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Tailored Sequential Drug Release from Bilayered Calcium Sulfate Composites

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Abstract

The current standard for treating infected bony defects, such as those caused by periodontal disease, requires multiple time-consuming steps and often multiple procedures to fight the infection and recover lost tissue. Releasing an antibiotic followed by an osteogenic agent from a synthetic bone graft substitute could allow for a streamlined treatment, reducing the need for multiple surgeries and thereby shortening recovery time. Tailorable bilayered calcium sulfate (CS) bone graft substitutes were developed with the ability to sequentially release multiple therapeutic agents. Bilayered composite samples having a shell and core geometry were fabricated with varying amounts (1 or 10 wt%) of metronidazole-loaded poly poly(lactic-co-glycolic acid) (PLGA) particles embedded in the shell and simvastatin directly loaded into either the shell, core, or both. Microcomputed tomography (MicroCT) images showed the overall layered geometry as well as homogenous distribution of PLGA within the shells. Dissolution studies demonstrated that the amount of PLGA particles (*i.e.*, 1 vs. 10 wt%) had a small but significant effect on the erosion rate (3% vs. 3.4% per day). Mechanical testing determined that introducing a layered geometry had a significant effect on the compressive strength, with an average reduction of 35%, but properties were comparable to mandibular trabecular bone. Sustained release of simvastatin directly loaded into CS demonstrated that changing the shell to core volume ratio dictates the duration of drug release from each layer. When loaded together in the shell or in separate layers, sequential release of metronidazole and simvastatin was achieved. By introducing a tunable layered geometry capable of releasing multiple drugs, CS-based bone graft substitutes could be tailored in order to help streamline multiple steps needed to regenerate tissue in infected defects.

Keywords

calcium sulfate; sequential drug release; bioceramic; simvastatin; metronidazole

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1. Introduction

Healing of infected bony defects, such as those resulting from periodontal disease, has become a major focus in the field of bone tissue engineering.[1] Onset of periodontitis, a bacterial infection affecting the gingiva, alveolar bone, periodontal ligament, and root cementum, leads to the initiation and propagation of chronic inflammation, eventually causing the destruction of surrounding connective tissue and bone.[2–7] Periodontal infections are first treated with extensive debridement and scaling of plaque.[4–6, 8] Some pathogens may not be susceptible to mechanical removal, however, because they often ‘hide’ deep in gingival pockets around compromised bony tissue.[5, 9] These bacteria can frequently trigger reoccurrence of the initial infection, which greatly affects restoration or preservation of lost bone.[8] Antibiotics systemically delivered orally or locally administered using topical gels, creams, or films are then used to eliminate the pathogens prior to implantation of grafting material.[1, 5, 8]

An infected periodontal pocket contains an abundance of microflora, such as *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*. [5, 10] These pathogens are often protected within a biofilm, which allows them to flourish and requires a substantially higher dose of antibiotic to fully eradicate the bacteria.[5, 10] Due to clearance in the blood during systemic administration, the resulting low local concentrations of antimicrobial agents at the infected site often do not meet the levels required to kill the microbes.[3, 6, 7, 11] As a result, long-term therapy with high systemic doses of antimicrobial agents may be needed to fully eliminate the infection. However, this treatment could potentially cause adverse effects, such as liver and kidney damage, or lead drug resistance.[12] Consequently, local administration of drug directly to the site of infection may prove more effective by providing a higher concentration while using a smaller dose.[5, 6] Of the different antibiotics used for dental applications, metronidazole significantly reduces periodontal infection when compared to others.[11, 13] In addition, using a biodegradable material capable of controlling the amount of metronidazole released may allow for higher sustained and more effective concentrations to be obtained at the site.

Following treatment of periodontal infections, regeneration of bony tissue can begin. Bone regeneration using autografts is considered the ‘gold standard’. Intra-oral donor sites are limited, however, and harvesting can lead to undesirable donor site morbidity and chronic discomfort.[14–28] Another option is the use of allografts harvested from cadaveric bone tissue. Although frozen, freeze-dried, and/or demineralized, patients receiving the grafts are still at risk of immunologic rejection or disease transmission.[28–30] Allografts have been combined with osteoinductive growth factors to make them more effective.[15] Because of the limitations, and even risks, involved with autogenous and allogeneic materials, much attention has turned to the development of innovative bone graft substitutes. Calcium sulfate (CS) represents a promising alternative.[15] CS becomes osteogenic in the presence of bone, and through dissolution, the material is completely absorbed without inducing a significant inflammatory response.[24, 31–34] Like many other synthetic grafting alternatives, however, the efficacy of CS can be further enhanced with the aid of bioactive agents.

A beneficial addition would be the incorporation of statin drugs. Statins are widely known as inhibitors of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase that help control cholesterol levels, but these pleiotropic drugs also have osteogenic activity.[29, 35–40] *In vitro* and *in vivo* studies have demonstrated the positive effects of simvastatin through stimulation of osteoblastic activity and inhibition of osteoclastic activity.[29, 35–39, 41] Simvastatin was shown to be effective when released from a CS matrix *in vivo*. [15]

A bone grafting device capable of sequentially releasing an antibiotic followed by an osteogenic agent may be able to treat infection while still being able to regenerate bone. Little research has investigated the release kinetics of antimicrobial and osteogenic drugs from the same grafting device.[1] In the present study, dual drug-loaded, bilayered CS composites comprising a shell and core geometry with embedded poly(lactic-co-glycolic acid) (PLGA) particles were developed. After evaluating the compressive strength and modulus, dissolution, and morphology of bilayered composites, the tunable sequential release kinetics of metronidazole and simvastatin from the composites were explored.

