Photo-crosslinked gelatin methacryloyl strengthened with nanoparticles for regeneration of rabbit calvarial defects

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Abstract

Background

Gelatin methacryloyl (GelMa) **hydrogel** has been regarded as a promising biomaterial in the field of bioprinting and biofabrication. The aim of this study was to investigate the efficacy of photo-crosslinked GelMa hydrogel and calcium phosphate nanoparticles (CNp) when applying different fabrication methods for bone regeneration.

Methods

Four circular defects were created in the calvaria of 10 rabbits. Each defect was randomly allocated to the following study groups: 1) the sham control group; 2) the GelMa group (defect filled with crosslinked GelMa hydrogel); 3) the CNp-GelMa group (GelMa hydrogel crosslinked with nanoparticles); 4) CNp + GelMa group (crosslinked GelMa loaded with nanoparticles). At two, four and eight weeks, samples were harvested, and histological and micro-computed tomography analyses were performed.

Results

Histomorphometric analysis showed that CNp-GelMa and CNp + GelMa groups at 2 weeks were significantly greater in total augmented area compared to the control group ($P < 0.05$). Greatest new bone area was observed in the CNp-GelMa group, however this was without statistical significance ($P > 0.05$). Crosslinked GelMa hydrogel with nanoparticles exhibited good biocompatibility with minimal inflammatory reaction.

Conclusion

Crosslinked GelMa hydrogel with calcium phosphate nanoparticles might enhance new bone formation. Further improvement in mechanical stability and resistance to enzymatic degradation is needed prior to application in bone regenerative procedures.

1. Introduction

The role of the scaffold **in tissue engineering** is to temporarily substitute the damaged tissue matrix during the regeneration process. The ideal characteristics of a scaffold materials include providing an environment for cell proliferation and functional differentiation, and mechanical support (1). Various types of biomaterials have been investigated and developed in tissue engineering to potentially mimic the roles of extracellular matrix (ECM), which regulates tissue organization, cell behavior and allows the distribution of metabolites and growth factors (2).

Recently, hydrogels have been widely studied as a scaffold due to their similar characteristics to the extracellular matrix (3). Hydrogels, containing hydrophilic groups or domains with three-dimensional structure can be made from synthetic or natural polymers (4). Synthetic hydrogels such as poly(ethylene glycol) (PEG), poly(vinyl alcohol)(PVA), and poly(2-hydroxyethyl methacrylate) (PHEMA) hydrogels have the advantages of controllable degradation rate and modifiable mechanical strength (5, 6). On the other hand, naturally derived hydrogels such as collagen, gelatin and hyaluronic acid have abilities of cell signaling, cellular interaction and biodegradability but can be limited by the low mechanical strength and uncontrolled degradation (6). Thus, combinations of synthetic and natural hydrogels have been attempted to complement the properties each other.

Gelatin methacryloyl (GelMa) has recently attracted attention as a versatile biomaterial in the field of bioprinting and biofabrication with functional bioinks (7). It can be synthesized by the reaction of free hydroxyl and amine groups on gelatin with methacrylate anhydride, preserving arginine-glycine-aspartic acid (RGD), which promotes cell adhesion (8) and matrix metalloproteinase (MMP), responsible for enzymatic cleavage (9). Then, by given UV radiation and photoinitiator, the side groups of gelatin chains are polymerized to produce covalently crosslinked gelatin network, which enhances mechanical rigidity and stability (10). GelMa hydrogels generally contain less than 5% methacrylic anhydride (11), which means that most of functional motifs of gelatin such as RGD and MMP don't react with methacrylic anhydride. Therefore, GelMa hydrogels can be produced and modified with other components while preserving the characteristic of cell attachment that RGD promotes (8).

The properties of GelMa hydrogel such as hydration, mechanical strength and degradability can be modified to satisfy the requirements of tissue engineering application by addition of materials to the hydrogel. Whether these additive materials can improve the mechanical properties of GelMa is still controversial; many studies have attempted to develop materials for further practical applications. Previous studies have shown that GelMa hydrogel loaded with nanoparticles, carbon nanotubes and graphene oxide to serve as a stem cell scaffold, was effective in increasing the stiffness (12). In addition, in vitro and vivo studies, construction of a scaffold based on a GelMa hydrogel by application of gold nanoparticles, had a significant influence on new bone formation (13). The results showed that gold nanoparticles promote proliferation, differentiation, and alkaline phosphate activities of human adipose-derived stem cell (ADSCs) and stimulate new bone formation. Inspired by previous works, we modified the structure of GelMa hydrogel by adding calcium phosphate-based nanoparticles with our own different fabrication strategy to reinforce its mechanical strength. There have been numerous in vitro studies and meta-analysis to characterize the mechanical properties of GelMa hydrogel (14). To the best of our knowledge, this was the first study to apply GelMa hydrogel loaded with nanoparticles in an in vivo model. We hypothesized that photo-crosslinked gelatin methacryloyl hydrogel with nanoparticles would enhance bone regeneration.

