel degradation was quantified by ImageJ (NIH, Bethesda, Maryland) after processing images to the binary mode and analyzing particles.

2.6. *In vitro* Cell Viability and Proliferation in Hydrogels

The mouse bone marrow stromal cell line (BMSC, CRL-12424, ATCC, Manassas, VA) were cultured in culture medium (CM) consisting of DMEM with 10% (volume per volume) FBS and 1% (volume per volume) P/S at 37 °C under 5% CO₂ humidified atmosphere. The BMSCs were encapsulated in hydrogels with 2×10^6 cells mL⁻¹ concentration. The hydrogels with different MeLyz concentrations (0, 0.1, and 1 mg mL⁻¹) were incubated in CM for two weeks at 37 °C. At day 1, 7, and 14, the viability of cells was pictured with Live/Dead staining kit (Invitrogen, Carlsbad, CA). To quantify the cell viability, the ratio of

live cells to total cells were calculated based on green and red color by ImageJ software (NIH, Bethesda, Maryland). The alamarBlue assay was performed to quantify the cell growth by following the manufacture's protocol. The relative cell growth was measured by the following equation (2).

$$\text{Relative cell growth} = \frac{F_e - F_b}{F_c - F_b} \quad (2)$$

where F_e , F_c , and F_b refer to fluorescence emission value at 585 nm after excitation at 570 nm of experimental, control (MeGC at day 0) and blank groups, respectively. To evaluate the change in mechanical properties of hydrogels by cell growth, the compressive modulus of cell-laden hydrogels was measured via an indentation test at day 1, 7, and 14 by Instron Electromechanical Testing Machine (Instron, Model 5564, Norwood, MA) using a 1.6 mm diameter indenter as described previously^{6,27}.

2.7. *In vitro* Cell Migration on Hydrogels

The BMSCs were seeded on the top surface of hydrogels with 2×10^4 cells per hydrogel (300 μ L, molded in 48-well plate) and cultured for 14 days to observe cell migration from the top to the bottom of hydrogel. At each time point, cells were stained with Live staining kit and imaged by Confocal Multiphoton STED microscope (Leica TCS-SP5 AOBS, Buffalo, IL) to track on Z direction.

2.8. *In vitro* Osteogenesis of Cells in Hydrogels

The BMSCs encapsulated hydrogels were cultured in osteogenic medium (OM) including CM, 10 mM of β -glycerophosphate, 50 μ g mL⁻¹ of L-ascorbic acid and 100 nM of dexamethasone for three weeks to induce osteogenic differentiation. After four days of culture, hydrogels were collected and fixed in 10% NBF for alkaline phosphatase (ALP) staining. The hydrogels were incubated in AP solution (100 mM Tris, pH 8.5, 50 mM MgCl₂, 100 mM NaCl) with NBT and 5-bromo-4-chloro-3-indoxylphosphate²⁸. For ALP activity test, the hydrogels were digested in 0.02% tween-20 solution in 1 $\times$ PBS, mixed with *p*-nitrophenol phosphate for 10 min and an absorbance at 405 nm was measured. ALP activity was normalized by total protein expression from Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). After three weeks of culture, hydrogels were collected and fixed for alizarin red S staining for mineral deposition measurement. The fixed hydrogels were incubated in 2% alizarin red S solution for 5 min and washed in 1 $\times$ PBS for 2 h. All stained hydrogels were imaged by Olympus SZX16 Stereomicroscope. The osteogenic genes, *Runx2*, *Colla* and osteocalcin (*OCN*), expression was measured by qRT-PCR. For *Runx2* and *Colla*, hydrogels were collected at day 4 and for *OCN*, they were collected at day 21. After incubation, total RNA was extracted using Trizol (Invitrogen, CA) and RNeasy mini kit (Qiagen, CA). Total RNA was reverse transcribed with cDNA transcription kit (Invitrogen, CA) and quantitative real-time polymerase chain reaction (qRT-PCR) was performed to evaluate the expression of osteogenic critical markers in a LightCycler 480 PCR (Indianapolis, IN) with SYBR Green. It was amplified 45 cycles and *GAPDH* was used for normalization. The primer sequence of each gene was as following:

GAPDH: AGGTCGGTGTGAACGGATTTG (forward), TGTAGACCATGTAGTTG AGGTCA (reverse); *ALP*: GTTGCCAAGCTGGGAAGAACAC (forward), CCCACCCCGCTATTCCAAAC (reverse); *Runx2*: CGGTCTCCTT CCAGGATGGT (forward), GCTTCCGTCAGCGTCAACA (reverse); *Colla*: AACCCGAGGTATGCTTGATCT (forward), CCAGTTCTTCATTGCATTGC (reverse); *OCN*: GGGAGACAACAGGGAGGAAAC (forward), CAGGCTTCCTGCCAGTACCT (reverse). All experiments were triplicated.

