Temporal Zinc Release from Hydrogels: Effects on Mechanics, Metabolic Activity, and Gene Expression

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INTRODUCTION: Fragility fractures are difficult to treat clinically with standard operative techniques. Localized delivery of zinc at surgical fracture fixation sites has promising therapeutic potential – especially at early timepoints [1]. It is known that zinc upregulates osteogenesis-related genes, inhibits osteoclast differentiation and regulates signaling pathways like RANKL/RANK/OPG which are involved in bone remodeling [2]. Gelatin methacryloyl (GelMA) is a photocrosslinkable hydrogel that is biocompatible, biodegradable, and has tunable mechanical properties. The stiffness and degradation kinetics of GelMA can be easily altered by adjusting the ratio of GelMA in the prepolymer solution [1]. However, little is known about GelMA as a zinc delivery vehicle. In this cell culture study, we doped GelMA with $ZnCl_2$ and examined degradation kinetics, metabolic activity, and changes in molecular pathway signaling. We hypothesized that the addition of zinc to GelMA would not affect degradation kinetics but would accelerate metabolic activity and increase gene expression levels related to osteoblast differentiation.

METHODS: This study consisted of 4 unique experiments that tested mechanical properties, degradation kinetics, metabolic activity, and gene expression. To create the test specimens, 5% (w/v) and 20% (w/v) GelMA hydrogels were fabricated by dissolving gelatin methacryloyl powder in Dulbecco's Phosphate-Buffered Saline (DPBS) and 0.5% (w/v) Irgacure 2959 at 37°C overnight. The solution was then pipetted into a mold and placed under 365nm UV light at the intensity of 65 mW/cm² for 300 seconds. GelMA samples of 5mm diameter and 3mm height were created with biopsy punches. 5% GelMA + 100µM $ZnCl_2$ (5% GelMA+Zn) hydrogels were created by adding 100µM $ZnCl_2$ in the prepolymer solution. The samples were incubated in DPBS for 24 hours before mechanical testing to ensure full swelling. In the first experiment, the equilibrium moduli of the GelMA hydrogels were obtained by performing an unconfined compression test that consisted of a 4.9N force held for 300 seconds, followed by cyclic loading to 1% strain at 0.5 Hz for 10 cycles. In a second experiment, degradation was measured by obtaining the dry weight of each sample everyday until day 7, then every 2 days until complete degradation. The percentage of mass remaining was calculated using the following equation:

$$\%\ \text{mass remaining} = \frac{W_{initial} - W_{dry}}{W_{initial}} \times 100\%.$$

In the final set of experiments, GelMA samples were incubated in Dulbecco's Modified Eagle Medium (DMEM)+10% fetal bovine serum, 1% penicillin-streptomycin for 24 hours. 100,000 bovine mesenchymal stem cells were seeded on the samples and cultured in DMEM at 37°C and 5% CO_2 on an orbital shaker. Alamar Blue assays were conducted on days 1, 3, 7, 14, 21, and 28 to measure metabolic activity. Fluorescence was measured using a 560-590nm wavelength setting on a plate reader. Realtime-qPCR was conducted on days 7 and 28 to measure mRNA expression of ALP and COL1a, with GAPDH as the housekeeping gene.

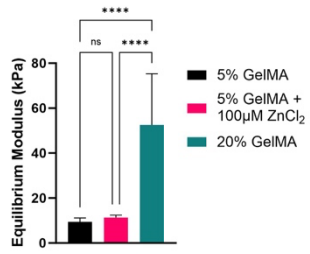

Fig. 1: *Mechanical testing of 5%, 5%+100µM $ZnCl_2$, and 20% GelMA. (n=8)*

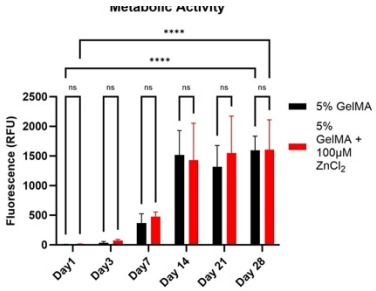

Fig. 2: *Results from Alamar Blue assays for 5% and 5%+100µM $ZnCl_2$ GelMA. (n=6)*

RESULTS: The equilibrium moduli of the 5% GelMA (9.43±1.72 kPa) and the 5% GelMA+Zn (11.36±1.10 kPa) were significantly less than the 20% GelMA (52.59±22.73 kPa; p<0.0001, **Fig. 1**). The 5% GelMA was completely degraded by day 22, and the 5% GelMA+Zn was degraded by day 31. The degradation rate of the 20% GelMA was slower, with more than 90% of its weight remaining at day 28. Because the goal of this therapeutic was to deliver zinc to a fracture healing site in the early phases of healing, the 20% group was excluded from the rest of the study. Alamar Blue assays did not show significant differences in metabolic activities between the 5% GelMA and 5% GelMA+Zn groups. An upward trend of metabolic activity was observed across all timepoints (**Fig. 2**). RT-qPCR showed no significant increase in ALP expression between the 5% GelMA and 5% GelMA+Zn groups across timepoints (**Fig. 3a**). COL1a expression showed a 2.22 fold decrease in the 5% GelMA+Zn group at day 28 compared to the 5% GelMA group (**Fig. 3b**).

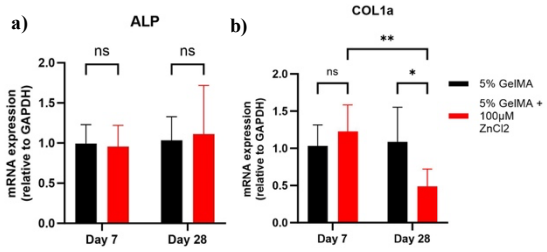

Fig. 3: *RT-qPCR results for 5% and 5%+100µM $ZnCl_2$ GelMA. **a)** Fold change for ALP. **b)** Fold change for COL1a. (n=5)*

DISCUSSION: The similarity in metabolic activity between 5% GelMA and 5% GelMA + Zn and an overall increase in metabolic activity demonstrated that the selected zinc dosage was noncytotoxic to bMSCs. The lack of change in ALP expression in the 5% GelMA + Zn group was likely due to a low overall dosage of $ZnCl_2$ in the GelMA, thus the amount of zinc released to bMSCs was insufficient to increase ALP levels. The gene expression levels of COL1a seems to support that the overall dosage of $ZnCl_2$ is insufficient. Zinc is known to upregulate RUNX2, an upstream gene that targets COL1a, at earlier timepoints of exposure. RUNX2 experiences a decrease in transcription levels in later timepoints of zinc exposure [2]. This is consistent with the slight increase of COL1a at day 7, then a decrease of COL1a at day 28. With a higher overall dosage of $ZnCl_2$, it is very likely that COL1a will see a higher expression level. This study had several limitations, including lack of interval timepoints for realtime-qPCR, failure to include 5% GelMA+Zn groups at different zinc concentrations, and lack of data for zinc release from the GelMA over time. Future studies will involve increasing the overall dosage of $ZnCl_2$ and subjecting the 10% and 20% GelMA groups to in vitro testing.

SIGNIFICANCE/CLINICAL RELEVANCE: This study provides preliminary findings that will help us understand the relationship between zinc dosage, zinc release rate, zinc exposure time, and osteogenesis. Such findings will guide the development of a scaffold/GelMA zinc delivery device used in bone regeneration.

REFERENCES: [1] K.H. Park, et al., Stem Cells and Development, 2018. [2] M. Sun, et al., Polymers, 2018.

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