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in the Treatment of Nonunion Fractures

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14. ABSTRACT A general set of hydrogel synthesis steps were defined which enables the incorporation of chitosan from multiple sources and suppliers and still produce a consistent material. Functional behavior of the hydrogel was confirmed with a new source of chitosan, inducing tissue ingrowth into a subcutaneously injected scaffold loaded with the composite xylan/chitosan hydrogel. Delivery of new hydrogel treatment for tibia fractures was accomplished with the xylan/chitosan composite showing bioactivity even in the absence of therapeutic proteins. Fractures treated with the xylan/chitosan composite showed accelerated remodeling of the fracture callus to normal bone compared to a hydrogel without the xylan component. No mineralization in response to treatment with BMP-4 has been observed in subcutaneous models, nor with the BMP-4/VEGF combination. A femoral fracture study was performed using the xylan/chitosan hydrogel treatment alone without protein delivery. The fractures treated with the xylan/chitosan healed while controls did not heal.					
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Introduction

Our hypothesis was that a degradable, thermally-responsive bone graft substitute, made from renewable sources, that effectively and simultaneously delivers osteogenic and angiogenic growth factors directly to the bone defect site can enhance repair of non-union fractures. In this study, a new chitosan/xylan composite hydrogel was studied as an improved bone graft substitute able to accurately deliver a combination of proven growth factors in a manner that is compatible with current surgical practice. This new bone graft substitute has immediate implications for clinical care of segmental bone loss and the acceleration of healing in traumatic bone injuries. The goal was to provide surgeons with an effective and reliable method for simultaneous delivery of synergistic growth factors. The thermally-responsive behavior of this new material allows surgeons to use stabilization methods in which they have confidence while at the same time enabling accurate delivery of precise quantities of synergistic proteins that are beneficial to healing. While the protein delivery aspect of this study did not succeed, treatment with the new xylan/chitosan hydrogel alone was enough to heal serious fractures that did not heal without treatment.

Body

During the first six months of the project, work focused on the goals of Task 1a. Even without animals, some characteristics of the hydrogel in question could be tested as well as performing *in vitro* cell work. The previous supplier of chitosan, the main polymer component of the hydrogel, no longer provides the product and so the hydrogel was redefined with chitosan from a new supplier. Because this is a naturally derived polymer and not a synthetic molecule, no two companies label the product in the same manner. We have found the best product from a new company that matches our specifications based on solubility and ability to form the composite hydrogel with xylan. *In vitro* cell culture experiments were performed to confirm its compatibility with osteoprogenitor cells and that the new source of polymer did not contain any unknown cytotoxic components. A thin layer of the polymer was used to coat the bottom of 24 well plates. Mouse osteoprogenitor cells (D1) were cultured on this layer in two groups, 1) New chitosan alone and 2) Xylan/chitosan composite with 12 wells per group. The cells were shown to survive after one week with both visualization using microscopes and colorimetric proliferation assays, which was the basis for concluding that the new source of chitosan was not cytotoxic. The new chitosan source still causes a large background reading using normal proliferation assays so that statistical differences between *in vitro* groups could not be seen, therefore new subcutaneous injections were performed to investigate tissue infiltration into the implant area.

The subcutaneous injection experiment used two groups with 5 mice each. Each mouse was injected with either pure chitosan or the xylan/chitosan composite under the skin of the back in four areas: front right, front left, back right, back left. This resulted in 20 subcutaneous injection samples. The results showed both positive and negative aspects of this proposed model which would be used to test mineralization and optimization of protein ratios. The negative aspect of the model was that at most, 9 of the 20 injections could be found for analysis after 1 week in either group. However, the implants that were located revealed the positive aspects of the xylan/chitosan composite compared to the pure chitosan injections. The implant sites with attached skin were dissected and mounted in paraffin blocks. Sections were then cut and stained with hematoxylin and eosin. Representative sections are seen in **Figures 1 and 2**. **Figure 1** is a pure chitosan hydrogel implant. The staining revealed that the pure chitosan hydrogel remains at the implantation site after 1 week. Tissue begins to break down the polymer at the surface, but cells have not penetrated into the interior in large numbers, leaving an acellular implant. **Figure 2** is from a xylan/chitosan composite implant. The staining revealed that the fat tissue has completely replaced the composite hydrogel and blood vessels that are present within that tissue. These results show that even though natural polymers can behave differently depending on the source, this composite hydrogel can be synthesized using more than one source of chitosan and retain its functional properties. These experiments also revealed new information. Vascularization of the replacement tissue was previously unseen, giving further weight to the claims of biocompatibility and functionality for this new xylan/chitosan composite hydrogel.