2. Materials and Methods

2.1. Metronidazole-loaded PLGA Particles

Poly(lactic-co-glycolic acid) (Durect Corp., Birmingham, AL; 50:50; inherent viscosity: 0.55–0.75 dL/g; carboxylate end group) particles loaded with metronidazole (Sigma-Aldrich, St. Louis, MO) were created by film-casting and hand-grinding. Initially, 25 mg of metronidazole were combined with 200 mg of PLGA and dissolved in 1 mL of dimethyl sulfoxide (DMSO; an FDA Q3C Class 3 solvent). The solution was poured into a circular Teflon mold, frozen quickly at -80°C , and lyophilized to remove the DMSO. The dried film was hand ground to obtain particle sizes between 150–250 μm . A small amount of CS was used to prevent the polymer from sticking during grinding. The particles were washed with ethanol to remove residual CS powder on the surface of the polymer. Ethanol was chosen for washing to prevent drug loss because the low solubility of metronidazole in this solvent. Washed particles were vacuum-filtered, rapidly air-dried, and stored at -20°C until used.

A short-term study of metronidazole-loaded microparticles was conducted to determine how much drug may be released during the setting phase of composite formation. For this purpose, 10 mg of washed PLGA particles were incubated at 37°C in 1 mL of phosphate-buffered saline (PBS), pH 7.4. Supernatant was collected and replaced with fresh PBS every 15 min for the first hr, every 30 min for the 2nd hr, every hr for the 3rd and 4th hr, and finally increased to every 2 hr for the 6th and 8th hr time points. Supernatants were filtered (0.45 μm) and the absorbance measured at 318 nm.

2.2. Bilayered Calcium Sulfate Composites

Fabrication of the bilayered composites is illustrated in Figure 1. The composites consisted of calcium sulfate hemihydrate (Sigma-Aldrich) as the structural matrix. First, blank CS samples without layers were produced by combining 1 g of CS with 800 μL of deionized (DI) water. The slurry was injected into a mold having a diameter of 6.3 mm and a height of

12.6 mm. The loaded mold was placed in a 43°C oven for 24 hr to allow for the CS to completely set.

To begin formation of bilayered composites, cylindrical cores were produced in Teflon molds having a diameter of 4.7 mm and a height of 10 mm. A small, 8 mm long metal peg with a 0.63 mm diameter was fitted precisely in the center of the mold, with about 2.5 mm of the peg embedded into the core. The pegs suspended and centered the cores for shell production later. To make blank CS cores, 800 μ L of DI water was added to 1 g of CS powder and mixed thoroughly in 3 mL non-sterile syringes fitted with a 16 gauge blunt needle. The slurry was loaded into the custom-fabricated Teflon mold and placed in a 43°C oven for 24 hr to set the CS. For cores loaded with simvastatin, the same process was used, however 20 mg of simvastatin (Haouri Pharma-Chem, Inc., Edison, NJ) were mixed along with the CS and DI water. Pegs were removed from the cores when they were dried, and the cores were stored at room temperature with desiccant until used.

To form the shell around the cores, another Teflon mold was created with cylindrical holes having the diameter of 6.3 mm and a height of 12.6 mm. The base plate was fabricated with 3.5 mm deep holes into which metal pegs were securely inserted. This depth allowed the cores to be positioned precisely in the center of the mold, thus allowing the shell to surround the core. Blank and simvastatin-loaded shells were created using the same method described above for the cores, where 1 g of CS was mixed with 800 μ L of DI water, and in the case of simvastatin-loaded shells, 20 mg of drug were directly added. For samples containing metronidazole-loaded PLGA, the particles were added to both the blank and simvastatin-loaded shells at either 1 or 10 wt% and then mixed with 850 μ L DI water in 3 mL syringes fitted with a blunt-tipped needle for easy, consistent filling of the mold. Using these formulations for the shells, bilayered composites were formed by filling the molds about half full. Next, prefabricated cores were quickly dipped in DI water to wet the surface, which allowed for smooth coverage of the shell slurry around the core, inserted into the mold, and pressed down onto the metal pegs until they stopped. The pegs positioned the cores and held them in place during setting of the shell slurry. The filled mold was placed into a 37°C oven and allowed to dry overnight. For simplification, the different types of samples were given abbreviated names (Table 1).

The shells and cores described had a volume ratio of 50:50. Two other ratios were tested with simvastatin loaded into the shell only (SSBC), core only (BSSC), or both layers (SSSC). These samples were used to demonstrate how a change in the volume ratio would affect drug release from the composites. Table 2 lists the volume ratios used and the dimensions of the respective core and shell components. Custom molds were created to accommodate the different sizes, but the rest of the fabrication process was the same as described previously.

