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Catherine Linh
University of Rhode Island, catherine_linh@my.uri.edu

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Ultrasonically Responsive Tissue Engineering Scaffolds for the Temporal Control over Osteo-Inductive Growth Factor Delivery

Catherine Linh¹ and Stephen Kennedy²,³
¹Department of Chemistry, ²Department of Chemical Engineering, ³Department of Electrical, Computer and Biomedical Engineering
University of Rhode Island, Kingston, RI 02881

Introduction
In 2012, approximately 6.8 million people were diagnosed with orthopedic injuries and diseases¹. Over 500,000 people had undergone bone grafting procedures, which cost 2.5 billion dollars per year⁵. The most common treatment for bone defects is autogenous bone grafting, which usually involves the removal of the host’s bone from the pelvis in order to fill the bone defect⁵. However, these traditional bone grafting treatments have complications, such as donor site morbidity, and can result in non-unions at the injury site⁵.

Figure 1. Traditional bone grafting has complications. Biological stimuli have an influence on bone regeneration. Delayed healing caused by inappropriate stimuli would result in a bone defect. Figure adapted from Mehta et al [5].

Because of these complications, synthetic bone grafting materials are of great interest. Polymer-based grafting scaffolds can provide the 3D structure required to facilitate 3D bone tissue growth. Moreover, these scaffolds can be loaded with bioactive molecules that direct bone tissue development, delivering them in a localized, sustained manner through polymer degradation and/or diffusion.

Bone regeneration requires biomaterial scaffold implantation and can be potentially enhanced by incorporating growth factors. Kolambkar conducted a study on bone regeneration of an 8 mm critical defect, which was filled with the biomaterial scaffold (alginate and nanofiber mesh) and bone morphogenic protein or BMP-2 (³). The bone regeneration that resulted from the (i) biomaterial scaffold and (ii) biomaterial scaffold with BMP-2 was analyzed by micro computed tomography (µCT) at 4 and 12
weeks. There is significant bone regeneration from the biomaterial and BMP-2, compared to the biomaterial scaffold alone. There was another study done by Lee on the bone regeneration of rat cranial defects by calcium phosphate cement (CPC). These defects had been filled with scaffolds incorporated with either no growth factor (i), BMP-2 (ii), or BMP-2 & transforming growth factor beta-1, or TGFβ-1 (iii) were analyzed by μCT. The CPC scaffold alone has some effect on bone regeneration. Accompanied with BMP-2, the defect is nearly filled in with bone tissue. Lee and his colleagues attempted to see if they could improve the bone regeneration by having BMP-2 and TGFβ-1 at the same time inside the scaffold. The bone regeneration actually worsened, compared to having BMP-2 alone. This happened because the cells were directed to proliferate and to osteo-differentiate at the same time. Once the stem cells become osteoblasts, these cells lose the ability to divide rapidly.

![Figure 2](image)

**Figure 2.** Bone regeneration requires biomaterial scaffold implantation and can be potentially enhanced by incorporating growth factors. (A) An 8 mm critical defect was filled with the biomaterial scaffold. (B) The bone regeneration that resulted from the biomaterial scaffold (i) and biomaterial scaffold with BMP-2 (ii) was analyzed by micro computed tomography (μCT). Figures adapted from Kolambkar et al [3]. (C) The rat cranial defects that have been filled with scaffolds incorporated with no growth factor (i), BMP-2 (ii), or BMP-2 & TGFβ-1 (iii) were analyzed by μCT. Figure adapted from Lee et al [4].

However, bone regeneration requires the orchestration of a sequence of events (i.e., bone progenitor cell recruitment, proliferation, and osteo-differentiation). The bone progenitor cells are first recruited into the cell. Then, these cells proliferate and multiply into many cells. At a later point in time, these cells undergo osteo-differentiation and become osteoblasts, which results in bone formation.
Figure 3. The natural bone healing cascade suggests that SDF-1α (recruiting factor) be delivered prior to delivery of BMP-2 (differentiation factor). (A) Schematic of the path of MSC from stem cell to osteocyte. (B) Schematic of native bone repair sequence. Figures adapted from Mehta et al [5].

