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14. ABSTRACT During this reporting period, a more 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, therefore activity is being tested with positive controls. Demonstration of non-union defect in rodent femur has been accomplished. Femoral fracture study in rats is now underway.					
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Introduction

Our hypothesis is 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 ongoing study, a new chitosan/xylan composite hydrogel is being 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 is 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

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. New methods continue to be investigated that will allow accurate proliferation measurements in the presence of these chitosan based hydrogels.

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 as described previously in the proposal application. 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 are being 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.

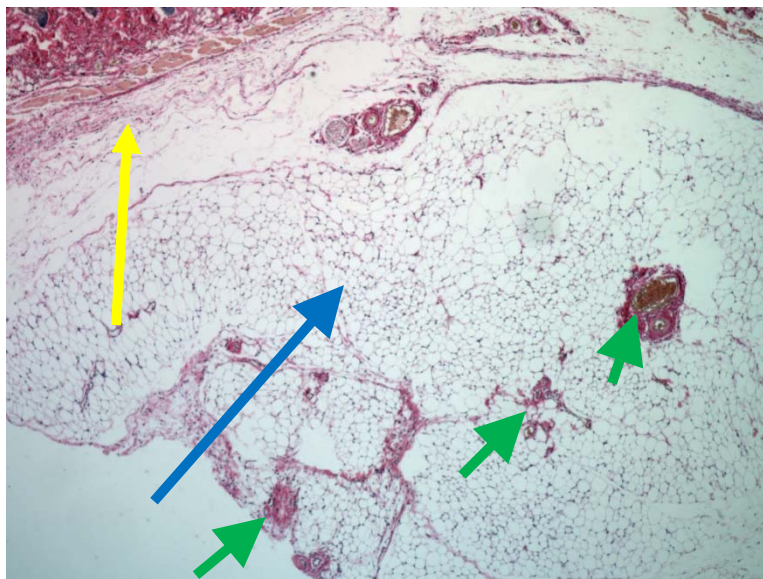


Figure 2. Chitosan/xylan composite hydrogel injected subcutaneously in the back of CD-1 mice. After 1 week the polymer has been replaced with fat tissue from nearby fat deposits at the mouse shoulder. Blood vessels can also be seen in the tissue. The yellow arrow points at the skin layer, the blue arrow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

As protein activity is being confirmed, work has begun on *in vivo* testing of the xylan/chitosan composite in a bone defect as control experiments to compare the efficacy of protein delivery with this material. We have confirmed that delivery of the composite hydrogel to the tibia fracture defect is repeatable and have confirmation that the injured bone can be stabilized. Animal and material purchase for this mouse tibia fracture model were not directly supported by this grant, however the results directly inform how the rat study in Task 2 of this award will proceed and so results are reported here.

Close 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.

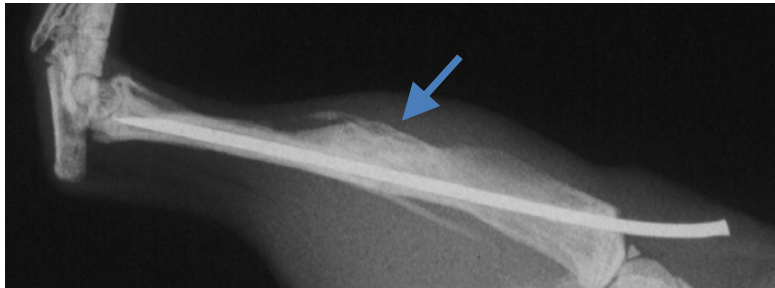


Figure 3. Pure chitosan hydrogel injected into fracture caused by blunt guillotine. A large callus is still seen at 4 weeks. Arrow points to site of fracture.



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. **Figures 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. Methods of analyzing bone density of the fracture callus in these images are being compared to determine which measurement is most consistent.