2.3. Composite Microarchitecture

To monitor the distribution of PLGA particles within the CS shell matrix as well as the interface between shell and core, microcomputed tomography (microCT) was employed. Using a Scanco Medical μ CT-40 scanner, specimens were evaluated at high resolution. Other parameters were set as follows: 156 μ m increments, 0° angle, 70 kVp, 114 μ A, 0.5

mm Al filter, and a voxel size of 8 μm . The raw images were qualitatively investigated for particle distribution trends, core orientation, and shell-core interaction. In addition, qualitative and quantitative assessment of the composites was conducted using a built-in 'bone trabecular morphometry' analytical tool with a lower threshold level of 130, gauss sigma of 3.0, and gauss support of 9. This script created a three-dimensional reconstruction that allowed visual assessment of cross-sections through the composite and provided the volume percentage of embedded polymeric particles and internal voids.

2.4. Composite Dissolution

Destructive testing was used to monitor dissolution of the bilayered composites. BSBC, 1-BSBC, and 10-BSBC samples were weighed, placed in separate 20 mL scintillation vials containing 12 mL of PBS and incubated on a plate shaker at 37°C. Every 4 d, replicate samples of each type were removed and dried at 43°C overnight. For the remaining samples, the PBS was replaced with fresh solution. The dried samples were weighed to determine the amount of material remaining, which was then used to calculate the percentage of residual mass.

2.5. Mechanical Properties

Compression testing was performed to investigate any effects on mechanical properties caused by the layered geometry, simvastatin directly loaded into CS as well as the introduction of PLGA particles into the shell of the composites. All samples types listed in Table 1 were evaluated. Testing was accomplished using a Bose ELF 3300 system. Contact surfaces were lightly sanded, if necessary, to create parallel surfaces in contact with the compression platens. All samples were loaded at a rate of 0.5 N/sec until failure. Compressive modulus (M) and ultimate compressive strength (UCS) were calculated.[42]

2.6. Drug Release from Bilayered Composites

2.6.1. Simvastatin Release—Release of simvastatin was measured for composites having shell to core volume ratios of 50:50, 70:30, and 85:15. BSBC, BSSC, SSBC, and SSSC samples for all volume ratios were prepared using the same simvastatin loading described in section 2.2. Samples were pre-weighed and submerged in PBS. Considering the overall size differences between the sample types, different volumes of PBS were used to maintain similar fluid volume to composite surface area ratios. To determine suitable sink conditions, the sample surface area to solution volume ratio of 50:50 samples was compared to those used in previous research [43]. A small pilot study showed that the dissolution rate of 50:50 samples remained constant for volumes above 10 mL (data not shown). To avoid saturation of CS or drug in PBS, a larger volume (12 mL) was used for the 50:50 samples. All other samples were placed in 4 mL, similar to previous release studies.[44] To determine how simvastatin would be released from bilayered composites dissolving in non-sink conditions, 50:50 samples were also immersed in 8 mL of PBS. All samples were incubated at 37°C on a plate shaker. Every 4 d, supernatant was collected and replenished with fresh PBS. Collected supernatant was treated with 100% ethanol in a 50:50 volume ratio to make sure all simvastatin was in solution. The mixture was then 0.45 μm -filtered and absorbance measured at 240 nm.

2.6.2. Multiple Drug Release—To investigate the kinetics of metronidazole and simvastatin release from bilayered CS composites, samples were pre-weighed, submerged in 12 mL of PBS, and incubated at 37°C while on a plate shaker. Supernatant was collected and replenished every 4 d. Two aliquots from each sample were kept for measurement of metronidazole and simvastatin separately. Metronidazole in syringe-filtered (0.45 µm) supernatant was assayed directly using UV spectroscopy at 318 nm. Simvastatin was measured using high performance liquid chromatography (HPLC) on a Hitachi Primaide system fitted with a Kinetix C18 column (5 µm, 4.6×150 mm). Prior to measurement, supernatant was mixed with 5 mM EDTA (pH 8.0) at a 50:50 volume ratio and allowed to sit overnight. EDTA, a common chelating agent, was used to remove calcium ions that could precipitate during HPLC analysis. Next, this mixture was mixed with 100% ethanol in a 50:50 volume ratio to ensure complete dissolution of the poorly soluble simvastatin. The final sample composition was 25% supernatant, 25% EDTA, and 50% ethanol. A 70:30 (acetonitrile : DI water + 0.01% trifluoroacetic acid) isocratic mobile phase at a flow rate of 1 mL per minute was used. Simvastatin was detected at 240 nm.

2.7. Statistics

Statistical analysis of the results was conducted using either a two-tailed unpaired t-test or one-way ANOVA. As appropriate, a Tukey-Kramer multiple comparison post hoc test was implemented. Linear regression was performed on sustained release profiles and the calculated slopes compared for significant differences using a two-tailed t-test. Differences between groups were considered to be significant with p-values <0.05.

3. Results

3.1. Metronidazole-loaded PLGA Particles

Metronidazole-loaded PLGA particles alone were first evaluated to determine how well the polymer controlled drug release. According to the results in Figure 2, metronidazole was released steadily during the first 3 hr at a rate of about 4 µg per minute. Only about 8% of the drug was released during the first hr. After 3 hr, 30% of the total loading of metronidazole had been released. From this point forward, the release of drug from the particles slowed, with roughly 35% of the loaded metronidazole released after 6 hr.

