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Improved small molecule drug release from *in situ* forming poly(lactic-co-glycolic acid) scaffolds incorporating poly(β -amino ester) and hydroxyapatite microparticles

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Abstract

In situ forming implants are an attractive choice for controlled drug release into a fixed location. Currently, rapidly solidifying solvent exchange systems suffer from a high initial burst, and sustained release behavior is tied to polymer precipitation and degradation rate. The present studies investigated addition of hydroxyapatite (HA) and drug-loaded poly(β -amino ester) (PBAE) microparticles to *in situ* forming poly(lactic-co-glycolic acid) (PLGA)-based systems to prolong release and reduce burst. PBAEs were synthesized, imbibed with simvastatin (osteogenic) or clodronate (anti-resorptive), and then ground into microparticles. Microparticles were mixed with or without HA into a PLGA solution, and the mixture was injected into buffer, leading to precipitation and creating solid scaffolds with embedded HA and PBAE microparticles. Simvastatin release was prolonged through 30 days, and burst release was reduced from 81% to 39% when loaded into PBAE microparticles. Clodronate burst was reduced from 49% to 32% after addition of HA filler, but release kinetics were unaffected after loading into PBAE microparticles. Scaffold dry mass remained unchanged through day 15, with a pronounced increase in degradation rate after day 30, while wet scaffolds experienced a mass increase through day 25 due to swelling. Porosity and pore size changed throughout degradation, likely due to a combination of swelling and degradation. The system offers improved release kinetics, multiple release profiles, and rapid solidification compared to traditional *in situ* forming implants.

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Disclosure Statement

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Keywords

in situ precipitation; injectable; PLGA; poly(β -amino ester); simvastatin; clodronate

1. Introduction

Controlled drug delivery systems offer a variety of potential advantages over traditional routes of administration, such as oral dosages and intravenous or subcutaneous injection of drug solutions, due to their spatial and temporal control over drug release [1]. Traditional dosage forms result in systemic circulation of drug, and the oral route is also subject to first-pass metabolism [2]. Injections introduce a bolus of drug at high concentrations, and long-term treatment requires repeated dosing, resulting in pulsatile concentration profiles [3]. Implantable drug-loaded scaffolds can be placed at the treatment site to minimize systemic exposure and can be designed to control release kinetics by varying the chemical and physical nature of the carrier [3, 4]. These systems, however, generally require a surgical procedure to implant the device, and in the case of a non-degrading material, require a removal surgery. As a result, significant effort in the drug delivery field has focused on injectable, biodegradable drug carriers, such as *in situ* gelling, polymerizing, or precipitating systems [5, 6]. A space-filling scaffold capable of forming into a biodegradable solid at the treatment site is an appealing option because it can penetrate a tissue network to deliver drugs at a fixed location with minimal invasiveness and no removal surgery.

Poly(lactic-co-glycolic acid) (PLGA) is an attractive material for an *in situ* forming delivery system because it is already present in FDA-approved injectable products (Atridox® and Atrigel® systems), it degrades hydrolytically, and its physical properties can be controlled by varying the lactide:glycolide ratio, molecular weight, and end species [7]. PLGA is dissolved in a water-miscible solvent and mixed with a pharmaceutical agent prior to injection to form a drug suspension or solution. The resulting mixture is injected into the treatment site, where it precipitates into a solid and gradually releases the drug [8]. These systems have been investigated for protein release [9, 10] as well as small molecule drugs [11], and have demonstrated biocompatibility [12].

Typically, injectable PLGA systems have a large initial burst release followed by sustained release over a period of hours to weeks [5]. The magnitude of this burst release as well as precipitation rate are both governed by the rate of solvent exchange [13, 14]. Therefore, systems with lower initial burst tend to undergo slower solidification due to gradual solvent exchange, while rapid precipitation corresponds to rapid solvent exchange and correspondingly large burst. Release kinetics following the burst are primarily diffusive until degradation of the polymer matrix becomes a contributing factor [14]. The burst can be reduced by increasing polymer content of the solution, increasing polymer molecular weight, or changing to a less water-miscible solvent [5, 15, 16]. However, increasing polymer content increases the viscosity of the solution, slows precipitation, and leads to the formation of a low-porosity “skin” around the scaffold [14, 17]. Increasing polymer molecular weight and/or hydrophobicity increases degradation time [16], and choosing a less water-miscible solvent dramatically slows precipitation [13, 15].

