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Gelatin Templated Polypeptide Co-Cross-Linked Hydrogel for Bone Regeneration

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Abstract

Polypeptides with short chains of amino acid monomers have been widely applied in the clinic because of their various biological functions. However, the easily-inactivated characteristics and burst releasing of the peptides limit their application in vivo. Here, a novel osteogenic polypeptide hydrogel (GelMA-c-OGP) is created by co-cross-linking template photo-cross-linked gelatin (GelMA) with photo-cross-linkable osteogenic growth peptides (OGP) using ultraviolet radiation. GelMA enables the formation of hydrogel with photo-cross-linkable OGP with good mechanical properties and also promotes bone regeneration. GelMA-c-OGP hydrogel accelerates the bone formation procedure of osteogenic precursor cells by significantly enhancing the expression of osteogenic-related genes BMP-2, OCN, and OPN, and increasing the precipitation of calcium salts in osteoblasts. Similarly, GelMA-c-OGP hydrogel promotes bone regeneration in vivo. Furthermore, it is observed that more collagen fibers connect cortical bones in the GelMA-c-OGP implanted group than the control group by hematoxylin-eosin and immunohistochemical staining of Collagen I and TGF-β. The co-cross-linked OGP polypeptide converts from liquid to solid hydrogel with transient UV light in situ, which also can strengthen the mechanical property of the defect bone and avoid burst osteogenic peptide, releasing during the bone defect healing period. Overall, this hydrogel delivering system has a significant impact on bone defect healing compared with traditional methods.

Introduction

Bioactive compound combining with scaffold materials have been widely used for bone regeneration with different application method. Physical loading or chemical conjugating of bioactive compound to scaffold are the most common ways. How-ever, these methods are not ideal ways for bioactive molecules function. They may lead to fast release or inactivation of the bioactive molecules.[1]

Osteogenic growth peptide (OGP) is a native molecule containing a highly con-served 14-amino acid motif. The C-terminal pentapeptide (Tyr-Gly-Phe-Gly-Gly) of OGP is the physiological active form of OGP after it is proteolytically cleaved. OGP can improve the proliferation and differentiation ability of osteoblasts in vitro,[2] and it can also accelerate the bone formation and enhance trabecular bone density in vivo.[3–5] In addition, OGP has a stable active structure that can endure high temperature and organic solvents. Thus, OGP has the potential to become part of the bioactive scaffold fabrication. Currently, OGP is mainly delivered by physical adsorption or self-assembling.

Zhao et al. grafted OGP to a biological scaffold, which promoted the osteo-genesis of osteogenic cells and accelerated the formation of bone defect.[6] However, OGP is sensitive to the surrounding medium and prone to be biodegraded when adsorbed on the surface of the stent.[7] Wei et al. developed a dual-layer mate-rial with NF and hydrogels in situ cross-linking GelMA. After cultivation of dermal fibroblasts and keratinocytes on the mate-rial, GelMA was coated with human dermal fibroblasts (HDF) by self-assembly. They demonstrated this self-assembly material has high expression of pan-keratin and showed high proliferation of dermal cells.[8] However, self-assembly is a noncovalent reaction that is weaker and fragile to surrounding environment compared to covalent cross-linking.[9,10] In addition, the grafted polypeptides have a short half-life time. Therefore, maintaining long-term effective biological activity of OGP is the primary objective in its application. To functionalize the scaffold material, the polypeptide modification usually utilizes the amino-terminus group or carboxyl-terminus group to chemically react with the scaffold material.[11] However, the active site (usually the amino or carboxyl group) of the polypeptide may also be involved in the modification process, which results in a decreasing in the biological function of scaffold materials.[12,13] Therefore, it is necessary to introduce a specific reactive group, such as polymerizable C=C or clickable C≡C, to minimize the activity loss during polypeptide modification.

