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Biomodulation of an implant for enhanced bone-implant anchorage

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Abstract

Aseptic loosening of implants is the major cause for revision surgery. By modulating the bone-implant interface, early bone-implant anchorage could be improved. Implant surface manipulation by the addition of osteopromotive molecules locally and systemically to promote implant integration has been described with limited success. This study describes a novel approach by making the implant capable of biologically modulating its surroundings. It was hypothesized that the early implant fixation would improve by filling the interior of the implant with a carrier providing spatio-temporal release of bone active drugs with known osteogenic effect. The implant consisted of a threaded polyether ether ketone (PEEK) hollow chamber with holes at the bottom. The implant was filled with a calcium sulphate (CaS)/hydroxyapatite (HA) carrier, delivering two bone active molecules; zoledronic acid (ZA) and bone morphogenic protein-2 (BMP-2). At first, a rat abdominal muscle pouch model indicated a sustained in-vivo release of both 125I-rhBMP-2 (57%) and 14C-ZA (22%) from the CaS/HA carrier over a period of 4-weeks. The biomodulated implant was then inserted in the proximal tibia in rats with the following experimental groups: G1) Empty implant, G2) Implant + CaS/HA, G3) Implant + CaS/HA + ZA and G4) Implant + CaS/HA + ZA + rhBMP-2. Significantly higher bone volume (BV) was seen around the implant in groups G3 (3.3 ± 0.7 mm³) and G4 (3.1 ± 0.7 mm³) compared to the control (1.3 ± 0.4 mm³) using micro-computed tomography and qualitative histology. Group G3, also exhibited significantly higher pull-out force and absorbed energy when compared to the control group G1. These findings indicate that a low dose of ZA alone, released in a controlled manner from within a fenestrated implant is enough to improve implant anchorage without the need of adding rhBMP-2. This simple method of using a fenestrated implant containing a ceramic carrier releasing bone active molecules improved bone anchorage and could clinically reduce prosthetic failure.

Statement of Significance

Aseptic loosening remains as a major cause for implant revisions and early reaction of surrounding bone to the prosthesis is important for longevity. A novel approach to enhance early bone-implant anchorage is presented.

The implant is filled with a carrier providing controlled release of bone active molecules. In an animal model, a calcium sulphate (CaS)/hydroxyapatite (HA) carrier was used to provide a spatio-temporal release of bone morphogenic protein-2 (BMP-2) and zoledronic acid (ZA). Significantly better bone-implant integration was achieved using ZA alone, thereby eliminating the need for adding BMP-2. The developed method of implant biomodulation holds potential to prevent implant loosening and is an alternative to prosthetic coatings or systemic drug treatment. Importantly, all constituents are approved for clinical use.

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1. Introduction

Despite technical advancements in fracture fixation and improved implants, complications such as prosthetic loosening or fracture fixation failure can cause demanding revision surgeries. Approximately 5–10% of joint arthroplasties and fracture fixations fail, causing increased morbidity and cost [1,2]. Early mechanical stability and bone anchorage of an implant appears to be important, both for long term survival of implants as well as for fracture fixation. Means to improve early fixation may potentially increase implant survival [3].

Attempts have been made to enhance peri-implant bone formation using anabolic bioactive molecules like fibroblast growth factor (FGF), transforming growth factor-β, bone morphogenic protein-2 (BMP-2), parathyroid hormone (PTH), extracellular matrix proteins such as fibronectin [4–7]. The bioactive molecules have been applied either locally by coating the implants directly or by means of systemic administration. Another way to enhance fixation is by using bisphosphonates, which work as anti-catabolics, reducing the resorption of traumatized bone around the implant. Bisphosphonates alone have shown significant potential in enhancing peri-implant bone formation in several different animal models [8–10] by preventing osteoclastic bone resorption [11]. Although the approach to manipulate implant osseointegration using anabolic and anti-catabolic agents is promising experimentally, little is known about the methods of delivering these molecules, their pharmacokinetics and the in-vivo biological consequences thereof. The most common local delivery route has been soaking the implant in a solution containing bioactive molecules. At best, the molecules stick to the implant by covalent bonds or they are just physically adsorbed onto the implant surface [4,9]. Sometimes the bioavailability of bioactive molecules is tested in vitro but this rarely reflects the in-vivo biological behavior [12]. The in-vivo bioavailability of these molecules during the course of bone regeneration is unknown. It is hypothesized that a spatio-temporal release would theoretically optimize the biological effect [13]. Recent studies have advocated local delivery of bioactive molecules, which besides providing high local doses, also reduces the total drug doses. This aids in circumventing the side effects of systemic delivery [14–16]. Due to a growing scientific interest in enhancing implant anchorage to surrounding bone, implant surface modification has been tried, predominantly by changing the implant macro structure or applying nanoscale surface coatings on implants [17]. Although moderately successful in experimental trials, clinical acceptance is still low and a cumbersome fabrication process makes clinical translation challenging. Therefore, there is a need of an implant, which can self-regenerate bone by controlled delivery of molecules, and is easy to use clinically.

In this study, a novel implant made of polyether ether ketone (PEEK) was biomodulated. The implant had a hollow core and the proximal tibia of rats. Peri-implant bone formation was studied using radiography, micro-computed tomography (micro-CT), mechanical testing and histology. It was hypothesized that the CaS/HA biomaterial will provide sustained delivery of rhBMP-2 and ZA due to affinity of these molecules to HA, thereby providing a simple yet efficient method for implant biomodulation [22,23]. By creating holes at the base of the implant, high local concentration of bioactive molecules would be achieved. Migration and differentiation of osteoprogenitor cells into osteoblast like cells is enhanced by the use of BMP-2 and secondary osteoclastic resorption prevented by ZA [24], thereby enhancing the peri-implant bone growth and anchorage.