Because so few implants were able to be located after just 1 week with a normal injection, the model was changed to use PLGA microsphere scaffolds as carriers for the hydrogels being tested. 200 micron PLGA microspheres were sintered together to create 5x5mm scaffolds. The large pores were filled with either pure chitosan, or the xylan/chitosan composite. To begin the protein delivery experiments described in Task 1a, six experimental groups were used with five mice in each group: 1) pure chitosan, 2) xylan/chitosan composite, 3) pure chitosan with BMP-4, 4) pure chitosan with VEGF/BMP-4 in 1.8 ratio, 5) composite with BMP-4, and 6) composite with VEGF/BMP-4 in 1.8 ratio. The animals were analyzed with *in vivo* microCT at 2 and 4 weeks, however, no bone formation at all was seen in any group. A smaller group of mice (one mouse from each

experimental group) was kept for 2 months, however even at 8 weeks, no bone was seen to form in any of the implants. The implants were harvested from all mice except the extended time point group at 4 weeks. All implants could be located. Tissue invasion into the xylan/chitosan composite group implants was highlighted by extensive integration of the implant with the skin and fat tissue found in the back of the mouse. These implants could not be separated from the adjacent skin. The chitosan only implants were easily removed. All implants were x-rayed again after removal to investigate whether new bone was masked by surrounding tissue in the *in vivo* model, but again no mineralization was apparent in any of the x-rays.

BMP-2 has previously been successfully delivered with this polymer, forming mineralized tissue in ectopic sites. BMP-4 was chosen for this proposal because the synergistic response with VEGF is larger and therefore more easily measured. To investigate whether BMP-4 is no longer active after incorporation in this hydrogel, further injections to study ectopic bone formation were performed to compare the mineralization response to BMP-2 and BMP-4 in both the studied xylan/chitosan composite and matrigel as a positive control.

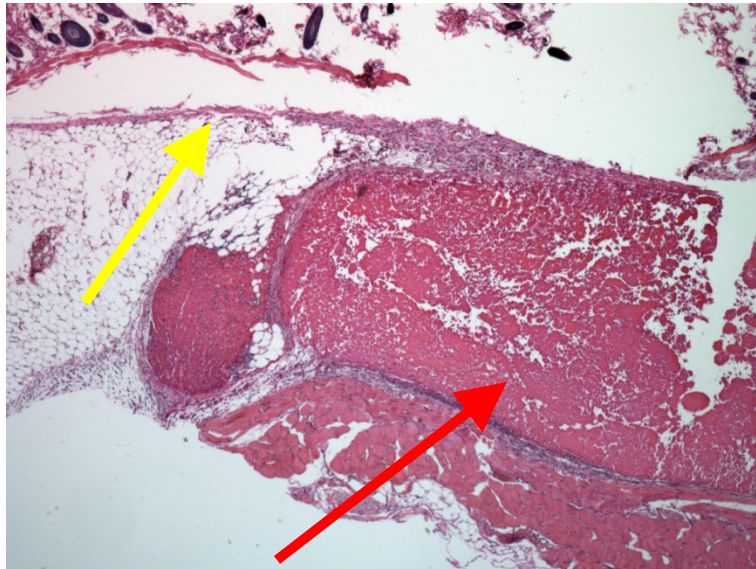


Figure 1. Pure chitosan hydrogel injected subcutaneously in the back of CD-1 mice. After 1 week the chitosan can still be seen. The red arrow points to the remaining chitosan and the yellow arrow points to the skin layer.

