Injectable High Viscosity Liquid Carriers for Bone Tissue Engineering

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Declaration of Contributions

The work presented in this thesis was conducted during my full time candidature to fulfil the requirements of Doctor of Philosophy through the Sydney Medical School, The University of Sydney. Unless otherwise stated, all experiments were conducted by myself at the Orthopaedic Research and Biotechnology Unit at the Children’s Hospital at Westmead. Animal ethics was approved by the CMRI/CHW Animal Ethics Committee. All rodent surgeries were conducted by Kathy Mikulec-Langton and Lauren Peacock, with assistance from myself. Confocal imaging methods were developed with Dr Laurence Cantrill, unless otherwise specified. Experimental conception, and manuscript and thesis editing was conducted in conjunction with my supervisors Prof David Little and Dr Aaron Schindeler.

In Chapter 2, SAIB synthesis was conducted at the School of Chemical and Biomolecular Engineering at the University of Sydney, Australia. Protocols were developed with Dr Peter Valtchev in the laboratory of Prof Fariba Dehghani. Cell culture methods were developed with Dr Ciara Murphy.

In Chapter 3, the microCT analysis methods were developed with Alyson Morse.

In Chapter 4, confocal imaging and cell culture methods was conducted with the assistance of Dr Ciara Murphy. Fracture grading was assessed using either Dr Aaron Schindeler or Prof David Little under blinded conditions, while I analysed the results.

In Chapter 5, the large animal surgery was approved by the Westmead Animal Ethics committee and performed by Prof David Little and Dr Clare Carpenter. DEXA imaging was conducted with assistance from Kathy Mikulec-Langton.
In Chapter 6, synthesis of new materials was conducted at the School of Chemical and Biomolecular Engineering at the University of Sydney, Australia. Protocols were developed with Dr Peter Valtchev in the laboratory of Prof Fariba Dehghani.

**Statement of Originality**

This is to certify that to the best of my knowledge, the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

Tegan L Cheng
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I would also like to thank coffee.
**Abstract**

The clinical management of critical sized defects and traumatic bone injuries continues to challenge the orthopaedic field. The gold standard of treatment involves the use of autologous bone grafting. While effective in many cases, an insufficient response can lead to a need for additional surgeries. Moreover, donor site morbidity is a common complication. The field of bone tissue engineering aims to develop viable alternatives to autologous bone graft. The bone graft substitute INFUSE® (bone morphogenetic protein-2 [BMP-2] in acellular collagen sponge) is currently in clinical use, however we and others have hypothesized that the collagen delivery system is suboptimal. This thesis investigates the use of sucrose acetate isobutyrate (SAIB) as an alternative high-viscosity liquid carrier system for BMP-2.

In Chapter 2, I describe how a mouse ectopic bone formation model was used to test the SAIB/BMP-2 delivery system. Compared to collagen sponge, SAIB was able to generate three-fold more bone for the same dose of BMP-2, as measured by micro-computed tomography (microCT). Next, we tested co-delivery with a range of other pharmaceutical agents that were hypothesized to augment net BMP-2 induced bone. Many of these led to significant increases in bone, with co-delivery of hydroxyapatite nanoparticle (HA) coated with the bisphosphonate Zoledronate (ZA) being the most effective. SAIB/BMP-2/HA/ZA was able to generate a 10-fold increase in bone formation over SAIB/BMP-2 alone.

In Chapter 3, the versatility of the SAIB carrier was further explored as I examined intraosseous injection of BMP-2 in a mouse model of brittle bone diseases (Osteogenesis Imperfecta [OI]). In combination with reaming, SAIB/BMP-2 were able to induce periosteal bone formation despite being delivered into the medullary canal.
In Chapter 4, the upscaling of SAIB/BMP-2 into a larger rodent model, the rat, was investigated. In the rat, the SAIB/BMP-2/HA/ZA combination was found to be similarly effective as previously shown in the mouse model. Next, SAIB/BMP-2 and SAIB/BMP-2/HA/ZA were trialled in a rat open fracture model, a more challenging model of orthopaedic repair. The SAIB/BMP-2/HA/ZA treatment showed similar overall union rates and mechanical strength to collagen/BMP-2, however the capacity of this system for minimally invasive delivery makes it nonetheless an appealing one for further translational development.

In Chapter 5, the model system was once again upscaled to a porcine (pig) model. In this instance, a model of idiopathic osteonecrosis of the hip (Perthes’ disease) was used. It was speculated that local co-delivery of SAIB/BMP-2 +/- ZA would improve stability and preserve sphericity of the femoral head. While this was only trialled in a limited number of pigs, SAIB/BMP-2/ZA prevented femoral head collapse in 50% of cases.

In Chapter 6, alternative novel sugar-based high viscosity carriers were developed. These included fructose acetate isobutyrate (pFAIB) and polycondensed deoxyribose (pdRAIB), both of which exhibited high viscosity and hydrophobicity. These materials were tested as BMP-2 carriers in a mouse muscle pouch model. As a carrier, pdRAIB was able to generate comparable bone to pdRAIB, while its degradation products have the potential to improve bone repair by also promoting angiogenesis.

In summary, this work describes a novel approach to minimally invasive/injectable delivery of BMP-2 that does not involve conventional hydrogels. With their superior physicochemical properties (viscosity, phase-transitioning) and likely benefits of their monosaccharide breakdown products, these biomaterials have significant translational potential.
Original publications

Over the course of this thesis, several studies have been written into articles, peer reviewed and accepted into scientific journals.

Publications related to this thesis

Original published articles related to the submission of this thesis:


Publications not related to this thesis

Original published articles that are not related to the submission of this thesis:


El-Hoss J, Cheng T, Carpenter EC, Sullivan K, Deo N, Mikulec K, Little DG and Schindeler A, 2014. A Combination of rhBMP-2 (Recombinant Human Bone Morphogenetic Protein-2) and


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<tr>
<td>(rh)BMP</td>
<td>(Recombinant human) Bone morphogenetic protein</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>AP</td>
<td>Anteroposterior</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BADGE</td>
<td>Bisphenol-A-diglycyl ether</td>
</tr>
<tr>
<td>BG45S5</td>
<td>Bioglass 45S5</td>
</tr>
<tr>
<td>BMPR</td>
<td>BMP receptor</td>
</tr>
<tr>
<td>BV</td>
<td>Bone volume</td>
</tr>
<tr>
<td>DEXA</td>
<td>Dual-energy X-ray absorptiometry</td>
</tr>
<tr>
<td>DFO</td>
<td>Desferoxamine</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dR1P</td>
<td>Deoxyribose-1-phosphate</td>
</tr>
<tr>
<td>dRAIB</td>
<td>Deoxyribose acetate isobutyrate</td>
</tr>
<tr>
<td>EQ</td>
<td>Epiphyseal quotient</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated protein kinase</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and drug administration</td>
</tr>
<tr>
<td>FFP</td>
<td>Farnesyl diphosphate</td>
</tr>
<tr>
<td>FG</td>
<td>Food grade</td>
</tr>
<tr>
<td>Gal-3</td>
<td>Galectin-3</td>
</tr>
<tr>
<td>GSK-3</td>
<td>Glycogen synthase kinase-3</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>HA</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>HDE</td>
<td>Humanitarian device exemption</td>
</tr>
<tr>
<td>HVLCM</td>
<td>High viscosity liquid carrier medium</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IKK</td>
<td>I-κB kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>I-κB</td>
<td>Inhibitor of NF-κB</td>
</tr>
<tr>
<td>JNK</td>
<td>c-jun N-terminal kinase</td>
</tr>
<tr>
<td>LS</td>
<td>Laboratory synthesised</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK kinase</td>
</tr>
<tr>
<td>microCT</td>
<td>Micro-computed tomography</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem/stromal cell</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor κB</td>
</tr>
<tr>
<td>OI</td>
<td>Osteogenesis imperfecta</td>
</tr>
<tr>
<td>ONFH</td>
<td>Osteonecrosis of the femoral head</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>pdRAIB</td>
<td>Polycondensed deoxyribose acetate isobutyrate</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>pFAIB</td>
<td>Polycondensed fructose acetate isobutyrate</td>
</tr>
<tr>
<td>PGA</td>
<td>Poly(glycolic acid)</td>
</tr>
<tr>
<td>PLA</td>
<td>Poly(lactic acid)</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactic-glycolic acid)</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>RUST</td>
<td>Radiographic union score of the tibia</td>
</tr>
<tr>
<td>Saf O</td>
<td>Safranin O</td>
</tr>
<tr>
<td>SAIB</td>
<td>Sucrose acetate isobutyrate</td>
</tr>
<tr>
<td>SPF</td>
<td>Specific pathogen free</td>
</tr>
<tr>
<td>TCP</td>
<td>Tricalcium phosphate</td>
</tr>
<tr>
<td>TGA</td>
<td>Therapeutic goods administration</td>
</tr>
<tr>
<td>TMD</td>
<td>Total mineral density</td>
</tr>
<tr>
<td>TP</td>
<td>Thymidine phosphorylase</td>
</tr>
<tr>
<td>TRAP</td>
<td>Tartrate resistant acid phosphatase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>TV</td>
<td>Total volume</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VOI</td>
<td>Volume of interest</td>
</tr>
<tr>
<td>vWF</td>
<td>vonWillebrands factor</td>
</tr>
<tr>
<td>ZA</td>
<td>Zoledronic acid/Zoledronate</td>
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</table>
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Bone biology

Bone anatomy and physiology

There are 206 bones in the adult skeleton, and these come in a variety of shapes and have a range of functions. Broadly speaking, bone is a rigid connective tissue and the major structural element of the human body, fulfilling roles including load transfer, soft organ protection, and mineral storage. Bone has a highly organised structure, and it is vital to understand the make-up and interaction between the hierarchal levels to understand tissue responses as a whole [1, 2]. These levels include organ level structure, macrostructure, microstructure, and sub-microstructure.

At the organ level, the diverse range of shapes that bones have reflect their functional role. For example, long bones such as the femur and humerus are important structural elements of the skeleton and, via their articulations and muscle action, act as levers for movement and bear the weight of the body. These bones are highly resistant against axial loading and bending forces. In contrast, bones that resist high compressive forces tend to be short, such as those of the vertebra.

At a macroscopic level, bone structure can be broadly divided into two forms; cortical (also known as compact bone) and trabecular (also known as cancellous or spongey bone), as shown in Figure 1.1. The shells of all bones, and in particular those of long bones, are made up of cortical bone. In humans, the thickness of this cortical bone ranges from less than a millimetre in vertebral shell [3] to millimetres and even centimetres in the case of long bones diaphyses [4, 5]. Cortical bone aims to resist the forces of bending and bucking, providing stability to our long bones. Areas in bones that are under high compressive stresses, such as vertebra or the femoral head and neck, are also filled with trabecular bone. Trabecular struts have a more consistent range in thickness of between one and two hundred micrometres [6], and this helps distribute compressive loads within the structure. The exterior surface of cortical bone is covered by a thin layer of fibrous and osteogenic
cells known as the periosteum, that acts as a transition area between the bone surface and overlying musculature and soft tissue [7]. The medullary canal of cortical bones is covered by a membrane that is known as the endosteum. Together, these two membranes bound cortical bone, defining the outer and inner sides of the bone as the periosteal and endosteal surfaces.

On a microstructural level, cortical and trabecular bone have varying amounts of total porosity as well as a range of pore sizes. Healthy cortical bone is a dense tissue, with a porosity less than 10% [8]. Larger pores are primarily occupied by blood vessels, but there are also smaller pores containing osteocytes, a type of bone cell (Figure 1.1). The blood vessels are surrounded by concentric rings of bone tissue, and this structure is known as an osteon (also known as Haversian system). Osteons are packed tightly together to form cortical bone, and within and between each layer the osteocytes within communicate through small canals known as canaliculi. Conversely, trabecular bone forms a lattice like structure that has a porosity of around 80% [9]. Trabecular struts are oriented in a direction which maximizes load distribution for that bone as a whole. The pores in trabecular bone are largely formed by the spaces between trabecular struts, but much like cortical bone, it also contains small pores that contain osteocytes. Unlike cortical bone however, trabecular bone does not contain blood vessels, and relies instead on nutrient diffusion from the surrounding marrow. Bone is a highly dynamic organ, and it is these osteocytes that are the mechanism behind bone’s response to loading.

The sub-microstructure of cortical and trabecular bone have similarities in composition, but vary in organization. At this level, bone is comprised of a composite material of organic collagen fibres that are contained within an inorganic calcium phosphate matrix, mostly in the form of hydroxyapatite crystals. This matrix is organized in regular planes known as lamellae. In cortical bone, it is these lamellae that are arranged around a blood vessel to form osteons, as seen in Figure
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1.1. Osteons are approximately 200-250 µm in diameter, and are aligned roughly parallel to the long axis of the bone. There are also instances when regular lamellar or structures do not form, occurring most commonly in healing or injured bone as osteoblasts are producing osteoid rapidly. If lamellar structures do not form, and the collagen fibres are not in a regular distribution, this is known as woven bone.

![Hierarchical structure of bone in humans](image)

**Figure 1.1:** The hierarchical structure of bone in humans, from [1]

*Bone matrix*

Bone is a composite material that consists of an organic and inorganic component, as well as water. The organic component is made up of type 1 collagen as well as other non-cartilaginous proteins and this makes up about 25% of bone and is also called osteoid. The inorganic mineral component
is known as hydroxyapatite and is made up of a crystalline complex of calcium and phosphate, making up about 70% of bone. Water makes up the remaining 5% of calcified bone.

**Bone cells: osteoblasts, osteoclasts, and osteocytes**

Bone is a highly dynamic tissue and at a healthy resting state it is in a constant cycle of bone formation and resorption. This resorption (catabolism) and bone formation (anabolism) occurs in a process termed remodelling, also known as bone turnover. Three main cell types have been identified as having key roles: osteoclasts, osteoblasts, and osteocytes. They work in coordination to ensure the normal function and maintenance of the skeleton. Conversely, dysfunction of one or more of these elements, or their coordination, can lead to bone disease.

Osteoclasts are cells that resorb or breakdown bone and have their origins in the haematopoietic lineage. Once matured on the bone surface, these large, multi-nucleated cells secrete acid to dissolve the mineral component of bone in what is known as a resorption pit, or Howship’s lacuna. These cells can be detected histologically as red cells lining the bone surface, using a tartrate-resistant acid phosphatease (TRAP) stain (Figure 1.2).
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![Image of osteoclasts and osteoblasts](image)

**Figure 1.2:** Osteoclasts are identified as TRAP positive (red) cells lying on the bone surface (arrowheads). The paraffin processed sample shows multinucleated osteoclasts surrounding a trabecular bone spicule, and is counterstained in 0.5% Methyl Green in 0.1 M sodium acetate. Scale bar represents 50 µm.

Osteoblasts are cells that produce bone, and they originate from a mesenchymal lineage. Mature osteoblasts produce and mineralize osteoid (organic extracellular matrix). Osteoid consists predominately of type I collagen, but also contains proteins including osteonectin and osteocalcin [10]. Calcium phosphate is deposited on osteoid in the form of hydroxyapatite crystals. Histologically, osteoblasts can be detected by a stain that targets alkaline phosphatase (ALP). ALP is an enzyme that is expressed by mature osteoblasts, and its expression is increased during osteoblast mineralisation and bone formation [11].
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Figure 1.3: Osteoblasts are identified by a stain for alkaline phosphatase (blue-purple as indicated by arrowheads), with nuclei highlighted in pink-red by a Nuclear Fast Red counterstain. These cells are seen lying on an unstained and undecalcified bone surface. Scale bar represents 50 µm.

Osteocytes are formed from osteoblasts that have become incorporated into the bone matrix they have produced around them (Figure 1.4). These terminally differentiated cells are the most ubiquitous cell type in the bone, and they communicate with other cells by canaliculi. Osteocytes facilitate the sensing of mechanical load, and in turn transduce this into a biological signal. Osteocytes release signalling molecules that modulate osteoclast formation or osteoblastic bone formation to regulate skeletal mass and/or structure.
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Figure 1.4: Osteocytes are cells that are embedded in bone, and can be seen here in a cross section of femoral cortical bone by their dark nuclei staining in a Haematoxylin and Eosin stain (arrowheads). Scale bar represents 50 µm.

In a healthy skeleton, these three cell types act in concert to ensure that bone stock is maintained during normal remodelling, as well as to respond to changing loading conditions and skeletal injuries. However, when these processes become uncoupled, pathologies, such as osteoporosis, can arise [12].

**Bone formation**

There are three main methods of bone formation: intramembranous ossification, endochondral ossification and periosteal apposition [13, 14]. The first instance of bone formation in the human body occurs during foetal development, and during this phase both intramembranous and endochondral ossification are active. During growth, endochondral ossification occurs at specialized organs known as growth plates, while intramembranous ossification forms lamellar bone in osteons. Growth from the outer bone surface is known as periosteal appositional bone
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formation, and is the main mechanism of increasing bone width [14]. Once the bones of the body have formed, endochondral ossification is primary method for bone healing in long bones.

Intramembranous ossification occurs during the embryonic formation of certain bones, including the flat bones of the skull. This method of ossification is characteristic in its lack of cartilage as an intermediate to bone formation [15]. Intramembranous bone formation can occur during fracture healing, and is thought to be seen only in highly stabilized fractures [16].

Endochondral ossification involves the ossification of a cartilage template. It is seen in the foetal formation of long bones, development of long bones, and in bone healing [17]. In this mode of bone formation, an initial cartilage model of the bone to be formed is created [18]. A primary ossification centre is formed, for example, in the centre of the shaft of the femur in the foetus. Here, osteoblasts begin to lay down osteoid, which is then calcified to form woven bone. The osteoblasts, together with osteoclasts, begin creating the initial structures of the bone, such as the cortex, trabeculae and medullary canal. The woven bone is also replaced by the much stronger lamellar bone. After birth, secondary ossification sites appear at the ends of the long bone, also forming a cartilage layer known as a growth plate between the primary and secondary ossification sites. Growth plates allow for longitudinal growth during a person’s development and close off that are transformed into bone during their late teens.

Periosteal apposition occurs through the action of osteoblasts in adding mineralised tissue to the outer surface of the bone [19]. In children, periosteal apposition occurs as their bones grow in width, while in adults, this process occurs as part of the normal remodelling [14]. It is through periosteal apposition that much of the diaphysis of long bones are formed [20].
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**Bone remodelling**

Bone remodelling is an essential component of maintaining a healthy skeleton, with the coupled processes of resorption and ossification occurring simultaneously. The remodelling process assists with growth and development of the skeleton, adjusting bone to its mechanical load, the repair of microdamage, as well as regulating systemic calcium levels. In the healthy skeleton, osteocytes, osteoblasts and osteoclasts must all work together to maintain and adapt bone stock [21].

In the first phase of remodelling, pre-osteoclasts migrate to the bone surface, where they form multinucleated osteoclasts. Here, after sealing of an area with an actin ring, they secrete proteolytic enzymes and hydrogen ions that demineralize and degrade the proteins lying beneath its ruffled border in the dominant direction of loading [22]. Following this, mononuclear cells appear at the bone surface to prepare it for the arrival of osteoblasts. These cells secrete factors that induce osteoblast differentiation and migration. Finally bone formation occurs when the osteoblasts are recruited to the surface and lay down new bone. These osteoblasts then either become embedded in the bone they have formed, and turn into osteocytes, apoptose, or form lining cells that cover the surface of the bone [23]. Lining cells cover the non-remodelling surfaces of bone and were originally thought to be dormant, however they can be stimulated to become active osteoblasts by intermittent parathyroid hormone [23] and mechanical loading [24]. Unlike the periosteum, which is the layered membrane that covers the external surface of most bones in the body, lining cells cover the inactive bone surfaces that are mostly on the endosteal surface [25].

**Mechanical properties of bone**

One of the vital functions of bone is to provide mechanical support for the body. To accomplish this, bone is a strong and resilient material that is constantly remodelled in response to loading
conditions. The strength of bone comes from two fundamental properties: its material composition and its structure [12]. The material properties of bone are defined by the tissue make-up, while the mechanical properties of the whole bone are dependent on the structure of the bone itself.

**Bone as a composite material**

Bone is a composite material that is both bendable and strong. The collagen component of bone provides the flexibility, while the mineral component provides stiffness. Stiffness is important for resisting deformation during loading, while flexibility is key for absorbing energy without failing. If bone is too brittle (stiff and not flexible), less energy can be absorbed before micro-cracks and fracture occur. If bone is too flexible it will deform excessively and fracture can occur. Thus bone must maintain an optimal level of mineral density to ensure normal function, with pathologies such as osteoporosis arising when this balance is disturbed.

Structurally, bones are tailored to their function. For instance, long bones such as the femur act as lever arms that transfer muscle loads across the body. Long bones have thick cortical shells that are very stiff to resist bending loads. During development, resorption in the marrow cavity along with bone formation on the periosteal surface of the bone increases the long bone’s resistance to bending [26]. This shifts bone away from the neutral axis, increasing the moment of inertia while decreasing the mass of the bone. This is well demonstrated in the tibia, which forms with greater periosteal bone on the anterior and posterior surfaces of the bone (Figure 1.5) [27]. The distribution of periosteal apposition in key areas, rather than the addition of more bone tissue uniformly, demonstrates the optimal pay off between bone mass and strength [28]. Four-fold more bone would be required to generate the same resistance to bending with uniform periosteal apposition [27].
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**Figure 1.5:** A cross-section through the tibia demonstrating the elliptical bone mass distribution around the centre of the bone (marked by the black dot) during growth. There is increased periosteal apposition in the antero-posterior plane over the in the medio-lateral plane. Image from Wang, Cheng [27]

*Wolff’s Law*

Bone is a highly dynamic structure and is able to remodel its structure and shape in response to the loading it receives. This principle was first described by Julius Wolff in the 19th Century [29] and is known as Wolff’s Law. This action is mediated by osteocytes, which act as mechanosensors and mechanotransducers transforming mechanical signals into chemical secretions [30]. Wolff’s Law is highly apparent in the trabecular region of the femoral head where trabeculae align in the direction of loading (Figure 1.6). Bone will adapt its structure to better resist the forces being applied to it, with the inverse also being true. An absence of loading forces on the skeleton, such
as those experienced during spaceflight or bedrest, lead to bone loss [31-34]. The loading conditions of bone highly influence the bone architecture.

![Image of femoral head cross section](image)

**Figure 1.6:** A cross section through the femoral head demonstrating the alignment of trabecular in the direction of loading as indicated by the arrow. Image from [30].

**Material properties of bone**

Difficulty arises when trying to define the exact material properties of bone, with reported values for properties such as Young’s Modulus varying. Bone material properties can differ for a variety of reasons. Firstly, bone is a biologically created material and there is an inherent variability between species, individuals, and bone sites. Secondly, bone is an anisotropic material, meaning that the properties are dependent on direction of loading. Thirdly, bone’s properties are viscoelastic and therefore dependant on the strain rate of testing. Thus, in humans, bone properties depend on the age of the person (as bone mineral, and therefore strength, decrease with age), site the bone was from, as well as the conditions under which it was tested. Despite these factors, reported values...
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of the Young’s Modulus of human cortical bone range from 5.4 – 25.0 GPa [35-38] and trabecular bone ranging 4.6 – 13.5 GPa [35, 37, 39].

While the absolute material properties of bone are difficult to define, the relative properties of whole bones can be examined experimentally. Whole bone properties can be a useful functional outcome in basic and translational research for example, to observe the effect of a treatment on a bone healing model in a rat. Under experimental conditions, parameters such as bone site and age of the animal can be controlled. Mechanical tests, such as compression and four-point bending, can be undertaken to see if an experimental treatment has resulted in a relative functional increase or decrease in bone strength.
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Bone repair

Bone has great regenerative potential, and can often repair itself fully without the formation of scar tissue. This property is due to the highly organised repair process of bone healing that can be seen during fracture repair. However, if this repair process is disrupted in some way, defects in bone healing can occur. These defects require a surgical intervention to encourage healing and prevent further disability to a patient.

Growth factor release during fracture healing

In a typical orthopaedic injury, fracture is followed by the secretion of a range of factors by both injured and recruited inflammatory cells, including cytokines and growth factors [40, 41]. These secretions are vital for cell recruitment, infection resistance, and initiating the events that lead to bone repair. The bone morphogenetic proteins (BMPs) are a key growth factor family involved in the bone repair process, and are crucial molecular regulators of bone healing.

The growth factors known as the BMPs in the transforming growth factor-β superfamily have essential roles in embryonic development in pattern forming and tissue specification as well as postnatal tissue repair [41-46]. Mice with global mutations in BMP-2 and BMP-4 are non-viable, and die in utero from complications in cardiac development [47] and mesodermal formation [48]. Deficiency in BMP-7 results in post-natal fatality, with defects in the skeleton, kidney and eyes [49]. Mice with limb specific BMP-2 conditional knock out spontaneously fracture their bones and are unable to initiate fracture healing [50]. However, conditional knock out of BMP-4 or BMP-7 do not affect bone formation or fracture repair [51, 52].
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The BMPs mechanism of action involves serine-threonine kinase receptors known as BMP receptors (BMPR), and act by binding to BMPR type I that then complexes with BMPR type II [53]. The activated receptor complex then phosphorylate the intracellular transcription factors SMAD 1, 5 and 8 [54]. These phosphorated SMADs then form a complex with the SMAD 4 protein and translocate to the nucleus, leading to an upregulation of number of osteoblast transcription factors [55]. BMP-SMAD signalling is further regulated by inhibitory SMADs (SMAD 6 and 7), SMAD binding proteins, and protein degradation [56-58]. Aside from the canonical BMP-SMAD signalling pathway, BMPs can also signal via the mitogen-activated protein kinase (MAPK) pathway [54]. Moreover, there is evidence of crosstalk between the BMP pathway and the TGF-β/activin, Wnt, Ca2+/Calmodulin, Erk-MAPK, and JAK-STAT pathways [54]. The complexity and crosstalk within the signalling pathways is not surprising considering BMPs are involved in a wide range of biological functions from embryonic development to fracture repair.

The cellular targets of the BMPs include pluripotent mesenchymal cells, bone marrow cells, pre-osteoblasts, myoblasts, and fibroblasts [59]. Osteoprogenitor cells express the BMP receptors on their surface [60]. Osteogenic BMP signalling activates the transcription factor RUNX2 and Osterix, master gene regulators of osteoblasts, resulting in cellular proliferation and osteoblastic differentiation [58, 61-63]. BMP signalling also activates osteoclasts through the RANK/RANK-L pathway [64].

During bone injury, a cascade of BMPs and other cytokines are released and these act to co-ordinate the repair process [65, 66]. There are more than 22 BMPs found in humans [67], with BMP-2, -4, -6, -7, and -9 purported to have the most pro-osteogenic effects [68-70]. BMPs are active in the relatively early phases of bone healing, with BMP-4 predominately active during days 0 to 5 post-injury, peaking closer to day 5. BMP-2 is active throughout the regeneration process, continuing
until woven bone is remodelled into lamellar bone. BMP-7 is most active after day 14 of the healing process.

**Histological characterisation of fracture healing**

Fracture healing is a process that can be generally categorised into four main stages that can be defined histologically [71]. However, while these phases are linear, they not necessarily discrete and various stages of the healing process can often be observed in the same sample. Typically, long bone fractures largely heal via endochondral ossification, however increasing the stability of fracture fixation can increase the proportion of intramembranous bone formation. The initial stage of fracture healing is inflammatory, with general wound healing pathways activated. This is followed by the formation of a fibrocartilaginous soft callus, which acts to bridge and stabilise the fracture. The soft callus is then replaced by a bony hard callus, which provides added stability. Finally, the hard callus is remodelled to resemble the original bone functionally and structurally.

**Phase 1: Inflammation**

The creation of a fracture is associated with damage to the local vasculature, soft tissues, periosteum, and disruption of the normal bone architecture. A haematoma forms and inflammatory cells such as macrophages, leukocytes and lymphocytes are recruited to the site (Figure 1.7) [72]. This process allows for blood clotting, removal of cellular debris, as well as recruiting cells required for the next stage of fracture healing, such as cartilage progenitor cells.
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Figure 1.7: The first stage of fracture healing is the inflammatory response, and can be seen in this Haematoxylin and Eosin stain as inflammatory cells infiltrating into the fracture site. The cortical bone is at the bottom of the image, with the infiltrating inflammatory cells above (arrows). Scale bar represents 100 µm.

Phase 2: Soft callus formation

The soft callus is primarily made up of cartilage and fibrous cells, and acts to bridge the broken bone ends. At the peak of soft callus formation, hypertrophic chondrocytes are the primary cell type (Figure 1.8) [71]. The soft callus acts as a template for the hard callus that will follow, where it will remodel into more structurally stable lamellar bone [46]. Vasculature begins to invade the mineralised matrix, bringing along with it osteoblast progenitors, as the callus undergoes endochondral ossification.
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**Figure 1.8:** The second stage of fracture healing is defined by the formation of the soft callus around the fracture site. This can be seen with blue staining of cartilage in the callus around the fracture site in an Alcian Blue/Picrosirius Red stain. The fractured cortical bone end can be seen at the bottom of the image, with proliferating (arrow) and hypertrophic (arrowheads) chondrocytes highlighted in blue. Scale bar represents 250 µm.

*Phase 3: Hard callus formation*

The hard callus is formed as the soft callus mineralises and is replaced with woven bone. This typically happens from the peripheral edge of the callus, moving towards the centre of the fracture. During this phase, woven bone completely replaces the cartilage matrix that was laid down, and forming a bony bridge across the fracture (Figure 1.9). This fracture would now be considered united. Once bony union has been achieved, the process of remodelling begins.
Figure 1.9: In the third stage of fracture healing, the formation of a hard callus replaces the soft callus. This is seen as the red staining of bone tissue, and the absence of blue staining cartilage, at the fracture site in this Alcian Blue/Picrosirius Red stain. The original cortical bone is at the bottom of the image, and the trabecular-like woven bone of the hard callus can be seen above it (arrowhead). Scale bar represents 250 µm.

Phase 4: Remodelling

For the final stage of fracture healing, the woven bone that was laid down during hard callus formation is replaced with the more regular lamellar bone, restoring the original structure of the bone (Figure 1.10). The osteoclast is the key cell type during this phase, and acts to resorb the irregular woven bone of the callus. Coupled with this process, osteoblasts then lay down regular lamellar bone to restore the bone function. When this process occurs undisrupted, bone healing can occur so well that no evidence of fracture can be seen.
Figure 1.10: In the final stage of fracture healing, the callus is remodelled to closely resemble the original structure of the bone. In this Alcian Blue/Picrosirius Red stain, the resorption of the callus into the cortical bone of the diaphysis can be seen. The lamellar cortical bone of the original cortex and remodelled lamellar callus bone are centred in the image, with the marrow space below, and the muscle above. Scale bar represents 250 µm.

Orthopaedic management of bone defects and fractures

While fracture treatment can be approached either conservatively or surgically, the aim of treatment remains the same: to reduce inter-fragmentary movement and restore anatomical alignment [73]. Conservative treatment involves placing the fracture in either traction, where a tensile force is applied externally to the broken limb to reduce the fracture in the limb axis, or by splinting, where the broken limb is realigned and stabilised using an external cast or splint. A fracture can be reduced via surgical methods using an implant, such as an external fixator, internal fixation, or an intramedullary nail. External fixators involve fastening a device through the skin to the broken bone. While fixators can be cumbersome and increase infection risk, they allow the surgeon to adjust the fixation without further surgery. Internal fixation involves the application of plates to the bone, and is especially used when there are multiple fragments of bone involved. Intramedullary nails are rods that are inserted into the medullary canal of the bone; they can be locked to resist
torsional forces. For simple long bone fractures, intramedullary nailing can be quite effective, with one centre reporting that nailing of closed tibial shaft fractures resulted in a 98% union rate [74].

More challenging bone defects, such as critical sized defects, can require more complex surgical interventions. A critical sized bone defect is one where the distance between the broken bone ends is so large that the bone will not heal spontaneously, and can be the result of congenital defects, tumour resection or trauma. Currently, the gold standard for the treatment of critical bone defects is the use of a bone graft, either harvested from the patient (autograft) or from a donor (allograft) [75]. However, autografts are associated with pain at the donor site, infection, haematoma, and the risks of an additional surgery [76, 77], as well as only supplying a limited amount of graft material. The use of allograft can increase the amount of graft material available, but it has a lower osteogenic capacity, has a higher resorption rate and a larger immune response [75, 78].

Worldwide, bone is the second most transplanted tissue with the most heavily cited estimate of over 2.2 million bone grafting procedures occurring annually in the early 2000s [79, 80].

With the issues and complications associated with current grafting techniques it is vital that a superior gold standard of treatment is found. The development of synthetic grafting materials, such as the ceramics hydroxyapatite and tricalcium phosphate, as well as biologic forms of bone regeneration like recombinant human bone morphogenetic proteins (rhBMPs), have grown in the past few decades. However, none of these solutions are adequately address issues such as sufficient bone formation or elimination of disease transfer risk.
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**Failures in bone repair**

Despite the advances of modern orthopaedics, complications in fracture healing do occur and can require further surgical intervention. In some cases, the broken ends of the bone do not achieve bony union and this is known as a delayed or non-union (Figure 1.11) [81]. While descriptions of non-union vary, the United States Food and Drug Administration (FDA) defines one as a fracture that has remained unhealed for nine months or more [82]. The long bones of the limbs are the most vulnerable, with the most common sites of non-union being the lower tibia, femoral neck, radius and ulna [81]. It has been reported that the 2.5% of closed fractures proceed to non-union, while delayed union occurs in 4.4% of open fractures [82]. In addition, a 2012 study of the Scottish population found that the incidence of non-union was 18.94 in 100 000 per annum [83].