Incorporating drug-loaded microparticles into an injectable PLGA system may provide a secondary means of controlling release kinetics. This concept has been previously investigated by mixing PLGA microspheres containing plasmid DNA into the PLGA solution prior to injection [18]. PLGA microspheres will dissolve in the organic solvents used in the system, however, which confers a time constraint on the mixing process and may be unsuitable for small molecule drugs that can easily escape from partially dissolved microspheres. By instead utilizing crosslinked microparticles that will not dissolve in NMP but are still hydrolytically degradable, the utility of the *in situ* forming PLGA system may be greatly expanded. Poly(β -amino ester) (PBAE) hydrogels are formed via step growth polymerization of a diacrylate and an amine followed by free radical crosslinking, and degradation time can be modified by changing the monomers to influence crosslink density and hydrophilicity [19]. Recently, PBAE microparticle and nanoparticle systems have been investigated for their drug delivery potential [20–22]. Incorporation of drug-loaded PBAE microparticles into an injectable PLGA system has the potential advantage of controlling and prolonging drug delivery while reducing initial burst.

Intraosseous injection in particular is a suitable application for an *in situ* forming system due to the interconnected porous network of the trabecular bone that makes placement of pre-formed scaffolds difficult and removal surgery impossible. Injectable PLGA systems can theoretically accommodate filler particles such as hydroxyapatite (HA) to influence microarchitecture, mechanical properties, or osteoconductivity. N-methyl-2-pyrrolidone (NMP), currently used in the FDA-approved Atrigel and Atridox delivery systems, is highly water-miscible and provides rapid solidification. Additionally, NMP has been shown to promote bone morphogenetic protein 2 (BMP-2) dependent alkaline phosphatase activity as well as reduce osteoclast activity [23, 24]. Bisphosphonates, such as clodronate, are used to prevent bone resorption via osteoclast apoptosis or inactivation [25]. Simvastatin is a drug that is primarily used for its inhibition of 3-hydroxy-3-methylglutaryl coenzyme A to treat high cholesterol, but it has recently been investigated for osteogenic applications due to numerous studies reporting that simvastatin promotes bone formation via BMP-2 upregulation [26, 27].

Clodronate and simvastatin are both small molecule drugs, and therefore they are prone to rapid release and high burst in a traditional *in situ* forming PLGA system. It was hypothesized that an *in situ* forming PLGA system containing drug-loaded PBAE microparticles and HA filler would provide superior simvastatin and clodronate release kinetics compared to existing systems using freely mixed drugs.

2. Materials and Methods

2.1 Materials

Poly(lactic-co-glycolic acid) (PLGA; 50:50 L:G, acid terminated, inherent viscosity 0.55–0.75 dL/g) was purchased from DURECT, Inc. (Birmingham, AL, USA). Simvastatin was purchased from Haorui Pharma-Chem (Edison, NJ, USA). Clodronate, hydroxyapatite, 2,2-dimethoxy-2-phenylacetophenone (DMPA), and N-methyl-2-pyrrolidone (NMP) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2 PBAE polymer synthesis

PBAE macromer was synthesized by reacting diethylene glycol diacrylate and isobutylamine at a 1.2:1 diacrylate:amine molar ratio at 85°C for 16 hours. Macromer was stored in an opaque vial under refrigeration until use. To create crosslinked hydrogels from macromer, 1 wt/wt% DMPA initiator dissolved in 50 wt/wt% ethanol was vortexed with macromer. The mixture was then pipetted between two glass plates with Teflon spacers, sealed and clamped, and then exposed to a UV flood source with an intensity of 12 mW/cm² for 5 minutes to form a crosslinked hydrogel slab. These PBAE polymer slabs were washed overnight in ethanol to remove unreacted monomer and initiator, and then stored in a desiccator to remain dry until use.

2.3 PBAE particle formation and drug loading

PBAE microparticles were formed by grinding dry PBAE slabs with a mortar and pestle, with HA added during the grinding process to coat particles and prevent aggregation. HA content was preliminarily tested at 66 w/w % and 75 w/w % weight ratios, and 75% (50 v/v %) was chosen due to ease of particle fabrication. PBAE particles were sieved to less than 250 µm, and larger particles were re-ground until all material was collected through the sieve. A Zeiss Evo MA 10 scanning electron microscope (SEM) (Carl Zeiss, Thornwood, NY) at 4 kV accelerating voltage was used to visualize particle morphology, and a Quantax energy-dispersive x-ray spectroscopy (EDS) detector was used for elemental analysis. A series of 10 microscope images of HA and PBAE particles were analyzed using freely available ImageJ software to calculate mean particle size.