2. Materials and methods

Zoledronic acid (ZA) (Novartis, concentration: 4 mg/5 mL), recombinant human bone morphogenic protein-2 (rhBMP-2) (supplied as part of the Medtronic C8 Inductos kit), Diazepam (5 mg/mL), Pentobarbital sodium (60 mg/mL), Ketamine hydrochloride (50 mg/mL) (Intervet), Xylazine hydrochloride (20 mg/mL) (Bayer) and Buprenorphine (0.3 mg/mL) (Schering Plough) were procured from the local pharmacy (Apotheke AB, Sweden). The CaS/HA biomaterial was provided by Bone Support AB, Sweden for research use. rhBMP-2 (Medtronic C8) and ZA were radiochemically coupled with $^{125}$I and $^{14}$C, respectively by Perkin Elmer (U.S.A). The respective specific radioactivity of the two bioactive molecules was 59.3 MBq/mL and 7.2 MBq/mL and the radiochemical purity was >95%. Medical grade, biocompatible polyether ether ketone (PEEK) block used for the manufacturing of the implant was purchased from Röchling Sustaplast (Germany). Male Sprague-Dawley rats (weight at operation: 373 ± 30 g) were procured from Taconic (Denmark).

2.1. In-vivo study design

The first part of the study was conducted to evaluate the in-vivo release of rhBMP-2 and ZA from the CaS/HA biomaterial in the abdominal muscle pouch model using single photon emission computed tomography (SPECT) and scintillation counting, respectively. During the second part of the study, a PEEK implant was used to functionalize the implant with bioactive molecules delivered via the CaS/HA biomaterial. Its osseointegration with the surrounding bone was assessed in the proximal tibia of rats using different analysis techniques including radiography, micro-CT, mechanical testing and histology.

2.2. In-vivo $^{125}$I-rhBMP-2 release from the CaS/HA biomaterial

To perform the rhBMP-2 release kinetics using SPECT-CT, CaS/HA biomaterial was directly mixed with $^{125}$I-rhBMP-2 and ZA at a dose of 5.25 μg/pellet for both biomolecules. Briefly, 500 mg of sterile CaS/HA powder (ratio: 6:4) was mixed with 210 μL of 0.2 mg/mL $^{125}$I-rhBMP-2 and 52.5 μL ZA (concentration: 0.8 mg/mL) under laminar air flow. The total liquid to powder ratio was higher (0.52 mL/g) compared to what has been used previously (0.43 mL/g) and needed to be modified due to the concentration of the stock solution of radiolabeled $^{125}$I-rhBMP-2 (0.2 mg/mL, specific radioactivity: 59.3 MBq/mL) [18]. Adding a higher amount of $^{125}$I-rhBMP-2 (10 μg/pellet) as reported previously [16] would increase the liquid to powder ratio significantly. Further, adding lower amounts of $^{125}$I-rhBMP-2 could potentially cause a weak signal during SPECT scanning rendering the images unusable. To avoid this, the liquid to powder ratio had to be adjusted for the in-vivo release study. After rigorous mixing for 1 min, the paste was poured into 8 different wells of a sterile cylindrical mold.
(Ø = 5 mm and height = 2 mm/well) and allowed to set for at least 1 h before implantation. After setting, 6 CaS/HA pellets containing 125I-rhBMP-2 and ZA were implanted in the abdominal muscle pouch of male Sprague-Dawley rats following a well-established protocol with one pellet/animal [12,15,18]. Each pellet had an approximate specific radioactivity of 1.3 MBq. In-vivo release of 125I-rhBMP-2 was performed by following the protocol established in a previous study [12]. Briefly, 12 h post-surgery (Day 1), the animals were anesthetized using a mixture of isoflurane (2%), O2, and N2O (flow rate: 0.4 L/min) and placed in the SPECT-CT scanner (NanoSPECT/CT, Mediso Medical Imaging Systems, Budapest, Hungary). Alongside the animal, a control tube containing a known amount of 125I-rhBMP-2 (specific radioactivity: 0.98 MBq) was placed parallel to the implant by securing it to the animal bed using adhesive tape. The tube was placed to correct for the decay in the isotope activity over time. A scout CT-scan was performed initially to ensure the location of the CaS/HA implant (projections: 360°, voltage: 65 kV, exposure: 1500 ms and final voxel size: 73 µm). With these settings, the anatomical location of radio-dense CaS/HA biomaterial could be verified on the micro-CT images. Based on the location of the CaS/HA biomaterial from the CT images, the SPECT region of interest (ROI) was chosen to detect the radioactivity in the implanted location as well as the control tube. The detector was calibrated for 125I and the obtained SPECT images were reconstructed (VivoQuant v2.5p3, inviCRO, U.S.A). During analysis, the CT and the SPECT images were superimposed and thereafter two ROIs of similar dimensions were drawn; one for the signal emanating from the implanted biomaterial (ROI 1) and the other from the control tube (ROI 2). A ratio of the radioactive counts between the two ROIs were used to normalize for radioactive decay. The same set of animals were followed longitudinally and scanned at days 3, 7, 14, 21 and 28. The measured counts on day 1 were considered to be the maximum signal and the release kinetics curve was plotted using the signal from day 1 as 100% followed by taking a ratio of signal obtained on a given test day with the signal on day 1.