Gelatin methacryloyl (GelMA) is an ideal choice for polymerizable C=C or clickable C≡C with OGP. GelMA has been widely used in various biomedical applications. The most relevant application fields of GelMA are bone and blood vessel regeneration. Good biocompatibility, biodegradable ability, strong hydrophilicity, high side chain reactivity, stable physical and chemical properties are major advantages of GelMA.[14–16] Therefore, GelMA is widely used as a solid phase carrier in tissue engineering scaffold materials.[17] In addition, gelatin has an important arginine-glycine-aspartate (RGD) sequence, which promotes cell adhesion,[18] differentiation[19] and proliferation.[20] There are several ways to modify gelatin, including patterning,[21] self-assembly[22] or microfluidic techniques.[23] By using microfluidic technology, Yang et al. prepared a multi-cellular vascular channel using a 3D printing template of GelMA hydrogel for studying cell invasion and vascular drug release.[24] However, the use of gelatin hydrogels in bone engineering is less studied, because gelatin lacks osteogenic factors or other ions that can promote mineralization of bones.

In our case, we synthesized a new photo-cross-linkable OGP. By using a photo-cross-linkable GelMA as a template, we constructed a co-photo-cross-linked GelMA-c-OGP hydrogel that can prolong the releasing time of OGP. This compound hydrogel can maintain osteogenic activity of OGP for a much longer time and extend its releasing time after slowly degradation. GelMA-c-OGP hydrogels provide an ideal microenvironment for cell adhesion, proliferation and osteo-blast differentiation. In vivo, the

co-cross-linked delivery system can sustain the release of OGP peptides and promote bone formation with bioactive RGD groups of GelMA synergistically, making GelMA-c-OGP a promising alternative for bone repair and regeneration.

Result and Discussion

In this experiment, we synthesized the photo-cross-linkable OGP peptide (Methylacrylamide-GGGGG-YGFYY) using a solid phase peptide synthesis strategy. The OGP were first characterized with electrospray ionization mass spectrometry (ESI-MS). As shown in Figure S1 (Supporting Information), the monoisotopic ion peak [M-H]− of the OGP peptide was detected at m/z 851.7, which is in good agreement with calculated mass of 852.88 Da. High performance liquid chromatography result showed that the purity was higher than 96% (Figure S2, Supporting Information).

The synthesized photo-cross-linkable OGP and GelMA were dissolved in an aqueous solution (Figure 1a) to prepare a mixed solution of GelMA and OGP. After irradiation for 30 s by ultra-violet light (UV), we constructed hydrogels (Figure 1b). In the GelMA-c-OGP hydrogel, both OGP-grafted photo-cross-linking and gelatin photo-cross-linking groups are formed by self-cross-linking and co-cross-linking (Figure 1b).

The mixed solution of GelMA and OGP had a good fluidity and was injectable. In liquid state, it can be directly administrated to the bone defect site and adhered to the damage interface by local injection and then form an osteogenic poly-peptide hydrogel with a certain elastic modulus by the local rapid UV irradiation (Figure 1g). As expected, we made a hydrogel scaffolds with various shapes through different molds (Figure 1c). From the Fourier transform infrared spectroscopy (FTIR) results, we found out that the FTIR spectrum of GelMA-c-OGP is similar to GelMA (Figure S3, Supporting Information).

Tissue engineering scaffolds require large and highly con-nected aperture, which can contribute to the growth of cells and circulation of nutrients.[25] After freeze-dried, we analyzed the structures of GelMA and GelMA-c-OGP. GelMA and GelMA-c-OGP had a porous-connected structure, and the appearance of the pores was irregular and mostly long fusiform. The internal connectivity of the pores was observed (Figure 1d). To detect the degradation of the hydrogels, we immersed saturated GelMA and GelMA-c-OGP hydrogel into PBS and measured the remaining mass at different time points. The degradation rates of the GelMA and GelMA-c-OGP hydrogel were almost the same (Figure 1e). It has been well reported that the scaffolds need to match mechanical properties with the host tissue.[26] Excessive strength and stiffness of the scaffold will damage the surrounding bone tissue and the

