Published in final edited form as:

Part Part Syst Charact. 2020 October ; 37(10): . doi:10.1002/ppsc.202000180.

Development of PLGA-PEG-COOH and gelatin-based microparticles dual delivery system and E-beam sterilization effects for controlled release of BMP-2 and IGF-1

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Abstract

The purpose of this study was to develop a PLGA-PEG-COOH- and gelatin-based microparticles (MPs) dual delivery system for release of BMP-2 and IGF-1. We made and characterized the delivery system based on its morphology, loading capacity, Encapsulation efficiency and release kinetics. Second, we examined the effects of electron beam (EB) sterilization on BMP-2 and IGF-1 loaded MPs and their biological effects. Third, we evaluated the synergistic effect of a controlled dual release of BMP-2 and IGF-1 on osteogenesis of MSCs. Encapsulation efficiency of growth factors into gelatin and PLGA-PEG-COOH MPs are in the range of 64.78% to 76.11%. E-beam sterilized growth factor delivery systems were effective in significantly promoting osteogenesis of MSCs, although E-beam sterilization decreased the bioactivity of growth factors in MPs by approximately 22%. BMP-2 release behavior from gelatin MPs/PEG hydrogel shows a faster release (52.7%) than that of IGF-1 from the PLGA-PEG-COOH MPs/PEG hydrogel (27.3%). The results demonstrate that the gelatin and PLGA-PEG-COOH MPs based delivery system could realize temporal release of therapeutic biomolecules by incorporating different growth factors into distinct microparticles. EB sterilization was an accessible method for sterilizing growth factors loaded carriers, which could pave the way for implementing growth factor delivery in clinical applications.

Graphical Abstract

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TOC

We develop and characterize PLGA-PEGCOOH- and gelatin-based microparticles dual delivery system that are capable of a broad range of delivery durations of biomolecules. E-beam sterilization is an effective and accessible method for sterilizing growth factors loaded carriers, which could pave the way for implementing growth factor delivery in clinical applications.

Keywords

Gelatin microparticles; PLGA-PEG-COOH microparticles; BMP-2; IGF-1; E-beam sterilization; dual delivery; osteogenesis of MSCs

INTRODUCTION

Therapeutic biomacromolecules such as growth factors have been used to enhance the regeneration of damaged tissues by stimulating cellular activities such as cell migration, proliferation, and differentiation [1]. However, they have short biological half-lives in physiological conditions due to rapid degradation and deactivation by enzymes and other chemical and physical reactions [2]. In addition, the course of tissue regeneration is complicated by the interactions of multiple factors [3]. There is a great need for drug delivery systems that allow for controlled release of multiple growth factors to enhance their therapeutic efficacy. Bone morphogenetic protein-2 (BMP-2) is one of the most important osteogenic factors, which plays a central role in the expression of osteogenic markers such as alkaline phosphatase (ALP) and osteocalcin [4]. Insulin-like growth factor-1 (IGF-1) is well known to promote cell proliferation and inhibit cell apoptosis, and it could stimulate osteoblast cells to respond with the synthesis of ECM proteins such as type-I collagen [5,6]. Studies showed that the effective concentration range of BMP-2 and IGF-1 for cell osteogenic differentiation were 10–100 ng/mL and 1–10 ng/mL [7–10]. Recent studies have

shown that the combined delivery of BMP-2 and IGF-1 enhances tissue regeneration compared to single growth factor delivery^[11]. Steven et al. investigated the use of biodegradable hydrogel composite scaffolds to deliver BMP-2 and IGF-1 for the repair of osteochondral tissue in a rabbit model^[12]. Their results suggested that the fast release of BMP-2 followed by a slow and sustained release of IGF-1 synergistically enhance the degree of subchondral bone formation. We have previously developed a chitosan gel/gelatin microsphere (MSs) dual delivery system for dual release of BMP-2 and IGF-1^[13]. This combinational delivery system provided an initial release of BMP-2 followed by a slow and sustained release of IGF-1. However, the dual delivery system has relatively short delivery duration, and the cumulative release amount of BMP-2 and IGF was about 70% (35 ng/mL) and 36% (18 ng/mL), which is insufficient for the long process of bone and tissue regeneration. In this study, the cumulative release amount of BMP-2 and IGF was 31% (46 ng/mL) and 12% (107 ng/mL) at day 7, which significantly extended release duration and enhanced early osteoblastic differentiation of mouse bone marrow stromal cells. We also developed the poly(L-lactide) (PPLA) MSs/poly(lactide-co-glycolide)(PLGA) composite scaffold for dual delivery of vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2) and platelet derived growth factor (PDGF)^[14]. Although the structural polymer scaffold could achieve the distinct release kinetics for multiple growth factors, the bioactivity of proteins was severely compromised due to their exposure to organic agents during double emulsion fabrication.