![An example of a non-union fracture in the femur of a rat. X-ray was taken six weeks after the creation of an internally fixed open fracture that receive no bone grafting or pharmacological treatment. The hard callus has failed to bridge the fracture site, and fracture union has not occurred.](image)

**Figure 1.11:**

There have been many factors associated with the appearance of a non-union fracture [84, 85]. These include factors associated with the cause and treatment of the original injury, those related to a patient’s general health, and compliance with treatment instructions. However, the cause is not
always known. The failure to unite and the presence of a delayed union fracture increases with the severity of an open fracture, where the fractured bone ends are exposed through the skin [82, 86]. Current treatment strategies include the introduction of bone graft materials, including autograft or allograft, as well the use of low-intensity ultrasound and bone morphogenetic proteins [84]. However, the autograft remains the gold standard of treatment for non-union fracture [87]. With the treatment of a tibial non-union estimated to cost on average twice that of a united fracture [88], more effective solutions need to be developed to prevent and better treat these fractures.
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**Disorders of normal bone function**

Pathologies of bone can arise from a range of factors, including disruption of normal genetic and metabolic function, as well as those from idiopathic aetiologies. Dysfunction in bone formation, remodelling, or regeneration can lead to pathological alterations in bone structure and impair its physiological roles. One of the most common bone diseases is osteoporosis, where bones become less dense and more prone to fracture [89]. Osteoporosis is the result of increased bone resorption compared to bone formation [90]. Osteoporosis affects 43% of women and 13% of men over 70 in Australia [91], and is of increasing concern with the ageing population. Another condition is known as Paget’s disease, which is a localised disorder of bone remodelling that occurs as a result of increased osteoclast activity and a compensatory increase in disordered new bone formation [92]. The newly formed and highly disorganised bone results in skeletal sites that are less compact, and more susceptible to deformity and fracture [93]. Paget’s disease generally presents in older people of western and southern European descent, with its prevalence falling in recent times [94].

While these conditions are of great concern to bone health and the population in general, this research project has focussed on two bone diseases that primarily affect a paediatric population. The genetic condition Osteogenesis Imperfecta affects the normal composition of bone, and results in bones that are highly fragile. Legg-Calve-Perthes disease describes an idiopathic degeneration of the femoral head, which can lead to a collapse of the femoral head.

**Osteogenesis imperfecta**

Osteogenesis Imperfecta (OI) is a genetically inherited disorder that is associated with low bone mass and increased bone fragility [95]. The high fragility that accompanies OI has led to it being known as the ‘brittle bone’ disease. OI is reported to affect 6 – 7 in every 100,000 live births [96]
and it has a broad range of severity that can range from perinatal fatality to much milder forms that don’t involve fractures [97]. Most commonly, OI is caused by mutations in the *COL1A1* or *COL1A2* genes that code for the chains that make up type 1 collagen [98, 99]. However, additional causative mutations in different genes have been discovered in recent years [100-102].

Diagnosis of OI can be based on a combination of skeletal and non-skeletal signs and symptoms. The non-skeletal manifestations are variably associated and can include blue sclera (whites of the eyes), dentinogenesis imperfecta (abnormal tooth development), hearing loss, skin hyperlaxity, and joint hypermobility [95, 97]. Skeletally, OI is associated with low bone mass, reduced bone material strength, bone fragility and deformity, growth deficiencies, and an increased risk of fracture. The skeletal signs and treatment regimens of OI depend on the severity of the disease and, due to this, classifications schemes focus on the clinical presentation. The Sillence classification was the first system that was introduced, with four groupings based on clinical and radiological findings as well as the mode of inheritance [103]. As OI cases were identified that did not include a mutation in type 1 collagen genes and presented with unique and distinctive phenotypes, the classification of the disease adopted additional groupings [96, 97, 104]. Currently, the classification system with the broadest clinical use is an expansion of the Sillence groups, now types I – V including subclasses, and is based on phenotypic characteristics and inheritance patterns [105, 106].

Treatments for OI aim to reduce fracture incidence and increase the mobility of affected individuals. The bone fragility in OI is due to a combination of poor initial bone quality as well as secondary osteoporosis [105]. While both bone formation and bone resorption are increased, the net effect is frequently progressive bone loss [97]. Several trials have shown that systemic bisphosphonate treatment is effective in treating this bone loss, and reduces the frequency of
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fractures [107-111]. While bisphosphonate use is considered to be the standard of care for OI sufferers [97, 112], concerns with bisphosphonate use in children highlights there is still space for complementary therapies that can increase bone mass.

**Legg-Calves-Perthes disease**

Legg-Calve-Perthes or Perthes disease is a condition that describes an idiopathic osteonecrosis of the femoral head (ONFH) in children, typically between the ages of four and ten [113]. There are many theories as to the underlying cause for Perthes disease, including its origins as an inflammatory disorder, a genetic mutation, a traumatic event, or a combination of multiple factors [114-116]. The common root of all these causes is that the osteonecrosis that develops in the femoral head results directly from a disrupted vascular supply [117]. Ischemic necrosis follows from the pathological imbalance between disrupted bone formation together with increased resorption [118]. This imbalance has been observed histologically in animal models of the disease, [119, 120] as well as in clinical biopsies [121].

Treatment for Perthes disease include conservative non-operative treatments as well as surgical approaches. Non-operative interventions such as bracing are common, but osteoarthritic degeneration and/or femoral head collapse can still result. In these cases total hip arthroplasty or resurfacing has been advised [122]. While a single total hip arthroplasty may suffice for treatment of ONFH in older patients, it is not an adequate treatment option in the paediatric field. These interventions typically require multiple revisions over a lifetime, which can lead to depletion of bone stock and the need for additional hip replacements. Currently, there are no treatments that address the regeneration of the femoral head.
Bone tissue engineering approaches to bone regeneration

Bone tissue engineering is an arm of regenerative medicine that aims to assist the body in restoring bone tissue form and function. It is the process of inducing and manipulating bone formation *in vivo* by enhancing and directing the body’s own repair mechanisms. Bone tissue engineering systems are being developed as a method of targeting bone healing in various applications where current treatments are insufficient, especially in instances that require autograft harvesting. Potential applications for bone tissue engineering solutions include replacing allograft and autograft use in large bone defects, spinal fusions, treatment of open fractures and non-union fractures. In addition, there are some applications for bone regeneration that are not addressed by current treatment regimes, such as regeneration of the femoral head in Perthes disease.

There are several critical elements required for a successful tissue engineering construct [123]. Known as the tissue engineering triad, these fundamental principles are the introduction of growth factors, the recruitment of progenitor cells, and the presence of a supportive scaffold (Figure 1.12).

![Figure 1.12: The tissue engineering triad: the fundamental elements required for a successful tissue engineering construct.](image-url)
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Osteogenic growth factors

The BMP growth factor family are strong drivers of bone development and repair. The ability to augment BMP availability at a particular site via tissue engineering approaches is a highly promising route towards improving bone healing. Exogenous BMPs can increase the mobilization of osteoprogenitors, increase the replication of local osteoprogenitors, and improve the differentiation of osteoprogenitors into functional osteoblasts [124]. Early investigations on two recombinant human BMPs (BMP-2, and OP-1/BMP-7) by industry, interested in the idea of harnessing the de novo bone formation, revealed two important conclusions. The first was that these single BMPs have osteoinductive capabilities and can induce bone formation on their own [58]. The second was that the resultant bone formed in a dose-dependent manner [125, 126]. In addition, there is an osteogenic threshold concentration of exogenously applied BMP that is required before bone formation can be induced.

However, the addition of excessive BMP results in a large amount of bone formation that is accompanied by an increase in osteoclastic action and increased bone resorption [64, 127, 128]. Excessive doses of BMP can also lead to a pathological inflammatory response, which has been associated with clinical complications [129]. Thus, the local BMP dose required for effective bone formation lies somewhere between the lowest amount needed for bone formation and the upper limit before excessive bone resorption and heterotrophic ossification occurs [130, 131]. With the high cost of production for these recombinant proteins, effort must be made to augment the effect of BMPs and generate sufficient bone, whilst reducing the required dose.

Most of the studies into the bone regeneration potential of BMPs have focused on BMP-2 and BMP-7. These two BMPs can induce de novo bone, as has been demonstrated in animal models of ectopic bone formation [132-134]. BMPs have also shown to accelerate healing in rat closed
fractures [135], rabbit [136], goats [137-139] and non-human primates [140]. In open fractures, BMPs were able to prevent non-union in the rat [141-143]. BMPs were also able to heal and restore function in challenging critical-sized defect models, such as in the long bones of rat [144], rabbit [145, 146], and sheep [147].

BMP-2 and BMP-7 have been approved for use in humans by the FDA. BMP-2, sold as Infuse® by Medtronic, was granted approval for use in lumbar spinal fusions in 2002, open tibial fractures in 2004, and sinus augmentations in 2007 [148]. BMP-7, sold as OP-1 putty by Stryker, was approved in 2001 for limited use in long bone non-unions [149] and 2004 for lumbar spinal fusion. Clinically, BMPs have proven to be a promising alternative to bone grafting in several applications.

In acute fractures, the goal of BMP use is to accelerate fracture healing and reduce the occurrence of non-union and the need for secondary surgery. In non-unions, the use of BMP aims to achieve bony union, which is particularly important in recalcitrant non-unions. For spinal fusions, the addition of BMP is intended to achieve fusion between adjacent vertebral levels.

One study investigating acute open tibial shaft fractures treated with intramedullary rodding and either BMP-7 or standard closure, found that BMP-7 significantly decreased the number of secondary interventions required [150]. In a seminal study involving 124 non-unions, Friedlaender et al. found that the use of BMP-7 in tibial non-unions with intramedullary rods had no difference when compared to autograft [149]. Indeed, there have been many studies showing the safety and efficacy of BMP-7 in non-union fractures [151-153]. In the spine, the use of BMP-7 in lumbar fusion was found to be comparable to autograft up to a four year follow up [154]. While BMP-7 was approved in 2004 for spinal fusion under a Humanitarian Device Exemption (HDE), for treatment of up to 4000 patients per year along with other conditions, it was found to not be non-inferior to standard of care, and in 2009 was not approved by the FDA for normal use. However,
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BMP-7 was not able to live up to its promising initial results, which may be due to its lower osteoinductive capacity, inappropriate dose or carrier [155], and has since been removed from the market [156].

In 2002, a study by Govender et al. examined BMP-2 use in open tibial fracture; standard of care was compared with two doses of BMP-2 in 450 patients. They published findings showing accelerated fracture healing and a dose dependant decrease in the rate of secondary interventions [157]. However, subsequent analysis identified that the groups were not equally distributed, with younger patients in the two BMP treated groups and a higher rate of reamed intramedullary nail fixation in the highest BMP treated group. Another later study combined the data of Govender et al. with additional clinical data (total of 501 patients) and found similar benefits. BMP-2 treatment resulted in fewer secondary procedures in the most severe fractures, as well as a decrease in infection rate [158]. But to account for the imbalances in patient assignment to BMP-2 vs control groups, Aro et al. compared standard of care treatment in 277 patients with acute open tibial fractures that involved only reamed intramedullary nail fixation. In this cohort, BMP-2 intervention did not accelerate fracture healing and they also found a significant increase in infection rate in the BMP-2 treated group [159].

A Cochrane review on the use of BMPs in fracture healing in adults concluded that there was only limited evidence for the effectiveness of BMP treatment in acute tibial fractures, and inconclusive evidence as to their effectiveness in non-union [160]. The authors also suggested that there may some economic gain in the use of BMPs, but only in the most severe fractures. This review also highlighted concerns regarding the involvement of industry in key trials. Authors of early studies were also admonished by Carragee et al., who drew attention to the chronic underreporting of adverse effects in industry sponsored trials [129].
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BMPs are potent bone growth stimulants, however the challenge in bone tissue engineering lies in improving the economic considerations for BMP use, optimising delivery, and reducing the required dose.

**Osteoprogenitor cells**

The osteoprogenitor cells required for bone formation can be recruited to the site from endogenous tissues or implanted in conjunction with the bone tissue engineering system. These scenarios will both be discussed.

There is evidence for a range of cell populations that reside within the body that have the capability to be recruited to act as osteoprogenitors. This is most clearly seen in the success of ectopic bone models used by this laboratory, where unseeded scaffolds containing exogenous BMP-2 are placed in a muscle pouch resulting in *de novo* bone formation [132, 161]. The source of these progenitor cells is not yet fully understood, and multiple cell lineages are thought to contribute. In instances where the bone forms within the muscle, such as in the muscle pouch model or in heterotrophic ossification, the cell origin is likely local. Although muscle satellite cells are able to be differentiated into osteoblasts in culture [162], it is unclear if this occurs *in vivo*. Other pluripotent cells in the muscle include pericytes, a progenitor cell residing near blood vessels, which have been shown to possess osteogenic potential [163]. Another known source is the bone marrow, with its store of undifferentiated stromal cells [164, 165]. There have been many studies that show that transplantation of either autologous or allogenic mesenchymal stromal cells (MSCs – also known as mesenchymal stem cells) can promote bone growth *in vivo* [166, 167]. The periostium is another valuable local source of osteoprogenitors [168-170]. Surgical cauterization or stripping of the periostium results in delayed bone repair, suggesting the importance of the periostium in normal
bone healing [171, 172]. It is well known that adequate vasculature is vital for healing bone, however there may be circulating cells may have a role in bone repair [173]. Several studies have shown that grafted limbs or parabiotic mice become repopulated with host derived cells, which have arrived via the circulation [174-176]. However, these circulating osteoprogenitors do not have a major involvement to normal defect healing.

Bone tissue engineering constructs that involve recruited osteogenic cells utilize cells from a variety of sources, including the bone marrow, periosteum, muscle, and circulation. The relative contribution from each of these sources is unknown, however the success of ectopic bone models reveal the validity of unseeded scaffolds. Were such systems able to be translated into clinical use, they would require less stringent handling and storage conditions, and additionally would lead an easier path to regulatory approval.

Alternatively, the seeding of live cells onto a scaffold ensures that a cell population is readily available to produce bone. But such approaches require significant optimization in regards to cell type and pre-treatment. Cells can either be harvested from the recipient or from a donor. Cell populations studied have included cells derived from the marrow (either a direct autologous or allogenic transplant [177, 178], or first differentiated down the osteogenic lineage [179, 180]), periosteum [181], amniotic fluid [182], and even induced pluripotent stem cells [183]. However, there is much debate as to the role of seeded cells; are they actively contributing to bone formation by differentiating into osteoblasts, or are they acting in a paracrine fashion by secreting growth factors that recruit osteogenic cells? Practically, the use of autologous cells would increase the time before the patient receives the scaffold, as cells would need to be harvested and then cultured. This increased time with cells in culture also dramatically increases the overall cost of the procedure. The use of allogenic or xenogenic (from an animal source) cells that are pre-seeded onto scaffolds
would further restrict handling and storage constraints. The use of cells that have been cultured will have to undergo a stringent approval process from the relevant regulatory bodies, such as the FDA in the United States, and the Therapeutic Goods Administration (TGA) in Australia.

**Bone tissue engineering scaffolds**

Bone tissue engineering scaffolds have been proposed in a variety of materials and manufacturing techniques. The hunt for the perfect combination of properties has led to the development of metal, ceramic, and polymer scaffolds, each with its own unique advantages and disadvantages. The ideal scaffold must meet several requirements, including suitable:

- Biocompatibility of the intact scaffold and its breakdown products
- Biodegradability
- If biologics or drugs are incorporated, the elution of these agents in a controlled manner
- Mechanical support

In addition, the scaffold must meet practical requirements such as low cost, sterilization compatibility, and ease of storage, handling, and manufacture. The elimination of animal-derived materials would reduce the risk of disease transfer and immunological issues.

Traditionally, bone tissue engineering scaffolds are solid constructs with defined shapes and sizes. These require surgical implantation, and may have drugs and/or biological agents incorporated during manufacture or adsorbed prior to implantation. Alternatively, there has been recent interest in minimally invasive scaffolds that can be injected, potentially avoiding the need for surgical intervention.
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**Solid scaffolds**

In addition to the conditions listed above, an idealised solid scaffold must also have a three-dimensional, highly porous structure, with interconnected pores [184]. While there is some discussion as to the optimal pore size and interconnectivity, it is acknowledged that these parameters allow for nutrient diffusion, waste removal, and cellular proliferation and migration [185, 186].

**Metals** are often used for clinical orthopaedic applications such as bone screws, plates, and other implants that are made of titanium, stainless steel, or cobalt chrome [187]. While metals have excellent mechanical properties, the Young’s Moduli of implants are up to ten fold above that of bone. If they are highly load bearing, this can result in stress shielding and subsequent bone loss [188, 189]. As metals are chemically stable and would not undergo significant degradation the body, they are generally considered for permanent or semi-permanent implants (which would require surgical removal). Hence, the majority of research in this area aims to improve the osseointegration of implants to bone [190, 191]. However, due to the biocompatibility, ease of manufacture and relatively low cost of using metals there has been recent interest producing metallic scaffolds for bone tissue engineering applications. Porous scaffolds manufactured from metals such as tantalum and titanium have been produced [192-194]. However, these scaffolds cannot have growth factors integrated into their structure, may induce stress shielding, and do not undergo significant degradation in vivo [195, 196].

**Ceramics** used for bone tissue engineering include hydroxyapatite (HA), tricalcium phosphate (TCP), and bioactive glass (Bioglass). These ceramics are currently used in clinical orthopaedics as bone void fillers and implant coatings [197-199]. They have excellent biocompatibility properties, are biodegradable, and have a good history with regulatory bodies. Ceramic materials
are quite versatile and can produced as pastes, granules, and blocks. However, ceramics have very poor fracture toughness and low mechanical strength, which prevent their use in areas of high tension, bending, and/or shear loading [123, 200]. Due to their poor properties in bulk solid forms, most of the use of ceramics in this field has been as part of a composite material or in particle/granule form [201-203].

**Biodegradable synthetic polymers** are an attractive option for solid scaffolds because they are easy to manufacture, shape, sterilize, and store [204]. Polymers are cost-effective and their properties can be tailored by altering the polymer choice and synthesis method. The incorporation of biologics and adjunctive agents is relatively simple and provokes a minimal immunological response in their intact state. The most prominent polymers are poly(lactic acid) or PLA, and poly(glycolic acid) or PGA, and their co-polymer poly(lactic-co-glycolic acid) or PLGA [205, 206]. PLA and PGA are currently FDA approved materials for a variety of uses, including dissolvable sutures and skin grafts [207]. The breakdown of polymers to their constituent monomers can produce an acidic local environment that can result in osteolysis [208, 209].

**Absorbable collagen sponge**

The current clinical gold standard scaffold for BMP-2 delivery is the absorbable collagen sponge. Collagen fibres form the regular networks that organise most tissues, and these extracellular matrices form the natural scaffolds that surround cells and other components. Tissue engineering scaffolds have historically aimed to biomimically recreate the three dimensional architecture of these collagen networks [210, 211]. Aside from bone tissue engineering, collagen sponge has also been investigated as a carrier in burns treatment [212], ureteral replacement [213], antibiotics delivery [214], dermal fillers [215, 216], and tooth-tissue engineering [217].
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The collagen that is used clinically for BMP-2 delivery, is a bovine type 1 collagen that is harvested from tendons, denatured, and freeze-dried to produce a sponge. While widely used, animal-derived materials are associated with potential immunological reaction (that may or may not affect orthopaedic outcomes) [218, 219], disease transfer including prions [220, 221], and animal-to-animal variability [222]. The costs of maintaining herds of disease free cattle also translate to higher costs of collagen sponge production [223, 224]. Any attempts to use recombinant collagens are stymied by high production costs, and the lack of strong biological activity [225].

As a carrier, collagen sponge is biocompatible, biodegradable, and is able to bind BMP-2 and release it over time, as well as acting as a matrix for infiltration of cells. The BMP-2 release profile from collagen sponge is a burst upon initial implantation, with 30% of the BMP-2 lost in the first 10 minutes [226, 227]. The strong burst release has been associated with clinical complications including heterotrophic ossification, inflammation, haematoma, bone resorption, and tumour formation [129, 228, 229].

Clinically, BMP-2 solution is soaked onto the collagen sponge at least 15 minutes prior to implantation. However, upon wetting the collagen sponge loses mechanical stability, and there are concerns with loss of BMP-2 solution through handling [230, 231]. The use of collagen sponge to deliver BMP-2 has been largely successful, despite the concerns listed above.

*Injectable scaffolds*

Of growing interest to the field of bone tissue engineering is the development of an injectable scaffold. An injectable system would allow for minimally invasive introduction of BMPs, with the goal of percutaneous delivery that could avoid open surgery. An injectable system would also allow the introduction of BMPs in awkward surgical procedures where the implantation of a solid
scaffold would be difficult. The use of injectable scaffolds could also reduce the risk of infection, due to the minimally invasive nature of delivery.

There have been several proposed injectable systems for bone tissue engineering, including hydrogels based on natural and synthetic polymers, which can incorporate microparticles. Injectable carriers have been based on naturally occurring polymers such as alginate [232-234], chitosan [235, 236], and hyaluronic acid [237, 238]. Synthetic polymers such as PLGA [239-241], poly(ethylene glycol) (PEG) [242, 243], and polyacrylamide [244] have also been proposed. Hydrogels that set in situ via pH [245, 246], temperature [245, 247], and UV light curing [248, 249] have also been explored. While some of these systems have yielded promising results in vivo, there have been many associated challenges including the release of polymerisation by-products, the exchange of organic solvents, or technical hurdles centred on tightly controlled gelation conditions or the need for an externally applied gelation trigger.

Sucrose Acetate Isobutyrate

One alternative to all the scaffolds proposed thus far is a material known as sucrose acetate isobutyrate (SAIB). SAIB is a highly viscous (100,000 mPa.s at 30°C) mixed sucrose ester that has been used as beverage emulsifier for many decades [250]. With a long history in the food industry, SAIB has been deemed safe for consumption by the FDA (GRN 000104, 25 April 2002; 78 FR 14665, 7 March 2013). SAIB is approved for use in food and beverages in at least 28 countries world-wide, including Australia, and is judged by the World Health Organisation as low risk [251].

While SAIB is a highly viscous material, the incorporation of a small amount of solvent can reduce the viscosity dramatically. When this mixture is injected intramuscularly or subcutaneously the solvent diffuses away rapidly, leaving behind a highly viscous, semi-solid depot of SAIB. This phase-transitioning property has led to the investigation of SAIB as a drug delivery system in a
variety of situations. SAIB has been proposed as system to deliver growth hormones in rats [252], the anti-psychotic Risperidone in rats [253], vaccinations in horses [254], and gonadotrophin releasing hormone analogues in mares [255-257]. Under the brand name SucroMate®, has been FDA approved since 2010 as the carrier component of a system to induce ovulation in horses [258]. Furthermore, the release of agents from SAIB has also been modulated by altering the SAIB concentration, solvent choice, and additives such as PLA microparticles [259-261].

In animal studies, the intramuscular delivery of SAIB in animals has been deemed well tolerated. The delivery of SAIB, ethanol and recombinant human growth hormone in rats resulted in localised inflammation after seven days, and was considered acceptable [252]. The intramuscular administration of SAIB, ethanol, PLA, and Risperidone in rats revealed a mild localised inflammatory response that had cleared by 28 days [253]. In regards to solvent choice, ethanol is generally regarded as acceptable for parenteral administration [262, 263].

SAIB has been previously suggested as part of a delivery system for the repair of bone tissue. In a 2005 study, a collagen-chondroitin sulfate disk coated with PLA and SAIB was investigated in a muscle pouch of a rat [264]. However, SAIB was only used to slow the release of BMP-2 from the collagen-chondroitin sulfate disks, and its phase-transitioning drug depot properties were not employed.

SAIB has been developed for use in humans as a carrier system for controlled drug release under the brand name SABERTM. SAIB is in clinical trials for the use as a systemic drug release depot for Risperadone (Phase 1, NCT01592110), as well as the local release of the anaesthetic Bupivacaine (Phase 3, NCT01052012). A Phase 3 randomised controlled trial for the use of SAIB for Bupivacaine release at the surgical site was reported as safe, with no detrimental effects on normal wound healing [265]. Compared to alternative polymeric injectable delivery systems, SAIB
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requires only small amounts of organic solvents for delivery and manufacture, can be administered using standard injection equipment, is low cost, and is already established for large scale pharmaceutical manufacture [259, 266]. The use of SAIB as a drug delivery system is highly promising avenue that warrants further investigation in the context of bone tissue engineering.
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Therapeutic modulation of bone

There are several ways to manipulate the formation of bone. Agents can be used to increase bone volume through the prevention of catabolism. There are also agents that can indirectly affect bone, such as by increasing vascularity.

Anti-catabolic agents

Bisphosphonates

Bisphosphonates are a class of synthetic anti-resorptive drugs first used clinically in the 1960s to prevent bone loss. Bisphosphonates have a similar structure to the endogenous inorganic pyrophosphate, which helps regulate bone mineralisation *in vivo* [267]. All bisphosphonates have a highly stable phosphate-carbon-phosphate (P-C-P) backbone, with two side chains that differ, defining the type of bisphosphate. Bisphosphonates have a very strong affinity for bone mineral, and act by binding strongly to solid-state calcium phosphate, and inhibiting further crystal formation, aggregation and dissolution. Once dosed, bisphosphonates are known to home to bone, particularly to areas of high osteoclastic activity, and are only released again when the bone they are bound to has resorbed [268, 269]. The half-life of bisphosphonates bound to bone can range from 1 to 11 years, depending on the bisphosphonate and the species [268, 270].

Bisphosphonates are often classified as either non-nitrogen containing or the more potent nitrogen containing compounds. Both types of bisphosphonate are taken up by osteoclasts during resorption, where the acidity of the resorption pit produced by the osteoclast dissociates the bisphosphonate from the bone mineral [271].
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The relative potency of some of the most commonly used bisphosphonates are as follows [272]:

\[
\text{Etidronate} < \text{Clodronate} < \text{Pamidronate} < \text{Aledronate} \leq \text{Ibandronate/Risedronate} < \text{Zoledronate}
\]

The non-nitrogen containing bisphosphonates are less potent than the nitrogen-containing bisphosphonates, with Zoledronate approximately 10,000 times more potent than Etidronate.

Non-nitrogen containing bisphosphonates, such as Clodronate, Etidronate and Tiludronate, inhibit bone resorption by being metabolised into cytotoxic analogues of adenosine triphosphate (ATP) [273-275]. The intracellular accumulation of these non-nitrogen containing bisphosphonate metabolites results in osteoclast apoptosis [276-279]. Nitrogen-containing bisphosphonates, such as Pamidronate, Ibandronate and Zoledronate, are much more potent than non-nitrogen containing bisphosphonates, and act by inhibiting the enzyme farnesyl diphosphate (FFP)-synthase [280-282].

There is a strong correlation with the ability of a bisphosphonates to inhibit FFP-synthase to its in vivo bone resorption capacity [282]. Inhibition of FFP-synthase prevents the prenylation of small signalling molecules known as GTPases, resulting in inhibition of downstream signalling and/or accumulation of unprenylated GTPases that inappropriately activates downstream signalling [283, 284]. Nitrogen-containing bisphosphonate action is not necessarily associated with osteoclast cell death, but rather with functional inactivation [284].

Due to the ability of bisphosphonates to prevent bone resorption, they have been widely used clinically in diseases involving low bone mass, such as osteoporosis [285, 286], Osteogenesis Imperfecta [287, 288], Paget’s disease [289, 290], and in oncology [291, 292]. The TGA currently has eight bisphosphonates approved for use in Australia and these are Alendronate, Etidronate, Pamidronate, Ibandronate, Risedronate, Clodronate, Tiludronate, and Zoledronate [293]. Generally, bisphosphonates are delivered systemically, either by oral or intravenous routes.
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However, oral delivery has been associated with poor bioavailability and gastrointestinal toxicities, such as esophagitis and diarrhoea [294, 295], and issues with intravenous delivery of bisphosphonates have included injection site reactions and flu-like symptoms [295, 296]. There have also been concerns raised with long term use of bisphosphonates and the incidence of osteonecrosis of the jaw [297, 298], as well as atypical fractures [299, 300]. Thus, while it is clear the bisphosphonates are highly potent and effective inhibitors of bone resorption, their use must be controlled to reduce the risk of systemic side effects and maximise their local effects.

*Cathepsin K inhibitor*

Osteoclasts secrete proteases during resorption to degrade the organic matrix of bone. One of these enzymes is known as Cathepsin K, and it is expressed primarily in osteoclasts [301]. Mutations in the Cathepsin K gene result in osteopetrosis (featuring larger and more dense bones) in both human disease [302-304] and transgenic mouse models [305, 306]. Inhibition of this protease using small molecule drugs was shown to reduce osteoclast activity and decrease bone resorption in rats [307, 308] and non-human primates [309]. Clinically, a cathepsin K inhibitor has been trialled as an osteoporosis treatment with promising results [310, 311]. There has also been interest in the role of cathepsin K inhibition in metastatic bone cancers to prevent osteoclast-mediated bone resorption [312].

*IκB kinase inhibitor*

Nuclear factor-κB (NF-κB) is a crucial regulator of osteoclastogenesis [313]. Mice that lack proper expression of NF-κB do not produce mature osteoclasts and have an osteopetrotic phenotype [314, 315]. The endogenous inhibitor of the NF-κB enzyme, inhibitor of NF-κB- (IκB), regulates the activity of NF-κB. IκB kinase (IKK) acts by phosphorylating IκB, leading to its dissociation from NF-κB, thus allowing activation of NF-κB [316]. Inhibitors of IKK have been shown to induce
osteoclast apoptosis as well as inhibiting bone resorption in vivo [317-320]. The role of NF-κB in immune signalling pathways has also led to interest in IKK inhibitors as a therapy for the treatment of inflammation induced bone loss [316, 321].

**Pro-vascular agents**

Whilst agents that modulate vascularity may not necessarily result in increased bone formation on their own, it is known that angiogenesis and osteogenesis are tightly coupled [322, 323]. A supportive vascular network is vital for the homeostasis of healthy bone tissue [324]. It is well known that the process of angiogenesis and an adequate blood supply are required for successful fracture healing [325-327]. Conversely, bone undergoes necrosis in the absence of sufficient blood supply (avascular necrosis) [328, 329]. The clinical success of vascularised fibular grafts in treating bony defects further confirms the close relationship between blood and bone [330-332].

**Vascular endothelial growth factor**

One of the most dominant mediators of angiogenesis is vascular endothelial growth factor (VEGF), which is secreted by many cell types [333]. VEGF mediates the process of endochondral ossification by stimulating the capillary invasion of cartilage cells, which is required before it can be transformed into bone [334]. The addition of exogenous VEGF has shown to improve bone repair [335, 336]. Conversely, chemical inhibition of VEGF has also been shown to impair bone formation and healing [337, 338]. However, the challenges of optimising the delivery of the short lived protein and high costs of production have held back the use of VEGF protein clinically. Studies into gene therapy have shown promising results in in vivo animal models of bone healing [339, 340], but such approaches have significant hurdles to clinical translation.
Deoxyribose

Deoxyribose is a monosaccharide sugar that has many roles in the body. The biologically common D-2-deoxyribose is a precursor to deoxyribonucleic acid (DNA) [341, 342]. Alongside its vital role in DNA, a deoxyribose precursor (deoxyribose-1-phosphate (dR1P)) is also released by platelets, and this has been found to have proangiogenic effects. This action is mediated by the enzyme thymidine phosphorylase (TP), by dephosphorylating dR1P into deoxyribose, which is then able to induce endothelial cell migration [343-347]. Platelet secreted deoxyribose has also been shown to increase endothelial cell motility and increase capillary formation in the chick chorioallantoic membrane assay [348, 349]. TP has also been implicated in tumour angiogenesis and aggressiveness [350], and high TP expression has been linked with poor survival [351, 352].

Agents that affect cell fate

Multicellular organisms contain many differentiated cell types that originate from stem cells and progenitor cells. These progenitor cells are multipotent and are slightly more differentiated towards a particular cell type than stem cells, but still require further signalling to become fully differentiated. Progenitor cells generally remain largely dormant, but act to replace cells that die as a result of natural turnover or are injured, ensuring the tissue is maintained over the lifetime of the organism. The cell type these progenitor cells differentiate into can be influenced by the application of drugs, growth factors, or biomaterials [353-356].

Of particular interest are osteoprogenitors, the precursors to bone forming cells. Osteoblasts are a mesenchymal cell type, with progenitor cells reported to reside in the bone marrow [357, 358]. Mesenchymal progenitor cells can also differentiate into cartilage and adipose cells [359-361]. For each of these lineages, there are key pathways that direct cell differentiation.
The peroxisome proliferator-activated receptor (PPAR) family is one of the major regulators of fat storage. The PPARs are ligand activated transcription factors that belong to the nuclear receptor family [362]. PPARs are activated by a wide range of endogenous and exogenous free fatty acids and their metabolites [363, 364]. PPAR exists in three isoforms, PPARα, PPARγ and PPARδ. The most heavily studied isoform is PPARγ, which has shown to have a critical role as a transcriptional regulator of adipogenesis [365, 366]. PPARγ activation results in the differentiation of mesenchymal progenitors into adipocytes rather than osteoblasts [367]. Mice with haploinsufficiency of PPARγ exhibit increased bone mass and enhanced osteoblastogenesis [368]. Further, administration of the PPARγ antagonist bisphenol-A-diglycyl ether (BADGE) in aging mice is able to increase the bone mass and reduce marrow adiposity [369]. Thus, there exists a reciprocal relationship between adipogenesis and osteogenesis, and it is hypothesised that pharmaceutical agents could promote one lineage at the expense of the other.

The mitogen-activated protein kinase (MAPK) signalling cascade is involved in a wide range of cellular functions, including proliferation, survival, and differentiation [370]. In classical MAPK signalling, upstream signals are transduced through intermediate kinases MAPK kinase (MEK) and extracellular signal-regulated protein kinase (ERK) [371]. The MEK inhibitor PD0325901 has been shown to improve bone volume and mineralisation in a genetically hypophosphatemic mouse [372], but the majority of studies with this inhibitor are in the context of cancer [373, 374]. Our group has examined PD0325901 in an orthopaedic setting. Treatment with this agent promoted soft and hard callus formation, but delayed endochondral ossification in a fracture model [375]. It is speculated that in the context of BMP-2 induced bone formation, where bone forms de novo with limited cartilage involvement, PD0325901 may be able to augment net bone formation.
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Related to the classical MAPK signalling pathway is the c-jun N-terminal kinase (JNK) pathway. JNK signals can be activated by inflammatory cytokines, which are abundant during the early stages of bone repair [376]. The JNK inhibitor **SP600125** has been shown to enhance the osteogenic differentiation of bone marrow stromal cells [377]. In this laboratory, JNK inhibition through SP600125 has shown to have adjunctive effects with BMP-2 *in vitro* [378]. In a tibial fracture model in a mouse, an alternative JNK inhibitor (CC-930) was found to give a modest improvement in time to union (N Deo, unpublished data).

