Published in final edited form as:

Adv Healthc Mater. 2015 December 9; 4(17): 2699–2708. doi:10.1002/adhm.201500531.

Hierarchical Nanofibrous Microspheres with Controlled Growth Factor Delivery for Bone Regeneration

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Abstract

The integration of controlled growth factor delivery and biomimetic architecture into a microsphere is a challenging but attractive strategy for developing new injectable biomaterials for tissue engineering. In this work, we developed a unique hierarchical nanosphere-encapsulated-in-microsphere scaffolding system for bone tissue regeneration. First, we synthesized heparin-conjugated gelatin (HG) that provides binding domains for bone morphogenetic protein 2 (BMP2) to stabilize this growth factor, protect it from denaturation and proteolytic degradation, and subsequently prolong its sustained release. Next, we developed a unique approach that includes a water-in-oil-in-oil (W/O/O) double emulsion process and a thermally induced phase separation to encapsulate BMP2-binding HG nanospheres into nanofibrous microspheres. The nanofibrous microsphere was self-assembled from synthetic nanofibers, and had superior surface area, high porosity, low density, and was an excellent carrier to support cell adhesion and tissue in-growth. BMP2 in the hierarchical microsphere was released in a multiple-controlled manner (by the binding with heparin and encapsulation of the nanosphere and microsphere) and retained its high bioactivity. An *in vivo* calvarial defect model confirmed that this unique hierarchical microsphere was an excellent osteoinductive scaffold for enhanced bone regeneration. By choosing different growth factors, this hierarchical microsphere system can be easily applied to other types of tissue regeneration. Our work expands the ability to develop new injectable biomaterials for advanced regenerative therapies.

Keywords

microspheres; nanospheres; nanofibrous; bone tissue engineering; controlled release

1. Introduction

The development of a suitable scaffolding system is pivotal in engineering tissue regeneration and repair.^[1, 2] In clinics, there are often irregular-shaped defects and wounds needing to be filled and repaired. In such cases, the use of injectable scaffolds is very attractive because they can easily fill irregular-shaped defects *in situ*, in a minimally invasive manner, and thus improve patient comfort and satisfaction. With the increasing demand for minimally invasive surgery in medicine, the development of injectable scaffolds is even more urgent.^[3] Hydrogels have been widely explored as injectable biomaterials for non-load-bearing bone regeneration.^[4] Despite their many advantages, hydrogels do have limitations.^[5] For example, they often do not possess a good tissue adhesive property, which could be disadvantageous for integration with the host tissue.^[6]

Besides hydrogels, biodegradable microspheres have also been studied as injectable cell carriers for bone tissue regeneration.^[7] The microspheres have the advantage of being used as micro-carriers for cell cultivation before injection into the body.^[8] The solid-interior structure of the microspheres, however, limits high density cell seeding and tissue in-growth after implantation, as well as generates large amounts of degradation by-products. Furthermore, the smooth surfaces of the spheres are not favorable for cell adhesion and proliferation.

The integration of nano-structures into microspheres is an effective way to create new cell carriers. Nano-structured biomaterials are appealing in tissue engineering because they mimic the architecture of the natural extracellular matrix (ECM) on a nanometer scale and are believed to contribute significantly to the growth of biological functions in the tissues.^[9] Nanofibrous scaffolds have been demonstrated to possess high surface area and porosity that facilitate cell adhesion and tissue in-growth.^[10] The overall low density of nanofibrous biomaterials also generates fewer by-products resulting from degradation.^[11] A variety of materials have been fabricated into nano-structured scaffolds.^[12] However, these scaffolds (except for hydrogels) usually have fixed geometries and are non-injectable.

Another important component in tissue engineering approaches is growth factors, which are essential for cellular signaling of migration, proliferation, differentiation, and maturation.^[1] The localized and temporally controlled delivery of growth factors is crucial to achieve optimal clinical efficacy.^[13] Encapsulating growth factors into biodegradable polymeric nano- or microspheres is a widely used approach to controlling the release profile and protecting unstable bioactive molecules from degradation.^[14] However, encapsulation is a simple physical entrapment process and often leads to high initial burst release of growth factors. A more advanced strategy is needed to overcome this limitation. To date, it remains a challenge in the development of biomimetic nano-structured microspheres that not only act as a cell carrier but also provide controlled growth factor delivery for tissue regeneration.

