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Functionalizing Titanium Implants with Bioactive Peptides to Increase Osseointegration

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Abstract:

Orthopedic surgeries have continued to increase, but the material of choice remains constant: titanium. Titanium has become the benchmark implant material due to its durability and non-immunogenic properties. However, while high rates of success are correlated with joint replacement surgery, certain patients with predisposed diseases such as diabetes or arthritis may have increased complications.[1] This can be due to lack of osseointegration due to decreased bone formation and mineralization,[2] which will lead to increased rates of infection or aseptic loosening of the implant from the bone. [3] Current methods to alleviate these complications include invasive revisional surgeries, which can be emotionally taxing and dangerous towards the patient. Fortunately, new research has shown that bioactive peptides are able to modify titanium surfaces and mimic natural proteins to increase osseointegration.[4] Previously, through solid phase peptide synthesis, a series of multivalent dendrons containing bioactive osteogenic growth peptide (OGP) domain and a titanium binding domain consisting of surface binding catechol groups were obtained. Dendrons containing 4 catechol units displayed increased binding strength to titanium oxide surfaces compared to the 2- and 1-unit analogs.[5] Titanium oxide functionalized with this peptide molecule and seeded with mouse calverial derived stem cells (MC3T3) showed up-regulation of osteogenic markers bone sialoprotein (BSP) and osteocalcin (OCN) by 3-fold and 60-fold relative to controls after 21 days. Furthermore, there was a 3-fold increase in calcium deposition.[6] These promising results suggest that these peptides may enhance bone regeneration *in vivo*. In this research, a study was performed *in vivo* to appraise this model and further apply it to other bioactive peptide mimics such as bone morphogenetic proteins (BMP) 2, 7, and 9. Surgical Grade 5 titanium (Ti6Al4V) pins were coated with the peptide

molecules and implanted into Sprague Dawley Rat femurs. Biomechanical and histological analyses showed an increase in bone growth from a period of two to five weeks, which may indicate therapeutic benefits for these bioconjugates in diabetic animal models.

Introduction:

Over the past twenty-years, total joint replacements have skyrocketed in the United States and by 2030, total hip and knee surgeries will reach an estimated four million procedures performed annually, which is a 601% increase from 2005.[1] While the number of procedures may be growing, the material being used, titanium, has not changed. The requirement of an orthopaedic implant to function correctly is its ability to osseointegrate, which means achieving adequate bone growth around the implant.[4,6] Due to titanium's durability and non-immunogenic properties, titanium has become known as the benchmark implant material. However, while high rates of success are correlated with joint replacement surgery, if osseointegration fails to take place and the implant becomes loose, this results in a failed surgery and many negative consequences. Micromotions of this loose implant will generate gaps between the bone and implant interface, which will significantly delay the healing process by causing infections[2] or wear-particles induced osteolysis.[3-4] Recent research has aimed to minimize these complications by focusing on biomaterials that employ bioactive peptides that are able to communicate with the body and promote a desired response of improving contact with bone.[5]

As stated earlier, by acquiring fast osseointegration of the implant, this will decrease the amount of complications later on. One possible solution for increased early implant osseointegration is the surface modification of titanium implants. Surface modifications help to enhance the biocompatible and osteoconductive properties; leading to better osseointegration.[6] Osteoconductive properties are modifications that allow for bone to grow on the surface and can be achieved by increasing the surface area and porosity at the surface of the implant[7] or by using peptides that promote cell adhesion. However, there have been certain ways to promote early onset of bone growth via osteoinductive approaches using growth hormones such as Bone Morphogenetic Protein-2 (BMP-2),[8] or by using zinc surface modifications.[9] These approaches have shown promising results *in vitro* and *in vivo*.

