Whitepaper

TITLE: **PROGRAMMED DRUG RELEASE OF rhBMP-2 FOR BONE HEALING**

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DISCLOSURES: [1] Employed by Locate Bio Ltd

REGULATORY STATUS: The product discussed in this whitepaper is not approved for human use.

1. Summary:

Non-union of bone fractures can cause pain and disabilities. Amongst certain fracture types, in specific age groups, the incidence of non-union is over 8% in the United States and over 3.6% of all fractures in the UK. Fracture non-unions have lifelong consequences and a debilitating impact in patients' life. Bone-grafts, allografts and xenograft are currently used to treat bone fractures; however, they have limitations. To overcome these limitations, we herein present a synthetic bone graft substitute composed of P_{DL}LGA and rhBMP-2. We have demonstrated the suitability of this bone graft substitute both in *in-vitro* and *in-vivo* models. In the *in-vitro* model, rhBMP-2 release was sustained for 28 days. In the *in-vivo* animal studies, the bone graft substitute showed both osteoinductive potential with ectopic bone formation in a rat muscle pouch, as well as osteogenic potential confirmed by the effective bone and mineral formation in a rabbit posterolateral vertebral fusion study.

2. Introduction:

Bone fractures are commonplace, with an estimated 8 million occurrences each year in the United States (US) alone. Amongst certain fracture types in specific age groups, the rate of non-union is over 8% and somewhat surprisingly, the higher non-unions rates occur mostly in young and middle-aged adults rather than in the elderly population (Mills et al., 2017). These non-unions can cause prolonged pain and disability and can have a profound negative impact in many aspects of patient's life (Johnson et al., 2019).

In the United Kingdom (UK), the cost of treatment of non-union has been estimated at £7,000 to £79,000, but these figures relate only to the cost of hospital treatment (Mills et al., 2014; Kanakaris and Giannoudis, 2007). In the US, the median cost of treating non-unions has been estimated at $25,556 USD per open tibial fracture, with increased healthcare utilisation, and increased prescription and longer duration of opioids in these cases (Antonova et al., 2013).

To reduce the incidence of non-union, a variety of bone grafts, such as autografts (using the patient's own bone from elsewhere in the body), allografts (bone from organ donors) and xenografts (animal bone) are used clinically. However, these have limitations such as limited quantity and quality, morbidity associated with the donor harvest site, the risk of animal or human-derived pathogen transmission and insufficient osteogenic potential (Gleeson et al., 2010).

To overcome these limitations, synthetic bone graft substitutes have been developed and are widely used. Calcium phosphate-based (CaP) biomaterials are the predominate form owing to their similar composition to bone (LeGeros & LeGeros, 2003). CaP bone graft substitutes have the advantages of biocompatibility, safety, availability, low morbidity, cost-efficiency, and

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osteoconductivity (Best et al., 2008). Recent advances have seen further optimisation of the surface topography of these CaP materials, which has been shown to improve the healing time of this class of bone graft substitute (Van Dijk et al., 2020).

An alternative strategy has been utilised for enhancing bone regeneration and reducing the healing period, especially in large and non-contained bone defects. Among the pharmacological agents used in bone regeneration, are growth factors and the most recognised is recombinant human bone morphogenetic protein 2 (rhBMP). rhBMP-2 is a powerful therapeutic protein which has been shown to play a significant role in new bone growth (Salazar et al., 2016).

The FDA has approved the use of rhBMP-2 delivered on a collagen sponge, however, there has been ongoing controversy surrounding potential side effects, believed to be related to the burst release of rhBMP-2 coupled with poor retention of the rhBMP-2 at the intended site (Han et al., 2021; Hustedt & Blizzard, 2014). This burst release results in an initial supraphysiological level of rhBMP-2 followed by suboptimal release and a lack of extended release The combined effect of the poor release profile can result in significant clinical complications (Han et al., 2021). In studies of normal fracture healing, native BMP-2 has been shown to be present for up to 28 days (Faßbender et al., 2014; Onishi et al., 1998), and therefore an extended-release profile of rhBMP-2 would provide a more physiologically relevant release.