In the long term of translational research, terminal sterilization of the scaffolds is inevitable for clinical applications. Growth factors such as BMP will become denatured by many terminal sterilization methods, such as dry heat and autoclaving^[15,16]. The microparticles are too large to be sterile-filtered and the ethylene oxide sterilization is not appropriate due to the undesirable residues^[17]. The gamma irradiation assured enough high energy for a powerful penetration in the materials, with consequent inactivation of microorganisms by damage of their nucleic acids. However, this radiation can induce simultaneous chain scission and crosslinking of polymeric chains, affecting the drug release properties and consequently the biological efficacy^[18]. Electron beam (E-beam) is a nontoxic and low temperature ionizing radiation-based method, and can be used for sterilizing heat-sensitive scaffolds by using of high radiation dose in a short exposure time^[16,19]. Several commercially available ceramic-based bone scaffold products are sterilized with E-beam irradiation^[16,20]. Hence, E-beam sterilization might be an effective and accessible method for growth factors loaded delivery systems. To the best of our knowledge, this is the first study to systematically evaluate the effect of clinically available E-beam sterilization on growth factors loaded carriers, which could pave the way for implementing growth factor delivery in clinical applications.

In this study, the purpose was to develop and characterize a gelatin and poly(lactic-co-glycolic acid)-poly(ethylene glycol)-carboxyl (PLGA-PEG-COOH) based microparticles (MPs) delivery system to achieve a broad range of delivery durations of proteins. We hypothesized that the quickly degradable, protein-laden gelatin MPs would allow for faster release of proteins compared with the slowly degradable, protein-loaded PLGA-PEG-COOH MPs. To this end, we first characterized the delivery system based on its morphology, particle size, encapsulation efficiency, loading capacity, and release kinetics using a model

protein, BSA. Then BMP-2 and IGF-1 were loaded into gelatin MPs and PLGA-PEG-COOH MPs for further validation, respectively. Moreover, the feasibility of the E-beam irradiation as a possible sterilization procedure for these particles was evaluated. The effects of E-beam sterilization on growth factors loaded MPs and their biological effects on osteogenesis of human mesenchymal stem cells (MSCs) *in vitro* were studied. We hypothesized that E-beam sterilization would be an effective method for growth factors loaded particles delivery system. Finally, in order to achieve a controlled dual delivery of BMP-2 and IGF-1, we incorporated fast-degrading BMP-2-gelatin MPs and slow-degrading IGF-1- PLGA-PEG-COOH MPs into PEG hydrogel scaffolds for sustained release. We also investigated the effects of dual delivery of dual growth factors on MSC osteogenesis by measuring alkaline phosphatase (ALP) specific activity and mineralized activity.

EXPERIMENTAL SECTION

Materials.

Type-B gelatin (MW 40000), ethanol, glyoxal, sodium metabisulfite, MES buffer, N-Ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-Hydroxysulfosuccinimide sodium salt (NHS), Poly(ethylene glycol) (PEG) (MW 1000), Alizarin red S (ARS) and tris hydrochloride (Tris-HCl) buffer were purchased from Sigma-Aldrich (St Louis, MO). PLGA-PEG-COOH (50:50, PLGA Mw10kDa, PEG Mw2000) was purchased from Nano soft polymers. Phosphate buffered saline (PBS, 0.01 M), Dulbecco's Modified Eagle's Medium (DMEM) with 1.0 g/L glucose, fetal bovine serum (FBS), penicillin/streptomycin, trypsin/ethylenediaminetetra-acetate (trypsin/EDTA) were purchased from Fisher Scientific (Fair Lawn, NJ). BMP-2 and IGF-1 were purchased from Peprotech (Rocky Hill, NJ). ALP assay kit was purchased from BioAssay Systems (Hayward, CA). BCIP/NBT Color Development Substrate was purchased from Promega (Madison, WI). Human BMP-2 Elisa kit and Human IGF-1 Elisa kit were purchased from Raybiotech (RayBio, GA). MSCs were cultured as per American Type Culture Collection (ATCC) instructions.

Fabrication of Gelatin MPs and PLGA-PEG-COOH MPs.

Schematics of fabrication of gelatin and PLGA-PEG-COOH MPs and growth factor loading are shown in Figure 1. For the gelatin MPs, 200 mg of gelatin was dissolved in 20 ml of deionized distilled water. The pH of the resulting solution was adjusted to 7.00 with 0.2 M sodium hydroxide. MPs were formed by gradual displacement of the water with ethanol under controlled stirring conditions. The prepared gelatin MPs were crosslinked by glyoxal for 10 h. The unreacted aldehyde groups of glyoxal were quenched with sodium metabisulfite aqueous solution. MPs were separated using centrifugation at 12,000g for 90 min, washed twice, and lyophilized. For PLGA-PEG-COOH MPs, a mixture of 50 mg PLGA-PEG-COOH dissolved in 8 ml DMSO was loaded in a dialysis tube and dialyzed against DI water for 24 h, with a change of water every 2–4 h. After self-assembly, the MPs suspension was freeze-dried to obtain a free-flowing powder.

Loading of Growth Factors.

COOH⁻ of MPs was activated in MES buffer supplemented with EDC and NHS salt for 1 h at room temperature [21]. According to the effective concentration of BMP-2 and IGF-1 [7–10], 10 mg of the activated MPs were dialyzed against DI water at 4 °C overnight, added to the BMP-2 solution (300 ng/mL) and IGF-1 solution (1500 ng/mL) respectively, and allowed to react overnight at 4 °C. The growth factors grafted MPs were centrifuged and freeze-dried. The encapsulation efficiency and loading capacity were calculated by using the formulas as follows:

$$\text{Encapsulation efficiency} = \frac{\text{Total amount of drug added} - \text{Leftover drug in supernatant and wash fluid}}{\text{Total amount of drug added}}$$

$$\text{Loading capacity} = \frac{\text{Total amount of drug added} - \text{Leftover drug in supernatant and wash fluid}}{\text{Total amount of particle added}}$$

Characterization of BMP-2-Gelatin MPs and IGF-1-PLGA-PEG-COOH MPs.