In this study, we developed a hierarchical scaffolding system for bone tissue regeneration using nanosphere-encapsulated microspheres. In this system, the growth factor bone morphogenetic protein 2 (BMP2) binds with heparin and is encapsulated into heparin-conjugated gelatin nanospheres, which are further immobilized in the injectable nanofibrous microspheres (MS) of poly(L-lactic acid) (PLLA) (Figure 1). This system allows the integration of nanofibrous architecture with controlled growth factor delivery into one injectable microsphere. On one hand, the MS is self-assembled with PLLA nanofibers that mimic the architecture of natural collagen fibers at a nanometer scale and possess many unique properties including superior surface area, high porosity, low density, and controllable degradation rate.^[15] The ECM-mimicking nanofibrous architecture has also been shown to enhance mesenchymal stem cell adhesion, proliferation, differentiation, and neo mineralized tissue formation.^[16] On the other hand, BMP2 has binding domains with heparin, and the binding of BMP2 to heparin stabilizes this growth factor, protects it from denaturation and proteolytic degradation, and subsequently prolongs its sustained release.^[17] In addition, the heparin-binding BMP2 is encapsulated in gelatin nanospheres and entrapped by the nanofibers of PLLA microspheres (Figure 1). Therefore, the BMP2 is released in a multiple-controlled manner from the scaffolding system. We hypothesized that this hierarchical injectable scaffolding system would provide an excellent environment for tissue regeneration.

2. Results and Discussion

2.1. Preparation and characterization of the hierarchical MS

Encapsulation of nanospheres into biomimetic MS is a challenge. We previously prepared nanofibrous MS using tetrahydrofuran (THF) as a solvent.^[15] However, this method cannot incorporate nanospheres into the MS. In this study, we developed a unique approach to encapsulate nanospheres into nanofibrous MS. Our approach integrated a water-in-oil-in-oil (W/O/O) double emulsion process, a chemical crosslinking, and a thermally induced phase separation technology to prepare the hierarchical nanofibrous MS. First, heparin-conjugated gelatin (HG) dissolved in poly(vinyl alcohol) (PVA) aqueous solution was sonicated in ethyl acetate (EA) to form a W/O emulsion. Next, the emulsion was added in a PLLA dichloromethane (DCM) solution by stirring. Glutaraldehyde was added to the emulsion in this step to slightly crosslink the HG liquid droplets. Then the W/O emulsion was transferred into glycerol to form a W/O/O double emulsion. Next, the W/O/O double emulsion was quenched in liquid nitrogen to induce phase separation. After solvent extraction and freezing drying, hierarchical nanofibrous MS (abbreviated as HG-MS) was obtained (Figure 2).

As shown in Figure 2B-2C, the MS was composed entirely of nanofibers with an average diameter of 366 ± 146 nm, which is on the same scale as natural collagen fibers. The nanofibrous MS had a porosity of $94.24 \pm 0.52\%$, which is beneficial to provide sufficient space for cell growth and ECM deposition. A higher porosity can be further achieved by decreasing the PLLA concentration. The average size of the MS can be controlled from a few micrometers to several hundred micrometers by varying the stirring speed and the polymer concentration. A higher stirring speed or/and a lower PLLA concentration leads to

the smaller size of the MS. The MS were sieved, and a size ranging from 106-150 μm was used for the rest of the experiments.

Heparin was conjugated onto gelatin by condensing the carboxyl groups of the heparin and the amino groups of the gelatin via the 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)/N-hydroxysuccinimide (NHS) coupling reaction, as we reported recently.^[18] The amount of heparin incorporated into HG was controlled by the heparin concentration in the reactants. Under our experimental conditions (see Experimental Section), the amount of immobilized heparin in the HG was $7.8 \pm 1.2\%$ (wt/wt) as determined by a toluidine blue assay. To visualize the encapsulated HG nanospheres in MS (abbreviated as HG-MS), we added tetramethylrhodamine (TRITC)-conjugated gelatin (5% wt/wt) to HG, and deposited fluorescein isothiocyanate (FITC)-conjugated bovine serum albumin (BSA) on the nanofibers of the MS. As shown in the confocal images (Figure 2D, 2E), the HG nanospheres (red) were uniformly encapsulated in the nanofibrous MS (green).

We chose DCM/EA solvent mixture for our system because: (1) DCM and EA are immiscible with water and glycerol, capable of forming a W/O/O emulsion after sonication of the polymer mixture; and (2) the DCM/EA solvent mixture can induce a phase separation of the PLLA. When the ratio of the DCM/EA ranged from 2/8 to 4/6, a typical nanofibrous MS was obtained after the phase separation (Figure 3). As the ratio of the DCM/EA increased from 2/8 to 4/6, the average diameter of the MS nanofibers decreased from 528 ± 159 nm to 281 ± 72 nm. Meanwhile, a lower molecular weight of the PLLA led to the formation of leaf-like plates (Figure 3D, 3H). Other structural parameters of the MS, including porosity, apparent density and fiber length, are listed in Table 1.