The potential exists for new biomaterials to be used to alter the rhBMP-2 release profile damping the initial burst, holds the promise of reducing unwanted side effects and achieving an extended release that will better match the physiological need. This combination of dampened initial burst coupled with extended release is called “Programmed Drug Release”. A material science method is herein proposed to achieve this outcome.

3. Design Considerations:

There are several approaches to achieving a programmed drug release, with dampened drug burst and an extended-release profile. One approach is to encapsulate the drug within a biomaterial, and whilst this approach is more complex than simple adhesion, it provides the ability to tune the release profile through specific design considerations. This design has been successfully used to provide the extended release of drugs by encapsulating them within biodegradable poly DL lactic acid-co-glycolic acid ($P_{DL}LGA$) (Fredenberg et al., 2011; Hines & Kaplan, 2013), for example Lupron Depot® which is available as a single injection every 24 weeks (LUPRON DEPOT 3.75 mg and –3 Month 11.25 mg. North Chicago, IL: AbbVie Inc.)

$P_{DL}LGA$ is a copolymer, previously cleared by the FDA for use in implantable medical devices. It is biocompatible, non-toxic, undergoes predictable degradation *in-situ* and the products of its degradation are fully cleared by the body via hydrolysis (Anderson & Shive, 1997).

$P_{DL}LGA$ is a suitable material for bone repair for various reasons, firstly, it can be easily processed using a variety of technologies. Secondly, by varying the ratio of lactic acid and glycolic acid the degradation profile can be adjusted to match that of bone remodelling. Thirdly, $P_{DL}LGA$ has an initial mechanical strength adequate to support bone integration in the early stages of bone growth. Fourthly, $P_{DL}LGA$ can be loaded with different bioactive factors or small molecules (Zhao et al., 2021).

However, there are potential disadvantages of using $P_{DL}LGA$ in the context of bone regeneration. Firstly, $P_{DL}LGA$ is not inherently porous like CaP-based materials. An absence of porosity can lead to areas of bone necrosis due the combined effect of inadequate blood supply through the graft area and reduction in bone marrow cell penetration (Murphy & O'Brien, 2010). Secondly, where proteins are encapsulated in the $P_{DL}LGA$, sterile manufacture cannot be performed and therefore terminal sterilisation must be applied which is a process known to degrade proteins (Ijiri et al., 1994).

To overcome these potential disadvantages, a proprietary manufacturing method has been developed using a PLGA base process to produce pellets that contain lumens, from a dry mixture containing (i) rhBMP-2, (ii) excipients to protect the rhBMP-2 during terminal sterilisation and (iii) $P_{DL}LGA$. These pellets take the form of hollow cylinders whose diameters are in the 0.5 – 1mm range and whose heights are in the 0.6 – 1.5mm, conferring an aspect ratio of circa 1.5 to optimise random packing and support intraparticle microporosity. Particle interconnectivity is facilitated by the pellet lumen and random packing.
The pellets surfaces are biocompatible and the pellets act as an osteoconductive scaffold, allowing stem cells from the host to migrate into the graft site and attach to them. We refer to these pellets as rhBMP-2 scaffold pellets in the remaining text.

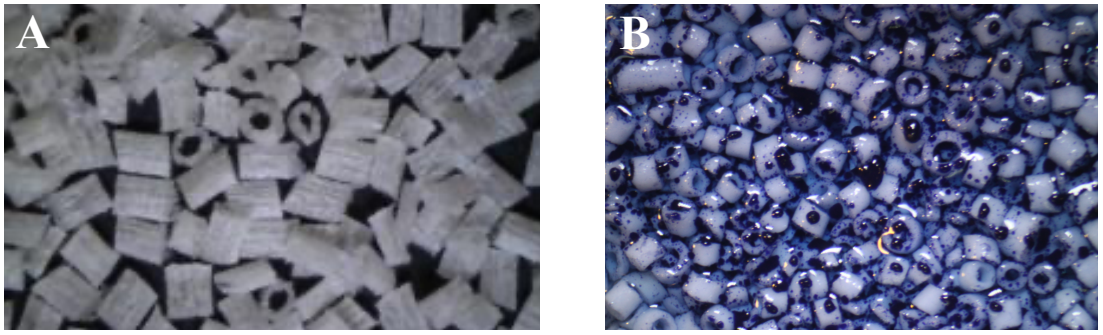

Figure 1. (A) rhBMP-2 pellets fabricated through a hot melt extrusion process. (B) Toludine blue cell staining, showing MSC attachment on the surface of rhBMP-2 scaffold pellets.

