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Biodegradable Polymerized Simvastatin Stimulates Bone Formation

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Abstract

Previous research from our labs demonstrated the synthesis of polymerized simvastatin by ring-opening polymerization and slow degradation with controlled release of simvastatin *in vitro*. The objective of the present study was to evaluate the degradation and intramembranous bone-forming potential of simvastatin-containing polyprodrugs *in vivo* using a rat calvarial onlay model. Poly(ethylene glycol)-*block*-poly(simvastatin) and poly(ethylene glycol)-*block*-poly(simvastatin)-*ran*-poly(glycolide) were compared with simvastatin conventionally encapsulated in poly(lactic-co-glycolic acid) (PLGA) and pure PLGA. The rate of degradation was higher for PLGA with and without simvastatin relative to the simvastatin polyprodrugs. Significant new bone growth at the circumference of poly(ethylene glycol)-*block*-poly(simvastatin) disks was observed beginning at 4 weeks, whereas severe bone resorption (4 weeks) and bone loss (8 weeks) were observed for PLGA loaded with simvastatin. No significant systemic effects were observed for serum total cholesterol and body weight. Increased expression of osteogenic (BMP-2, Runx2, and ALP), angiogenic (VEGF), and inflammatory cytokines (IL-6 and NF-κB) genes was seen with all polymers at the end of 8 weeks. Poly(ethylene glycol)-*block*-poly(simvastatin), with slow degradation and drug release, controlled inflammation, and significant osteogenic effect, is a candidate for use in bone regeneration applications.

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Conflict of interest Statement

DAP has an equity interest in Regenera Materials, LLC, a start-up company for commercializing novel biomaterials for tissue regeneration.

Data Availability

Data will be made available upon request.

Graphical Abstract

Keywords

controlled release; degradable biomaterials; osteogenesis; prodrugs; simvastatin

1. Introduction

Simvastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, was first reported to upregulate the expression of bone morphogenetic protein (BMP) family members, including BMP-2, by activating the promoter region [1]. The osteogenic effect of simvastatin was subsequently demonstrated in numerous cell-based and *in vivo* studies [2-4]. Simvastatin promotes osteoblast differentiation from stem cells by increasing the expression of osteocalcin and osteonectin genes [5] and via the membrane-bound Ras/Smad/Erk/BMP-2 pathway [6]. Statins also inhibit osteoclast differentiation by inhibiting Src [7] and reactive oxygen species (ROS) [8] mediated signaling pathways, and they promote angiogenesis by increasing the expression of vascular endothelial growth factor (VEGF) in a dose-dependent manner [9].

Oral administration of simvastatin results in increased accumulation at the liver, where it is converted to its active dihydroxy acid form. It undergoes significant first pass metabolism resulting in very low systemic availability (<5%) [9]. With the current oral regime for simvastatin, it is not possible to achieve the desired bone growth at any target site because the therapeutic effect depends on the local concentration. Injecting simvastatin locally was found to increase both osteogenesis and angiogenesis around titanium alloy mini-screws in ovariectomized rats, but the risk of rhabdomyolysis restricts the amount of simvastatin that could be delivered locally [10].

Several approaches have been explored for developing carrier systems that could release the drug continuously at the target site, prevent foreign body response and fibrous capsule formation, act as a scaffold for infiltration of cells and cell growth, and promote bone formation and regeneration [9]. For example, single application of 2.2 mg of simvastatin in methylcellulose gel entrapped within a polylactide membrane significantly increased bone growth, but the animals exhibited ulceration, edema, and scab formation because of the high

dose [11]. Similarly, the combination of simvastatin with calcium sulfate enhanced bone regeneration, in spite of significant inflammation (e.g., bone growth observed only after soft tissue inflammation caused by the burst release of simvastatin was reduced after ~5 weeks) [12].

Previous research from our lab demonstrated the bone-forming ability of lower doses (5-10 µg) of simvastatin hydroxyacid, the active form of the drug. The hydroxyacid was delivered locally to the rat calvarium with either an intermittent or sustained release pattern using an association polymer system. Interestingly, intermittent release stimulated a greater response in bone thickness and area than did sustained release. Limitations of this system, however, include erosion of the drug delivery component by 28 days, limited total drug loading, and the need for a slow-degrading shell (poly(lactic-co-glycolic acid)) to provide directional release elicited an inflammatory response [13].