While the size and the encapsulation efficiency of the immobilized gelatin nanospheres were affected by many factors, including sonication output power, PVA concentration, solvent composition, and polymer concentration, we focused on the ratio of HG/PLLA in this study. As the ratio of the HG/PLLA in the emulsion increased from 1% (wt/wt) to 6% (wt/wt), the average diameter of the HG nanospheres increased from several hundred nanometers to a few micrometers (Figure 4C). Meanwhile, the encapsulation efficiency of the HG nanospheres in the MS increased from $53.9 \pm 9.4\%$ to $91.0 \pm 2.3\%$ as the ratio of the HG/PLLA increased from 1% (wt/wt) to 6% (wt/wt) (Figure 4D). This is because an increase in the amount of HG raises the probability that the dispersed HG nano-droplets collide and merge into large liquid droplets in the PLLA emulsion. After the HG liquid droplets are crosslinked and solidified, large HG nanospheres are formed in the MS. In addition, large-sized HG spheres can easily be entrapped in the nanofibrous MS, leading to a high encapsulation efficiency at high ratio of HG/PLLA.

2.2 In vitro release of BMP2 from the MS and bioactivity assay

We first used BSA as a model protein to examine the encapsulation efficiency and the release behavior of the growth factor from the hierarchical MS. BSA was loaded into the HG-GS using a soaking process as described in the Experimental Section. For comparison, BSA was also loaded into gelatin nanospheres (the resulting hierarchical MS was abbreviated as G-MS) and deposited directly onto the nanofibrous MS nanofibers without nanospheres. The same amount of BSA was added to the MS, G-MS, and HG-MS. As

shown in Figure 5A, the MS adsorbed a significant amount of BSA ($42.7 \pm 1.3 \mu\text{g}/\text{mg MS}$) onto its nanofibers owing to the high surface areas. The encapsulated BSA in the G-MS and the HG-MS increased with the ratios of the gelatin/PLLA and HG/PLLA. For example, the encapsulated BSA in the HG-MS increased from $43.5 \pm 1.1 \mu\text{g}/\text{mg MS}$ to $61.1 \pm 1.0 \mu\text{g}/\text{mg MS}$ as the ratio of the HG/PLLA increased from 1% (wt/wt) to 6% (wt/wt). This result indicates that the amount of encapsulated protein in the hierarchical MS can be controlled by tailoring the composition of HG and PLLA in the HG-MS.

The BSA release profiles from the microspheres are shown in Figure 5B. An initial burst release of up to $64.2 \pm 0.7\%$ was detected in the MS group at the first day. In contrast, the initial burst release at day 1 decreased to below 30% in both the G-MS and the HG-MS groups. After the first day, a sustained release of the BSA from both hierarchical MS was retained for several days. This result indicates that encapsulation of BSA into the nanospheres provides a better control of the protein release from the MS. At each time point, there was no significant difference ($p > 0.05$) of the released BSA between the HG-MS and the G-MS, suggesting that there is no specific interaction between BSA and heparin.

Next, we loaded BMP2 into these three microspheres and examined their release profiles. Two kinds of BMP2 concentrations ($500 \text{ ng}/\text{mg MS}$ and $50 \text{ ng}/\text{mg MS}$) were included in the study. For the high BMP2 concentration group (Figure 5C, 5D), the burst release on the first day was $20.9 \pm 4.3\%$, $26.3 \pm 3.5\%$, and $31.0 \pm 2.9\%$ for the HG-MS, G-MS, and MS groups, respectively (Figure 5C). Apparently, loading BMP2 into gelatin and heparin-conjugated nanospheres significantly decreased ($P < 0.05$) the initial burst release of the protein from the microspheres. Furthermore, the BMP2 in the HG-MS was released in a more sustained way than from the other two groups. From the 2nd week to the 4th week, over 17% of the BMP2 ($36.6 \pm 1.6 \text{ ng}$) in the HG-MS was released, while only about 10% ($17.0 \pm 1.6 \text{ ng}$) and 7% ($10.0 \pm 1.8 \text{ ng}$) of the BMP2 were released from the G-MS, and MS, respectively (Figure 5C, 5D). Although we loaded the same amount of BMP2 into each type of microspheres, higher total amount of BMP2 (BMP2 released from the microsphere + BMP2 still in the microsphere) was detected from the HG-MS than from the MS and the G-MS (Figure 5D). This result indicates that heparin in the HG-MS bound with BMP2 and the binding provided protection of BMP2 from degradation/denaturation during the release period. Similar release profiles were observed when a lower amount of BMP2 ($50 \text{ ng}/\text{mg MS}$) was loaded into the microspheres (Figure 5E, 5F). The burst release of BMP2 from the HG-MS at day one was only $17.1 \pm 4.8\%$, which was significantly lower than the results from the MS ($28.9 \pm 3.0\%$) and the G-MS ($25.0 \pm 1.8\%$) (Figure 5E). Over 70% of the total BMP2 in the MS was released within 1 week, while these amounts were 49.3% and 31.2% for the G-MS and HG-MS. The release rate of the MS reached a plateau after 1 week, and only 9.1% of the total BMP2 in the MS was released during the last 3 weeks of the experiment. In contrast, more BMP2 was released from the G-MS and HG-MS during 1 week to 4 weeks (Figure 5F). Specifically, the HG-MS released BMP2 consistently at a rate of approximately 1 to 2% per day (1-2 ng) from day seven to the four-week endpoint of the experiment. As mentioned previously, BMP2 has a binding domain to heparin. Therefore, during the release process, the BMP2 in the HG-MS needs to: (a) be dissociated from the binding with heparin, (b) escape the entrapment of the nanosphere, and (c) overcome the physical adsorption of the

