Supporting Information

Nanostructured Mineral Coatings Stabilize Proteins for Therapeutic Delivery

*Xiaohua Yu, Adam H. Biedrzycki, Andrew S Khalil, Dalton Hess, Mark D. Markel, William L. Murphy**

Dr. Xiaohua Yu, Prof. William L. Murphy, Department of Orthopedics and Rehabilitation, University of Wisconsin, Madison, WI 53705
E-mail address: wlmurphy@wisc.edu (W.L. Murphy)

Mr. Andrew S Khalil, Mr. Dalton Hess, Prof. William L. Murphy , Department of Biomedical Engineering, University of Wisconsin, Madison, WI 53706, USA

Prof. Adam H. Biedrzycki, Prof. Mark D. Markel, Comparative Orthopedic Research Laboratory, School of Veterinary Medicine, University of Wisconsin-Madison, Madison, WI 53705

Materials and Methods

Materials: Poly (Lactide-co-glycolide) (PLG, lactide:glycolide = 85:15, aveage Mw = 50, 000-70, 000) was from Sigma Aldrich (St. Louis, MO). Bovine serum albumin was from Fisher Scientific (Hampton, NH). Horseradish peroxidase (HRP) was from Sigma Aldrich (St. Louis, MO). Basic fibroblast growth factor (bFGF) was from R&D system (Minneapolis, MN). 125I-labeled bFGF was purchase from Perkin Elmer (Waltham, MA). Hydroxyapatite microparticles were obtained from Plasma Biotal LTD (Derbyshire, UK). NaCl, KCl, $MgSO_4$, $MgCl_2$, $NaHCO_3$, $CaCl_2$, KH_2PO_4, Tris. HCl, poly (vinyl alcohol) (PVA) were from Sigma Aldrich (St. Louis, MO). TMB stabilized substrate for horseradish peroxidase and CellTiter Blue Cell Viability Assay were from Promega (Madison, WI). Tranwell® Permeable Support was from Corning (Tewksbury, MA). Dulbecco's Modified Eagle's Medium (DMEM) was from CellGro (Mannassas, VA). UltraCulture serum-free medium was purchase from Lonza (Allendale, NJ). Fetal calf serum, trypsin, penicillin/streptomycin, and L-glutamine were from Hyclone (Logan, UT). Live/dead Cell Viability Assays were from Life Technologies. All the solvents and general use chemicals used for this study were purchased from Sigma Aldrich (St. Louis, MO).

PLG microspheres fabrication: PLG microspheres were fabricated using a water-in-oil-in-water (W/O/W) double-emulsion technique. Briefly, 100 µL of protein (10 mg/mL HRP or 1.0 mg/mL bFGF) in PBS (pH 7.40) was added into 1.0 mL 10% PLG in ethyl acetate. The mixture was sonicated using a Sonifer 250 (West Chester, PA) for 15 s. 1.0 mL of 1% PVA/7% ethyl acetate in DI water was immediately added into the resulting first emulsion and mechanically vortexed for 15 s to form the second emulsion. The resulting solution was then transferred in to a beaker containing 200 mL of 0.3% PVA in 7% ethyl acetate and further rigorously stirred for 4h to harden the microspheres. The PLG microspheres were collected by filtration through a 0.22 µm filter and then washed three times with DI water. The resulting microspheres were finally lyophilized for at least 2 days and stored at -20 ºC freezer. The plain PLG microspheres were prepared without addition of protein likewise.

Mineral coating of PLG microspheres: PLG microspheres were coated with a layer of mineral coating by incubating in mSBF with 100 mM NaHCO3 for 7 days (see mSBF receipt in Table S-1). mSBF was prepared bydissolving NaCl, KCl, $MgSO_4$, $MgCl_2$, $NaHCO_3$, $CaCl_2$, KH_2PO_4, and Tris.HCl in DI water, and pH was adjusted by adding HCl and NaOH solution to 7.40. The resulting mineral coated PLG microspheres were filtered through a 35 μm cell strainer to exclude the large aggregates. The collected mineral coated PLG microspheres was lyophilized and stored at -20 ºC freezer.

Fabrication of bone-mimic mineral coating and protein binding on MCM: Hydroxyapatite microparticles with diameter between 3~5 μm (Plasma Biotal LTD, UK) were incubated in mSBF (pH 6.80) to form a layer of MCMs covering the microparticles. Each 100 mg hydroxyapatite microparticles were incubated in 50 mL mSBF at 37 ºC for 7 days with daily solution refreshment. All MCM except nanostructure screen were grown in mSBF with 100 mM $NaHCO_3$. For MCM nanostructure screening, the concentration of $NaHCO_3$ was systematically changed from 4.2 to 150 mM (Table S-1). The resultant MCM were washed in de-ionized water and lyophilized.