As an alternative to these carrier-based systems, we polymerized simvastatin into a degradable polymer by ring-opening polymerization and demonstrated slow degradation of the simvastatin-containing polyprodrug *in vitro* [14]. These polyprodrugs provide high drug loading efficiency, molecular weight, and mechanical strength. In light of this, the objective of the present study was to evaluate the degradation and intramembranous bone-forming potential of simvastatin-containing polyprodrugs in the absence of a bone defect *in vivo* using a rat calvarial onlay model.

2. Experimental Section

2.1 Simvastatin containing polyprodrug synthesis

Poly(ethylene glycol)-*block*-poly(simvastatin) was synthesized by reacting monomethyl ether poly(ethylene glycol) (1 eq, Mw: 5k, Sigma-Aldrich, St. Louis, MO) with simvastatin (100 eq, Haorui Pharma-Chem, Edison, NJ) with triazabicyclodecene (1 wt%, Sigma-Aldrich, St. Louis, MO) as catalyst [15]. Poly(ethylene glycol)-*block*-poly(simvastatin)-*ran*-poly(glycolide) was prepared by adding glycolide (40 eq, Polysciences Inc, Warrington, PA) to the poly(ethylene glycol)-*block*-poly(simvastatin) reaction mixture [16].

2.2 Implant disks

Non-porous disks of poly(ethylene glycol)-*block*-poly(simvastatin) and poly(ethylene glycol)-*block*-poly(simvastatin)-*ran*-poly(glycolide) were formed by compressing (at 6000 lb_f for 5 mins) the copolymer in a cylindrical dye (2 X 6 mm) using a Carver press. Samples were then annealed at 30 °C and 60 °C for 24 hr each to form disks with a final mass of 50 mg. Poly(lactic-co-glycolic acid) (PLGA; 75:25, M_w 56.2 kDa) and simvastatin-loaded PLGA (5 wt% simvastatin and 10 wt% PLGA in dichloromethane) was solvent cast into disks (2 X 6 mm; 100 mg final mass) and used for comparison.

2.3 Implantation on the rat calvarium

This animal study was conducted at the University of Kentucky in accordance with a protocol approved by the Institutional Animal Care and Use Committee (2014-1288). Following administration of anesthesia and skin preparation, a transverse incision was made

at the caudal end of the calvarium of male Sprague-Dawley rats (250-275 g). The periosteum was reflected anteriorly using a periosteal elevator to create a pocket that exposed the calvarium. Polyprodrug and PLGA implants were placed directly on the calvarium beneath the periosteum, and the incision was closed with clips (Supplementary Figure S1). A total of 144 rats were used, six for each formulation and time point. Implants and adjacent tissues were retrieved and analyzed at 1, 2, 3, 4, 6, and 8 weeks. Separate samples were used for gel permeation chromatography (n=3) and microcomputerized tomography (n=3).

2.4 Gel permeation chromatography

After drying, retrieved samples were dissolved in tetrahydrofuran (10 mg/ml), filtered (0.45µm filter) and analyzed for the weight average molecular weight using two ResiPore columns (300 X 7.5 mm, 3 µm particle size; Agilent Technologies) in series in a Shimadzu Prominence LC-20 AB HPLC system with Waters 2410 refractive index detector. Polystyrene (Mw: 162 Da -364 kDa, Agilent, Santa Clara, CA) was used as standard and samples were injected at flow rate of 1mL/min at 40 °C [14].

2.5 Microcomputerized tomography

MicroCT was used to evaluate the morphology of the calvarium under the implant. After euthanasia, calvaria were removed and fixed in 10% buffered formalin. Samples were analyzed using a Scanco µCT 40 (70kVp, 114µA, 8W) under high resolution, and 3D images were reconstructed using the manufacturer's software Version 6.1.

2.6 Histology

Following microCT, the calvaria were decalcified using 40% formic acid in 10% formalin for 7 days. Each calvarium was cut into two halves at the mid-coronal plane and embedded in paraffin with the implant side facing up. Tissues were cut coronally (5 µm) and the sections were stained with hematoxylin and eosin.

Measurement of the woven bone thickness was performed on images captured using an Olympus IX51 inverted microscope under bright field. The region between the fibrous capsule surrounding the implant and calvarium at the right and left extremities was assessed. Six different slides for each group and time point were analyzed.