4. Materials and Methods:

4.1 Programmed Drug Release using ALP production in an *in-vitro* assay

To assess the release profile of the drug from the rhBMP-2 scaffold pellets, an *in-vitro* assay measuring ALP activity in the mouse muscle myoblast cell line, C2C12, was used. For the assay rhBMP-2 pellets were suspended above a C2C12 monolayer in an insert with a porous (8 µm) PET membrane. Following 3–4-day exposure the inserts were transferred to fresh cells for a further 3-4 days for a period of up to 28 days. Cells were assayed for ALP activity following each transfer step.

4.2 Osteoinductive potential using an *in-vivo* model

To assess the effectiveness of the rhBMP-2 scaffold pellets, they were evaluated for osteoinductivity in a rodent ectopic model. In this study, the capacity of rhBMP-2 scaffold pellets to promote osteogenesis was assessed 4 weeks after intermuscular implantation in nude rats. For the study, rhBMP-2 scaffold pellets were sterilised by irradiation (X-Ray 25kGy) and compared to Infuse™ (Medtronic).

This model meets the European Directive 2010/63/UE, September 22nd 2010, and was authorised by local and national ethics committees.

For the study, three rats were used, and each received a rhBMP-2 scaffold pellet implant in the left leg and an Infuse™ implant in the right leg. For each implantation, a longitudinal skin incision parallel to the femur was made. Approximately midway between the hip and the knee, the membrane between the two leg muscles near the femur (biceps femoris and gluteus superficialis) was punctured and a pocket was opened between the muscle groups, without damaging muscle fibres (blunt dissection). The implant was inserted into the pocket. The pocket was sutured to prevent implant migration and the skin wound was closed using surgical staples. Both groups received rhBMP-2 doses of 127.5µg from implants that were all 0.175 cm^3 in volume. Infuse™ was prepared as per the Instructions for Use.

4.3 Functional testing in a rabbit PLF model

To evaluate the *in-vivo* performance of the rhBMP-2 scaffold *pellets, a proof-of-concept study employing a widely used industry standard animal model was performed. In this study, the performance of the rhBMP-2 scaffold pellets was assessed against Infuse™. A rhBMP2-free version of the scaffold pellets was used as a negative control. Two doses of rhBMP2 were included as test groups. Test Article 1 contained 10% (0.04mg/cc) and Test Article 2(0.28mg/cc) contained 70% of the dose in the Infuse™ Positive Control group (0.40mg/cc) respectively.

Table 1. The table below highlights the composition of the various implants with the corresponding assigned group.

Group	**Implants**
Negative Control (rhBMP-2 free)	Calcium Sulfate/P_{DL}LGA scaffold pellets
Test Article – 1 (0.04mg rhBMP-2/cc)	Calcium Sulfate/P_{DL}LGA/rhBMP-2 scaffold pellets
Test Article – 2 (0.28mg rhBMP-2/cc)	Calcium Sulfate/P_{DL}LGA/rhBMP-2 scaffold pellets
Positive Control (0.40mg rhBMP-2/cc)	Infuse™

*The pellets in this study contained calcium sulfate as they were an earlier version of what is currently described as rhBMP-2 pellets

A single-level bilateral lumbar (L4-L5) posterolateral vertebral fusion in rabbit was used as animal model.

The study design was based upon the guidance provided in ASTM F3207-17: Standard Guide for *in-vivo* Evaluation of Rabbit Lumbar Intertransverse Process Spinal Fusion Model, ASTM International, West Conshohocken, PA, 2017, www.astm.org.