The two most common bone forming growth factors are Osteogenic Growth Protein (OGP) and Bone Morphogenic Protein (BMP). BMP has started to become frequently used in orthopedic surgery and has been approved by the FDA for certain procedures such as anterior lumbar interbody fusion, open tibial shaft fractures, and recalcitrant long bone nonunions.[1-2] Additionally, it has achieved relatively widespread off-label use in a number of operative settings which necessitate a bone fusion. Comparable to BMP, Osteogenic growth protein (OGP) has similar growth factor potential and can be used in practical applications within orthopedics. OGP can be found in micromolar concentrations in the human blood serum[10] and has been found to enhance proliferation, differentiation, and matrix mineralization of osteoblasts.[11] The active sequences of both OGP (YGFGG, 10-14) and various BMP molecules have shown success in bioactivity regarding orthopaedic implants.

Recently, we have shown that bioactive molecules containing the short active peptide sequences attached to the PEG-Catechol binding domains are able to modify the titanium surface.[5] A series of multivalent dendrons containing bioactive osteogenic growth peptide (OGP, 10-14) domain and four surface binding catechol groups were obtained. The catechol groups tethered to the titanium oxide surface via coordination bonds and it was shown that the peptide remained on the surface for up to 14 days *in vitro*. In addition, the two bone forming markers bone sialoprotein (BSP) and osteocalcin were upregulated 3-fold ad 60-fold. There was also an 3-fold increase in calcium deposition relative to controls. From these results, it showed that OGP (10-14) was bioactive and was an appropriate surface modification of titanium to enhance osteogenesis *in vitro*.

For this study, Grade 5 titanium (Ti6Al4V) implants were coated multiple versions of the 4-catechol unit dendron molecule bearing different bioactive peptide sequences (BMP 2/7/9 and OGP). They were then implanted into Sprague Dawley rat femurs in order to assess the performance of the bioactive molecules in promoting bone growth. Non-functionalized pins were also used *in vivo* as a control method. We hypothesize that when compared to the control, the four peptides will enhance osteoblastic differentiation, resulting in increased bone growth around the implant site.

Materials and Methods:

The first goal of the project was to create and synthesize the bioactive peptide coating that would be used on the pins. In addition, the binding properties of this peptide dendrimer conjugate would have to be characterized for titanium functionalization.

Previously published by the Becker group[5] were the steps needed to create the peptide dendrimer conjugate via Fmoc solid-phase peptide synthesis (SPPS) using a

Liberty 1 peptide synthesizer (CEM Corporation, Matthews, NC). To start, 0.25 mmol of Fmoc- amino acid - Wang resin of the C-terminal amino acid of the peptide sequence was loaded into the synthesizer. For example, a Fmoc-Gly-Wang resin was used for the synthesis of OGP. The N-terminus of this amino acid was made available for amino acid addition via deprotection of the Fmoc group, which was done under microwave irradiation. The four conjugates created will be peptide mimics of OGP and BMP, which as discussed previously, are growth factors involved in bone morphogenesis. These peptide mimics will be the active sequences of OGP, BMP-2, BMP-7, and BMP-9 (see table). These have been shown to have high success with inducing bone morphogenesis and are more stable and easier to synthesize than the full native protein.

Peptide	Sequence
OGP (10-14)	YGFGG
BMP-2 (73-92)	KIPKASSVPTELSAISTLYL
BMP-7 (89-117)	TVPKPSSAPTQLNAISTLYF
BMP-9 (68-87)	KVGKASSVPTKLSPISILYK

Once created in the synthesizer, the resin with peptide sequence was transferred to a peptide synthesis reaction vessel for the remainder of the reactions. The synthesized peptide was then washed three times each by dimethylformamide (DMF), methanol (MeOH), and dichloromethane (DCM), and then swelled in dimethylformamide (DMF) for 15 minutes under argon bubbling. The terminal Fmoc group was deprotected using a 25 mL cocktail of a 20% Piperidine in DMF and Hydroxybenzotriazole (HOBt) solution for 1 hour. Afterwards, the deprotection solution was drained, and the resin was washed again using DMF, MeOH, and DCM. A solution of Fmoc-NH-PEG_6-Propionic acid (4 equiv, 1 mmol) (AAPPTEC, Louisville, KY) in DMF was then added to the resin

along with a solution of HOBt (4 equiv, 1 mmol) (AAPPTEC, Louisville, KY) in DMF. To start the addition, Diisopropylcarbodiimide (DIC, 4 equiv, 1 mmol) was added. This coupling reaction was run for 3 hours to yield the peptide with six-repeat unit PEG spacer group.