2.3. In-Vitro and In-vivo 14C-ZA release from the CaS/HA biomaterial

2.3.1. In-vitro 14C-ZA release

0.36 mL 14C-ZA (concentration: 1 mg/mL, specific radioactivity: 7.2 MBq/mL) was mixed to a tube containing 0.36 mg rhBMP-2 and 0.93 mL saline. The contents of the tube were thoroughly pipetted and mixed with 3 g CaS/HA powder (weight ratio: 6:4) and rigorously mixed for 1 min using a sterile spatula to achieve a homogenous distribution of the bioactive molecules in the biomaterial slurry. The slurry was then transferred to three 1 mL syringes and the contents of the syringes were extruded into the wells of a sterile plastic mold. A total of 37 such pellets could be casted with each pellet containing approximately 9.7 µg rhBMP-2 and 9.7 µg 14C-ZA. The pellets were then allowed to reach rubber like consistency (waiting time: 5 min) allowed to reach rubber like consistency (waiting time: 5 min) before the day of the assay, n = 7 unused pellets of CaS/HA biomaterial taken from the stock of pellets described above were placed individually in scintillation vials and mixed with 1 mL PBS. At the day of the assay, n = 7 unused pellets of CaS/HA biomaterial taken from the stock of pellets described above were placed individually in scintillation vials and mixed with 1 mL PBS. The pellets were homogenized using a tissue disruptor (IKA Ultra Turrax® Germany) at 13,500 rpm for 1 min and mixed with 3 mL of scintillation cocktail (Perkin Elmer, USA) and vortexed for 15 s immediately before the test. Frozen fractions of PBS containing 14C-ZA fraction released from the CaS/HA biomaterial at different time points were thawed to room temperature and transferred to scintillation vials. 3 mL of scintillation cocktail was added to each vial and the contents were vortexed for 15 s. Samples were analyzed for radioactive counts on a Wallac 1414 liquid scintillation counter (Perkin Elmer, USA) for a total of 2 min/sample. Only scintillation fluid (3 mL) mixed with 1 mL of PBS was used to compensate for background activity. The average counts (disintegrations/min (DPM)) were corrected by total subtraction. The radioactivity detected at each time point was compared with the radioactivity in the 7 control pellets to get a % release at each time point. Released 14C-ZA was summed up in a cumulative fashion at each time point to get a release kinetics curve.

2.3.2. In-vivo 14C-ZA release

24 pellets of CaS/HA biomaterial containing 14C-ZA and rhBMP-2 (as described in Section 2.3.1) were implanted in the abdominal muscle pouch of 24 male Sprague-Dawley rats (1 pellet/rat). At each time point (days 1, 7, 14 and 28), 6 animals were sacrificed. The harvested pellets were cleaned of the surrounding soft tissue and transferred into scintillation vials containing 2 mL of 5 M HCl for decalcification/softening of the scaffolds for a total of 48 h. Samples were then homogenized as described in Section 2.3.1 and mixed with the scintillation cocktail at a dilution of 1:10. The radioactivity from each pellet was measured using a scintillation counter (Wallac 1414, Perkin Elmer, USA) and compared to the un-implanted control pellets (described in 2.3.1) to calculate the % release of 14C-ZA at each experimental time point.

2.4. Implant design

The implant consisted of a biocompatible PEEK hollow chamber with a conical base to fit into the proximal tibia of rats. The cylindrical part of the chamber was threaded and contained 3 equally spaced holes at its distal end. The end of the chamber was kept open to allow for filling the implant with the CaS/HA biomaterial with or without bioactive molecules. The triangular end of the chamber was closed and had a pointed end to ensure firm grip with the posterior cortex. A schematic of the chamber, chamber dimensions and its functionalization with the CaS/HA biomaterial are depicted in Fig. 1A–C.

2.5. Experimental groups and doses

A total of 4 experimental groups were used in the implant integration model. The experimental groups and dosage ofZA and rhBMP-2 are detailed in Table 1.

2.6. Sample preparation and in-vivo implantation in the implant integration model

All PEEK implants were disinfected by dipping them in 70% EtOH for 1 h followed by fresh EtOH change for 1 h under aseptic conditions. Implants were then dried by treating them with 2 changes of 99.5% alcohol for 10 min each and allowed to dry for 30 min. In the empty group G1, the implant did not contain any filling. For the CaS/HA biomaterial only group (G2), 500 mg of the CaS/HA powder (premixed at a ratio of 6:4) was mixed with 215 µL of loxehol (iodine-based contrast agent) and mixed with a spatula for 1 min. The paste was then transferred to a 1 mL syringe and extruded on the lid of a 24 well plate into 12 equal portions measured using markings on the syringe. The material was then allowed to reach rubber like consistency (waiting time: 5 min) before each portion was further cut into smaller pieces to be able to fit into the chamber hole. Once the contents of one portion were completely transferred into the chamber, a sterile steel rod was used to impact the contents together. In treatment group G3,
500 mg of the CaS/HA powder was mixed with 150 mL ZA solution (concentration: 0.8 mg/mL, total ZA: 120 mg) and 65 mL of Iohexol and thoroughly mixed for 1 min to achieve 12 equal portions of the CaS/HA material containing ZA. The PEEK implants were then filled with the CaS/HA biomaterial containing ZA (10 mg/implant) in a similar manner as described above for G2. The final treatment group (G4) consisted of PEEK implant containing CaS/HA + ZA + rhBMP-2. A total of 60 µg rhBMP-2 was mixed with 150 µL ZA solution (concentration: 0.8 mg/mL, total ZA: 120 µg) and 65 µL of Iohexol in a lo-bind Eppendorf tube. The contents were then pipetted on 500 mg of CaS/HA powder and mixed for 1 min to achieve homogenous distribution of the bioactive molecules. A 1 mL syringe was used to obtain 12 volumetrically equal portions of the paste. Contents were loaded into the PEEK chambers as described above for G2 and G3. The waiting time to achieve rubber like consistency in G3 and G4 was longer (~15 min).