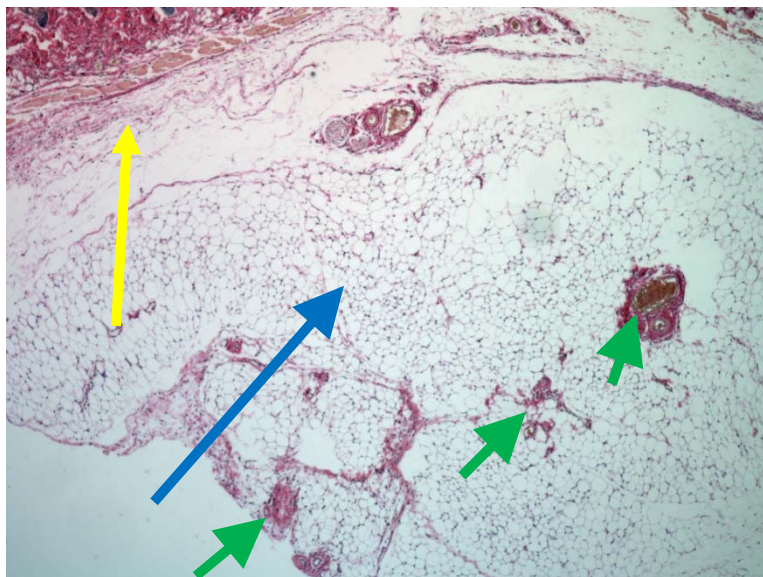


Figure 2. Chitosan/xylan composite hydrogel injected subcutaneously in the back of CD-1 mice. After 1 week the polymer has been replaced with fat tissue from nearby fat deposits at the mouse shoulder. Blood vessels can also be seen in the tissue. The yellow arrow points at the skin layer, the blue arrow points at the fat tissue that has replaced the hydrogel implant, and the green arrows point to blood vessels that run through the tissue.

Twelve mice were used in the study to confirm protein activity in the subcutaneous models. There were four groups of three mice each, BMP-2, BMP-2/VEGF, BMP-4, BMP-4/VEGF, with a ratio of 1:2 used in the BMP/VEGF groups. Each mouse had two implants under the skin on its back near the rear legs. On the left side, the proteins were injected with matrigel and on the right side the same protein group was injected with the xylan/chitosan hydrogel. These groups were chosen so that BMP-2 and matrigel would provide a positive control. Both BMP-2 and BMP-2/VEGF in matrigel showed ectopic mineralization as expected. However, the same BMP-2 groups in the xylan/chitosan hydrogel showed no mineralization. The BMP-4 alone treatments showed no mineralization in either implant. The BMP-4/VEGF group showed a small amount of mineralization in the matrigel implants, but none in the xylan/chitosan implants. These experiments that included the BMP-2 positive controls seem to indicate that the xylan/chitosan implant is not suitable for delivery of therapeutic proteins. Further, they also seem to indicate that BMP-4 alone even in matrigel is not a good model for ectopic mineralization. However, in vivo control groups using this new material in fracture models showed promising results even without delivery of proteins.

Closed tibia fractures were created in mice using the blunt guillotine method to be used in the rat study. Fractures were treated with either a pure chitosan hydrogel, or the xylan/chitosan composite hydrogel, with five mice in each group. A 300 gram weight was dropped from 36 cm onto the left tibia of anesthetized mice, with the opposite leg left uninjured. The hydrogels were injected into the fracture site 3 hours after injury to allow small skin tears and abrasions to close so that the injected material would not simply leak out of the skin. The animals were x-rayed at 2, 3, 4, and 6 weeks to determine the progress of healing. Efficacy of treatment with the hydrogels is initially determined by the size of the mineralized callus and how soon it is remodeled to normal bone. Representative x-ray images are seen in **Figures 3 and 4**. The largest difference in fracture callus size was seen between week 3 and week 4. At week 3, no difference in callus size between the groups can be seen in the images. However at week 4, the fractures treated with the xylan/chitosan composite showed almost complete remodeling of the callus while the fracture callus remained even up to 6 weeks in the group treated with the chitosan hydrogel.