The Wnts are a secreted glycoprotein family that activate cell surface receptors, and through signalling pathways modulate a range of cellular activities throughout life [379]. The Wnt/β-catenin intracellular signalling cascade is the canonical Wnt pathway that can influence cell fate, proliferation and survival [380]. The Wnt/β-catenin pathway is heavily involved in the differentiation of osteoblast progenitors, and is constitutively supressed by glycogen synthase kinase-3 (GSK-3) [381]. Transgenic mice with a heterozygous deficiency of GSK-3β exhibit increased bone mass and improved bone repair [382]. Pharmacological inhibition of GSK-3 through **SB216763** has been shown to promote osteoblastic differentiation and suppress osteoclastic differentiation *in vitro* [381]. In a drill hole defect in the mouse, administration of an alternative GSK-3 inhibitor Li$_2$CO$_3$ resulted in accelerated bone regeneration [381].

Modulation of differentiation fate of osteoblastic precursor cells is a potential route to enhance bone formation. Direction of mesenchymal cells towards an osteogenic fate as opposed to lineages such as adipose or cartilage could be achieved through pharmaceutical interventions that block cell signalling pathways. The use of agents that affect cell decision to therapeutically augment bone formation is of great interest, however significant research is required before they can be translated into clinical usage.
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**Project objectives**

The main objective of this work was to develop a BMP-2 delivery system that has superior properties to the current clinical standard, absorbable collagen sponge. This project investigated the sugar based molecular SAIB as a phase-transitioning carrier for BMP-2 for bone tissue engineering. This follows SAIB development from initial *in vivo* implantation models through to its use in different models of bone healing (Figure 1.13). The SAIB delivery system was initially compared to collagen and then optimised through the co-delivery of adjunctive agents, with the goal of augmenting bone formation.

Several animal models were used to explore the delivery of BMP-2 in the injectable carrier SAIB. Initially, a mouse model was developed and optimised for screening of SAIB with other adjunctive drugs and biomaterials. Subsequently, SAIB formulations were then utilised in more challenging bone repair scenarios in a transgenic mice, a rat open fracture model, and in the porcine femoral head. Lastly, alternative novel sugar based compounds were synthesised and tested as BMP-2 carriers in a mouse model.
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**Figure 1.13:** Development of a novel injectable bone tissue engineering carrier for BMP-2.

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</tr>
</thead>
<tbody>
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<td>Use of the SAIB/BMP-2 delivery system in a transgenic OI mouse</td>
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Chapter 2. The development and optimisation of the injectable bone tissue engineering carrier SAIB in mouse models

Work from this chapter has been published in:


Chapter 2: Development of SAIB for bone tissue engineering

Introduction

Drug screening techniques

Advances in modern drug discovery, through innovations in molecular chemistry and synthesis techniques has generated large libraries of compounds that have the potential to effect biological changes. The identification of new molecular targets and targeted drug design using combinatorial chemistry and small molecule design methods have resulted in the generation of numerous potential therapeutics. This has necessitated the development of efficient methods for high throughput screening that allow for the exploration of potential effects in vitro, and ultimately in vivo.

There are five reported criteria that are essential for an effective drug discovery screen [383], and these are:

1. **Relevance.** The model used for drug screen should try to correlate with the proposed in vivo property of the drug.

2. **Effectiveness.** The criteria used to define effectiveness should be harsh enough to eliminate some of the candidates. Otherwise, too many agents progress through the screen, which can increase costs and delay progress.

3. **Speed.** The procedure used to screen should be quick in both the time taken to prepare the assay, as well as the time the assay requires.

4. **Robustness.** The screen must be applicable to a range of agents, and not limited by chemical structure.

5. **Accuracy and reproducibility.** The screen should generate reliable results without false positives or false negatives and excessive retesting.
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While *in vitro* drug screening is typically relatively low-cost and quick to execute, it may not accurately represent the drug effects *in vivo*. In addition, the results of *in vitro* drug screening assays do not always translate into *in vivo* efficacy [384]. In the context of bone, newly developed drugs that have the potential to enhance bone formation, either in the absence or in synergy with BMPs are of great interest. Thus, due to the limited use of *in vitro* techniques, an *in vivo* screening method is required to screen potential synergistic agents effectively.

Of great utility to the study of *in vivo* bone formation is the ectopic bone formation model, where bone is induced at a non-skeletal site. When compared to orthotopic (bone formation at a skeletal site) models, ectopic bone assays feature reduced endogenous osteogenic cytokines and native bone cells [385]. Ectopic bone formation is a useful model to examine the osteogenic influence of growth factors, adjunctive agents, and/or new carriers. There are three main sites that can be used for the induction of ectopic bone: subcutaneous, intramuscular, and the kidney capsule [385].

The subcutaneous model is the simplest method for bone induction, and requires implantation of the material beneath the skin of the animal. The muscle pouch model involves the implantation of the material in the intramuscular compartment. The subcutaneous compartment does not contain cells that naturally form bone, whereas the muscle pouch contains progenitor cells that can be induced down the osteogenic lineage [386].

Generally, the muscle pouch has a superior bone forming capacity than the subcutaneous site [387]. The muscle pouch model has been used in many animal models including mice [388, 389], rats [390, 391], dogs [392], goats [393], sheep [394] and pigs [395]. Rodent models are commonly used due their fast breeding, high stocking density and relatively low cost. In rodents, the hind limb muscle pouch (in the quadriceps) is often used, as it provides the most space.
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The kidney capsule method is the least common due to its technical difficulty. It involves placement of the material between the fibrous capsule of the kidney and the renal parenchyma. The main advantage of this system is that it can be readily used in combination with transplanted cells due to the high blood flow.

Assessment of bone via MicroCT

The quantification of bone formed in the ectopic hind limb muscle pouch is the primary outcome of this in vivo assay. Thus having reliable methods for quantification is key. Microcomputed tomography (microCT) is an imaging technique that is used to study the architecture of cortical and trabecular bone in three dimensions. To try and achieve consistency across the literature, the Journal of Bone and Mineral Research published a consensus in 2010 on the microCT assessment of bone structures in rodents [396]. Our laboratory aims to follow these guidelines as closely as possible to improve consistency within the studies conducted within the laboratory, as well as in the wider scientific literature.

In regards to the studies conducted within this thesis, it is important to note that image acquisition settings remained constant within each of the studies. Each study was also calibrated using a hydroxyapatite phantom that was scanned using the same settings as the study. These phantoms were used to calibrate the scans, and allow for reporting of the mineral density of the tissue. In addition, the knowledge of the mineral density of the tissues scanned allows a constant threshold value that was used to define bone tissue across all studies. For ectopic bone nodules, a value of 0.3 g/cm³, and for cortical bone a value of 0.4 g/cm³ was used. The following the standardised guidelines ensure confidence of the microCT quantifications of bone volume acquired throughout
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In this thesis, these levels were chosen as they reliably picked up obvious bone elements but minimal to no background noise in the soft tissue.

In this chapter, I explored the use of SAIB as a carrier for BMP-2 in the hind limb ectopic muscle pouch model in the mouse. Initially, SAIB was compared with the current clinical standard for BMP-2 delivery, the absorbable collagen sponge. Following this, SAIB was used as a carrier to screen a range of potentially adjunctive agents in this same muscle pouch model. Finally, the optimal SAIB delivery construct for maximal bone formation was proposed.
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Materials and Methods

Pharmaceuticals

Food grade (FG) SAIB was purchased from SAFC, a branch of Sigma Aldrich (Missouri, USA) and used for the majority of experiments to ensure batch consistency. BMP-2 and porous collagen sponge were purchased as a part of the Infuse® bone graft kit from Medtronic (Minnesota, USA). ZA was purchased from AXXORA, LLC (San Diego, USA). Radiolabelled carbon-14 ZA ($^{14}$C-ZA, specific activity 6.573 MBq/mg) was a gift from Dr Jürg Gasser (Novartis AC, Basel, Switzerland). Pre-calcined HA microparticles were from Plasma Biotal Ltd (Product P149) (United Kingdom). Bisphenol A diglycidyl ether (BADGE), SB216763, SP600125, and PS1145 were purchased from Sigma Aldrich (Missouri, USA). PD0325901 was purchased from Selleck (Texas, USA). AFG495 was a gift from Novartis (Basel, Switzerland). Pamidronate (Cipla, Bombay, India) was fluorescently labelled with the commercially available AlexaFluor 555 (Life Technologies, Victoria, Australia) according to the protocol provided by Life Technologies. The resultant fluorescently labelled bisphosphonate was termed AlexaPam. β-TCP particles (average particle size 5-10 µm) were ground from stock supplies purchased from Sigma-Aldrich. Bioglass 45S5 particles were ground from stock (average particle size 5-10 µm) purchased from Biometric (NSW, Australia).

Laboratory synthesis of SAIB

The synthesis of SAIB was conducted in collaboration with the School of Chemical and Biomolecular Engineering at the University of Sydney, Australia. Synthesis protocols were developed with Dr Peter Valtchev, in the laboratory of Prof Fariba Dehghani.

A magnetic stirrer, 60 mM acetic anhydride and 240 mM isobutyric anhydride were added to a dry round-bottom flask fitted to a reflux condenser, and this was stirred at 500 rpm for the duration of
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the synthesis. 10 g of sucrose and 2.1 g of sodium acetate, were added to the flask, and heated to 120°C for 30 minutes. The temperature was then dropped to 90°C, where excess ethanol was added (approximately 150 mL) for 20 minutes. The flask was then allowed to cool, and the contents poured into a 1 L Schott bottle filled with ice cold water. The bottle was shaken and allowed to chill in the refrigerator overnight, whereby the product precipitated and fell to the bottom. The water was decanted and the contents transferred to a 50 mL falcon tube. Water was added, the suspension shaken vigorously, and then centrifuged at 6000 rpm for 3 minutes. Water was decanted again the wash process repeated 4 to 5 times until the product was sufficiently viscous. The final product was allowed to dry at 50°C in a vacuum oven.

Animal purchase, housing, and ethics

8 week old female C57BL6 mice were purchased from the Animal Resources Centre (Perth, Australia) kept in an on-site specific pathogen free (SPF) animal facility. Mouse chow and water were provided ad libitum. Animals were allowed to acclimatize for a week prior to intervention. Ethics was approved by the CHW/CMRI Animal Ethics Committee (K294).

Intramuscular injection of SAIB

An SAIB:ethanol (80:20) stock solution was prepared and allowed to mix overnight. Prior to surgery, BMP-2 and any other agents were mixed into the stock solution of SAIB. Animals receiving an injection were pre-dosed with Buprenorphine at least 30 minutes prior to surgery, where they were anesthetized using Isofluorane gas. Using a syringe, 20 µL of the SAIB mixture was injected into the quadriceps of the mouse (Figure 2.1). Mice were closely monitored post-operatively.

Mice were monitored regularly over the three week experiment, and euthanized using a CO₂ chamber. Sites of interest were harvested and x-rayed. Specimens were preserved with 4%
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paraformaldehyde for four hours at room temperature, and at 4°C overnight, and then stored in 70% ethanol.

All in vivo experiments, barring the fluorescent ZA biodistribution study, were conducted using groups of five mice injected bilaterally (n=10/group). A bilateral model was utilised to reduce animal numbers required.

![Image of mouse injection](image)

**Figure 2.1:** The surgical procedure for the injection of SAIB and other injectable carriers. A syringe is used to inject 20 µL into the quadriceps of a mouse.

**Surgical implantation of collagen**

Collagen pellets were prepared from collagen sponges (5 mm height) provided in the BMP-2 Infuse® kits using a 3 mm diameter biopsy punch in a sterile environment. 10 µl of a 0.5 mg/mL (5 µg per pellet) BMP-2 solution was applied slowly to the sponge in a drop-wise fashion, and allowed to sit for 20 minutes prior to implantation. Animals (n=5) were anaesthetized by 35 mg/kg Ketamine and 0.5 mg/kg Xylazine. The operative site was shaved and wiped with Povidine-Iodine solution. An incision was made in the quadriceps of the mouse, in which the collagen pellet was
placed (Figure 2.2). The incision was sutured closed, the animals were allowed to recover on a heat pad, and given post-operative analgesics and saline.

Figure 2.2: Surgical procedure for the implantation of a solid, but porous, collagen sponge into the quadriceps of a mouse.

**Experimental groups**

*A comparison of SAIB and the current clinical standard for the delivery of BMP-2*

To compare SAIB delivery of BMP-2 with the current clinical standard, both SAIB and collagen sponge were used to deliver BMP-2 in the muscle pouch of the mouse (Table 2.1). This study also compared the commercially purchased (FG) and laboratory synthesised (LS) SAIB. The same dose of BMP-2 (5 µg) was used for each pellet. A previous dose-response study had shown that BMP-2 delivered in SAIB results in robust bone formation with a 5 µg dose [397].

**Table 2.1:** Experimental groups for the experiment comparing SAIB to collagen sponge as a carrier for BMP-2

<table>
<thead>
<tr>
<th>Group</th>
<th>Left Leg</th>
<th>Right Leg</th>
<th>BMP-2</th>
<th>N=</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Commercial SAIB (FG)</td>
<td>Medtronic Collagen</td>
<td>5µg/pellet</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>Homemade SAIB (LS)</td>
<td>Medtronic Collagen</td>
<td>5µg/pellet</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>Commercial SAIB (FG)</td>
<td>Homemade SAIB (LS)</td>
<td>5µg/pellet</td>
<td>5</td>
</tr>
</tbody>
</table>
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The high throughput screening of potential adjunctive agents in SAIB

After proving to be an effective carrier for BMP-2, SAIB was utilised for a high throughput assay for potential adjunctive agents to enhance bone formation in conjunction with BMP-2 (Table 2.2). The agents tested could be broadly split into two categories, those that affect cell fate decision (BADGE, SB216763, PD0325901 and SP600125) and anti-resorptives (ZA, PS1145, and AFG495). Briefly, BADGE is a competitive agonist for PPARγ, and inhibitor of the adipogenic pathway [398]. SB216763 is a Wnt pathway agonist [399], while PD0325901 is a MEK inhibitor [375], and SP600125 is a JNK inhibitor [377]. All of these pathways have been implicated in osteoblastic differentiation. The remaining drugs affect bone resorption and were used in an attempt to preserve the BMP-induced bone. ZA is a potent bisphosphonate, a class of bone anti-resorptive drugs that inhibit osteoclast function [284]. PS1145 is an IKK inhibitor, which blocks osteoblast formation [400]. AFG495 is a rodent specific inhibitor of Cathepsin K, which is a protease critical for osteoclastic bone resorption. The human Cathepsin K inhibitor AFG495 was reported to be in development for clinical applications [401].

These pathways and their relationship with osteogenic pathways are described in more detail in Chapter 1: Therapeutic modulation of bone.

Table 2.2: Experimental groups for the experiment screening potential adjunctive agents in the SAIB/BMP-2 carrier system

<table>
<thead>
<tr>
<th>Group</th>
<th>BMP-2/pellet</th>
<th>Adjunctive agent</th>
<th>Adjunctive dose (µg)/pellet</th>
<th>N=</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 µg</td>
<td>-</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>5 µg</td>
<td>BADGE</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>5 µg</td>
<td>SB216763</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>5 µg</td>
<td>PD0325901</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>5 µg</td>
<td>SP600125</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>5 µg</td>
<td>ZA</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>5 µg</td>
<td>HA 2% (w/v)</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>5 µg</td>
<td>ZA + HA 1 + 2% (w/v)</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>9</td>
<td>5 µg</td>
<td>PS1145</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>5 µg</td>
<td>AFG495</td>
<td>25</td>
<td>5</td>
</tr>
</tbody>
</table>
Investigation of the systemic biodistribution of locally delivered bisphosphonate in SAIB

To study the extent of the systemic biodistribution of bisphosphonates that were locally delivered in SAIB, a unilateral muscle pouch model was utilised. This study was conducted in groups of five mice that were injected unilaterally (n=5/group) (Table 2.3). To determine the release and visualise the subsequent biodistribution of bisphosphonate in vivo, Pamidronate was fluorescently tagged with a commercially available Alexa Fluor (555) according to the manufacturer’s protocol. The resultant fluorescent bisphosphonate was termed AlexaPam. It is hypothesised that AlexaPam has limited biological function as the conjugation with Alexa Fluor would diminish the inhibitory effect of Pamidronate on FFP-synthase, the key mechanism of nitrogen-containing bisphosphonate action. However, this conjugation would not compromise the ‘bone hook’ that is involved in Pamidronate binding with bone mineral. Further study with AlexaPam found that not all of the Pamidronate was fluorescently tagged [402]. However, Pamidronate is estimated to be 100x less potent than ZA [272], and at the doses used would be unlikely to have significant effects on bone turnover.

As an additional measure for bisphosphonate biodistribution, radiolabelled ZA (\(^{14}\)C-ZA) was used. The \(^{14}\)C-ZA was admixed with standard ZA (1:9), ensuring that ZA would have a functional effect on bone turnover.

In this study, two doses of locally delivered and admixed bisphosphonate were used 0.1 and 1 µg with and without HA (Table 2.3). For groups where local bisphosphonates were delivered in combination with HA, these were adsorbed and air-dried prior to addition to the SAIB mixture. Animals receiving systemic bisphosphonate were dosed at 0.1 mg/kg (equivalent to ~2 µg per mouse) 1 week post-surgery and sacrificed as normal (3 weeks post-surgery).
Table 2.3: Experimental groups to investigate the biodistribution of locally delivered bisphosphonate in SAIB

<table>
<thead>
<tr>
<th>Group</th>
<th>BMP-2/pellet</th>
<th>Bisphosphonate</th>
<th>HA (w/v)</th>
<th>N=</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>0.1µg of 14C-ZA 0.1µg of AlexaPam</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>0.1µg of 14C-ZA 0.1µg of AlexaPam</td>
<td>2% pre-adsorbed</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>1µg of 14C-ZA 1µg of AlexaPam</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>1µg of 14C-ZA 1µg of AlexaPam</td>
<td>2% pre-adsorbed</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>0.1mg/kg 14C-ZA 0.1mg/kg AlexaPam (systemically)</td>
<td>2% locally</td>
<td>5</td>
</tr>
</tbody>
</table>

The influence of ceramic type on bisphosphonate binding and bone formation

The potential for β-tricalcium phosphate (TCP) and bioactive glass 45S5 (BG45S5) as alternative ceramics in the SAIB/BMP-2/ZA system were investigated in the mouse muscle pouch model. The ceramics were ground to size from stock by Roya Ravarian. Ectopic bone was induced by 5 µg BMP-2 delivered in SAIB, alongside TCP and BG45S5 with and without bisphosphonate (Table 2.4). The bisphosphonate component involved equal amounts of AlexaPam and ZA, to allow for investigation into bisphosphonate retention in the pellet.

Table 2.4: Experimental groups investigating ceramic type and particle size, with and without the addition of bisphosphonate

<table>
<thead>
<tr>
<th>Group</th>
<th>Delivery</th>
<th>Ceramic</th>
<th>AlexaPam/ZA per pellet</th>
<th>n=</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SAIB+BMP-2</td>
<td>None</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>SAIB+BMP-2</td>
<td>2% TCP</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>SAIB+BMP-2</td>
<td>2% TCP</td>
<td>1 µg</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>SAIB+BMP-2</td>
<td>2% BG45S5</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>SAIB+BMP-2</td>
<td>2% BG45S5</td>
<td>1 µg</td>
<td>5</td>
</tr>
</tbody>
</table>
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**Radiography**

Bone nodule placement was monitored and checked by digital X-ray (Faxitron X-ray Corp, Illinois, USA) relative to the whole limb (25 kV, 2x magnification). Samples were then scanned by micro-computed tomography (microCT) using a SkyScan 1174 compact microCT scanner (Kontich, Belgium). All samples were scanned in 70% ethanol, using a 0.5 mm aluminum filter, 50 kV X-ray tube voltage, and 800 µA tube electric current. Bone nodules and tibiae were scanned at a pixel resolution of 14.8 µm. The scanned images were reconstructed using NRecon (SkyScan), and analysed using CTAnalyser software (SkyScan). A global threshold to define bone tissue in pellets was set at a mineral density of 0.3 g/cm³, and for trabecular analysis at 0.4 g/cm³. Samples that had fused to the femur were excluded, resulting in final test items of n=5-10 specimens in each group for all analyses.

**Brightfield histology**

After fixation in 4% paraformaldehyde, samples were stored in 70% ethanol for radiological analysis, and then transferred into decalcification solution (0.34 M EDTA, pH 8.0). For this process, samples were stored on an orbital shaker at 4° for 3-4 weeks with solution changes 3 times a week. Once decalcification was confirmed, samples were processed for paraffin histology and embedding. Samples were cut to 5 µm sections on a Leica RM 2155 microtome (Leica Microsystems, Wetzlar, Germany).

**Haematoxylin and eosin**

Haematoxylin and eosin (H&E) staining was conducted using standard protocol. Briefly, samples were dewaxed and brought to water, then stained in commercially purchased haemotoxylin (Harris; ProSciTech, QLD, Australia) for 1-5 minutes. Slides were then dipped in water, then in saturated lithium carbonate (2.5 g lithium carbonate in 100 mL distilled water), dipped in water, then stained
in eosin (500 mg aqueous eosin Y, 50 mg phloxine, 75 mL distilled water, 370 mL ethanol, 2 mL glacial acetic acid). Slides were then washed in pure ethanol, and brought to xylene and mounted. Slides were scanned using an Aperio ScanScope CS slide scanner (Aperio Technologies, CA, USA).

**Fluorescent tracking of in vivo ZA**

Following fixation, samples were cut using a diamond saw, either bisected down the centre of the pellet or along the growth plate for the femurs. Samples were imaged directly on a Leica SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany), AlexaPam was detected using the 561 laser line and with 570-670 nm emission bands, and muscle autofluorescence was detected using 488 laser line and with 500-560 nm emission bands. Bone surfaces were scanned with a 5× objective using the Tile Scan (9-12 images) and Z stack (4-5 slices of a 6µm optical depth that spanned between 30-200 µm) functions. High Z stacks were to account for uneven bone surfaces, with maximum intensity projections used to reconstruct a view of the bone surface in two dimensions. Images were processed with Leica Application Suite Advanced Fluorescence software and Adobe Photoshop CS5.

**Cell culture**

MC3T3-E1 cells (subclone 4), a commonly used pre-osteoblastic cell line [403], were cultured in standard tissue culture flasks using α-MEM supplemented with 10% foetal bovine serum, 1% L-glutamine and 2% penicillin/streptomycin. Media was changed every 4 days and cells were removed from flasks using trypsin-EDTA solution. Cell number was calculated using a haemocytometer. To determine the cytotoxic dose response to ZA, cells were seeded in 24-well plates at a density of 2x10⁴ cells per well in 500 µl and cultured in a range of ZA concentrations (1-200 µM). A dose of 50 µM was found to be highly cytotoxic and used for subsequent ceramics.
(HA, TCP, BG45S) rescue experiments. Transwell inserts (1 µm pore size; Merck Millipore) were inserted into each well containing 500 µl of media of the following treatments: media alone, 2% ceramic, 50 µM ZA or 50 µM ZA + 2% ceramic. Preliminary experiments where 10µg (2%) HA was added directly to cells showed high cell death and this was overcome by physically containing the HA within transwell inserts.

For the HA rescue experiments, viability was measured at 4 day and 7 days post seeding. Cellular viability was assessed using the CellTitre 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Wisconsin, USA) according to the manufacturer’s instructions. Briefly, cells were incubated with the viability solution for 30 min at 37°C and read using a spectrophotometer at 595 nm. All samples were assayed in triplicate and the results are representative of n=4. Data is represented as a percentage of the control cell viability value for that time point.

For the TCP and BG45S5 rescue experiments, viability was measured at 7 days post seeding using the Muse cell count and viability assay (Merck Millipore) as per the manufacturer’s instructions. Briefly, cells were trypsinized and re-suspended in media, then added to the assay media. Samples were then vortexed immediately prior to reading by the Muse Cell Analyzer.

**Quantification of $^{14}$C-ZA**

To determine the binding affinity of the bisphosphonate ZA for the ceramics, a range of doses (1-50 µM) of carbon-14 bound ZA ($^{14}$C-ZA) was added to 2% HA particles in saline and agitated. After two hours, samples were spun down in a centrifuge, and the supernatant carefully extracted and added to scintillation fluid. Readings from the precipitate were taken by dissolving in 10N HCl, neutralising with equal amounts of 10N NaOH, and added to scintillation fluid. To determine the affinities of ZA for the alternative ceramics, a 50 µM dose of $^{14}$C-ZA was added to 2% w/v of either TCP or BG45S5 particles in saline and agitated. After two hours, samples were spun down
in a centrifuge and the supernatant carefully extracted and added to scintillation fluid. Radio counts were read in triplicate using a 1900CA Tri-carb liquid scintillation analyser (Packard Bioscience, Connecticut, USA). Scintillation fluid alone was used as blank control, and a 50 µM dose of $^{14}$C-ZA was added directly to scintillation fluid as a positive control. The values measured in the supernatant values subtracted from positive controls to generate the amount of $^{14}$C-ZA that was left bound to the ceramics.

For quantification of $^{14}$C-ZA in tibia, tibiae were carefully stripped of any soft tissue and then dissolved in 10N HCl and homogenized using a TissueRuptor homogeniser (Qiagen, Germany). This was then neutralized using equal amounts of 10N NaOH. This was added to scintillation fluid, and read in triplicate using the 1900CA Tri-carb liquid scintillation analyser.

**Statistical analysis**

Statistical analyses on cell culture results were carried out using student’s t-test or ANOVA. To determine sample sizes for *in vivo* experiments, a power calculation was conducted using bone volumes seen in previous pellet studies conducted in this laboratory, yielding a group size of 10 (five mice treated bilaterally). A bilateral model was used to reduce the numbers of animals required for each experiment. Unlike cell culture assays, the *in vivo* ectopic bone formation have not been demonstrated to follow a normalised distribution. Samples that had fused to the femur were also excluded from analysis, as these samples proved difficult to isolate from cortical bone and were not able to be accurately quantified. In addition, fused samples were hypothesized to be subject to a periosteal response, and generally resulted in increased pellet size. Data was subject to non-parametric testing, initially with a Kruskal Wallis test. If there was a significant difference found, groups were compared using a Mann Whitney U test, and there were no adjustments made for multiple comparisons. All tests were performed using GraphPad Prism (GraphPad Software,
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California, USA). Statistical significance was set at $\alpha < 0.05$. Unless otherwise stated, these methods were used for all studies described in this thesis.

The approach taken for data analysis of model development and drug screening studies were consistent with the principles of exploratory data analysis. This was based on the philosophy that trying to visualize and efficiently interpret the data are more important for exploratory studies than complex statistical modelling. Thus complications such as bilateral implantation of specimens were not modelled using generalized linear models and highly rigorous multiple correction adjustments such as Bonferroni were not applied.

For the purposes of statistical analysis, multiple specimens from the same animals (from bilateral procedures) were treated as independent samples. Post-hoc examination specimens with the same treatment from left and right limbs showed a low degree of correlation (average correlation coefficient = 0.48 in n=20 groups, n=1 total specimens). Thus more complex generalized linear modelling was deemed to be unnecessary, particularly due to the preliminary hypothesis generating nature of these studies.
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Results

*BMP-2 delivery by SAIB produces more bone than delivery by porous collagen*

In this study SAIB was compared head to head against the commercial porous collagen sponge as the current clinical gold standard for BMP-2 delivery. Ectopic bone formation was induced by a single local dose of 5 µg BMP-2 delivered either by SAIB or collagen. For SAIB, two preparations were compared: (a) commercially-available food grade quality grade (FG SAIB) and (b) laboratory synthesized SAIB prepared in-house (LS SAIB).

After 3 weeks, specimens were harvested and X-rayed, and bone volume of the ectopic bone nodules quantified by microCT. The primary outcome measure was bone volume formation for each group (Figure 2.3A). With an equivalent dose of BMP-2, commercial SAIB produced increased bone volume (+190%, p<0.01) versus collagen sponge. SAIB synthesized in house also led to increased BV (+100%, p<0.01). Representative X-rays (Figure 2.3B) reveal that nodules produced by the collagen sponge were more regularly shaped than those produced by SAIB, and this was seen across all samples.

Histological examination of the nodules formed, show similarities across the three carriers (Figure 2.4). All show evidence of cortical bone surrounding the edge of the nodule, with trabecular bone inside the nodule and the formation of bone marrow.
Figure 2.3: BMP-2 induced bone growth as delivered by porous collagen sponge purchased as part of the Medtronic Infuse® kit, commercially purchased SAIB (food grade, FG), and SAIB synthesized in the laboratory (laboratory synthesized, LS). A Bone volume of the resultant pellet quantified by microCT. *, p<0.05. B Representative radiographs of specimens with the median bone volume.
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Figure 2.4: Haematoxylin and eosin staining of representative nodules of ectopic bone formation in response to 5 µg BMP-2 delivered in three carriers. Scale bar, 200 µm for all panels.

**Augmentation of BMP-2 induced bone formation**

The co-delivery of BMP-2 with agents that can increase bone progenitor differentiation, improve mature osteoblast function, or suppress bone resorption has the potential for synergistic increases in bone formation. A variety of small molecule drugs were trialled in a method for rapid *in vivo* screening. Drugs were delivered in FG SAIB by direct injection in combination with BMP-2 as an osteogenic stimulus.

The agents tested could be broadly split into two groups; those affecting cell fate decisions (BADGE, SB21676, PD0325901, and SP600125) and anti-resorptives (ZA, PS1145, and AFG-496). The primary outcome measure was bone volume formation in the ectopic bone pellets as measured by microCT. The most striking result was the +900% increase in bone volume in the BMP-2/HA/ZA group (*p*<0.01) compared to the BMP-2 only controls (Figure 2.5A). The ZA group showed an increase of +400% in bone volume (*p*<0.01), and the HA group increased by +125% (*p*<0.01). To confirm that the HA particles were not themselves being detected as additional bone, SAIB containing 2% HA was injected into animals and immediately scanned. The HA was not measurable by microCT using the settings utilized for subsequent bone scanning (Figure 2.6). For
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the other anti-catabolic agents, PS1145 gave a +70% increase (N.S.), and AFG495 resulted in a +82% increase (p<0.01) over BMP-2 alone.

The agents that affected cell fate produced more modest results. BADGE and SP600125 increased BV by +25% and +23%, respectively, while SB216763 decreased BV by -5% (all N.S.). Only the MEK inhibitor PD0325901 yielded a significant +105% increase in BV (p<0.05). Figure 2.5B displays the representative X-rays from each group. Most notable was the ZA + HA group, which showed an increase in both radio-opacity and bone pellet size.
Figure 2.5: Bone formation induced by 5 µg BMP-2, and a range of potential adjunctive agents including BADGE (15 µg), SB216763 (15 µg), PD0325901 (10 µg), SP600125 (20 µg), ZA (1 µg) and/or HA (2% w/v), PS1145 (10 µg) and AFG495 (25 µg). A Bone volume (BV) of the ectopic pellets was quantified after three weeks in vivo by microCT. *, p<0.05; **, p≤ 0.01, increased compared to the control group. B Representative X-rays showing the samples with the median BV from each group.
ZA rapidly and avidly binds to HA in vitro

Using $^{14}C$-ZA, the binding of bisphosphonate to HA was examined in vitro. A range of ZA doses were tested and the amount of unbound ZA was determined after 2 hours. While the absolute amount of unbound $^{14}C$-ZA increased with increasing $^{14}C$-ZA doses, the relative ratio of bound/total ZA remained constant at ~90% (Figure 2.7). The consistent ratio suggested that HA and ZA binding had reached equilibrium, with on and off binding rates being equal. These data suggest that the ZA dose used in the earlier in vivo experiment was appropriate, as the HA would not have been saturated by bisphosphonate.
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Figure 2.7: Detection of $^{14}$C-ZA in the supernatant after binding with 2% HA in saline for 2 hours. 
A Total unbound $^{14}$C-ZA detected at increasing doses and B percentage of $^{14}$C-ZA found bound to HA

Successive experiments examining the binding of 50 µM ZA to HA over a time series revealed that an equivalent proportion of ZA was bound at all of the time points (15 min - 24 h) (Figure 2.8). These data suggest a rapid and stable binding equilibrium between HA and ZA \textit{in vitro}.

Figure 2.8: Scintillation counting revealing percentage of bound $^{14}$C-ZA to HA after increasing lengths of binding time
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**HA yields protection against the cytotoxic effects of ZA in vitro**

It was hypothesized that the presence of HA could act as a sink for ZA to prevent unwanted cytotoxic effects on cultured cells. To identify a cytotoxic ZA dose, the mouse derived MC3T3 pre-osteoblast cell line was cultured with a dose range of ZA (0 µM, 1 µM, 5 µM, 10 µM, 50 µM, and 200 µM) and the effects on cell viability were measured. Doses of 50 µM and 200 µM ZA were cytotoxic, with over 90% cell death at days 4 and 7 (Figure 2.9).

![Figure 2.9: MC3T2 cell viability response to increasing doses of ZA (1 – 200 µM) at two time points, as measured by CellTitre viability assay. Data is normalised to cell viability values of control cells cultured in normal conditions at the relevant time point. *, p<0.05 compared to day 4 control; #, p<0.05 compared to day 7 control.](image)

Using the cytotoxic concentration of 50 µM ZA, the ability of HA to sequester the bisphosphonate and rescue cell viability was tested. Again, in the presence of 50 µM ZA there was significant cell death, but this was found to be attenuated by the presence of 2% HA (Figure 2.10). By day 7, cells treated with 50 µM ZA showed an 87% drop in viability compared to normal media controls, whereas 50 µM ZA/2% HA treated cultures were not significantly different from controls. These data suggest that HA can have a protective effect against ZA *in vitro*. 
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**Figure 2.10:** Mouse derived MC3T3 cell viability in the presence of ZA and/or HA. At two time points, 2% HA has a protective effect against 50 µM ZA on cell proliferation. Data is normalised to cell viability values of control cells cultured in normal conditions at the relevant time point. *, p<0.05 compared to day 4 control; #, p<0.05 compared to day 7 control.