The morphology of the gelatin MPs and PLGA-PEG-COOH MPs were observed using scanning electron microscopy (SEM) (JEOLT330A, JEOL, Ltd, Peabody, MA, USA). Particle size was measured by using a Zetasizer at a light-scattering angle of 90 at 25°C (Malvern, Nano ZS, USA). Encapsulation efficiency (EE) and Loading capacity (LC) of MPs were detected as follows. For the PLGA-PEG-COOH MPs, EE and LC were determined via extraction with methylene chloride. Freeze-dried loaded MPs were dissolved in 500 µL of methylene chloride, and 1 mL of PBS (pH 7.4) was then added. The mixture was vigorously shaken for 2 min to extract IGF-1 into PBS. After centrifuging at 4000g for 15 min, the aqueous solution was withdrawn and the IGF-1 content measured using an IGF-1 ELISA kit. For the gelatin MPs, the BMP-2 loaded gelatin MPs were immersed in PBS with a concentration of 1 mg/mL collagenase until MPs were dissolved. The tubes were centrifuged at 12,000g for 15 min. The supernatant was collected to analyze BMP-2 loaded in MPs via a BMP-2 ELISA kit.

Effects of E-Beam sterilization on BMP-2 and IGF-1 activity.

BMP-2-gelatin and IGF-1-PLGA-PEG-COOH MPs were sterilized by E-beam with the dosage of 25 kGy (ISO 11137). Effects of E-beam sterilization on encapsulation efficiency, loading capacity, and release were detected. After the release assay, microparticles were dried for 2 days in a vacuum desiccator and residual growth factors were extracted and analyzed. The biological effects of BMP-2 and IGF-1 from MPs after E-beam sterilization on osteogenesis of MSCs were tested by measuring ALP activity and mineralized activity. The ALP activity of cells was assessed through ALP quantitative assay (days 0, 4, 7 and 14), using ALP assay kit (BioAssay). The biomineralization of MSCs was measured by ARS staining (days 14 and 21). After staining, the samples were imaged, and the mineralization levels were quantified by using 10% cetylpyridinium chloride for 1h. The solution was collected and the absorbance at 570 nm was measured.

Dual Release of Growth Factor from PEG hydrogel.

10 mg BMP-2-gelatin and IGF-1-PLGA-PEG-COOH MPs were loaded into 20%PEG hydrogels and exposed to UV light (365 nm, 300 mW/cm², EFOSUltracure 100 ss Plus, UV

sport lamp, Mississauga, Ontario, Canada) for 60 seconds to obtain the PEG hydrogel-incorporated BMP-2-gelatin and IGF-1-PLGA-PEG-COOH MPs scaffolds. Growth factors loaded scaffolds were incubated in 1.0 mL PBS solution and shaken at 100rpm at 37°C and at predetermined time points (day 1, 3, 5, 7, 9, 14, 21, and 28). 0.5 mL of PBS solution containing released growth factors was collected, and the amount of released growth factors was determined by the ELISA kit.

Cell Culture and Seeding.

MSCs were cultured in DMEM, 10% v/v FBS, and 1 % v/v antibiotic/antimycotic solution and were incubated at 37 °C in 5 % CO₂ and 95 % air. The cells (1.0×10^4 cells/mL) were then seeded onto each well in the 24-well plates followed by addition of 1 mL of culture medium after 30 min. Growth factor-loaded hydrogels were then placed on top of cells. Cell culture media were replaced every 3 day and MSCs were cultured for 21 days.

ALP Activity and ALP Staining.

ALP activity was measured using the p-nitrophenol phosphate (pNPP) assay, which is based on the fact that the colorless pNPP is hydrolyzed by ALP, producing yellow pNPP. At designated time points (1, 4, 7 and 14 days) the medium was removed, the cell layers were washed twice with PBS (pH 7.4), lysed with 1 ml of 0.2% Triton X-100 and three freeze-thaw cycles, which consisted of freezing at -80°C for 30 min immediately followed by thawing at 37°C for 15 min. 50µL aliquots of the cell lysates were sampled and added to 50µL of working reagent (ALP assay kit, BioAssay) in a 96-well assay plate. The samples then were incubated for 1 h at 37°C in the dark. After incubation, the reaction was stopped with 100µL of 1 N sodium hydroxide on ice. ALP activity was determined from the absorbance using a standard curve prepared from pNPP stock standard (Sigma). The absorbance was measured at 405 nm using a microplate reader (TECAN Infinite F50). To observe ALP activities optically, an ALP detection kit was used according to the manufacturer's instructions. The cells on the plates were washed with PBS and fixed with 4 % paraformaldehyde in PBS for 2 min. Fixed cells were rinsed with rinse buffer (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.05 % Tween 20) and stained with 200 µL solution consisting of BCIP and NBT (ALP staining kit) in a dark room for 30 min. The staining solution was aspirated, and the cells were rinsed with rinse buffer. Finally, the cells were visualized using optical microscopy.

Matrix Mineralization.