nanofibers of the microsphere. Therefore, compared to MS and G-MS, the HG-MS provided the best control of the release of the BMP2 from the microsphere.

2.3 In vitro bioactivity assay

Rat bone marrow stem cells (BMSCs) were used to test the bioactivity of the BMP2 released from the microspheres. A total of 5×10^4 BMSCs were mixed with 2 mg microspheres and cultured in a 12-well plate. Figure 6A is a confocal image of the BMSCs adhered to the HG-MS after culturing for 48 h. Due to the low apparent density and nanofibrous architecture, the HG-MS was easily “grabbed” by the BMSCs to form an aggregation of microspheres/BMSCs. This aggregation is beneficial for its *in vivo* application because it prevents the migration of the BMSCs and microspheres to other areas after the injection. To examine the effect of the released BMP2 on the proliferation and differentiation of the BMSCs, we loaded 500 ng/mg BMP2 into the MS and HG-MS. Compared to the blank control, loading BMP2 into the microspheres (both MS and HG-MS) did not significantly affect the proliferation rate of the BMSCs ($P > 0.05$) (Figure 6B). However, the differentiation and mineralization of the BMSCs were significantly different when the BMSCs were cultured on these three microspheres for two weeks (Figure 6C). Many more mineral nodules were deposited on the HG-MS than on the other two groups. The real time-polymerase chain reaction also indicated that the expression of the osteogenic differentiation markers (OCN, DMP1, and ALP) on the BMP2-loaded HG-MS and MS was significantly higher ($P < 0.05$) than the control, indicating that the BMP2 released from the microspheres retained its bioactivity and enhanced the BMSC differentiation. Furthermore, the expression of these genes on the HG-MS+BMP2 group was significantly higher ($P < 0.05$) than on the MS +BMP2 group, consistent with the alizarin staining images in Figure 6C.

2.4 In vivo bone regeneration

To further investigate the effect of the hierarchical microspheres to enhance bone formation, the BMP2-loaded microspheres (including MS and HG-MS) were implanted at the site of calvarial bone defects of Sprague Dawley® rats. After 6 weeks, the specimens were retrieved from the rat skull, and X-ray, μ -CT, histological and immunohistochemical evaluations were performed. The X-ray (Figure 7A) and μ -CT (Figure 7E) images showed that there was only very small amount of thin bone formation in the blank control defect at the native bone margins. A small amount of bone appeared at the native bone margins and the center of the defect on the MS group (Figure 7B, 7F). A considerable amount of new bone had covered more than half of the defect on the BMP2-loaded MS groups (Figure 7C, 7G). In contrast, all the defect areas had been covered by new regenerated bone on the BMP2-loaded HG-MS (Figure 7D, 7H). Quantitative analysis confirmed that the HG-MS +BMP2 had the highest ratio of bone volume/total volume (BV/TV) than all the other three groups (Figure 7I).

The above findings were further confirmed by histological analyses. H&E and Masson's trichrome staining images showed that the defective area in the blank group was entirely filled with fibrous soft tissues (Figure 8A, 8E, 8J, 8N). On the MS group, most of the defect areas were occupied by fibrous soft tissues, although a small amount of new bone had also formed there (Figure 8B, 8F, 8K, 8O). More new bone formed on the BMP2-loaded MS

group than the MS and blank control (Figure 8C, 8G, 8L, 8P). However, most of the regenerated bone was located on the dura mater side, and the middle area of the defect was still filled with fibrous tissue. For the BMP2-loaded HG-MS group, the newly formed bone filled the entire defect site, and the regenerated collagen fibers were much denser than in other groups (Figure 8D, 8H, 8M, 8Q). Also, bone marrow cavities enclosing new blood vessels were clearly observed in the regenerated bone. To further examine the content of the bone mineralization, immunohistochemistry was performed to determine the expression levels of dentin matrix acidic phosphoprotein 1 (DMP1) and collagen type 1 (Col1).