The aim of this study was to investigate the efficacy of photo-crosslinked GelMa hydrogel and nanoparticles for bone regeneration. Furthermore, biocompatibility and degradation kinetics of GelMa hydrogel with the addition of nanoparticles will be assessed.

2. Materials And Method

2.1. Experimental animals

Ten 8-week-old male New Zealand white rabbits (2.8-3.2kg) were used in this study. The sample size was determined in accordance to the guideline for minimizing the number of experimental animals, refining procedures for improving animal welfare and replacing the use of animals with alternative protocols (Kilkenny, Browne, Cuthi, Emerson, & Altman, 2012). The study received ethical approval from the Animal Institution Animal Care and Use Committee of Yonsei Medical Center, Seoul, South Korea (approval number 2020 – 0285), The animal management, preparation and surgical procedures were performed in a laboratory which was accredited by International Association for Assessment and Accreditation of Laboratory Animals.

2.2. Preparation of GelMa

Synthesis of gelatin methacrylol (GelMa) has been described in detail in a previous study (15). In brief, pure gelatin methacrylol (GelMa) were synthesized by adding gelatin isolated from bovine skin (Sigma Aldrich, USA) and methacrylic anhydride (Sigma Aldrich, USA) in alkaline condition. Then, the obtained compound was dialyzed in distilled water for 4 days and lyophilized to gain pure GelMa.

Nanoparticle reinforced GelMa (NP-GelMa) was synthesized using a patented technique (KR 10-2020-0096101, 2020 (Applied: 15 July 2020)). This is a one-pot synthesise technique to produce the nanocomposite hydrogel in situ. For NP-GelMa, CaCl_2 was dissolved in distilled water. Gelatin was added to it and stirred in oil bath at 50°C at 400rpm for 6 hours to dissolve completely under pH 8. Na_2HPO_4 was dissolved in distilled water separately and added with stirring for 2 hours. The temperature was increased to 60°C and stirring continued for 30min. Methacrylic anhydride (Sigma Aldrich, USA) was added slowly drop by drop and stirring continued for 3hours. Phosphate buffered saline was added in the reaction flask and then it was cooled down and dialyzed in distilled water for 4 days. After dialysis, it was freeze-dried to obtain the nanoparticle reinforced GelMa. Table 1 shows the amounts used.

Each sample was made into a gel by mixing the above materials and putting them in a mold shape and then crosslinking them by irradiating UV light for about 30 minutes. These materials were made in a diameter of 6mm and a thickness of 2mm (Fig. 1). All samples were carefully manufactured in a sterile laboratory to avoid contamination.

2.3. Study design

Ten rabbits were divided into three groups according to the healing period of two, four, eight weeks and each group including four, four and two rabbits. Four circular defects having diameter of 6mm were formed in the calvarium and randomly allocated to the following study groups (Fig. 2):

1) Empty control group

2) GelMa group: cross-linked GelMa hydrogel

3) CNp-GelMa: GelMa hydrogel mixed with nanoparticles and crosslinked

4) CNp + GelMa: GelMa hydrogel crosslinked and then mixed with nanoparticles

At two, four and eight weeks, rabbits were sacrificed and the samples collected for analysis.

2.4. Surgical Procedure

The surgical procedures were followed in detail based on previous publications (16). The surgeries were performed under general anesthesia induced with inhalation of 2.5% isoflurane and subcutaneous injection of alfaxan (5mg/kg) and medetomidine (0.25mg/kg). The surgical sites were shaved and disinfected with povidone iodine, and infiltration anesthesia was performed using 2% lidocaine with 1:80,000 epinephrine. The incision was made along the midline of the rabbit calvarium and then a full-thickness flap was elevated. Four circular bone defects were formed using a trephine bur with a diameter of 6mm under saline irrigation without damaging the underlying dura mater or cerebral tissue. These defects were randomly allocated to the control group, GelMa group, CNp-GelMa group and CNp + GelMa group and filled according to the study design. Flap were carefully repositioned and sutured with absorbable 4 – 0 suture material (4 – 0 Vicryl, Ethicon, Somerville, NJ, USA).