2.7 Quantitative Real time Polymerase Chain Reaction (qRT-PCR) analysis

Fibrous capsule surrounding the implant was used for qRT-PCR. Total RNA from each capsule (n=36, 3 samples/polymer) for weeks 1, 4, and 8 was isolated using The RNeasy® Plus Mini Kit. cDNA was synthesized using The Transcriptor First Strand cDNA Synthesis Kit from Roche following the manufacturer's protocol [17]. The primers for each gene analyzed include: glyceraldehyde 3-phosphate dehydrogenase (GAPDH): Forward: ACTCCATTCTTCCACCTTTG, Reverse: CCCTGTTGCTGTAGCCATATT, bone morphogenetic protein 2 (BMP-2): Forward: TGTGAGGATTAGCAGGTCTTTG, Reverse: TTGTGGAGTGGATGTCCTTTAC, Runt-related transcription factor 2 (Runx2): Forward: CGCATTCCTCATCCAGTAT, Reverse: CTGACTCTGTCCTTGTGGATTA, alkaline phosphatase (ALP): Forward: AACAACTGACTGACCCTTC, Reverse: ATCCTGCCTCCTTCCACTA, vascular endothelial growth factor (VEGF): Forward:

GATGACGACGACGAAGAGATG, Reverse: TCTTCGATGATGCTGACCAC, interleukin 6 (IL-6): Forward: GAAGTTAGAGTCACAGAAGGAGTG, Reverse: GTTTGCCGAGTAGACCTCATAG, nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B): Forward: AGACATCCTTCCGCAAACCTC, Reverse: TAGGTCCATCCTGCCCATAA.

2.8 Systemic effect

Body weights were measured every week post-surgery to monitor animal health and also to determine systemic effect of the released drug. Total cholesterol in the serum was estimated by using cholesterol reagent in Beckman Coulter AU5400®.

2.9 Statistical analysis

Quantitative measurements are expressed as mean $\pm$ standard deviation (SD). Differences in response to different polyprodrugs and the response over time were evaluated by one-way and two-way ANOVA using IBM SPSS Statistics Version 20. A $p < 0.05$ was considered statistically significant. Turkey HSD post-hoc test was performed for statistically significant ANOVA results.

3. Results

Poly(ethylene glycol)-*block*-poly(simvastatin) and poly(ethylene glycol)-*block*-poly(simvastatin)-*ran*-poly(glycolide) of Mw 36.7 and 17.4 kDa, respectively, were synthesized by ring-opening polymerization (Figure 1A). Disks of simvastatin-containing polyprodrug, PLGA loaded with simvastatin, and pure PLGA were used for *in vivo* analysis (Figure 1B).

3.1 Visual observations

Rats used in the current study tolerated all the implants with no adverse effects, except for PLGA loaded with simvastatin. Fibrous capsules were found surrounding all implants beginning at 1 week post-implantation until the final time point analyzed (Supplementary Figure S1). Hematomas were initially observed with all four types of implants, which resolved more quickly from 2 weeks post-implantation for poly(ethylene glycol)-*block*-poly(simvastatin) compared to the other implants. Skin ulceration was observed for a small number of rats ($n=3/36$) implanted with poly(ethylene glycol)-*block*-poly(simvastatin)-*ran*-poly(glycolide) at 1 week post-implantation, although the lesions healed by 3 weeks without any treatment. Severe inflammation (edema and a dense fibrous capsule) was observed at 4 and 8 weeks post-implantation for all rats with PLGA loaded with simvastatin. Most poly(ethylene glycol)-*block*-poly(simvastatin)-*ran*-poly(glycolide) implants were degraded by the end of 8 weeks.

3.2 Degradation of polymers in vivo

Figure 2 shows changes in the molecular weight of the implant polymers in relation to time of implantation. The rate of degradation was high for PLGA loaded with simvastatin (~ 3.1 kDa/d) followed by PLGA (~ 2.6 kDa/d), poly(ethylene glycol)-*block*-poly(simvastatin)-*ran*-poly(glycolide) (~ 294 Da/d) and poly(ethylene glycol)-*block*-poly(simvastatin) (~ 26 Da/d).

Based on the measured molecular weights and our previous analysis of the polymeric prodrugs [16], each sample of poly(ethylene glycol)-*block*-poly(simvastatin) and poly(ethylene glycol)-*block*-poly(simvastatin)-*ran*-poly(glycolide) contained about 39 mg and 30 mg simvastatin, respectively. From the molecular weights decreases over 8 weeks, we calculated release of about 10.0 and 1.71 μg of simvastatin from poly(ethylene glycol)-*block*-poly(simvastatin) and poly(ethylene glycol)-*block*-poly(simvastatin)-*ran*-poly(glycolide), respectively. In comparison, the PLGA samples released tens of mg simvastatin (loaded with 50 mg) over the same time course.