Simvastatin was either loaded into PBAE slabs prior to particle formation (pre-loaded) or loaded directly into PBAE microparticles (post-loaded), and each method was assessed for loading efficiency as well as the percentage of drug that was weakly bound to particle surface. Figure 1A graphically represents the processing method for creating PBAE microparticles, as well as the differences between pre- and post-loaded particles. Post-loaded particles were prepared by dissolving simvastatin in ethanol at a concentration of 100 mg/mL and pipetting drug solution over particles at a ratio of 2 µL per mg of particles. This ratio allowed particles to swell without excess solution remaining, and expected drug loads were calculated under the assumption that all drug solution was imbibed by the microparticles. Particles were lyophilized overnight, briefly re-ground and sieved to break up aggregates, and stored in a vacuum chamber with desiccant. Pre-loaded microparticles were prepared by immersing PBAE slabs in simvastatin solution, allowing the hydrogel to swell for 24 hours, and then removing the hydrogel and lyophilizing overnight to evaporate ethanol. Drug-loaded PBAE slabs were ground with 75 wt% HA into particles and stored in a vacuum chamber with desiccant. Predicted values for drug loading using the pre-loading method were obtained by calculating swelling of PBAE in solution based on mass increase and assuming drug was homogeneously present in swollen hydrogels as well as surrounding solution. The density and concentration of the drug solution was then used to calculate expected drug loading based on mass change of the PBAE samples. Clodronate was loaded into separate batches of microparticles using an identical pre-loading technique with a 50 mg/mL clodronate solution in deionized water.

2.4 Measurement of drug loading into PBAE

Simvastatin loading into PBAE microparticles was measured by immersing drug-loaded particles in ethanol, vortexing, and allowing the particles to swell for 24 hours. The mixtures containing ethanol and swollen PBAE microparticles were then centrifuged, and supernatants were analyzed using HPLC to detect simvastatin as described in 2.7 *Measurement of drug concentrations*. Clodronate loading was measured with an identical technique using 50% ethanol, and was detected by absorbance as described below. A mass balance indicated that all drug was successfully removed from the particles during 24 hours of immersion in ethanol for simvastatin or 50% ethanol solutions for clodronate, and subsequent immersions extracted no additional drug. Loading efficiency was defined as the ratio of the total mass of drug loaded into the particles to the initial mass of drug exposed to the particles.

In addition to loading efficiency, the percentage of drug present on the surface of particles was determined by washing drug-loaded particles with 5 mL ethanol over a filter to remove loosely surface-bound simvastatin or with 5 mL deionized water to remove loosely surface-bound clodronate. The remainder of drug present in the bulk of the microparticles was extracted by immersing particles for 24 hours in ethanol for simvastatin or 50% ethanol for clodronate. Drug detected in the initial wash was deemed loosely bound to the surface of particles, while drug detected after the 24 hour soak was determined to be imbibed into the bulk of the particles.

2.5 Formation of injectable scaffold system

PLGA was added to NMP and stirred overnight until fully dissolved to create a 20 wt/wt% PLGA solution. Simvastatin-loaded PBAE microparticles pre-loaded using 100 mg/mL simvastatin were added at 5 wt% and mixed homogeneously prior to injection. HA was also mixed homogeneously prior to injection to bring the final mixture to 30 wt% HA. Clodronate-loaded PBAE microparticles pre-loaded with 50 mg/mL clodronate were added using an identical technique. Samples without microparticles were prepared by freely mixing 1 wt% simvastatin or clodronate into 20% PLGA solution to simulate a traditional *in situ* forming PLGA system. Figure 1B illustrates the differences between the proposed system and a traditional injectable system. Simvastatin and clodronate were measured as described in 2.7 *Measurement of drug concentrations*.

2.6 In vitro drug release

The scaffold mixture was injected dropwise through a 16 gauge, blunt-tipped dispensing needle into PBS at 5% wt/vol. Upon contacting PBS, surface PLGA immediately began to precipitate, forming semi-spherical scaffolds approximately 3 mm in diameter that sank to the bottom of the vial. Samples were kept in an incubator at 37°C on a plate shaker for the duration of the study. Supernatant was collected and replaced at each time point, and these samples were preserved at 4°C until analysis. Clodronate release was measured for three loading conditions: freely mixed clodronate without HA filler, freely mixed clodronate with HA filler, and clodronate pre-loaded into PBAE microparticles with HA filler. Because PBAE microparticles were fabricated with HA as a dispersing agent, drug release from loaded particles was not investigated without HA filler. Simvastatin release was measured

for three loading conditions: freely mixed simvastatin without HA filler, freely mixed simvastatin with HA filler, and simvastatin pre-loaded into PBAE microparticles with HA filler.