stability of the bone-to-implant interface. On the contrary, too low strength scaffolds cannot provide the conditions for bone repair.[27] To investigate the mechanical properties of GelMA-c-OGP hydrogel, we tested the mechanical properties, as shown in Figure 1f. The compression modulus of GelMA-c-OGP was even higher (92.69 ± 0.4 kPa) than GelMA (74.5 ± 0.7 kPa) (Figure 1f). We also examined the swelling ratio of the GelMA and GelMA-c-OGP, which indicated the capacity to absorb water. On the contrary, the swelling ratio was a little bit higher in GelMA than in GelMA-c-OGP (Figure S4, Supporting Information), which suggests that double cross-linking in GelMA-c-OGP hydrogel may lead to a tighter internal structure and increased mechanical strength, thus had better compression modulus and better mechanical properties than GelMA.

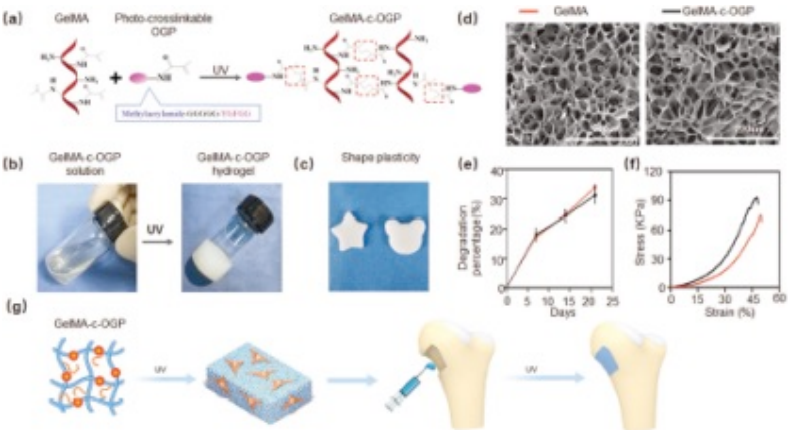

Figure 1. Flow chart of GelMA-c-OGP hydrogel construction and its mechanical properties. a) The chemical molecular structure of the methacrylated OGP polypeptide and gelatin (GelMA), and the structure of GelMA-c-OGP with ultraviolet (UV) light. b) Photograph of GelMA-c-OGP solution and GelMA-c-OGP hydrogel formation after UV light. c) A fixed-shaped gel block after photo-cross-linking of GelMA-c-OGP hydrogel. d) SEM images of GelMA and GelMA-c-OGP hydrogel. e) Degradation percentage of GelMA and GelMA-c-OGP hydrogel (n = 3). f) Stress of GelMA and GelMA-c-OGP hydrogel (n = 3). g) The pipeline of GelMA-c-OGP applied for bone regeneration.

Since OGP has been proved to promote osteoblastic osteogenesis,[28] we first investigated the dose of the OGP in GelMA-c-OGP hydrogel. Alkaline phosphatase (ALP) can hydrolyze organic phosphorus, increase local PO_4^{3-} concentration and promote Ca^{2+} deposition, and then regulate the process of bone mineralization.[29] We generated GelMA-c-OGP hydrogel, which contained different dose of OGP (10, 20, and 40 μg), and found the conditional medium from GelMA-c-OGP hydrogel contained 20 μg OGP had the best effects on increasing the mouse osteogenic precursor cells MC3T3-E1 cells' ALP activity (Figure S5, Supporting Information). Thus, we next have chosen the 20 μg OGP dose for further experiments.

In order to detect the amount of OGP in the hydrogel material, we first evaluated the cumulative release protein in leachate of the GelMA/OGP and GelMA-c-OGP hydrogel, using a bicinchoninic acid (BCA) assay. We found out that the total released protein level was higher in the GelMA/OGP group compared with the GelMA-c-OGP group for each time point. The amount of the released protein gradually increased in GelMA/OGP group, while it did not change significantly in the GelMA-c-OGP group over time (Figure S6, Supporting Information). This is probably due to the large amount of gelatin in GelMA, which prevents the distinction of the release of OGP in the BCA protein assay. However, the data suggests that the protein release in GelMA-c-OGP is slower than GelMA.