Once all the implants were ready, animals (44 male Sprague-Dawley rats) were anesthetized using a combination of ketamine hydrochloride (90 mg/kg) and xylazine (12 mg/kg) via the intra peritoneal route and randomized based on the treatment groups specified in Table 1. The surgical site (right knee) was shaved and disinfected using chlorohexidine spirit. A 1.5 cm long incision along the antero-medial aspect of the knee joint was made starting from the tibial epiphysis (Fig. 1D(i)). A thin layer of underlying muscle was cleared from the operating field and the periosteum was scraped to expose the flat surface of the tibia (Fig. 1D(ii)). A 3.2 mm Ø hand held drilling burr with a pointed tip was used to drill the cortical bone and the underlying cancellous bone (Fig. 1E(i)). Once the posterior cortex was reached, drilling was continued gently to create a small hole in the cortex to allow for easy placement of the implant. The wound was cleaned using a sterile gauze and saline flushed to remove bone fragments from the canal (Fig. 1E(ii)). Based on the treatment groups, chambers were inserted into the defect in a press-fit manner using a custom-made screw driver that aided in affixing the chambers (Fig. 1F(i)). Approximately 2–3 mm of the implant was kept outside the bone (green arrow) and covering the top opening of the implant with a bioinert, non-resorbable cap (green arrow) to prevent infiltration of soft tissue into the implant threads, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Bioactive Molecule Doses</th>
<th>Sample Size (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>PEEK Implant</td>
<td>–</td>
<td>11</td>
</tr>
<tr>
<td>G2</td>
<td>PEEK Implant + CaS/HA</td>
<td>–</td>
<td>11</td>
</tr>
<tr>
<td>G3</td>
<td>PEEK Implant + CaS/HA + ZA</td>
<td>ZA = 10 µg</td>
<td>11</td>
</tr>
<tr>
<td>G4</td>
<td>PEEK Implant + CaS/HA + ZA</td>
<td>rhBMP-2 = 2 µg</td>
<td>11</td>
</tr>
</tbody>
</table>

500 mg of the CaS/HA powder was mixed with 150 µL ZA solution (concentration: 0.8 mg/mL, total ZA: 120 µg) and 65 µL of Iohexol and thoroughly mixed for 1 min to achieve 12 equal portions of the CaS/HA material containing ZA. The PEEK implants were then filled with the CaS/HA biomaterial containing ZA (10 µg/implant) in a similar manner as described above for G2. The final treatment group (G4) consisted of PEEK implant containing CaS/HA + ZA + rhBMP-2. A total of 60 µg rhBMP-2 was mixed with 150 µL ZA solution (concentration: 0.8 mg/mL, total ZA: 120 µg) and 65 µL of Iohexol in a lo-bind Eppendorf tube. The contents were then pipetted on 500 mg of CaS/HA powder and mixed for 1 min to achieve homogenous distribution of the bioactive molecules. A 1 mL syringe was used to obtain 12 volumetrically equal portions of the paste. Contents were loaded into the PEEK chambers as described above for G2 and G3. The waiting time to achieve rubber like consistency in G3 and G4 was longer (~15 min).

Once all the implants were ready, animals (44 male Sprague-Dawley rats) were anesthetized using a combination of ketamine hydrochloride (90 mg/kg) and xylazine (12 mg/kg) via the intra peritoneal route and randomized based on the treatment groups specified in Table 1. The surgical site (right knee) was shaved and disinfected using chlorohexidine spirit. A 1.5 cm long incision along the antero-medial aspect of the knee joint was made starting from the tibial epiphysis (Fig. 1D(i)). A thin layer of underlying muscle was cleared from the operating field and the periosteum was scraped to expose the flat surface of the tibia (Fig. 1D(ii)). A 3.2 mm Ø hand held drilling burr with a pointed tip was used to drill the cortical bone and the underlying cancellous bone (Fig. 1E(i)). Once the posterior cortex was reached, drilling was continued gently to create a small hole in the cortex to allow for easy placement of the implant. The wound was cleaned using a sterile gauze and saline flushed to remove bone fragments from the canal (Fig. 1E(ii)). Based on the treatment groups, chambers were inserted into the defect in a press-fit manner using a custom-made screw driver that aided in affixing the chambers (Fig. 1F(i)). Approximately 2–3 mm of the chamber was left outside the bone to ensure that a mechanical testing jig component could be screwed to the upper part of the chamber at the time of mechanical testing (Fig. 1F(ii)). To prevent the ingrowth of fibrous tissue into the chamber from top or into the chamber threads, a biocompatible nylon cap was used to cover the top of the chamber (Fig. 1F(iii)). The muscle and the soft-tissue in close proximity to the defect were sutured using resorbable sutures (5–0, Vicryl) following which the skin was sutured using non-resorbable sutures (5–0, Ethicon). Animals were checked weekly to ensure that the chambers had not come loose by manually palpating the defect site. The animals were euthanized by CO₂ asphyxiation 6-weeks post implantation.