2.7 Measurement of drug concentrations

Simvastatin was measured on a Hitachi Primaide HPLC system equipped with a C18 column using a mobile phase composed of 70% acetonitrile and 30% water with 0.1% trifluoroacetic acid at a flow rate of 1 mL/min, and peaks were observed at 240nm. Clodronate from collected supernatants were measured using a Powerwave HT (Biotek; Winooski, VT, USA). *In vitro* release supernatant was pipetted into a UV-grade 96-well plate (Greiner Bio-One, Frickenhausen, Germany), and baseline absorbance was measured at 240 nm. On its own, clodronate does not exhibit a distinct absorption peak, so concentration was measured by mixing supernatant with a solution of 1.5 mM copper sulfate and 1.5 mM nitric acid at pH 2 to form a clodronate-copper complex that exhibits absorption at 240 nm [28]. A pilot experiment confirmed that HA did not significantly interfere with clodronate readings in the working range.

2.8 Characterization of release kinetics

Drug release from polymeric systems is often analyzed using an adaptation of the Higuchi model by Peppas et al., widely known as the power law, to characterize release kinetics [29]:

$$\frac{M_t}{M_\infty} = kt^n \quad (1)$$

Here, M_t is the mass of drug at time t , M is the total mass of drug in the system, $\frac{M_t}{M_\infty}$ represents fractional release of drug at time t , k is a constant encompassing scaffold and drug properties, and n is the release exponent used to characterize drug release. In the case of the spherical geometry (consistent with scaffolds formed via dropwise injection), $n = 0.43$ corresponds to pure Fickian diffusion, $n = 0.85$ corresponds to pure case II (polymer relaxation-based) transport, and values in between are combinations of the two, termed anomalous transport [29]. Plots of $\log \frac{M_t}{M_\infty}$ versus $\log t$ were used to determine n for each drug. The power law is applicable when $\frac{M_t}{M_\infty} < 0.6$, so release data were truncated to below 60% cumulative release when calculating n .

2.9 Mass loss and degradation

The injectable scaffold system was prepared as described previously, using unloaded microparticles. Non-destructive degradation analysis was performed on hydrated scaffolds in order to observe the mass change of wet samples. Scaffolds were injected dropwise into PBS and incubated at 37°C on a shaker for 80 days. Initial injected mass was recorded for each scaffold. At intervals, scaffolds were removed from PBS, gently blotted dry, and weighed. Destructive analysis of dried samples was used to analyze scaffold dry mass change. Scaffolds were injected dropwise into PBS and incubated at 37°C on a plate shaker until analysis. At each time point, scaffolds were lyophilized for 24 hours, and dry weight was measured. Lyophilized scaffolds were also scanned using a Scanco MicroCT 40

(Scanco Medical, Switzerland) at 55 kV and 145 mA with 6 μm voxel size. The built in bone trabecular morphometry tool was used to create 3D reconstructions to visualize microarchitecture and quantify porosity and pore size at various time points during the degradation process.

2.10 Statistical analysis

All data are presented as mean $\pm$ standard deviation. Release data were analyzed in JMP 10 software, using one-way analysis of variance (ANOVA) to determine differences between release curves followed by Tukey-Kramer mean comparison tests as necessary.

Comparisons between individual pairs of samples for loading efficiencies were performed using a student's two-tailed t-test. Differences were considered significant for $p < 0.05$.

3. Results

3.1 Drug loading into PBAE particles

Drug loading into PBAE increased linearly with drug solution concentration for simvastatin (Figure 2A) and clodronate (Figure 2B and 2C). Clodronate became insoluble at 20 mg/mL in 50% ethanol, while the drug remained soluble through 100 mg/mL in DI water. At 100 mg/mL clodronate, however, it was observed that hydrogel would swell to a greater degree and fracture upon handling, leading to high variability between samples (Figure 2C), so 50 mg/mL clodronate in DI water was used for future experiments. Simvastatin was loaded into gels or particles using a 100 mg/mL solution in 100% ethanol.

HA possessed a mean particle diameter of 10 μm , while the PBAE particles produced by grinding PBAE hydrogels together with HA had a mean diameter of 68 μm . PBAE microparticles exhibited a composite structure consisting of spherical particles consistent with HA sizes embedded in an amorphous material consistent with the hydrogel nature of PBAE (Figure 3A). The spherical HA particles exhibited a rough, granular surface morphology, compared to the smooth PBAE component. EDS elemental mapping showed calcium and phosphorous localized to the spherical particles (Figure 3B). Point analysis of presumed HA and PBAE regions indicated higher calcium and phosphorous levels in spherical HA particles, and higher carbon and oxygen content in the PBAE region (Figure 3C). When these microparticles were immersed in a mixture of 20% PLGA solution in NMP with 30 wt% HA added to simulate pre-injection conditions, they underwent a 47% mass increase due to swelling (Figure 3D), with a 28% increase occurring within 5 minutes.

