# Fabrication of Crosslinked Carboxymethylchitosan Microspheres and Their Incorporation Into Composite Scaffolds for Enhanced Bone Regeneration

B Reves, JA Jennings, JD Bumgardner, WO Haggard
University of Memphis, Department of Biomedical Engineering

**Statement of Purpose:** Our laboratories have previously fabricated chitosan (CS) microspheres and scaffolds to augment fracture healing.[1] Although our technology has shown much promise, faster scaffold degradation is desired. Ideally, the scaffolds should degrade at the same rate at which new bone is deposited so that complete regeneration can occur in a timely manner. In addition, the elution of rhBMP-2 from our chitosan scaffolds has previously displayed a significant burst release, and we believe an extended delivery profile may be more beneficial.[1] The purpose of this study was to fabricate carboxymethylchitosan (CMCS) beads and incorporate them into composite CS-CMCS scaffolds. We hypothesized that the addition of CMCS beads to our scaffolds will result in composite scaffolds with increased degradation and enhanced rhBMP-2 elution.

**Methods:** CS microspheres were prepared using a precipitation method as previously described.[1] CMCS beads were prepared by reacting the CS microspheres with monochloroacetic acid.[2] The CMCS beads were then crosslinked using two different methods: 1) amine-amine crosslinking using genipin (Gen-X CMCS) and 2) amide bond formation using carbodiimide chemistry (X-CMCS). A ninhydrin assay was used to measure the number of free amine groups and determine the extent of crosslinking.[3] The swelling ratio of the beads was determined in 1x PBS at 24 hours. Composite scaffolds (Gen-X CMCS/CS and X-CMCS/CS) were prepared by fusing the microspheres together using a 1% acetic acid wash. Scaffold degradation was determined in a 100µg/mL lysozyme solution. An elution study was performed by loading scaffolds with 30µg of rhBMP-2 and placing them in release buffer containing 1x PBS. The amount of rhBMP-2 eluted was quantified using ELISAs, and the activity of the released growth factor was measured by the W-20-17 assay.[4] The cytocompatibility of the composite scaffolds with SAOS-2 osteosarcoma cells was determined using the CellTiter-Glo assay. In addition, Live/Dead staining was performed.

**Results:** Gen-X CMCS and X-CMCS microspheres were successfully fabricated as described. The ninhydrin assay demonstrated that crosslinking occurred, as shown by the reduction in the number of free amine groups (Table 1). Crosslinking method was found to have a significant impact on the properties of the microspheres and scaffolds. As seen in Table 1, the X-CMCS beads had much increased swelling capacity (> 600%). The X-CMCS/CS scaffolds had greatly increased degradation of 14.5 ± 6.6% compared to 0.5 ± 0.4 (CS-only) and -2.7 ± 0.3% (Gen-X CMCS/CS). The X-CMCS/CS composite scaffolds eluted more rhBMP-2 at every timepoint. Furthermore, the W20-17 assay demonstrated that the eluted rhBMP-2 was active and that the X-CMCS/CS scaffolds eluted more bioactive growth factor at every timepoint (Figure 1).

| | µmole amine/g beads | Swelling Ratio (%) |
|---|---|---|
| CS | 44.4 ± 7.9 | 176.3 ± 18.1 |
| Gen-X CMCS | 32.0 ± 4.3 | 398.1 ± 8.1 |
| X-CMCS | 22.6 ± 2.5 | 612.1 ± 3.2 |

**Table 1.** Results of ninhydrin and swelling studies

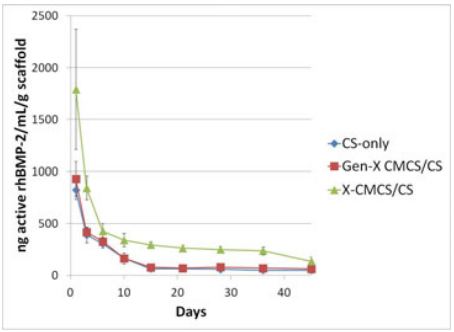


**Figure 1.** Bioactive rhBMP-2 eluted from scaffolds

The composite scaffolds were able to support the attachment and proliferation of SAOS-2 cells. Interestingly, the cells maintained a flattened morphology and spread well on the X-CMCS/CS scaffolds; whereas, the cells assumed a more rounded morphology on the Gen-X CMCS/CS scaffolds and did not spread as well (Figure 2).

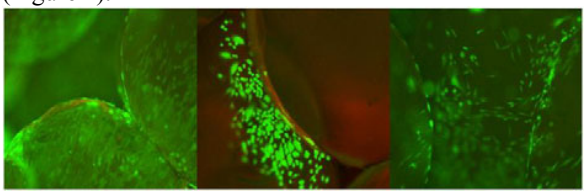

**Figure 2.** Live/Dead staining of SAOS-2 cells on scaffolds. L-R: CS-only, Gen-X CMCS/CS, and X-CMCS

**Conclusions:** Our research demonstrated that monochloroacetic acid can be reacted with chitosan microspheres to prepare CMCS beads. CMCS crosslinking method had profound impacts on the microsphere characteristics. X-CMCS beads were successfully incorporated into composite X-CMCS/CS scaffolds, and these constructs displayed increased degradation and an enhanced elution profile. Our hypothesis was confirmed, and X-CMCS/CS scaffolds are expected to augment bone healing in severe fractures.

**References:**

1. Reves BT. JBMR Part B. 2009; 90:1-10.
2. Shi ZL. Biomacromolecules. 2009; 10(6): 1603-11.
3. Yuan Y. Carbohydrate Polymers. 2007; 68(3): 561-7.
4. Thies RS. Endocrinology. 1992; 130(3): 1318-24.

**Acknowledgement:** Medtronic for donation of rhBMP-2.