The next step involved the dendron coupling to the PEGylated short peptide sequence. Like previous steps, the terminal Fmoc group of the Pegylated peptide sequence was deprotected using a 25 mL cocktail of a 20% Piperidine in DMF and Hydroxybenzotriazole (HOBt) solution for 1 hour. Afterwards, the deprotection solution was drained, and the resin was washed 3x DMF, 3x MeOH, and 3x DCM. A solution of Fmoc-Lys(Fmoc)-OH (4 equiv, 1 mmol) (AAPPTEC, Louisville, KY) in DMF was added to the resin along with a solution of HOBt (4 equiv, 1 mmol) (AAPPTEC, Louisville, KY) in DMF. Two additions of lysine will occur in order to obtain the four-armed amine. To start the addition, Diisopropylcarbodiimide (DIC, 4 equiv, 1 mmol) was added. This coupling reaction was run for 4 hours total with the second addition of lysine occurring at the 2-hour timepoint to yield the bioconjugate containing four-armed lysine groups.

Upon completion of this addition, the four Fmoc protecting groups of these lysine groups were deprotected using a 50 mL cocktail of a 20% Piperidine in DMF and Hydroxybenzotriazole (HOBt) solution for 1 hour. Afterwards, the deprotection solution was drained, and the resin was washed 3x DMF, 3x MeOH, and 3x DCM. A solution of acetal-protected 2,3 dihydroxyphenylpropionic acid (4 equiv, 1 mmol) (AAPPTEC, Louisville, KY) in DMF was added to the resin along with a solution of HOBt (4 equiv, 1 mmol) (AAPPTEC, Louisville, KY) in DMF. To start the addition, Diisopropylcarbodiimide (DIC, 4 equiv, 1 mmol) was added and run for 3 hours.

Now that the bioconjugate was fully complete, acid cleavage and deprotection from the resin was performed by first washing with 3x DMF, 3x MeOH, and 3x DCM. Next, the cleavage cocktail was added to the resin, which consisted of 15 mL of trifluoracetic acid (TFA), 0.75 mL Triisopropylsilane (TIPS), and 0.75 mL H_2O. This cleavage reaction was run for 2 hours total with a second identical aliquot of cocktail added at the 1-hour timepoint. Once complete, the solution was separated from the resin and the cleavage solution was removed under reduced pressure. After the peptide was obtained, it was redissolved in a small amount of TFA and then precipitated in cold diethyl ether three times. After being centrifuged, the precipitate was dried and dialyzed against 70% ethanol for one day. The product obtained was dissolved in DMF and further purified by a Waters 1525 HPLC system using an XBridge Peptide BEH C18 Column (300Å, 5 µm, 10 mm X 250 mm). The gradient used was from 20-80% methanol with 0.1% TFA with a flow rate of 3.3 mL/min. Finally, the purified products were freeze dried and analyzed by MALDI mass spectrometry. The steps for synthesis of the peptide-PEG-$(cat)_4$ molecules can be seen in figure 1.