For the study, aseptic surgery was performed bilaterally to decorticate the transverse processes of the fourth and fifth lumbar vertebrae (L4-L5). Approximately 3cc of test or control material were deployed onto the paraspinal beds between the decorticated transverse processes. The wounds were closed with standard surgical techniques and animals were recovered from surgery with appropriate analgesic and post-operative care. Radiography was performed immediately prior to surgery to identify the L4-L5 space, after surgery to verify implant placement, 12 weeks after surgery to monitor progress, and 32 weeks after surgery following sacrifice and lumbar spine harvest.

5. Results:

5.1 Programmed Drug Release

The activity of C2C12 cells in the presnce of rhBMP-2 scaffold pellets was assessed for 28 days. pNPP substrate was detected for up to 28 days, suggesting C2C12 cells respond to the rhBMP2 released from the scaffold pellets for a continous 28 days.

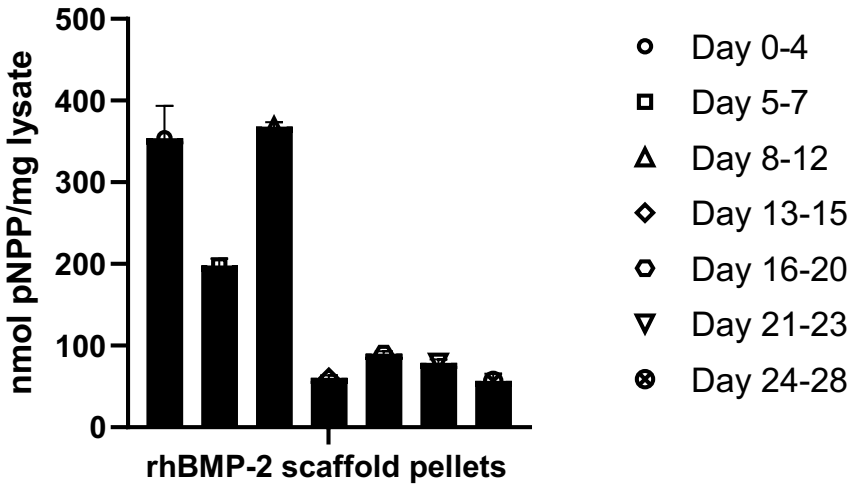

Figure 2. **Plot representing C2C12 ALP activity.** C2C12 cells were cultured in the presence of rhBMP-2 scaffold pellets. pNPP was detected for 28 days.

5.1 *In-vivo* Testing – rat ectopic study

At 28 days, both the rhBMP-2 scaffold pellets and Infuse™ showed clear bone mineralisation quantifiable by µCT.

Table 2. Table showing the parameters assessed from quantitative µCT to determine bone formation and mineralisation.

	Mean Total Volume	***Mean Bone Volume***	***Mean Bone Mineral Density***
rhBMP-2 scaffold pellets	269 mm^3	48 mm^3	0.3 $g.cm^{-3}$
Infuse™	42 mm^3	13 mm^3	0.3 $g.cm^{-3}$