Figure 3. Pure chitosan hydrogel injected into fracture caused by blunt guillotine. A large callus is still seen at 4 weeks. Arrow points to site of fracture.



Figure 4. Composite chitosan/xylan hydrogel injected into fracture caused by blunt guillotine. No large callus is evident after 4 weeks, indicating the healing progression is enhanced when the composite hydrogel is used. Arrow points to site of fracture.

As a quantitative measurement of callus size, the percent area of bone in a characteristic area centered on the fracture site was measured using ImageJ software. The characteristic area was a rectangle large enough to encompass the largest callus seen and then kept the same for every other image in all groups.

Figure 5 shows the bone area data. The data from week 3 in **Figure 5** was not distinguishable from that measured from the week 2 images. The data from week 4 in **Figure 5** were similar to that measured from the week 6 images, with the only difference being a that the average callus size of the xylan/chitosan group was slightly smaller. These results imply that the xylan component of the hydrogel has positive impacts on the fracture healing process even apart from the delivery of proteins.

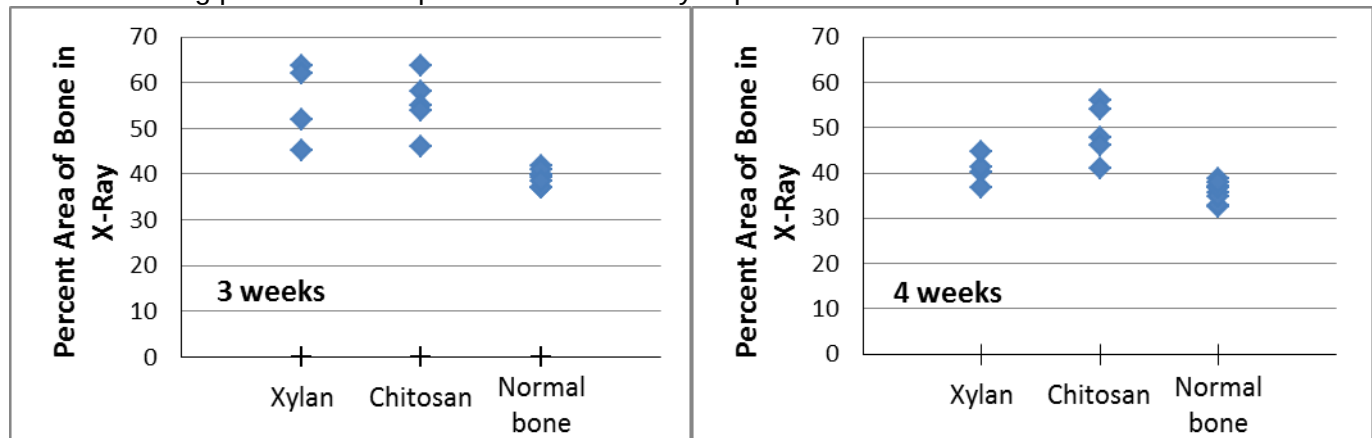


Figure 5. Size of fracture callus was compared by measuring the area of the bone in x-rays of the fracture site. Two groups, the xylan/chitosan composite and a pure chitosan hydrogel, were compared to uninjured bone. At three weeks there is no difference between groups, however at 4 weeks there is a significant reduction in size of callus in the Xylan group.

This could imply that the progression in healing due to proteins could be masked or overshadowed by the improvement caused the Xylan/chitosan composite itself. To investigate the healing potential of the hydrogel compsite alone, the rat femoral fracture model was performed using only the control groups without protein delivery. A small group of 15 rats were tested with three groups: no treatment, composite hydrogel, pure chitosan hydrogel. The fracture was created in a similar manner to the tibia model with the blunt guillotine, however the severity of impact was increased so that the injury created was a displaced comminuted fracture that would not heal without treatment. **Figure 6** shows the difference between no treatment and treatment with the composite hydrogel after one and six weeks. With no treatment the severe injury remained as a non-union while the composite hydrogel was able to provide an environment that allowed the fracture to heal. Even after only one week the x-ray images of the composite hydrogel group showed more opaque tissue at the injury site than the no treatment group, indicating the beginning of a healing callus. In pure chitosan hydrogel group a fracture callus was still evident at six weeks, indicating that even though the fracture was still not healed, perhaps it could be classified as a delayed healing rather than non-union at the six week stage. Neither of the other two experimental groups showed such complete healing as that seen in the xylan/chitosan composite group.