3.3 Tissue responses

MicroCT analysis (Figure 3) demonstrated significant new bone growth at the circumference of poly(ethylene glycol)-*block*-poly(simvastatin) disks beginning at 4 weeks. Conversely, severe bone resorption (4 weeks) and bone loss (6 and 8 weeks) were observed with PLGA loaded with simvastatin. No notable effect was observed with the PLGA and poly(ethylene glycol)-*block*-poly(simvastatin)-*ran*-poly(glycolide) disks.

Hematoxylin and eosin staining revealed a fibrous capsule surrounding the implants, vascularization, and osteoid formation with all polymer groups (Figure 4). Significant bone growth was observed for poly(ethylene glycol)-*block*-poly(simvastatin), which contrasted with bone loss and necrosis for PLGA loaded with simvastatin. More blood vessels were seen within the newly formed bone for poly(ethylene glycol)-*block*-poly(simvastatin). The thickness of woven bone formed between the fibrous capsule and the calvarium at the right and left edges of the implant was measured (Figure 5). There was a significant difference between the right and left sides ($p < 0.05$) as well as between polymers ($p < 0.001$) at week 1 and 8. Post hoc analysis showed a significant difference ($p < 0.05$) between the polymers except for PLGA and poly(ethylene glycol)-*block*-poly(simvastatin)-*ran*-poly(glycolide) at weeks 1 and 8, and between PLGA loaded with simvastatin and poly(ethylene glycol)-*block*-poly(simvastatin) at week 8. Thickness of the woven bone was greater with PLGA loaded with simvastatin at week 1 (Left: $217 \pm 15 \mu\text{m}$; Right: $206 \pm 19 \mu\text{m}$) (Figure 5A) and poly(ethylene glycol)-*block*-poly(simvastatin) at week 8 (Left: $219 \pm 16 \mu\text{m}$; Right: $276 \pm 24 \mu\text{m}$) (Figure 5B).

3.4 Expression of osteogenic, inflammatory, and angiogenic genes

Changes in the expression of molecular markers of inflammation, angiogenesis, and osteogenesis are shown in Figure 6. Levels of individual genes were normalized relative to GAPDH for each animal, and gene expression in response to simvastatin-containing polymers was normalized with respect to control PLGA. There was an increase in the expression of the osteogenic genes BMP-2, Runx2, and ALP in response to all polymers. Expression of the angiogenic gene VEGF was also in line with the osteogenic genes, and the inflammatory cytokines IL-6 and NF- κ B were high with all polyprodrugs. However, there were no significant differences between the type of polymer and its effect over time for each gene.

3.5 Systemic response

Systemic effects of the released simvastatin were assessed by measuring the body weight (Figure 7A) and serum total cholesterol (Figure 7B). There was a steady increase in the body weight in response to all the polyprodrugs, and differences were not statistically significant. Similarly, no significant differences were observed for serum total cholesterol level, although levels trended slightly lower with poly(ethylene glycol)-*block*-poly(simvastatin).

4. Discussion

Statins are well studied for their pleiotropic effects aside from lowering cholesterol, including osteogenesis, angiogenesis, anti-inflammatory, nitric oxide bioavailability, and endothelial cell function [18]. To increase the local concentration and achieve the desired therapeutic effect at the target we synthesized polyprodrugs. With polyprodrugs, it is the rate of degradation that determines the therapeutic efficiency of the released simvastatin. Gel permeation chromatographic analysis confirmed the slow degradation of poly(ethylene glycol)-*block*-poly(simvastatin) and poly(ethylene glycol)-*block*-poly(simvastatin)-*ran*-poly(glycolide), which reflects slow release of simvastatin and other degradation byproducts from the polyprodrugs.

For the present study, an onlay model was preferred over a bone defect model because it helps evaluate the challenge of vertical bone growth where defects do not exist, e.g., ridge augmentation, sinus lift, and spinal fusion [19, 20]. The supracalvarial site tests whether the materials stimulate progenitor cells in the periosteum for intramembranous bone formation. Moreover, postoperative stress and prolonged use of analgesics to control pain following creation of critical-sized defects can modify host responses and thereby alter bone healing [19, 21]. These effects would confound analysis of the polyprodrug-induced bone growth for a pleiotropic molecule such as simvastatin.