Project Aims
The problem is that bone regeneration needs a scaffold to deliver signals in sequence to cells. We hypothesized that an ultrasonically responsive hydrogel-based biomaterial system that can potentially deliver growth factors in sequence. Specifically, we aimed to design a polymer scaffold that can release one payload diffusively at early time points, followed by ultrasonically triggered release of a second payload. The ability to deliver sequential payloads on demand (using ultrasonic stimulation) can more accurately mimic natural biological responses and afford the ability to clinically alter the course of therapies after scaffold implantation. Calcium-crosslinked alginate hydrogels have self-healing properties, allowing them to potentially recover from damage caused by ultrasonic exposures. The crosslinking between the calcium and alginate is disrupted in the presence of ultrasound stimulation and reforms in the absence of ultrasound.

Figure 4. Ultrasonically responsive biomaterials have potential in sequential delivery. Schematic of the disruption of the crosslinking in the presence of ultrasound and its self-healing properties in the absence of ultrasound. Figure adapted from Huebsch et al [2].
Methodology

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<tr>
<td>Neutral</td>
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<td>FITC-Dextran 20 kDa</td>
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<tr>
<td>Charged</td>
<td>FITC-DEAE-Dextran 3-6 kDa</td>
<td>FITC-DEAE-Dextran 20 kDa</td>
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Figure 5. We used four model drugs of different sizes and charges.

The calcium alginate hydrogels were loaded with four model drugs (fluorescently labeled dextrans) of different charges and sizes in order to characterize drug release due to diffusion (controls, no ultrasonic signal applied), compared to when stimulated by different ultrasonic signals (different amplitudes and durations of 20 kHz signals). The figure below depicts the process of making and testing calcium-crosslinked alginate hydrogels. Release of these dextrans was quantitatively measured on a plate reader against a standard curve by measuring fluorescence at 525 nm.

Figure 6. Process of making and testing calcium-crosslinked alginate hydrogels. (i) 2.5% wt alginate was cast with a model drug and calcium sulfate on a glass plate. (ii) Individual gels were cut. (iii) Gels were rinsed in 5 mL of PBS containing CaCl$_2$ and MgCl$_2$. (iv) Gels before ultrasound stimulation. (v) Gels were ultrasonically stimulated at different amplitudes and durations. (vi) 1 mL samples were extracted after stimulation and reserved for quantification.
Results and Discussion

The optimal ultrasound stimulation for triggering release was a 20 kHz signal at 20% amplitude for 3 minutes due to a combination of release, thermal, and structural considerations.