Figure 6. Displaced comminuted fractures did not heal without treatment. However, when the xylan/chitosan composite hydrogel is injected into the injury the bone heals.

The observed bioactivity of the xylan/chitosan hydrogel even without therapeutic proteins has been the source of two further NIH R03 grant applications and an NIH STTR application. The first, which was not

funded, used the data generated here in the subcutaneous implant model showing fat tissue completely replacing the hydrogel implant as the basis for delivery of 3D adipose-derived stem cell aggregates to non-union defects. The second, which is now under review, focuses on the remodeling of the fracture callus and tries to understand the role xylan plays in the formation and remodeling of the callus to normal bone. The STTR application seeks to study the fracture healing response in an osteoporosis disease model. Xylan plays a role in fibrogenesis in the secondary cell walls of plants and it is hypothesized that it plays a similar role in collagen fibrogenesis during callus formation, leading to a callus that is more easily remodeled to normal bone.

Key Research Accomplishments

- Defined a more general set of hydrogel synthesis steps able to incorporate chitosan from multiple sources and suppliers and still produce a consistent material
- Confirmed functional behavior of xylan/chitosan composite hydrogel – inducing tissue ingrowth into a subcutaneously injected hydrogel and healing of fractures
- Delivery of new hydrogel treatment for tibia fractures and femur fractures has been accomplished with the xylan/chitosan composite showing bioactivity even in the absence of therapeutic proteins
- Demonstrated that chitosan based hydrogels are not suitable for therapeutic protein delivery

Reportable Outcomes

- PCT patent application (PCT/US2010/058272) and U.S. patent filing was begun in June 2012
- Oral presentation of research at 2011 American Institute of Chemical Engineers Annual Meeting
- Applied for NIH R03 and STTR funding based on accelerated remodeling of the fracture injuries
- Research resulted in a semi-finalist entry in the 2011 SEBIO BIO/Plan competition
- One manuscript submitted to *Biomaterials* in June 2012 based on subcutaneous implants and femur fracture model with a second manuscript planned based on tibia fracture model and polymer characterization

Conclusion

A new chitosan/xylan composite hydrogel was studied as an improved bone graft substitute that is compatible with current surgical practice. The original hypothesis that this material would be suitable for therapeutic protein delivery was proved incorrect. However, the synthesis steps have been improved to allow for multiple sources of chitosan, tissue ingrowth has been demonstrated in two different implant locations, non-union defects in the femur have been successfully created and healed with the composite hydrogel, and accelerated remodeling of fracture callus in the tibia has been demonstrated compared to treatment with pure chitosan. These experiments imply that xylan is an active component that in the composite that is able to improve the bone healing response in fracture injuries even without the problematic issue of protein delivery.

In the United States there are approximately 6.2 million bone fractures per year. Of this number, 5-13% will result in a failed repair known as a non-union.^{1,2} There are over 500,000 bone grafting procedures performed to treat these non-unions as well as other large needs such as spinal fusions. However, in 2007 the number of tissue donors was estimated to be only slightly higher than 25,000 which highlights the need for effective bone graft substitutes. A large market has grown around this need with sales of graft substitute materials and regenerative proteins that were estimated to top \$3 billion in 2009. Sales of synthetic bone substitutes alone were over \$700 million.³ The successful development of this chitosan/xylan composite hydrogel as a bone graft substitute has immediate implications for clinical care of segmental bone loss and the acceleration of healing in traumatic bone injuries.