Efficiency of incorporating simvastatin into post-loaded particles ranged from 52% to 77% (Figure 4B), and efficiency ranged from 89% to 96% in pre-loaded particles (Figure 4D). A surface wash indicated that between 69% and 77% of simvastatin was loosely surface-bound to post-loaded particles, with no differences between simvastatin concentrations (Figure 4C). Using the pre-loading technique, a significantly lower amount ($p < .05$), between 46% and 51% of simvastatin, was loosely surface-bound, again with no concentration dependence (Figure 4A). Pre-loaded clodronate yielded particles with 74% to 86% of drug loosely surface-bound (Figure 4E). Clodronate loading efficiency ranged from 91% to 97% at concentrations up to 50 mg/mL, and dropped to 59% at 100 mg/mL.

3.2 *In vitro* release

Freely mixed simvastatin was 81% released within 1 day and 95% released within 10 days, with no difference between scaffolds prepared with or without HA (Figure 5A). In both cases, there was a gradual decrease in release rate as fractional release (M_t/M_∞) reached 100%. Pre-loaded simvastatin microparticles mixed into the system reduced the 1 day burst to 39% ($p < 0.05$), followed by sustained release of 1.3%/day for 30 days. The sustained release from day 1 through day 30 was roughly linear, with no appreciable decrease in rate until completion of drug release.

Freely mixed clodronate prepared without HA exhibited a 49% burst within one day of release, while both freely mixed and pre-loaded clodronate prepared with HA produced 32% burst ($p < 0.05$) (Figure 5B). Freely mixed clodronate without HA gradually released at a rate of 0.6%/day through day 19, while clodronate mixed with HA or pre-loaded into microparticles released at 1.3%/day through day 19. By day 20, there was no difference in total clodronate release between each curve. All clodronate release curves showed a distinct increase in release rate to 3%/day at day 20 that continued through day 31, after which nearly all drug was released.

To characterize release kinetics, plots of $\log \frac{M_t}{M_\infty}$ vs $\log t$ were used to calculate n for pre-loaded simvastatin and clodronate through the first day of release. After the first day, release rates tended to be linear and were therefore expressed as a daily release rate. For initial release during the burst, simvastatin and clodronate release exhibited $n = 0.47$ and $n = 0.49$, respectively.

3.3 Mass loss and degradation

Lyophilized scaffolds exhibited an initial mass loss of 50% in the first hour, followed by an additional gradual decrease of 5% over the first day (Figure 6A). Mass fraction remained unchanged at approximately 45% for 15 days, followed by a linear decrease in mass at a rate of 0.2%/day until day 50. Mass fraction did not change significantly after day 50, ranging from 25% to 29% through day 80. Lyophilized samples collected at day 40 and beyond were primarily powder that crumbled upon handling.

Non-destructive mass change of wet scaffolds showed an initial 12% decrease in mass over the first day, followed by a linear increase of 1.4%/day over the next 14 days (Figure 6B). At day 15, mass fraction became more variable as some samples began to decrease in mass, while others swelled through day 30. Linear mass loss was observed from day 30 through day 55, when the remaining mass fraction stabilized at 20% until day 80.

Qualitative assessment of microCT cutplanes roughly bisecting samples revealed a uniformly porous microstructure through day 10 (Figure 7). By day 15, denser regions had developed in the middle and at the edges of scaffolds. By day 20, large macropores were present in the core of the scaffolds. At day 25, scaffold cores appeared more uniformly porous, and the denser regions near the edges of scaffolds had become more porous. The dense regions appeared to migrate towards one side of the scaffold by day 30, and by day 35, scaffolds had significantly decreased in size, leaving behind a uniformly porous pellet.

After day 35, scaffolds were too fragile to handle, and a representative sample of residual material collected at day 40 was nonporous. MicroCT morphometry revealed an increase in porosity from 31% through day 20 to 47% by day 30, followed by a return to 30% at day 35 (Figure 8A). Average pore size ranged from 40 to 100 μm , except for samples at day 25, which had average pore size of 231 μm (Figure 8B).