### 2.7. Radiography and Micro-CT

Radiography and micro-CT imaging was performed on a NanoScan CT scanner (Mediso Medical Imaging System, Budapest,
The volume of the chamber and its internal content were measured in ROI 1 was expressed as ROI ImEx. BV was measured between the solid and the dotted line. Middle shows the dimensions used for the intermediate ROI while image on the right shows ROI 2 which was obtained by summing the BV obtained in ROI 1 and the intermediate ROI. BV was measured between the solid and the dotted line.

Fig. 2. A schematic of the region of interest (ROI) selection for micro-CT quantifications. Left shows the dimensions used for ROI 1. Bone volume (BV) was measured between the solid and the dotted line. Middle shows the dimensions used for the intermediate ROI while image on the right shows ROI 2 which was obtained by summing the BV from ROI 1 and the intermediate ROI. BV was measured between the solid and the dotted line.

2.9. Representative histological evaluation

After biomechanical testing of all specimens, the specimens that did not fracture at the bone-implant interface or the ones that loosened from the brass connector were chosen for histology. The samples were fixed in 4% neutral buffered formaldehyde overnight followed by decalcification in 10% ethylene diamine tetra acetic acid (EDTA) adjusted to pH 7.4 at room temperature for 5-weeks with regular EDTA changes twice/week. Implant removal was performed on specimens that contained the implant after biomechanical testing by gently unscrewing the implants. Samples were embedded in paraffin and cut to a thickness of 5 μm. Hematoxylin and Eosin (Thermo Scientific, USA) and scanned using a histology slide scanner (Hamamatsu, Japan).

2.10. Statistical methods

Data were checked for normality using the Shapiro-Wilk Normality test by using GraphPad Prism 7 for Mac OS X (V 7.0a). Normally distributed data were tested using ANOVA with Dunnett’s multiple comparisons test for comparing all treatment groups to control group G1. Treatment groups (G2-G4) were compared using ANOVA with Tukey’s HSD multiple comparison post-hoc test. For non-normally distributed data, Kruskal-Wallis test with Dunn’s multiple comparison was used to test the difference of treatment groups with respect to the control group G1 or differences within the treatment groups. Data is presented as mean ± SD.
2.11. Animal ethics statement

Both the abdominal muscle pouch model (permit number: M124-14) and the tibia defect model (permit number: M79-15) were approved by the Swedish board of agriculture. Animals had free access to food pellets and water throughout the duration of the experiments. Animals were housed two/cage with 12 h light/darkness cycles.

3. Results

3.1. In-vivo \(^{125}\text{I}-\text{rhBMP}-2\) release from the CaS/HA biomaterial

The assessment of the release kinetics of \(^{125}\text{I}-\text{rhBMP}-2\) from the CaS/HA biomaterial in the ectopic muscle pouch model was performed using SPECT-CT (Fig. 3A–C). The CaS/HA biomaterial exhibited a gradual release of rhBMP-2 with approximately 57\% \(^{125}\text{I}-\text{rhBMP}-2\) released from the CaS/HA biomaterial at the end of 4-weeks (Fig. 3D).

3.2. In-vitro and In-vivo \(^{14}\text{C}-\text{ZA}\) release from the CaS/HA biomaterial

In-vitro, the CaS/HA biomaterial released 2.0\% of \(^{14}\text{C}-\text{ZA}\) on day 1, which increased to 10\% after 4-weeks displaying a slower release of \(^{14}\text{C}-\text{ZA}\) compared to rhBMP-2 (Fig. 4A). The CaS/HA material released slightly higher amount of \(^{14}\text{C}-\text{ZA}\) with a burst release of 10.7\% on day 1 in-vivo. On days 7, 14 and 28, the CaS/HA biomaterial released 16.0\%, 17.1\% and 22.2\% \(^{14}\text{C}-\text{ZA}\), respectively (Fig. 4B).

3.3. Radiographic assessment of peri-implant bone formation

A very similar peri-implant reaction could be observed in the control group (G1) and the group with the implant filled with only CaS/HA biomaterial (G2). Neither showed any significant enhancement of radio density (Fig. 5A, white arrows). In all CaS/HA treated groups G2-G4, the CaS/HA biomaterial could be seen as a dense cylindrical lump within the hollow implant (Fig. 5A, B). In the groups where the implant was filled with CaS/HA + ZA (G3) and CaS/HA + ZA + rhBMP-2 (G4), the area immediately around the implant appeared dense on the radiographs throughout the depth of the medullary canal (Fig. 5A, B, white arrows) with a similar response in both groups.

3.4. Quantification of peri-implant bone formation using micro-CT

Within ROI 1 i.e. bone formed immediately around the implant holes (within the medullary cavity), the bone volume (BV) fraction was significantly higher in G3 (mean: 3.3, 95\% CI: 2.8–3.8) \((p < 0.001)\) and G4 (mean: 3.1, 95\% CI: 2.7–3.6) \((p < 0.001)\) when compared with the empty control group G1 (mean: 1.3, 95\% CI: 1.1–1.6) (Fig. 5B and C, Left). Groups G3 \((p < 0.001)\) and G4 \((p = 0.012)\) also performed significantly better than group G2 (mean: 1.5, 95\% CI: 1.3–1.7) in terms of the measured BV in ROI 1. No differences between implants functionalized with ZA (G3) or ZA + rhBMP-2 (G4) could be observed (Fig. 5B and C, Left).