2.9. *In vivo* Bone Forming Ability of Hydrogels

All operations were executed by the Guide for the Care and Use of Laboratory Animals of the University of California, Los Angeles approved by the Chancellor's Animal Research Committee. To decrease the host immune response to implanted cells, the adult nude mice (CD-1, 2 months old, Charles River Laboratories) were used. The adult nude mice ($n = 4$ per groups) were anesthetized and 3 mm diameter craniotomy defects were created with trephine drill following our previous study²⁹. Each defect was washed with saline solution, filled with hydrogels containing MSCs and crosslinked with VBL for 40 s. After surgery, all animals were placed on a warm sheet and moved to the vivarium for postoperative care. All mice received subcutaneous injection of buprenorphine with 0.1 mg kg^{-1} for three days and drinking water including trimethoprim-sulfamethoxazole for seven days.

2.10. Three-Dimensional Microcomputed Tomography Scanning

After six weeks (for bone formation study) of implantation, all mice were sacrificed and calvarial tissues were collected. The calvarial tissues were fixed in 4% formaldehyde for 48 h at room temperature, washed with PBS and stored in 70% ethanol at 4°C till imaging. The resolution of high-resolution microcomputed tomography (μCT40 ; Scanco USA, Inc., Southeastern, PA) was $10 \mu\text{m}$ (57 kVp and $180 \mu\text{A}$ radiation source with 0.5 mm aluminum filtration). Reconstruction of μCT images was done by OsiriX MD (Pixmeo SARL, Switzerland) and new bone growth area was analyzed by ImageJ. The percent bone volume (BV TV^{-1}) and trabecular number (Tb.N) were assessed by CTAn (Bruker MicroCT).

2.11. Histological Examination

For *in vitro* cell migration study, hydrogels at day 0, 7, and 14 were fixed in 10% neutral buffered formalin (NBF) for 16 h and embedded in paraffin to cut into $5 \mu\text{m}$ thickness. The sections were imaged after deparaffinizing and hematoxylin and eosin (H&E) staining. For *in vivo* bone healing study, the fixed calvarial tissues were decalcified in 10% EDTA solution for a week under gentle shaking. The decalcified tissues were embedded in paraffin to cut into $5 \mu\text{m}$ thickness, deparaffinized, and stained with H&E and Masson-goldner trichrome to investigate new bone formation.

2.12. Statistical Analysis

The one-way analysis of variance (ANOVA) with Tukey's *post hoc* test was used for statistical analysis. A value of $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Preparation of Lysozyme Incorporated Hydrogels

For lysozyme incorporated hydrogel fabrication, methacrylated lysozyme (MeLyz) was prepared by the reaction of lysozyme with glycidyl methacrylate (GMA), and integrated into MeGC hydrogel¹⁵ by visible blue light crosslinking (Figure 1a). Under various concentrations of MeLyz (0, 0.01, 0.1, 0.5, 1, and 5 mg mL⁻¹), the survival rate of *S. aureus* was similar for natural lysozyme and MeLyz, which confirmed that the bioactivity of MeLyz is comparable to natural lysozyme (Figure 1b). Then, MeLyz was incorporated in MeGC at the concentration of 0, 0.1, 1, and 10 mg mL⁻¹ respectively, for hydrogel degradation study. The introduction of MeLys in MeGC accelerated the degradation rate in a dose dependent manner (Figure 1c).

3.2. Morphological Observations of Hydrogels

Cryo-freezing and cryo-SEM allows the study of dynamic process of hydrogels degradation with improved ultra-structural preservation and nanometer resolution. Variety of pores were generated in hydrogel scaffold when the degradation occurred. The degradation of the three hydrogels was examined by measuring pore size from cryo-SEM micrographs (Figure 2a). At day 0, all three hydrogels yielded comparable pore sizes of 39 ± 14 , 37 ± 15 , and 38 ± 14 nm respectively for MeGC, MeLyz0.1, and MeLyz1; thereafter increased to 68 ± 27 , 86 ± 49 , and 409 ± 396 nm respectively, after 10 days (Figure 2b). A clear trend was observed where both the average pore size and size distribution increased as MeLys dose increases for MeLys0.1 and MeLys1. Yet, MeLys1 hydrogel showed the greatest distribution in pore size with a larger fraction of outliers, reaching up to 1,200 nm at day 10.