ARS staining was used to measure the biomineralization of MSC. After 14 and 21 days of MSC culture, the cells were fixed with 4 % paraformaldehyde for an hour at room temperature. Then, 40 mM of ARS solution was added and incubated for 5 min at room temperature with gentle shaking. The solution was aspirated and washed with 50 v/v % ethanol. The stained sample was visualized using optical microscopy. To quantify the mineralization, the cells were desorbed using 10% cetylpyridinium chloride for 1h. The solution was collected and the absorbance at 570 nm was measured.

Statistical Analysis.

All data are presented as mean±standard deviation. For comparing two groups of data, a Student's t-test was performed. For comparing multiple groups of data, one-way ANOVA was performed followed by Dunnett's test. The differences in groups and experimental time points were considered significant if $p < 0.05$, and the data were indicated with (*) for $0.01 < p < 0.05$ and (**) for $p < 0.01$, respectively. Error bars represented standard deviation.

RESULTS AND DISCUSSION

Characterization of BMP-2-Gelatin MPs and IGF-1-PLGA-PEG-COOH MPs.

Figure 2a and b showed the SEM images of BMP-gelatin MPs and IGF-1-PLGA-PEG-COOH MPs before E-Beam sterilization. BMP-gelatin MPs showed a flaky structure with irregular edges. The gelatin morphology resembled a demineralized bone matrix or collagen matrix. The IGF-1-PLGA-PEG-COOH MPs exhibited spherical particles with smooth surfaces but obvious aggregation. Particle size measurements showed that the average particle size of BMP-2-gelatin and IGF-1-PLGA-PEG-COOH MPs were 44.85 μm and 53.70 μm , respectively.

As shown in Table 1, the encapsulation efficiency of BMP-2 and IGF-1 loaded in gelatin MPs and PLGA-PEG-COOH MPs was 64.78 % and 76.11 %, respectively, which was significantly higher compared with the literature. Kuo et al. synthesized the gemcitabine-loaded gelatin nanoparticles by nanoprecipitation and the encapsulation efficiency of gemcitabine was about 10% [21]. Kaul et al. prepared poly(ethylene glycol) (PEG)-modified gelatin nanoparticles via the pH and temperature controlled ethanol-water solvent displacement technique [22]. The loading efficiency of TMR-dextran in gelatin and PEGylated gelatin nanoparticles was 51% and 48%, respectively. King et al. developed the biodegradable PLGA/PEG microparticles loading with VEGF by the solid-encapsulation/single-emulsion/solvent extraction technique [23]. The loading efficiency of VEGF using this technique was calculated to be 16%. Kanget al. prepared BSA-loaded microparticles by the double emulsion solvent evaporation method. The encapsulation efficiencies of BSA within microparticles were 41.62% and 38.82% for PLGA/PEG ratios 2:1 and 1:1, respectively [24]. In these studies, growth factors were loaded in nano/microparticles during the process of nano/microparticles preparation. The activity of growth factors may have decreased due to the temperature and solvents involved in the preparation process. However, in our study, the processes of microparticles preparation and growth factors loading were separated. Gelatin MPs and PLGA-PEG-COOH MPs were prepared, and then BMP-2 and IGF-1 were grafted to the gelatin MPs and PLGA-PEG-COOH MPs. Therefore, the effect of microsphere preparation on the activity of growth factors was greatly reduced.

Effect of E-Beam sterilization on BMP-2 and IGF-1 activity from MPs.

Several techniques are available for the removal of microbial contamination, including filtration, autoclaving and irradiation, as well as formaldehyde, ethylene oxide and gas plasma treatments [17]. Of these sterilization methodologies, filtration may potentially remove microbial contamination without altering the physicochemical properties of the carrier nanoparticles or affecting their toxicity and functionality [25]. However, use of 0.22

μm or $0.45\ \mu\text{m}$ filters may not always be possible if the particles are larger than or close to the pore size of the filters, since clogging can occur and result in a decreased yield^[26,27]. For example, filtration of 200–300 nm PLGA nanospheres prepared using the standard Emulsion Solvent Diffusion (ESD) method resulted in less than 10% of the nanospheres passing through the membrane filter^[28]. In this study, we also tried to remove microbial contaminate from gelatin MPs and PLGA-PEG-COOH MPs by filtration with $0.45\ \mu\text{m}$ cellulose acetate membrane filters. However, approximately 56% of PLGA-PEG-COOH MPs and 82% of gelatin MPs were lost during the process of filtration (Supplement Table 1). Sterile filtration may therefore present itself as a reasonable method for the removal of bacterial contamination, only provided that a sufficiently high percentage of particles can be recovered following filtration. No single process may be applied to all nanoparticle preparations and, therefore, it is recommended that each nanoparticle-drug system be validated on a case-by-case basis. In our study, we used E-beam irradiation to sterilize BMP-2-gelatin MPs and IGF-1-PLGA-PEG-COOH MPs. We studied the effects of the processes on the properties of the MPs as well as on the loaded drugs, including the physicochemical properties of MPs, the bioactivity of the loaded growth factors, and the growth factors -release profiles.