In ROI 2, i.e. the bone formed along the entire depth of the implant (including cortical bone), groups G3 (mean: 7.7, 95\% CI: 6.7–8.7) and G4 (mean: 7.0, 95\% CI: 5.9–8.2) also showed significantly higher bone formation compared to groups G1 (mean: 4.6, 95\% CI: 4.3–4.9) and G2 (mean: 5.1, 95\% CI: 4.5–5.6) corroborating the radiographic assessment (Fig. 5B and C, Right).

3.5. Pull-out testing to assess implant osseointegration

Pull-out testing was used as a surrogate for implant osseointegration. The peak pull-out force was significantly higher in the group in which the implant was functionalized with CaS/HA + ZA (G3) (mean: 171.2, 95\% CI: 140.7–201.7) when compared to the control group G1 (mean: 118.9, 95\% CI: 106–131.8) \((p < 0.05)\) corresponding to an increase of 44\% (Fig. 6A, B). An increase of 84\% in absorbed energy between the two groups was also observed (mean G3: 114.9, 95\% CI: 81.0–148.7 vs. mean G1: 62.5, 95\% CI:
Furthermore, the new bone formed was characterized in low magnification histological slices and verified in micro-CT slices. In both groups functionalized with bioactive molecules (G3 and G4), extensive peri-implant bone formation was observed in-vivo (Fig. 8) using picrosirius red staining.

Likewise, minimal collagen deposition was observed as well as in the histology images stained with hematoxylin and eosin. Very limited bone formation could be seen in the region proximal and distal to the implant. At higher magnification, it could be observed that the bone matrix was laid out as one big unit in G3 with less inter-trabecular spacing and less bone marrow deposition (Fig. 8). The bone matrix in the BMP-2 + ZA group appeared to be more remodeled, characterized by the presence of large inter-trabecular spacing as well as abundant deposition of bone marrow like tissue between the trabeculae (Fig. 8). Remnants of the CaS/HA biomaterial could not be seen in the medullary cavity in any of the treatment groups G2-G4.

4. Discussion

The aim of this study was to functionalize a novel implant with bioactive molecules in order to enhance peri-implant bone formation and osseointegration. Rather than just dipping the implant in a solution of bioactive molecules or modifying the implant surface, a hollow implant was created. The implant was filled with a CaS/HA based biomaterial carrier delivering locally ZA or ZA + rhBMP-2, with an aim to provide a balance of anti-catabolic-anabolic responses, respectively. The release kinetics of the bioactive molecules from the CaS/HA carrier as well as subsequent peri-implant bone formation was evaluated in two separate animal models.

4.1. Ectopic muscle pouch model: release kinetics of rhBMP-2 and ZA from the CaS/HA biomaterial

The ectopic muscle pouch model was used for release kinetics experiment because it provides most relevant representation of the carrier properties of a biomaterial [12]. Orthotopic implantation sites possess the risk of rhBMP-2 or ZA reuptake in the surrounding bone making a clear assessment of release kinetics impossible. The CaS/HA biomaterial used in this study is approved for clinical use. The efficacy of the CaS/HA biomaterial for local delivery of rhBMP-2 and ZA has been reported earlier in the ectopic muscle pouch model [12,18,20]. The injectable and biphasic properties of the biomaterial make it an attractive carrier. CaS phase resorbs quickly within 6–8 weeks in vivo leaving behind an osteoconductive matrix of HA, which integrates with the new ingrowing bone over time [25]. HA is the key component for the prolonged release of both rhBMP-2 and ZA due to their affinity to HA [22,23]. In an earlier in-vitro study, it was demonstrated that the biphasic CaS/HA biomaterial displayed a rather rapid release of rhBMP-2 in-vitro with 90% of the protein being released in one week [18]. In-vitro ZA release kinetics was also studied using an indirect approach by studying the cytotoxic effects of ZA released from the CaS/HA biomaterial on A549 cells and a total of 10% ZA was released from the material in a week [18]. To verify the ZA release results in-vitro over a longer period of time, a direct release assay was performed in this study using 14C-ZA mixed with the CaS/HA biomaterial. The in-vitro release results corroborated well with the previous results and 10% of 14C-ZA was released in 4-weeks in this study. Compared to the in-vitro release of rhBMP-2 reported earlier [18], more prolonged release of the protein could be observed in-vivo (57%). This is contrary to the more accepted notion of porous polymeric biomaterials releasing more rhBMP-2 in-vivo compared to in-vitro [12,13,26]. It is speculated that the in-vitro dissolution of the CaS phase is much faster in the release buffer than during in-vivo implantation, which could explain higher release of rhBMP-2 in-vitro. In terms of release kinetics of rhBMP-2, an early burst release followed by a sustained release of the molecule during the entire course of healing is suggested to be optimal and is also coupled with biomaterial degradation as reported by Seo and co-workers [26,27]. Their recent article suggests that a fast resorbing biomaterial with burst rhBMP-2 release or a slow resorbing biomaterial with slow rhBMP-2 release can both reduce the biological action of rhBMP-2 [27]. The only FDA approved collagen sponge for the delivery of rhBMP-2 releases approximately 90% rhBMP-2 in 2-weeks, which is considered sub-optimal for rhBMP-2 activity in-vivo [28]. Our results suggest that the CaS/HA biomaterial can potentially be used as a more efficient...
Fig. 5. Radiography and micro-CT based assessment of peri-implant bone formation in the implant integration model 6-weeks post implantation. (A) Representative radiographs from each treatment group with white arrows emphasizing the extent of peri-implant bone formation. (B) 3D micro-CT images of representative samples showing the peri-implant bone formation assessed via micro-CT. Notice the abundant bone formation in G3 and G4 both in radiographs (5A, G3 and G4, white arrows) and 3D micro-CT reconstructions (5B, G3 and G4). (C, Left) The amount of bone (BV) regenerated around the implant in ROI 1 (inset) in various treatment groups, and in ROI 2 (inset) in various treatment groups (C, right). Scale bar represents 2 mm. ** indicates p < 0.01 and *** indicates p < 0.001.