Mineral coating on absorbable collagen sponge (ACS): Absorbable collagen sponge was purchased from Medtronic as part of INFUSE® bone graft. ACS was cut into disks (5 mm in diameter, 1 mm thick) and soaked in mSBF with 100 mM $NaHCO_3$ at 37 ºC for 7 days with daily solution refreshment. Mineral coated ACS (MCACS) and ACS was examined by SEM.

Protein binding on MCMs: For BSA, each of 5.0 mg MCMs was incubated in 1.0 mL 100 μg/mL of BSA in PBS at 37 °C for 4 h with rotation. After binding, the remaining concentration of BSA in the solution was measured by microBCA protein assay (Thermo Fisher, Rockford, IL).

For HRP, each of 5.0 mg MCMs was incubated in 1.0 mL 10 μg/mL of HRP in PBS at 37 °C for 4 h with rotation. After binding, the remaining concentration of HRP in the solution was measured by microBCA protein assay (Thermo Fisher, Rockford, IL).

For bFGF, radiolabeled bFGF (^{125}I-labeled bFGF) was used to determine the binding efficiency. Briefly, each of 5.0 mg MCMs was incubated in 1.0 mL 10 ng/mL or 100 ng/mL bFGF (with 0.1% ^{125}I bFGF) in PBS at 37 °C for 4 h with rotation. The MCMs were then centrifuged at 11,000 g for 2 min and washed with 1.0 mL PBS once. The radioactivity in the supernatant and washing PBS was measured by a Packard Cobra II Gamma Counter (Perkin Elmer, Waltham, MA) to determine the remaining bFGF concentration after binding.

All protein binding efficiencies were calculated from the protein concentration change before and after binding.

BSA circular dichroism (CD) spectra measurement: The secondary structure of BSA after organic solvents treatment was measured by a circular dichroism spectrometer (Model 420, Aviv) (Lakewood, NJ) at 20 ºC. BSA recovered from various treatments was quantified and diluted to 100 μg/mL in PBS. CD spectra were recorded with a 1 mm path length quartz curvette. Data were acquired at bandwidth of 1.0 nm, response of 3 s and each measurement was repeated at least three times and the average value was plotted.

Stability study of proteins bound on MCM against external stresses: 800 μL organic solvent was added into 5.0 mg protein bound MCM or 200 μL protein solution in PBS in a 1.5 mL centrifuge tube and the mixture was kept vortexing for different time periods. Control groups without addition of solvent were prepared accordingly. The solvent was then removed by vacuum drying after treatment and all the samples were reconstituted with PBS.

In another experiment, 1.0 mL 0.05% trypsin was added into 5.0 mg bFGF bound MCM or 10 μL 100 μg/mL bFGF in PBS in a 1.5 mL centrifuge tube and the mixture was kept rotating at 37oC for 1 h. Control groups without trypsin treatment were prepared to calculate the loss of bFGF activity. After the treatment, trypsin was removed from MCM-containing groups and MCM was resuspended in medium. bFGF in PBS was diluted to 100 ng/mL using medium and added to hDF culture at a final concentration of 1.0 ng/mL.

Reconstituted proteins samples were then subjected for their activity measurement using specific assay for each type of protein. An enzymatic assay was used to evaluate HRP activity. A human dermal fibroblast proliferative assay was performed to assess the bioactivity of bFGF (see Supporting Information for details). The protein activity recovered from solvent exposure was calculated with respect to each of their own control group activity (no solvent treatment).

BSA precipitation and secondary structure characterization after organic solvent exposure: BSA solution and BSA bound MCMs were treated with organic solvents for 12 h via vortexing. After removal of organic solvents, the protein bound on MCMs was eluted by soaking in 500 mM Na_2HPO_4 (pH 7.40) with rotation for 1 h. BSA was then transfer into PBS through a buffer exchange and quantified using micro BCA kit (Thermo Scientific, NJ). For BSA in PBS, the remaining protein after solvent exposure was quantified. The secondary structure of BSA after organic solvent treatment was characterized with a circular dichroism spectroscopy (Model 420, AVIV, NJ) at 20 ºC with a 1 mm path length cuvette.