The BMP-2-gelatin MPs and IGF-1-PLGA-PEG-COOH MPs showed similar size and structure at the surface before and after E-Beam sterilization (Fig.2). BMP-gelatin MPs exhibited a flaky structure with irregular edges (Fig.2c). The smooth surface and size of the IGF-1-PLGA-PEG-COOH MPs seemed to remain unchanged but still in obvious aggregation (Fig.2d). As shown in Table 1, the total amount of loaded BMP-2 after E-Beam sterilization was 150.86 ng per 10 mg of gelatin MPs, which corresponds to an encapsulation efficiency of 50.29 % and loading capacity of 1.50×10^{-3} . The total amount of loaded IGF-1 was 892.15 ng per 10 mg of PLGA-PEG-COOH MPs, which corresponds to an encapsulation efficiency of 59.48 % and loading capacity of 0.89×10^{-3} . After E-Beam sterilization, loading efficiency of BMP-2-gelatin MPs and IGF-1-PLGA-PEG-COOH MPs decreased in concentration to approximately 22.4% and 21.9% after E-Beam sterilization.

This study confirmed that microparticle shape was not obviously impacted by E-Beam irradiation. In addition, the drug content analysis confirmed that the E-Beam irradiation was able to induce a few changes in the drug content of the microparticles. Although approximately 22% of BMP-2 and IGF-1 loaded in MPs were lost in the process of E-Beam sterilization, it was far less than the amount lost in filtration processes. Alice et al. investigated effects of gamma irradiation on spray-dried Methotrexate (MTX)-loaded PLGA microparticles^[29]. The sphericity of non-loaded PLGA microparticles decreased and formed some toroidal microparticles, which was attributed to free radical formation after irradiation. However, the smooth surface and size of the microparticles seems remained unchanged. Katharina et al. fabricated the Oligodeoxynucleotide (ODN)-loaded gelatin nanoparticles (GNPs) and loading efficiency was dropped from 96.6% to 68.5% after sterilization by γ -irradiation (25 kGy)^[30]. The decrease in activity approximately 29% by γ -irradiation was in the range of the bioactivity loss of growth factors 22% by E-beam sterilization in our study. Our group has studied the effect of E-Beam sterilization on 3D-printed PCL/ β -TCP scaffolds for bone tissue engineering^[31]. The results also showed that E-beam irradiation did not alter the surface properties of scaffolds. No significant change in

the strut size, void size, or surface morphology was observed after the E-beam irradiation, which was in agreement with the results of the study.

We investigated the effects of E-beam irradiation on the release kinetic profile of the BMP-2-gelatin and IGF-1-PLGA-PEG-COOH MPs by measuring the cumulative amounts of growth factors over time. As shown in Figure 3, the red line and black line indicated the release behaviors of growth factors from sterilized and non-sterilized MPs, respectively, and growth factors were released from microparticles for 28 days. Both of BMP-2 and IGF-1 showed a similar initial burst release within 1 day before and after E-beam sterilization. As shown in Table 2, before E-beam sterilization, the cumulative release of BMP-2 and IGF-1 after 28 days was about 74% and 55%, respectively. After E-beam sterilization, the cumulative release of BMP-2 and IGF-1 was about 63% and 44%, respectively. Therefore, E-Beam sterilization was able to reduce release amount of BMP-2 and IGF-1 from gelatin and PLGA-PEG-COOH MPs, which may be related to the increase of crosslink density of MPs.

Previous studies showed that aliphatic polymers (PGA, PLA, and PCL) with oxygen in their backbone exhibit a high sensitivity toward radiation [17]. The general trend is that the crosslinking of PLGA chains increased with increasing doses of E-beam radiation [32]. Upon irradiation, free radicals are formed in the excited polymeric material and induce chemical changes. These changes are the cause of crosslinking reactions or shortening of the chain length by a chain scission or unzipping mechanism, and both the mechanisms may occur simultaneously [33]. It was found that the number-average molecular weight decreased whereas the weight-average molecular weight increased (e.g., on the whole, the chain got smaller but a proportion also got larger). This is a combined result of scission and crosslinking. Crosslinking could increase the tensile mechanical strength and make it more resistant to degradation [34]. Further, both gamma and E-beam irradiation caused dual scission and cross-linking reactions of the polymer chains as well as an increase in the polydispersity of PCL chains [35,20]. This indicates that E-beam irradiation did not affect the biocompatibility but improved the mechanical strength of PCL. Therefore, the decreased release profile of BMP-2-gelatin and IGF-1-PLGA-PEG-COOH MPs after E-beam sterilization can be attributed to the increased crosslinking density of gelatin and PLGA-PEG-COOH chains. It is worth mentioning that the sum of release and retention of growth factors in MPs is less than the initial loading amount by 8 to 13% before E-beam, and by 15 to 23% after E-beam. First, the losses between the initial loading and the sum of release and retention of growth factors, regardless of E-beam sterilization, are probably a result of the combinatory detrimental effects of sample preparation for bioassays and of bioassays themselves on proteins. Sagrario et al. investigated the half-life and activity of FGF and they found that FGF is remarkably unstable with a half-life for inactivation of only 15 min. It showed that the activity of FGF was decreased to less than 10% of initial activity after incubating for 2–4 hours in serum-free medium, pH 7.4, 37°C [36]. Additionally, the further losses of growth factors after E-beam sterilization could be the chain scissoring effect of proteins, which makes growth factors more susceptible to detrimental effects of sample preparation and bioassays.