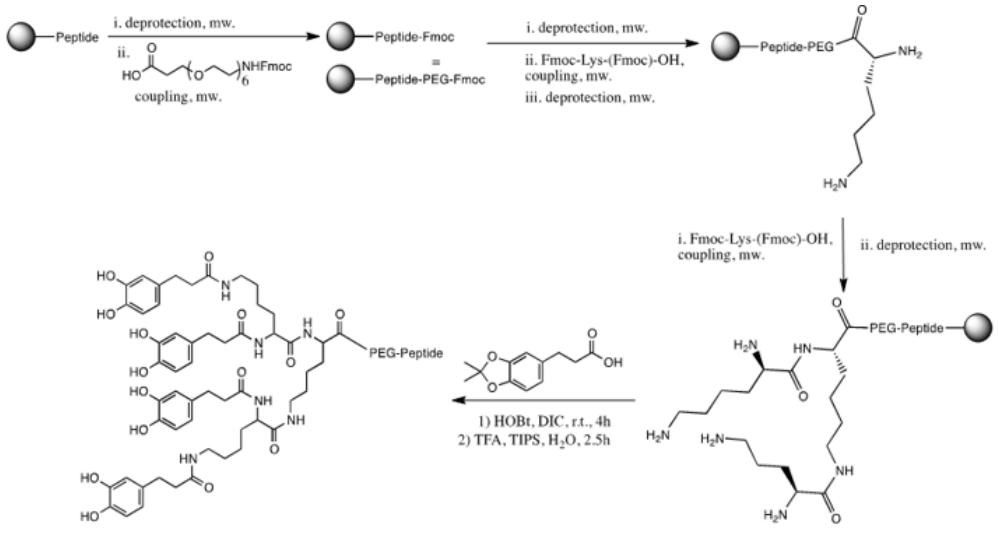

Figure 1: Steps for the synthesis of the peptide-PEG-catechol biomolecule used in the study.

Fluorescent analogs of these bioactive conjugates were also made by adding Fmoc-Lys(Mca)-OH (4 equiv, 1 mmol) (AnaSpec, Fremont, CA) to the N-terminus of the peptide sequence. The steps for synthesis were identical to the regular bioconjugates.

To begin the animal studies, approval by the University of Akron Institutional Animal Care and Committee (IACUC) was acquired. Eighty-four, 11-week-old, 350-450 gram Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) were used. All of the rats were housed in a climate-controlled room with set 12-hour dark and light cycles. In addition, there were two rats per cage, and each of them were given free access to food and water. Treatment of the animals strictly adhered to the IACUC protocols and no variance from the guidelines occurred.

Two titanium implants were used per rat and placed in both femurs. 168 custom TiAl6V4 pins, measuring 3/64” (1.19 mm) in diameter and 3/16” (4.7625 mm) in length were used (United Titanium, Wooster, Ohio). At the top of the pin, a 1/64” hole (0.396 mm) was drilled, which would be used later during mechanical pull tests. The titanium pins were then washed three times each with hexane, methanol, and water and then autoclave sterilized.

After sterilization, the pins were ready to be coated with the specific peptide conjugate. A 50 μM solution of bioconjugate in DMSO (Pharmaceutical grade, Sigma Aldrich) was created, and the sterilized pins were immersed in 10 mL of this solution. The titanium pins were placed in this solution 12 hours before surgery and stirred overnight. Prior to the surgery, the pins were removed from solution, washed once with DMSO, and washed 3x with PBS without magnesium and calcium.

Now that the coated pins were ready, implantation of the pins into the rat femurs was set to occur. There were five total groups for the implants. The implants without the bioconjugate coating or non-functionalized titanium were used as controls. The other four functionalized implants were coated with OGP, BMP2, BMP7, and BMP9 based conjugates. Rats were anesthetized via intramuscular injection using a cocktail of 0.57 mL/kg of ketamine (100 mg/mL): xylazine (20 mg/mL): acepromazine (10 mg/mL) (29.6:5.95:0.553). A subcutaneous injection of buprenorphine (0.02 mg/kg) was also applied for pain relief. Once the rats were unconscious, bilateral lower extremities were shaved completely and prepped using Betadine®. On the operating table, the rat was draped in a sterile fashion with only the lower extremity of interest exposed. An orthopaedic surgeon from Summa Hospitals performed these surgeries with assistance, and both individuals followed strict sterilization techniques. To access the femur, a longitudinal incision was made along the mid-femur, and blunt dissection was carried down to bone. Once exposed, the surgeon drilled a 3/64” (1.19 mm) hole in the femur, and the specific titanium pin was inserted. Once the pin was inserted into the femur, it was made sure that it could not be easily removed or disturbed. The incision was then closed with 4-0 nylon sutures. As stated earlier, each rat received one pin in each femur, and total surgery time for one rat was about one hour. Post-operatively, the rats were placed in individual cages with heating mats underneath and kept under close supervision. Once fully awake, they were provided with food and water and were given two additional doses of buprenorphine (0.02 mg/kg) every 12 hours.