Following implantation of the polyprodrug, a series of events is triggered, including nonspecific adsorption of proteins, foreign body response, and fibrous capsule formation [22]. The fibrous capsule initially formed around poly(ethylene glycol)-*block*-poly(simvastatin) and poly(ethylene glycol)-*block*-poly(simvastatin)-*ran*-poly(glycolide) resolved into a thin layer over the 8 week study period. This could be attributed to the anti-fibrotic effects of the released simvastatin. Earlier reports have shown that simvastatin reduces capsular fibrosis around silicone implants when administered orally, by down-regulating the expression of connective tissue growth factor (CTGF) and transforming growth factor (TGF) β_1 genes in rodents [23]. Further, resolution of hematoma could be attributed to the fact that simvastatin facilitates its absorption via phagocytosis by increasing the expression of CD36 (cluster of differentiation 36) [24]. The molecular degradation of poly(ethylene glycol)-*block*-poly(simvastatin)-*ran*-poly(glycolide) and poly(ethylene glycol)-*block*-poly(simvastatin) was slow *in vivo*.

A thicker capsule was observed with PLGA loaded with simvastatin and pure PLGA. It has been reported that thick mature capsule (acellular, avascular, and with dense collagen fibers) was formed around fast-degrading PLGA materials, whereas immature capsule (collagen with fibroblasts and capillaries) was observed with the slow-degrading counterparts [25].

The present GPC measurements showed a larger and faster decrease in the molecular weight of PLGA loaded with simvastatin compared to pure PLGA and the polyprodrugs. The presence of drug affects the degradation kinetics of poly(lactic-co-glycolic acid) systems by affecting the water diffusion coefficient [26, 27]. Of particular relevance is that the presence of a hydrophobic drug (haloperidol, Log D 2.81) slowed the degradation of PLGA 50:50 by altering its bulk-eroding mechanism into surface degradation [26]. Conversely, in our study the presence of hydrophobic simvastatin (Log D = 1.5-1.75) [28] increased polymer degradation in PLGA loaded with simvastatin compared to pure PLGA. This could be attributed to the difference in hydrophobicity of the drugs. Hydrophobic drugs accelerate degradation, but highly hydrophobic drugs (high Log D values) reduce water penetration and polymer degradation [29]. Accelerated degradation of PLGA led to faster release of simvastatin, i.e., milligrams of the drug compared to micrograms from the polymeric prodrugs. The combined effects of acidic degradation byproducts and large amounts of drug from simvastatin-loaded PLGA thereby stimulated an osteolytic reaction that compromised the calvarium, in contrast to responses to the poly(simvastatin) polymers. PLGA alone had negligible effect.

Multiple injections of simvastatin gel (0.5 mg of simvastatin in methylcellulose gel under a polylactic acid dome) have an additive effect that results in increased bone thickness compared to single dose [30]. With our polyprodrugs, however, significant bone growth was observed without any intervention after implantation, and we infer that the released drug is pharmacologically active even at the end of 8 weeks from this result.

Irrespective of degradation rate, increased expression of genes involved in bone formation (BMP2, Runx2, and ALP) was observed with all polymers relative to control PLGA through 8 weeks. Simvastatin induces osteogenesis primarily by the Ras-PI3K-Akt/MAPK signaling pathway, which increases expression of BMP-2, and secretion of BMP-2 subsequently activates the series of genes specific for osteoblast differentiation and bone formation through Runx2 [31]. Simvastatin has been reported to induce bone mineralization by enhancing the production of alkaline phosphatase by increasing the ALP gene [6, 32]. Increased levels of VEGF were observed with all polymers. A biphasic effect of statins on angiogenesis is well studied: they are pro-angiogenic at nanomolar concentrations but anti-angiogenic at micromolar concentrations [33]. Other hand, nano- and micromolar concentrations of simvastatin increase the expression of VEGF in osteoblasts via the phosphatidylinositide-3 kinase pathway [32]. While protein detection may have provided additional information, assuming levels were sufficient for detection, a mechanistic investigation of action was not designed into the experiment.

The severe inflammatory response observed for PLGA loaded with simvastatin discs can be attributed to their faster degradation, together with increased concentration of the drug released. Higher concentrations of simvastatin ($>10^{-7}$ M) are toxic to osteoblasts *in vitro*, [6] and high implanted doses have also resulted in edema, ulceration, and scab formation [11, 12]. We observed all these signs with PLGA loaded with simvastatin disks.