Fig. 6. The pull-out testing of the PEEK implants after 6 weeks of in-vivo implantation. (A) The setup used for the pull-out testing including the sample holder, loading direction and the brass connector. B-D shows the peak force, stiffness and absorbed energy, respectively, across different treatment groups. * indicates p < 0.05.
carrier for rhBMP-2 delivery in-vivo. ZA on the other hand has a much stronger affinity to apatite particles, and as a result, less ZA is released both in-vitro and in-vivo when compared to rhBMP-2. Less investigations have been carried out on in-vivo local ZA release from other biomaterials, but when comparing to a macro-porous CaS/HA scaffold combined with gelatin [12,15], less (50%) in-vivo release of ZA was measured from the CaS/HA biomaterial used in this study. The presence of a porous structure and a limited interaction between ZA and collagen or gelatin could be the reason. The biological effect of the differences in release kinetics of ZA from the two biomaterials is being investigated further in our laboratory at present.

4.2. The implant model: Drug doses and experimental groups

The implant design used in this sub-study is a modification of the bone conduction chamber described earlier by Aspenberg et al. [29] but with three large holes added at the base of the implant within the intramedullary canal, emphasizing on peri-implant bone formation rather than osteoconduction within the chamber [29,30]. Furthermore, instead of using traditional bone grafting materials like autograft or allograft, in this study the aim was to functionalize a CaS/HA biomaterial with bone active molecules to act as a bone graft substitute. The timelines in the study were also based on the experimental set-up from the bone

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Fig. 7. Representative overview of micro-CT and histology slices verifying the extent of peri-implant bone formation. Left panel shows representative micro-CT slices from each treatment group. Middle panel shows H&E stained low magnification overview of specimens from each treatment group and right panel shows Picosirius red stained section from each treatment group. Right side of the histological images represents the proximal tibia and left side represents the distal tibia. Scale bar represents 1 mm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 8. Representative H&E stained slices at high magnification showing the tissue immediately around the PEEK implant within the medullary canal across different treatment groups. * indicates bone tissue characterized by the presence of osteocytes and # indicates marrow like tissue. Scale bar represents 100 µm.
conduction chamber model [29,30]. PEEK was chosen over traditional metal alloys as a base material for the implant to avoid artefacts produced by metals during x-ray based micro-CT imaging, which would make the accurate evaluation of bone growth around the bone-implant interface difficult, even with subtraction programs for metals [31,32]. Furthermore, the local ZA dose used in this study was 3–5 times lower than reported systemically, yet a significant effect on bone formation was observed [24,33]. Although different concentrations of ZA were not tested, the selected ZA dose of 10 μg/animal was taken from the literature [12,14]. Detrimental effects of very high local concentration of ZA delivered via a CaS/HA biomaterial have been reported earlier and the concentration of ZA used in this study is within the reported working window [34]. Further lowering the doses of ZA delivered locally and its effect on bone formation is currently being evaluated in our laboratory. The doses of rhBMP-2 used experimentally in rats vary depending on the anatomical location. The literature does not provide a general consensus, which by speculation could be due to variable release properties of different carrier materials. In flat bone defects, studies indicate rhBMP-2 doses ranging from 0.125 to 6 μg to be effective in rats, with a broader range in long bones ranging between 1 and 50 μg [26]. High rhBMP-2 doses (>22.5 μg/rat) have been associated with abnormal bone structures resembling cyst like tissue [35]. In a tibia defect model, our group has shown that 5 μg rhBMP-2 delivered via a microporous CaS/HA carrier or a macroporous porous gelatin-CaS/HA carrier is sufficient for cancellous bone defect regeneration [15,20]. The implant model used in this study is similar to the tibia defect model in terms of the anatomical location and process of bone regeneration. Thus, the rhBMP-2 doses were based on the results from our earlier studies [15,20].

4.3. The implant model: Impact of controlled local delivery of rhBMP-2 and ZA

The micro-CT results demonstrated that the addition of ZA or the combination of rhBMP-2 + ZA led to significantly higher peri-implant bone when compared to the controls. However, somewhat surprisingly, no difference was seen between using ZA alone or combining ZA with rhBMP-2. Increased peri-implant bone formation also translated into increased pull-out strength and absorbed energy in the ZA treated group but not in the group with combined delivery (G4). A similar finding was reported by Baas and co-workers earlier in a canine implant osseointegration model [8]. The ZA treated implants were better integrated with the native bone compared to ZA + rhBMP-2 treatment and the result was attributed to an increased resorption drive induced by rhBMP-2 despite the presence of ZA. Another similar model of implant integration has been described in rats by Andersson and co-workers [9]. They covalently coupled ZA to the surface of an implant by chemical modification of the implant surface and observed an increase in peak pull-out force. The mechanical testing results from this study are thus in line with the existing relevant literature in the field. Interestingly, the extent of bone formation in groups G3 and G4, as seen by histology, was much higher than in earlier reports, for instance by coating an implant with extracellular matrix proteins [5]. A continuous supply of rhBMP-2 is required for differentiation of progenitor cells into mature bone cells to optimally regenerate bone. Subsequently, it is desired for ZA to stay within the new forming callus to continue its osteoclast inhibiting effect [36]. These release kinetics of rhBMP-2 and ZA from the CaS/HA biomaterial demonstrated a congenial trend, optimal for enhancing the peri-implant bone formation.