4. Discussion

PBAE microparticles were successfully formed by co-grinding PBAE hydrogels with HA, forming individual PBAE particles coated with multiple, smaller HA particles. Elemental analysis confirmed that the smaller, spherical coating consisted of HA, and the underlying particles were hydrogel. Simvastatin pre-loaded into PBAE hydrogels showed both higher loading efficiency and bulk imbibition of drug, which are favorable conditions to prolong drug release relative to freely mixed drug. Swelling data were found to be an accurate predictor of drug loading into gels at various concentrations, consistent with previous drug loading results with hydrogels [30]. It was determined that processing of particles does not appreciably affect simvastatin loading efficiency using the pre-loading technique while still providing favorable imbibition ratios of surface to bulk drug. A decrease in loading efficiency as well as a higher percentage of simvastatin present loosely bound to the surface of particles using a post-loading technique may be attributable to the free HA powder used to coat the PBAE particles absorbing a percentage of drug solution, preventing its complete penetration into the hydrogel and resulting in a majority of drug sequestered outside the hydrogel microparticles. The high levels of surface drug seen for clodronate pre-loading may be due to the fact that the PBAE used in these experiments swells to a significantly larger degree in ethanol than in water (data not shown), allowing less penetration of drug into the bulk of the gels. Additionally, clodronate was more favorably loaded into PBAEs in aqueous solutions rather than 50% ethanol solutions because its solubility was limited. The swelling kinetics of PBAEs in the injection mixture (30 wt% HA in 20% PLGA solution) suggest that particles swell appreciably due to NMP exposure prior to injection, but the magnitude of swelling is negligible compared to the amount observed in pure NMP (47% at equilibrium vs. 470% in pure NMP). This difference is due to the presence of PLGA, which dissolves readily in NMP [9] and prevents hydrogels from fully swelling as they would in pure NMP. Additionally, the particles may be immersed in PLGA solution for only a matter of minutes before injection, so realistically, particles may swell by only 23% if the system is injected 5 minutes after addition of microparticles to the PLGA solution.

Simvastatin release was strongly affected by its loading state. A comparison of release kinetics between injectable systems loaded with freely mixed simvastatin and simvastatin-loaded PBAE microparticles indicated that burst release can be significantly reduced and duration of release can be significantly extended by loading simvastatin into PBAE microparticles. Because simvastatin is soluble in NMP, freely mixed drug presumably was dissolved in NMP and the majority of it exited into the aqueous phase during the solvent exchange process, resulting in a large burst release. The residual 20% of simvastatin was released in a sustained manner over the next 10 days, similar to previous work on such systems using freely mixed drug [31, 32]. PBAE microparticles experience some swelling in the NMP-PLGA solution during the mixing stage prior to injection, but burst is attenuated

for pre-loaded PBAE microparticles. The 39% burst release through day 1 using pre-loaded particles may be attributable to loosely surface-bound simvastatin that was likely dissolved by NMP and released during solvent exchange, similarly to freely-mixed simvastatin. Because simvastatin was loaded into PBAE with high efficiency, the release kinetics are likely a combination of the swelling, degradation, and diffusion of drug through the PBAE material as well as the PLGA phase.

Clodronate release appeared to be unaffected by loading state of the drug in PBAE microparticles, but it was instead dependent on the presence of HA in the injection mixture. This can be attributed to the formation of a complex between the bisphosphonate drug and hydroxyapatite, specifically between the two phosphonate groups of clodronate and the divalent calcium cations present in HA crystals [33]. Freely mixed clodronate without HA filler was 49% released within 1 day, compared to 32% for both compositions with HA. Because clodronate is insoluble in NMP, this initial burst is likely due to free clodronate suspended in the PLGA solution being dissolved by water during solvent exchange. The increase in release rate observed around day 20 corresponded to the maximum swelling state of scaffolds, indicating that scaffolds had begun to appreciably degrade and more water could enter the system to promote more rapid clodronate dissolution. Samples prepared with HA had a lower burst and higher release rate between days 1 and 20 due to the clodronate being initially retained in the system by complexation, after which the drug was released diffusively from the solidified system. Clodronate release kinetics are consistent with the classic three-stage release profiles observed in rapidly-precipitating systems, in which an initial burst is followed by slow diffusion, and then a more rapid swelling- or degradation-mediated release occurs [34]. Interestingly, the slow diffusion stage was accelerated by the addition of HA, likely because the larger amount of drug retained in the scaffold provided a higher concentration gradient to drive diffusion.