We further studied the biological effects of BMP-2-gelatin and IGF-1-PLGA-PEG-COOH MPs after E-beam sterilization on osteogenesis of MSCs *in vitro*. Osteogenic differentiation was assessed through ALP activity assay and staining of ARS, an early and late osteogenic marker. ALP is known as an early marker for osteoblastic differentiation. ARS is commonly used to quantify the mineralization of MSCs because it binds to calcium salts selectively and the formation of calcium phosphate is the late stage marker for osteogenesis. For all groups, the ALP level increased significantly from days 4 to 7 and from days 7 to 14. IGF-1-PLGA-PEG-COOH MPs before and after E-beam sterilization showed significant biological effects on ALP staining and ALP activity of MSCs compared to the control group and when IGF-1 was added directly in solution ($p<0.01$) (Fig.4a). Mineralization of MSCs with IGF-1-PLGA-PEG-COOH MPs after E-beam sterilization was significantly higher than control groups ($p<0.01$) (Fig.4c), but no significant difference was observed between IGF-1-PLGA-PEG-COOH MPs groups before and after E-beam sterilization. BMP-2-gelatin MPs exhibited significantly higher ALP activity and MSC mineralization after E-beam sterilization ($p<0.01$) (Fig. 4b and d), but there was also no significant difference after E-beam sterilization.

No statistical difference was observed for the osteoinductive assay of BMP-2 and IGF-1 released from MPs with and without E-beam sterilization, indicating that the bioactivity of growth factors after E-beam irradiation and the biocompatibility of E-beam irradiated constructs. The osteogenic differentiation of MSCs in the presence of osteoinductive IGF-1 and BMP-2 has been shown elsewhere [13,37,38]. In this study, additive IGF-1 and BMP-2 containing medium did not significantly increase ALP and mineralization compared to IGF-1 loaded MPs and BMP-2 loaded MPs. This may be because the growth factor dosage in medium was too low, which was based on the release concentration from Figure 3 at the same period. BMP-2 and IGF-1 was added into the media at the concentration of 8 and 35 ng/mL, respectively. The cell culture medium was changed every 2 days and the cell culture duration was 21 days. Nevertheless, MPs with low doses of growth factors was more effective in localized delivery than medium with the same doses of growth factors. This may be because while the localized delivery of growth factors through MPs can induce sustained stimulation on cell differentiation, growth factors in medium can only induce fluctuating stimulation [36].

BMP-2-gelatin and IGF-1-PLGA-PEG-COOH MPs experienced decreased bioactivity of growth factors to approximately 22% after E-beam sterilization, but were still effective in significantly promoting osteogenesis of MSCs. E-beam treatment is thus safe and appropriate for sterilization of BMP-2-gelatin MPs and IGF-1-PLGA-PEG-COOH MPs for bone tissue engineering applications. The effect of E-beam sterilization on the biological response *in vivo* remains to be determined in the future, though some studies have shown biocompatibility of PLGA and gelatin microparticles sterilized using radiation-based methods [28,30].

Dual Release of Growth Factor from PEG hydrogel encapsulated gelatin MPs and PLGA-PEG-COOH MPs.

Fig. 5 shows the *in vitro* release behaviors of BMP-2 and IGF-1 from PEG hydrogel encapsulated with E-beam sterilized gelatin MPs and PLGA-PEG-COOH MPs for 4 weeks. BMP-2 release behavior from gelatin MPs/PEG hydrogel shows a higher initial burst release (8.7%) within 1 day, followed by a slow release for 4 week of incubation. IGF-1 release from the PLGA-PEG-COOH MPs/PEG hydrogel exhibited a lower initial burst (4.6%) and amore sustained release pattern afterward (17.6 % at day 9). After 28 days, the cumulative release of BMP-2 and IGF-1 from PEG hydrogel was about 52.7% and 27.3%, respectively. Although these results didn't show the release behaviors of BMP-2 and IGF-1 after four weeks, it suggested that the release of IGF-1 was much slower than that of BMP-2, and a greater amount of BMP-2 was released from the delivery system during the release periods.

The *in vitro* release behavior of IGF-1 and BMP-2 from the delivery system showed that the cumulative release of BMP-2 from the gelatin MPs/PEG hydrogel was significantly higher than that of the IGF-1 from the PLGA-PEG-COOH MPs/PEG hydrogel during 28 days of incubation ($P < 0.05$), and the encapsulation of MPs into a PEG gel provided a moderate, sustained release. The fast-degrading, protein-loaded gelatin microparticles could allow for the faster release of proteins compared with the slow-degrading, protein-loaded PLGA-PEG-COOH microparticles. Therefore, a dual release of BMP-2 followed by IGF-1 can be achieved by the BMP-2 loaded gelatin MPs and IGF-1 loaded PLGA-PEG-COOH MPs from a PEG gel. Recent studies suggested that dual release of multiple growth factors significantly enhances osteoblastic differentiation compared to the simultaneous release of growth factors [39,40]. Our delivery system had the capability to control the release of multiple growth factors at different time periods, which had the potential to synergistically enhance cell response and tissue regeneration. Similar behaviors were observed for the release of BMP-2 and IGF-1 from a PEG hydrogel containing only BMP-2-gelatin MPs or IGF-1-PLGA-PEG-COOH MPs (data was showed in supplement Figure 1), compared with release of each growth factor from PEG hydrogel containing both BMP-2-gelatin MPs and IGF-1-PLGA-PEG-COOH MPs (shown in Figure 5). This result demonstrates that presence of one growth factor had no effect on the release of the other growth factor. One important advantage of our dual delivery system is that various release profiles can be obtained independently for each growth factor by changing the properties of the gelatin MPs and the PLGA-PEG-COOH MPs separately. For example, the release of growth factors from PLGA-PEG-COOH MPs could be controlled by changing the molecular weight or the ratio of lactide to glycolide, and the release from gelatin MPs could be controlled by using different chemistries and processes for the preparation of the microparticles (volume ratio of gelatin solution to ethanol or the crosslinking density, supplement figure 2.).