Local release of simvastatin from the polymers placed on the calvarium did not systemically affect total cholesterol, which would result from simvastatin inhibiting the synthesis of

mevalonate via 3-hydroxy-3-methylglutaryl coenzyme A reductase [34]. An oral dose of 20-40 mg/d (for humans)[35] and 80 mg/kg/d (for rats) [36] is needed to lower low density lipoprotein cholesterol (LDL-C) when systemic bioavailability is <5% [9]. Polymerized simvastatin allowed stimulation of only local tissue response without other effects.

4. Conclusions

Poly(ethylene glycol)-*block*-poly(simvastatin) degraded *in vivo* to stimulate osteoprogenitor cells in the periosteum to form bone via the intramembranous pathway even in the absence of a large bone defect. In contrast, severe inflammation-induced bone resorption occurred with conventional PLGA-simvastatin. Slow degradation of the polymeric prodrug controls inflammation and also aids in bone growth. The osteogenic potential of poly(ethylene glycol)-*block*-poly(simvastatin) merits further attention for applications in bone regeneration, including fabrication of porous formulations that can be used to regenerate bone defects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- [1]. Mundy G, Garrett R, Harris S, Chan J, Chen D, Rossini G, Boyce B, Zhao M, Gutierrez G.I., Stimulation of bone formation in vitro and in rodents by statins, *Science*. 286(5446) (1999) 1946–1949. [PubMed: 10583956]
- [2]. Garrett IR, Mundy GR, The role of statins as potential targets for bone formation, *Arthritis Res*. 4(4) (2002) 237–40. [PubMed: 12106493]
- [3]. Shah SR, Werlang CA, Kasper FK, Mikos AG, Novel applications of statins for bone regeneration, *Natl. Sci. Rev* 2(1) (2015) 85–99. [PubMed: 26543666]
- [4]. Mori M, Nishikawa T, Masuno K, Okamura T, Tanaka A, Shikimori M, Statins: candidates for promoting bone formation via BMP-2, *Oral Med. Pathol* 14(3) (2010) 81–87.
- [5]. Pagkalos J, Cha JM, Kang Y, Heliotis M, Tsiroidis E, Mantalaris A, Simvastatin induces osteogenic differentiation of murine embryonic stem cells, *J. Bone. Miner. Res* 25(11) (2010) 2470–8. [PubMed: 20564244]
- [6]. Chen PY, Sun JS, Tsuang YH, Chen MH, Weng PW, Lin FH, Simvastatin promotes osteoblast viability and differentiation via Ras/Smad/Erk/BMP-2 signaling pathway, *Nutr. Res* 30(3) (2010) 191–9. [PubMed: 20417880]
- [7]. Yamashita M, Otsuka F, Mukai T, Yamanaka R, Otani H, Matsumoto Y, Nakamura E, Takano M, Sada KE, Makino H, Simvastatin inhibits osteoclast differentiation induced by bone morphogenetic protein-2 and RANKL through regulating MAPK, AKT and Src signaling, *Regul. Pept* 162(1–3) (2010) 99–108. [PubMed: 20346376]
- [8]. Moon HJ, Kim SE, Yun YP, Hwang YS, Bang JB, Park JH, Kwon IK, Simvastatin inhibits osteoclast differentiation by scavenging reactive oxygen species, *Exp. Mol. Med* 43(11) (2011) 605–12. [PubMed: 21832867]