3.3. *In vitro* Cell Viability and Proliferation in Hydrogels

The BMSCs were mixed with hydrogel solution (MeGC, MeLyz, and RF) before visible blue light (VBL) crosslinking for cell encapsulation (Figure 3a). The viability of BMSCs encapsulated in hydrogels was observed over two weeks, and both fluorescent images and the quantitative analysis demonstrated that cell viability in all hydrogels was above 90% over two weeks (Figure 3b). This indicated that regardless of MeLyz incorporation, hydrogels have biocompatible environments. In spite of the high viability of cells, fluorescent images at day 14 showed larger green-colored areas for MeLyz0.1, and MeLyz1 compared with those for MeGC. This result can be clarified by the cell proliferation result (Figure 3c) pointing out the higher cell proliferation of MeLyz0.1, and MeLyz1 than that of MeGC, with intensified the green fluorescence in cell viability images (Figure 3b). To investigate the change of mechanical strength of hydrogel *via* cell encapsulation, a compressive modulus test was performed with or without the encapsulation of BMSCs (Figure 3d). The initial compressive modulus of MeGC, MeLyz0.1, and MeLyz1 without cells at day 1 was 7.9, 7.4, and 7.1 kPa, which are similar around 7.5 kPa. However, after two weeks, the modulus of MeLyz0.1 and MeLyz1 without cells decreased to 2.6 and 1.4 kPa, which are significantly lower than that of MeGC, 6.9 kPa. We already observed the mass loss of hydrogels with degradation (Figure 1c), hence the decrement of compressive modulus can also be explained as a consequence of degradation. However, with the encapsulation of cells, all MeGC, MeLyz0.1, and MeLyz1 showed enhanced compressive

modulus over the time compared to that of hydrogels without cells. Particularly, MeLyz0.1 and MeLyz1 at day 14 had significantly risen, which represented that the production of ECM from the proliferated cells as a result of hydrogel degradation supported the mechanical strength.

3.4. *In vitro* Cell Migration on Hydrogels

The BMSCs were seeded on the surface of VBL-crosslinked hydrogels (Figure 3a), and migration from the upper surface to the inside of hydrogels was monitored by the confocal microscope for two weeks. Cells were observed only on the surface of MeGC hydrogels, instead spread throughout the hydrogel bulk in MeLyz1 over time (Figure 4a). To further investigate the migrated cells on the hydrogels, histological analysis was processed (Figure 4b). For MeGC, cells seeded on the surface of hydrogels did not penetrate inside of hydrogels. On the other hand, cells seeded on MeLyz1 permeated into hydrogels over two weeks. The surface of both hydrogels changed from smooth to rough structure, possibly due to contractile force on the surface with the proliferation of cells². Specifically, the surface of MeLyz1 at day 14 was entangled with the migrated cells and exhibited more uneven structure than that of MeGC.

3.5. *In vitro* Osteogenesis of Cells in Hydrogels

The BMSCs were encapsulated in hydrogels (Figure 3a) and osteogenic differentiation of cells was evaluated. The alkaline phosphatase (ALP) expression, an early stage osteogenic marker, was assessed by staining and the activity test (Figure 5a). The intensified staining and increment of ALP activity normalized by total protein expression was observed in MeLyz incorporated hydrogels in a dose dependent manner. Next, mineral expression, a late osteogenic marker, was examined by alizarin red S staining and the calcium production assay (Figure 5b). Similar with ALP expression, darker staining and elevated calcium production were shown in MeLyz incorporated hydrogels with higher dosage compared with those in MeGC. The early osteogenic genes, *Runx2*, an essential controller of osteogenesis, and *Col1a*, a gene for type I procollagen, were upregulated at day 4 with the incorporation of MeLyz (Figure 5c). In addition, the late osteogenic gene, *OCN*, expression was also enhanced at day 21 in MeLyz incorporated hydrogels. All three osteogenic genes were highly produced in a MeLyz dose dependent manner.