Then, we evaluated the amount of released OGP, using indirect functional experiments. We cultured the MC3T3-E1 cells in conditional medium from GelMA/OGP or GelMA-c-OGP hydrogels to confirm whether the leachate of the two different hydrogels retained the osteogenesis efficacy, and set the osteogenic culture medium as control. From the ALP activity assay, two conditional media from the hydrogel had better osteogenic properties than the control group, but the GelMA-c-OGP hydrogel group had better osteogenic properties compared to the GelMA/OGP hydrogel group (Figure S7, Supporting Information). Altogether, these results demonstrate that the co-cross-linking GelMA-c-OGP hydrogel is stable and has better osteogenic properties after 7 d of degradation compared to the GelMA/OGP hydrogel.

Cell adhesion, growth status, and proliferation rate are the main parameters for evaluating the biological properties of scaffold materials. We cultured MC3T3-E1 cells on the GelMA, GelMA/OGP, and GelMA-c-OGP hydrogel. After 24 h incubation, there had no significant difference in the number of dead cells (in red) among three groups, and most of the cells were alive (in green), measured by a live/dead cell assay (Figure 2a,b). CCK-8 kit was used to quantify the effect on cell growth at different time. The results showed that the OD values of each group increased as the cell culture time prolonged. After 1, 3, 5, and 7 d culture, there were no statistically significant differences between the control, and GelMA, GelMA/OGP, and GelMA-c-OGP groups at the same time point, respectively. This suggests that the scaffold had no cytotoxicity after cross-linking and it did not inhibit cell proliferation. (Figure 2c). These results demonstrate that GelMA-c-OGP had a good biocompatibility similar to GelMA.

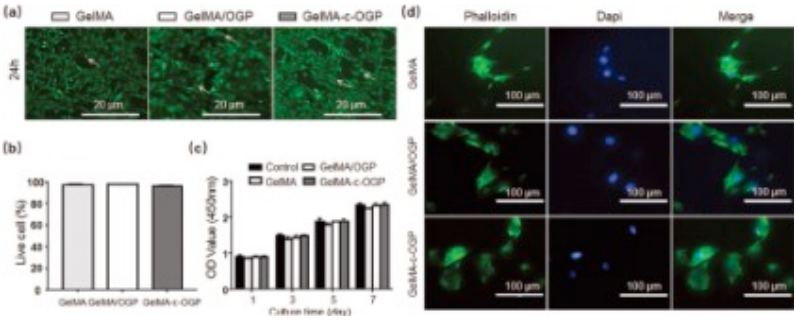

Figure 2. Biocompatibility of GelMA-c-OGP hydrogel detected by MC3T3-E1 cells. a) Live/dead staining of MC3T3-E1 cells on the three types of hydrogel (GelMA, GelMA/OGP and GelMA-c-OGP) after 24 h incubation, and b) the percentage of live cells (n = 5). c) Proliferation of MC3T3-E1 cultured directly on the different hydrogels detected by using the CCK-8 kit (n = 5) (control, cells cultured on the tissue culture plate). d) Microfilament skeleton of MC3T3-E1 cultured on the surface of the different hydrogels stained by Phalloidin staining.

GelMA contains RGD active motifs that can facilitate cell adhesion.[30] GelMA-c-OGP should have the same capability. In order to verify our hypothesis, we seeded MC3T3-E1 cells on the surface of the GelMA, GelMA/OGP, and GelMA-c-OGP hydrogels. Cell adhesion was detected by phalloidin staining after 24 h culture. Compared with the GelMA and GelMA/OGP groups, the cells in GelMA-c-OGP group adhered to the scaffolds and grew well. As observed under the fluorescence microscope, the pseudopod stretched longer, the cells were bright, the structure was uniform, and the nuclei of the cells were smooth and clear (Figure 2d, Figure S8, Supporting Information).