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Appendices

AICChE abstract from 2011 annual meeting and International patent application (PCT/US2010/058272):

Composite hydrogel from chitosan and hemicellulose for musculoskeletal tissue engineering

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A thermally-responsive composite hydrogel has been developed and synthesized from the natural polymers chitosan and xylan. The new material is a viscous liquid at room temperature, but turns to a solid gel at physiological temperature (37 °C). Rate of gelation is controlled with addition of a salt solution. Applications are for tissue engineering and local delivery of therapeutic agents including proteins and other drugs. The specific application that has been tested is for delivery of the protein BMP-2 for creation of new bone and 2D cell culture for osteogenic stimulation of mesenchymal cells.

The polymer composite remains a viscous liquid for more than 2 hours at room temperature after the salt solution is added. This allows for addition of the desired mass of growth factors and ample time to complete the surgical procedures. Once the polymer solution reaches physiological temperature it undergoes a phase change to become a solid gel in less than 10 minutes. This system allows for a known mass of therapeutic agent(s) to be accurately delivered to injuries of complex shape. *In situ* gelling lets the material match the complex geometries of injuries that prefabricated scaffolds and carriers are unlikely to match. The material is delivered with commonly used techniques, requiring only a common syringe to administer the liquid solution to the correct target area. This gelling material can be used in combination with current surgical techniques without disrupting the procedure. For example, a non-union fracture is usually fixed in place with titanium rods or fixation plates. This thermally-responsive composite hydrogel can be injected after placement of the more traditional healing aids, filling in the areas of likely non-union to accurately provide a precise dose of growth factor and medium in which the cells can move and bridge the defect.

A thermally-sensitive hydrogel (thermogel) graft substitute made from ultrapure chitosan has been previously used to treat critical sized unicortical defects in a rat femur.¹ The thermogel successfully delivered osteogenic peptides that induced healing over an 8 week time period. The pure chitosan thermogel was successful at delivery of osteogenic factors, however it did not promote ingrowth of tissue or cells before complete degradation. The hydrogel material did not allow cells to penetrate into the interior or allow tissue growth into the area until the chitosan was completely removed from the defect site. One reason for this is that the chitosan only displays positive charges in the thermogel matrix. This has a large potential to impede the healing at an injury site as cell movement is blocked at the boundary of the hydrogel.

A multi-ion environment is more conducive to cell migration within biomaterials. To address this issue, chitosan was blended with another natural polymer, xylan, which is a hemicellulose that displays negative charges on its side chains. We have developed this hydrogel to behave as a thermally-sensitive hydrogel, or thermogel, based on the results from the pure chitosan thermogel. Natural polymers were chosen for this hydrogel composite because of a large emphasis in engineering circles to increase sustainability in all areas of research from biofuels to biomaterials. Chitosan is already a renewable material as it is found in crustacean shells. Hemicellulose can be up to 90% of the biomass material from plants used as feed stock in other applications (sugarcane, switchgrass, wood, algae, etc.).

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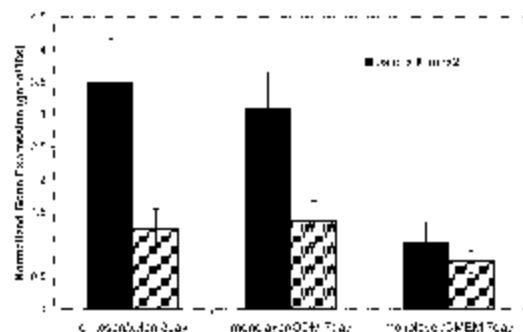


Fig. 6

(57) Abstract: The present invention provides a method for preparing chitosan and xylan composite, thermosetting solutions to allow regulating the conditions in which the chitosan and xylan solution will gel. The present invention also provides methods for using chitosan/xylan composites and for using chitosan/xylan solutions in vivo and in vitro. A thermosensitive chitosan composite hydrogel has been developed and synthesized from the natural polymers chitosan and xylan. The new material is a viscous liquid at room temperature but turns to a solid gel at physiological temperature (37°C). Rate of gelation is controlled with addition of a salt solution. Applications include tissue engineering and local delivery of therapeutic agents, including proteins and drugs, as well as cells.

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