As shown in Figure 9, the expression of DMP1 and Col1 in the BMP2-loaded HG-MS group was much stronger than in all other three groups. Taken together, the BMP2-loaded HG-MS was an excellent cell and growth factor carrier for enhanced osteogenesis and mineralization.

It should be noted that we used BMP2 in this study to illustrate our strategy of building a hierarchical nanofibrous microsphere system for bone regeneration. Furthermore, this strategy can be easily expanded to other tissue regeneration. For example, vascular endothelial growth factor (VEGF) also has a binding domain with heparin,^[18] and VEGF-loaded HG-MS can be used as a new injectable microsphere system for induced angiogenesis. In addition, multiple growth factors can be loaded into our hierarchical microspheres (either immobilized in nanospheres or directly adsorbed on the nanofibers of the microsphere) to achieve sequential delivery of these growth factors for tissue regeneration. This work expands our ability to design suitable injectable scaffolding systems, which will lead to new advanced regenerative therapies.

3. Conclusions

In this work, we designed and synthesized a unique hierarchical scaffolding system using nanospheres encapsulated in microspheres. In this system, growth factor BMP2 binds with heparin and is encapsulated in the nanosphere and the nanofibers of the microsphere. This system not only protects the growth factor from denaturation and degradation, but also provides better control of its sustained release. In addition, the nanofibrous microsphere itself integrates the ECM-mimicking architecture with a highly porous injectable form, efficiently accommodating cells and supporting tissue formation. The *in vivo* study showed that the BMP2-loaded HG-MS was an excellent carrier for enhanced bone regeneration. The combination of controlled growth factor delivery with an injectable biomimetic scaffold provides new insights into the design and fabrication of cell-instructive scaffolds.

4. Experimental Section

Materials

Gelatin (Type B, from bovine skin, 225 g Bloom), heparin (sodium salt from porcine intestinal mucosa, MW~17-19 kDa), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), tetramethyl thodamine iso-thiocyanate (TRITC), bovine serum albumin (BSA), fluorescein isothiocyanate labelled bovine serum albumin (FITC-BSA), NaCl, and glutaraldehyde (GTA, 25% aqueous solution), collagenase (type I), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Poly(vinyl alcohol) (PVA, 88%

hydrolyzed, MW 88000), ethyl acetate (EA), and dichloromethane (DCM) were purchased from Acors Organics. Dialysis membrane (MWCO: 10 kD and 50 kD) were purchased from Spectrum® Laboratories (Dallas, TX, USA). Poly(L-lactide acid) (PLLA) was a gift from PURAC® America, Inc. Recombinant human bone morphogenetic protein 2 (BMP2) was purchased from R&D Systems Inc. (Minneapolis, MN, USA).

Preparation of heparin-conjugated gelatin (HG)—The HG was synthesized as we previously reported with minor modifications.^[18] Briefly, 0.125 g heparin and 0.50 g gelatin were dissolved in 12.5 ml aqueous solution that contained 50 mM MES and 0.2 M NaCl. Next, 0.138 g EDC and 0.033 g NHS were added into the heparin solution under magnetic string for 15 minutes. The two solutions were mixed and reacted for 12 h at room temperature under gentle stirring. The final product was dialyzed for 3 days in 0.2 M NaCl solution before being transferred into deionized water. The purified HG was lyophilized and stored in a desiccator for later use. The amount of heparin conjugated to the gelatin was determined by a toluidine blue assay as we reported recently.^[18]

Fabrication of hierarchical MS (HG-MS and G-MS)—The hierarchical MS was prepared using a combination of the water-in-oil-in-oil (W/O/O) double emulsion process, chemical crosslinking, and thermally induced phase separation. First, HG (or gelatin) aqueous solution (2%) with 0.5% PVA was added into EA and emulsified for 60 s. The W/O emulsion was poured into a PLLA solution of DCM and stirred for 120 s. The emulsion was further sonicated for another 60 s. Next, glutaraldehyde (20 µl) was added to the W/O emulsion. Under rigorous mechanical stirring, the W/O emulsion was gradually poured into glycerol to form a W/O/O emulsion. After that, the emulsion was quenched in liquid nitrogen to induce phase separation. After 10 min, pre-cooled ethanol (-80°C) was added for solvent exchange for 24 h. The hierarchical MS were incubated in 100 mM glycine for 1h to neutralize the unreacted glutaraldehyde. The MS were washed with distilled water three times and sieved to obtain different ranges of sizes. Finally, the hierarchical MS were lyophilized and stored in a desiccator for later use. The porosity, diameter and length of the nanofibers, and the apparent density of the hierarchical MS were calculated as we reported previously.^[15]

Encapsulation efficiency of the HG nanospheres in the HG-MS—The MS with un-crosslinked HG was used to test the encapsulation efficiency. First, the hierarchical MS (10 mg) was dissolved in DCM (0.5 mL). One milliliter of distilled water was added to the solution. After 30 min, the mixture was centrifuged at 10,000 rpm for 5 min. The supernatant was used to test the gelatin content using a MicroBCA™ Protein Assay Kit (Thermos Scientific) according to the manufacturer's protocol.