HRP activity measurement: HRP activity was measure by catalyzing the reaction of 3,3',5,5'-tetramethylbenzidine (TMB) to 3,3',5,5'-tetramethylbenzidine diimine. To measure HRP activity in PBS after various treatments, the recovered HRP was quantified by a MicroBCA kit and diluted to 1.0 μg/mL in PBS. 50 μL HRP sample was added into 150 μL TMB substrate in each well of 96-well plate to develop the color. After incubate at room temperature in dark for 5 min, the reaction was stopped by addition of 50 μL 2 N H_2SO_4. The absorbance of each sample was measured at 450 nm on a Molecular Device microplate reader (Sunnyvale, CA). To measure the activity of HRP bound on MCMs, 200 μL TMB was added into 1.0 mg of HRP bound MCMs in a microcentrifuge tube and incubated on a rotator for 5 min after vortexing. The tube was then centrifuged at 10,000 g for 30 s and 100 μL of the supernatant was transferred into a well of 96-well plate. The absorbance was measured at 450 nm after adding 50 μL of 2 N H_2SO_4. The HRP activities of both PBS and MCMs without any treatment were set as 100%.

bFGF bioactivity evaluation: Human dermal fibroblast (hDF) was maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2% fetal calf serum, 100 unit/ml penicillin and 100 μg/ml streptomycin at 37 °C with 5% CO_2. The medium was changed every other day and hDF were used up to passage 11.

To facilitate hDF adhesion in serum free medium, 24-well plate was coated with 0.1% gelatin for 2 h before cell seeding. hDF were harvested from T75 flasks after trypsinization and resuspended in UltraCulture serum-free medium supplemented with 2 mM L-glutamine, 100 unit/ml penicillin and 100 μg/ml streptomycin. The cells were seeded into gelatin coated 24-well plates at 10,000/well and incubated for overnight to allow cell attachment. For untreated/treated bFGF in PBS, final bFGF concentrations of 1.0 and 10.0 ng/mL were used to induce hDF proliferation. For untreated/treated bFGF bound on MCMs, 3.0 mg MCMs containing different amount of bFGF was suspended in 200 μL serum-free medium and added into a transwell placed on top of hDF (Fig S-3). After 72 h of culture, CellTiter-Blue Viability Assay (Promega, WI) was conducted to evaluate the proliferation of

hDF. The group received only blank medium was used as the control group and all the experimental groups were normalized to the control group. Each group contains four replicates.

Evaluation of bFGF activity released from different particles: bFGF encapsulated PLG microspheres (~13 ng/mg) were fabricated using a W/O/W double emulsion[26]. Plain PLG microspheres were coated with MCMSs using the same procedure as previously described. bFGF was bound to MCMs at ~13 ng/mg calculated based on bFGF binding efficiency. 3.0 mg of each type of bFGF loaded microspheres was re-suspended in 200 µL UltraCulture medium and added into a transwell insert in a 24 well seeded with 10,000 hDF cells/well. At each time point, the Transwell insert was transferred into a new well seeded with the same number of hDF 12 h in advance. CellTiter Blue assay was performed to evaluate the growth of hDF in each group. hDF proliferation was normalized to the blank control which only received UltraCulture medium. Live/dead staining was conducted at each time point following CellTiter Blue assay. Micriparticles were collected at the end of release study and subject to scanning electron microscopy. Significant microparticle agglomeration was observed in PLG, while MCM showed no noticeable agglomeration after 35 days of release (Fig. S-10), indicating MCM helped maintain integrity of the microparticle during release while PLG accelerate protein denaturation via polymer degradation.

Evaluation of bFGF activity released from ACS and MCACS: 10 ng bFGF was absorbed on ACS and MCACS, respectively by dripping bFGF solution on the scaffolds. The scaffolds were then dried in biosafety cabinet for overnight. The two types of scaffolds were then soaked in ethyl acetate for 2h with gentle agitation. Then the scaffolds were taken out of the solvent and dried in vacuum dryer. The bioactivity of bFGF released from the scaffold before and after entyle acetate treatment was evaluated using human dermal fibroblast proliferation assay described previously.

Evaluation of growth factor release from tendon tissue ex vivo: Nylon braided sutures of size 4-0 and 6-0 USP were generated using the mSBF and incubated at two time points with two different growth factors (VEGF and bFGF). The radioactive isotope for the growth factors used in suture incubation was at a concentration of 0.015%. After the formation of the first layer mineral coating, the first growth factor was applied by incubating the 5 cm coated suture in 10 mL growth factor solution in 15 mL conical tube for 4 h; then the suture was incubated in mSBF for another 6 days to form the second layer. The second growth factor was then loaded onto the superficial layer of the coating. A 5 cm section of suture material was passed through samples of rabbit lateral gastrocnemius tendon six times via a tunneling technique. The suture was then knotted with a single throw. The suture (passage and knotted) was then incubated in SBF. The tendon tissue, through which the 5 cm section of suture was passed through, was also placed in SBF. Radioactive release from the and from the tissue was monitored using a scintillation counter (Packard Gamma Counter, Cobra II, GMI-Inc, Ramsey, MN). SBF was changed with each radioactivity reading. Fresh SBF was generated on a per use basis. Readings were made for each level at 2,4,6,8,10,14,18,21,28,34, 42 and 48 days.