2.5. Micro-CT Analysis

All harvested specimens were fixed with 10% formalin for 7 days and scanned with a high-resolution micro-computed tomography system (Sky-Scan 1173, SkyScan, Kartuizersweg 3B 2550 Kontich, Belgium) at a pixel size of 13.93 μm (130 kV, 60 μA). The scanned data sets were processed in Digital Imaging and Communications In Medicine (DICOM) format, and reconstructed with three dimensional (3D) reconstruction software (Nrecon reconstruction program [Ver 1.7.0.4], Bruker-CT, Kontich, Belgium). The region of interest(ROI) was defined by (i) the lateral boundaries of defect sites formed by the trephine bur, (ii) the superiorly; periosteum, (iii) inferiorly; dura mater. The measurements were performed by using an CT analyzing software (CTan (version;1.17.7.2; Bruker micro-CT)). Within the region of interest, the following volumes were measured.

- Total augmented volume (TAV; mm^3): Total augmented volume of the ROI, including mineralized tissue, graft material, and fibrovascular connective tissue within the ROI.
- New bone volume (NBV; mm^3): Sum of volumetric measurements of newly-formed bone within the ROI.

2.6. Histologic and histomorphometric Analysis

The block samples were decalcified in 5% formic acid for 14 days and embedded in paraffin. Then, 5- μm -thick sections were cut along the middle of each circular defect. Each tissue slide was stained with Hematoxylin-eosin and Masson trichrome staining. The slide images were captured by a digital slide

scanner (Panoramic 250 Flash III, 3D HISTECH, Budapest, Hungary). The histomorphometric measurements of defects were performed with a computer-aided slide image analysis program (CaseViewer 2.1, RTM_v2.1.2.69595) and a computer software (Photoshop® CS6; Adobe System, San José, CA, USA). The region of interest (ROI) was defined by (i) the lateral boundaries of defect sites formed by the trephine bur, (ii) the superiorly; periosteum, (iii) inferiorly; dura mater. Within the ROI, the following areas were measured.

- Total augmented area (TAA; mm²): the overall area of new bone, mineralized tissue, graft material, and fibrovascular connective tissue within the ROI.
- New bone area (NBA; mm²): Sum of areas of newly-formed bone within the ROI.

2.7. Statistical Analysis

The statistical analysis was performed by using a software program (IBM SPSS statistics 26.0; SPSS Chicago, IL, USA). Total augmented volume, New bone volume measurements from micro-CT and Total augmented area, New bone area measurements from histomorphometric analysis were summarized by median, mean values and standard deviations. The significance of differences between groups with different materials was determined by the Kruskal-Wallis test followed by the Bonferroni test. The Kruskal-Wallis test was performed for comparisons between different materials within each healing period, and the Mann-Whitney test was used to evaluate the data between each experimental group. Statistical significance was considered at the $P < 0.05$ level.

3. Results

3.1. Clinical observations

The wound healing was uneventful. None of the sites showed any signs of material exposure or infection throughout the entire study period.

3.2. Micro-CT Analysis

The results from the statistical analysis are presented in Table 2 and Fig. 3. All 8-week groups had significantly increased in NBV compared to each of their corresponding 2-week and 4-week groups except the GelMa group. The GelMa and CNp-GelMa groups at 8 weeks showed an increase in NBV compared to the corresponding groups at 2 and 4 weeks. At 8 weeks, highest NBV was found in the CNp-GelMa group ($15.34 \pm 3.12 \text{ mm}^3$). At both 2 and 8 weeks, the GelMa, CNp-GelMa and CNp + GelMa group showed greater NBV compared the control group. At 4 weeks, the CNp-GelMa and CNp + GelMa group showed greater NBV compared the control group. However, there were no significant differences in NBV and TAV in experimental groups compared to the control group between 2, 4 and 8 weeks.

3.3. Histomorphometric Analysis

The results from the histomorphometric analysis are summarized in Table 3 and Fig. 7. At 2 weeks, the CNp-GelMa ($4.93 \pm 1.89 \text{ mm}^2$, $p = 0.029$) and CNp + GelMa groups ($5.05 \pm 1.11 \text{ mm}^2$, $p = 0.029$) showed significantly higher TAA compared to the control group ($2.35 \pm 0.80 \text{ mm}^2$), but there was no significant difference between the GelMa group ($4.00 \pm 1.03 \text{ mm}^2$, $p = 0.057$) and the control group. In measurements for NBA, there were no significant differences compared to the control group. At 4 weeks, the CNp-GelMa group exhibited the highest TAA ($6.18 \pm 1.94 \text{ mm}^2$) and the CNp + GelMa group had the highest NBA ($2.68 \pm 0.75 \text{ mm}^2$), however, the differences were not significant. At 8 weeks, all experimental groups showed higher TAA and NBA compared to the control group but there were no significant differences between groups.