Incorporation of BSA and BMP2 into HG-MS—Lyophilized BSA powder was reconstituted to 10 mg/mL using Dulbecco's phosphate-buffered saline (DPBS) and lyophilized BMP2 powder was reconstituted to 5 µg/mL by 4 mM HCl containing 0.5% BSA. The hierarchical MS were sterilized by soaking in 70% ethanol overnight. The MS were washed 6 times with DPBS. Under gentle magnetic stirring, the BSA or BMP2

solution was loaded into the MS at 4°C for 12 h. The BSA-loaded MS were washed once and lyophilized while the BMP2-loaded MS were lyophilized directly.

***In vitro* release test**—To determine the protein (FITC-BSA or BMP2) release profile, protein-loaded MS were immersed in 1 mL DPBS incubated at 37 °C as we described previously.^[19] At designated time points, the samples were centrifuged to collect the supernatant, which was replaced with equal amounts of fresh DPBS. For the final collection, 0.5 mL DCM was used to dissolve the MS, and 1 mL collagenase (type I, 30 units/mL) was added to degrade the gelatin. The mixture was centrifuged at 10,000 rpm for 5 min, and the supernatant was collected and defined as the unreleased protein in the MS. To measure the amount of released FITC-BSA from the MS, the absorbance of the collected medium at 495 nm was measured using a spectrophotometer. To measure the amount of released BMP2 from the MS, a BMP2 Quantikine ELISA Kit was used according to the manufacturer's instructions. All samples for each group were performed in triplicate, and the experimental data were presented as the means±standard deviation.

***In vitro* bioactivity assay**—Bone marrow stromal cells (BMSCs) were isolated from the femurs and tibias of Sprague Dawley (SD) rats as described.^[20] The BMSCs were cultured in α -modified essential medium (α -MEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Sigma Aldrich) and 1% penicillin–streptomycin (Gibco) in a humidified incubator with 5% CO₂ at 37°C. The culture medium was changed every 2 days. A total of 5×10^4 BMSCs were mixed with 2 mg MS (G-MS, HG-MS) and cultured in a 24-well plate. The culture medium was changed every 2 days. During the medium change, cell/micropsphere constructs with culture medium were transferred to a centrifugal tube (Eppendorf) and the cell/micropsphere constructs were collected at 1000 rpm for 5 min. After removing the medium, the same amount of fresh culture medium was added. The cell/micropsphere constructs with fresh culture medium were then transferred back to the plate for further culture. The proliferation of the BMSCs on the MS (G-MS, HG-MS) was determined as we described previously.^[15] For cellular morphology observation, cell-micropsphere constructs were rinsed with DPBS and fixed with PFA after culturing for 48 hours. After blocking with 5% BSA for 30 min, the construct was stained with phalloidin 633 (Invitrogen) and mounted with ProLong® gold antifade mountant (Invitrogen). The construct was observed under a confocal laser scan microscope (TCS SP5, Leica, Buffalo, USA). To analyze the differentiation and biomineralization, the BMSCs on the MS (G-MS, HG-MS) were cultured in differentiation medium (with 50 mg/mL ascorbic acid, 5 mM β -glycerophosphate, and 10 nM dexamethasone). After 2 weeks, the construct was fixed with 4% paraformaldehyde and stained with 2% alizarin red. The specimens were washed with DPBS, and images taken using a photo scanner (Epson).

Real-time reverse transcription-polymerase chain reaction (qRT-PCR)—The qRT-PCR was performed as we reported previously.^[21] Briefly, the total RNA was extracted using RNeasy plus MiniKit (Qiagen, USA) and first-strand cDNA was reverse-transcribed with a QuantiTect Rev. Transcription Kit (Qiagen, USA). Human-specific primers were designed and synthesized as follows: osteocalcin (OCN, sense 5'-ACTGTGACGAGTTGGCTGAC-3'; antisense 5'-CAAGGGCAAGAGGAAAGAAG-3');