To further determine the osteogenicity effect of GelMA-c-OGP, we cultured the MC3T3-E1 cells in the medium contained GelMA/OGP or GelMA-c-OGP leachate (collected on d5) and set the leachate from GelMA as Control. 7 d after osteo-induc-tion, we examined the expression and enzyme activity of ALP by the ALP staining and ALP activity assay. There were more ALP positive cells in GelMA-c-OGP group, and the staining color of the cells were deeper compared with the other two groups (Figure 3a,b). The ALP activity also significantly increased in the GelMA-c-OGP group (477.71 ± 17.69 U g−1 protein) com-pared with the GelMA/OGP group (337.92 ± 23.7 U g−1 protein) and the GelMA group (262.36 ± 18.11 U g−1 protein) ($p < 0.05$, Figure 3d).

We also observed the formation of calcified nodules in each group by alizarin red staining. There were more reddish-brown calcium nodules in the GelMA-c-OGP group after 21 d of culture (Figure 3c). The quantitative results of alizarin red also demonstrated that GelMA-c-OGP group had a better osteogenic effect compared with the other two groups ($p < 0.05$, Figure 3e), which further verified that GelMA-c-OGP had a durable osteo-inductive capacity.

We also detected the expression of osteogenic related genes, such as runt-related transcription factor 2 (Runx2), bone morphogenic protein (BMP-2), osteocalcin (OCN), and osteopontin (OPN) in MC3T3-E1 cells cultured directly on the GelMA, GelMA/OGP, or GelMA-c-OGP hydrogel by qRT-polymerase chain reaction (PCR) (real-time fluorescent quantitative PCR). Compared with the cells of the GelMA group, the expression levels of BMP-2, OCN and OPN were significantly increased in the cells of GelMA/OGP and GelMA-c-OGP group (Figure 3f–h). Normalized to the gene expression of GelMA group, the gene expression level of BMP-2 was 2.56 ± 0.56 fold, OCN was 1.54 ± 0.22 fold, and OPN was 4.13 ± 0.78 folds in the GelMA/OGP group. Compared with the GelMA group, the expression level of BMP-2 was increased by 15.21 ± 1.56 fold, OCN by 5.74 ± 0.67 fold, and OPN by 10.89 ± 1.89 fold in the GelMA-c-OGP group. OPN is a secreted glycosylated phosphoprotein that serves as an early marker of osteoblast differentiation, and has a more wide-spread role in the regulation of bone growth and mineralization. However, the expression of Runx2 showed no statistical significant differences between these three groups, probably because that the Runx2 is an early and master transcription factor for osteogenesis, which may increases to the high level after 7 d of osteogenic induction cell culture in the GelMA group (data not shown).[31] The gene expression level of osteo-related proteins further demonstrated that GelMA-c-OGP had the efficiency and capability of promoting osteogenic differentiation. It was demonstrated that the OGP and GelMA retained their biological activity after cross-linking, and the GelMA-c-OGP promoted osteogenesis of osteoblast precursor cells.