3.6. *In vivo* Bone Forming Ability of Hydrogels

The benefit of MeLyz incorporated hydrogels for *in vivo* bone formation was studied after six weeks of hydrogel implantation. The microCT images revealed that the defect area was filled more with the incorporation MeLyz, specifically there was more bone formation near the edge of the defect (Figure 6a). The bone growth area was quantified, 9, 37, and 52% for blank, MeGC, and MeLyz1 respectively, and this result demonstrated that MeLyz incorporated hydrogels promoted significant new bone formation (Figure 6b). The quantified percent bone volume (BV TV⁻¹) of hydrogels were 8, 19, and 50% for blank, MeGC, and MeLyz1 respectively, which indicated the significantly enhanced bone volume with MeLyz incorporation (Figure 6c). The trabecular number was also significantly increased up to 1.8 and 5.3 mm⁻¹ for MeGC and MeLyz1 from 0.06 mm⁻¹ for blank (Figure 6d). The microCT results were further supported by histological examinations of H&E and Masson-goldner

trichrome staining. The development of osteoid near the boundary of the defect was observed more in MeLyz1 compared to that in the blank and MeGC samples as well as the size of defect was decreased (Figure 6e).

4. Discussion

Lysozyme-induced degradation of chitosan was well studied^{20,30}, but the potential of a lysozyme-chitosan construct as a degradable tissue engineering scaffold was not fully explored yet. First, we investigated whether lysozyme can influence growth or differentiation of BMSCs at various lysozyme concentrations. The viability of BMSCs was well maintained over a wide range of lysozyme concentrations up to 100 mg mL⁻¹, which is well above the highest lysozyme concentration (10 mg mL⁻¹) used in this study (Figure S1a). In addition, the minimal osteogenic effect was observed with lysozyme as confirmed by *ALP* and *Runx2* gene expression at various lysozyme concentrations (Figure S1b). These results indicated the minimal toxicity and unwanted adverse effects of lysozyme.

Next, the fine-tuned degradation profiles were successfully achieved by varying MeLyz concentrations (Figure 1c). The rate of degradation was observed to be in proportion to the concentration of lysozyme in the hydrogels. However, hydrogels underwent significant mass loss with high MeLyz incorporation such as MeLyz10, which provided an inadequate structure for scaffold applications. An increase in lysozyme concentration from 1 to 10 mg mL⁻¹ led to significant reduction in cell growth due to early loss of mechanical integrity of the fast degrading hydrogels (Figure S2). For this reason, MeLyz10 was excluded for further investigation.

The tissue-engineered hydrogels are replaced by host tissues with multicellular invasion following degradation³¹. The hydrogel swelling ratio correlated with degradation, is a key parameter to calculate theoretical mesh size³². For example, the theoretical initial mesh size of polyethylene glycol (PEG) hydrogels is about 25 nm, which is 2-3 orders smaller than MSCs and potentially restricted cell migration³. The internal network structure of hydrogels started to cleave with degradation, potentially increasing water uptake (Figure S3a) as well as swelling ratio (Figure S3b). The computed theoretical hydrogel mesh sizes of MeGC, MeLyz0.1, and MeLyz1 based on swelling ratio were 49, 52, and 77 nm respectively at day 0. These values increased up to 64, 389, and 592 nm at day 14, respectively (Figure S3c). From cryo-SEM image analysis, the average pore size of hydrogels was comparable to theoretical mesh sizes at day 0. The calculation of theoretical mesh size was hypothesized with a uniform distribution³³, however, it hardly matched with the non-homogeneously cleaved network observed in our hydrogels. Therefore, the distribution of pore diameters was much broader in actual hydrogel samples. This corresponds to our observation using cryo-SEM that the attenuated and cleaved network with a wide range of pore sizes was detected from MeLyz1 at day 10 (Figure 2a, b). In addition, the interconnectivity of pore structure was increased with the incorporation of MeLyz, and exhibited disconnected and attenuated polymer network, which was different with homogeneously enlarged networks from MeGC. Although the increased pore size with a more open structure is desired, the inhomogeneous porous hydrogels with a large variation of pore size may limit uniform

nutrient diffusion and homogeneous cell distribution throughout the hydrogel. Future studies will need to create more homogeneous structure.

Hydrogel degradation could provide room for cell migration and deposition of ECM molecules which can interact with cells to induce specific signaling pathways for cell binding, migration, and differentiation^{34,35}. We noticed higher proliferation of encapsulated MSCs and longer migration of seeded MSCs with enhanced hydrogel degradability (Figure 3c, and Figure 4a and b). The ECM production and accumulation were enhanced with the elevated cell proliferation, and increased compressive modulus of MeLyz incorporated hydrogels (Figure 3d)³⁶. The stored ECM not only improved physical properties of hydrogels, but also enhanced osteogenic differentiation. Elevated *Colla* gene expression was observed, which produced a component of type I collagen, a bone specific ECM and an essential factor for osteogenesis³⁷. During the ossification process, type I collagen secreted by osteoblasts could initiate mineral deposition, and this was further confirmed by high mineral accumulation (Figure 5b)³⁸.