alkaline phosphatase (ALP, sense 5'-CCACGTCTTCACATTTGGTG-3'; antisense 5'-AGACTGCGCCTGGTAGTTGT-3'); dentin matrix protein 1 (DMP1, sense 5'-TGGGGATTATCCTGTGCTCT-3'; antisense 5'-TACTTCTGGGGTCACTGTCG-3'); and collagen $\alpha(1)$ I (Col1, sense 5'-AAAAGGAAGCTTGGTCCACT-3'; antisense 5'-GTGTGGAGAAAGGAGCAGAA-3'). GAPDH (sense 5'-AGCCGCATCTTCTTTTTCGTC-3'; antisense 5'-TCATATTTGGCAGGTTTTTCT-3') was used as an internal control. The RT-PCR was carried out using a Bio-Rad C1000™ Thermal Cycler and the conditions for the RT-PCR were as follows: 94°C (30 s), 56°C (30 s), and 72°C (60 s) for 40 cycles, with a final 10 min extension at 72°C. The RT-PCR products were separated by electrophoresis (EC105, Apparatus Corporation) on a 1.5% agarose gel and visualized using a Kodak EL logic 100 imaging system. The conditions for qRT-PCR were as follows: denaturation at 95°C (10 min), 45 cycles at 95°C (15 sec), 55°C (1 min, annealing) followed by a final dissociation step. The qRT-PCR reactions were performed using power SYBR Green QPCR Master Mix (Applied Biosystems) and the Bio-rad qRT-PCR Detection System (Bio-rad, USA).

In vivo surgical procedure—The animal surgical procedure was approved by the University Committee on Use and Care of Animals (UCUCA) of Texas A&M University Baylor College of Dentistry. SD rats (180-200 g) were anesthetized by intraperitoneal injection of ketamine/xylazine. A 1.5 cm long mid-sagittal incision was made with a sterile scalpel blade. The full thickness skin and periosteum were raised to expose the calvarial bone. A 5-mm diameter trephine was used to drill a standard surgical defect on the right side of the midline. After the bleeding was controlled, microspheres were carefully implanted on the top of the dura. The periosteum and skin were closed with absorbable polyglycolic acid (PGA) sutures. Sixteen animals were divided into four groups: (1) without any implants (blank control group); (2) implanted with MS (MS group); (3) implanted with BMP2-loaded MS (MS+BMP2 group); and (3) implanted with BMP2-loaded HG-MS (HG-MS+BMP group). After 6 weeks, the animals were euthanized and the skull was harvested for an X-ray, micro-computed tomography (μ -CT), histological and immunohistochemical examination.

X-ray radiography and μ -CT—The tissues at the calvarial bony defects were analyzed by X-ray radiography (Faxitron) and μ -CT35 imaging system (Scanco Medical). The μ -CT analysis was a high-resolution scan at the defect, and the acquired data were used for quantitative analyses. The bone density of bone volume to total volume ratio (BV/TV) was obtained using a Scanco software.

Histological and immunohistochemical staining—The specimens were fixed and demineralized in 10% ethylenediaminetetraacetic acid (EDTA) for 7 days at room temperature, dehydrated through graded alcohol and subsequently embedded in paraffin. The sections (5 μ m thick) were processed for histologic observation by Hematoxylin and Eosin (H&E), Masson's trichrome staining. For immunohistochemical analysis, samples were de-paraffinized using xylene and ethanol and reacted with primary human collagen type I (Col I 1:50 dilution) (Abcam, MA, USA), and dentin matrix acidic phosphoprotein (DMP1: 1:400 dilution).^[22] All the immunohistochemistry experiments were detected with a

3, 3-diaminobenzidine kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions.

Statistical analysis—All data were presented as means±standard deviations. To test the significance of observed differences between the study groups, an unpaired Student *t*-test was applied. A value of $P<0.05$ was considered to be statistically significant.

Acknowledgments

This study was supported by NIH/NIDCR 1R03DE22838-01A1, Texas A&M-Weizmann Collaborative Program, and Texas A&MNSFC Collaborative Program. We would like to thank Jeanne Santa Cruz for her assistance with the editing of this paper.

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Figure 1. Schematic illustration of the hierarchical MS structure. Growth factors bind with heparin and are encapsulated into heparin-conjugated gelatin nanospheres, which are further immobilized in a nanofibrous PLLA microsphere.

Figure 2.

(A) SEM image of hierarchical HG-MS. (B) SEM image of a typical HG-MS, showing that the microsphere was self-assembled with nanofibers. (C) A high-magnification image of (B), showing the nanofibers on the surface of the HG-MS. (D) A 2D cross-sectional confocal image of HG-MS, showing the gelatin nanospheres (red) encapsulated in nanofibrous microspheres (green). (E) Confocal image of a typical HG-MS showing the size and distribution of gelatin nanospheres in the nanofibrous microsphere. To visualize the nanospheres and microspheres, TRITC-conjugated gelatin (5% wt/wt) blended with HG was used and FITC-conjugated BSA was adsorbed on the nanofibers of the microspheres.