The data from this study also suggests that the local delivery of ZA at an optimal dose is enough for cancellous bone formation around the implant to allow an enhanced integration with the surrounding bone. The usage of rhBMP-2 is already under scrutiny and, besides being costly, means to circumvent the clinical side-effects of rhBMP-2 or even completely avoid its usage are desirable [37]. Although the molecular mechanism of a putative anabolic action of local ZA delivery is unknown, von Knob et al., indicated a dose dependent osteoinductive effect of ZA on human bone marrow stromal cells in-vitro [38]. This positive effect of local ZA treatment on implant integration was supported by other in-vivo experimental studies wherein ZA was applied in the form of an implant coating or topical administration using bone grafts/irrigation [8,39,40]. Our primary aim of using an implant with a biomaterial carrier filled core was to locally deliver ZA and create an off-the-shelf solution for bone regeneration. This could possibly eliminate the need of traditional bone grafting. Moreover, systemic administration of ZA is associated with side effects [41] including reduced bone remodeling [42] and in one of our earlier studies, an onsite effect of local ZA delivery was demonstrated [16]. It is important to avoid the side effects of systemic ZA delivery especially in young patients, because altered bone remodeling due to long term bisphosphonate administration can have negative effects on bone quality [42]. The representative histology from this study also suggests a different histological pattern of peri-implant bone formation despite no differences in the micro-CT data. The peri-implant bone formed in the ZA group appeared to be less remodelled, characterized by thicker trabeculae and less bone marrow deposition within them and is most likely due to a strong effect of ZA on osteoclast-based remodeling. On the contrary, the rhBMP-2 + ZA group (G4), indicated more remodelled bone despite the presence of ZA. This difference in the pattern of bone formation did not have an effect on osseointegration in this model and this mode of local ZA treatment holds the promise of not interfering with physiological bone remodeling process at other anatomical sites.

4.4. Clinical implications

An implant containing a carrier inside, releasing bioactive molecules in a controlled fashion, could increase peri-implant bone formation, reduce micro motion and in particular prevent later loosening or failure in fracture fixation in the important early stages. Due to an impaired implant-bone bed, revision cases are even more challenging [43]. An implant that can accelerate peri-implant bone formation to provide initial stability could be one of the applications of the bioactive molecule functionalized implant described in this study. The findings from this study can also potentially be applied to other orthopedic devices such as cannulated or fenestrated screws and open cages used for spinal fusions [44,45].

4.5. Limitations

Due to weak material properties of the PEEK implant and the surrounding threads, the brass holder used to test the anchorage of the bone-implant interface caused bending of the threads at higher forces, thereby causing slipping in some cases. This rendered the samples unusable for analysis because any amount of force applied to the bone-implant interface would incur micro-architectural damage leading to unreliable readings. This reduced the overall sample size for pull-out testing, especially in the treatment groups, G2–G4. However, our prime objective was to perform robust analysis of the peri-implant bone formation due to which we were inclined to choose a base material that did not interfere with micro-CT quantifications. Despite this shortcoming, an increase in the mechanical forces was demonstrated when the implant was functionalized with local ZA. Arguably, an experimental group with CaS/HA + rhBMP-2 was missing but we have...
previously, in several studies, shown that co-delivery of rhBMP-2 + ZA is superior than delivering rhBMP-2 due to a substantial secondary osteoelastic activation by rhBMP-2 [12,15,18,46]. Only one time point was used to evaluate the extent of bone formation and the very early response of ZA on osseointegration was not investigated, which would need further experimentation.

5. Conclusion

In conclusion, the CaS/HA biomaterial provided a sustained release of both rhBMP-2 and ZA in-vivo. Contrary to the ZA release, more rhBMP-2 was released in-vivo, indicating a stronger affinity of ZA to HA. A very strong effect on peri-implant bone formation was observed when a fenestrated PEEK implant was filled with the CaS/HA biomaterial containing ZA or a combination of rhBMP-2 + ZA. The results from the implant integration model clearly indicate that local controlled delivery of ZA alone is sufficient to enhance bone implant anchorage without the need of adding rhBMP-2. The results have the potential to be translated into the clinical setting to reduce implant micromotion in primary orthopaedics, enhance peri-implant bone formation in poor bone quality revision surgery or accelerating mechanical stability of other orthopedic hardware used for fracture fixation.

Author contributions

MT, DBR, LL and HI designed the study, developed and optimized the PEEK implant design and the animal model. DBR, MT, LL and HI were involved in the in-vivo release of rhBMP-2 and ZA experiments. DBR performed functionalization of the implant with bioactive molecules. DBR, MT and DL performed animal surgeries. DBR and HI analyzed the micro-CT images. DBR, HI and EAS performed pull-out testing. DBR and EAS prepared specimens for histology. Manuscript was drafted by DBR and revised based on the inputs from all authors.

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Disclosures

LL is a board member of Bone Support AB, Lund, Sweden and Ortho Cell, Australia. All other authors have nothing to disclose.

Data availability

The corresponding author or the senior author can provide data upon reasonable request.

References