In Vitro Osteogenic Differentiation of MSCs.

For the effect on the differentiation of MSCs, we examined six groups: control (in absence of MP and growth factors in medium); PEG hydrogel containing gelatin MPs and PLGA-PEG-COOH MPs without growth factors (gelatin & PLGA MPs/PEG gel); PEG hydrogel containing only BMP-2 loaded gelatin MPs (BMP2-Gelatin MPs/PEG gel); PEG hydrogel containing only IGF-1 loaded PLGA-PEG-COOH MPs (IGF1-PLGA MPs/PEG gel); PEG

hydrogel containing both BMP-2 loaded gelatin MPs and IGF-1 loaded PLGA-PEG-COOH MPs (BMP-2-Gelatin & IGF-1-PLGA MPs/PEG gel); BMP-2 and IGF-1 solution in DMEM medium.

To investigate the osteogenic differentiation of MSCs cultured at different conditions, we stained the cells with alkaline phosphatase (ALP) after 7 and 14 days. After cultured for 7 and 14 days, faint ALP staining was detected on the groups without growth factors (control and gelatin & PLGA MPs/PEG gel, Figure 6a). It was also observed that the group of PEG hydrogel containing both BMP-2-Gelatin and IGF1-PLGA-PEG-COOH MPs showed higher levels of ALP staining than the groups of PEG hydrogel containing only BMP-2 or IGF-1 loaded MPs group. The quantitative analysis of ALP levels was showed in Figure 6b. At 4 days of cell culture, the ALP activity considerably increased only in the BMP-2 or IGF-1 MPs containing gel, indicating that both BMP-2 and IGF-1 alone could contribute to the early osteogenesis of MSCs. More importantly, from 4 to 14 days of cell culture, ALP specific activity was significantly greater for dual release of BMP-2 followed by IGF-1 from the MPs-loaded PEG gel than all other treatments ($P < 0.01$). This result indicated the dual delivery of BMP-2 followed by IGF-1 by the gelatin MPs/PLGA-PEG-COOH MPs/PEG gel induced significantly greater ALP activity of MSCs than single BMP-2 or IGF-1 delivery or simultaneous administration of BMP-2 and IGF-1 in DMEM medium.

As shown in Figure 6c, at 14 and 21 days of cell culture, dual treatment with BMP-2 followed by IGF-1 was more strongly stained than any other treatments ($P < 0.01$). Quantitative analysis of ARS staining also supported those results as shown Figure 6d, which indicated the significantly higher calcium deposition in BMP2-Gelatin & IGF1-PLGA MPs/PEG gel group than other groups including BMP-2 or IGF-1 only groups. These results confirmed that MSCs cultured with the dual delivery system of IGF-1 and BMP-2 showed stronger osteogenic commitment (especially in mineralization). It should also be noted that significant cell aggregates were observed only in the group of BMP-2-Gelatin & IGF-1-PLGA MPs/PEG gel, as shown in Fig 6c. Formation of cell aggregates is a positive result for the osteogenesis of MSCs because it promotes mineralization without reducing the differentiation capability^[37]. Although MSCs cultured with the groups of PEG hydrogel containing only BMP-2 or IGF-1 loaded MPs showed evenly distributed ARS stained region (Fig 6c and d), there was slight or no definite cell aggregates. Therefore, treatment with BMP-2 combined with IGF-1 increased the number of cell aggregates. This result is consistent with our previous study^[13,37], in which the dual releases of BMP-2 followed by IGF-1 first promoted MSC differentiate into osteoprogenitor cells and then promoted fast proliferation of these osteoprogenitor cells, leading to enhanced mineralization. In addition, both a burst and a sustained release of BMP-2 are needed for new bone formation^[33,34]. For large critical-sized bone defects, a burst release helps to attract osteoprogenitor cells into the defects and increase cell aggregation while a sustained release promotes osteoblastic differentiation^[42,43]. Thus, the formation of cell aggregates in this study can be attributed to the initial release of BMP-2 from the system. On the other hand, the sustained delivery of BMP-2 enhances bone regeneration by affecting a larger population of osteoprogenitor cells at the fracture and promoting vasculogenesis^[42,44]. As such, it is to be determined if the initial faster release of BMP-2 followed by the sustained releases of both BMP-2 and IGF-1 may synergistically enhance bone regeneration and outperform the release of single growth

factor of BMP-2 by combination of burst and sustained release. We will test the effects of dual release and single growth factor releases of BMP-2 and IGF-1 on bone formation in vivo in future.

