The Role of Inorganic Polyphosphates in the Formation of Bioengineered Cartilage

Incorporating a Zone of Calcified Cartilage In Vitro

by

Jean-Philippe St-Pierre

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Graduate Department of Materials Science and Engineering

University of Toronto

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The development of bioengineered cartilage for replacement of damaged articular cartilage has gained momentum in recent years. One such approach has been developed in the Kandel lab, whereby cartilage is formed by seeding primary articular chondrocytes on the top surface of a porous biodegradable calcium polyphosphate (CPP) bone substitute, permitting anchorage of the tissue within the pores of the substrate; however, the interfacial shear properties of the tissue-substrate interface of these biphasic constructs are 1 to 2 orders of magnitude lower than the native cartilage-subchondral bone interface. To overcome this limitation, a strategy was devised to generate a zone of calcified cartilage (ZCC), thereby mimicking the native architecture of the osteochondral junction; however, the ZCC was located slightly above the cartilage-CPP interface. Thus, it was hypothesized that polyphosphate released from the CPP substrate and accumulating in the tissue inhibits the formation of the ZCC at the tissue-substrate interface. Based on this information, a strategy was devised to generate biphasic constructs incorporating a properly located ZCC. This approach involved the application of a thin calcium phosphate film to the surfaces of porous CPP via a sol-gel procedure, thereby limiting the accumulation of polyphosphate in the cartilaginous tissue. This
modification to the substrate surface did not negatively impact the quality of the in vitro-formed cartilage tissue or the ZCC. Interfacial shear testing of biphasic constructs demonstrated significantly improved interfacial shear properties in the presence of a properly located ZCC. These studies also led to the observation that chondrocytes produce endogenous polyphosphate and that its levels in deep zone cartilage appear inversely related to mineral deposition within the tissue. Using an in vitro model of cartilage calcification, it was demonstrated that polyphosphate levels are modulated in part by the inhibitory effects of fibroblast growth factor 18 on exopolyphosphatase activity in the tissue. Polyphosphate also appears to act in a feedback loop to control exopolyphosphatase activity. Interestingly, polyphosphate also exhibits positive effects on cartilage matrix accumulation. The potential implication of polyphosphate in the maintenance of articular cartilage homeostasis is intriguing and must be investigated further.
ACKNOWLEDGEMENTS

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACI</td>
<td>autologous chondrocytes implantation</td>
</tr>
<tr>
<td>ANK</td>
<td>progressive ankylosis</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>Arp</td>
<td>actin-related protein</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>β-GP</td>
<td>β-glycerophosphate</td>
</tr>
<tr>
<td>C</td>
<td>coated</td>
</tr>
<tr>
<td>CaP</td>
<td>calcium phosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CIAP</td>
<td>calf intestinal alkaline phosphatase</td>
</tr>
<tr>
<td>CPP</td>
<td>calcium polyphosphate</td>
</tr>
<tr>
<td>CPPD</td>
<td>calcium pyrophosphate dihydrate</td>
</tr>
<tr>
<td>DAPI</td>
<td>4'-6-Diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>DZ</td>
<td>deep zone of articular cartilage</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EDX</td>
<td>energy dispersive x-ray spectroscopy</td>
</tr>
<tr>
<td>ENPP-1</td>
<td>ectonucleotide pyrophosphatase/phosphodiesterase family member 1</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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</table>
FGF  fibroblast growth factor
FGFR  FGF receptor
GAG  glycosaminoglycan
GDP  guanosine diphosphate
GTP  guanosine triphosphate
HA  hydroxyapatite
M  mineralization medium
MAPK  mitogen activated protein kinase
MGP  matrix gla protein
MM  mineralization medium
mTor  mammalian target of rapamycin
MZ  mid-zone of articular cartilage
NC  non-coated
NM  non-mineralizing conditions or non-mineralized
OA  osteoarthritis
OCP  octacalcium phosphate
PBS  phosphate buffered saline
PD173074  1-t-Butyl-3-(6-(3,5-dimethoxyphenyl)-2-(4-diethylaminobutylamino)-pyrido[2,3-d]pyrimidine-7-yl)urea (FGFR tyrosine kinase inhibitor)
PHB  poly(3-hydroxybutyrate)
Pi  orthophosphate
PPi  inorganic pyrophosphate
PP45  inorganic polyphosphate (average chain length of 45 orthophosphate residues)
PPK  polyphosphate kinase
PPN  endopolyphosphatase
PPX  exopolyphosphatase
PTHrP  parathyroid hormone related peptide
qPCR  quantitative polymerase chain reaction
RNA  ribonucleic acid
RQ  relative quantification
rRNA  ribosomal RNA
SAED  selected area electron diffraction
SEM  standard error or the mean or scanning electron microscopy
Sox 9  Sry-type HMG box-containing transcription factor 9
SZ  superficial zone of articular cartilage
TEM  transmission electron microscopy
TNAP  tissue non-specific alkaline phosphatase
ZCC  zone of calcified cartilage
CHAPTER 1: INTRODUCTION
1.1 Structure and function of articular cartilage

Articular cartilage is an avascular and aneural connective tissue that covers the articulating ends of bones in synovial joints. It is a load-bearing tissue that absorbs and distributes the compressive and shear forces applied to the joint while permitting low friction articulation. Articular cartilage fulfills these functions through a complex depth-dependant zonal architecture, each with a distinct composition and extracellular matrix organization. A closer look at the development, maturation and aging of articular cartilage can provide insight for the design of bioengineered cartilage suitable for the treatment of diseased or damaged cartilage.