Depending on the group, the rats were sacrificed at either two or five-week time periods. Sodium Pentobarbital solution (Fatal-Plus) was administered via intraperitoneal

injection followed by cutting of the diaphragm. The femurs were then harvested, cleared of any tissue, and stored in either 40% ethanol or 1x PBS at 4^{o} C until use. The femurs stored in 40% ethanol were used for histological analysis and the femurs stored in 1x PBS were used for biomechanics.

The bone samples used for histological analysis were analyzed using histomorphometric analysis using color thresholding. This was used in order to see bone remodeling over the two and 5-week time-points. The samples were stained with Sanderson's rapid bone stain with Van Gieson counterstain. They were then imaged on a IX81 microscope (Olympus) and color thresholding was performed using the microimaging Olympus cellSens software. These images helped to analyze bone area (BA) and bone-to-implant contact (BIC). BA was defined as the area percentage of the newly formed bone within an area 100 μm outside the implant to the whole area of interest. BIC was defined as the percentage of osseointegration on the implant.

Bone-to-implant contact or bone growth around the implant was assessed using a biomechanical pull-out test. The pull-out force of each pin was measured by an Instron 5567 (Canton, MA). To start, the femurs were secured to a custom holding device that allowed to titanium pin to be exposed to the direction of the pulling-force. As stated earlier, there was a small hole in each implant. A 0.007-inch diameter piano wire was threaded through this hole and attached to a 100 N load cell on the Instron instrument. Once the sample were properly fixed, a 2 N pre-load was applied to ensure proper and identical wire tautness among implants. Once acquired, the pins were pulled-out at 2 mm/min until the implant failed or slipped from the bone. By determining the maximum force before failure in a load displacement curve (load vs extension), the

pullout force (N) was determined for the implants. The results for the five implant groups were then compared.

The final step was analyzing the data acquired from the biomechanical and histological tests. Statistical analysis was performed using IBM SPSS Statistics 24 (Armonk, New York). The data that was presented as box-and-whisker plots showed only the median, quartiles, whiskers, and outliers. Before any of biomechanical data could be tested for significance, a log transformation was performed. Afterwards, a one-way analysis of variance (ANOVA) following multiple comparisons with the Tukey-Kramer method at 95.0% confidence level was performed to show which means were significantly different from each other. For the acquired histology data, no transformation was applied, and a one-way ANOVA was performed followed by multiple comparison with the Tukey-Kramer method at 95.0% confidence level.

Results:

In previous studies conducted by the Becker group, we have shown that the bioactive conjugate OGP-PEG-$(Cat)_4$ was synthesized successfully using solid phase peptide synthesis (SPSS). This molecule was able to have distinct peptide and binding domains that retained their intended function. In this study, we further expanded this design by applying and using identical methods to synthesize short peptide mimics of bone morphogenetic proteins 2,7, and 9. These products were confirmed by Matrix-assisted laser desorption/ionization (MALDI) mass spectroscopy and analytical HPLC.

As mentioned earlier, the binding affinity of the bioconjugates to Grade 5 Titanium (Ti6Al4V) was quantified using fluorescence spectroscopy. The same

molecular structure of the bioconjugates was used for the fluorescence studies, except there was an addition of a coumarin-containing lysine amino acid (Fmoc-NH-Lys(Mca)-OH) to the N-terminus of the peptide sequence. After coating the titanium implants, the concentration of the solution was compared to the concentration of the original solution before coating. As a result, the implant surface concentration of the peptide bioconjugates were measured and analyzed by fluorescence. These results from figure 2 showed that the surface concentration of the peptide bioconjugates were all relatively the same.