CONCLUSION

We developed gelatin and PLGA-PEG-COOH based microparticles delivery system with a broader spectrum of delivery duration with a model protein, BSA, and growth factors, including BMP2 and IGF-1. The encapsulation efficiency of BMP-2 and IGF-1 loaded in gelatin MPs (about 65%) and PLGA-PEG-COOH MPs (about 76%) was significantly higher compared with previous studies. Although bioactivity of growth factors in BMP-2-gelatin and IGF-1-PLGA-PEG-COOH MPs was decreased after E-beam sterilization (approximately 22%). E-beam sterilized growth factors delivery systems were effective in significantly promoting osteogenesis of MSCs. The decreased release of growth factors after E-beam sterilization by 10% is probably the result of the increase of crosslink density of MPs. Dual delivery of BMP-2 followed by IGF-1 was achieved by taking advantage of the different degradation rates of gelatin MPs and PLGA-PEG-COOH MPs. The enhanced effect of dual administration of BMP-2 and IGF-1 on osteogenic differentiation markers activity of MSCs was clearly confirmed by the simultaneous administration of IGF-1 and BMP-2 in medium and single delivery of either IGF-1 or BMP-2 under the same conditions. The results demonstrate that the gelatin and PLGA-PEG-COOH based microparticles are effective dual delivery carriers for therapeutic biomolecules. E-beam sterilization was an effective and accessible method for growth factors loaded carriers, which could pave a way of growth factor delivery for clinical applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS:

This research was funded through financial support from NIH grants R01AR057837, R01AR057837S, U01AR069395, R01AR072613, R01AR074458 from NIAMS, Fundamental and advanced research projects of Chongqing city (cstc2017jcyjAX0029) and Discipline talents development projects of School of Pharmacy (YXY2019XSGG10).

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Figure 1. Schematics of fabrication of gelatin (a) and PLGA-PEG-COOH (b) microparticles and growth factor loading.

Figure 2. SEM images of (a) BMP-Gelatin MPs and (b) IGF-1-PLGA-PEG-COOH MPs before E-Beam sterilization. (c) BMP-Gelatin MPs and (d) IGF-1-PLGA-PEG-COOH MPs after E-Beam sterilization (scale bar: 100 µm).

Figure 3.

In vitro release kinetics of BMP-2 and IGF-1 before and after exposition to E-beam irradiation. PBS was used as the release medium. (a) Release of BMP-2 loaded gelatin MPs. (b) Release of IGF-1 loaded PLGA-PEG-COOH MPs. Each value represents the mean $\pm$ SD (n = 3). * indicates statistical significance at the same time point (**P < 0.01; *P < 0.05).

Figure 4.

Effects of E-Beam sterilization on biological activity of BMP-2-gelatin MPs and IGF-1-PLGA PEG-COOH MPs. (a) Effects of IGF-1-PLGA-PEG-COOH MPs on ALP activity of MSCs. (b) Effects of BMP-2-gelatin MPs on ALP activity of MSCs. (c) Effects of IGF-1-PLGA-PEG-COOH MPs on mineralized ability of MSCs. (d) Effects of BMP-2-gelatin MPs on mineralized ability of MSCs. Each value represents the mean $\pm$ SD ($n = 3$). The means of the four experiments were compared using One-way ANOVA at a single timepoint. * indicates statistical significance relative to the control at the same time point (** $P < .01$; * $P < .05$).

Figure 5.

In vitro cumulative release of BMP-2 and IGF-1. (a) Comparison of in vitro cumulative release of BMP-2 and IGF-1 from the same types of microparticles and from two different microparticles. (b) In vitro cumulative release profiles of BMP-2 and IGF-1 from E-beam sterilized gelatin MPs and PLGA-PEG-COOH MPs contained in PEG hydrogel over four weeks period. PBS was used as the release medium. Each value represents the mean $\pm$ SD ($n = 3$).

Figure 6.

Effects of the dual delivery system on osteogenic differentiation of hMSCs. (a) ALP staining of hMSCs cultured after 7 and 14 days. (b) Quantitative analysis of ALP activity on day 0, 4, 7 and 14. (c) ARS staining of hMSCs cultured after 14 and 21 days. (d) Quantitative analysis of the mineral deposition on day 14 and 21 by ARS staining. Each value represents the mean $\pm$ SD (n = 3). * and ** denotes significant difference between groups at the same time point ($P < 0.05$, $P < 0.01$).

Table 1.

The characterization of BMP-2-gelatin and IGF-1-PLGA-PEG-COOH MPs

	BMP-2 loaded Gelatin particles			IGF-1 loaded PLGA-PEG-COOH particles		
	TC (ng)	EE (%)	LC (%)	TC (ng)	EE (%)	LC (%)
Before EB	194.34	64.78	1.94×10^{-3}	1141.59	76.11	1.14×10^{-3}
After EB	150.86	50.29	1.50×10^{-3}	892.15	59.48	0.89×10^{-3}

TC: Total content of growth factor in 10 mg MPs

EE: Encapsulation Efficiency of growth factor in 10 mg MPs

LC: Loading Capacity of growth factor in 10 mg MPs

Table 2.

Release amount of BMP-2-gelatin and IGF-1-PLGA-PEG-COOH MPs

	BMP-2 loaded Gelatin particles			IGF-1 loaded PLGA-PEG-COOH particles		
	TC (ng)	CRA (ng)	URA (ng)	TC (ng)	CRA (ng)	URA (ng)
Before EB	194.34	143.11 (74%)	35.34	1141.59	627.87 (55%)	364.07
After EB	150.86	94.47 (63%)	34.29	892.15	392.55 (44%)	293.29

TC: Total content of growth factor in 10 mg MPs

CRA: Cumulative release amount of growth factor in 10 mg MPs over 28 days

URA: Un-released amount of growth factor in 10 mg MPs over 28 days