Rabbit Achilles tendon healing model: The animal study was approved by the UW-Madison Animal Care and Use Committee (Protocol V01554). 4 month-old New Zealand while rabbits with average weight of 2-3 kg were used for this study. After anesthesia with isoflurane, a 15 mm skin incision was made centered 15 mm proximal to the calcaneus on the lateral aspect of the tibia, the lateral tendon was sharply transected 15 mm proximal to the calcaneus with a #15 scalpel blade. The transection was immediately repaired with a single locking loop of 4-0 braided nylon (Surgilon, Covidien, Mansfield, MA) coated with bone-like mineral containing different growth factor combinations. Once the body of the tendon was repaired, a circumferential repair of the paratenon was applied in a Silfaverskiold epitendinous reapir using using 4-0 braided polyglactin 910 (Vicryl, Ethicon, Cincinnati, OH) (also coated with same coating containing growth factors identical to the central core suture). The subcutaneous fascia was sutured using 6-0 polyglactin 910 (Vicryl, Ethicon, Cincinnati, OH) in a

simple continuous pattern and the skin was sutured using 3-0 polypropylene (Prolene, Ethicon, Cincinnati, OH) in a cruciate pattern.

Biomechanical testing: tendons used for biomechanical tests were harvested four weeks after the surgery. The entire calcaneal-tendon-muscle unit was then placed in a 50 ml sealed tube and filled with saline solution until mechanical testing. Collected tendons were tested in uniaxial tension using a MTS 858 Bionix Test System (MTS Systems Corp., Eden Prairie, MN) with a 2225 N load cell. Tendons were preconditioned with 10 cycles to 3% strain at a rate of 2.5 mm/s. Tendons were stretched at a rate of 2.5 mm/s to failure. Force and elongation data were continuously sampled at a rate of 50 Hz. Elongation was used to compute the average strain along the tendon. Ultimate stress was taken as the peak stress reached during loading.

Histological analysis: The excised portion of the tendon was then placed in OCT solution (Tissue-Tek, VWR, Radnor, PA), in a cryoblock (Tissue Path™ Disposable Base Molds, 34 mm x 24 mm x 5 mm, Fisher Scientific, Pittsburgh, PA). The tissue was then cryo-sectioned with thickness of 7μm. After fixation of the tissue in 10% formaldehyde, slides were washed in distilled water and then stained in alcian blue solution (Sigma-Aldrich Corp. St. Louis, MO) for 30 minutes. Slides were then placed under running tap water for 2 minutes, followed by rinsing in distilled water. A nuclear counterstain in fast red solution (Sigma-Aldrich, St. Louis, MO) was then performed for 5 minutes, followed by rinsing in tap water for 1minute. The dehydration procedure through 95% ethanol, 3 changes of 100% ethanol for 1 minute and 10 dips each occurred, as previously specified. Slides were then cleared with xylene and mounted with cytoseal 60 and cover slipped.

Two random longitudinal samples from each rabbit section were subjected to CD31 antibody for evaluation of neovascularization. Briefly, slides were blocked by using 1% normal donkey serum (Abcam, Cambridge, MA). Primary antibody anti-CD1 (Abcam, Ab52128) was applied to the sections. After washing with PBS, the staining signals were visualized with the secondary antibody was a polyclonal IgG donkey anti-mouse heavy and light chain (Alexa Fluor 488) with at 1:200 dilution in PBS and 1.5% normal donkey serum. Slides were then again washed in 1X TBS for 15 minutes each time. Fluoroshield mounting media with 4',6-diamidino-2-phenylindole (DAPI) was then applied, the slides were coverslipped and placed in a dark container until visualization under a florescence microscope for 30 minutes at room temperature.

Statistical analysis: All quantitative results are expressed as means $\pm$ standard deviations. The statistical difference was analyzed using one-way analysis of variance (ANOVA), and $p<0.05$ was considered significant.