- [9]. Park J-B, The use of simvastatin in bone regeneration, *Med. Oral Patol. Oral Cir. Bucal* 14(9) (2009) e485–488. [PubMed: 19415061]
- [10]. Tan J, Yang N, Fu X, Cui Y, Guo Q, Ma T, Yin X, Leng H, Song C, Single-dose local simvastatin injection improves implant fixation via increased angiogenesis and bone formation in an ovariectomized rat model, *Med. Sci. Monit* 21 (2015) 1428–39. [PubMed: 25982481]
- [11]. Thylin MR, McConnell JC, Schmid MJ, Reckling RR, Ojha J, Bhattacharyya I, Marx DB, Reinhardt RA, Effects of simvastatin gels on murine calvarial bone, *J. Periodontol* 73(10) (2002) 1141–8. [PubMed: 12416771]
- [12]. Nyan M, Sato D, Oda M, Machida T, Kobayashi H, Nakamura T, Kasugai S, Bone formation with the combination of simvastatin and calcium sulfate in critical-sized rat calvarial defect, *J. Pharmacol. Sci* 104(4) (2007) 384–6. [PubMed: 17721043]
- [13]. Jeon JH, Piepgrass WT, Lin YL, Thomas MV, Puleo DA, Localized intermittent delivery of simvastatin hydroxyacid stimulates bone formation in rats, *J. Periodontol* 79(8) (2008) 1457–1464. [PubMed: 18672996]
- [14]. Asafo-Adjei TA, Dziubla TD, Puleo DA, Synthesis and Characterization of a Poly(ethylene glycol)-Poly(simvastatin) Diblock Copolymer, *RSC. Adv* 4(102) (2014) 58287–58298. [PubMed: 25431653]
- [15]. Asafo-Adjei TA, Dziubla TD, Puleo DA, Tuning Properties of Poly(ethylene glycol)-block-poly(simvastatin) Copolymers Synthesized via Triazabicyclodecene, *React. Funct. Polym* 119 (2017) 37–46. [PubMed: 29217967]
- [16]. Thilanga Liyanage AD, Chen AJ, Puleo DA, Biodegradable simvastatin-containing polymeric prodrugs with improved drug release, *ACS. Biomater. Sci. Eng* 4(12) (2018) 4193–4199. [PubMed: 30631799]
- [17]. Ferrin J, Ebersole JL, Kirakodu S, Jensen D, Al-Attar A, Peyyala R, Novak MJ, Dawson D, Al-Sabbagh M, Stromberg AJ, Orraca L, Gonzalez-Martinez J, Burgos A, Gonzalez OA, Gene expression analysis of neuropeptides in oral mucosa during periodontal disease in nonhuman primates, *J. Periodontol* (2018).
- [18]. Kavalipati N, Shah J, Ramakrishan A, Vasawala H, Pleiotropic effects of statins, *Indian J. Endocrinol. Metab* 19(5) (2015) 554–62. [PubMed: 26425463]
- [19]. Kisiel M, Klar AS, Martino MM, Ventura M, Hilborn J, Evaluation of injectable constructs for bone repair with a subperiosteal cranial model in the rat, *PLoS. One* 8(8) (2013) e71683. [PubMed: 23967235]
- [20]. Takiguchi S, Kuboyama N, Kuyama K, Yamamoto H, Kondoh T, Experimental study of bone formation ability with the periosteum on rat calvaria, *J. Hard Tissue Biol* 18(3) (2009) 149–160.
- [21]. Thomas MV, Puleo DA, Infection, inflammation, and bone regeneration: a paradoxical relationship, *J. Dent. Res* 90(9) (2011) 1052–61. [PubMed: 21248364]
- [22]. Morais JM, Papadimitrakopoulos F, Burgess DJ, Biomaterials/tissue interactions: possible solutions to overcome foreign body response, *AAPS. J* 12(2) (2010) 188–96. [PubMed: 20143194]
- [23]. Chung KJ, Park KR, Lee JH, Kim TG, Kim YH, Simvastatin Reduces Capsular Fibrosis around Silicone Implants, *J. Korean Med. Sci* 31(8) (2016) 1273–8. [PubMed: 27478339]
- [24]. Chen Q, Shi X, Tan Q, Feng Z, Wang Y, Yuan Q, Tao Y, Zhang J, Tan L, Zhu G, Feng H, Chen Z, Simvastatin Promotes Hematoma Absorption and Reduces Hydrocephalus Following Intraventricular Hemorrhage in Part by Upregulating CD36, *Transl. Stroke. Res* 8(4) (2017) 362–373. [PubMed: 28102508]
- [25]. Grayson AC, Voskerician G, Lynn A, Anderson JM, Cima MJ, Langer R, Differential degradation rates in vivo and in vitro of biocompatible poly(lactic acid) and poly(glycolic acid) homo- and co-polymers for a polymeric drug-delivery microchip, *J. Biomater. Sci. Polym. Ed* 15(10) (2004) 1281–304. [PubMed: 15559850]
- [26]. Siegel SJ, Kahn JB, Metzger K, Winey KI, Werner K, Dan N, Effect of drug type on the degradation rate of PLGA matrices, *Eur. J. Pharm. Biopharm* 64(3) (2006) 287–93. [PubMed: 16949804]
- [27]. Li S, Girod-Holland S, Vert M, Hydrolytic degradation of poly (DL-lactic acid) in the presence of caffeine base, *J. Control. Release* 40(1–2) (1996) 41–53.