3.2. Composite Microarchitecture

3.2.1. Qualitative Evaluation—MicroCT images showed the CS cores embedded within the layered composites and their interaction with the CS shells, as well as the distribution of PLGA particles embedded in the shells (Figure 3). For comparison, results for a CS sample without layers (NL) are included. Minor defects (bubbles and other discontinuities) were found throughout the CS matrix in all samples. These defects were nearly spherical in nature and, thus, easily distinguishable from PLGA particles, which were irregularly shaped. The blank (drug-free) layered samples, BSBC, showed the CS core embedded within the CS shell and oriented parallel to the outer walls. The distribution of 1 wt% PLGA particles in the shells of 1-BSBC samples appeared to be homogeneous but sparse (Figure 3, top right). In 10-BSBC samples with 10 wt% loading of particles in the shells, there was a homogeneous but more frequent distribution of particles throughout the CS shell (Figure 3,

bottom right). Similar to BSBC, both 1-BSBC and 10-BSBC had cores that were embedded in the center of the composite with a parallel orientation to the outer surface. Additionally, all layered samples had some minor defects located at the shell/core interface, with most of these discontinuities occurring near the top and bottom of the samples.

3.2.2. Quantitative Evaluation—Morphometric analysis was conducted to better assess the overall microarchitecture of bilayered CS samples. As shown in Figure 4, the average volume percentage of voids in NL samples (0.96%) was significantly lower than that for all others ($p<0.001$). Furthermore, the particle content of 10-BSBC samples, 7.59%, was significantly higher ($p<0.001$) than that of both BSBC and 1-BSBC. Percentages for BSBC (3.30%) and 1-BSBC (4.52%) were not significantly different.

3.3. Composite Dissolution

Loading 1 wt% of PLGA particles into the shells of bilayered composites did not have a significant effect on the dissolution rate ($-3.1\%/d$) (Figure 5). Increasing the loading to 10 wt% PLGA, however, significantly increased the dissolution rate to $-3.43\%/d$ ($p<0.001$), even though the time for complete dissolution differed by only about 4 d.

3.4. Mechanical Properties of Layered CS

Fabrication of layered structures significantly affected the mechanical properties of CS composites (Figure 6). The ultimate compressive strength of NL (5.40 ± 0.38 MPa) samples was not significantly higher than that of BSSC (4.14 ± 0.75 MPa) and 1-BSBC (4.33 ± 0.72 MPa), but it was significantly greater than that for BSBC (3.80 ± 0.46 MPa, $p<0.01$) and all other bilayered samples ($p<0.001$) (Figure 6A). Within subgroups for PLGA particle loading, the strength of 1-BSBC (4.33 ± 0.72 MPa) was significantly higher than that for both 1-SSSC (3.03 ± 0.2 MPa) and 10-BSSC (3.03 ± 0.4 MPa) ($p<0.05$). There were no other significant differences seen for layered samples, both loaded with PLGA and without.

The compressive elastic modulus of NL (712 ± 139.6 MPa) was significantly higher ($p<0.001$) than that for SSBC (250 ± 37.6 MPa), 1-BSBC (172 ± 96.9 MPa), 1-SSSC (162 ± 70.4 MPa), 10-BSBC (237 ± 84.2 MPa), 10-BSSC (154 ± 58.4 MPa), 10-SSBC (216 ± 87.8 MPa), and 10-SSSC (184 ± 29.6 MPa) bilayered samples (Figure 6B). The average modulus of the blanks was also significantly greater than those of SSSC (306 ± 157.6 MPa, $p<0.01$) and 1-SSBC (391 ± 282 MPa, $p<0.05$). BSSC (495 ± 184 MPa) samples had a significantly higher ($p<0.05$) modulus than did 1-BSBC (172 ± 97 MPa), 1-SSSC (162 ± 70 MPa), and 10-BSSC (154 ± 58 MPa). There were no other significant differences between layered samples and their subgroups.

3.5. Drug Release from Bilayered Composites

3.5.1. Simvastatin Release—To demonstrate temporally controlled release of simvastatin using bilayered composites, several experiments were conducted using different shell to core volume ratios. Figure 7A shows the cumulative release of simvastatin from composites that had an 85:15 shell to core volume ratio. The sustained release of drug from samples with simvastatin loaded into both shell and core (SSSC), 0.055 mg/d, was significantly faster ($p<0.001$) than for SSBC (0.043 mg/d) and SSBC+BSSC (0.046 mg/d).

Minimal drug loaded into the core only (BSSC) was released during the first 24 d. From that point until the samples dissolved, however, release of simvastatin from CS cores increased to 18 µg/d, whereas release from SSBC samples was finished. The total amount of simvastatin released from BSSC (shell) and SSBC (core) samples was 0.24 ± 0.05 mg and 1.19 ± 0.01 mg, respectively. The rate of release from BSSC was significantly slower than that for both SSSC ($p < 0.05$) and SSBC+BSSC ($p < 0.01$).

Figure 7B shows the results for simvastatin released from composites consisting of 70% shell volume and 30% core volume. Over the first 20 d, the drug release rate from SSBC (0.046 mg/d) was significantly faster ($p < 0.01$) compared to 0.041 mg/d for SSSC. Little to no drug was released from BSSC samples during the first 20 d, followed by an upward shift to a rate of 0.022 mg/d, which was significantly slower than the rates for both SSSC and SSBC+BSSC ($p < 0.01$). For BSSC samples, 0.32 ± 0.03 mg of drug was released. After 20 d of simvastatin release from SSBC samples, the shells had completely dissolved and released 1.30 ± 0.07 mg of simvastatin.