**The biodistribution of locally delivered ZA**

It was hypothesized that sequestration of bisphosphonate by HA was a key mechanism underlying the synergistic effects of ZA+HA on BMP-2 induced bone. This effect was further investigated using fluorescently-labelled Pamidronate to track the bio-distribution of bisphosphonates after implantation. Equal amounts of AlexaPam and $^{14}$C-ZA were implanted with 5 µg BMP-2 in SAIB/2% HA. Two doses of 0.1 µg AlexaPam and $^{14}$C-ZA and 1 µg Alexa-Pam and $^{14}$C-ZA were trialled. One group received a systemic dose of 0.1mg/kg Alexa-Pam and 0.1mg/kg $^{14}$C-ZA one week post-surgery. With the average size of the mice of 20 g, this equated to a systemic dose of ~2 µg of each bisphosphonate per mouse.
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Three weeks after implantation, the distribution of bisphosphonate in the ectopic bone pellet and the long bones (contralateral femora or tibiae) were examined by fluorescent confocal microscopy. Confocal images were taken from cross sections through centres of the pellets. A significant retention of AlexaPam in the SAIB/2% HA groups was observed but not for the SAIB alone groups (Figure 2.11). In the SAIB/2% HA groups the staining was shown to be focal, with retention at the pellet and on the borders of the local femur. A higher fluorescent signal was observed in the high bisphosphonate groups, although this was not quantified.

**Figure 2.11**: Equal amounts of fluorescently tagged pamidronate (AlexaPam) and radiolabelled Zoledronate were delivered in SAIB with and without 2% HA into the mouse quadriceps. Images are representatives, with the panels showing local delivery of the agents and the final panel shows bisphosphonates delivered by systemic injection (0.1 mg/kg, equivalent to ~2 µg per mouse) one
week following implantation of SAIB/BMP-2/HA. Images were taken from specimens bisected through the bone pellet and femur. Images show fluorescent AlexaPam (red) overlaid over background muscle autofluorescence (white). P = Pellet, F = femur, all panels at same magnification, scale bar indicates 1 mm.

MicroCT analysis of the proximal trabecular bone architecture was undertaken on the contralateral tibia as a measure of effects of systemic effects on bone turnover. The 0.1 µg bisphosphonate dose groups had comparable bone volumes to the control group. The 1 µg dose locally delivered bisphosphonate groups showed elevated bone volumes in the proximal tibia comparable to the 0.1 mg/kg systemic bisphosphonate dose (Figure 2.12A).

![Graph A and B](image)

**Figure 2.12:** The effects of locally delivered bisphosphonate from SAIB depots on the contralateral limb was investigated. A Quantified bone volume data of the proximal trabecular of the contralateral tibia from microCT. B $^{14}$C-ZA counts detected in the whole contralateral tibia

The contralateral tibiae were also examined for binding of $^{14}$C-ZA by scintillation counting. While $^{14}$C-ZA counts were only found to be significantly increased in the systemic dosing group (Figure 2.12B).
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2.12), analysis of AlexaPam distribution by fluorescent confocal microscopy showed evidence of bisphosphonate from the SAIB implant reaching the contralateral tibiae in the 1 µg dose locally delivered bisphosphonate groups (Figure 2.13).

![Figure 2.13](image)

**Figure 2.13:** Representative images of fluorescently labelled bisphosphonate detected in cross sections of the contralateral tibia. Images show fluorescent AlexaPam (red) overlaid over background muscle autofluorescence (white). Scale bar indicates 1 mm across all panels

**Investigations into alternative bioceramics**

Two alternate bioceramics, β-tricalcium phosphate (TCP) and Bioglass 45S5 (BG45S5), were selected as alternatives to HA in the SAIB/BMP-2/ZA system. Both TCP and BG45S5 are commonly used as bone void fillers and substitutes in clinical orthopaedics. Initially, the capability for bisphosphonate binding was tested using fluorescent AlexaPam (Figure 2.14). AlexaPam was added to the ceramics, and imaged using fluorescent microscopy before and after being washed in water. In this assay, AlexaPam appeared to bind well to TCP and BG45S5 even after being washed.
Figure 2.14: Visualisation of binding shows fluorescent AlexaPam (red) present on the surface of TCP and BG45S5 particles, both before and after washing with water.

**TCP and BG45S5 were not able to significantly increase bone formation nor bind strongly with ZA**

The capacity of alternative bioceramics TCP and BG45S5 to enhance BMP-2/SAIB induced bone formation was measured in the murine muscle pouch model. Ectopic bone induced by 5 µg BMP-2 delivered by SAIB were quantified by microCT. The effects of incorporating 2% (w/v) TCP or BG45S5, ±1 µg ZA were assessed based on bone volume (Figure 2.15A).

TCP and TCP + ZA did not significantly influence the volume of bone produced, compared to SAIB/BMP-2 controls (-30%, p=0.2743; and +33%, p=0.3121 respectively). BG45S5 alone also did not augment bone formation (-43%, p=0.1728), however BG45S5+ZA led to an increase that was significant vs BG45S5 alone (p<0.05), but not when compared SAIB/BMP-2 controls. X-ray
images of representative samples with the median bone volume values per group are shown in Figure 2.15B. Strikingly, the addition of TCP or BG45S5 delivered in SAIB/BMP-2 did not generate significant increases in bone formation when compared to SAIB/BMP-2 alone, unlike HA which alone resulted in a 125% increase (Figure 2.5).

![Figure 2.15](image)

**Figure 2.15:** The bone volume (BV) formed in the ectopic mouse muscle pouch model in response to the addition of 5 µg BMP-2 is not significantly increased by the addition of either 2% w/v TCP or BG45S5 particles, even with the addition of 1 µg ZA. A Bone volume of the ectopic nodules quantified by microCT, B Representative X-rays from each group.*, p < 0.05.

The co-delivery of the fluorescently labelled AlexaPam within the SAIB/BMP-2/ZA system allowed for the study of bisphosphate retention by TCP and BG45S5 after three weeks *in vivo*. In stark contrast to the effects seen with HA (Figure 2.11), there was limited AlexaPam binding to either TCP or BG45S5 in the bone nodules (Figure 2.16). The limited fluorescent signal seen in these samples led to speculation that the limited synergy seen was due to a poor affinity of these ceramics to ZA.
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Figure 2.16: Confocal imaging of fluorescently tagged bisphosphonate co-delivered with the alternative bioceramics TCP and BG45S5 in BMP-2 induced bone nodules. Images show fluorescent AlexaPam (red) overlaid over background muscle autofluorescence (white).

The effects of adding TCP or BG45S5 on SAIB/BMP-2 induced bone formation were significantly less than those previous seen with HA (Figure 2.15). TCP in particular showed a lack of synergy with ZA. It was previously demonstrated that HA could bind and sequester ~90% of the added ZA (Figure 2.7B), so comparable $^{14}$C-ZA binding assays were undertaken with TCP and BG45S5. Following incubation with 50 µM $^{14}$C-ZA, the ceramics TCP and BG45S5 bound 12% and 6% respectively (Figure 2.17A).

As a functional test for bisphosphonate sequestration, the ZA-mediated cytotoxicity cell culture assay was utilised. MC3T3 cells were cultured in the presence or absence of 50µM ZA, with and without TCP or BG45S5. It was previously found that the addition of 2% HA was able to rescue MC3T3 cells from ZA mediated cytotoxicity (Figure 2.10). In wells without ZA, the addition of TCP alone led to a 33% reduction in the numbers of viable cells compared to untreated controls, and BG45S5 led to a 27% reduction (Figure 2.17B). In wells with 50µM ZA, considerable cell death was seen at day 7. TCP particles led to no rescue of the 50µM ZA treated cells, consistent
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with $^{14}$C-ZA binding data. BG45S5 particles showed a trend towards increased cell survival with ZA treatment. However, data interpretation was confounded by the decreased cell numbers seen with BG45S5 alone.

Figure 2.17: *In vitro* binding assays of TCP and BG45S5 reveal weaker binding than previously seen with HA. The ceramics TCP and BG45S5 show A decreased $^{14}$C-ZA measured as bound, and B limited ability to protect cells against ZA mediated cytotoxicity.
Discussion

The work in this chapter describes the first use of the high viscosity material SAIB as a delivery system for BMPs in an orthopaedic context. Bone tissue engineering carriers are required to deliver BMP-2, create a potential space for new bone formation, support cell proliferation, as well as provide an environment for nutrient, oxygen and waste transfer.

When compared to a porous collagen sponge, the current clinical standard of BMP-2 delivery, SAIB exhibited simpler implantation and superior ectopic bone volume formation (Figure 2.3A). It is hypothesised that SAIB may have resulted in more bone formation due to a different BMP-2 release profile than collagen. It has been reported that SAIB releases drugs in a burst release fashion [253], while BMP-2 adsorbs onto collagen resulting in a more sustained release [231]. In a study investigating various formulations involving BMP-2 delivered on collagen pellets, it was found that the BMP-2 release profile has an effect on ectopic bone formation [404]. They suggested that the most effective profile was that of an initial burst with sustained release. It may be that our SAIB more fits this profile than the collagen sponge. Infection is a major issue for many situations where BMP-2 is currently used. The use of a collagen sponge requires the creation of an open wound to deliver BMP-2, which carries with it the risk of infection [129]. An injectable scaffold could mitigate this risk.

The avoidance of open wounds associated with the SAIB construct also has practical benefits with respect to bone drug screening in animal models. Compared to an injectable matrix, the implantation of a solid scaffold in an animal model typically requires anaesthesia, increased surgical and recovery time, as well as more intensive post-surgical monitoring and a higher infection risk. It also eliminates the use of sutures in animal studies and subsequent re-suturing.
In these experiments, we demonstrated that injected SAIB scaffold could reliably act as a carrier for BMP-2. In the murine model, ectopic bone nodules were present after three weeks (Figure 2.3). In mouse models, 10 µg BMP-2 is a standard dose to induce robust formation of ectopic bone, when delivered via porous collagen [405]. However, bone growth has been seen with as little as 1 µg of BMP-2 on a collagen sponge in a mouse [204]. For alternative delivery systems such as poly-(α hydroxy acid) polymers, higher doses are required [406]. In a mouse, SAIB was shown to result in bone nodule formation with as little as 2 µg BMP-2, and bone volume was increased in a BMP-2 dose dependent manner.

For an equivalent dose of BMP-2, SAIB delivery increased the resultant bone by up to 3-fold when compared to collagen. While there was no significant difference between bone volume formation between the commercially purchased food grade SAIB and the laboratory synthesized version, there appeared to be a trend towards increased bone formation in the FG SAIB. This may be attributed to residual solvents in the laboratory synthesized SAIB, which will require further optimization of the process. However, the success of this in-house manufactured SAIB further validates the synthesis process.

Injected BMP-2/SAIB offers limited control over the shape of the resultant bone formation. In the mouse heterotrophic ossification model, the bone nodules that formed were highly irregular and likely affected by the forces they receive within the muscle (Figure 2.3B). If a regular shape is required, some method of containing the injected SAIB may be required.

The development of SAIB construct in this study has allowed for high throughput screening of putative adjunctive agents. In regards to its use as a drug screen, the use of SAIB in the hind limb muscle pouch fulfils the five essential criteria described in the introduction. The assay is relevant as the bone is induced within the muscle, which is a similar environment to where bone is normally
Chapter 2: Development of SAIB for bone tissue engineering

contained. The assay also effectively highlighted the most promising candidates for further study, as only five out of nine candidates resulted in significant increases in bone volume when compared to control. The ectopic muscle pouch was also a fast assay in a few respects. The use of SAIB as a delivery system meant that it was quick to prepare, as agents were simply required to be mixed in prior to delivery. Our group has previously described a solid, polymer delivery system that required up to a week for manufacture [400, 406]. The surgical injection of the SAIB mixtures was also fast, as mice were anaesthetised using Isofluorane gas so their recovery was also expedited when compared to surgical implantation under full anaesthetics. The muscle pouch model was robust, as a wide range of drugs was able to be tested as well as ceramic nanoparticles. The screen was also accurate and reliable, with highly significant results produced that were able to be replicated. This construct has also enabled a reduction in the dose of BMP-2 required, increasing the economy of these studies. Previously, a dose of 10 µg BMP-2 per pellet was used to generate bone formation [400, 406].

In screening of potential adjunctive agents to BMP-2, significant increases in bone formation were found with the addition of the MEK inhibitor PD0325901, the Cathepsin K inhibitor AFG495, and HA, ZA, and their combination (Figure 2.5). Following this drug screen, the combination of HA and ZA was chosen for further optimisation as it increased bone volume by the greatest magnitude. The performance of other adjunctive agents may be further improved by dose optimization, although in most cases the doses used were maximal as limited by the loading capacity of the SAIB carrier.

While the addition of HA to the formulation in combination with ZA resulted in the greatest synergy (+900%), on its own it still resulted in a +125% increase in bone formation (Figure 2.5). In part, this response can be explained by the fact that HA may act as a nidus for bone formation.
Chapter 2: Development of SAIB for bone tissue engineering

HA is highly osteoconductive, and induces appositional bone growth, where osteoid is laid down directly onto the mineral surface [407]. This effect can be seen in the increase in bone formation in the HA treatment group. However, it is hypothesized that HA also acts to sequester locally delivered bisphosphonate, retaining it for action at the local site. It has been noted that locally delivered bisphosphonate has an inhibitory effect on bone healing at high doses [408]. Thus, this sequestration reduces the local unwanted cytotoxic effects of the bisphosphonate on local vascular and osteogenic cells, while targeting its action to osteoclasts.

An in vitro binding assay using radiolabelled ZA revealed that ZA binds to HA immediately and at a constant rate (Figure 2.7). Thus, the methods of the previous in vivo experiment would have been sufficient to bind 90% of the available ZA to the 2% HA that was added. Further, when this binding was examined in cell culture, it was found that HA was able to rescue ZA mediated cell death (Figure 2.10). It appears that the strong affinity of ZA for HA is able to protect against its cytotoxic effects to surrounding cells. This data further confirms previous data from this laboratory showing that when ZA has been bound to HA, it is toxic to only osteoclasts, and not osteoblasts [409].

This sequestration was investigated in vivo using fluorescently labelled bisphosphonate (Figure 2.11). Confocal images showed that after three weeks, bisphosphonate was only retained locally in the dual administered HA/bisphosphonate groups. Thus HA can sequester locally delivered bisphosphonate, as evidenced by our in vitro and in vivo biodistribution studies. This sequestration may improve the efficacy and availability of bisphosphonate at the desired site of action [410].

While HA acts to retain bisphosphonate at the local site, it does not totally prevent its release into the circulation (Figure 2.12, Figure 2.13). Although ZA was not detected using the $^{14}$C-ZA assay (Figure 2.12B), this could be due to insufficient specific activity of the radiolabelled agent or due
to subtle differences in affinity between the bisphosphonates. Despite the effects of locally delivered bisphosphonate on ectopic bone formation, in the absence of HA there is no evidence of high levels of bisphosphonate retention at the site. It is possible that these increases are due to low levels of bisphosphonate retention that are below levels of detection. This is in contrast to the high levels of retained bisphosphonate observed with HA co-delivery. Moreover, this is associated with the large increases in ectopic bone volume seen in these groups. It is clear that the combination of BMP-2, HA and ZA demonstrates strong synergy.

The use of BMP-2 in excessive doses can lead to side effects that range from pathological inflammation and/or heterotrophic ossification to premature bone resorption [129, 411, 412]. The ability to reduce the amount of BMP-2 needed may ameliorate these side effects without sacrificing the resulting bone formation. For the same dose of BMP-2, SAIB results in almost three times the bone production when compared to the current clinical standard for rhBMP-2 delivery (Figure 2.3). With the addition of some adjunctive agents, most notably the combination of HA and ZA, this bone formation is even further boosted (Figure 2.5). This is highly relevant clinically, as a reduction in BMP-2 needed for a procedure reduces the overall cost of the procedure, the potential side effects, as well as infection risk all whilst using currently approved agents.

It was hypothesised that the ceramics TCP and BG45S5 could be used as HA alternatives in the SAIB/BMP-2/ZA construct. However, the addition of TCP and BG led to no significant increases in bone volume in the murine model (Figure 2.15). This is in stark contrast to what was seen when HA was used in this system (Figure 2.5). There are conflicting studies within the literature on osteoinductive potential of β-TCP [413-415], but it is noteworthy that its degradation products can cause inflammation and impair subsequent bone formation [416]. Prior studies have indicated that BG is not highly osteoinductive de novo [417], but our study tested it in the context of a potent
Chapter 2: Development of SAIB for bone tissue engineering

BMP-2 bone formation scenario. Aside from the lack of increased bone seen with TCP and BG alone, neither bioceramic showed significant synergy in combination with a bisphosphonate. Imaging of the fluorescent bisphosphonate showed little to no retention of bisphosphonate after three weeks in vivo (Figure 2.16), leading to speculation that a lack of ZA binding may underlie this phenomenon.

The $^{14}$C-ZA binding assay is an extremely sensitive assay for the measuring the affinity of the interaction between the bisphosphonate and a bioceramic. Such assays can be challenging for ZA compared to other bisphosphonates due to the potency of ZA and the low concentrations it is used at. The binding $^{14}$C-ZA to TCP and BG microparticles (Figure 2.17A) was significantly less than previously observed for HA (Figure 2.7). Bisphosphonates have a high affinity for bone mineral and the nitrogen-containing bisphosphonates act by inhibiting the enzyme FPP synthase [284, 418, 419]. In these assays, HA showed significantly higher sequestration of $^{14}$C-ZA than TCP and BG particles. These data show that for ZA the “bone hook” component of the inhibitor shows a higher affinity for mineral intrinsic to bone (hydroxyapatite) than TCP and BG as bone graft substitutes.

This may be due to the fact that bisphosphonates were designed to bind strongly to bone mineral, which is composed of hydroxyapatite, rather than alternative ceramics. This finding has important implications for the uses of these bioceramics in conjunction with bisphosphonates. Nevertheless, it is acknowledged that this study has examined only a single bisphosphonate (ZA) this result would be strengthened by a comparison using other bisphosphonates.

The sequestration of ZA by HA was effectively demonstrated in a biological setting using a cell culture model where MC3T3-E1 osteoblasts are exposed to lethal doses of bisphosphonate (50 µM ZA). When HA is incubated in a transwell, it was able to bind ZA and prevent cell death (Figure 2.10). However, neither TCP nor BG were able to affect rescue in this assay system (Figure 2.17B).
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This finding is consistent with the low in vitro binding seen in the $^{14}\text{C-}Z\alpha$ assay, but the negative effects of the alternative bioceramics on cell survival in the absence of ZA were also notable. This may be due to the production of a basic extracellular environment as these bioceramics solubilized over time [420].
Chapter 2: Development of SAIB for bone tissue engineering

**Summary and conclusions**

In this chapter, I describe the novel use of the compound SAIB to deliver BMP-2 in a bone tissue engineering context. Utilising a mouse muscle pouch model, SAIB was shown to generate significantly more bone than collagen (the current clinical standard) for the same dose of BMP-2. This model was then used as a relatively high throughput screening tool for a variety of potential adjunctive agents. The formulation of SAIB/BMP-2/HA/ZA was shown to generate 10-fold more bone than BMP-2 in SAIB alone, and this was attributed to the binding between the HA and ZA. It should be noted that it is extremely rare to find synergies that result in such immense fold changes in bone volume, even in the low order model of the mouse. Thus, the use of SAIB could then be considered as a carrier for delivering BMP-2 in a range of functional models of bone healing. The SAIB carrier was compared with collagen sponge delivery of BMP-2 in standard models, and was further tested in novel settings that are unachievable for a solid scaffold.

Although the SAIB formulation has been optimised to maximise bone formation for a given dose of BMP-2 (SAIB/BMP-2/HA/ZA), this formulation may be not applicable or appropriate for all bone healing scenarios. In these instances, the SAIB formulation could be tailored to the needs of the bone regeneration. For example, the injectable nature of SAIB allows for delivery to epiphyseal or metaphyseal regions of bone, areas that are currently inaccessible to solid BMP-2 delivery systems. In these instances, it may be appropriate to omit the HA component of the formulation as surrounding trabecular bone can bind the ZA, as well as allowing for easier delivery and distribution within the bone structures.
Chapter 3. Intraosseous injections of SAIB containing BMP-2 to improve bone strength in a mouse model of Osteogenesis Imperfecta.
Chapter 3: Intraosseous injections of SAIB for OI

**Introduction**

*Bisphosphonate treatment for Osteogenesis Imperfecta*

As noted previously, Osteogenesis Imperfecta (OI) is a genetic disorder that involves low bone mass and increased bone fragility [95]. OI commonly involves a mutation in the gene that codes for collagen type 1 (COL1A1 or COL1A2) [98, 99]. These mutations disrupt the normal entwining of the collagen triple helix, and affected cells produce a mixture of normal and abnormal collagen resulting in a variable phenotype [421, 422]. The bone from patients of OI has a higher average mineralisation density [423]. The long bones are also smaller and have thinner cortices than age matched controls, and this has been attributed to poor periosteal bone formation [424]. The rate of bone turnover rate is increased, due to both an increase in bone resorption by osteoclasts, as well as an increased bone formation rate despite decreased activity of individual osteoblasts [425]. The bone fragility in OI is due to a combination of poor quality bone in combination with a secondary osteoporosis [105].

Currently, the treatment of sufferers of OI focus on improving functional outcomes and ability, such as ambulation by improving joint motion and muscle strength, and reducing fracture risk [426, 427]. Physical therapy, rehabilitation and orthopaedic surgery are used in combination to improve patient quality of life. Physical exercises are encouraged to prevent contractures and disuse osteopenia [427]. Implantation of intramedullary rods assist in straightening the femora and tibiae, encouraging standing and walking, as well as reducing further fracture risk [97, 427, 428].

The treatment approaches involving physical therapy and surgery aim to improve quality of life outcomes, but do not address the fragility of bone in patients with OI. Currently, the use of systemic bisphosphonates is the only widely used strategy employed, and is considered the standard of care.
Chapter 3: Intraosseous injections of SAIB for OI

[97, 112]. While the quality of bone cannot be improved through this method, the risk of fracture can be decreased by improving the bone mass of OI patients. Bisphosphonate use inhibits osteoclastic action, preventing the increased bone resorption that is characteristic of OI [429, 430]. Several trials have shown that systemic bisphosphonate treatment (including Pamidronate, Zoledronate, and Risedronate) have proven effective at preventing bone loss [107-111, 431]. Clinically, bisphosphonate use in children with moderate to severe OI has been promising, with reports of a decrease in chronic bone pain, enhanced sense of well-being, and increased grip strength [97, 432]. However, there are concerns of the effects of systemic bisphosphonate treatment on the growing skeleton. Bisphosphonate treatment has been shown to delay the healing of osteotomy in children with OI [433]. In addition, bisphosphonate treatment results in cortical thickening through reducing endosteal resorption rather than periosteal bone formation [434]. While this increase in cortical thickness is beneficial, it does not further increase the bone’s size with the child’s growth. Aside from bisphosphonates, there are emerging anti-resorption agents from the osteoporosis field that are of interest to OI. The RANK ligand antibody Denosumab has shown reduced fracture rates and increased bone mineral density in a subset of recessive OI patients [435, 436]. However much like bisphosphonates, these anti-resorption therapies are unlikely to improve bone formation on the periosteal surface.

The use of anabolic treatments to promote new bone formation in OI has also been explored. The use of recombinant human growth hormone has been proposed as a method of improving bone strength and stature [437]. The administration of growth hormone in children with OI showed increased height velocity and bone density in some small studies [438, 439]. Histomorphometry of bone biopsies showed increased bone turnover [439], and this is likely not appropriate in a condition of high bone turnover [425]. The use of recombinant human parathyroid hormone
Chapter 3: Intraosseous injections of SAIB for OI

(Teriparatide) has shown promising increases in bone volume and thickness in adults with osteoporosis [440]. Teriparatide has shown some anabolic effect in adults with OI [441, 442], but use in children is not recommended due to worries of osteosarcoma [443]. Ultimately, gene and cell based therapies that replace or restore defective collagen would be the ideal treatment strategy for OI. However, these methodologies are still in their infancy, and there is still potential for treatment of OI that involves currently approved techniques.

In 2013, Seeherman et al reported that a single intraosseous injection of BMP-2 in a calcium phosphate matrix into the femoral neck of non-human primates resulted in the formation of endosteal and periosteal bone [444]. The appositional bone growth on the periosteal surface of the femoral cortical bone was present despite chronic ovariectomy, which mimics post-menopausal bone loss in humans. Ovariectomy induced osteopenia is the result of increased bone turnover, and increased osteoclastic activity [445, 446], and increased bone turnover is also seen in patients who suffer OI [425].

Increasing periosteal bone formation is of great interest in conditions that involve weak bones, such as those seen in OI. Treatments that act on the periosteal surface would increase bone formation, as well as increasing bone size in children. Increasing bone on the outer surface of the cortex increases the value of a material property known as moment of inertia. A low moment of inertia is associated with increased fracture risk, and vice versa [447, 448]. Therefore, there is interest in treatments that attempt to shift bone mass to the periosteal surface of bone, that in turn increase the moment of inertia, and reduce the risk of fracture. Thus, this study aimed to utilise SAIB to deliver BMP-2 intraosseously in a transgenic mouse model of OI in order to induce periosteal bone formation.
**The challenges of using transgenic mouse models of OI**

Heritable human diseases can be studied by creating transgenic mouse models that carry similar genotypes and phenotypes as their human counterparts. The BrtlIV mouse, which carries a G349C substitution in the COL1A1 gene, is a model of OI type IV [449, 450]. These mice carry a moderate to severe phenotype, with a variability that ranges from perinatal fatality to full life spans [449]. The other mouse of note is the oim, which carries a mutation in the COLIA2 gene [451-453]. This naturally occurring mutation displays a moderate to severe phenotype, and homozygous mice presentation clinical features consistent with Type III OI [451]. However, the oim mouse has been found to exhibit a very rarely observed mutation, with an atypical biochemical phenotype [454]. Both the oim and the BrtlIV mice display moderate to severe phenotypes, and are subject to fracture under normal laboratory and animal housing procedures [453, 455].

The transgenic OI mouse used for this study has a G610C mutation in the COL1A1 gene. This gene variant was chosen as it was carried by a large number of OI sufferers who belonged to an Old Order Amish community in Pennsylvania, USA [454], leading the mouse to be known as the Amish mouse. Phenotypically, both the human and mice have reduced bone mineral density, bone strength and body mass, consistent with a mild to moderate variant of OI (Type I/IV). The Amish OI mouse was chosen for this study because it has an autosomal dominant inheritance pattern, and a mild to moderate disease phenotype.

The Amish OI mouse has a mild to moderate disease phenotype, and requires significant care when handling to prevent bone fracture over the course of the study. This presents a challenge for breeding and studies involving this mouse, for instance, the study presented in this chapter. However, these challenges were addressed by small changes to handling procedures, as well as by close communication with the transgenic animal facility which housed these mice. All animal staff
Chapter 3: Intraosseous injections of SAIB for OI

were made aware of the phenotype of these mice, and OI mice were indicated as being fragile on cage cards, reducing the risk of fracture during handling and cage changing. Female OI mice were not used for breeding, to spare their bodies of the physical and metabolic burden of pregnancy. For systemic dosing, traditional restraint methods for subcutaneous administration could prove too rough for some mice. As a result, mice were generally placed under light anaesthesia using inhaled Isoflurane gas to reduce the risk of fracture during dosing. With these protocols in place, mice were able to be utilised for study without excessive distress or harm.
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Materials and Methods

*Transgenic OI mice*

Amish OI transgenic mice were sourced from John Bateman, at the Murdoch Children’s Research Institute. Mice were bred and housed in the Transgenic Facility on site. Mice were allowed *ad libitum* access to food and water. At 8 weeks, mice were transferred to the experimental room. Ethics was approved by the CHW/CMRI Animal Ethics Committee (K315).

*Mechanical testing of tibiae*

Tibia were mechanically testing by four-point bending to failure on an Instron 5944 (Massachusetts, USA), with data collected using BlueHill 3 software. Tibiae were harvested from female OI mice, aged between 12 and 16 weeks. Bones were stored at -80°C prior to testing, and allowed to thaw to room temperature prior to testing. Tibiae were positioned so that the medial side was resting across the bottom spans (Figure 3.1). The support span was 10 mm, and the upper span measured 5 mm. Samples were pre-loaded at 0.25 mm/min until a load of 1N was reached, at which point the loading rate increased to 0.5 mm/min until failure.
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![Image of four point bend testing jig for mouse tibiae on the Instron 5944. Tibiae were aligned so that the medial side of the bone rests across the bottom span (10 mm), and the top span (5 mm) came to rest across the lateral surface.](image)

**Figure 3.1:** Four point bend testing jig for mouse tibiae on the Instron 5944. Tibiae were aligned so that the medial side of the bone rests across the bottom span (10 mm), and the top span (5 mm) came to rest across the lateral surface.

**Surgical procedure for intraosseous injections**

At the time of surgery, mice were anaesthetised with by 35 mg/kg Ketamine and 0.5 mg/kg Xylazine. The operative site was shaved and wiped with povidine-iodine solution. The right tibias of the experimental mice are initially reamed from the proximal end using a 27 G needle and flushed with saline, then the tibial cavity is filled with a radiopaque dye (Ultravist 240; Bayer Health Care, NSW, Australia). The presence of this dye at the distal end of the medullary cavity is then confirmed by intraoperative X-ray (Figure 3.2). Reamed tibias were then given intraosseous injections of either saline, or 5 µg BMP-2 in 20 µL 80:20 SAIB:ethanol and X-rayed once more. The absence of the contrast agent indicated that the cavity was now filled with treatment solutions. Mice were subcutaneously dosed with 10 mg/kg calcein (Sigma, NSW, Australia) at day -10 and -3 before cull. Mice were culled 2 weeks after surgery.
Figure 3.2: Intraoperative X-rays which demonstrate the effective reaming and flushing of the tibia, where contrast agent is replaced with intraosseous injections. Note the increased opacity of the medullary canal (arrowheads) all the way down to the ankle (arrow) when the contrast agent is present.

For this study, there are three experimental groups for each genotype. One group received no surgical interventions, and was called the Nil treatment group. The remaining two groups underwent surgery, and were subject to tibial reaming, and received intraosseous injections of either saline (Sham) or SAIB containing BMP-2 (BMP-2).
Table 3.1: Experimental design for investigating the effect of intraosseous injections of SAIB containing BMP-2 in OI mice, and their WT littermates.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>WT Mice</th>
<th>OI Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Sham injection</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>SAIB/BMP-2 injection</td>
<td>8</td>
<td>7</td>
</tr>
</tbody>
</table>

**MicroCT Analysis**

After fixation in 4% paraformaldehyde, samples were stored in 70% ethanol. Samples then underwent microCT scanning on a SkyScan 1174, using standard settings described earlier (Chapter 2), and a pixel resolution of 9.4 µm. Analysis of scans focused on a region of interest (ROI) that is shown in Figure 3.3. This ROI focused on the mid diaphysis of the tibia, but was distant from the surgical site at the proximal tibia. Once the ROIs were isolated on the microCT scan, multi-level thresholding was used to select only low density new bone on the periosteal surface.
**Figure 3.3:** MicroCT analysis of new periosteal bone formation in response to reaming and intraosseous injections. **A** Schematic showing the region of interest (ROI) that was chosen; a 1.2mm region located 0.5mm above the tibial-fibular junction. **B** A representative microCT slice showing a cross section through an ROI. **C** The same microCT slice showing how CTAn software applies a new ROI selecting only new periosteal bone for quantification.

**Confocal imaging of calcein dosing**

Tibia were then bisected in coronal plane using a diamond saw, in the upper ROI chosen for microCT analysis, then fine grit sandpaper was used to create a flat surface. The bone cross section imaged directly on a Leica SP5 confocal microscope (Wetzlar, Germany), with calcein detected using the 488 nm laser line and 500-560 nm emission bands. Image capture settings were retained for all samples. The bone surface was imaged using a 10x objective and composed using Tile Scan (four to nine images) and Z-stack functions (two to four slices). Maximum intensity projections were used to recreate the two dimensional bone surface.
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The raw confocal image had to be manipulated to prepare it for quantification. This was done in Photoshop (Version CS6), and the process is shown in Figure 3.4. The raw image is shown in A. To isolate the response on the periosteal surface, the endosteum and medullary canal is removed from the picture, and shown in B. At this stage, and large speckles and imaging artefact are removed. The image is then converted into grayscale, and shown in C. Finally, the image is then inverted for easier image analysis, as shown in D. For analysis of endosteal and medullary bone response, the analysis procedure was repeated with isolation of these areas in the original confocal images at stage B.

The inverted images were then imported into ImageJ for fluorescence quantification. A greyscale threshold value of 230 was used for all images, and the positive thresholded area quantified.

![Image manipulation process](image)

**Figure 3.4:** Image manipulation process for analysis of fluorescent calcein signal from a cross section of a tibia visualised by confocal microscopy. A Raw image. B Endosteal region removed, and manually despeckled. C Image in changed to a grayscale image. D Image is inverted, and ready for quantification in Image J.
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Results

Validation of the OI phenotype in bone

The Amish OI mice under our care were confirmed to have reduced bone strength when compared to their wild type littermates (Figure 3.5). Compared to WT, the OI tibia exhibited a significant 10.8% decrease in the maximum load to failure under four-point bending (p<0.01). However, no difference in stiffness was seen between the two genotypes. These results validated that the OI mice used for subsequent experiments display a marked bone weakness phenotype.

Figure 3.5: Validation of the weak bone phenotype in the OI transgenic mouse compared to their wild type littermates (WT). A Maximum load to failure of the tibia under four point bend testing. B Stiffness of the tibia under four point bend testing to failure. **, p < 0.01

X-rays taken at cull reveal no obvious bone formation

Two weeks after intraosseous injection into the tibiae, mice were culled. Gross X-ray of the whole tibia revealed no obvious signs of bone formation on the periosteal surfaces (Figure 3.6). The opacity of the medullary canals and thickness of the cortical bone in the distal tibiae appeared
consistent across treatment groups. However, the appearance of new bone close to the surgical site in some BMP treated samples were noted (indicated by arrow in Figure 3.6).