Supplementary Figure

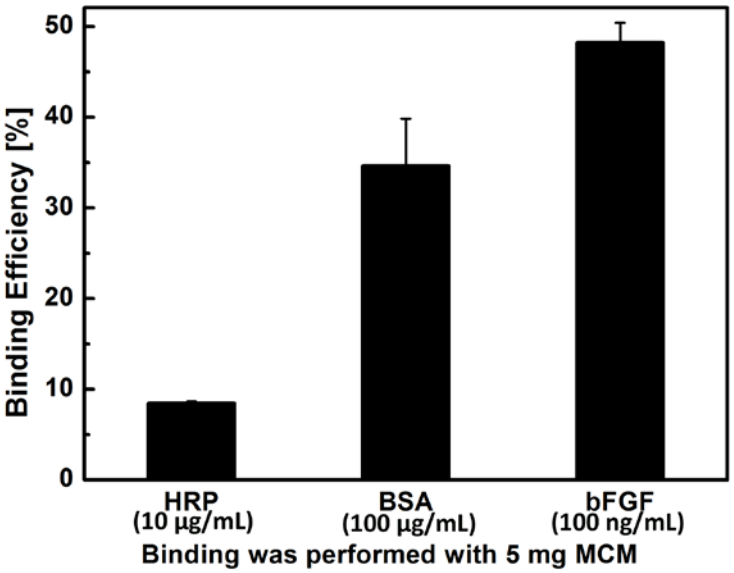

Figure S-1 Binding efficiencies of various proteins on MCM.

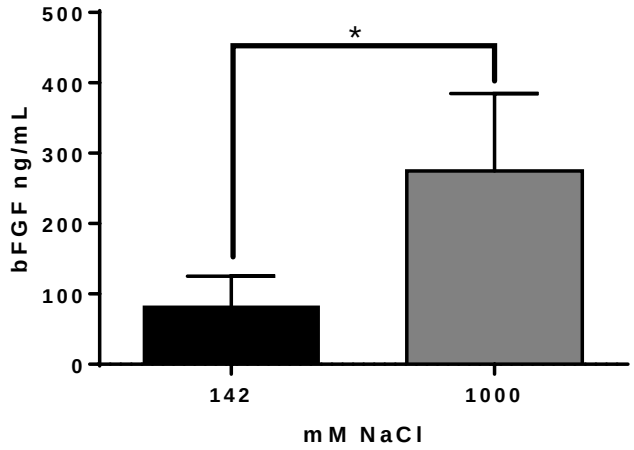

Figure S-2. 3mg 4.2mM bicarbonate MCMs were incubated in 1 mL of 1 µg/mL bFGF for 1 hr. bFGF was released in either SBF (142mM NaCl) or mSBF containing 1000mM NaCl. After 3 days, the release media was sampled and bFGF concentration was assayed via ELISA. Increase in NaCl concentration from 142mM to 1000mM resulted in a 330% increase in bFGF release.

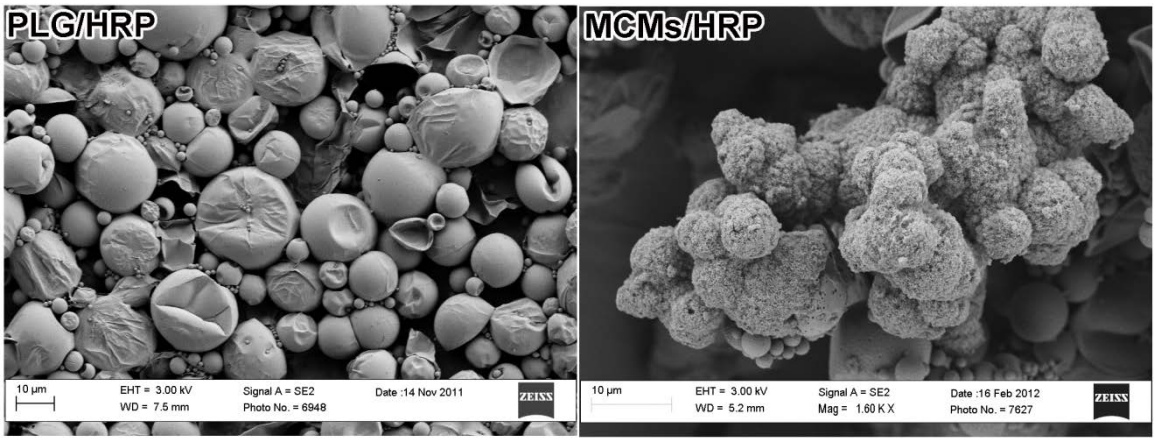

Figure S-3 SEM images of HRP loaded microparticles: PLG/HRP: HRP encapsulated PLG microspheres. MCMs/HRP: HRP bound on mineral coated plain PLG micrespheres