The rapid precipitation implied by the initial burst of clodronate and simvastatin as well as the early mass loss is indicative of rapid NMP exchange, which demonstrates the potential of the system for rapid solidification. Fast precipitation is favorable for quick delivery of NMP for an initial osteogenic stimulus, and rapid formation of a solid drug delivery depot may have advantages over gradually precipitating systems with persistent gel or liquid cores for days or weeks after injection due to NMP retention [32]. Specifically, this has implications for intraosseous injection, where the system can completely precipitate within days while continuing to release drug over a period of weeks to months. In systems with freely suspended or dissolved drug, precipitation rate strongly influences burst release [13, 35], and the addition of drug-loaded PBAE microparticles can allow for prolonged release and decreased burst without prolonging precipitation of the system. The rapid precipitation may also allow filler particles, such as hydroxyapatite, to provide mechanical support in future iterations of the system. After the initial burst, pre-loaded simvastatin exhibited prolonged delivery over the entire 30 day period. The lack of change in simvastatin release kinetics upon addition of 30 wt% HA was expected, because HA should not appreciably interact with statins, which lack the phosphonate groups that provide binding sites for the calcium in HA. The successful demonstration of sustained release of both hydrophilic and

hydrophobic small molecule drugs with reduced burst from this system shows promise for new applications of *in situ* forming PLGA systems where rapid precipitation is required.

By fitting the power law to initial drug release, the release exponent n can be used to classify the mechanism of drug release from the system. For simvastatin, n of 0.47 indicates primarily Fickian diffusion based on the standard n values for a spherical scaffold of $n=0.43$ for pure Fickian diffusion and $n=0.85$ for pure Case II transport. The slightly higher release exponent compared to the pure Fickian value can be attributed to several factors, including the swelling of the PBAE hydrogel microparticles and minor swelling of the surrounding PLGA matrix. Similarly, n of 0.49 for clodronate corresponds to primarily Fickian diffusion as well. Both clodronate and simvastatin are therefore released via diffusive mechanisms prior to their sustained release, which follows a more linear trend. This data supports the idea that the burst was composed of a fraction of the loaded drug dissolved or suspended in NMP, and the rapid solvent exchange was likely responsible for the initial release. The sustained release was likely a combination of diffusion, erosion, and swelling-based mechanisms, and the superposition of these mechanisms produces release curves that are most simply expressed in daily release rates. Because simvastatin is almost insoluble in water but highly soluble in NMP, the larger burst compared to clodronate is unsurprising, as dissolved simvastatin may be transported out of the scaffold during the solvent exchange phase. Future iterations of the system may be able to further reduce burst by reducing access of drug to the NMP phase to limit the initial diffusive component of the system.

PLGA is a bulk-eroding material that degrades via hydrolysis of ester bonds, resulting in a progressive decrease in molecular weight until monomeric or oligomeric species of sufficiently low molecular weight are generated and cleared when molecular weight drops to approximately 1.5 kDa [36, 37]. The *in situ* forming scaffold has the additional property of a rapid precipitation event upon injection, and due to the high aqueous miscibility of NMP, a rapid initial dry mass loss was observed within the first hour following injection as NMP entered the aqueous phase and the highly hydrophobic PLGA precipitated. By 24 hours, 46% dry mass remained, corresponding to the loss of the majority of the NMP content that composed 54 wt% of the system. The subsequent lack of dry mass change suggests that hydrolytic degradation was unable to produce small enough molecular weight species to be cleared into the surrounding aqueous environment until day 15. After day 15, the linear decrease in dry mass corresponded to clearance of degradation byproducts until all PLGA was removed from the system. The remaining 29% dry mass can be attributed to remaining HA content that composed 30% of the original mass. For wet scaffolds, the initial 12% decrease in wet mass throughout the first day of degradation supports the replacement of NMP with water due to precipitation of PLGA. The 20% increase in wet mass over the following 14 days indicates swelling of the PLGA matrix. Following this brief swelling, individual sample behavior diverged as swelling reached a maximum between 110–120% between days 20–30. The linear decrease in mass was delayed compared to dry samples because the small losses in polymer degradation byproducts were offset by increased water content as the system swelled. The lack of mass change beyond day 55 was consistent with the complete clearance of PLGA observed in dry mass studies, and the lower mass fraction remaining (20% compared to 29%) was likely due to repeated handling of samples during

nondestructive measurements resulting in small amounts of HA being resuspended and removed with supernatant at each time point.

Based on microCT images, it was evident that scaffold microarchitecture underwent dramatic changes throughout the degradation process. The first indicator of change was the development of denser regions in the core and near the edges of scaffold by day 15, which may be attributable to the cessation of NMP release and the hydrophobicity of PLGA causing solid regions to condense together. At day 20, the highest pore size was observed as large, closed macropores. This phenomenon corresponds to the scaffolds at their maximum swelling state based on wet mass, and was accompanied by further densification of the core and edge regions. At day 25, the core remained dense, but macropores were no longer evident, and the edges of scaffolds were less dense and more porous, which is consistent with reports of PLGA of this molecular weight beginning to lose mass to clearance after 3 weeks [37]. At day 30, it is possible that enough polymer had been lost to allow mobility of HA content through the PLGA, which settled at the bottom of scaffolds during incubation. By day 35, scaffold density and pore distribution appeared more uniform, and scaffolds were noticeably smaller in size due to further degradation of the highly porous regions seen at day 30.