---

**Figure 3.6**: Representative X-rays of tibia receiving either no treatment (Nil), tibial reaming and either saline injections (Sham) or SAIB containing BMP-2 (BMP-2) in OI mice or their WT littermates. Arrow indicates the presence of bone formation near the surgical site.
**MicroCT analysis reveals new periosteal bone formation as a result of intraosseous injections**

MicroCT was used to quantify periosteal bone in the chosen ROI. Reconstructions of this ROI from the microCT scans revealed that new periosteal bone could be detected in Sham and BMP-2 treated tibia (Figure 3.7). When the periosteal bone was quantified, the trends between the treatment groups was mirrored in the two genotypes (Figure 3.8). Sham treatment significantly increased the measured periosteal bone compared to Nil treatment, in the WT (+110%; p<0.01) and the OI (+54%; p<0.01). Intraosseous BMP-2 significantly increased periosteal bone compared to Nil treatment, in the WT (+179%; p<0.01) and OI (+89%, p<0.01) genotypes. There was an increase in periosteal bone between the Sham and BMP-2 treatment, however this did not reach significance.

![MicroCT Reconstructions](image)

**Figure 3.7:** MicroCT reconstructions of representative samples taken from the upper ROI of tibia.
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Figure 3.8: Periosteal bone formed in the tibial ROI following intraosseous injection with either saline (Sham) or SAIB containing BMP-2 (BMP-2) as measured by microCT.* p<0.05; ** p<0.01

The perimeter of the tibia in the ROI was also examined using microCT analysis. When the average perimeter of the bone over the ROI was quantified both Sham and BMP-2 treatment was able to significantly increase the perimeter of the tibia in the WT mice (Sham +17%, BMP-2 +12%; p<0.01 for all) (Figure 3.9). This same trend was not seen in the OI groups, which all had a comparable perimeter.
Figure 3.9: The mean total cross sectional perimeter of the tibia in the ROI as measured by microCT. *, p<0.05; **, p<0.01

**Calcein quantification reveals new periosteal bone formation in the tibia**

Calcein was administered subcutaneously at two time points during the study to allow measurement of dynamic bone formation. Initial plans to calculate the mineral apposition rate, as per standard protocol, were disrupted by the greater than expected periosteal response that resulted in callus-like woven bone formation on the outer surface of the cortex (Figure 3.10). Thus, the total Calcein-labelled area was quantified as a measure of new bone formation. The images taken were manually despeckled, and divided into either periosteal region or endosteal and medullary region.
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Figure 3.10: Representative confocal images showing calcein labelling on a tibial cross-section highlights the increase in signal seen in the treatment groups.

In the periosteal region, there was significantly more fluorescent label detected when comparing the WT Nil treatment to the Sham (+2328%, p<0.01) and BMP-2 (+3262%, p<0.01) (Figure 3.11). This trend was also seen in the OIs, which showed a significant increase in the Sham (+1420%, p<0.01) and BMP-2 (+2452%, p<0.01) treated groups compared to the Nil.
Figure 3.11: Significantly more fluorescent calcein was detected on the periosteal surface of tibae treated with either Sham or BMP-2, than Nil. Confocal imaging was used to capture calcein signal on cross-sections of tibae, which was quantified in ImageJ as fluorescent area. *, p<0.05; **, p<0.01.

When the endosteal and medullary compartment was analysed, a different trend was seen (Figure 3.12). The highest level of calcein signal was detected in the Sham treated groups of both genotypes, which showed increases of +175% (N.S.) and +1004% (p<0.05) for WT and OI respectively when compared to Nil treatment. In the WT mice, BMP-2 treatment actually led to a 57% reduction in calcein signal detected when compared to Nil treatment (p < 0.05). However, in the OI mice, BMP-2 treatment led to an increase in fluorescence detection (+441%) that did not reach significance.
Figure 3.12: An increase in calcein signal is seen on the endosteal surface and medullary canal in the Sham treated group compared to Nil and BMP-2 treated. Confocal imaging was used to capture the fluorescent signal, and ImageJ quantified the area of fluorescent signal in this region. *, p<0.05; **, p<0.01.
Discussion

The bone response to reaming

In the two mouse genotypes studied, intramedullary reaming resulted in an increase in new periosteal bone formation in both ROIs studied, as measured by microCT and Calcein labelling. Clinically, the reaming of long bones is a method that can be used alongside the implantation of an intramedullary nail for fixation of diaphyseal fractures. There has been some controversy in the literature regarding the contribution of reaming to intramedullary fixation, as it has been associated with thermal necrosis [456], fat embolism [457, 458], and pulmonary complications [459]. However, reaming and intramedullary nail exchange has been proposed as a technique for the treatment of aseptic non-union fractures [460-463]. The mechanism of new bone formation in this procedure is proposed to be due to reaming products, as reaming can mobilise cancellous bone from inside the medullary canal [464], as well multipotent progenitor cells [465, 466] and growth factors [467].

Our laboratory has previously published work using this reaming model in mice, which revealed a robust endosteal, but not periosteal, bone formation response at 10 days post-surgery [406]. As shown in Figure 3.6, new periosteal bone was not easily detectable on X-ray even after two weeks. Thus responses may have been overlooked in this previous study. In the literature the periosteal bone formation in response to reaming has been noted in a range of animal models in the absence of intramedullary nailing [468, 469]. In these studies, the bone formation was hypothesized to be in response to bone marrow products being moved under the periosteum [470]. While reaming causes injury to the endosteal surface and blood supply, it greatly increases the periosteal blood flow [471, 472]. It may be that the marrow products moving to the periosteal surface, as well as
the increased blood flow increasing the contact of progenitor cells to the periosteal space result in
the periosteal bone formation in Sham treated animals.

A possible counter-hypothesis is that reaming may result in biophysical strain sensed by the
osteocytes, which is translated into biochemical signals. Osteocytes constitute 90 to 95% of the
cellular component of mature bone, and are characterised by highly interconnected dendritic
processes that span bone via canaliculi [473]. Osteocytes have critical roles in regulating bone
remodelling and maintaining the bone matrix, and they do this by transforming chemical and
mechanical stimuli into cell signals that control osteoblast and osteoclast activity [474]. Osteocytes
can sense mechanical signals directly as forces through the bone matrix [475, 476], or indirectly
through fluid pressure [477] and shear stresses [478] accrued from load induced fluid flow [479].

Reaming can result in extensive increases in mechanical strain on the bone, and the injection of
either saline of SAIB/BMP-2 may increase the fluid pressure in the lacunar-canalliculi channels.
The periosteal response may be mediated by osteocytes signalling to the osteoblasts to increase
bone volume on the outer surface, in an attempt to improve the bone’s resistance to strain. This
hypothesis would be experimentally challenging to test using the current mouse models in this
laboratory. However, there has been a range of inducible transgenic mouse models reported in the
literature that affect osteocyte signalling, which could be used to test the response to reaming.
These include mice with inducible and conditional knock outs in the gap junction protein
Connexin43 [480], the Wnt co-receptor Lrp5 [481], the metalloproteinase MT1-MMP [482], as
well as controlled ablation of osteocytes [483]. While these lines show dysregulated
mechanotransduction, the responses to loading are often inconsistent between the models. Thus it
remains challenging to select the most appropriate model(s) for investigation in the context of
reaming.
Chapter 3: Intraosseous injections of SAIB for OI

**Effects of intramedullary BMP-2 treatment**

It was initially hypothesized that intramedullary injection of BMP-2 would lead to increased periosteal bone formation as previously reported in a primate model [444]. In our study Sham animals undergoing reaming without BMP-2 was an internal control. Contrary to the expected result, the BMP-2 injection group showed no significant increase in periosteal bone volume compared to the Sham group.

However, secondary measures did suggest that the BMP-2 did produce a local effect. In Sham treated tibiae, the periosteal bone formation was close to the bone surface (Figure 3.10). This appears to be appositional bone formation that has grown just offset from the periosteal bone surface, similar to the prior literature [470]. In the BMP-2 treated groups, the bone appeared more like woven bone or an un-remodelled hard callus. Thus it is possible that the mechanisms behind the additional bone formation may differ between the groups, with reaming resulting in increased appositional bone growth, and BMP-2 resulting in new endochondral bone around the existing bone midshaft. Additional studies looking at earlier time points would be required to test this hypothesis to look for a cartilage intermediate. Later studies looking at subsequent remodelling may also be instructive. Further, bone formation in the medullary canal differed between the Sham and BMP-2 treated bones, which may be due to BMP-2 mediated resorption [484]. Additional studies investigating the effect of bisphosphonate treatment could confirm this hypothesis.
Methods of measuring dynamic bone growth

One method to measure dynamic bone growth is through the histological analysis of fluorescent bone markers that were administered during the study. The discovery in the 1950s that the antibiotic tetracycline binds to bone, and can be studied using fluorescent microscopy allowed scientists to investigate *in vivo* bone formation dynamics [485, 486]. Currently, the standard protocol in this laboratory involves the subcutaneous administration of the marker Calcein at set time points [487, 488]. Post-harvest, the samples are fixed and embedded and processed for resin histology in a practice that can take up to three months. Sections must then be cut for each sample, which are then analysed by hand using specialised analytical software (Bioquant measure 32 Nova Prime; TN, USA) connected to a fluorescent microscope.

For this thesis, a more efficient system for analysing calcein labelling was attempted. In this process, samples were fixed and then bisected in the plane of interest using a diamond saw. Samples were then laid down on a glass dish and imaged directly using confocal microscopy. These images are saved, and analysed later using Image J. For this method, it was assumed that the confocal microscope would be able to take an accurate image of an intact sample. Although, unlike a uniformly thick microtome sectioned slide, sometimes the samples would not sit flush with the dish and a Z-stack was utilised to compensate. In addition, the image capture is permanent, and reanalysis of images is possible without further imaging. It was also assumed that ImageJ was an adequate software package to analyse the fluorescent signals, as only total fluorescent area was required.

This process of imaging greatly reduces the time between sample harvest and first possible image down to a few days, compared to a few months for resin based methods. While this process does
Chapter 3: Intraosseous injections of SAIB for OI

not present a novel approach to imaging or analysis, it has adapted currently available technology
to accelerate analysis of bone labelling. This protocol has now been accepted within the laboratory.
Summary and conclusions

In this chapter, SAIB was found to be a feasible method of delivering BMP-2 into the medullary canal. At the time point studied, reaming with and without SAIB/BMP-2 was found to increase the generation of new periosteal bone in the tibia of both WT and OI mice. Further study involving mechanical testing would be required to verify if this periosteal bone correlated with increased resistance to loading. However, SAIB proved capable of delivering BMP-2 in a minimally invasive method, to a site unavailable through currently approved delivery methods.

Clinically, reaming is not typically utilised in isolation as a method of building bone mass, but rather in additional to intramedullary fixation, or as a side effect of bone marrow harvest. Thus, there is limited scope for speculation as to whether reaming alone or in combination with BMP-2 would suffice as a beneficial treatment option in the context of OI. However, as one surgical treatment option to assist in ambulation in OI patients is the insertion of nails into the long bones of the lower limb, the decision to ream may be beneficial.
Chapter 4. The use of the SAIB carrier to treat a challenging model of bone healing in rats

Work from this chapter has been published in:


Introduction

Challenges of upscaling

One of the challenges in BMP, and most biologics, research is the extrapolation of the dose required for clinical efficacy. Animal research is critical in demonstrating the effectiveness of a treatment, but there is a well-established steepening of the dose-response curve with the progression into higher order animals [58]. The conventional research movement from small animals, such as mice, into larger animals, such as pigs and sheep, allow for screening of treatment strategies that do not show translational potential. This results in a pyramid-like schematic of effective treatments, as unsuccessful approaches do not advance into the next higher animal (Figure 4.1). With the goal of translation into humans, the transition and success of a treatment into a higher animal is critical step.

Figure 4.1: Successful treatment strategies are reduced as you move into higher order animals, and finally humans.
Clinically, BMPs are applied at supraphysiological doses, which are orders of magnitude greater than found endogenously. These high BMP doses are an attempt to mimic the effect sizes seen in previous animal models. Small animals, especially mice, have a lower osteogenic threshold, and respond readily to BMP treatment (Chapter 2). Movement into larger animals is associated with an increased dose of the BMP required to necessitate the same effect. One study found that more than 200-times more BMP was required to generate heterotrophic bone in non-human primates than rats [489]. The supraphysiological doses that need to be used in humans may also be due to suboptimal release kinetics from the carrier.

Practically, upscaling into larger animal models also has some advantages. SAIB experimentation so far in mice has been limited by their small size, restricting the needle gauges and injection volumes that can be used. Difficulty was experienced in injecting some of the SAIB compositions seen in Chapter 2, especially those that contained ceramic particles. The transition into rat models allows for the use of larger gauge needles than those used in mice, allowing for the easier injection of ceramic particles. In addition, it was hypothesized that the large bone volume variability seen in some of the mouse studies, in particular those groups that included HA (Figure 2.5), could be attributed to the difficulty in injecting small volumes. With their larger size, rats also allow for injection of larger volumes of SAIB than can be used in mice, hypothetically reducing the variability seen in these models so far.

**Open fracture treatments**

Open fractures can be challenging to treat, occurring when the broken ends of the bone are exposed through the skin, and the associated periosteal disruption and soft tissue injury. Acute treatment
strategies aim to reduce the fracture, and prevent infection through surgical debridement and intravenous antibiotics [73]. However, due to the periosteal and soft tissue injury, open fractures are highly likely to go on to delayed or non-union. Delayed union has been reported to occur in up to 4.4% of all fractures [82], but when considering open fractures alone this rate increases dramatically. In the presence of considerable soft tissue injury, the rate of delayed union from open fractures can be 13-16% [490, 491], and when extensive vascular injury is involved the rate of delayed union has been as reported to be as high as 46% [492]. A non-union, where re-operation is required to achieve healing, can be highly debilitating for the patient, leading to substantial pain and disability, and resulting in substantial healthcare costs [88].

Since 2004, BMP-2 has been offered as an alternative to bone grafting for the treatment of acute open tibial fractures to prevent the occurrence of non-unions [148]. The initial randomized clinical trial for treatment of open tibial fractures with BMP-2 reported accelerated healing and a reduction in secondary interventions compared to the standard of care [157]. This study subsequently received criticism as the randomized groups had a statistically significant difference in the use of reamed nails. A follow up trial by Aro et al. where all fractures received reamed nails reported no acceleration in healing rates in BMP-2 treated acute open tibial fractures and an increase in infection rate [159]. A Cochrane review on the use of BMPs in adult fracture healing suggested that the economic gain of BMP use was favorable only in the most severe fractures [160]. Further, one study found that costs of autograft and BMP treatment for non-union were comparable, but the main cost of BMP treatment was in the protein itself [493].

Clinically, BMP-2 is delivered to the fracture site adsorbed onto a collagen sponge. As previously mentioned in Chapter 1, one of the main issues with collagen sponge use arises from its derivation from animal tissues, and its accompanying costs, variability [222], and disease transfer risks [220].
Chapter 4: Treating open fractures with SAIB and BMP-2

There have also been concerns raised in the literature on the lack of mechanical stability of wetted collagen, and potential loss of BMP-2 solution through handling [230, 231]. In order to generate bone, the levels of BMP-2 delivered must surpass the osteogenic threshold in the local environment, and ideally sustain delivery over a period of time [494]. Collagen sponge delivers BMP-2 in a burst release upon initial implantation [495], requiring implantation of higher doses than required to initiate bone formation. Thus, there is a potential benefit in developing alternative synthetic carriers for BMP-2 that avoid the use of animal derived proteins, have improved usability and handling properties, reduces the required BMP-2 dose, and the opportunity for minimally invasive delivery even during open surgery.

In this study, the use of SAIB to deliver BMP-2 was investigated in the rat. Initially, an ectopic hind-limb muscle pouch model is used to generate a dose-response to BMP-2 in SAIB. Following this, the ability to replicate the synergy of the optimal SAIB formulation (SAIB/BMP-2/HA/ZA) found in mice was examined in the same muscle pouch model. Two particle sizes of HA, nano and micro, were tested in the SAIB/BMP-2/ZA system to examine if HA particle size would also need to be increased in response to upscaling. Once an optimised SAIB formulation was found for the rat, the feasibility of this system to deliver BMP-2 to an established open fracture model was then tested. Finally, the effectiveness of the SAIB/BMP-2 system in treating the open fracture model was compared to the current clinically standard, collagen sponge.

**Animal models of non-union**

In order to study potential therapeutic approaches to the treatment of non-unions, a reliable animal model must be generated. There have been many factors that have been attributed to the formation
of non-union fractures. The severity of an open fracture, the energy absorbed during the injury, the patient’s general health and infection have all been reported to contribute to delayed union [82]. However, it is most likely that it is a combination of factors that lead to the delayed union of a fracture. Animal models of non-union are largely generated via surgical trauma. While challenging due to their small size, mouse models have been developed. Segmental defects [496], periosteal cauterisation [497], unstabilised fixation [498], and hind limb ischaemia [499] have all been used to generate non-union fractures in mice. Similar methods have been utilised in rats, with segmental defects [500], periosteal cauterisation [501], and unstabilised fixation [502] recorded in the literature.

Clinically, stripping of the periosteum membrane as a result of either the initial injury or during surgical fixation is a factor that contributes to non-union [503]. In animal models, periosteal stripping is a relatively simple addition to an open fracture surgery that has been shown to lead to non-union [336, 504]. In this laboratory, periosteal stripping along with open fracture is an established model for delayed union in both mice [505] and rats [506]. For this study, rats were chosen over mice due to their larger size, and open fractures with periosteal stripping were conducted as per established protocols.
Fracture grading

While the phase of fracture healing can be readily identified using histological methods, such as those described in Chapter 1, it can be challenging to recognise these stages using non-invasive and longitudinal methods. Standard clinical outcomes of fracture healing such as pain and weight-bearing are difficult to assess in rodent models, and as such X-rays are commonly used to monitor healing progression. In both clinical and research settings, the ability to assess fracture union is critical, as this represents an ideal clinical end-point, as well as a vital outcome measure in research. The evaluation of union via X-ray can be highly subjective, and guidelines must be established to grade fracture healing more objectively. Scores such as the Lane and Sandhu and the RUST (Radiographic Union in Tibia) have been proposed clinically, however the translation into small animal research is not always relevant. In animal studies, a general impression of the fracture progression is often used, however a standardised scale improves reproducibility and accuracy of assessment [507]. This is especially important when considering that radiographic union is a key outcome measure in many studies, including one described in this chapter.

RUST scoring of fracture healing

The RUST score was originally used to describe fracture healing radiographically in the tibia [508]. This method utilises the addition of scores to assess the degree of union in a fracture. Two X-rays are needed, one of the anterior-posterior, and one of the lateral plane. From these X-rays four cortices are visible, each of which is scored from 1-3, where 1 means the fracture line is still visible with no callus, 2 means visible fracture line with callus, and a score of 3 means bridging callus with no fracture line visible (Figure 4.2). The scores are summed, with a minimum score of 4 and a maximum score of 12. In the context of rodent models of fracture healing, RUST scoring has been validated as an accurate and reliable method for grading union [507].
Figure 4.2: Anteroposterior (A/P) and lateral X-rays of a rat femoral fracture showing the three RUST scoring levels.
Chapter 4: Treating open fractures with SAIB and BMP-2

Materials and Methods

Measuring extrusion force of SAIB

An Instron 5944 (Massachusetts, USA) was used to test disposable 1 mL Luer lock syringes in compression, with data collected using BlueHill 3 software. A 100N load cell was used, with either 27G, 25G, 23G, or 19G needles attached to the syringes (Figure 4.3). The syringe was supported by a hollow metal support with a hole at the top, which allowed free flow of the extruded material. Mixtures are extruded at rates of 0.2mm/s, and this was chosen as it was estimated to match the extrusion rate for surgical injection.

![Image of the jig used to measure the extrusion force of SAIB mixtures through a syringe and needle on an Instron 5944.]

**Figure 4.3:** The jig used to measure the extrusion force of SAIB mixtures through a syringe and needle on an Instron 5944.
Intramuscular injections of SAIB in rats

The procedure for ectopic bone formation model in the rat is very similar to that described for the mouse in Chapter 2. An 80:20 SAIB:ethanol stock solution is prepared and allowed to spin overnight. Rats received an analgesic injection of Buprenorphine 30 minutes prior to surgery. Rats were kept under anaesthesia by inhaled Isofluorane gas. A disposable BD 1 mL Luer lock syringe with a 25 G needle was used to inject bilaterally into the muscle pouch of the rat (Figure 4.4). For the initial BMP-2 dose response study, 400 µL was injected. This volume was then dropped to 200 µL for the subsequent HA particle size study. Rats were regularly monitored and given post-operative pain relief. Rats were culled four weeks after surgery.

Figure 4.4: Percutaneous intramuscular injections of SAIB into the quadriceps of the rat. A BD 1 mL Luer lock disposable syringe with a 25 G needle is used to introduce the SAIB construct into the muscle pouch of the rat.
**In vitro binding assay**

The translation of the SAIB/BMP-2 carrier system from mice into rats allowed for the co-delivery of larger particle sizes of HA. For this study, three HA particles were used, the first was P149 HA particles purchased from Plasma Biotal Ltd (Derbyshire, UK), which have been used for all experiments in mice until this point. However, with supplies limited, new sources of HA needed to be found. HA was purchased from Berkeley Advanced Biomaterials (CA, USA) in two particle sizes: nano (average particle size 100 nm), and micro (particle size range 50-150 µm).

To determine the affinities of the bisphosphonate ZA for HA, a 50 µM dose of $^{14}$C-ZA was added to 2% w/v ceramic particles in saline and agitated. After two hours, samples were spun down in a centrifuge and the supernatant carefully extracted and added to scintillation fluid. Radio counts were read in duplicate using a 1900CA Tri-carb liquid scintillation analyser (Packard Bioscience, Connecticut, USA). Scintillation fluid alone was used as blank control, and a 50 µM dose of $^{14}$C-ZA was added directly to scintillation fluid as positive control. The values measured in the supernatant values subtracted from positive controls to generate the amount of $^{14}$C-ZA that was left bound to the ceramics.

**Cell culture experiments**

The ability for the different HA particles to bind ZA and protect cells from ZA-mediated cytotoxicity was evaluated in the pre-osteoblastic cell line, MC3T3-E1. Cells were cultured in αMEM supplemented with 10% foetal bovine serum, 1% L-glutamine, and 2% penicillin/streptomycin. Cell media was changed every 4 days, and cells were lifted from flasks using trypsin-EDTA.
Chapter 4: Treating open fractures with SAIB and BMP-2

Previous experiments have shown that a ZA dose of 50 µM is cytotoxic to cells in vitro (Figure 2.9), and this dose was used for the following experiments. Transwell inserts (pore size of 1 µm) were used to allow the ZA-containing media to interact with the ceramics particles without coming into direct contact with the pre-osteoblasts. The inserts were introduced into the well containing cells and media, and contained the following treatments: media alone, 2% ceramics, 50 µM ZA, or a combination of the ceramic and ZA treatment. Viability was measured at 7 days post seeding using the Muse cell count and viability assay (Merck Millipore; Hesse, Germany) as per the manufacturer’s instructions. Briefly, cells were trypsinized and re-suspended in media, then added to the assay media. Samples were then vortexed immediately prior to reading by the Muse Cell Analyzer.

**SAIB injections around an open fracture**

Rats were anaesthetised using 70 mg/kg Ketamine and 10mg/kg Xylazine administered intraperitoneally. An incision is made on the lateral mid-thigh, and the muscle dissected around the femur. The femur is cut using an oscillating bone saw (Stryker, MI, USA) at the midshaft, then the periosteum is disrupted 10 mm around the fracture site. A small incision is made at the knee, and a sterile Kirschner wire is introduced to stabilise the fracture. Any treatment is then introduced from the lateral incision (Figure 4.5). The two incisions are then sutured, and the rats allowed to recover on a heated mat. Subcutaneous 0.01 mg/kg Buprenorphine (Temgesic, Reckitt Benckiser) was administered for pain relief, and rats were monitored closely for the duration of the study.

For collagen sponge implantation, a 1.6 x 0.5 cm rectangle is cut from the collagen sponge that accompanies the BMP-2 Infuse® kit. These sponges are kept sterile prior to surgery, during which 90 µL of BMP-2 solution is slowly dripped on 20 minutes prior to implantation. For implantation, the sponge is carefully wrapped around the fracture site, after which, the muscle is then sutured.
Chapter 4: Treating open fractures with SAIB and BMP-2

For SAIB injections, a 24 G catheter sheath is wrapped around the fracture site, and the muscle loosely sutured. A BD 1 mL Luer lock syringe is then attached to the catheter sheath, and the SAIB mixture is injected upon withdrawal. For the pilot study, 100 µL was injected around the site, and in the full study 50 µL was used. For both studies, an 80:20 SAIB:ethanol stock solution was used.

![Collagen implantation](image1.png)  ![SAIB injection](image2.png)

**Figure 4.5:** The introduction of BMP-2 on either collagen or SAIB carrier (using a 24 G catheter sheath) around an open fracture in the rat.

For the first week after surgery, animals received antibiotics (0.125 mg Enrofloxacin per mL of water; Troy Laboratories, NSW, Australia) in their drinking water. At six weeks after surgery, animals were culled via CO₂ asphyxiation. For samples undergoing mechanical testing, these were wrapped in saline soaked gauze and stored at -80°C. Samples for histological analysis were fixed in 10% formalin, and then stored in 70% ethanol.
Chapter 4: Treating open fractures with SAIB and BMP-2

**Experimental designs**

*The dose response of BMP-2 delivered in SAIB in a rat muscle pouch*

To establish BMP-2 doses for further studies, a BMP-2 dose response was examined in nine week old male Sprague-Dawley rats. Four doses of BMP-2 were used, 0, 2, 5 and 10 µg BMP-2 per pellet (Table 4.1). The BMP-2 was mixed in 400 µL of SAIB stock solution, and the mixture was injected bilaterally into the quadriceps.

**Table 4.1**: Experimental design for a study exploring BMP-2 dose response in the rat muscle pouch

<table>
<thead>
<tr>
<th>Group</th>
<th>BMP-2 dose (µg)</th>
<th>N=</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>5</td>
</tr>
</tbody>
</table>

*The effect of HA particle size on bone formation*

To examine the effects of particle sizes of HA on SAIB/BMP-2 induced bone formation and ZA binding, two particle sizes of HA were studied, nano and micro (Berkeley Advanced Biomaterials; CA, USA). A bilateral muscle pouch model was conducted in nine week old male Wistar rats. A dose of 5 µg of BMP-2 per pellet was chosen, and this was co-delivered with 2% w/v HA, with and without 2 µg of ZA in 200 µL of SAIB (Table 4.3). For groups with both HA and ZA, these were pre-bound prior to addition to SAIB. For this, HA and ZA were mixed in saline, centrifuged with the supernatant removed, and then dried. As ZA is suspended in saline, this process removed all the water prior to addition to SAIB, which is highly hydrophobic.
Chapter 4: Treating open fractures with SAIB and BMP-2

Table 4.2: Experimental design to explore the effect of HA particle size and ZA binding on bone formation

<table>
<thead>
<tr>
<th>Group</th>
<th>BMP-2 dose</th>
<th>HA</th>
<th>ZA</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 µg</td>
<td>-</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>5 µg</td>
<td>2% nano HA</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>5 µg</td>
<td>2% nano HA</td>
<td>2 µg</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>5 µg</td>
<td>2% micro HA</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>5 µg</td>
<td>2% micro HA</td>
<td>2 µg</td>
<td>5</td>
</tr>
</tbody>
</table>

SAIB delivering BMP-2 to an open fracture – pilot study

For this pilot study, open fractures were created in the femora of nine-week old male Sprague-Dawley rats. Rats were allocated into one of three treatment groups (Table 4.3). The control group consists of SAIB vehicle alone being injected around the fracture site. One of the treatment groups, received BMP-2 was delivered to the site in SAIB. The final study group received BMP-2 in SAIB, as well as 2% nano HA that has been pre-bound to 2 µg ZA. All BMP-2 treated rats received 10 µg per fracture site. For this study, an original group size of n = 5 was planned, however four rats were excluded. Three rats were culled early due to pin slip (1 from SAIB group, 2 from SAIB/BMP-2/HA/ZA group) and one was culled due to detection of osteomyelitis in the femur (from SAIB group).

Table 4.3: Experimental design for a pilot study investigating SAIB delivery to an open fracture

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Initial N=</th>
<th>Final N=</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SAIB alone</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>SAIB + BMP-2</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>SAIB + BMP-2 + HA + ZA</td>
<td>5</td>
<td>3</td>
</tr>
</tbody>
</table>

SAIB delivering BMP-2 to an open fracture – full study

For the full study looking at SAIB delivery of BMP-2 to open fractures, nine week old male Sprague-Dawley rats were used. All rats were allocated into one of five treatment groups (Table
4.4). For the negative control group, open fractures were left untreated. A collagen sponge containing BMP-2 was wrapped around the fracture site, and used to model the current clinical standard of care. Another group received SAIB alone injected around the fracture site. Two formulations of SAIB, SAIB containing BMP-2 as well as SAIB containing BMP-2, 2% HA and 2 µg ZA, were also investigated. For all BMP-2 treated groups, a dose of 10 µg per fracture site was used. The study was planned for an N = 15 for each group, however there was three exclusions. Two rats were culled early due to pin slip (1 from SAIB alone group, 1 from SAIB/BMP-2 group) and one rat was culled due to wound irritation (from collagen/BMP-2 group).

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Initial N=</th>
<th>Final N=</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Untreated</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>Collagen sponge + BMP-2</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>3</td>
<td>SAIB alone</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>SAIB + BMP-2</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>5</td>
<td>SAIB + BMP-2 + HA + ZA</td>
<td>15</td>
<td>15</td>
</tr>
</tbody>
</table>

**Radiographic analysis**

Bone nodule formation, as well as fracture placement and fixation was monitored weekly using digital X-ray (Faxitron X-ray Corporation; IL, USA). For the open fracture pilot study, radiographic union grading was judged by supervisor AS, who was blinded to treatment. Fractures were judged as either non-united, partially united or fully united. For the full open fracture study, radiographic union grading was conducted by an orthopedic surgeon (DGL) who was blinded to treatment. At 3 week bony union was judged from a single anterior-posterior X-ray. At the 6 week end point, union was judged from two X-ray images showing anterior-posterior and lateral angles of the femur. The Radiographic Union Score in Tibia (RUST) was used to grade fractures for
union. In this context, at the 3 week time point each fracture is assigned a score out of 6 (one view, two cortices visible), and the 6 week time point out of 12 (two views, 4 cortices visible).

MicroCT analysis was conducted using a SkyScan 1174 (SkyScan NV, Kontich, Belgium). Samples were scanned with a 0.5 mm aluminum filter, 50 kV X-ray tube voltage, 800 μA tube electric current, and 4500 ms exposure time. Muscle pouch samples were scanned at a resolution of 17.8 μm, and fractures scanned at a resolution of 28.6 μm. For muscle pouch studies a 0.3 g/cm³ and for fractures a 0.4 g/cm³ threshold were used to define bone tissue, and this was calibrated using phantoms of known density.

For fractures, a volume of interest (VOI) was selected that spanned 6 mm from the center of the fracture (12 mm in total). From this VOI, bone volume (BV), tissue volume (TV), BV to TV ratio, and total mineral density was calculated. Reconstructions were generated using Mimics Medical (Version 17; Materialise NV, Leuven, Belgium).

The investigation into HA particle size was scanned on a Quantum FX microCT scanner (PerkinElmer, Massachusetts, USA). Samples were scanned using 90kV X-ray, and 80 μA current, at a field of view of 20 mm, resulting in an image pixel size of 40 μm. The scanned images were reconstructed using NRecon (SkyScan), and analysed with the accompanying software package CTAnalyser (version 1.13.5.0, SkyScan).

**Brightfield histology**

*Alcian blue and picrosirius red*

Sections were cut from decalcified and paraffin embedded samples on a Leica RM2155 microtome. Slides were dewaxed and brought to water, stained in Weigert’s haematoxylin (equal parts Goldner’s solution A (1.0 g haematoxylin in 100 mL 95% ethanol) and Goldner’s solution B
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(1.16 g ferric chloride and 1.0 g hydrochloric acid in 100 mL distilled water) for 15 minutes. Slides were then washed in tap water for 5 minutes, then stain with 1% Alcian Blue in 3% acetic acid for 10 minutes, then wash in distilled water. Stain with 1% aqueous phosphomolybdic acid for 2 minutes, then wash in distilled water. Slides were then stained with 0.1% Sirius Red in saturated picric acid for 90 minutes. Slides were then brought to Xylene and mounted with Ultramount.

**Fluorescent TRAP staining**

Non-decalcified samples were bisected and embedded face down using Tissue Tek OCT Compound (Sakura, Japan) in preparation for cyrosection. Sections 5 µm thick were cut using cryofilm (Type 2C, Section-Lab, Japan).

Slides were rehydrated for 15 minutes in PBS, then incubated in 1M Tris-HCl (pH 9.4) for 5 minutes, and then for 10 minutes in 1M Sodium acetate (pH 5.0). This was followed by a 4 minute incubation in 37°C TRAP solution (Add 5 mg tartaric acid in 5 mL sodium acetate buffer and mix. In a separate tube dissolve 7 mg Naphthol AS-MX phosphate (Sigma Aldrich, #855) in 50 µL DMF, and then add to tartaric acid solution. Add 20 µL 4% sodium nitrate solution to the previous solutions. Then add 2 mg Fast Red TR salt (Sigma Aldrich #F8764) to the solutions. Mix well and filter). Wash in three changes of wash buffer (9.3g EDTA in 1 L distilled water, pH 8.0) to remove any crystals. Counter stain in DAPI (1:10 000 in PBS), wash in PBS and then mount in CC mount.

Fluorescence was visualised using Leica SP5 Confocal microscope, at an emission wavelength of 555 nm. Image capture settings were retained for all samples.