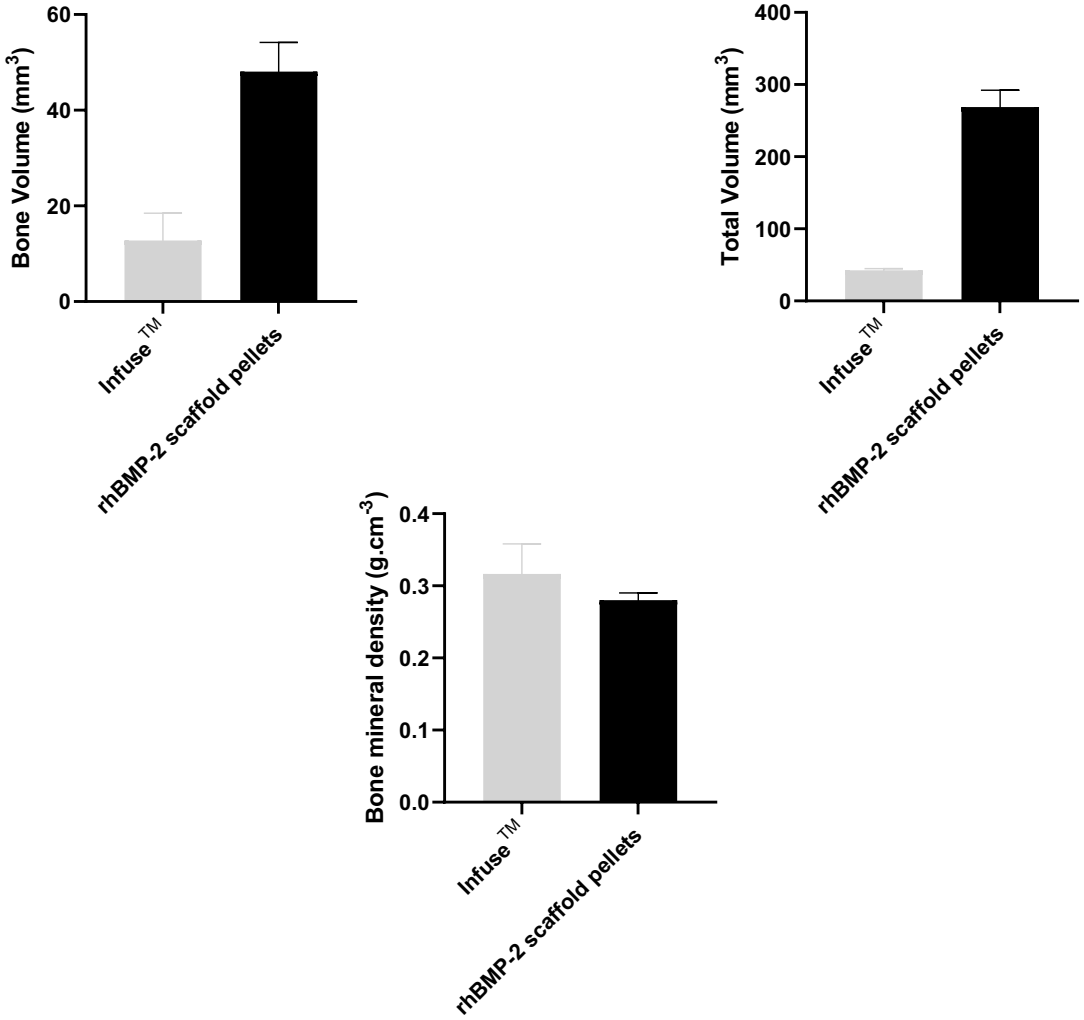

Figure 3. **Evaluation of bone volume and mineral density in the mineralised structures.** Bone volume, total volume, and bone mineral density were measured within each region of interest, defined around the mineralised structure. Results are presented as average mean.

5.2 Testing - rabbit PLF model

At 12 weeks, the animals were assessed for spinal fusion using µCT and the following scoring parameters.

Table 3. Table representing spinal fusion score parameters.

Spinal fusion scoring parameters by µCT	
Score	**Observations**
0	No bone mass on either side
1	Bone formation only on one transverse process
2	Bone formation on both adjacent transverse processes with bilateral radiolucent features
3	Bone formation on both adjacent transverse processes with radiolucent features on one side,
4	Continuous radiopaque bridging between adjacent transverse processes

Table 4. Table showing the radiographic fusion assessment for each group.

Group	Occasional trabecular elements	Partial fusion assessment	Complete fusion assessment	Mean Fusion Score
Negative Control	8/8	0/8	0/8	5.6
Test Article Low (0.04mg/cc)	0/8	1/8	7/8	14.4
Test Article High (0.28mg/cc)	0/8	0/8	8/8	15.6
Infuse™ Positive Control (0.40mg/cc)	0/8	0/8	8/8	15.0