- [28]. Fong CW, Statins in therapy: understanding their hydrophilicity, lipophilicity, binding to 3-hydroxy-3-methylglutaryl-CoA reductase, ability to cross the blood brain barrier and metabolic stability based on electrostatic molecular orbital studies, *Eur. J. Med. Chem* 85 (2014) 661–74. [PubMed: 25128668]
- [29]. Rabin CR, Siegel SJ, *Delivery systems and dosing for antipsychotics*, Current Antipsychotics, Springer 2012, pp. 267–298.
- [30]. Lee Y, Schmid MJ, Marx DB, Beatty MW, Cullen DM, Collins ME, Reinhardt RA, The effect of local simvastatin delivery strategies on mandibular bone formation in vivo, *Biomaterials*. 29(12) (2008) 1940–9. [PubMed: 18255137]
- [31]. Ruan F, Zheng Q, Wang J, Mechanisms of bone anabolism regulated by statins, *Biosci. Rep* 32(6) (2012) 511–9. [PubMed: 22799752]
- [32]. Maeda T, Matsunuma A, Kawane T, Horiuchi N, Simvastatin promotes osteoblast differentiation and mineralization in MC3T3-E1 cells, *Biochem. Biophys. Res. Commun* 280(3) (2001) 874–7. [PubMed: 11162604]
- [33]. Weis M, Heeschen C, Glassford AJ, Cooke JP, Statins have biphasic effects on angiogenesis, *Circulation*. 105(6) (2002) 739–745. [PubMed: 11839631]
- [34]. Aguirre L, Hijona E, Macarulla MT, Gracia A, Larrechi I, Bujanda L, Hijona L, Portillo MP, Several statins increase body and liver fat accumulation in a model of metabolic syndrome, *J. Physiol. Pharmacol* 64(3) (2013) 281–8. [PubMed: 23959724]
- [35]. Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S), *Lancet*. 344(8934) (1994) 1383–9. [PubMed: 7968073]
- [36]. Rajyalakshmi RA, Rajesham V, A comparative antihyperlipidemic activity of atorvastatin with simvastatin in rats, *Internet. J. Pharmacol* 6(2) (2009) 9.

Statement of Significance

Traditional drug delivery systems, e.g., drug encapsulated in poly(lactic-co-glycolic acid) (PLGA), are typically passive and have limited drug payload. As an alternative, we polymerized the drug simvastatin, which has multiple physiological effects, into macromolecules (“polysimvastatin”) via ring-opening polymerization. We previously demonstrated that the rate of degradation and drug (simvastatin) release can be adjusted by copolymerizing it with other monomers. The present results demonstrate significant new bone growth around polysimvastatin, whereas severe bone loss occurred for PLGA loaded with simvastatin. This degradable biomaterial with biofunctionality integrated into the polymeric backbone is a useful candidate for bone regeneration applications.

Figure 1.

A) Predicted structures of simvastatin-containing polymeric prodrugs. B) Images of the polymer disks (2 ×6 mm) implanted: poly(lactic-*co*-glycolic acid) (PLGA); poly(lactic-*co*-glycolic acid) loaded with simvastatin (PLGA-S); poly(ethylene glycol)-*block*-poly(simvastatin) (PS); poly(ethylene glycol)-*block*-poly(simvastatin)-*ran*-poly(glycolide) (PSG).

Figure 2. Changes in the weight average molecular weight (M_w) of polymers over the 8-week period of implantation in the calvarial onlay model.

Figure 3. MicroCT images showing the dorsal surface of rat calvaria at 1, 4, 6, and 8 weeks post-implantation polymers. The arrowheads point to new bone growth. Scale bar: 1 mm.

Figure 4. Representative coronal histological images of the entire rat calvarium at 6 weeks post-implantation. Higher magnification images of the boxed regions show immature/woven bone formed at interface of capsule and calvarium in response to the polyprodrugs. The arrowhead points to blood vessels. Scale bar: 100 μm.

Figure 5.
Thickness of the newly formed woven bone formed in response to different polymers after (A) 1 and (B) 8 weeks of implantation.

Figure 6. Real time PCR analysis of expression of osteogenic (BMP2, Runx2, ALP), angiogenic (VEGF), and inflammatory cytokines (IL-6, NF-κB).

Figure 7.
A) Body weight and B) serum total cholesterol for rats treated with different polymers during the 8 week study.