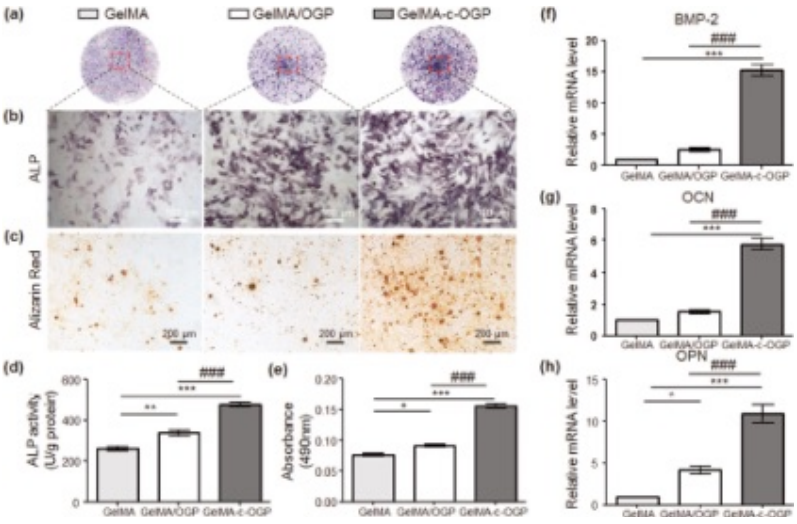

Figure 3. The osteogenesis properties of GelMA-c-OGP hydrogel in vitro. MC3T3-E1 cells were cultured with the GelMA, GelMA/OGP, or GelMA-c-OGP hydrogel. a) General view of ALP staining after 7 d of osteo-induction. b) ALP staining images under microscope. c) Alizarin red staining images after 21 d of osteo-induction. d) Quantitative results of ALP staining (n = 5). e)

Quantitative results of Alizarin red staining (n = 5). f−h) Osteogenic related genes expression of cells, which were cultured on different hydrogel were examined, including BMP-2, OCN and OPN (n = 5). *p < 0.05, **p < 0.01, ***p < 0.001 compared with GelMA group, ###p < 0.001 compared with the GelMA/OGP group.

OGP can enhance the bridging of the fracture space and modulates callus structural and mechanical properties, thereby promote the new bone formation of male rabbits after fracture.[32] To explore the co-cross-linking effect of photo-cross-linkable OGP on osteogenesis, SD rats were subjected to a bone defect surgery at the distal femur and then were implanted with different group of hydrogels into the defect site. The implanted rats were scanned imageologically 8 weeks post-surgery. X-ray displayed that in all three groups, the callus were filled in the bone defect area. Furthermore, compared to the GelMA and GelMA/OGP group, the density of the defect site in the GelMA-c-OGP group was higher and the cortical bone was more continuous (Figure 4a).

From the 3D reconstruction of the surface of the distal femur defect by micro computed tomography (μ-CT), the GelMA-c-OGP group had more bone mass than the ones in the GelMA and GelMA/OGP group. For the GelMA-c-OGP group, the bone defect was almost invisible and the cortical bone was continuous. On the contrary, the bone defects and discontinuous cortical bone in the GelMA and GelMA/OGP group were only partially visible (Figure 4b). We further quantified the new bone formation in the defect area. The bone mineral density (BMD) was statistically higher in GelMA-c-OGP group (0.66 ± 0.03 g cc−1) than in the GelMA/OGP (0.54 ± 0.02 g cc−1) and GelMA groups (0.49 ± 0.03 g cc−1) ($p < 0.05$, Figure 4d). The bone tissue volume per tissue volume rate (BV/TV) was the highest in the GelMA-c-OGP group (50.48 ± 4.45%) com-pared with the control, GelMA/OGP (41.03 ± 3.97%) and GelMA groups (31.65 ± 4.47%), with significant statistically differences (GelMA vs GelMA-c-OGP, $p < 0.001$; GelMA/OGP vs GelMA-c-OGP, $p < 0.001$) (Figure 4c). The image analyses demonstrate that GelMA-c-OGP can repair the bone defect more effectively.

In accordance with the μ-CT results, hematoxylin and eosin (H&E) staining evidenced that the lateral cortical bone was discontinuous in control GelMA group (Figure 4f), and rare trabecular bone structures were seen. Bone defects were basically repaired in the GelMA/OGP group. In the GelMA-c-OGP group, the bone defect was completely regenerated, and there were many new trabecular bone formation in the defect site. The osteoid was evenly distributed and surrounded with large number of osteoblasts. Corresponding to the H&E staining results, Masson staining also displayed that bone defect area was filled with new bone, and there were more collagen fibers in the GelMA-c-OGP group (Figure 4e).