3.4. Histologic observations

Control group

At 2 weeks, little or none of new bone formation was detected at the margin of the defect. Fibrous tissue and brain tissue invaded into the defect area. Inflammatory cells were observed around the margin of the defect (Fig. 4A). At 4 and 8 weeks, new bone formation could be found from margin of circular defect to the center.

GelMa group

At 2 weeks, GelMa hydrogel was mostly resorbed. Some residual materials remained in a few of the samples. There were no inflammatory cells surrounding the residual materials (Fig. 4B). The defect areas were filled with loose connective tissue. Until 4 weeks, woven bone formation originated from the margins of the defect. At 8 weeks, immature bone tissues over the defects were visible which was similar to the natural bone formation in the control group.

CNp-GelMa group

At 2 weeks, most of CNp-GelMa hydrogel had been resorbed, but small amounts of residual material were observed. Inflammatory reaction to the remaining materials appeared minimal (Fig. 4C). In the high-magnification view, osteoblasts could be observed around the newly formed bone. At 4 weeks, CNp-GelMa hydrogel was completely degraded (Fig. 5C). At 8 weeks, almost complete closure of the defects was achieved.

CNp + GelMa group

At 2 weeks, residual materials could be detected, and infiltration of inflammatory cells were scarcely observed around the residual material. At 4 weeks, residual materials were completely resorbed. New bone formation originated from the native bone at the margin of defect was observed (Fig. 5D). At 8 weeks, the defect area was still not fully closed by bone bridge (Fig. 6D).

4. Discussion

Hydrogels have shown potential as a scaffold for tissue engineering due to its physical characteristic that mimics the extracellular matrix. For bone regeneration, however, it has been typically lacking in stability and space maintenance. The current crosslinked GelMa hydrogel has been developed in combination with nanoceramic particles to enhance these drawbacks. In this study, we evaluated the efficacy of GelMa hydrogels for bone regeneration and compared different methods of fabrication in combination with nanoparticles. The main findings revealed that, (i) in terms of NBV and TAV, greatest bone regeneration was achieved by the CNp-GelMa group at 8 weeks; (ii) GelMa hydrogel combined with nanoparticles were able to maintain space inside the defect up to 2 weeks; and (iii) the GelMa hydrogel exhibited excellent biocompatibility with low immune response.

In this study, the greatest new bone formation was achieved by the CNp-GelMa group. Although there was no statistically significant difference, a close inspection of the histologic representations revealed that there were greater number of sites with complete defect closure in the CNp-GelMa group compared to the other experimental groups. The addition of inorganic nanoparticles to the GelMa hydrogel has been shown to increase the expression of osteogenic genes such as bone sialoprotein, osteocalcin and runt-related transcription factor 2 (13). Furthermore, the nanoparticles were shown to produce other beneficial effects including reinforced structural stability, improved cell proliferation, stimulation of extracellular matrix production, and enhancement of cell-to-cell communication (17). The various nano-biomaterials can be classified as ceramics, polymers, carbon, and noble metals. Nanoparticles that we used in this study are calcium phosphate-based materials, which are widely utilized in skeletal tissue engineering as they are the major composition of ECM for strengthening bone (18). Although the specific mechanisms are not clear, it is presumed that the nanoparticles added to GelMa hydrogel can promote new bone formation by mediating cell signaling related to osteoinduction.

The GelMa hydrogel containing nanoparticles was able to maintain space within the defect up to 2 weeks in this study. This was confirmed by the histomorphometric analysis, in which CNp-GelMa and CNp + GelMa groups exhibited significantly greater augmented volume compared to the control group. Notably, GelMa group without the nanoparticles showed no significant difference compared to the control group, which could imply that greater stability might be achieved when GelMa hydrogel is combined with the nanoparticles. Previous studies have shown rapid degradation characteristics of hydrogel derived materials. GelMa hydrogel was completely resorbed by collagenase after 14 days, in vitro (19), and polyethylene glycol (PEG) hydrogel was degraded within 10 days in vitro (20). Rapid resorption of hydrogel was owed to the enzyme-sensitive sequences within its molecular chains which makes it susceptible to degradation by matrix metalloproteinases (MMPs). The results of this study demonstrated that nanoparticles within the crosslinked GelMa hydrogel might behave as a potential stabilizing agent to slow down enzymatic degradation. Nevertheless, the period of stabilization achieved by the addition of nanoparticles in this study was insufficient. In general, the scaffold material should provide space within the defect for at least two months during which early bone formation occurs (21). In large-sized bone defects in the oral cavity requiring extensive bone regeneration, considerably longer healing periods are