The results for release of simvastatin from composites with a 50:50 shell to core volume ratio are depicted in Figure 7C. For the first 16 d of the experiment, the rate of release from SSBC composites, 0.095 mg/d, was significantly slower ($p < 0.05$) than that for SSSC (0.13 mg/d) and SSBC+BSSC (0.12 mg/d). During the same period, a small amount of drug was released from BSSC at a slow rate of 0.025 mg/d. After 16 d and until the composites dissolved, the rate of drug release from SSBC samples gradually slowed as the shell finished dissolving, a trend similar to what was observed in both Figures 7A and 7B. A total of 1.52 ± 0.3 mg of drug was released from the shell. At its peak, the rate of release from BSSC samples, 0.10 mg/d, was not significantly different from the rates observed for SSSC and SSBC+BSSC samples, ultimately releasing a total 1.44 ± 0.24 mg of simvastatin.

The total amount of simvastatin released from the cores (BSSC) for 50:50 (1.44 ± 0.24 mg) samples was significantly different ($p < 0.001$) than that for samples with either a 70:30 (0.32 ± 0.03 mg) or 85:15 (0.24 ± 0.05 mg) shell to core volume ratio. There was no significant difference in the total drug release between the 70:30 and 85:15 BSSC samples. For 50:50 SSBC samples, the total amount released (1.52 ± 0.3 mg) from the shells was significantly different ($p < 0.001$) than for 70:30 SSBC (1.30 ± 0.07 mg) and 85:15 SSBC (1.19 ± 0.1 mg) samples. In addition, the total amount of drug released from 70:30 SSBC samples was significantly different than from 85:15 SSBC samples ($p < 0.01$). When comparing the total amount of drug released when SSBC and BSSC results are combined (SSBC+BSSC) to the total amount of drug released from SSSC samples, SSBC+BSSC specimens with an 85:15 shell to core volume ratio had a total combined release that was significantly lower ($p < 0.01$) than the total amount of simvastatin released from the SSSC with the same volume ratio.

By reducing the volume of PBS from 12 mL to 8 mL, the dissolution time of samples having a 50:50 shell to core ratio was doubled (Figure 8). During the first 32–36 d of incubation, simvastatin was released from both SSSC and SSBC samples at a rate of 0.074 mg/d and 0.077 mg/d, respectively. Also, a small amount of drug from BSSC samples was released during the same time frame at a rate of 0.009 mg/d. From day 36 until the end of the

experiment (68 d), the rate for the SSSC samples continued steadily, however the rate of release from SSBC decreased to zero around 48 d. Additionally, the rate of release from BSSC samples increased to 0.051 mg/d and remained at this rate until the samples finished eroding.

3.5.2. Multiple Drug Release—Figure 9 shows results for release of metronidazole from PLGA particles embedded in CS shells as well as the release of simvastatin directly loaded in the shell or core of bilayered composites. The samples used for this experiment had a 50:50 shell to core volume ratio. Based on this ratio and the dissolution results presented in Figure 5, the shell and core portions of the composites were predicted to dissolve completely in 14–16 d of the 28–32 d dissolution period for the complete composite. For composites with 1 wt% PLGA particles embedded in CS, a large amount of metronidazole (65%) was initially released from the shells during the first 4 d at a rate of 16.3%/d (Figure 9A). After the initial burst of metronidazole, the release of drug slowed to a rate of 4.0%/d and continued to slowly decay to zero until the shells completely dissolved. Composites with 10 wt% PLGA particles showed a similar burst of metronidazole during the first 4 d, with as much as 55% of the total drug released (Figure 9B). The rate of release of metronidazole decayed from 13.7%/d through the first 4 d, to 6.7%/d from days 4–8, and finally down to 0% by d 12 of the release. The results in Figure 9 were normalized based on the amount of drug loaded into the respective layer rather than the complete composite. This allowed for direct comparison of the temporal release observed between metronidazole and simvastatin. When simvastatin was loaded into only the shell for 1 wt% and 10 wt% PLGA composites, metronidazole was initially released at a higher rate than simvastatin (2–6.3%/d). Because of the slow initial rate of simvastatin release, a short lag in the release profile developed, creating separation from the metronidazole profile and allowing for simvastatin to be released for up to 8 d longer. When the drugs were separated by keeping metronidazole-containing PLGA particles in the shell and loading simvastatin in only the core, 80–90% of the metronidazole was released over approximately 12 d before trace amounts of simvastatin were detected. After 16 d, the shells had completely dissolved, and metronidazole was no longer detected. In addition, the majority of simvastatin, isolated to only the core, was released starting after 12 d. Due to the layers separating the two drugs, a sequential release was observed with all metronidazole drug released prior to simvastatin.