We also evaluated the osteogenic potency of MeLyz incorporated hydrogel using a mouse calvarial critical-sized defect model. We verified promoted cell migration in MeLyz incorporated hydrogel *in vitro* (Figure 4a, b), and reflected this study *in vivo* by observing cell infiltration. Our MeLyz incorporated hydrogel system showed great efficacy to recruit surrounding cells into the defect area at an early time point, week 1 (Figure S4). The bone healing efficacy of encapsulated cells in MeLyz incorporated hydrogel was confirmed by bone ingrowth from the edge as well as osteoid matrix formation at week 6 (Figure 6a, e). The observed improvement of bone formation in the MeLyz incorporated hydrogel may be due to increased proliferation and osteogenic differentiation of encapsulated cells in the fast degrading hydrogels as demonstrated by *in vitro* characterization as well as accelerated endogenous cell infiltration as confirmed in calvarial defects.

The lysozyme incorporated hydrogel system developed in this study showed that it is promising to be used as bone regeneration biomaterial platform. Moreover, this hydrogel platform could be applied in different areas of tissue engineering such as wound healing. Lysozyme is a well-known antimicrobial enzyme, which cleaves the peptidoglycan in gram positive bacteria cell wall³⁹. The bacterial infection is one of the main issues from the surgical practice, which can be potentially solved by our lysozyme incorporated hydrogel system. In addition, this system can be used as a drug release carrier in a sustained manner based on tunable degradation^{40,41}. Lastly, it has favorable features for blood vessel regeneration, which can create an enlarged network from degradation to induce angiogenesis⁴².

5. Conclusions

In this work, enzymatically degradable hydrogel systems were designed to allow hydrogel degradation in a cell-independent manner. We have selected a unique enzyme-substrate pair as a hydrogel where the hydrogel is designed with photocrosslinkable chitosan and lysozyme. Our study demonstrated that the enzyme-functionalized hydrogel successfully modulated the degradation rates of the hydrogel and aspects of cellular behavior and

supported functional tissue formation. The findings demonstrate that the strategy of combining lysozyme with chitosan is a promising approach to increase the efficacy of current material-based therapeutics for tissue repair.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

a) The scheme of degradable hydrogel preparation with MeLyz incorporation. Lysozyme degrades chitosan by cleaving 1,4-beta-glycosidic bond in chitosan backbone. b) Bioactivity of MeLyz in comparison to lysozyme tested by antibacterial effect on *S. aureus* for 5 h. c) Degradation kinetics of hydrogels for 14 days by measuring dry weight.

Figure 2.

a) Morphological observations of MeGC, MeLyz0.1, and MeLyz1 hydrogels by Cryo-SEM at day 0 and 10. b) The box graph of pore diameter from Cryo-SEM analysis.

Figure 3.

a) The scheme of BMSCs encapsulation and seeding to hydrogels. b) Live/Dead staining images of BMSCs encapsulated in MeGC, MeLyz0.1, and MeLyz1 for 14 days and quantified cell viability. c) Relative cell growth of BMSCs encapsulated in hydrogels evaluated by alamarBlue assay. d) Compressive modulus of MeGC, MeLyz0.1, and MeLyz1 hydrogels compared with hydrogels with BMSCs encapsulation for 14 days (* $p < 0.05$).

Figure 4.

In vitro cell migration on hydrogels. a) BMSCs seeded on hydrogel surface at day 0 and monitored cell movement for 14 days. b) Histological evaluation (H&E staining) of cell migration from hydrogel surface for 14 days.

Figure 5.

In vitro osteogenesis of cells in hydrogels. a) ALP staining images of BMSCs in hydrogels and quantified ALP activity normalized by total protein production at day 4. b) Alizarin red S staining images of BMSCs in hydrogels and its quantified calcium production at day 21. c) Osteogenic gene expression of BMSCs analyzed by qRT-PCR at day 4 (*Runx2*, *Col1a*) and day 21 (*OCN*) (* $p < 0.05$).

Figure 6.

In vivo bone formation. a) MicroCT images of calvarial defects treated with hydrogels after 6 weeks. b) Quantification of percent bone growth area. c) Quantification of percent bone volume over tissue volume (BV TV⁻¹). d) Quantification of trabecular number (Tb. N, mm⁻¹). e) Histological evaluation of regenerated bone in calvarial defects. H&E (left) and masson-goldner trichrome (right) staining of calvarial defects. Two vertical lines were drawn for easy observation (**p* < 0.05)