**Four-point bend testing**

Femora were tested in four point bending to failure on an Instron 5944 (Massachusetts, USA), data collected using BlueHill 3 software. Bones were stored at -80°C prior to testing and allowed to
thaw to room temperature prior to testing. Femurs were positioned so that the posterior surface rested on the bottom span, with the upper fixture resting on the mid diaphysis (Figure 4.6). The support span was 20 mm, with the upper span measure 10 mm. Samples were pre-loaded at 0.5 mm/min until a load of 2 N was reach, at which point the loading rate increased to 2 mm/min until fracture.

Figure 4.6: The four-point bending set up for either intact or 6 week post fracture rat femora on the Instron 5944 with custom jig. Note the fracture callus lies between the upper anvils.

In the instance of a poorly united fracture, the test was stopped prior to failure if it was deemed to no longer be testing the properties of the fracture site, or for possibility of jig damage (Figure 4.7).
Figure 4.7: A representation of a poorly united fracture showing high plastic deformation, and an instance of mechanical testing that was stopped prior to failure.

**Statistical methods**

In the open fracture study, mechanical data was analysed using a Kruskal-Wallis test for non-parametric data initially, followed by a post-hoc Dunn’s multiple comparisons test.
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Results

The force required to extrude SAIB increases with smaller needle size and lower dilutions

The delivery of SAIB dilutions, especially at low dilutions and with the incorporation of various agents, had proven challenging in certain situations. It is evident that increasing the size of the needle that the mixture is extruded through decreases the force required to extrude it (Figure 4.8A). In addition, increasing the solvent component of the mixture reduces the force required to extrude the mixture through a needle (Figure 4.8B). By upscaling from a mouse model into a rat model, it is hypothesized that the variability seen in the mouse models will be reduced. Upscaling into the rat model also allows for larger gauge needles to be used to deliver SAIB mixtures, allowing for variables such as ceramic particle size to be explored.

![Figure 4.8](image-url): The force required to extrude SAIB from a 1 mL Luer lock syringe at a rate of 0.2 mm/s varies with SAIB dilution and needle size. A. Decreasing needle gauge increases the force required to extrude 90:10 SAIB. B. Decreasing the solvent ratio in the SAIB mixture increases the force required to extrude the SAIB mixture through a 27 G needle.
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**BMP-2 dose response in the rat**

Initially, a dose finding study was conducted in the muscle pouch of the rat to find the optimal dose of BMP-2 as delivered by SAIB for further studies. Four doses of BMP-2 were investigated, 0, 2, 5 and 10 µg BMP-2 per pellet. The group containing no BMP-2 resulted in no bone formation (Figure 4.9). The delivery of BMP-2 resulted in the generation of a comparable volume of bone, regardless of the dose administered.

![Bone formation response to BMP-2 doses](image)

**Figure 4.9:** The bone volume response to increasing doses of BMP-2 delivered in SAIB in the ectopic bone formation model in the rat. **A** Quantified bone volume as detected by microCT. **B** Representative X-rays from each group.

**HA particle size affects ZA binding**

Previous studies in the mouse model used to optimise SAIB formulations found that the co-delivery of ZA and nano-HA resulted in the maximal bone formation (Figure 2.5). To investigate
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whether this synergy would be affected with the upscaling into the rat, an additional HA particle size was studied (micro-HA).

Radiolabelled ZA was used to assay the binding affinities of nano HA that has been used in studies to this point (Biotal HA), as well as newly purchased HA in two particle sizes (Berkeley HA). In this in vitro assay, it was found that the nanoparticle sized HA from both suppliers were able to bind a comparable percentage of $^{14}$C-ZA (Biotal HA – 94%, Berkeley HA – 92%) (Figure 4.10). The microparticle sized HA was only able to bind 43% of the $^{14}$C-ZA. The comparable binding between the two nano-sized HA particles resulted in confidence to proceed with further studies. From this point forward, the Berkeley HA particles were used and are referred to as nano-HA and micro-HA.

![Figure 4.10: The binding affinity of radiolabelled ZA ($^{14}$C-ZA) for three particles of HA; Biotal HA that has been used for previous experiments, and newly purchased Berkeley HA in two particle sizes.](image-url)
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Following this, the ability for the two particle sizes of HA to prevent ZA mediated cytotoxicity was tested in the MC3T3 cell culture assay (Figure 4.11). On their own, both nano and micro HA alone had little effect the growth of cells (95% and 101% of cell control respectively). The addition of ZA to cell culture media is able to completely diminish viable cells (8% of control readings). Delivering ZA in the presence of micro HA results in a similar effect (11% of control readings). However, when ZA is administered alongside nano HA, cell viability is brought back up to 102% of control readings, providing a rescue effect for the cells.

![Graph showing cell viability with and without ZA](image)

**Figure 4.11**: Cultures of MC3T3 cells reveal that only nano HA is able to rescue cell viability in the presence of 50 µM ZA. Viability is presented as live cells a percentage of the control MC3T3 group, as measured by flow cytometry.

**SAIB/BMP-2 induced bone formation is enhanced with nano HA and ZA**

Ectopic bone formation was induced by 5 µg BMP-2 delivered in SAIB to the muscle pouch of the rat (Figure 4.12A). Both particle sizes of HA were able to induce a significant increase in bone formation when compared to BMP-2 control (nano HA - 126%, micro HA - 278% increases in BV; p<0.05). The co-delivery of ZA with micro HA led to a significant 530% increase in ectopic
bone volume when compared to control (p<0.01), although there was no significant increase when compared to micro HA alone. The delivery of nano HA with ZA led to an 889% increase in bone volume when compared to control (p<0.01), and a 337% increase when compared to nano HA alone (p<0.01). The increase in bone volume, both spatially and in opacity, can be seen in the representative X-rays of Figure 4.12B.

![Figure 4.12: Bone volume (BV) response to 5 µg BMP-2, as well as the addition of two particle sizes of HA with and without ZA in the muscle pouch of a rat. A BV quantified by Quantum microCT, upper threshold used to exclude HA particles. B Representative X-rays showing samples from each group.](image)

Slices from the centre of the bone nodule were stained for TRAP, counterstained in DAPI, and imaged (Figure 4.13). Qualitative analysis suggests that micro HA, whether co-delivered with ZA or not, has similar levels of TRAP staining as control. However, the addition of nano HA, especially when co-delivered with ZA, leads to increases in levels of TRAP positive cells detected. Nearby sections that were paraffin embedded and stained using Haematoxylin and Eosin shows
increased trabecular bone formation and a corresponding decrease in marrow space in the samples treated with ZA (Figure 4.13).

**Figure 4.13:** Representative histology on bone nodules formed in response to 5 µg BMP-2 delivered in SAIB, along with two particle sizes of HA, with and without ZA. A Fluorescent TRAP histology as imaged by confocal microscopy. Red, TRAP positive cells; blue, autofluorescence. B Haematoxylin and eosin staining of similar regions. Dotted line represents the bone pellet border with the muscle pouch. Scale bar represents 250 µm for all panels.

**SAIB is a feasible means to delivery BMP-2 to the site of an open fracture**

The muscle pouch study demonstrated that the strong synergy in the SAIB/BMP-2/nano HA/ZA system was still exhibited in the rat model, resulting in a 10-fold increase in bone formation over SAIB/BMP-2 alone. As a result, the use of the SAIB carrier in a functional and challenging model of bone healing, that of the open fracture, was explored.

Initial testing for the feasibility of utilising SAIB to deliver BMP-2 to the site of an open fracture of a rat was investigated in a small pilot study. In this study, SAIB alone, SAIB with 10 µg BMP-2, and the combined treatment group SAIB with 10 µg BMP-2, 2% nano HA and 2 µg ZA, were
delivered to the site. Rats underwent weekly X-ray over the six week study, for both monitoring purposes as well as for union assessment. Longitudinal X-ray reveal bony callus formation in both groups treated with BMP-2 from week two, with SAIB vehicle resulting in frustrated callus that did not heal by six weeks (Figure 4.14; representative samples shown).

**Figure 4.14**: Representative X-rays of open fractures treated with SAIB alone, SAIB and BMP-2, and SAIB, BMP-2, HA and ZA across the 6 weeks of the study.

X-rays were assessed for bony union at two time points, three and six weeks, by supervisor AS who was experienced in fracture grading. Fractures were judged as either united, partially united...
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or non-united at each of these time points. At three weeks, fractures treated with SAIB alone were not united, while fractures treated with SAIB and BMP or SAIB, BMP-2, HA and ZA were all either partially or fully united at this time point (Figure 4.15). By six weeks, all the BMP-2 treated fractures had progressed to full union, while two out of three vehicle treated fractures showed no signs of callus bridging.

![Figure 4.15: Union rates for rat open fractures treated with SAIB at A three weeks, and B six weeks.](image)

MicroCT analysis of the fracture callus, including the original cortex showed comparable values for bone volume (BV), total volume (TV), BV/TV and total mineral density (TMD) across all groups (Figure 4.16). Despite these similarities, representative microCT slices and reconstructions reveal very different stages of fracture healing (Figure 4.17). SAIB treatment results in frustrated callus, which does not bridge the fracture gap and is very typical of delayed union. BMP-2 treatment results in united calluses and healed fractures. However, the callus formation is slightly irregular in these groups, most clearly seen in the microCT reconstructions in Figure 4.17.
Figure 4.16: MicroCT analysis of rat open fracture pilot study. **A** Bone volume (BV), **B** total volume (TV), **C** bone volume to total volume ratio (BV/TV), and **D** total mineral density (TMD).
Figure 4.17: Representative samples from the open fracture study in a rat, treated with SAIB and BMP-2. First row, microCT slice through the centre of the callus. Second row, reconstruction of fractures, highlighting callus formation around the fracture.

Alcian Blue and Picrosirius Red staining was used to histologically investigate the effects of SAIB, SAIB/BMP-2, and SAIB/BMP-2/HA/ZA treatments on open fracture repair. At six weeks post fracture, substantial cartilage remained in SAIB vehicle treated calluses as visualized by Alcian
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Blue staining (Figure 4.18). In the SAIB/BMP-2 treated fractures, evidence of bony union was seen. In the combination treatment of SAIB/BMP-2/HA/ZA, the callus was highly trabecular, and no delay in union or non-mineralized cartilage could be detected. The bubble-like structures seen in the two BMP-2 treated groups on the edge of the callus were speculated to be filled with unresorbed SAIB prior to paraffin processing (Figure 4.18).

Figure 4.18: Alcian blue and picrosirius red staining of representative samples from the rat open fracture pilot study. Second row shows higher magnification of area indicated by the square. Distal end to the right; first row scale bar 2 mm; second row scale bar 1.5 mm.
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The use of SAIB/BMP-2/HA/ZA was an effective treatment for open fractures

With the promising results of the pilot study investigating SAIB as a delivery system for BMP-2 for the treatment of open fractures, a full study was conducted. For this study, the volume of SAIB injected (but not the amount of BMP, HA or ZA) was reduced from 100 µL to 50 µL, due to concerns that an excessive amount of SAIB was injected for the space around the fracture. Two more control groups were also added; an untreated fracture group to serve as negative control, and a BMP-2 delivered on collagen sponge acting as the positive control.

Fracture healing was assessed by grading of bone repair in X-rays taken at the three-week midpoint and at cull with the intramedullary fixation removed (Figure 4.19; representative X-ray shown). Examination of longitudinal X-rays showed a stimulation of bony callus formation from 2 weeks in collagen/BMP-2, SAIB/BMP-2 and SAIB/BMP-2/ZA/HA groups with some subsequent reduction in callus size via remodelling (Figure 4.20). Overall, fractures without treatment showed poor healing with very few showing evidence of robust union, while the majority of BMP-2 treated specimens were united by six weeks (Figure 4.19).

![Figure 4.19: Representative samples from each group from an open fracture study showing healing and bridging in each of the treatment groups.](image-url)
The degree of radiographic fracture union was graded at two time points by supervisor DGL. At the three week time point, the majority of the fractures were poorly united in the untreated and SAIB alone groups. In the untreated group, 0% had fully united (a RUST score of 6), while 33% were considered partially united (RUST score of 5), and 67% were considered to be not united (RUST score of 3 or 4). For the SAIB group, 14% were graded as fully united at this time point, 7% were partially united, and the remaining 79% were not united (Figure 4.21A).
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In the fractures that were treated with collagen/BMP-2, 93% of fractures were graded as fully united, and 7% were considered partially united. SAIB/BMP-2 treatment resulted in a 36% of fractures considered fully united, with 50% partially united, and 14% considered not united. The use of SAIB/BMP-2/HA/ZA saw 47% of fractures were united, 20% partially united, and 33% not united. At this three week time point, the collagen/BMP-2, SAIB/BMP-2 and SAIB/BMP-2/HA/ZA groups showed significant increases in union compared to untreated controls (p<0.05).

At the six week study end point, untreated and SAIB treated open fractures remained mostly non-united (RUST score of 8, 53% and 64% respectively) (Figure 4.21B). For the BMP-2 treated groups, most fractures in each group achieved full bony union, and these were significantly increased compared to untreated controls (p<0.05). In the collagen/BMP-2 group, 100% of the fractures were judged to have a RUST score of 12, representing complete union. In the SAIB/BMP-2 group, 71% achieved full union, and the remaining 29% were deemed partially united. In the SAIB/BMP-2/HA/ZA group 87% are fully united at this time point, while 7% are partially united, and 7% non-united.
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MicroCT analysis was conducted over a 12 mm region that was centered on the fracture line. The highest BV recorded was in the SAIB/BMP-2/HA/ZA group, which was significantly higher than the untreated, collagen/BMP-2 and SAIB/BMP-2 groups (Figure 4.22A; p<0.01 for all). Collagen/BMP-2 treatment had the lowest BV values, which was also significantly lower than the SAIB group (p<0.05). The SAIB/BMP-2/HA/ZA had the highest TV, which was significantly higher than the untreated and SAIB/BMP-2 groups (Figure 4.22B; p<0.01 and p<0.05 respectively). The untreated group had the lowest TV values, which were also significantly lower than collagen/BMP-2 (p<0.01). Comparing the BV to TV ratio, the collagen/BMP-2, SAIB/BMP-2 and SAIB/BMP-2/HA/ZA groups are all significantly lower than untreated fractures (Figure 4.22C; p<0.01, p<0.01 and p<0.05 respectively). The collagen/BMP-2 treated fractures had significantly lower BV/TV than all other groups. TMD was highest in the collagen/BMP-2 treated fractures, and lowest in the SAIB/BMP-2/HA/ZA group (Figure 4.22D; p<0.01). Reconstructions of representative fracture calluses are shown in Figure 4.23.

Figure 4.21: Radiographic union grading ratings at two time points A three and B six weeks as assessed by DLG using RUST scoring.
Figure 4.22: MicroCT analysis of open fractures treated with either SAIB alone, collagen sponge with BMP-2, SAIB with BMP-2, or a combination of SAIB, BMP-2, HA and ZA. A Bone volume (BV), B tissue volume (TV), C bone volume to tissue volume ration (BV/TV), and D bone mineral density (BMD). *, p < 0.05; **, p < 0.01.
Figure 4.23: MicroCT images of representative calluses formed six weeks after the generation of an open fracture that was treated with SAIB alone, collagen sponge with BMP-2, SAIB with BMP-2, or a combination of SAIB, BMP-2, HA and ZA. The top panels show representative microCT slices through the centre of the callus, and the bottom panel shows three-dimensional reconstructions.

The fractured and intact contralateral femora were subjected to four-point bend testing as a functional outcome of the open fracture treatments. The untreated, SAIB vehicle and SAIB/BMP-2 treated fractures showed significantly reduced maximum loads when compared to the intact contralateral bones (Fig 4A; -60% (p<0.01), -67% (p<0.01) and -22% (p<0.01) respectively) (Figure 4.24A). The collagen/BMP-2 and SAIB/BMP-2/HA/ZA treated fractures had calluses that were of comparable strength to the intact femora. In contrast, the stiffness values were increased
compared to control and SAIB vehicle treatment, but still significantly reduced compared to intact bones (Figure 4.24B). Only the collagen/BMP-2 intervention showed no difference in the energy absorbance prior to failure when compared to the intact contralateral femora, with all other groups not affect the mechanical testing of the fracture, and were seen to resorb over time (Figure 4.20).

**Figure 4.24**: Four-point bend testing of femora compared intact (contralateral) controls analysing for A maximum load to failure, B stiffness, and C energy to maximum load. Notably, the maximal load was improved in the Collagen/BMP-2 and SAIB/HA/BMP-2 groups (* p<0.05 vs untreated), with the two groups showing restoration to the strength of intact bones (a, p<0.05 vs contralateral
controls). Significant differences were also seen in comparisons vs SAIB alone (# p<0.05) and vs Collagen/BMP-2 (^ p<0.05).

Figure 4.25: An example of abnormal callus formation that was seen in many open fractures that were treated with SAIB/BMP-2 and SAIB/BMP-2/HA/ZA. The upper panel shows an X-ray taken at the six week end point, and the lower panel shows a photograph that was taken after the surrounding soft tissues were removed.
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Discussion

This chapter describes the upscaling of the SAIB carrier system into the higher rodent model, the rat. Initial dose response studies in the muscle pouch model in the rat found that all doses of BMP-2 studied formed comparable bone nodules after four weeks in vivo (Figure 4.9). This result was unexpected, as previous SAIB/BMP-2 dose response studies in mouse muscle pouch models found increased bone formation with increasing doses of BMP-2 [397]. However, a robust bone nodule was formed in each BMP-2 dose group, which contrasts data from this laboratory that the low dose of 2 µg BMP-2 delivered on collagen sponge results in unreliable bone formation in a rat muscle pouch (A Schindeler, unpublished). It is hypothesised that the lack of dose response found in this rat model was due to an excessive SAIB injection volume. In mouse models, a SAIB injection volume of 20 µL is used. The volume chosen to inject into the rat muscle pouch was estimated by a direct scale up from the mouse. As the average mouse weighed 20 g and received a 20 µL injection, the injection volume of 400 µL was chosen as the average rat used weighed 400 g. At the time of surgery tremors were seen in the muscle being injected, however this did not affect the rat’s recovery. Following this study, the injection volume was halved for the subsequent muscle pouch study, which led to significant differences found between study groups.

It was hypothesized that as part of the upscaling from mouse to rat, that the HA within the optimised SAIB formulation (SAIB/BMP-2/HA/ZA) would also need to be upscaled. It is well noted that the magnitude of treatment responses tend to decrease as higher order models are used [58]. The synergy in the SAIB/BMP-2/HA/ZA system in the mouse has been attributed to the strong binding and sequestration of the ZA to the HA. However, in vitro experiments have shown that the strong binding (Figure 4.10) and cytoprotective effects (Figure 4.11) are limited to nano-HA. The stronger affinity is most likely due to the higher surface area to volume ratio of nano-
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HA, allowing for an increase in binding sites for ZA. Most notably, the \textit{in vivo} rat muscle pouch model was able to reconfirm previous mouse studies that showed that SAIB/BMP-2/nano-HA/ZA was able to generate 10-fold more bone than SAIB/BMP-2 alone (Figure 4.12). There was a significant increase in bone formation when ZA was added to SAIB/BMP-2/nano-HA, however this trend was not seen in micro-HA. The lack of synergy is most likely due to inadequate sequestration of ZA, as seen in prior \textit{in vitro} assays.

Study of histology found an increase in fluorescent TRAP+ cells in the nano-HA group. Nano-HA has been previously associated with an increase in osteoclast like cells when compared to a larger particle sizes \textit{in vitro} [509]. Moreover, a study that investigated particle sizes of a HA/TCP mixture in rat drill holes found that the smallest particle size (10-20 µm) induced the strongest inflammatory response, but also the highest new bone volume [510]. Thus it could be postulated that nano-HA may yield increased bone formation linked with inflammation, but that the associated increases in resorption can be attenuated by pre-adsorption of bisphosphonate.

Confident that the SAIB/BMP-2/nano-HA/ZA was the optimal formulation for study in the rat, the feasibility of using the SAIB system to deliver BMP-2 at a fracture site in the rat was trialled. A pilot study was designed to investigate SAIB, SAIB/BMP-2 and SAIB/BMP-2/HA/ZA introduction intra-operatively around an open fracture. Promising results, including high union rates (Figure 4.15), strong microCT (Figure 4.17) and histological (Figure 4.18) outcomes gave confidence to proceed to a full study that included the addition of positive and negative controls (collagen/BMP-2 and untreated fractures, respectively). The presence of abnormally shaped calluses in the pilot study led to the volume of SAIB being halved from the 100 µL to 50 µL.
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The use of SAIB as a carrier for BMP-2 and other agents to the site of an open fracture was compared against the current clinical standard of BMP-2 delivery, collagen sponge. In this study, the SAIB was well tolerated around the fracture site in this study, mirroring Phase 3 clinical trial data that reported SAIB use at a surgical site was safe, with no detrimental effects on normal wound healing [265]. The previous muscle pouch study had exhibited synergy between BMP-2 and HA/ZA that resulted in a 10-fold increase in net bone (Figure 4.12). While the ectopic bone formation model is an effective screening methodology, it fails to recapitulate the complex micro-environment of a fracture. However, the large increases in bone volume seen in the muscle pouch, which was the primary outcome, may not be as important in challenging fracture models, where the main outcome is union rates and mechanical strength. The periosteal stripping leads to impaired healing, and aptly recapitulates the conditions associated with a major orthopedic injury [506]. While the Einhorn closed fracture model is often used to screen interventions, this osteotomy model was selected to better represent a challenging clinical scenario. Some therapeutic interventions that improve healing in closed fractures have been subsequently found to be ineffective in this open fracture model, for example PTH(1-34) [506].

In this study, SAIB was compared with the absorbable collagen sponge that currently constitutes the clinical delivery system for BMP-2. In terms of bone volume formed, the SAIB delivery system was similar to collagen sponge. Collagen sponge, while the current standard, still has major limitations with its use including (1) infection risk, (2) suboptimal drug release profile in humans, and (3) poor biomechanics.

Firstly, the risk of infection is increased by the use of a solid carrier for BMP-2 that must be manually handled and inserted into an open wound. In this study, BMP-2 infused collagen sponge was wrapped around the fracture created in the femur of the rat, which required the use of multiple
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Instruments that had contact with the open wound (Figure 4.5). In contrast, the SAIB solution was loaded into a syringe prior to use, and a single pair of forceps fed the catheter sheath around the fracture site, minimizing the instrument contact with the open wound. All animals received antibiotics were administered through drinking water for 1 week post-surgery to minimize infection risk, and in this study there were no infections in any of the animals. While it is hypothesized that SAIB use could reduce infection risk in open fractures or when injected into a delayed union, this would be both experimentally and ethically challenging to demonstrate in an animal model.

Secondly, collagen sponge shows a rapid burst release of BMP-2 in vivo, where up to 30% of the BMP-2 is eluted in the first 10 min [226]. Although this burst release seems to produce favourable results in animal models (where healing is rapid), it has been linked to clinical complications including heterotrophic ossification, hematoma, and excessive bone resorption [228, 229]. It has been speculated that an ideal BMP-2 delivery system for humans would release in a manner that reaches the local osteogenic threshold but yields a sustained release. Previous in vivo pharmacokinetic studies have reported that SAIB releases small molecule agents in such a manner [252, 253]. SAIB is a highly hydrophobic compound, making in vitro BMP-2 release assays challenging to perform and interpret. However, in previous chapters the 3-fold increase in BMP-2 induced bone with SAIB compared to collagen in an ectopic bone formation assay has been attributed to a superior BMP-2 release profile (Chapter 2).

Thirdly, the absorbable collagen sponge has poor mechanical properties that limits its usability. The wetted collagen sponge lacks structural integrity, and in this study there was concern that the forces applied during handling and implantation could lead to loss of BMP-2 solution. This issue has been previously raised in the literature [230, 231]. The use of an injectable system for BMP-2
delivery largely avoids the issues of scaffold integrity, however injected SAIB is subjected to the forces and compressions of the muscle around it. In some cases, this resulted in abnormal callus shape formation (Figure 4.25). While the mode of delivery requires further refinement, the primary concern was bony union of the fracture, and positively, these abnormal calluses, even those treated with ZA, were seen to undergo callus remodeling (Figure 4.14 and Figure 4.20) with no indication of heterotrophic ossification. Future studies will focus on better containment of the delivery system, and control of the spatial formation of bone.

Some limitations on experimental design are noteworthy. The mass of the sponge made the size of the SAIB depot and the collagen implant differ, however the total dose of BMP-2 added to both groups as well as the delivery volumes of the SAIB and the saline used to load the collagen sponge were comparable. For ethical and practical reasons, exhaustive internal controls for tissue histology were not performed, and specimens were compared to historical collagen/BMP-2 treated fractures as previously published [511]. The overall histological appearance of the collagen/BMP-2 and SAIB/BMP-2 and SAIB/BMP-2/HA/ZA groups were analogous, and in addition the delayed/non-unions in the SAIB group were akin to untreated open fractures also showing delayed union [511].

It was hypothesized that the addition of the potent bisphosphonate ZA co-delivered with the ceramic HA would lead to significant improvements in union rate, bone volume, and bone strength. Notably, ZA/HA only led to a non-significant trend towards improved union (Figure 4.21) and did not achieve significant increases in maximum load to failure (Figure 4.24A). However, as the union rates at the 6 weeks with collagen/BMP-2 and SAIB/BMP-2 were high, it would be challenging to obtain further significant improvements. Synergy between ZA and BMPs have been reported in the past in rat models of fracture repair [512, 513]. However, these approaches utilized
systemic delivery, the side effects of which were avoided in this study by delivering ZA locally to the fracture site. Although it has been reported that local bisphosphonate treatment can inhibit bone healing [408], in this study ZA was targeted to the location and cell type by co-delivery with HA [397], with no detrimental effects on bone regeneration observed. It is clear that there are osteoclast-independent pathways that are involved in endochondral ossification [514], and that fractures can remodel even in the presence of ZA [515], as was seen by the shrinking calluses seen in this study (Figure 4.14 and Figure 4.20).
Chapter 4: Treating open fractures with SAIB and BMP-2

Summary and conclusions

In this chapter, I demonstrated the successful upscale of the SAIB carrier into the rat, as well as its use to treat a challenging model of fracture healing. In the muscle pouch model in the rat, the delivery of SAIB/BMP-2/nano-HA/ZA was able to replicate the striking 10-fold increase in bone formation over SAIB/BMP-2 alone seen in the mouse. In the open fracture model, SAIB/BMP-2 and SAIB/BMP-2/HA/ZA were able to be applied to an open fracture, with comparable outcomes to the clinical standard of collagen sponge. With similar fracture healing outcomes as collagen, the SAIB delivery system also has additional benefits including usability and delivery. Development on containment strategies has the potential to further extend the versatility of the SAIB system.
Chapter 5. The use of the injectable SAIB carrier system in a porcine model of surgically induced Legg-Calve-Perthes disease

Work included in this chapter has been published in:

Chapter 5: SAIB in a piglet model of Perthes disease

Introduction

Animal models of Perthes disease

Perthes disease describes the paediatric presentation of idiopathic osteonecrosis of the femoral head (ONFH) [113]. As the aetiology of the disease is unknown, the development of animal models with comparable pathology has been difficult. The osteonecrosis seen in Perthes disease has been attributed to an altered blood supply to the femoral head, and as such, most animal models attempt to interrupt the vascular support to the femoral head. Small animal models, while challenging to pursue due to their size, have been explored. Mouse models are prohibitively small, but osteonecrosis of the femoral head in rats has been seen in spontaneously hypertensive rats [516, 517], in rats forced into standing positions [518], or when induced via surgery [519]. Treatment of osteonecrosis in rats resulting from both the spontaneously hypertensive state [520] and surgical trauma [488] have been explored in this laboratory. While rats are a preferred model to utilise, with less housing space required and short breeding cycles, they do not adequately represent the clinical issues. Large animal models more closely represent the bone sizes, growth rates, and weight loads on the femoral head seen in children.

A variety of large animals have been investigated as models for Perthes disease. A study in young dogs belonging to breeds that are susceptible to femoral head collapse, found that artificially induced effusion in the joint capsule resulted in an immediate reduction of the blood supply to the femoral head [521]. Further, sustained extreme pressure in the capsule by injection of ethylene glycol wax into the joint space resulted in signs of femoral head deformity after 20 weeks [522]. However, high joint capsule pressure is not thought to be a significant causative factor in Perthes disease [523], and the requirement of high pressures would limit the ability to apply interventions. In an attempt to mimic the radiographic feature seen in Perthes clinically, the capital femoral
Chapter 5: SAIB in a piglet model of Perthes disease

Epiphysis was ablated in young goats, however this did not lead to femoral head collapse or changes typical of Perthes disease [524]. The injection of ethanol into the femoral heads of sheep found partial necrosis after 12 weeks that was consistent with early stage ONFH [525]. The models listed so far have only included quadrupeds, however there has been some interest in using bipedal flightless birds, such as the emu, with insult to the femoral head induced by ischaemia, extreme cold, and corticosteroids [526, 527]. Despite the advantages of a bipedal stance, the bone structures in birds are very different to those of mammals and the pathology seen in this model was much greater than what is seen clinically.

One model for Perthes disease that is well described in the literature is a surgically induced ischaemia in the femoral head of growing mini pigs. In this model, surgically applied ligature around the femoral head results in avascular necrosis, with comparable pathology over eight weeks to clinical Perthes [119, 528]. In addition, the pig is considered to have comparable bone anatomy, morphology, healing and remodelling to humans, and the use of mini pigs limits the growth rate and allows for easier handling [529]. Thus, this model was chosen for further study, and supervisor DGL was able to travel to the laboratory of Harry Kim to study and acquire the surgical procedure.

**Bisphosphonate and BMP-2 therapy for Legg-Calve-Perthes disease**

Perthes disease is characterised by ischemia to the femoral head, leading to necrosis of the bony tissues. It has been proposed that the use of bisphosphonate therapy may ameliorate the risk of collapse by preventing bone loss while the vasculature becomes re-established and bone anabolism recovers [530, 531]. There is limited clinical evidence that systemic intravenous bisphosphonate treatment can reduce the deformity of the femoral head in this condition [532-534]. However, the presence of a vascular insult at the vascular head draws question as to whether systemic bisphosphonate treatments can adequately penetrate the femoral head.
Chapter 5: SAIB in a piglet model of Perthes disease

Previous preclinical studies have modelled the efficacy of systemically delivered bisphosphonate in animal models of Perthes disease. In a model of spontaneously hypertensive rats that develop osteonecrosis, weekly and monthly systemic bisphosphonate treatment was able to significantly improve epiphyseal quotient (EQ), the main measure for severity, after 11 weeks [520]. In a surgical model of Perthes disease in rats, systemic ZA treatment was able to increase bone volume fraction and bone mineral density in the femoral head when compared to saline [488]. In these models, it is important to note that repeated dosing of bisphosphonate was required. There is limited vascularity to the femoral head, especially at early time points, and it is believed that multiple doses are required to accumulate enough bisphosphonate in the necrotic bone [117, 535]. However, an alternative would be to develop a local bisphosphonate dosing system, and this could also allay concerns associated with the long-term effects of systemic bisphosphonate treatment in children and their growing skeletons.

While bisphosphonate treatment could prevent the necrotic bone resorption that occurs during periods of avascularity, promoting anabolism could further strengthen the femoral head and stimulate bony regeneration. Currently, BMP-2 is delivered on a solid, porous absorbable collagen sponge, which is appropriate for wrapping around fracture sites or packing into spinal fusion cages, but poorly suited for use in metaphyseal bone regions. This highlights the potential for a locally deliverable carrier for BMP-2 that can distribute the protein into the femoral head without excessive soft tissue or bone resection. The concept of a system that locally delivers a bisphosphonate such as ZA and the anabolic protein BMP-2 would be highly applicable to Perthes disease.

Insight into local dosing of bisphosphonates can be gained studies of the surgically induced ischemia in the growing mini pig described earlier. In this model, a systemic dosing regimen of the bisphosphonate Ibandronate led to significant improvements after 8 weeks [536]. In 2011,
Chapter 5: SAIB in a piglet model of Perthes disease

Vandermeer et al published a report describing a combination of BMP-2 and the bisphosphonate Ibandronate being co-delivered locally to the femoral head by percutaneous intraosseous injection [537]. This led to a significant increase in epiphyseal quotient compared to saline controls.

It was hypothesized that superior infiltration and drug delivery could be achieved by using an alternative to saline for delivering BMP-2 into the femoral head. SAIB was proposed as a feasible injectable delivery system for BMP-2, and had previously shown the ability to result in robust bone formation in small animal models. In this large animal model of surgically induced Perthes disease, it was hypothesized that the phase transitioning properties of SAIB would allow it to be injected into the femoral head, it would then disperse and be contained within the femoral head.

This study describes the results of treating the piglet hip avascular necrosis model with BMP-2 in combination with the potent third-generation bisphosphonate Zoledronate. In addition, fluorescently labelled Pamidronate was included to allow the biodistribution of bisphosphonate to be examined at the experimental endpoint. The model was examined at an endpoint of 8 weeks by a range of measures including X-ray, dual-energy X-ray absorptiometry (DEXA), tissue histology and fluorescent bisphosphonate tracking.
Chapter 5: SAIB in a piglet model of Perthes disease

Materials and Methods

Pharmaceuticals

SAIB was purchased from SAFC, a branch of Sigma Aldrich (Missouri, USA). BMP-2 was purchased as part as of the INFUSE bone graft kit from Medtronic (NSW, Australia). Purified ZA was sourced from AXXORA, LLC (San Diego, USA). Pamidronate (Cipla, Bombay, India) was fluorescently labelled with a AlexaFluor 555 labelling kit (Life Technologies, Victoria, Australia) using the manufacturer’s instructions to generate AlexaPAM. While the covalent attachment of dye moiety could compromise the anti-resorptive activity of Pamidronate, the ‘bone hook’ region responsible for mineral avidity is unable to be labelled.

SAIB preparation

SAIB was prepared as an 80:15 stock solution and allowed to spin on a rotary spinner overnight. Prior to surgery, BMP-2 was mixed into the SAIB solution in ethanol to yield an 80:20 SAIB/ethanol ratio. This solution was then loaded into a 1mL syringe prior to surgery.