4. Discussion

4.1. Metronidazole-loaded PLGA Particles

A previous study showed that loading a hydrophilic drug into carrier particles prior to embedding into a CS matrix significantly reduced the burst release witnessed when the drug was directly loaded into CS.[44] Therefore, biodegradable PLGA microparticles were employed for the current study to assist with the sustained release of metronidazole from bilayered composites. Because the particles were exposed to water during the setting phase of CS, a release study was conducted using a larger volume of water than present during composite formulation to monitor the potential for premature release of drug from PLGA. No initial burst of drug was observed, indicating that the majority of the drug was contained within the PLGA microparticles during the formation of the composites.

4.2. Composite Microarchitecture

Qualitative assessment of the morphology of bilayered composites showed good distribution of PLGA microparticles embedded into CS shells at 1 and 10 wt%. The initial CS slurry was kept sufficiently fluid to prolong the working phase and allow for easy filling of the molds yet viscous enough to suspend PLGA particles during the setting phase. This trend has been shown in previous research in which hydrogel particles were uniformly distributed throughout a CS matrix using similar powder to liquid volume ratios.[43] Another study, showed the exposure of particles at or near the surface before dissolution followed the pitting of CS resulting from the release of particles from those locations after a short duration submerged in PBS.[44] With the aid of small, metallic pegs, which were removed after fabrication, preformed blank and simvastatin-loaded cores were positioned in the center of the composites. Small defects were observed along the shell/core boundary. Many of these were air bubbles trapped as the CS set. Larger bubbles that appeared to accumulate near one end of the samples along the shell/core interface were most likely caused by an air pocket created when the cores were pressed into CS slurry.

Quantitative measurements assessed the particle volume fraction of bilayered CS composites. Although a significant increase in porosity was seen between samples containing 1 wt% and 10 wt% PLGA, the difference was not 10-fold. This lack of separation could be due to other defects, such as air pockets found along the shell/core interface. The script used to calculate the porosity is limited to only distinguishing between what is solid and what is not. The introduction of bubbles adds error to the calculations because these imperfections show up as radiolucent spaces similar to PLGA particles. Comparing the solid and layered samples demonstrates that defects strongly influenced on the porosity calculations.

4.3. Composite Dissolution

Calcium sulfate is a dense material that dissolves via surface erosion.[31, 43] Although the embedded PLGA particles were distributed throughout the CS matrix, they did not appear to be interconnected, which otherwise would have allowed for fluid to seep into the composite. During erosion, closed pockets with PLGA particles near the surface were exposed, releasing the polymer particles and increasing the surface area for further dissolution. Consequently, as the composites dissolved and PLGA particles were released, the surface area to volume ratio increased, allowing for faster dissolution of CS. Furthermore, because embedded PLGA particles did not change the dissolution characteristics of CS, the shorter lifespan when 10 wt% particles were added was related to the smaller overall volume of CS per sample that needed to dissolve. These trends have also been witnessed in a previous study in which dissolution of CS was observed after hydrogel particles were uniformly distributed throughout the composite.[43] Thus, the presence of polymer particles, even having different chemistries, affected only the duration of dissolution, depending on the amount of particles loaded, and not the nature of the dissolution process itself.

4.4. Mechanical Properties of Layered CS

To be a suitable alternative to autologous bone, the ideal synthetic material would have characteristics similar to those of tissue at the implantation site. CS has been described as

having properties being similar to cancellous or trabecular bone.[45] Investigating the mechanical properties of human trabecular bone from the mandible, Misch et al. measured an ultimate compressive strength of 3.9 ± 2.7 MPa and elastic modulus of 96.2 ± 40.6 MPa. [46] The introduction of a bilayered geometry significantly affected properties compared to solid CS samples, with up to a 44% and 78% reduction in the strength and elastic modulus, respectively. Note, however, that the properties of the layered composites were comparable to those of trabecular bone. The decrease in strength compared to the samples without layers could be due to the small air pockets along the shell/core interface acting as discontinuities within the composite, both at the interface parallel to the central axis of the cylinder as well as at the ends of the core. These stress concentrators contributed to a 17–44% decrease in the overall strength of the composites. The addition of either 1 or 10 wt% PLGA particles to CS shells did not have an effect when compared to blank bilayered samples. In previous research, as much as a 50–60% reduction in strength was seen following the addition of 10 wt% of gel particles to the monolithic CS matrix.[43] In the present study, the PLGA particles were isolated to only the shell of the composites. Also, the presence of a solid blank core may have provided reinforcement for the composite, which may allow a greater range of PLGA particle loading that would provide greater control over the drug dose within the composites.

The loading of simvastatin directly into the shell, core, or both layers generally did not significantly affect the strength of the bilayered composites, even though isolated differences were observed (1-BSBC versus 1-SSSC and 10-BSSC). In another experiment conducted by Orellana et al., loading of simvastatin directly into monolithic CS did not have a significant effect on the strength of the samples.[44] Another group determined that up to 10% loading of simvastatin into calcium phosphate samples did not significantly affect the compressive strength.[47] The present study had a lower loading of simvastatin (i.e., 2 wt %), however. Thus, direct loading of simvastatin does not affect the overall strength of different materials.

4.5. Drug Release from Bilayered Composites

The layered geometry used for the CS samples provided a unique platform for achieving a customizable sequential release of therapeutic agents. Loading of PLGA microparticles into the CS matrix allows for further tailoring of drug release.