Figure 3.

SEM images of the HG-MS prepared with different ratios of DCM/EA. (**A, E**) DCM/EA=2/8. (**B, F**) DCM/EA=3/7. (**C, G**) DCM/EA=4/6. The HG-MS were fabricated from PLLA with an inherent viscosity of $M\eta=2.3$. (**D, H**) DCM/EA=3/7; this HG-MS was fabricated from PLLA with an inherent viscosity of $M\eta=1.0$.

Figure 4. Confocal images of HG-MS fabricated with different ratios of HG/PLLA. (A) 0%; (B) 1%; (C) 3%; and (D) 6%. (E) The effect of the HG /PLLA ratio on the encapsulation efficiency of HG nanospheres in the HG-MS. (F) The effect of the HG /PLLA ratio on the diameter of the HG nanospheres in the HG-MS. (* $P < 0.05$).

Figure 5.

(A) The encapsulation percent of BSA in microspheres fabricated with different ratios of HG/PLLA. (B) The release profiles of BSA from MS, G-MS, and HG-MS. (C, D) The release profiles of BMP2 (500 ng/mg MS) from MS, G-MS, and HG-MS. (E, F) The release profiles of BMP2 (50 ng/mg MS) from MS, G-MS, and HG-MS. In (B), (C), (D), (E) and (F), the HG-MS were fabricated with a HG/PLLA ratio of 6%. (* $P < 0.05$).

Figure 6.

(A) A confocal image of BMSCs adhering to HG-MS. The actin of the BMSCs was labeled red and the nuclei of the BMSCs were labeled blue. The HG nanospheres encapsulated in the microsphere were labeled green due to the adsorption of FITC-conjugated BSA. (B) The proliferation of BMSCs on the control (2D culture dish), BMP2-loaded MS and BMP2-loaded HG-MS. There was no significant difference of the proliferation rate among the three groups at each time point ($P>0.05$). (C) Alizarin red staining of the BMSCs cultured on control (2D culture dish), BMP2-loaded MS and BMP2-loaded HG-MS in conditioned media for 2 weeks. (D) Relative gene expression after the BMSCs were cultured on the control (2D culture dish), BMP2-loaded MS and BMP2-loaded HG-MS in conditioned media for 2 weeks.

Figure 7.

X-ray and μ -CT images of the calvarial bony defects 6 weeks after implantation. (**A, E**) Blank control group. (**B, F**) Implanted with MS (MS group). (**C, G**) Implanted with BMP2-loaded MS (MS+BMP2 group). (**D, H**) Implanted with BMP2-loaded HG-MS (HG-MS+BMP2 group). The amount of loaded BMP2 in (C) and (D) was 500 ng/mg MS. (**I**) The ratio of bone volume to total volume (BV/TV) ($n=4$). (* $P<0.05$).

Figure 8.

The H&E staining images (A-H) and Masson's trichrome staining images (J-Q) of the calvarial bony defects 6 weeks after implantation. (A, E) H&E staining of the blank control group. (B, F) H&E staining of the MS group. (C, G) H&E staining of the BMP2-loaded MS group. (D, H) H&E staining of the BMP2-loaded HG-MS group. (J, N) Masson's trichrome staining of the blank control group. (K, O) Masson's trichrome staining of the MS group. (L, P) Masson's trichrome staining of the BMP2-loaded MS group. (M, Q) Masson's trichrome staining of the BMP2-loaded HG-MS group. Green arrows in (F, G, H) indicate microspheres, and yellow arrows in (O, P, Q) indicate new formed blood vessels.

Figure 9.

The immunohistochemical staining images of the calvarial bone defects 6 weeks after implantation. (A) DMP1 in blank control. (B) DMP1 in MS. (C) DMP1 in BMP2-loaded MS. (D) DMP1 in BMP2-loaded HG-MS. (E) Col1 in blank control. (F) Col1 in MS. (G) Col1 in BMP2-loaded MS. (H) Col1 in BMP2-loaded HG-MS.

Table 1
Comparison of structural parameters among HG-MS with different processing parameters

Microspheres (DCM/EA)	Porosity (%)	Apparent density (g/cm ³)	Fiber diameter(nm)	Fiber length (nm)
2/8 ^a	94.40±0.61	0.070±0.007	528±159	3306±778
3/7 ^a	94.24±0.52	0.072±0.006	366±146	3875±692
4/6 ^a	91.04±0.64	0.112±0.008	281±72	2013±703
3/7 ^b	90.30±0.14	0.121±0.002	735±141	3944±1319

^aThe MS was fabricated using PLLA (4% wt/v) with $M\eta = 2.3$ dl/g.

^bThe MS was fabricated using PLLA (10% wt/v) with $M\eta = 1.0$ dl/g.