Surgical procedure

12 large white/Landrace cross piglets with weights ranging from 10-15kg (6-8 weeks old) were allowed to acclimatize for one week in the vivarium. Animals were fed twice daily, and allowed free access to water but were fasted overnight prior to surgery. Animals were pre-anaesthetized using Zoletil 4.4mg/kg, Atropine 0.05mg/kg, and Xylazine 2.2mg/kg delivered intramuscularly and maintained under general anaesthesia via Isoflurane following intubation. Pigs underwent the operative procedure described by Kim et al. [7]. Briefly, the joint capsule was opened, the ligamentum teres was cut and a suture tied around the base of the femoral head. An EZ-IO cannula (Vidacare, Texas, USA) was used to deliver the treatments to the femoral head (Figure 1). Three
groups (n=4/group) received treatment as outlined in Table 5.1. The first group received Saline, the second group SAIB/BMP-2 and the third group SAIB/BMP-2/bisphosphonate.

<table>
<thead>
<tr>
<th>Group</th>
<th>N=</th>
<th>BMP-2 (200 µL)</th>
<th>Local BP (200 µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Saline</td>
<td>4</td>
<td>SAIB only</td>
<td>Saline only</td>
</tr>
<tr>
<td>2. BMP-2</td>
<td>4</td>
<td>200 µg</td>
<td>Saline only</td>
</tr>
<tr>
<td>3. BMP-2 + local BP</td>
<td>4</td>
<td>200 µg</td>
<td>250 µg</td>
</tr>
</tbody>
</table>

At the completion of surgery, animals were given saline and the antibiotic Enrofloxacin 50mg/kg intravenously. Piglets were allowed to recover on a heat mat. Post-operatively, animals received the analgesic Buprenorphine 0.01mg/kg two to three times daily. Calcein (10 mg/kg) was delivered via subcutaneous injection at weeks 6 and 7 post surgery. Calcein labelling is a commonly used technique that becomes incorporated into new bone, and is used to identify newly mineralised bone. Pigs were culled at 8 weeks. All experiments were carried out with approval from the South Western Area Health Service (SWAHS) Animal Ethics Committee (Protocol number 5086, issued April 2011).

**Epiphyseal Quotient (EQ) and Area Analyses**

Area calculations were taken using Image J from flatbed scanned images of bisected femoral heads. The value for each sample was an average of three separate measurements of the femoral head, bounded by the growth plate. Epiphyseal quotient was calculated as described by Gong et al. as a ratio of the height of the femoral head from the centre of the growth plate to the length of the growth plate [15]. In a healthy femoral head this ratio is ~0.5, with a value of <0.4 representing significant collapse.
Chapter 5: SAIB in a piglet model of Perthes disease

**Radiography**

Samples were digitally X-rayed (Faxitron X-ray Corp, Illinois, USA) at 25kV, 1.5x magnification. X-rays were used to image the hard tissues, and identify collapse of the femoral head. Soft tissues of the joint were imaged using dual-energy absorptiometry (DXA, Piximus, Wisconsin, USA) to identify any instances of heterotrophic ossification (HO).

**Confocal imaging**

Imaging was conducted on a Leica SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) using the 488nm, and 633nm laser lines and with 500-560nm, and 640-720nm emission bands, respectively. Bone surfaces were scanned with a 5x objective using the Tile Scan (300-400 images) and Z stack (4-5 slices at 150µm intervals) functions, and images were processed with Leica Application Suite Advanced Fluorescence software (Version 2.5.1-6757) and Adobe Photoshop.
Chapter 5: SAIB in a piglet model of Perthes disease

Results

**Ease of percutaneous injection of SAIB into the femoral head**

Following cannulation, manual infusion of SAIB containing agents was a rapid and simple procedure (Figure 5.1). Injection of the SAIB:solvent solution did not require excessive pressure to infuse the head. After removal of the cannula, no leakage of SAIB into the operative space was observed. In contrast, saline was poorly contained after removal of the cannula.

One pig from the control group was culled early due to excessive limping. All of the SAIB infused animals survived without complications during monitoring for the 8 week duration of the study.
Figure 5.1: Surgical procedure to induce osteonecrosis of the femoral head. (A) Division of the short abductor muscle (B) Joint capsule is opened, ligamentum teres cut, and suture placed around the base of the femoral head (C) Sutures tied (D) EZ-IO cannula is used to deliver bisphosphonates and SAIB containing BMP-2 into the femoral head
**Prevention of femoral head collapse with SAIB/BMP-2/bisphosphonate**

Based on calculations of EQ, the n=3 surviving saline treated animals all showed an EQ<0.4 in the operative side indicating collapse at 8 weeks after surgical intervention. In contrast, the contralateral hips of all groups retained their sphericity (Figure 5.2).

![Figure 5.2: Quantification of femoral head collapse in the porcine model of Perthes disease as measured by epiphyseal quotient (A). Representative measurement taken from flatbed-scanned image and quantified in Adobe Photoshop is shown in B. Circles (•) represent un-collapsed samples; diamonds (♦) represent collapsed samples.](image)
Chapter 5: SAIB in a piglet model of Perthes disease

Figure 5.3: Quantification of femoral head collapse in the porcine model of Perthes disease as measured by femoral head area (A). Representative measurement taken from flatbed-scanned image and quantified in ImageJ shown in B. Circles (●) represent un-collapsed samples; diamonds (♦) represent collapsed samples.

In the BMP-2 intervention group, one hip showed an apparent prevention of collapse with an EQ value of 0.42. However, subsequent analysis showed that this corresponded to the hip with the lowest 2D cross-sectional area (Figure 5.3) and that this hip showed histological evidence of significant collapse. In comparison, two pigs from the SAIB/BMP-2/bisphosphonate treatment group showed a lack of collapse both histologically and by EQ > 0.4. This group demonstrated a higher mean EQ and femoral head area than the saline and BMP-2 treated groups.

X-rays of the operated femoral heads showed clear radiological evidence of collapse and deformity, and this was reduced with SAIB/BMP-2/bisphosphonate treatment (Figure 5.5 A-D). An acute effect of bisphosphonate treatment is a horizontal line of radiodense sclerotic bone at the metaphysis, termed bisphosphonate lines, and these were seen in ZA/AlexaPAM-treated specimens. Flatbed scans of the bisected femoral heads revealed thickened articular cartilage,
Chapter 5: SAIB in a piglet model of Perthes disease

particularly in the saline and SAIB/BMP-2 groups (Figure 5.4 E-H). These groups were also the most irregular in respect to shape of the femoral head and growth plate. The SAIB/BMP-2/bisphosphonate treated hips were the most similar to the non-operated side.

**Fluorescent tags for new bone growth and local bisphosphonate delivery**

Fluorescent labelling of new bone formation using Calcein and the distribution of bisphosphonate in the femoral head were examined by confocal microscopy (Figure 5.4 I-L).

Bisphosphonate was only observed in the SAIB/BMP-2/bisphosphonate group, and a robust signal was observed in the hip at 8 weeks after injection indicating long-term retention in the hip. Systemic Calcein dosing demarking bone formed between 6-8 weeks after operative insult showed a limited signal the saline and BMP-2 treated groups; this indicated minimal new bone formation. In contrast, the SAIB/BMP-2/bisphosphonate group revealed a strong Calcein signal peripheral to the bisphosphonate line indicating robust new bone formation.
Figure 5.4: Visualisation of femoral head collapse in a surgical model of Perthes disease in the pig. Samples from each group are shown as X-ray (A-D), flat bed scan (E-H), and confocal images (I-L). In the confocal images: Green, calcein; blue, local BP. Arrows indicate collapsed head, arrowhead indicates bisphosphonate line.
**SAIB/BMP-2 induces limited heterotrophic ossification in the joint capsule**

HO was observed in the soft tissues of the joint in a number of specimens treated with BMP-2. The bone area of the soft tissues was quantified by DEXA and a bimodal distribution was noted corresponding with the presence of heterotopic bone by X-ray (Figure 5.5). HO was absent in 50% of animals receiving SAIB/BMP-2 suggesting that further optimization of this procedure may be able to minimize this potential complication.

**Figure 5.5:** Quantification of heterotrophic ossification in the joint capsule in treated hips. (A) X-ray example of bone formation in the soft tissue of the joint. Arrowheads highlight bone nodules. (B) Quantification of bone area in the soft tissue of the joint.
Chapter 5: SAIB in a piglet model of Perthes disease

Discussion

In this study, we were able to replicate a surgical model of osteonecrosis of the femoral head in the pig [117] and demonstrate that the local delivery of BMP-2 and bisphosphonates in the sugar-based carrier SAIB was able to prevent collapse in half of the cases. Clinically, the severity and prognosis of Perthes disease correlates with the sphericity of the femoral head [538]. A femoral head with an EQ ≤ 0.4 commonly progresses to osteoarthritis [539], and treatments therefore, aim to maintain the sphericity.

Injectable solutions have been previously investigated for the treatment of ONFH, where an injectable calcium based ceramic combined with core decompression showed promising results clinically [540]. The injectable carrier SAIB has been previously employed to delay BMP-2 release from a collagen-chondroitin scaffold by infusing it into the scaffold similar to our injection into the femoral head [264]. This system was selected based on its capacity to phase transition allowing it to both be injected and infuse the femoral head, but minimize its leakage into the joint capsule. While some heterotopic bone was observed with the SAIB/BMP-2 groups, we predict that this would be a more substantive issue if BMP-2 was delivered via saline and was poorly contained within the femoral head as previously reported by Vandermeer et al. who injected BMP-2 and ibandronate in saline [537]. HO is a major concern with the use of BMP-2, especially in joint spaces, as they can decrease the flexibility and structural properties of the soft tissues surrounding the joint. It is possible that further optimization of the SAIB system and/or injection technique may reduce or eliminate this potential adverse event.

We demonstrated that labelled Pamidronate injected in SAIB was retained within the femoral head out to 8 weeks at the experimental end point. In a comparable pig model, bisphosphonate distribution was examined at 1 week after surgery [541] and showed that $^{14}$C tagged Ibandronate
delivered locally to the femoral head was retained. We hypothesize the release of BMP-2 may be more advantageous from a slow release carrier such as SAIB, however as bisphosphonates can bind the bone of the femoral head it is unclear whether there is specific advantage to delayed release. Nevertheless, rapid loss of bisphosphonate into the joint capsule would likely be less favourable than remaining in the femoral head, thus we speculate that use of a phase-transitioning carrier such as SAIB may be advantageous in this regard.

Treatment of femoral head osteonecrosis typically targets preventing resorption of the dead bone that can result in collapse. In this study, we noted that anabolic intervention with SAIB/BMP-2 was unable to restore femoral head sphericity. Treatment with BMP-2 has been associated with increased osteoclast activity and bone catabolism [484]. Clinically, BMP-2 has been associated with inflammation, which has been associated with adverse events [542]. In a segmental defect model in a rat, high doses of BMP-2 were seen to cause a significant inflammatory response with an associated increase in osteoclast-like cells [543]. These data suggest that combined anabolic and anti-resorptive treatments may yield improved outcomes. Our group has previously described a synergistic relationship between BMPs and bisphosphonates in other animal models [144, 161, 544].

The selected growing pig model reflects the intention of developing a superior intervention for Perthes disease, the results of this study could also be applicable to traumatically induced or adult onset ONFH. Adult onset ONFH has many causes [545, 546], however much like Perthes disease, the femoral head presents with necrosis of the bone that may lead to collapse. In such a setting our system could be used on its own, or perhaps in combination with a surgical procedure such as core decompression.
Summary and conclusions

In this chapter, I describe the successful use of SAIB in delivering local BMP-2 intraosseously in a large animal model of surgically induced Perthes. The co-delivery of local BMP-2 and bisphosphonate was able to prevent half of the pigs from continuing onto femoral head collapse. Further, the local bisphosphonate was also demonstrated to be retained in the femoral head 8 weeks after a single dose, with evidence of new bone formation in this group. Although, it is likely not clinically realistic that a treatment could be delivered at the same time as the vascular insult, this study highlights the feasibility of SAIB delivery of BMP-2 into trabecular bone regions that are inaccessible to traditional BMP-2 carriers.
Chapter 6. The synthesis and validation of novel high viscosity liquid carrier mediums for bone tissue engineering
Chapter 6: Novel carriers for BMP-2

Introduction

**Angiogenesis and osteogenesis are tightly coupled**

An adequate blood supply is critical for bone regeneration and repair [547]. There is a long-established clinical association between angiogenesis and osteogenesis. The presence of a compromised blood supply is one of the major contributors to the delayed union of fractures [492, 548]. Open fractures in particular often show extensive damage to the surrounding soft tissues that is associated with a decreased ability to vascularise the callus; such fractures are highly susceptible to progress to non-union. Conversely, the use of vascularised bone grafts to ensure both adequate blood supply and pro-osteogenic environment has been shown to greatly improve bone regeneration [549, 550].

The potent angiogenic growth factor VEGF has been experimentally shown augment bone regeneration in multiple models [336, 551, 552]. In a rabbit model of open fractures, treatment with VEGF delivered in hyaluronic acid resulted in union that had comparable biomechanical properties to autograft treatment [336]. Continuous delivery of VEGF through an osmotic pump for 7 days post-surgery resulted in the bridging of a critical sized radial defect in the rabbit [553].

However, recombinant VEGF protein has not been marketed clinically in orthopaedics. The therapeutic range of VEGF is quite narrow, and the dose applied must be tightly controlled as excess could lead to haemangioma or tumour formation. The use of VEGF is limited by the unstable nature and short biological half-life of the protein *in vivo*, as well as the high cost of production. One strategy to extend the *in vivo* efficacy of VEGF is the use of gene therapy, which has been shown to enhance the healing the healing of segmental defects created in rabbits [554, 555]. However, the use of gene therapies clinically still must overcome significant scientific and regulatory hurdles before it is a readily used treatment strategy. Regardless of the delivery method,
Chapter 6: Novel carriers for BMP-2

when used in combination with BMP-2 is also important to consider the ratio between the factors [556] as well as the temporal release of each protein [557].

The use of small molecule pro-angiogenic drugs can ameliorate some of the challenges associated with VEGF usage. Desferoxamine (DFO) is a drug that activates the hypoxia inducible factor pathway, in turn leading to the downstream stimulation of VEGF expression. The delivery of DFO in bone healing scenarios has been shown to enhance bone regeneration in animal models of fracture repair [558], distraction osteogenesis [559], and segmental defects [560]. However, the use of DFO or similar agents to stimulate angiogenesis during bone healing in a clinical setting has yet to be translated.

**Rationale for synthesis of new materials**

For many decades, the SAIB molecule has been utilised for a range of applications, including in the food and pharmaceutical industries. Its long standing use in food consumption has lent a well-documented safety record, both in animals and humans. In this thesis, I have described the novel application of SAIB as a bone tissue engineering carrier in a range of applications (Chapters 2–5). There are two critical physical properties made SAIB appealing as a bone tissue engineering carrier: hydrophobicity and viscosity. The high viscosity allows an implanted material to form a depot at the injection site and trap any drugs or proteins that have been delivered. The hydrophobicity of SAIB facilitates the rapid diffusion of solvent into the surrounding tissues *in vivo*, creating the phase transition phenomenon. Further, these two properties are exploited during the synthesis procedure to purify the material. Thus, any new high viscosity liquid carrier medium (HVLCM) synthesised should optimally exhibit these two properties.

It was hypothesised that a SAIB-like carrier based on an alternative sugar could be used to deliver BMP-2 or other agents while simultaneously stimulating an angiogenic response. SAIB itself is a
Chapter 6: Novel carriers for BMP-2

relatively neutral material that has been reported to have no effect on either promoting or inhibiting angiogenesis [561]. In comparison, the pentose monosaccharide deoxyribose has well recognized native roles in regulating angiogenesis (as described in Chapter 1: Therapeutic modulation of bone). Fructose-based polymers known as levans are reported to be biocompatible with applications in drug delivery [562], and wound healing [563]. Thus HVLCMs based on fructose were also explored.

The SAIB molecule is synthesised by the esterification of sucrose with acetic and isobutyric anhydrides. Although this esterification procedure is controlled, the final SAIB product is a mixed ester that has previously showed a ratio of 2 acetate:6 isobutyric side chains covalently bound to the sucrose backbone [250]. Thus SAIB has acetic and isobutyric sidechains replacing all hydroxyl groups of the sucrose monomer (Figure 6.1). In-house synthesis of SAIB was performed as a part of this thesis, and the protocol is described in Chapter 2: Materials and Methods.

Figure 6.1: The chemical structure of the SAIB molecule. Image modified from [564]

Esterification and polycondensation were identified as key potential synthesis techniques for novel HVLCMs. To generate an esterified material (such as SAIB), the hydroxyl groups (-OH) on the sugar are replaced by acetic and isobutyric side chains. Again, for the example of SAIB, the sucrose molecule is made up of two different monosaccharides, the hexose glucose and the pentose
fructose. Sucrose contains eight hydroxyl groups that are available for esterification (Figure 6.2). Unlike sucrose, deoxyribose is a pentose monosaccharide and has only three available hydroxyl sites for esterification (Figure 6.2). Fructose, one of the monomers of sucrose, is also a pentose sugar. However, fructose has five hydroxyl groups available for esterification (Figure 6.2).

Another method that can be utilised to increase the viscosity of products is polycondensation. This reaction involves the step-growth polymerisation to create polymers of the base sugars. The polycondensation joins two sugar monomers via an ether bridge by dehydrating two hydroxyl groups. This reaction then generates a heterogeneous mix of linkages between the base sugars (e.g. position 1-6, 1-4, 2-6 etc.) that are expected to form branching structures. After polycondensation the sugar polymers are able to undergo esterification, however there are reduced hydroxyl sites available for esterification (Figure 6.2).

This chapter describes the synthesis of new HVLCMs to act as potential carriers for bone tissue engineering. The synthesis techniques of esterification and polycondensation were utilised to generate new materials. Significant optimisation of the synthesis procedures was required to
generate the best carriers possible. Following synthesis, the materials were tested for two critical biological properties essential for successful translation of a bone tissue engineering carrier: biocompatibility and BMP-2 delivery. These properties were tested in vivo in a muscle pouch implantation model in the mouse. In this model, the capability and effectiveness of the new materials to deliver BMP-2 and form ectopic bone nodules was investigated.
Chapter 6: Novel carriers for BMP-2

Materials and Methods

Synthesis of novel high viscous carriers

The synthesis of sugar-based materials was conducted in collaboration with the School of Chemical and Biomolecular Engineering at the University of Sydney, Australia. Synthesis protocols were developed with Dr Peter Valtchev, in the laboratory of Prof Fariba Dehghani. The protocols described have undergone optimisation, and were used for the production of carriers used in subsequent in vivo testing.

Optimised protocol for synthesis of esterified deoxyribose

The initial attempts to synthesise a new sugar-based carrier for bone tissue engineering was based on a modification of the procedure used to manufacture SAIB (Chapter 2). The methods aimed to esterify individual deoxyribose molecules, mimicking the sucrose esterification that forms SAIB.

For this method, 10 g of deoxyribose (Carbosynth; Berkshire, UK) and 2.1 g sodium acetate was added to a three necked round bottom flask. This was mixed using a magnetic stirrer and heated to 95°C in a silicon oil bath until melted. Following this 6.6 mL acetic anhydride and 38 mL isobutyric anhydride was added to the flask, and allowed to esterify for 30 minutes. Ethanol was added to purify the product, and allowed to mix for an additional 30 minutes. The contents of the flask was then poured into ice cold water, and the precipitate at the bottom of the flask was collected. The product was then washed by dissolving in ethanol and then adding water which caused the product to precipitate. The product was then isolated by centrifugation, with this process repeated three times. The product was then vacuum dried at 40°C for at least three days.

The resultant product was termed deoxyribose acetate isobutyrate (dRAIB).
Optimised protocol for synthesis of polycondensed and esterified sugars

A modified method for synthesis of sugar-based carriers for bone tissue engineering was developed. These methods attempted to increase viscosity of the final product by initially polymerising the base sugars, creating a polycondensed sugar backbone that was subsequently esterified. For these methods, two pentose sugar bases were chosen, deoxyribose and fructose.

To synthesize the products, 10 g of either fructose (Sigma Aldrich; Missouri, USA) or deoxyribose was added to a three necked round bottom flask, along with 10 mL acetic acid and 50 mg citric acid. A magnetic stirrer was added and a condenser mounted onto the flask, which as then heated to 90°C in a silicon oil bath (Figure 6.3A). Once the sugar was dissolved, a mild vacuum was applied for 10 minutes. At this point the vacuum is released, a vessel with Schliff valve was attached to the flask and 6.6 mL acetic anhydride and 38 mL isobutyric anhydride were added to the flask dropwise, along with 2.1g sodium acetate. Once all the anhydrides had been added, the reaction was allowed to occur for 15 minutes. To purify the product, 100 mL ethanol was added to the flask, and allowed to mix for 30 minutes. The flask was then lifted from the heat, allowed to cool down, then poured into ice cold water (Figure 6.3B) and the precipitate collected (Figure 6.3C). The product was then washed by dissolving in ethanol and then adding water which caused the product to precipitate. The product was then isolated by centrifugation, with this process repeated three times. The product was then vacuum dried at 40°C for at least three days.

The compound created with fructose is then called polycondensed fructose acetate isobutyrate (pFAIB), and the compound synthesized with deoxyribose is called polycondensed deoxyribose acetate isobutyrate (pdRAIB).
Figure 6.3: Synthesis of novel viscous materials based on the sugars fructose and deoxyribose.
A The equipment set up for synthesis of polycondensed and esterified sugars. B Following the synthesis procedure, the materials are poured into ice cold water to allow for removal of solvents and collection of the material which forms as a precipitate C The viscous new material on the bottom of the bottle.

**Viscosity testing of new materials**

Samples were tested using a Physica MCR 301 (Anton Paar; Graz, Austria) viscometer. Temperature was kept stable at 37°C, and samples were measured using plates 50 μm apart.
Samples were tested at a range of solvent concentrations; SAIB was tested diluted using 10%, 20%, 30% and 50% ethanol (v/v), while pFAIB was tested at dilutions of 10%, 20%, 30% and 50% ethanol (w/w). The pdRAIB was too viscous to be tested at 10% ethanol (w/w), and was measured at 20% ethanol (w/w).

**Surgical implantation of new materials**

The biocompatibility and bone formation capability of the new materials was tested in the muscle pouch model in the mouse. The procedure was the same as described in Chapter 2. Briefly, the carriers were diluted with ethanol and mixed with BMP-2. The mixtures were then loaded into a 1 mL Luer lock syringe and injected into the muscle pouch of the mouse.

**Experimental design**

*The use of SAIB to deliver angiogenic compounds*

SAIB was utilised to study the effects to potentially angiogenic compounds on ectopic bone volume. For the addition of the angiogenic compounds DFO (Sigma Aldrich) and deoxyribose sugar, these were admixed into SAIB along with the BMP-2 (Table 6.1). The newly synthesized dRAIB had a low viscosity, and was thus tested undiluted as well as a diluent for SAIB.

<table>
<thead>
<tr>
<th>Group</th>
<th>BMP-2/pellet</th>
<th>Pro-vascular Agent</th>
<th>Dose/pellet</th>
<th>N=</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 µg</td>
<td>DFO</td>
<td>25µg</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>5 µg</td>
<td>Deoxyribose</td>
<td>25µg</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>5 µg</td>
<td>dRAIB</td>
<td>20% (v/v) SAIB</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>5 µg</td>
<td>dRAIB</td>
<td>30% (v/v) SAIB</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>5 µg</td>
<td>dRAIB</td>
<td>20µL</td>
<td>6</td>
</tr>
</tbody>
</table>

**Table 6.1**: Experimental groups for investigating an esterified deoxyribose compound. Due to its low viscosity, it was used in combination with SAIB in some cases.
Chapter 6: Novel carriers for BMP-2

*Study design investigating the safety of new polycondensed and esterified carriers*

The newly synthesized carriers were initially tested for *in vivo* toxicity effects in the muscle pouch of a mouse (Table 6.2). The main outcome of this study was to assess if any of the new carriers generated adverse effects in the mouse, including inflammation, signs of distress or sudden death. SAIB was diluted with 20% ethanol (v/v) as per previous studies while the new carriers, pFAIB and pdRAIB, were diluted with 20% ethanol (w/w). One mouse was injected bilaterally per group, and culled one week post-surgery.

**Table 6.2:** Experimental design for investigating potential toxicity of new carriers

<table>
<thead>
<tr>
<th>Carrier</th>
<th>BMP-2</th>
<th>N=</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAIB</td>
<td>5 µg</td>
<td>1</td>
</tr>
<tr>
<td>pFAIB</td>
<td>5 µg</td>
<td>1</td>
</tr>
<tr>
<td>pdRAIB</td>
<td>5 µg</td>
<td>1</td>
</tr>
</tbody>
</table>

*Comparison of SAIB and pdRAIB as carriers for bone tissue engineering*

The newly synthesised carrier pdRAIB was then investigated as to its capacity to deliver BMP-2 to generate bone nodules in the muscle pouch model in the mouse. SAIB was used as the control (Table 6.3), and as per the previous study, pdRAIB was diluted with 20% ethanol (w/w). Mice were culled three weeks after surgery.

**Table 6.3:** Experimental design comparing SAIB and pdRAIB as carriers for bone tissue engineering

<table>
<thead>
<tr>
<th>Carrier</th>
<th>BMP-2</th>
<th>N=</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAIB</td>
<td>5 µg</td>
<td>5</td>
</tr>
<tr>
<td>pdRAIB</td>
<td>5 µg</td>
<td>5</td>
</tr>
</tbody>
</table>
Chapter 6: Novel carriers for BMP-2

**Histological analysis**

*Safranin O*

Safranin O (Saf O) binds to the proteoglycans present in collagen matrix, and stains collagenous tissue red [565]. Nuclei are stained in purple, while bone and soft tissues are stained in green.

Paraffin slides were dewaxed and brought to water. Slides were stained in 5% iron haematoxylin for 4 minutes, and washed under running tap water for 5 minutes. Samples were then stained in 0.4% aqueous light green for 6 minutes, and rinsed in two changes of aqueous 1% acetic acid. Slides were then stained in 0.1% aqueous SafO for 5 minutes. Samples were then dehydrated by dipping in 95% ethanol five times, followed by three dips in three changes of 100% ethanol, and then three changes of Xylene. Coverslipped and mounted using Ultramount.

*IHC against von Willebrand factor*

Von Willebrand factor (vWF) is a blood glycoprotein that is expressed in the endothelium, but also in megakaryocytes and sub-endothelial connective tissue [566].

Paraffin slides were dewaxed (without heat) and brought to water, then soaked in PBS. Antigen retrieval was conducted using 0.4% pepsin (Sigma Aldrich) at 37°C for 25 minutes. Slides were then washed in PBS, then blocked for unspecific binding (5% horse serum in PBS) for 1 hour at room temperature. Without washing, the primary antibody (Rabbit-anti-mouse vWF (AB7356, Merck Millipore, Massachusetts, USA) diluted 1:200 in dilution buffer (1% bovine serum albumin, 0.1% Tween 20 in PBS) and allowed to incubate at 4°C overnight. Slides were then washed in PBS, and endogenous peroxidase quenched with 3% hydrogen peroxide in PBS for 30 minutes at room temperature, and washed again in PBS. The secondary antibody (ECL donkey-anti-rabbit; #NA934V, GE Healthcare, Little Chalfont, UK) was then added at a 1:300 dilution in dilution buffer for 30 minutes at room temperature. After this, slides were washed in PBS, and then slides
were developed using a DAB substrate kit (#SK-4100; Vector Laboratories Inc, California, USA), and counterstained with haematoxylin and coverslipped using aqueous mounting medium (CC mount, Sigma Aldrich).

**IHC against Galectin-3**

Galectin-3 (Gal3) is a member of the lectin family, is also known as LGALS3, galactose-specific soluble lectin 3, Mac-2, and L-29. Gal3 is expressed by many cells, including macrophages [567], and is indicative of an acute and chronic inflammatory responses [568]. Gal3 is reported to have a critical role in phagocytosis [569].

Paraffin slides were dewaxed (without heat) and brought to water, then soaked in PBS. Antigen retrieval was conducted using 0.4% pepsin at 37°C for 25 minutes. Slides were then washed in wash buffer (0.05% Tween 20 in PBS), then blocking buffer was added (0.1% Tween 20, 10% goat serum, 1% bovine serum albumin in PBS) for 1 hour at room temperature. Without washing, the primary antibody (Rat-anti-mouse Galactin-3 (#14-5301, eBioscience, California, USA)) was diluted 1:200 in dilution buffer (10% goat serum, 0.05% Triton X-100 in PBS), added to the slide and allowed to incubate at 4°C overnight. Slides were then washed in wash buffer, which was used for all subsequent washes. Endogenous peroxidase quenched with 3% hydrogen peroxide in PBS for 20 minutes at room temperature, and slides were re-washed. The secondary antibody (donkey-anti-rat HRP; #AP189P, Merck Millipore) was then added at a 1:250 dilution in dilution buffer for 30 minutes at room temperature. After this, slides were washed and then streptavidin-peroxidase (#S5512, Sigma-Aldrich) diluted 1:100 in PBS was added for 30 minutes at room temperature. Slides were washed a final time and then developed using a DAB substrate kit. Tissue sections were counterstained with haematoxylin and aqueous mounted.
Chapter 6: Novel carriers for BMP-2

Results

Delivery of potentially angiogenic compounds in SAIB

Esterification of the base sugar deoxyribose led to the synthesis of the material denoted as dRAIB. This material was light in colour and it had a viscosity that was close to water. It was hypothesised that dRAIB would be angiogenic due to the properties of its constituent monomer, deoxyribose. A standard muscle pouch model was used to investigate the ability for this material to augment bone formation. dRAIB was tested as a carrier on its own and in combination with SAIB (70/30 and 80/20 SAIB/dRAIB formulations). Additionally, SAIB/BMP-2 was admixed with two pro-angiogenic compounds: DFO and the unaltered sugar deoxyribose.

The group receiving BMP-2 delivered in dRAIB alone suffered an adverse reaction. Mice were found in a catatonic state as part of normal post-procedural monitoring at day 1, and these animals were euthanized. This adverse event was subsequently attributed to the circulation of residual solvents from synthesis, and is later discussed in detail. The remaining groups did not display any signs of distress at any time in the study.

After three weeks the bone formation in the remaining groups was quantified using microCT. There were no differences seen in mean bone volume formed between any of the groups studied (Figure 6.4). All groups, irrespective of treatment generated comparable bone nodules.
Chapter 6: Novel carriers for BMP-2

**Figure 6.4:** Bone volume formed in response to 5 µg BMP-2 delivered in SAIB in combination with potentially angiogenic substances. **A** Bone volume quantified by micro CT and **B** X-rays of representative samples from each group.

*The newly synthesized materials are viscous*

The polycondensation of the base sugar prior to esterification led the successful synthesis of new viscous materials denoted as pdRAIB and pFAIB. During the synthesis procedure, the longer the materials were allowed to polycondense, the more viscous the final product. The final protocol involved polycondensation time of 10 minutes at 90°C under vacuum, which was found to be the optimal balance between extending the sugar chain before depletion of –OH groups available for esterification.

The newly synthesised materials were variable in colour, ranging from a dark brown to a light yellow (Figure 6.5). This browning was generally more pronounced during pFAIB synthesis. In addition, it was found that when older batches of the base sugar deoxyribose were used, a deeper colour developed in the pdRAIB during synthesis.
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Figure 6.5: The colour range of the synthesised products pdRAIB and pFAIB. Commercially produced SAIB was compared against three runs of pdRAIB and two runs of pFAIB.

The new materials then underwent dynamic viscosity testing at a constant shear rate at 37°C. In relation to SAIB, pdRAIB and pFAIB showed a similar steep drop in viscosity in response to increasing proportions of ethanol (Figure 6.6). Despite the dilutions in ethanol between the carriers being calculated differently (SAIB was w/w, and pdRAIB and pFAIB were v/v) the overall response of the new carriers to dilution in ethanol is more important. For muscle pouches, SAIB is generally used at an 80:20 dilution with ethanol that is calculated by volume, which is calculated using the specific gravity described by the supplier (1.146 g/cm³). However, as we have not yet fully characterised the new carriers, these dilutions were based on volume. Importantly, small proportions of ethanol (10-50%) were able to result in great reductions in viscosity of pdRAIB and pFAIB. Critical to the success of SAIB as an injectable carrier is the small amounts of ethanol required to decrease the viscosity and allow for injection. Thus, pdRAIB and pFAIB continue to be considered as candidates for novel BMP-2 carriers.
Figure 6.6: Comparison of dynamic viscosity of HVLCMs across different ethanol dilutions at a shear rate of 50/s at 37°C. The viscosity of pdRAIB was too high to attain a value at 10% solvent.

**pdRAIB and pFAIB show in vivo biocompatibility**

In light of the adverse events seen with dRAIB, a preliminary *in vivo* toxicity study was conducted using the newly synthesised pdRAIB and pFAIB. In addition, the materials were left to dry in a vacuum oven to ensure the majority of residual solvents from the manufacture were removed.

In this study, a single mouse received bilateral injections containing each of the carriers admixed with BMP-2. The mice were monitored closely over the course of the week long study. The mouse injected with pFAIB developed some swelling in the hind limbs, which had abated by 36 hours. The pdRAIB and SAIB mice were unaffected. After one week, the mice were culled. At the harvest, it was observed that there was an increase in the subcutaneous vascular density of the pdRAIB mouse (Figure 6.7).
Figure 6.7: Areas of increased vascular density seen on the back of the mouse that received pdRAIB injections at the one week cull point.

X-rays taken at the cull point showed the presence of early bone formation in each of the mice (Figure 6.8). Discrete regions of increased radio-opacity were noted in some of the X-rays, potentially consistent with initiation of ectopic bone formation.
Figure 6.8: X-rays of both hind muscle pouches from each mouse that received BMP-2 delivered in SAIB, pFAIB or pdRAIB one week post injection. Arrowheads indicate regions of new bone formation.

Bone nodules then underwent paraffin histology and were stained with haematoxylin & eosin, and Safranin O. The red staining seen in the Safranin O highlighted the cartilage that appeared to largely comprise the nodules; the darker purple areas in both stains indicated bone formation was occurring (Figure 6.9).
Figure 6.9: Histology of nodules formed by SAIB, pFAIB, and pdRAIB with 5 µg BMP-2 one week after implantation into the quadriceps of a mouse. Paraffin histology stained with haematoxylin & eosin (H&E) and Safranin O (Saf O). Scale bar indicates 300 µm for all images.
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Immunohistochemical staining against von Willebrand factor (vWF) was used to identify endothelial cells that line blood vessels. Qualitative vWB staining on each of the nodules revealed the most signal seen in the pFAIB group, and more restricted signal seen in the SAIB and pdRAIB samples (Figure 6.10). The increased staining in the pFAIB was speculated to be likely associated with the post-operative swelling seen in this group.