indicated, which can vary between six to nine months depending on the defect size (22). In this respect, the mechanical stability and durability of GelMa hydrogel need to be improved. A previous study has shown that the degradation rate of GelMa could be slowed down by increasing the crosslinking density of the methacryloyl group (23). The mechanical properties are affected by the different types and dose of additives, concentration of GelMa as well as the degree of substitution and UV dose (24). The current outcomes indicate that such measures need to be taken further in future studies to improve the stability of GelMa.

In this study, two different manufacturing methods were used to prepare nanoparticles reinforced GelMa hydrogel. The CNp-GelMa was prepared by one pot synthesise technique, which means GelMa and the nanoparticles were chemically crosslinked, whereas the CNp + GelMa was simply a mixture of the nanoparticles and GelMa that have been separately crosslinked. According to previous studies, the chemical crosslinking of nanoparticles to GelMa hydrogel enhanced the mechanical stiffness and resistance to enzymatic degradation (14, 25). Histologically, remnants of CNp-GelMa and CNp + GelMa were observed at 2 weeks, but they were not maintained at 4 weeks. Therefore, these materials need to be improved in terms of stiffness and stability, and further study should be performed to find the optimal crosslinking method.

In this study, minimal inflammatory reactions were observed in the experimental groups containing GelMa and the nanoparticles, which indicates that GelMa has good biocompatibility. It has been shown in previous studies that such good biocompatibility is exhibited by hydrogels in general (19). Biocompatibility is an essential characteristic of any biomaterial and is one of the main reasons why GelMa hydrogel has been considered for bone tissue engineering.

Another advantage of GelMa hydrogel is that it can be produced by three-dimensional bioprinting. Since the chemical, physical and mechanical characteristics of GelMa hydrogel can be easily modified by manipulating its components, it is feasible to fabricate cell-laden materials to mimic the structure of native tissues. Also, during the printing process, construction of hybrid hydrogels can be conjugated with various nanofillers suitable for the target tissue (4, 26). In that respect, GelMa hydrogel is a promising material for expanding spectrum of applications in many other biomedicines.

The critical sized circular defect in the rabbit calvarial defect model has been shown to be 8 mm in diameter (16). However, for the present study, the diameter of the defects was 6 mm. Although 6 mm is smaller than the critical size indicated in the literature, a smaller dimension has been recommended to evaluate shorter healing period (27). In addition, the healing period of 2 and 4 weeks were chosen to observe the early healing response while a healing period of 8 weeks was chosen to assess the late healing at which the extent of defect regeneration can be observed (27).

The limitation in this study was the small sample size in the 8-week group. Since the hydrogel has been known to have short duration when applied in vivo, greater number of animals were allocated to the 2- and 4-week groups to observe the degradation kinetics in the short term.

5. Conclusion

In summary, photo-crosslinked GelMa hydrogel with nanoparticles could maintain space within a bone defect up to 2 weeks. Further studies should aim to enhance the mechanical stability and resistance to enzymatic degradation prior to application in bone regenerative procedures.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

S.-H.C. conceived the ideas, and reviewed/revised the manuscript; D.-N.L. and J.-Y.P. collected and analyzed the data and drafted the manuscript; D.-N.L., Y.-W.S. and X.J. performed the animal experiments; and J.-M.H., A. B. and I.-S.N. manufactured the materials; S.-H.C., J.-Y.P. and I.-S.N. critically reviewed and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Tables

Table 1 to 3 is available in the Supplemental Files section.

Figures

Figure 1

Clinical photograph of samples

Figure 2

Experimental design in the rabbit calvarium.

Figure 3

3-dimensional reconstructed micro-computed tomography images of rabbit calvaria at 2, 4 and 8 weeks of experimental groups

Figure 4

Histological view of group at 2 weeks

Figure 5

Histological view of group at 4 weeks

Figure 6

Histological view of group at 8 weeks

Total augmented area

Figure 7

In histomorphometric measurements, the total augmented area of CNp-GelMa ($4.93 \pm 1.89 \text{ mm}^2$) and CNp+GelMa ($5.05 \pm 1.11 \text{ mm}^2$) group showed a significant difference compared to the control group at 2 weeks ($p=0.029$, $p=0.029$ each)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Table01.png](#)
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