4.5.1. Simvastatin Release—The present experiments were designed to demonstrate how the release of simvastatin can be tailored depending on which layer the drug was loaded in, whether it was the shell only, core only, or both. Furthermore, to illustrate the ability to adjust the duration or even the delay of drug release from either the shell or core, the shell to core volume ratio was altered. However, there were limitations to how much the volume ratio could be adjusted. For instance, the 50:50 shell to core ratio was considered the maximum. With the present dimensions, increasing the core volume beyond this point would create a thin and unstable shell. On the other hand, if the ratio was made so the shell would be greater than 85% of the total volume, the local concentration of drug released from the core could be too low to be therapeutically relevant.

For all of the release profiles that had simvastatin loaded in only the cores, there was a small amount of drug released from the start of composite dissolution up to when the core was completely exposed. Because the cores were suspended using a small metal peg that was later removed after the samples were fabricated, the hole that remained may have been large enough to allow a noticeable, but statistically insignificant, amount of simvastatin to be released.

In addition to investigating the effects of adjusting the shell to core volume ratio, the volume of PBS used for the release study was reduced from 12 mL to 8 mL. Because of the possible diverse environments in various implantation sites, it is likely for the implant to encounter different fluid volumes and/or turnover rates that may not allow for sink conditions. A small study demonstrated how release of simvastatin would change under non-sink conditions. To conduct the comparison, the 50:50 volume ratio was used. Interestingly, even with the reduction in the volume of PBS to 8 mL, the transition after shell depletion to core only erosion occurred around the halfway point, similar to the results seen under sink conditions. In previous work, the effects of fluid volume on the dissolution of CS were investigated.[43] It was determined that the change in volume of fluid or even the turnover rate could have a large effect on the dissolution of CS.[43] The duration of drug release can be greatly prolonged (doubled in the present study) using different fluid volumes. McLaren et al. also observed a large difference in the rate of drug release from calcium sulfate pellets when the fluid was completely refreshed at each time point versus exchanging only a fraction of the fluid volume.[48] In the present study, although the rate of release slowed, the mechanism of drug release remained dependent on dissolution of CS. This could allow for tailoring of drug loading and/or the sample geometry according to the physiological conditions expected at the implant site.

4.5.2. Multiple Drug Release—A multiple drug release study was conducted to investigate the release kinetics of bilayered composites loaded with an antimicrobial agent and an osteogenic agent. Findings for samples with metronidazole in PLGA particles and simvastatin directly loaded into CS demonstrated sequential release. Polymeric particles loaded with metronidazole and embedded into the shell were exposed as CS experienced surface erosion, and sustained release of the drug occurred as the PLGA particles subsequently degraded. Release from both 1 and 10 wt% particles embedded into the CS shell was sustained until the shell portion of the bilayered composites completely dissolved. Controlled release of metronidazole from PLGA microspheres after an initial burst has been described as a possible treatment for periodontal disease.[49] By embedding particles into a CS matrix, the release of metronidazole was controlled throughout the first 16 d of the present study. In addition, previous research has demonstrated that drug-loaded hydrogel particles homogeneously distributed throughout a CS matrix allowed for a controlled release of drug, which only occurred due to the breakdown of particles exposed at the surface of the dissolving composite.[43, 44]

With the intended use of these composites as a grafting substitute for alveolar bone augmentation, the oral cavity presents challenges for proper tissue regeneration due to the environment being rich with bacteria that can colonize natural and synthetic substrates. Thomas and Puleo described the implications of infection and inflammation in periodontal

disease and tissue regeneration.[50] Currently, the standard treatment for infected periodontal defects has antimicrobial agents being administered, either systemically or locally, prior to implantation of grafting material, which only delays the overall recovery of lost tissue.[1, 5, 8] Administration of antimicrobial agents allows for better bone formation. Chen et al. investigated the effects of two different growth factors in a chronically infected bony defect in rat femurs.[51] Although some healing occurred in the infected sites, the extent of bone formation was greater with the systemic administration of antibiotics.[51] To further enhance the process of fighting infection and then regenerating lost tissue, release of an anti-bacterial agent from the graft starting at the time of implantation may prove beneficial to help reduce the overall healing time.

Many studies have investigated dual purpose implantable scaffolds, but none have employed a concentric cylindrical CS system as described in the present studies. Reis et al. developed drug-free, bilayered membranes comprising a continuous outer layer of PLGA with a porous calcium phosphate inner layer for the regeneration of lost periodontal tissue.[52] For infected sites, Nguyen et al. developed a co-culture model using methicillin-sensitive *Staphylococcus aureus* and mouse bone marrow stromal cells to investigate the dual effects of an antibiotic, vancomycin, along with bone morphogenetic protein-2 (BMP-2).[1] Separately, the two agents were not effective, but when delivered together, the needed concentration of vancomycin was significantly reduced, suggesting that lower, non-toxic doses could be used.[1] An *in vivo* study in which vancomycin and BMP-2 were delivered simultaneously from a biodegradable polyurethane scaffold demonstrated that bone formation could be regenerated within an infected defect.[53] However, these systems release the drugs simultaneously. Considering the intent for the current device to help streamline the existing treatment process, it was encouraging to see metronidazole released before simvastatin, even when loaded into the shell together. The difference in the release kinetics can be explained primarily by the way the two drugs were loaded. Previous work has shown that release of drug from polymer particles embedded into a CS matrix had a rapid, initial burst followed by decay in the rate of release.[43, 44] The lower rate is attributed to the decrease in surface area as CS degrades, leading to a smaller volume of particles exposed at the surface over time.[43, 44] Simvastatin, on the other hand, is directly mixed with CS during sample formation, and due to the hydrophobic nature of the drug, it does not become segregated to the surface during the setting of CS. The release of simvastatin is, therefore, governed by the surface erosion characteristics of CS, which were shown to be linear. This allowed for a near constant rate of release of simvastatin. These differences in release kinetics between the two drugs and their means of loading allowed for enough separation for all the metronidazole to be released 4 d sooner than simvastatin. When simvastatin was loaded into only the core while PLGA particles loaded with metronidazole remained in the shell, there was a much greater lapse in time for a fully separated sequential release to occur, which may be useful for mimicking the clinical sequence of events for treating infection and subsequently restoring lost or damaged tissue.