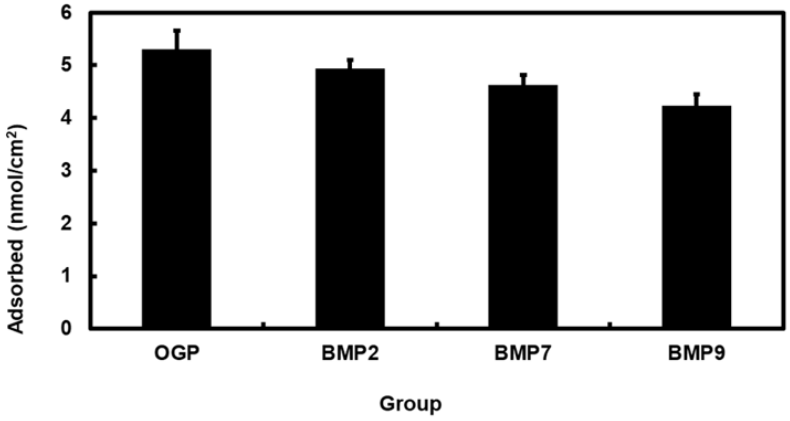

Figure 2: Binding affinity of bioconjugates to grade 5 titanium quantified using fluorescence spectroscopy. Absorbance was similar for each bioconjugate meaning binding affinity was similar for all.

The rats were sacrificed at the timepoints of two weeks and five weeks after the implantation of the titanium pins. Immediately following, implants were pulled out from the femurs. No statistical difference ($p>0.05$) was seen between the bioconjugate groups at two weeks. However, at five weeks, the Control and OGP groups were statistically higher than all the other groups at two and five weeks respectively. As noted earlier, the samples were preloaded with a 2N force to ensure identical wire tautness before the test. Some of the samples did fall out of the bone before the 2N force was

reached and this data was still included for the box plots and statistic calculations (Figure 3).

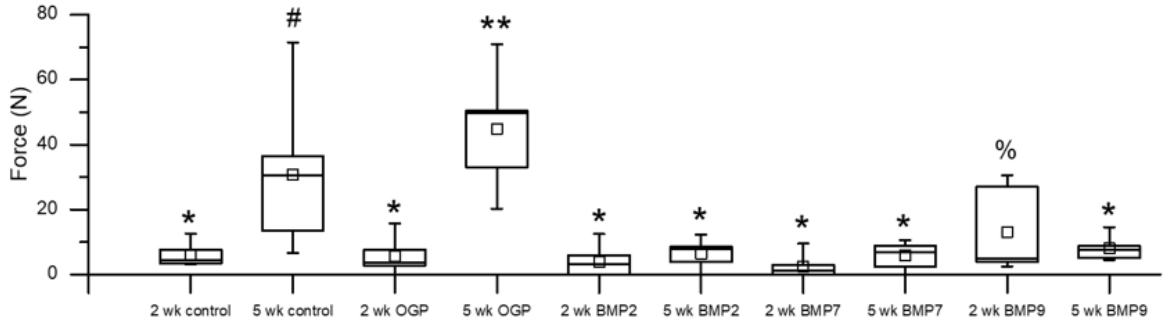

Figure 3: Pullout force after 2 and 5 weeks in N/mm^2 (box plot): arithmetic mean (square), median (horizontal line), upper and lower quartile (box), and whisker (vertical line). Groups labeled with two asterisks (**) are significantly different than groups with one asterisk (*). Groups labeled with a pound symbol (#) are significantly different than all one asterisk labeled groups (*), except 2 wk OGP. Groups labeled with a percentage sign (%) are not significantly different than any of the groups. Data were $\log_{10}$ transformed before analysis via one-way ANOVA, using a Game's Howell post-hoc test ($p < 0.05$).

Using light microscopy, we were able to visualize the contact been the titanium pin and the bone. Histology was analyzed in three ways: the whole pin-bone interface (femur-bone interface), the spongy-bone interface, and the cortical-bone interface. The femurs were stained using a Sanderson's Rapid Bone stain with a Van Gieson counterstain, which stained mineralized bone pink and osteoid blue. Three cutting axes of the implant were also utilized (figure 4). By selecting certain regions of interest, the amount of bone formation around the implant was quantified (figure 5). Bone area (BA) was quantified by measuring the area of bone within a 100 μm and was represented as a percentage (figures 6-8). All samples were normalized by the area of the interface of interest. By analyzing the BA for the Spongy-bone, cortical-bone, and femur-bone interface, there was a correlated increase from 2 to 5 weeks, however, there were very few significant differences. When the control pin was compared to the other four peptide

mimics, there was not a significant amount of extra bone growth for the functionalized pins.