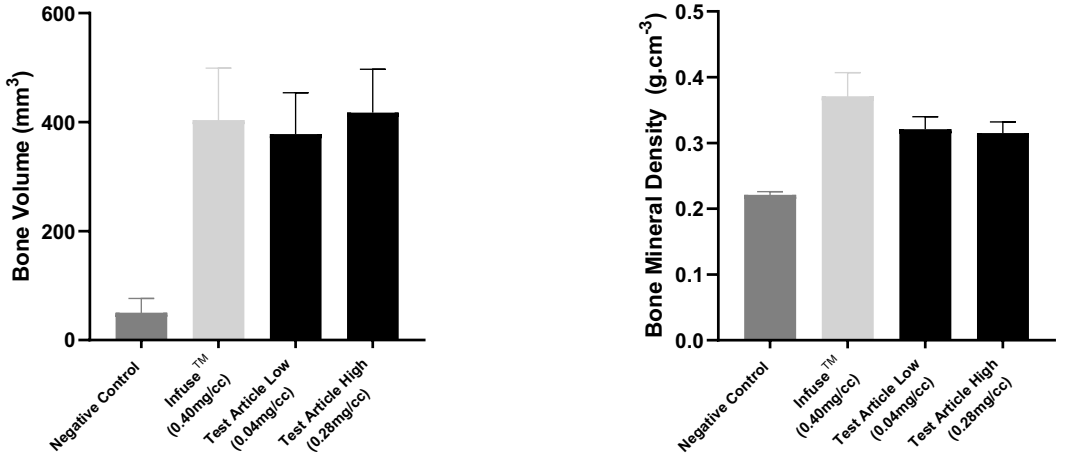

Figure 4. **Evaluation of bone volume and mineral density in the whole implant volume of interest**. Bone volume calculated in each whole 3D implant confirms the observations of spinal fusion scoring (Table 4.) made on coronal and sagittal 2D images.

6. Discussion:

6.1 Programmed Drug Release

C2C12 cells were cultured in the presence of rhBMP-2 scaffold pellets. ALP assay was able to detect the activity of the cells for 28 days. This result suggests that rhBMP-2 activity released from scaffold pellets was maintained and sustained for 28 days. This rhBMP-2 extended-release profile is more physiologically relevant.

6.2 Osteoinductive Potential

Both the Test Article (rhBMP-2 $P_{DL}LGA$ composite) and Control (Infuse™) showed clear ectopic bone formation in the muscle pouch. It is difficult to draw material comparisons based on bone volume in this model. This is because with the Programmed Drug Release the Test Article continues to release new rhBMP-2 for longer than 28 days, which is the sacrifice date in this study. Furthermore, the rhBMP-2 most recently release will not have yet produced a mineralisation response, whereas the rhBMP-2 on ACS of the Control will have been bioavailable for a full 28 days.

It is noteworthy to compare the differences in total volume produced. Both materials included the same implant size ($0.175cm^3$) but produced significantly different total volumes of graft. The Control group reduced in size from $175mm^3$ to $42.4mm^3$. The Test Article increase volume from $175mm^3$ to $269mm^3$. We can postulate that this is a result of the contraction of the collagen sponge in the Control group and its lack of compressive strength relative to the $P_{DL}LGA$ containing composite material.

6.3 Functional Study

In this discriminatory preclinical model, the Test Articles at both 10% and 70% rhBMP-2 relative dose, showed effective bone formation. Bone volume and bone mineral density for all three Test Article Low Dose, Test Article High Dose and Infuse™ groups were equivalent, and all three were statistically superior to the negative control group.

7. Conclusion and Outlook:

Herein, we have shown the potential to achieve a programmed drug release of the powerful therapeutic protein, rhBMP-2. This has been achieved through the encapsulation of the protein within a biodegradable $P_{DL}LGA$ using a proprietary manufacturing process. This white paper has shown that the rhBMP-2 has been protected through the manufacturing and terminal sterilisation process. The drug burst has been damped and release has been extended to 28 days. In the functional study, we have further shown that the controlled release allows for a reduction in total rhBMP2 dose, with equivalent bone volume being created at both 10% and 70% of the Infuse™ dose.

The functional study utilised a basic osteoconductive scaffold comprised of Calcium Sulfate encapsulated within $P_{DL}LGA$. The focus of this work has been on testing a proprietary manufacturing process to achieve the Programmed Drug Release of the rhBMP-2. We can

speculate that further enhancements to the overall product should be possible by substituting the Calcium Sulfate:$P_{DL}LGA$ composite with an improved osteoconductive scaffold which utilise Calcium Phosphate materials. The next step will be to evaluate the safety and effectiveness of the product, or the enhanced Calcium Phosphate version, in a large animal model.

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