**Figure 6.10:** Immunohistochemical staining for von Willebrand factor on nodules formed by SAIB, pFAIB, and pdRAIB with 5 μg BMP-2 one week post implantation. Panels focus on the pellet border (dashed line) with the muscle (M). Scale bar indicates 200 μm in all panels.

Further immunohistochemical staining was conducted against Gal3, which is a marker of inflammatory macrophages. Qualitative analysis showed strong staining in the SAIB and pFAIB
nODULES, WITH MUCH LESS SIGNAL SEEN IN THE PDRAIB SAMPLE (FIGURE 6.11). AS EXPRESSION OF GAL3 IS INDICATIVE OF ACTIVATED MACROPHAGES, AND IT APPEARS THAT SAIB AND PFAIB HAVE STIMULATED A FOREIGN BODY REACTION. AT THIS TIME POINT, THERE APPEARS TO BE MINIMAL SIGNAL IN THE PDRAIB SAMPLE, HOWEVER THIS COULD BE THE RESULT OF AN INFLAMMATORY RESPONSE THAT OCCURRED EARLIER IN TIME THAT HAS NOW DISSIPATED. FURTHER TIME COURSE STUDIES WOULD BE REQUIRED TO FULLY UNDERSTAND THE INFLAMMATORY RESPONSE TO INTRAMUSCULAR PDRAIB.

**Figure 6.11**: Immunohistochemical staining for Galectin-3 on nodules formed by SAIB, pFAIB, and pdRAIB with 5 µg BMP-2 at one week post implantation. Panels focus on the pellet border with the muscle. Scale bar indicates 150 µm in all panels.

In response to the results seen from the one week toxicity study, it appears that both pFAIB and pdRAIB are non-toxic. In response to the co-delivery of BMP-2, signs of early bone formation are evident. However, with the strong inflammatory reaction in response to the intramuscular delivery of pFAIB, this compound was not chosen for further study at this time. The use of pdRAIB as a carrier of BMP-2 was selected for studies investigating bone formation.
**pdRAIB is an effective delivery system for BMP-2**

The capacity for pdRAIB to deliver 5 µg BMP-2 in the mouse hind limb muscle pouch was investigated in a 3 week study. MicroCT quantification revealed that both pdRAIB and SAIB generated comparably sized nodules, with no significant difference found between the carriers (Figure 6.12A). Radiographs and microCT reconstructions revealed bone nodules that are indistinguishable, with representative samples shown in Figure 6.12B and C.

![Figure 6.12](image)

**Figure 6.12**: Bone volume in response to 5 µg BMP-2 delivered in either SAIB or pdRAIB to the muscle pouch of the mouse. **A** Bone volume quantified by micro CT, **B** representative X-rays from each group, and **C** microCT reconstructions of the representative pellets from each group.
Figure 6.13: Histological analysis of bone nodules formed in response to BMP-2 delivered in either SAIB or pdRAIB after three weeks. Paraffin histology was stained with haematoxylin and eosin (H&E), for tartrate resistant alkaline phosphatase (TRAP), and immunohistochemistry against Galectin-3 (Gal3 IHC). Scale bar represents 400 µm for all panels.
Haematoxylin & eosin staining of bone nodules formed by BMP-2 delivery in SAIB and pdRAIB groups demonstrated very similar ectopic bone tissue morphology (Figure 6.13). Bone nodules formed as a cortical shell within the muscle tissue bed. The interior of the nodule contained trabecular bone spicules along with adipose and marrow cells. There also appeared to be no clear difference in TRAP positive cells lining the bone surfaces (Figure 6.13). Immunohistochemistry for Gal3 showed the presence of positive staining cells in ring formations surrounding what was posited to be bubbles of unresorbed carrier (Figure 6.13).
Chapter 6: Novel carriers for BMP-2

Discussion

In this chapter I describe the successful synthesis of alternative carriers to SAIB that are highly viscous and capable delivering recombinant BMP-2 to produce new bone. Initially, utilising the same protocol as used for the synthesis of SAIB, the deoxyribose sugar was esterified to produce a material that was labelled dRAIB. This resulted in a product that was largely inviscid. In contrast, when the deoxyribose was polycondensed prior to esterification (pdRAIB), the resultant product was highly viscous (Figure 6.6). The same was true for the polycondensed and esterified version of the sugar fructose, pFAIB (Figure 6.6).

In liquids, a key factor affecting viscosity is the interactions between molecules. It is hypothesised that the viscosity of SAIB is due to the internal resistance between the ester chains on the sucrose molecule. Although it appears that sucrose is highly unique molecule in this respect, as the simple esterification of a sugar is not sufficient to produce a viscous material (i.e. dRAIB). The polycondensation of the sugar produced a branching backbone that allowed for increased esterification sites at a single molecule. This is hypothesised to increase the internal resistance between molecules, thus increasing the viscosity of the material. Further, an increase in the time allowed for polycondensation during synthesis generated a more viscous product. However, increased polycondensation must be balanced against leaving enough –OH groups for esterification. If the product is polycondensed excessively, it will generate a viscous material that will not be hydrophobic, and thus does not fulfil our criteria for a BMP-2 carrier.

While our first generation carrier (dRAIB) showed unfavourable physicochemical properties and poor in vivo biocompatibility, the second generation carriers, pdRAIB and pFAIB, showed significantly greater promise. These materials exhibit the two critical physical properties - hydrophobicity and viscosity - that I hypothesise are behind the success of SAIB as BMP-2 carrier.
Further, pdRAIB was found to form a comparable bone nodule to SAIB, in regards to bone volume (Figure 6.12) and morphology (Figure 6.13). Thus, pdRAIB has great potential to be a successful carrier for BMP-2.

Despite the hypothesised benefits of the co-delivery of angiogenic agents with BMP-2 on bone formation, this was not seen in this study. Neither the delivery of an established pro-angiogenic drug, such as DFO, (Figure 6.4) nor the use of pdRAIB/BMP-2 (Figure 6.12) were able to augment BMP-2 induced bone formation. Although DFO has been reported to have some positive effects on bone healing, there is some controversy in the literature about its efficacy. In one study, DFO was found to increase vascularity in a rat segmental defect [560]. However, the combination treatment of DFO and BMP-2 did not significantly improve functional bone healing outcomes such as load to failure over BMP-2 alone.

The variability in the synergistic effects of these agents reported in the literature may be due the carrier, or alternatively that the effects of pro-vascular agents are minimal in areas of adequate blood supply. The influence of pro-vascular agents in an orthopaedic setting may also be location specific. One study reported significant increases in bone volume with the scaffold co-delivery of VEGF and BMP-2 over BMP-2 in a subcutaneous ectopic bone formation model in a rat [557]. However when this same system was used in a critical defect model in the rat, there was no significant differences in bone volume or vessel density in the combined VEGF/BMP-2 group when compared to BMP-2 alone. Despite the results of that study, the effectiveness of VEGF in ectopic models of bone formation also appear to be limited. The use of VEGF in conjunction with BMP-7 was found to have no significant difference in new bone formation when compared to BMP-7 alone delivered on ceramic scaffolds in the subcutaneous mouse ectopic model [570]. In
Chapter 6: Novel carriers for BMP-2

an ectopic dorsal muscle pouch model, bone formation induced by BMP-2 delivered on ceramic blocks was not increased by the addition of VEGF [571].

It may be that the addition of agents with pro-angiogenic properties may not be able to augment bone formation at a site that has an established and adequate vascular supply. While the ectopic bone formation models are suitable for optimising new carrier formulations, they may only show advantage in models that have a vascular challenge. A HVLCM with angiogenic properties may be most beneficial when delivering BMP-2 to treat bone defects under conditions where the vascular environment has been challenged. As the ectopic model used in these assays was conducted in the hind limb, a hind limb model featuring tissue ischemia could be compared. The use of hind limb ischemia has been utilised to test angiogenic agents, and can be induced through arterial ligature and excision [572, 573] or \emph{ex vivo} bands [574]. The administration of anti-angiogenic agents has also been used in the literature, with agents such as nicotine [575], the VEGF antagonist sFlt1 [337] and TNP-470 [338] shown to impair bone healing. These models of vascular challenge could be used in conjunction with ectopic bone nodule model to further elucidate the angiogenic potential of new carriers.

The previously described models focus on vasculature deficiencies in soft tissues, however the use of pdRAIB also has potential to deliver BMP-2 in challenging bone healing environments. There are conditions where a bone defect is accompanied by an insult to the surrounding blood supply. Tissues that have undergone radiation therapy, for instance in the treatment of cancers, have a compromised vascularity in surrounding tissues that can impair healing. The use of VEGF to induce angiogenesis in areas of irradiated bone has been shown to improve regeneration in calvarial defects in rats [551, 576]. Clinically, open fractures that are subject to significant vascular damage are highly susceptible to delayed union [492], and fractures of the lower limb that are accompanied
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by vascular damage experience higher rates of amputation [577]. Experimentally, the use of electrocautery to damage the fracture site can create a severe non-union that remains non-united after 8 weeks [501]. In this case, pdRAIB could be used to deliver BMP-2 to the fracture, as I have already demonstrated in this thesis the feasibility of using a liquid carrier to deliver BMP-2 to the site of an open fracture (Chapter 4). Further, pdRAIB could also be used in composite models where a bone defect is created in conjunction with vascular trauma. Hind limb ischemia has been shown to lead to delayed union of mouse fractures [499], while the addition of BMP-7 was able to encourage fracture union [578].

The use of the materials that had been synthesised in the laboratory also brought risks of contamination of the carriers with by-products of manufacturing. In the first study, the use of dRAIB resulted in an adverse reaction in the mice receiving this depot injection, leading to humane euthanasia. This event highlighted the importance of ensuring any synthesised materials are cleared as much as possible of contaminants. In addition, the method of screening and validating new carriers was adjusted to include an in vivo safety study. These toxicity studies aimed to ensure the new carriers were safe and biocompatible prior to measuring bone formation. This method mirrors the way in which clinical trials are conducted in humans, with the first phase of trials assessing safety and the second phase evaluating efficacy. As a result of the inflammation in response to the delivery of pFAIB seen in the toxicity trial this material was relegated from further study. The use of pFAIB caused inflammation that was visible on the mouse as well as in histological analysis (Figure 6.11). The inflammation may have been in response to the browning of the pFAIB that occurs during synthesis, which I hypothesise is due to Maillard reactions, or non-enzymatic browning. Maillard reactions occur in vivo both endogenously [579] and in response to exogenous diet [580] and are linked to inflammation in the body, thus the body is capable of clearing these
products. Most promisingly though, the toxicity study gave confidence that pdRAIB would be safe for study in a bone formation assay.
Chapter 6: Novel carriers for BMP-2

**Summary and conclusions**

In this chapter, I have successfully synthesised and validated new high viscosity liquid carriers for BMP-2 delivery. Applying the techniques of polycondensation and esterification, I generated pdRAIB as a viscous and potentially angiogenic carrier that exhibits a comparable bone forming capacity to SAIB. This project was facilitated by collaboration with chemical engineers to develop and optimize reaction conditions.

Most promisingly, the use of pdRAIB was able to generate comparable BMP-2 induced bone nodules as SAIB. The pdRAIB carrier has many potential benefits for bone healing scenarios where the surrounding vasculature is compromised, for instance irradiated bone or open fractures. In addition, these studies have led to the establishment of a carrier discovery pipeline for assessing the safety and efficacy newly synthesised biomaterials.
Chapter 7. Discussion
Introduction

Orthopaedic medicine conventionally holds autologous bone grafting as the gold standard of treatment for bone defects [79, 87]. However, the harvesting of autologous bone grafts is associated with side effects that include donor site morbidity and pain. The field of bone tissue engineering is driven by the goal of reducing the need for autologous bone grafting by using engineered implants to guide and support the body in regenerating lost tissues and function. The field has largely focused on the use of solid scaffolds, with the most advanced construct with FDA approval being the INFUSE® kit [148]. This tissue engineering system involves the delivery of recombinant BMP-2 protein via a porous sponge manufactured from bovine collagen. Initial trials with INFUSE® showed highly promising results [157], however, there have been issues and adverse reactions associated with the clinical use of this product. These have largely been attributed to the properties of the collagen carrier [129, 228, 229]. The collagen sponge that accompanies the INFUSE® kit is known commercially as HELISTAT®, and was originally developed as a haemostatic agent [581]. HELISTAT® was not initially developed for the delivery of BMP-2, and there is a need for materials specifically developed for BMP-2 delivery.

Another challenge with INFUSE® was highlighted in a clinical trial by Aro et al. This study revealed a significant increase in infection rate in BMP-2 treated open fractures when compared to standard of care [159]. This is possibly due to the physical manipulation and manual insertion of the BMP-2 soaked collagen sponge. The development of a minimally invasive delivery system for BMP-2 that would minimise this infection risk has great potential as deep infections require secondary procedures and debridement. The BMP-2 protein is a potent stimulus for bone formation, and there are significant gains to be made by improving its delivery.
Injectable biomaterials for bone tissue engineering

The use of solid scaffolds for the delivery of BMP-2 is an established paradigm within the tissue engineering field. When utilising scaffolds that are made from solid materials, physical properties such as pore size and interconnectivity, surface microstructure, and macroscopic shape all require consideration [186]. With an increasing array of manufacturing and synthesis protocols that can be employed to generate solid scaffolds, the ability to alter these parameters is ever increasing. More recently, injectable carrier systems have gained momentum in a tissue engineering context. The use of an injectable carrier allows for the potential for minimally invasive delivery to the site of a defect, as well as increasing the versatility and applications of the carrier system.

Injectable systems used for BMP-2 delivery

In the context of bone tissue engineering, there are several criteria for an appropriate injectable carrier for BMP-2. As an injectable system must exist in some liquid state prior to use, it must be able to transition to a solid or semi-solid state in situ, and be able to gel under physiological conditions at an appropriate rate with no harmful by-products [582]. The rate at which the injectable material sets must be slow enough to practically allow for delivery, but rapid enough that it won’t disperse from the site. No matter the mechanism by which the injectable system transforms into a solid or semi-solid, this should ideally occur without the release of any cytotoxic products, such as UV photoinitiators [583].

Injectable calcium phosphate cements are commonly used in orthopaedics and as such were a rational choice to act as a carrier for BMP-2. These cements are non-compressible and will set into a solid shape when delivered in vivo. The use of a calcium phosphate matrix (CPM) to deliver BMP-2 has been shown to accelerate the healing of a number of animal models of bone regeneration. A single percutaneous injection of BMP-2/CPM was able to accelerate healing when
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compared to CPM alone or no treatment in both canine and non-human primate osteotomy models [140, 584]. With these promising pre-clinical results, a phase I trial of BMP-2/CPM was then investigated as a treatment for closed internally fixed tibial fractures [585]. The fractures were treated with percutaneous injections of BMP-2/CPM, with a reduction in time to radiographic union when compared to historical controls.

However, when the BMP-2/CPM progressed into phase II/III, the trial was stopped prematurely due to a lack of significant improvements in two key clinical outcome measures of fracture healing: radiographic union or time to full weight bearing [586]. The lack of effect has been proposed to be due to inefficient release of BMP-2 at the fracture site, as well as dispersion of the CPM away from the fracture site. The CPM/BMP-2 study series further highlights the importance of the BMP-2 release dynamics from the carrier. Further, it demonstrates the market space for a percutaneously deliverable BMP-2 system that can enhance fracture healing and reduce the need for secondary surgeries.

One advantage of the CPM carrier is its non-compressibility, such that once it has been injected it transitions into a solid that cannot be deformed. However, this also raises the concern of a poorly positioned carrier that could be placed inappropriately, which is likely to be applicable to all percutaneous systems including SAIB. The ability to position the injectable system during delivery will ensure that bone formation is induced at the correct site and reduce the risk of heterotrophic ossification. One approach to address this issue while maintaining minimal invasiveness is the incorporation of a radiopaque dye that can be used to guide and track the injectable using fluoroscopy. Another option is the use of a stent or balloon that can be delivered percutaneously, much like a cardiac stent, that is then inflated using the BMP-2 containing injectable. The issue of positioning is one that is common to all percutaneous injectables, and requires further development.
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As an injectable BMP-2 construct, the CPM carrier has been the most advanced in terms of clinical translation. However, there is also a large body of work investigating the use of hydrogels for bone drug and growth factor delivery. Hydrogels are three-dimensional cross-linked networks that are hydrophilic, enabling them to contain large amounts of water [587]. Hydrogels can be based on synthetic and natural polymers, and there is a small subset of studies on their ability to deliver BMP-2 \textit{in vivo}.

An injectable PLA-PEG co-polymer hydrogel has been examined in mouse muscle pouch [242] and canine spinal fusion models [588]. However, this system is not truly \textit{in situ} forming as the cross-linking occurs \textit{ex-vivo}; the hydrogel is heated until its viscosity drops allowing it to be injected. \textit{In vivo} synthetic polymers such as PLA and PGA (and their co-polymers) are hydrolysed into their monomers of lactic and glycolic acids, which can create an acidic local environment. The acidity of the breakdown products can impede bone formation and result in local inflammation [208, 209] [589]. One strategy to neutralise the local acidity caused by the degradation of these polymers is the co-delivery of ceramics, which generate a basic local environment as they break down [590]. There have been several \textit{in vitro} studies found that the use of calcium carbonate was able to neutralise the drop in pH caused by the degradation of PLA [591, 592]. However, this has generated conflicting results in the literature, with one study reporting that the addition of calcium phosphates to PLA did not alter the pH of the local environment \textit{in vivo} and extended the resorption time compared to PLA alone [593].

Hydrogels that are based on natural polymers have also been investigated in a tissue engineering context. Hyaluronic acid based hydrogels have proven to be effective carriers of BMP-2 in a variety of animal models, including rat muscle pouch [594] and calvarial defects [595, 596], and cranial defects in pigs [597]. However, the release rates for BMP from these hydrogels range from 75-
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100% within 24 hours [596] to less than 20% at 5 days [595] depending on the preparation technique. An *in situ* gelling injectable hydrogel based on another natural polymer, chitosan, was able to generate ectopic bone in the rat muscle pouch when delivering 150 µg BMP-2, which was further enhanced with the addition of β-TCP particles [598]. However, when this construct was then piloted to treat a critical radial defect in the rabbit, it was found that there was incomplete bone regeneration despite a very high dosage of BMP-2. The authors attributed the failure to leakage of the injectable construct into soft tissue voids created during surgery.

The failure to transition this injectable system highlights the difficulty of applying a single BMP-2 system uniformly to all bone regeneration scenarios. In this thesis, the difference in local tissue environment on bone formation was seen when translating from an ectopic muscle pouch model into the orthotopic open fracture model. It is acknowledged that the fold increases in bone volume between SAIB/BMP-2 and SAIB/BMP-2/HA/ZA were seen in the muscle pouch were not recapitulated in the open fracture model. However, in the fracture model there were nonetheless improvements in the functional outcomes of union and mechanical strength. The success of the SAIB system in the open fracture is likely due in part to adapting the delivery by utilising a flexible catheter sheath (Figure 4.5), ensuring that the SAIB mixture coated the broken bone ends. Further, for the SAIB system to be successful in a critical defect model, some form of containment would be required to ensure that the SAIB remains within the defect space. It is most likely that there is no injectable tissue engineering system that can be homogenously applied to all bone defects, but rather success will rely on the tailoring of these systems.

Injectable composite systems that combine elements of the different material types described have also been explored, and arguably constitute the majority of research in the bone tissue engineering field. These constructs combine materials either chemically or additively to overcome the
limitations of an individual material, or to enhance and tailor the performance of the materials used. One example is the use of the synthetic polymer PEG to modify the properties of the natural protein fibrinogen, and control the physical properties of the hydrogel [599]. BMP-2 delivered in PEGylated fibrinogen increased the bone formed in a critical sized cranial defect in the rat. However, this hydrogel requires UV light to gel and thus cannot be percutaneously delivered. Another injectable based on hyaluronic acid and polyvinyl alcohol that gels within minutes mixing was tested in the ectopic muscle pouch of a rat [600]. This study examined the effect of various bioceramics on BMP-2 induced bone formation, however even with nano-HA they found no significant increases in bone volume.

Another prevalent strategy is the use of microspheres to encapsulate and deliver growth factors. The advantage of using microsphere delivery is an ability to control the release of the proteins, allowing for sustained delivery and reducing burst release. One study delivered sequential release of BMP-2 and the angiogenic basic fibroblast growth factor (bFGF) in gelatin microspheres into a femoral condyle defect in the rat [601]. This study found that the delivery of BMP-2 alone was able to increase new bone volume in this defect, bFGF alone had no effect, while the co-delivery of BMP-2 and bFGF actually had an inhibitory effect on bone formation. BMP-2 has also been loaded into microspheres made of HA, which were injected into a radial bone defect in the rabbit [602]. The BMP-2/HA microsphere treated defects had a higher load to failure when compared to empty HA microspheres. It would be interesting to see if the addition of ZA to this system would result in the synergy seen in with SAIB/BMP-2/HA/ZA (Figure 2.5 and Figure 4.12). Despite some of the positive bone formation capacities seen with hydrogel use in animal models, no hydrogels have made it into clinical orthopaedic use at this time.
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Despite the advantages associated with minimally invasive delivery, there are benefits to designing containment systems for injectable BMP-2 carriers. As previously noted, percutaneous injection of BMP-2 in SAIB led to a 3-fold increased bone volume compared to implanted collagen (Chapter 2). However, there may be advantages to also guiding the shape and size of the bone that is formed, which can be challenging even with semi-solid carriers. Indeed, the SAIB/BMP-2 induced bone was often irregularly shaped compared to collagen/BMP-2 induced bone. 3D printing potentially allows for generating bone of specific shapes and sizes, with the ability to tailor the scaffold to be compatible with loading an injectable carrier, such as pdRAIB or SAIB.

One notable question raised by our studies is whether a solid scaffold is even required for tissue engineering. SAIB depots lack a porous internal structure, but can nevertheless be superior to porous collagen. It is often noted that porosity and pore size are critical elements of a solid scaffold for tissue engineering, allowing for nutrient diffusion and cell transport [603, 604]. The concept behind porous scaffolds is that cells are able to migrate along the struts within the negative space of the scaffold, and that the speed of cell migration is dependent on strut and pore size [605]. While the method of cellular invasion of SAIB is unclear, it is speculated that cells may be able to infiltrate the depot and both initiate vascularization and SAIB resorption. Nevertheless, has been technically challenging to assess, as no practical method has been elucidated to perform histology on the hydrophobic semi-solid depot. Efforts are ongoing to develop cryohistology technique to obtain sections containing SAIB and examine any incorporated cellular constituents.

Hybrid systems for BMP-2 delivery

The use of hybrid systems to guide injectable bone systems is concept that has been explored in the literature. The hybrid systems described involve the use of a solid component to contain the injectable carrier for BMP-2 in some way. Such systems have been explored inadvertently by
several groups that have contained their injectable constructs within inert tubing for muscle pouch models [594, 606]. More targeted approaches have included the use of a calcium phosphate/polymer nanofiber mesh which delivered BMP-7 in platelet-rich plasm in a rat critical defect [607]. After 12 weeks, treated defects had healed and comparable mechanical properties to intact bones, although group sizes were small. Another approach utilised a polymer based electrospun nanofiber mesh tube that contained an alginate based hydrogel containing BMP-2 in a critical sized defect in the rat [608]. After 12 weeks, defects treated with a perforated mesh tube with BMP-2 containing hydrogel healed with equivalent mechanical properties to intact femurs. While the perforations in the tube were able to improve bone healing over unperforated tubes in this model, they did so without increasing vascularity. In a critical defect, the treatment of which would require open surgery, it may be more appropriate to have a hybrid system that involves both an implantable sheath or cage with the BMP-2 delivered in the injectable. To enable SAIB to be used to treat a critical sized defect, a hybrid system is most likely to be appropriate to contain it within the defect. Ideally, SAIB/BMP-2 would be contained within a solid scaffold that would have the least material possible while maintaining the potential space for new bone formation.

Another strategy for the use of injectable carriers to treat defects is the coating of bone grafts or bone graft substitutes. Allografts and bone graft substitutes such as TCP are commonly used in orthopaedic practice to fill defects. However, these materials have limited potential to stimulate bone regeneration [79]. Coating these graft substitutes may represents a novel method of improving the osteogenic potential of these grafting materials. One group has reported coated TCP/HA ceramic granules with BMP-2 using a polyelectrolyte multilayer film [609]. This coating led to reduced new bone formation than free BMP-2 loaded onto the ceramic granules in a dorsal muscle pouch model. This poor response may be due to poor release kinetics of BMP-2 from the film, which remains a challenge with coating-based approaches. Another study coated deproteinised
bovine bone, most commonly used in dentistry, with calcium phosphate and BMP-2, and found strong bone formation that was comparable to autologous grafts in an ovine critical sized defect [610]. In the context of SAIB and pdRAIB, further study would be needed to identify if the release kinetics that make them successful BMP-2 carriers as a depot system would also work as a coating system. But, if successful, this could prove to be an innovative approach to enhancing bone graft materials using BMP-2.

Finally, one element that has been potentially underappreciated in tissue engineering is the concept of supplying an energy substrate for the tissue regenerating cells. Currently, solid scaffolds focus on optimising interconnectivity of their porous structure to ensure adequate nutrient and oxygen diffusion. However, this is only relevant once a vascular supply has been established within the scaffold. Prior to this, cells can be limited in their ability to migrate into the scaffold by the diffusion limit of oxygen from surrounding blood supply.

I hypothesise that one reason for the success of the SAIB system lies in its sugar base, as cells are able to breakdown the SAIB molecule into sucrose that can be utilised for anaerobic glycolysis prior to the arrival of vasculature. The degradation of intramuscular SAIB has not been described, but oral administration results in hydrolysis by non-specific esterases [611]. Sucrose is a disaccharide of glucose and fructose, and in skeletal muscle glucose can be used to generate ATP in the absence of oxygen [612, 613]. I postulate that recruited cells can invade the SAIB depot, and generate energy anaerobically prior to the formation of blood vessels. Further study will be required to validate whether intramuscular administration of SAIB allows it to be fully hydrolysed into its monosaccharide components that then are utilised anaerobically by cells.

The hydrophobic nature of SAIB makes conducting in vitro assays, such as those studying the pattern of SAIB degradation, technically challenging. When placed into water, SAIB will
accumulate at the bottom of the solution, and due to an extremely low solubility in water it will remain undissolved. This is contrast to what occurs *in vivo*, as it is clear that SAIB depots are largely degraded after three to four weeks in a rodent muscle pouch. Simple *in vitro* models are unable to take into account the body’s ability to break down SAIB enzymatically, and as such make elution studies poor representations of the *in vivo* response. One method to investigate the breakdown could utilise radiolabelled sucrose that could be synthesised into SAIB; the radiolabelled compound could then tracked *in vivo* over time. The concept of providing an anaerobic energy supply to nourish cells within a tissue engineering construct is highly deserving of further research. If this theory is true, it would represent a paradigm shift within the tissue engineering triad, altering the approach of future strategies.
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**Pipeline for biomaterial development**

This thesis describes the initial optimisation and subsequent use of the SAIB carrier to treat bone defects, as well as the concurrent synthesis and development of the alternative carriers, pFAIB and pdRAIB. The use of SAIB was the initial foundation of this work and, as it is available at food-grade levels through commercial vendors, there was no requirement to synthesise this product. SAIB was readily available for experimental use, allowing for the development of its applications in more functional and complex models of bone regeneration (Chapters 3-5).

However, in addition to the use of SAIB in a range of animal models, there was interest in concurrently developing new sugar based carriers that could be used for tissue engineering. This led to a new collaboration with the School of Chemical and Biomolecular Engineering, at the University of Sydney. This process has spanned the fields of chemical engineering, basic and translational science, and orthopaedic medicine. Active collaboration between all of these groups ensured that novel materials could be synthesised, and tested in translationally relevant animal models of bone regeneration with a clear clinical direction.

Through the work conducted in this thesis, a pipeline for biomaterial development has been established. This pipeline is ongoing and involves the continuous manufacture and testing of new materials (Figure 7.1).

![Figure 7.1: The pipeline for biomaterial development](image-url)
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In this pipeline, new sugar-based materials are first synthesised and screened for the required physical properties of viscosity and hydrophobicity (as described in Chapter 6). Materials that display the required properties can then be tested for biocompatibility and *in vivo* toxicity using the mouse muscle pouch model. For this model, animals are closely monitored for any signs of distress or adverse reactions to the new carrier over a week. Group sizes are small with one animal per material, and SAIB is used as the control. The main outcome of this experiment is to ensure that the material is well tolerated by the body, as seen by monitoring of animal welfare and post-operative tissue histology.

Once a material is considered biocompatible, the ability of that new material to deliver BMP-2 and result in bone formation is tested in the mouse ectopic muscle pouch. Once again, SAIB is used as the control, and bone formation is quantified after three weeks. If the new material generates bone nodules that are comparable or greater than SAIB, that new material is a candidate for use in more challenging models of bone formation. The challenging models of bone formation are dependent on the proposed advantages of the new material. For example, the ability of pdRAIB to promote bone healing in models of vascular challenge.

In the literature it is common to see tissue engineering constructs that have been developed in a vacuum of input from end-users, particularly clinical professionals. Novel materials are produced and intensely characterised using *in vitro* techniques such as electron microscopy and cell culture models, but often fail to advance into *in vivo* testing. To produce translationally relevant biomaterials, a clear vision for the ultimate clinical application must be maintained. Ensuring strong collaborations between basic scientists and clinicians is the most efficient way to bring new technologies to the patients in need.
Potential future applications of SAIB/pdRAIB technology

This thesis describes the novel bone tissue engineering application of the sugar-based ester SAIB to deliver BMP-2 *in vivo*. The use of the BMP-2/SAIB drug-biomaterial combination was explored and optimised using bone formation assays in the mouse, and later validated in more advanced orthopaedic models in mice, rats, and pigs. This research on SAIB has significantly added to the field of bone tissue engineering; the properties of SAIB as a liquid, non-polymeric, and non-hydrogel system make it a highly innovative approach. Further, this thesis describes the synthesis of novel and highly viscous sugar based carriers that were similarly utilised as delivery systems for BMP-2.

There are many potential uses for SAIB and pdRAIB both within the bone tissue engineering field and beyond:

- SAIB or pdRAIB could be used to deliver BMP-2 into personalized scaffolds to recreate large bone defects. 3D printing can be used to create models of bone defects that can mirror the contours of the original site that can help to restore aesthetics and symmetry. One application of SAIB or pdRAIB is to deliver BMP-2 into 3D printed scaffolds of bone defects, such as a femur as shown in Figure 7.2. The liquid nature of the carriers enables them to fill the required shape, and the loaded scaffold can be implanted at the site of the defect. Another alternative is to implant the scaffold into a muscle pouch in the patient, such that they act as their own bioreactor, forming living bone that can then be transplanted into the defect site.
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Figure 7.2: A 3D printed femur

- The SAIB and pdRAIB systems for delivering BMP-2 could have applications in the dental field. Bone grafting systems are used in this field to repair bone stock in preparation for the surgical insertion of dental implants [614]. Rather than the use of collagen sponge to release BMP-2 and stimulate bone growth, SAIB or pdRAIB could be used as a more minimally invasive delivery system.

- The SAIB and pdRAIB systems could be utilized for the regeneration of osteoarthritic defects that span both bone and cartilage within a joint. This thesis has shown that BMP-2 delivery in either SAIB or pdRAIB results in robust bone formation. Low doses of exogenous BMP-7 have shown to promote cartilage repair [615, 616], and it is feasible that SAIB could also be used to deliver BMP-7. Thus, the SAIB and pdRAIB systems could be used to deliver growth factors to targeted locations, such as BMP-2 into bone and BMP-7 into cartilage.
• SAIB or pdRAIB could be used to coat metal implants with BMP-2 to enhance osseointegration. Metal implants have previously been coated with a combination of BMP-2 and prophylactic antibiotics [617]. This laboratory has reported on the synergistic effects of a novel antibiotic, CSA-90, with BMP-2 [618]. SAIB or pdRAIB could be used to coat metallic implants, such as joint replacements, with the combination of these two agents, BMP-2 and CSA-90, to prophylactically treat infection and promote bone integration.

• Further to this, BMP-2 coatings have also been used to enhance the osseointegration of metallic screws [619]. SAIB or pdRAIB could be used to coat bone screws with BMP-2 to increase stability when used in either a dental or orthopaedic setting.
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Conclusions

This thesis explores the use of the injectable *in situ* forming SAIB depot as a carrier for the potent bone stimulus BMP-2. Although it has been used as a food and beverage emulsifier for many decades [250], the use of SAIB as bone tissue engineering carrier is highly novel.

In this thesis, I have described the development, optimisation and validation of this novel phase transitioning liquid carrier for BMP-2 in a number of models of bone healing and regeneration. I have shown that bone formation can be maximised with the delivery of SAIB/BMP-2/HA/ZA in a mouse assay for bone formation (Chapter 2). I have demonstrated the versatility of the SAIB carrier by delivering BMP-2 in a range of models of bone regeneration. SAIB was used to delivery bone intraosseously, both within the medullary canal (Chapter 3) as well as the trabecular bone of the epiphysis (Chapter 5). These sites are currently inaccessible to the current clinical standard for BMP-2 delivery, the solid and porous acellular collagen sponge. Further, I was able to show the feasibility and effectiveness of using SAIB at an orthotopic site to enhance the union of an open fracture (Chapter 4). Finally, the new carrier pdRAIB that was based on the sugar deoxyribose was developed and shown to be capable of generating robust BMP-2 induced bone (Chapter 6).

I have successfully demonstrated that SAIB and pdRAIB are an effective carriers for drug delivery in the field of bone tissue engineering. Furthermore, these phase-transitioning minimally invasive constructs have demonstrated potential for use in orthopaedic as well as broader medical fields.
Chapter 8. References


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