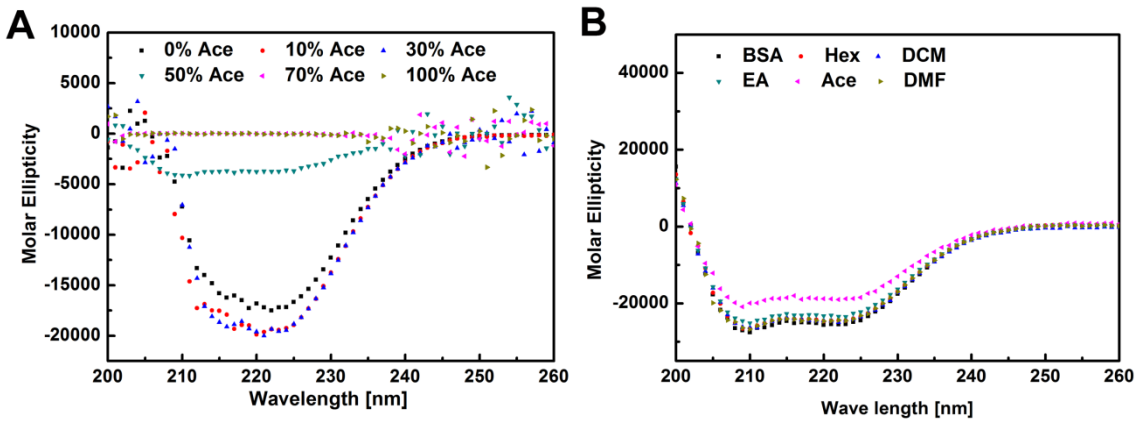

Figure S-4 CD spectra of BSA after organic treatments: A) CD spectra of BSA with varying acetone concentration present in PBS during CD measurement. B) CD spectra of BSA recovered after various solvent treatments (BSA recovered from solvent treatment and reconstituted in PBS).

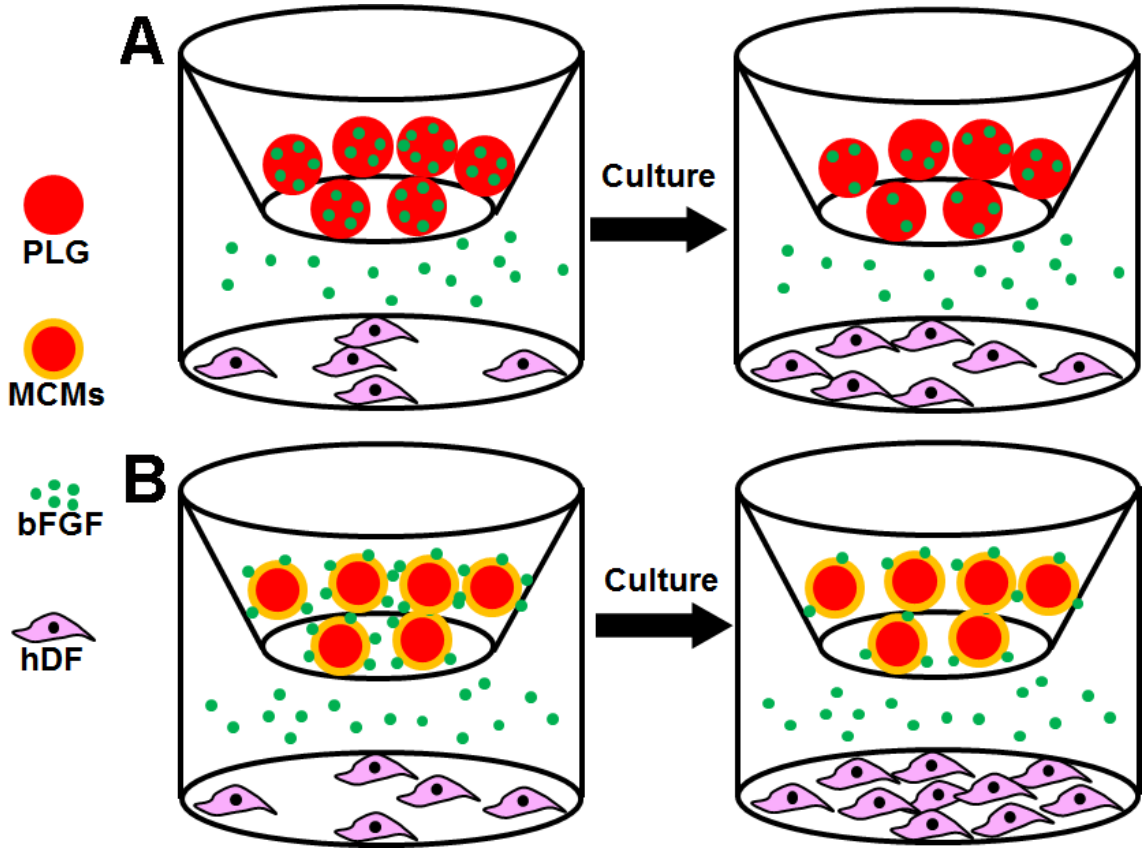

Figure S-5 hDF transwell proliferation assay comparing bFGF bioactivity from different deliver vehicles: A) bFGF was encapsulated in PLG microspheres and released to hDF on the bottom of the well through the transwell membrane, then stimulated hDF growth. B) bFGF was bound on the mineral coating surface of PLG microsphres and released to hDF seeded on the bottom of the well, then stimulated hDF growth.