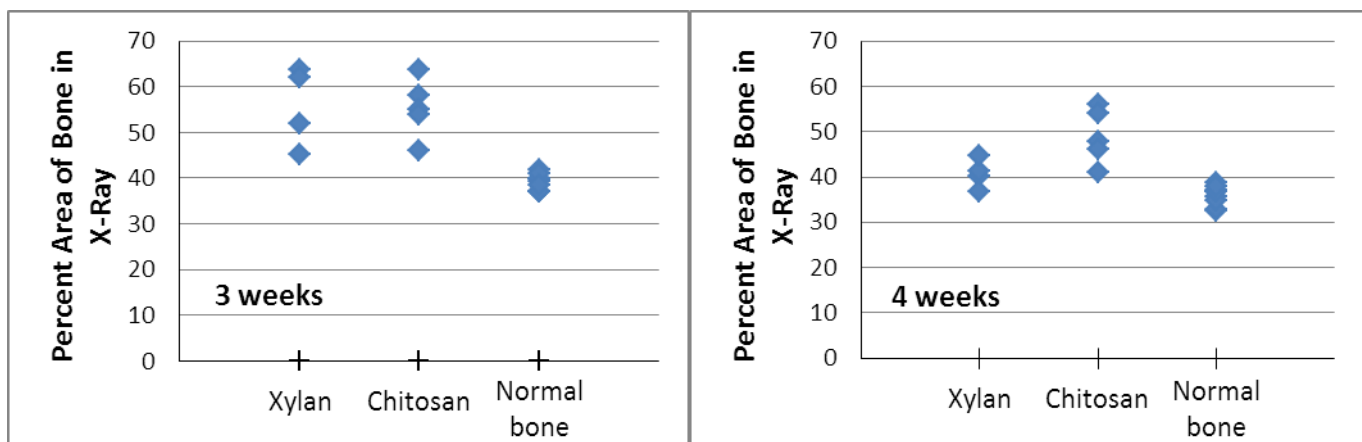


Figure 5. Size of fracture callus was compared by measuring the area of the bone in x-rays of the fracture site. Two groups, the xylan/chitosan composite and a pure chitosan hydrogel, were compared to uninjured bone. At three weeks there is no difference between groups, however at 4 weeks there is a 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 whether this will be the case in the rat femoral fracture, a small group of 15 rats are currently being tested with these same treatments. The femoral fracture may be more severe and not respond in the same way. However, in the case that the Xylan/chitosan composite has a large healing effect in the femoral fracture an alternate method of creating a non-union defect with less healing potential has been demonstrated. **Figure 6** shows that such a non-union can be created using two pins and a bur to make a segmental defect in the femur that can then be treated with the hydrogels and synergistic proteins.

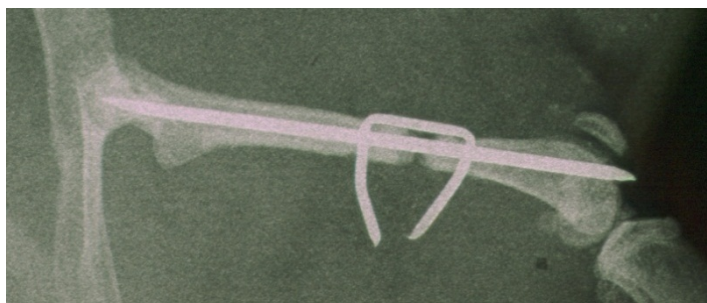


Figure 6. The alternate method of creating a non-union fracture in the rodent model using two pins and a bur to create a segmental defect.

The observed bioactivity of the xylan/chitosan hydrogel even without therapeutic proteins has been the source of two further NIH R03 grant applications. 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 being submitted for 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. 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 hydrogel with new source of chitosan – inducing tissue ingrowth into a subcutaneously injected hydrogel
- Delivery of new hydrogel treatment for tibia fractures has been accomplished with the xylan/chitosan composite showing bioactivity even in the absence of therapeutic proteins
- Demonstration of non-union defect in rodent femur has been accomplished
- Femoral fracture study in rats is now underway
- Testing the efficacy of protein delivery made more robust by inclusion of positive controls

Reportable Outcomes

- PCT patent application (PCT/US2010/058272)
- Oral presentation of research at 2011 American Institute of Chemical Engineers Annual Meeting
- Applied for NIH R03 funding based on accelerated remodeling of the fracture callus in tibia fractures
- Research resulted in a semi-finalist entry in the 2011 SEBIO BIO/Plan competition

Conclusion

In this ongoing study, a new chitosan/xylan composite hydrogel is being 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. The synthesis steps have been improved to allow for multiple sources of chitosan, tissue ingrowth has been demonstrated in two different implant locations, experiments studying the efficacy of delivering synergistic growth factors with this material are underway and now include positive controls, non-union defects in the femur have been successfully created as an alternative surgical option, and the rat femoral fracture study is now underway.

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

AIChE 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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(54) Title: METHODS FOR REGULATING GELATION OF HYDROGEL SOLUTIONS AND USES THEREOF

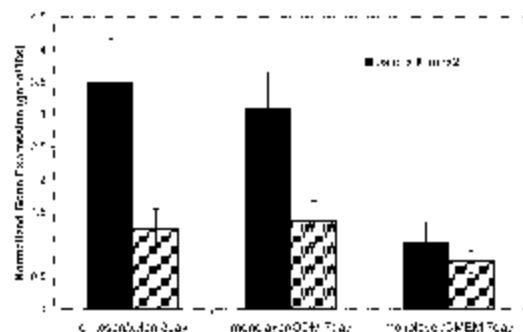


Fig. 6

(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 thermosetting 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 temperatures (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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