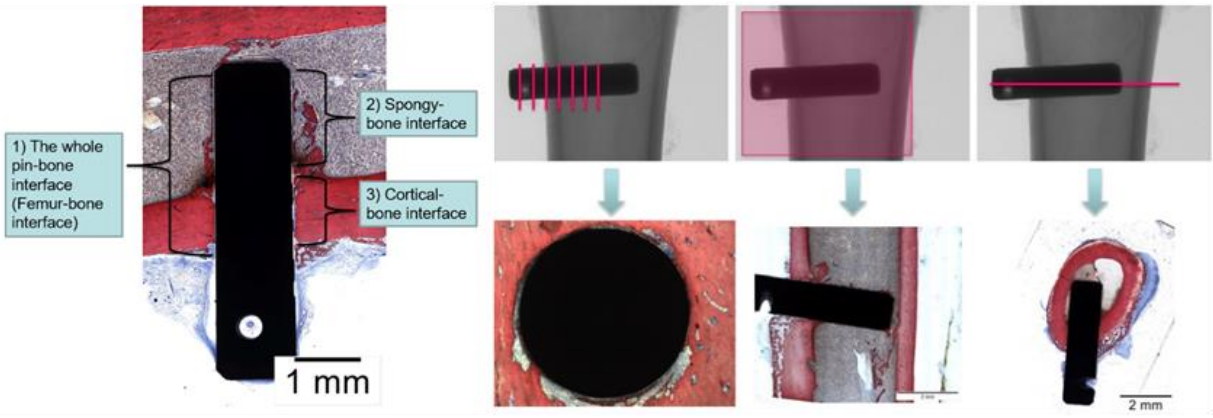

Figure 4: Image on the left shows the three regions that were analyzed for bone growth. The image on the right shows the three different axes that the pin was cut and analyzed.

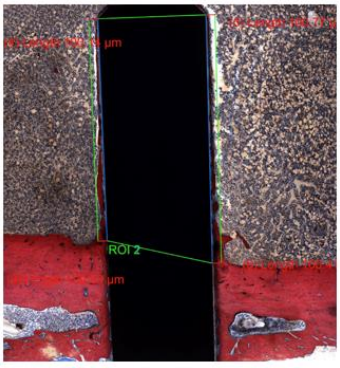

Figure 5: Regions of interest chosen 100 microns around the implant. ROI (green) – ROI (Blue) = ROI.

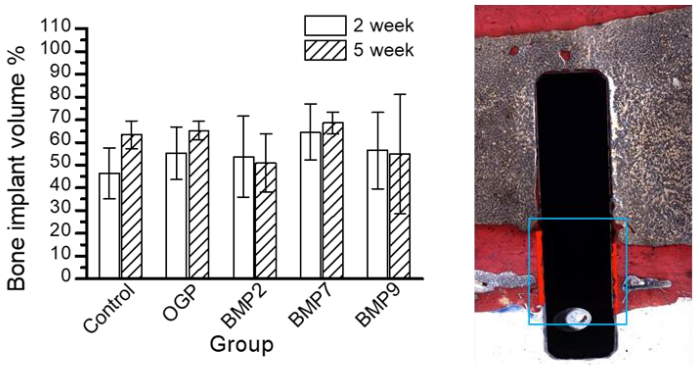

Figure 6: Bone implant volume in % after two and five weeks for cortical bone interface. Groups with an asterisk (*) are significantly different from each other at $p<0.05$ (One-way ANOVA using Tukey-Kramer post-hoc test).