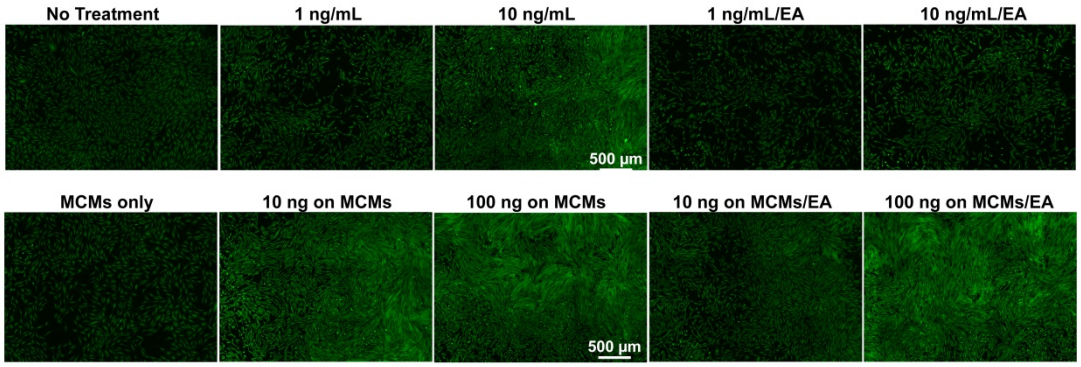

Figure S-6 Live/dead staining of hDF treated with different bFGF conditions for 72 h.

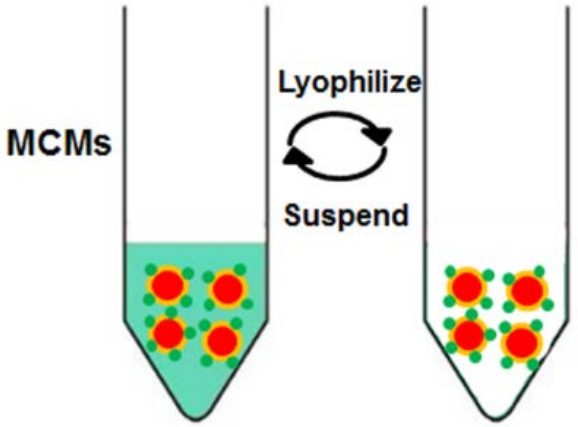

Figure S-7 Impact of freeze drying cycles on stability of bFGF loaded on MCMs: bFGF bound on MCMs was lyophilized then re-suspened in PBS; this cycle was repeated for multiple times.