(A) Percent release (i) and rise in temperature (ii) after ultrasonic stimulation at various amplitudes (0, 20 and 40%) and durations (1 and 3 min; blue and orange, respectively). (B) Temperature achieved at the indicated ultrasonic stimulations (i) and percent of released due to diffusion at the maximum temperature achieved during stimulation (ii). (C) Photograms of the ultrasonically responsive hydrogels after the indicated ultrasonic stimulations: 1 minute (top row) and 3 minute (bottom row) exposures. In parts A, B, and C, ‡ indicates the optimized ultrasonic stimulation condition. n.s., *, **, and *** indicate statistical comparisons that were not statistically significant (n.s.), or significant with $p < 0.05$, 0.01, or 0.001, respectively. N = 4.
The optimal ultrasound stimulation for triggering release was a 20 kHz signal at 20% amplitude for 3 minutes due to a combination of release, thermal, and structural considerations. In figure 7 part A, percent release (i) and rise in temperature (ii) were measured after ultrasonic stimulation at various amplitudes (0, 20 and 40%) and durations (1 and 3 min; blue and orange, respectively). There is a significant release of the model drug from 20% and 40% amplitude at 3 minutes, compared to the controls. Ultrasound stimulation also increases the temperature of the solution. Because of the 14 °C temperature increase, 40% amplitude for 3 minutes was not chosen as the optimal stimulation. Temperature is crucial because proteins denature and lose their bioactivity at 45 °C. Since 20% amplitude for 3 minutes resulted in a 5 °C temperature increase, proteins would still have their bioactivity. In part B, the temperature achieved after each ultrasonic stimulation (i) and percent of released due to diffusion at the maximum temperature achieved during stimulation (ii) were measured. There was no significant difference in drug release at room temperature and elevated temperature, indicating that the release is solely due to the ultrasonic stimulation and not heat. In part C, photographs of the ultrasonically responsive hydrogels were taken after each ultrasonic stimulations, where the top row shows 1 minute exposure and the bottom row shows 3 minute exposure. After being exposed to ultrasound at 20% amplitude for 3 minutes, the morphology of the gel was intact, where the 3D structure of the scaffold was preserved. In comparison to the gel after 40% amplitude for 3 minutes, the gel was no longer firm and was starting to lose its 3D structure.
Figure 8. Charged model drugs could be retained in the hydrogels prior to ultrasound stimulation. (A) Percent release due to 3 minutes of ultrasonic stimulation at 0% amplitude as a function of molecular charge for small (purple) and large (aqua) dextrans. (B) Percent release due to diffusion (0%) and after being exposed to 20% ultrasonic amplitude for 3 minutes for small (purple) and large (aqua) cationic dextrans. (C) Cumulative release vs. time for 10 kDa uncharged dextran (blue) and 3-6 kDa cationic dextran (red). Red rectangle indicates when gels were exposed to ultrasound. (D) Cumulative release vs. time of SDF-1a (blue) and BMP-2 (green). Gray rectangle indicates when gels were exposed to ultrasound. For all parts, n.s., *, **, and *** indicate statistical comparisons that were not statistically significant (n.s.), or significant with \( p < 0.05 \), 0.01, or 0.001, respectively. \( N = 4 \).

Charged model drugs could be retained in the hydrogels prior to ultrasound stimulation. In part A, the percent releases due to 3 minutes of ultrasonic stimulation at 0% amplitude are represented as a function of molecular charge for small (purple) and large (aqua) dextrans. The neutral drugs tended to diffuse out of the scaffold prior to stimulation, while the charged drugs were retained in the scaffold prior to stimulation. In part B, the percent release due to diffusion (0%) and after being exposed to 20% ultrasonic amplitude for 3 minutes was measured for small (purple) and large (aqua) cationic dextrans. The small charged drug was retained and then was ultrasonically released in an on-demand manner. The large charged drug was still retained after ultrasound stimulation. In part C, the cumulative release vs. time for 10 kDa uncharged dextran (blue) and 3-6 kDa cationic dextran (red) was measured. Red rectangle indicates when gels were exposed to ultrasound for 3 minutes at 20% amplitude for every hour. The
uncharged dextran was released at a faster rate than the charged dextran through diffusion. The charged dextran was retained in the scaffold until it is released by ultrasonic stimulation, resulting in a cumulative release of approximately 3 μg. The uncharged dextran was also released by ultrasonic stimulation where the cumulative release was approximately 12 μg. For both uncharged and charged dextrans, there was a significant difference in the release by diffusion and ultrasound stimulation. In part D, the cumulative release vs. time of SDF-1α (blue) and BMP-2 (green) was measured. Gray rectangle indicates when gels were exposed to ultrasound for 3 minutes at 20% amplitude for every 15 minutes. SDF-1α was released at a faster rate than the charged dextran through diffusion. The BMP-2 was retained in the scaffold until it is released by ultrasonic stimulation, resulting in a cumulative release of approximately 58 ng. The SDF-1α was also released by ultrasonic stimulation where the cumulative release was approximately 75 ng. For both SDF-1α and BMP-2, there was a significant difference in the release by diffusion and ultrasound stimulation.

The publication of this study is pending.

**Conclusion**

An ultrasonically responsive calcium-crosslinked hydrogel system could provide delayed release of small, charged model drugs and differentiating BMP-2. This system can be used to investigate how the timing and sequence of drugs based on their size and charge and can be applied to bone regeneration in the delivery of growth factors.

**References**


2 Huebsch et al. PNAS. 2014; 111: 9762-9767.