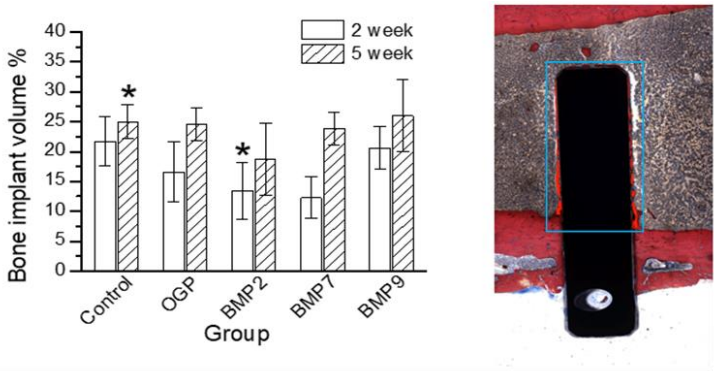

Figure 7: Bone implant volume in % after two and five weeks for spongy bone interface. Groups with one (*) or two (**) asterisks are significantly different from each other at $p<0.05$ (One-way ANOVA using Tukey-Kramer post-hoc test).

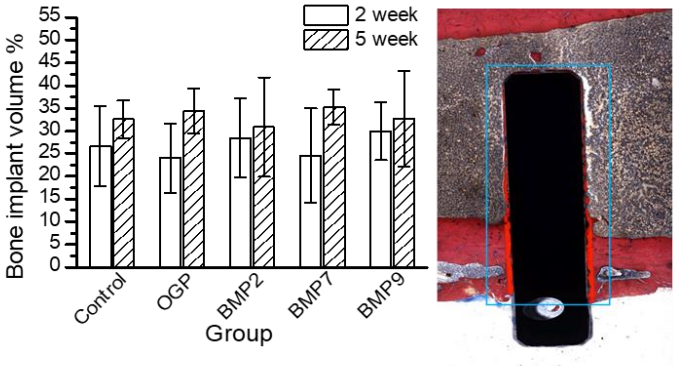

Figure 8: Bone implant volume in % after two and five weeks for femur bone interface. No significance detected.

Discussion:

Previous results from our *in vitro* studies done with MC3T3's showed promising results with increased bone formation and osteoblast differentiation. This established reason to apply these bioconjugates *in* vivo in a rat model. However, after biomechanical testing and histomorphometric analysis, results from this *in* vivo experiment suggest that the functionalized coating performs similarly to the uncoated, control pins.

The biomechanical testing appeared to show no significantly promising results for the pin coatings. The uncoated, control pins and the OGP bioconjugate coating performed similarly with increased bone growth from two to five weeks. When the two groups were statistically compared, there was no significant differences at either timepoint, leading us to believe that something must have gone wrong due to the promising results from the *in vitro* study. As for the BMP coated samples, there was no significant bone growth from two to five weeks, which included the samples that fell out of the bone before the 2N preload force was applied. After five weeks recovery, it was not expected to have some of the pins not be anchored in the bone. Some possible speculations for these results could be that the coating itself was providing negative feedback to the surrounding tissue, resulting in decreased bone formation and anchorage around the implant. In addition, upon euthanasia of some of the rats, hematomas were found around the implants, which lead us to believe that the poor performance *in vivo* may be concentration dependent. It has been shown that high concentrations of Bone Morphogenetic Proteins, specifically BMP-2, can result in hematomas and ectopic bone formation. Analysis via a microarray could have been

done from the cells at the implant site. This would have helped to look at gene expression and the inflammatory response around the implant, which could have helped us better understand why the bioconjugate coatings did not perform as expected. Further analysis was needed to understand these unexpected results.

Histological analysis yielded similar results to the biomechanical testing. The pins were cut along the short and long axes of the bone, while maintaining the long axis of the pins (see figure 4). Once cut, three different interfaces were assessed for the bone: the while pin-bone interface (femur-bone interface), the spongy or cancellous-bone interface, and the cortical-bone interface (ctb-pin) (see figure 4). These three different interfaces were then quantified for total bone growth around the interface. Bone volume percent for all three analyses yielded a trend of increased bone growth from two to five weeks, but with few significant differences ($p<0.05$). Further analysis using *in vitro* studies could help us to better understand these results.

While the study yielded few significant differences between groups, it should be noted that these were done in healthy rats. As mentioned previously, titanium based joint replacement surgeries have a high rate of success, and a majority of failures were only seen in patients with predisposed conditions such as diabetes. Therefore, future work will be done with diabetic rat models to see if there are any clinical benefits in using these bioconjugates.

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