Transforming growth factor beta (TGF-β) is a secreted pro-tein that plays an important role in embryonic development, wound healing, matrix formation, bone formation and regen-eration. We found that the expression of TGF-β was high in GelMA-c-OGP group by immunohistochemical staining (Figure 4h). Type I collagen (col-I) is an important indicator of the formation of bone mechanical strength. The more col-lagen fibers are arranged in order, the stronger strength of newly formed bone. Therefore, we detected the levels of Col-I to clarify the osteogenic ability of the hydrogels. As expected, the expression of Col-I was also higher in the GelMA-c-OGP group than in the other two groups (Figure 4g). The hydrogel's degradation-mediated cellular traction affects the migration and differentiation of human mesenchymal stem cells.[33] Double cross-linking in the GelMA-c-OGP hydrogel may further affect cell behavior. However, GelMA-c-OGP hydrogel had better effects on bone defects repairing when compared with the GelMA/OGP hydrogel from the in vivo experiments.

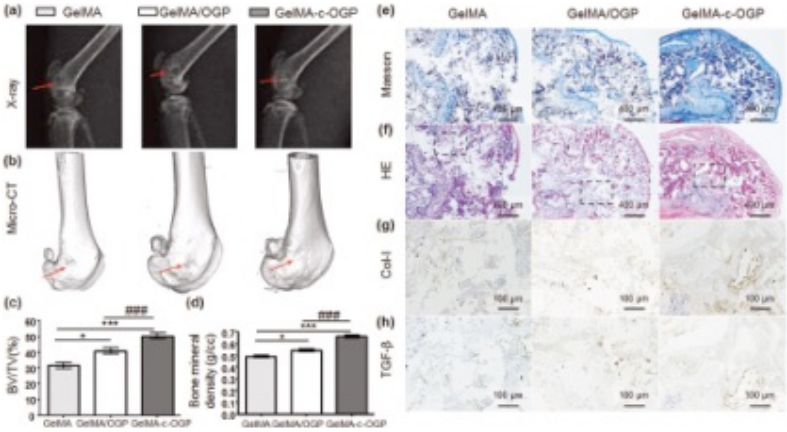

Figure 4. The osteogenesis properties of GelMA-c-OGP hydrogel in vivo. SD rats were subjected to the distal femoral defect surgery, implanted with the GelMA, GelMA/OGP, and GelMA-c-OGP hydrogel, and irradiated by UV light. a) X ray of distal femoral defected after 8 weeks of treatment. b) 3D reconstruction image after μ-CT scanning of distal femoral defected area. c) BMD and d) BV/TV of distal femoral defected site quantified by μ-CT. e–h) Histo-morphological examination of bone defect region with different treatment. e) Masson's trichrome staining (25×), f) H&E staining 25×), g) immunohistochemical staining of Col-I (100×), and h) TGF-β (100×). The results are shown as mean ± SD (n = 10/group). *$p < 0.05$, ***$p < 0.001$ compared with GelMA group, ###$p < 0.001$ compared with the GelMA/OGP group.

Overall, we synthesized a photo-cross-linkable OGP peptide, and then generated the GelMA-c-OGP by co-cross-linking OGP with GelMA. The GelMA-c-OGP hydrogel can be solidified in situ by co-cross-linking under UV light. The mechanical compression capability of GelMA-c-OGP reaching 90Kpa, and is better than GelMA. GelMA-c-OGP can degrade, which is beneficial for the release of

OGP. In situ photo-cross-linking GelMA-c-OGP hydrogels can promote cell adhesion and have good biocompatibility, which is supported by the enhanced osteogenesis in mouse MC3T3-E1 cells. In vitro, GelMA-c-OGP increased the proliferation and osteogenic differentiation of mouse MC3T3-E1 cells and enhanced the expression of osteogenic related genes. It can also promote the repairment of rat distal femur defects in vivo. Further-more, GelMA-c-OGP hydrogel scaffold promoted the differentiation of osteoblast in vitro, increasing the formation of new bone and repaired bone defects in vivo. Both in vitro and in vivo experiment show that the material is beneficial for cell adhesion, good biocompatibility, enhance the expressions of osteogenic-related genes and accelerate bone regeneration. Therefore, the GelMA-c-OGP hydrogel provides a potential carrier for the clinical application of arthrodesis, bone defect, and bone regeneration.

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