5. Conclusion

In the present study, novel bilayered CS composites were investigated for their ability to provide tailored release of therapeutic agents as well as a sequential release of different

drugs. Such a system may be useful as a bone graft substitute for treating infected bony defects, e.g., periodontal pockets. Although the shell and core geometry reduced mechanical strength of the composites, the properties were similar to those for mandibular trabecular bone. This may be an important trait that could allow for these implants to better mimic the surrounding target tissue being treated. Changing the shell to core volume ratio dictates the duration of drug release from each layer. When metronidazole and simvastatin were loaded together in the shell or in separate layers, temporal separation of the two drugs was achieved. Being able to tune such a system may help streamline the multiple steps needed to regenerate tissues more efficiently.

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Highlights

- Bilayered CS composites were fabricated as potential bone graft substitutes.
- The shell and core geometry allows for tunable sequential release of drugs.
- The bilayered devices are mechanically similar to mandibular trabecular bone.
- Controlled release of drug and carrier particles is governed by CS dissolution.

Figure 1. Schematic depicting the process of forming a bilayered CS composite. From left to right: core formation; insertion of core into shell mold; final composite showing core encased within a CS shell. Images are to scale.

Figure 2. Cumulative release of metronidazole from 150–250 µm PLGA particles. Data are mean $\pm$ standard deviation (n=3).

Figure 3.

Representative microCT images of CS/PLGA composites: raw X-ray slices and cross-sections of 3D reconstructions. Closed arrows mark bubbles, and open arrows with circles indicate PLGA particles. Scale bars denote 1 mm.

Figure 4. Volume percentage of voids/particles in CS/PLGA composites determined by microCT. Data are mean $\pm$ standard deviation (n=5). Symbols (*) indicates significant differences (p<0.001).

Figure 5. Mass loss profiles for bilayered blank CS and composites with 1 and 10 wt% PLGA loaded into the shells. Data are mean $\pm$ standard deviation (n=3).

Figure 6.

Mechanical properties of CS/PLGA composites with directly loaded simvastatin: A) ultimate compressive strength and B) compressive modulus. Data are mean $\pm$ standard deviation (n=5). Symbols indicate significant differences: $p < 0.001$ (*), $p < 0.01$ (#), and $p < 0.05$ (Δ).

Figure 7.

Cumulative release of simvastatin from bilayered samples having different shell to core volume ratios: (A) 85:15; (B) 75:25; and (C) 50:50. Data are mean $\pm$ standard deviation (n=5).

Figure 8.

Cumulative release of simvastatin from bilayered samples incubated below sink conditions. The samples tested had a 50:50 shell to core volume ratio. Data are mean $\pm$ standard deviation (n=5).

Figure 9.

Cumulative release of simvastatin and metronidazole from bilayered composites.

Normalized profiles of directly loaded simvastatin and metronidazole loaded into PLGA particles released from composites with (A) 1 wt% and (B) 10 wt% PLGA particles loaded in shells. Data are mean $\pm$ standard deviation (n=5).

Table 1

Abbreviations for the different sample types fabricated. Codes are read: BSBC » (B)S(B)C » (Loading) in SHELL, (Loading) in CORE.

Shell Composition	Core Composition	Code
Blank	Blank	BSBC
Blank	Simvastatin	BSSC
Simvastatin	Blank	SSBC
Simvastatin	Simvastatin	SSSC
1 wt% PLGA	Blank	1-BSBC
1 wt% PLGA	Simvastatin	1-BSSC
1 wt% PLGA & Simvastatin	Blank	1-SSBC
1 wt% PLGA & Simvastatin	Simvastatin	1-SSSC
10 wt% PLGA	Blank	10-BSBC
10 wt% PLGA	Simvastatin	10-BSSC
10 wt% PLGA & Simvastatin	Blank	10-SSBC
10 wt% PLGA & Simvastatin	Simvastatin	10-SSSC
No Layer Blank (No Drug)		NL

Note: PLGA particles contained metronidazole.

Table 2

Shell to core volume ratios and dimensions of the bilayered composites

Volume Ratio	Shell Height	Shell Diameter	Core Height	Core Diameter
50:50	12.6 mm	6.3 mm	10 mm	4.7 mm
70:30	9.6 mm	4.7 mm	6.2 mm	3.2 mm
85:15	9.6 mm	4.7 mm	6.2 mm	2.4 mm