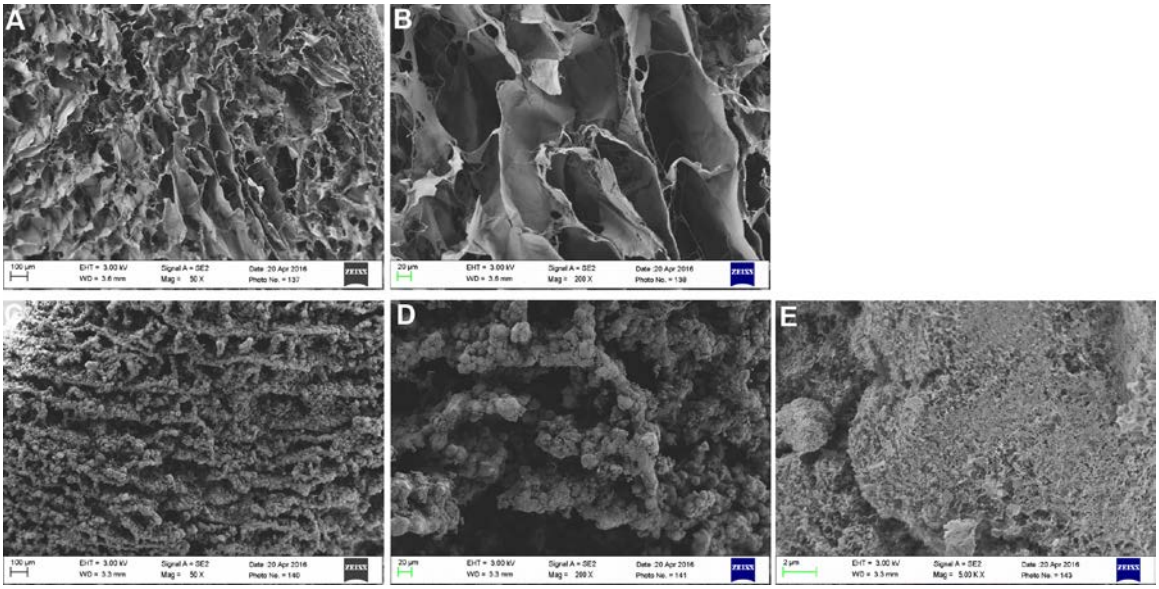

Figure. S-8 SEM micrographs of mineral coated absorbable collagen sponge (ACS): A) uncoated ACS, low mag; B) uncoated ACS, high mag; C) Mineral coated ACS, low mag; B) Mineral coated ACS, high mag; E) Morphology of mineral on ACS is similar to microspheres.

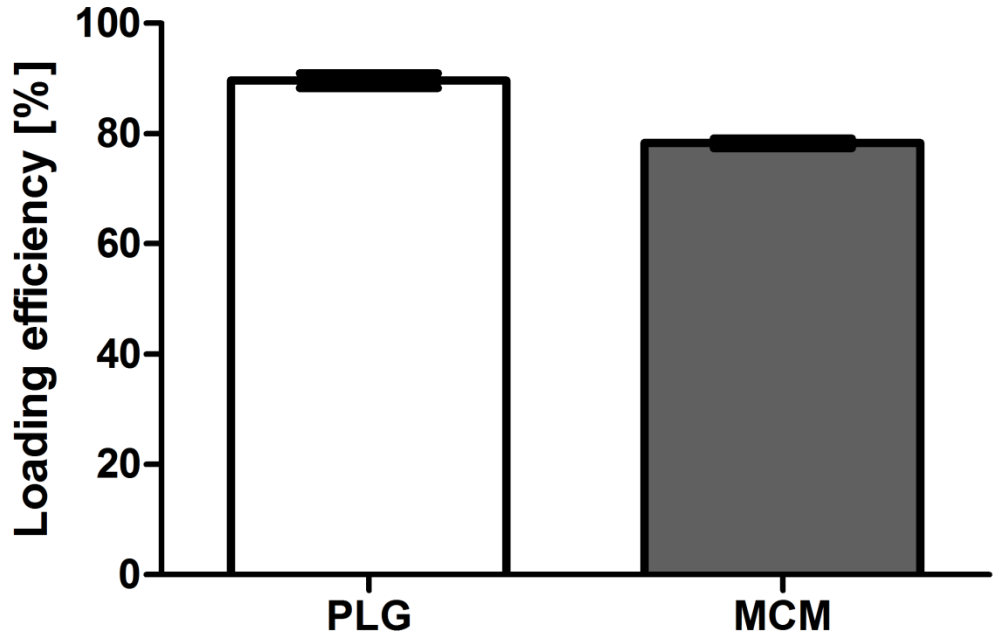

Figure. S-9 Loading efficiency of two types of microparticles: PLG prepared with w-o-w double emulsion and bFGF was incorporated into PLG during emulsion; for MCM, bFGF was bound on to MCM via incubation in bFGF solution.

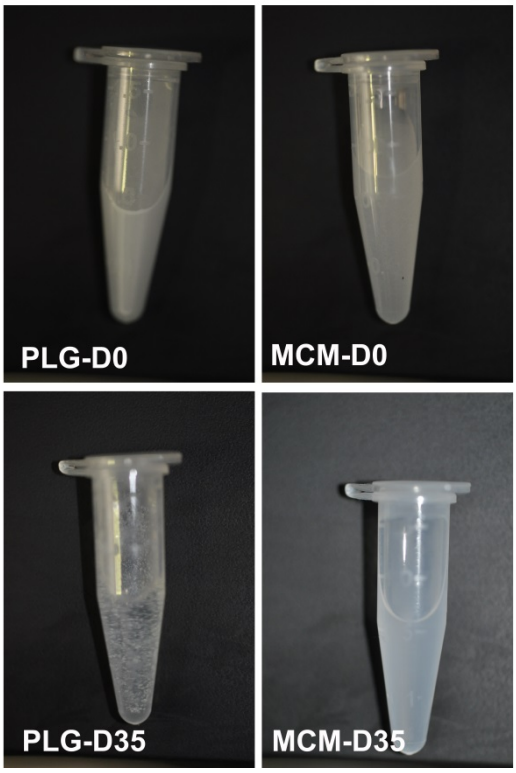

Figure. S-10 Agglomeration of microparticles after release *in vitro*: PLG microparticles severely agglomerated into big particles; MCM did not show noticeable agglomeration after 35 days of release.

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