In situ forming PLGA systems have been popularized due to their ease of use and unique ability to solidify at the point of injection, forming a drug delivery depot in the tissue of interest. However, because drug release from these systems is highly dependent on solvent exchange and polymer chemistry [13], release of multiple agents with unique release profiles is challenging. Additionally, low molecular weight drugs tend to exit during the solvent exchange, resulting in high initial burst [14]. Multiple drug release platforms for small molecule drugs, therefore, tend to be pre-formed scaffolds or injectable suspensions of micro- or nanoparticles [38–41]. The addition of PBAE microparticles to the *in situ* forming PLGA system combines the ease of use of injectable systems, the controlled release of microparticle systems, and the spatial control of solid scaffolds. Even in a rapidly-solidifying system, PBAE microparticles are capable of prolonging release of both hydrophilic and hydrophobic drugs over a 30 day period with significantly less burst than traditional injectable PLGA systems using freely mixed drug.

5. Conclusions

An *in situ* hardening, PLGA-based system offers a unique drug delivery platform due to its injectability and space-filling properties, and the addition of drug-loaded microparticles introduces the possibility for tunable release profiles for multiple pharmaceutical agents. In addition to the potential for multiple release profiles, the addition of drug-loaded PBAE microparticles enables the sustained release of water-soluble, small molecule drugs, such as clodronate, as well as lipophilic drugs, such as simvastatin, independent of PLGA precipitation. The biodegradability of the system makes this a favorable candidate for applications where a secondary surgery to remove the depleted scaffold is undesirable or impossible. Release kinetics can be multiphasic simply by loading drug freely into the PLGA solution for burst release, or into PBAE microparticles for sustained release with reduced burst. This injectable scaffold provides the simplicity of a targeted injection with

the utility of an implantable drug delivery scaffold without compromising desirable release kinetics.

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Figure 1. PBAE microparticle and injectable scaffold fabrication process. A) Technique for formation of PBAE hydrogels and processing them into drug-containing pre- or post-loaded microparticles. B) Comparison of a traditional injectable PLGA system (right) to the proposed system (left).

Figure 2.

Drug loading into PBAE hydrogel. A) Simvastatin loading after soaking in 100% ethanol solutions for 24 hours. B) Loading after soaking in 50% ethanol clodronate solution for 24 hours. C) Loading after immersion in aqueous clodronate solution for 24 hours. Data are mean $\pm$ standard deviation (n=3).

Figure 3.

Morphology, composition, and swelling of PBAE microparticles. A) SEM image of a PBAE microparticle, with EDS performed on the highlighted portion. B) Top left: Region chosen from (A) for EDS analysis, with arrows indicating points of spectral analysis performed in (C). Bottom left: Composite overlay of calcium and phosphorous on that region. Top right: EDS detection of calcium. Bottom right: EDS detection of phosphorous. C) EDS spectra of an HA particle (blue) and PBAE (red). D) PBAE swelling kinetics expressed as a percentage of mass increase in injection mixture (30 wt% HA mixed into 20% PLGA solution). Data are mean $\pm$ standard deviation (n=3).

Figure 4. Surface vs. bulk loading in microparticles: A) simvastatin post-loaded, C) simvastatin pre-loaded, and E) clodronate pre-loaded. Loading efficiency: B) post-loaded simvastatin, D) pre-loaded simvastatin, and F) pre-loaded clodronate. Data are mean $\pm$ standard deviation (n=3).

Figure 5.

Cumulative release profiles. A) Release of simvastatin loaded freely into the PLGA solution with () or without HA (○), or loaded into PBAE (□) (n=3). B) Release of clodronate freely loaded into PLGA solution with () or without HA (○), or loaded into PBAE microparticles (□) (n=4). Data are mean $\pm$ standard deviation.

Figure 6.

Degradation of *in situ* forming PLGA scaffolds. A) Destructive mass loss showing dry mass change expressed as a fraction of pre-injection mass (n=3). B) Non-destructive mass loss showing total hydrated scaffold mass change, expressed as a fraction of pre-injection volume (n=5). Data are mean $\pm$ standard deviation

Figure 7. Representative microCT cut-plane images of lyophilized scaffolds showing internal microarchitecture throughout the degradation process. Scale bars are 1 mm.

Figure 8. Morphometric parameters during degradation. A) Porosity and B) average pore size measured by microCT evaluation of samples through 35 days.