1.1.1 Development of articular cartilage

During embryogenesis, the skeleton develops from the condensation of mesenchymal cells and their subsequent differentiation into chondrocytes, which accumulate collagen type II within their matrix [1,2]. The shape of the condensation and subsequent cartilaginous anlagen is dependent on the type of bone being formed, with opposing long bone elements generally originating from a single uninterrupted condensation [3]. The first observable stage of joint formation within the cartilaginous anlagen is the appearance of a thin interzone consisting of elongated mesenchymal cells at the presumptive joint site, creating a demarcation between two opposing cartilage elements [4]. The mechanism by which the joint location is specified is not fully understood. The cavitation process that allows for the separation of two opposing surfaces of a joint and the eventual formation of a fluid-filled capsule appears to be caused partly by the accumulation of hyaluronan at the interzone [5-7], in combination with muscle-induced
motion and extracellular matrix reorganization, which lead to a loss of tissue integrity [8]. The process of morphogenesis by which the varied three-dimensional joint shapes are achieved is complex and remains poorly understood.

Away from the developing joint surface, a growth plate is established to permit lengthening of the cartilaginous template for bone through endochondral ossification [9]. In this process, chondrocytes proliferate rapidly before undergoing maturation towards a hypertrophic state upon arrest of the proliferative state. Hypertrophic chondrocytes mineralize their extracellular matrix and modulate the vascular invasion of the mineralized matrix, from which progenitor cells that resorb cartilage and replace it with bone are delivered. The absence of a visible demarcation between growth plate and articular cartilage in humans following birth has supported the hypothesis that articular cartilage is a remnant of the growth plate following the arrest of endochondral ossification. However, with the advent of a secondary ossification center this division is established suggesting that articular cartilage is distinct from the growth plate [10]. Further supporting this new hypothesis is the observation that articular cartilage arises by appositional growth from a specific stem cell-like sub-population in the interzone distinct from cells that participate in the growth of the cartilage elements [11-13]. It has also been suggested that articular surfaces are completely reformed shortly after birth [14]. Additionally, the presence of pericellular collagen type V in the interzone during joint formation and later in articular cartilage, but not in epiphyseal growth plate cartilage, suggests major differences in the matrix composition of articular and growth plate cartilage [15]. Similarly, the morphology of hypertrophic chondrocytes close to the articular surface of joints differs from that in the growth plate [10]. As well, the degree of
mineralization in the growth plate is significantly higher than that observed within mature articular cartilage [16]. This report also notes that the zone of calcified cartilage (ZCC) in articular cartilage is delimited by the presence of a tidemark; this feature is not observed in growth plate cartilage. These distinctions between the two types of cartilage stress the need to be careful in transferring knowledge from studies of the growth plate to articular cartilage biology.

1.1.2 Articular cartilage composition

The composition of articular cartilage is critical to its ability to accomplish its specialized functions. In addition to chondrocytes, which occupy less than 10% of the tissue and act to regulate and maintain the integrity of the tissue [17], cartilage is comprised of an extracellular matrix composed mainly of collagens, proteoglycans and other non-collagenous proteins, as well as a fluid phase. The interactions between these components determine the mechanical properties and functions of the tissue.

Collagens are the main component of the articular cartilage extracellular matrix (50-70%, dry weight) [18]. Collagens are composed of three α chain polypeptides, each chain containing at least one domain of repeating glycine-X-Y where X is often proline and Y hydroxyproline, to facilitate their self-assembly into triple-helix. Collagen type II is the most abundant collagen in articular cartilage but other collagens including type III, V, VI, IX, X, XI, XII, XIII and XIV are also found [18,19]. Collagen type II is a homotrimer of α1(II) chains synthesized as procollagen precursors with amino and carboxy peptides that must be enzymatically cleaved to permit further fibrillar assembly [20]. Collagen type IX is covalently bound to the surface of collagen type II and may play
a role in cross-linking of the fibrillar collagen network together and with non-collagenous proteins [21]. Collagen type XI is present within and at the surface of the collagen type II fibrils, where it acts to initiate fibril self-assembly and control their diameter [21]. Collagen type X forms hexagonal networks and has been localized to the deep zone in articular cartilage, where it is hypothesized to play a role in the mineralization process [22]. Potential functions for collagen type X in the cartilage mineralization process will be discussed in more detail in a later section.

Proteoglycans are the second largest components within the articular cartilage extracellular matrix. These macromolecules are glycoproteins comprising a central core protein branched covalently with glycosaminoglycans. Aggrecan is the most abundant proteoglycan within articular cartilage and consists of a core protein to which highly negatively charged keratin sulphate and chondroitin sulphate chains are attached [23]. Aggrecan molecules bind to hyaluronan via the link protein to form large aggregates within the collagen network. A variety of small leucine-rich proteoglycans including biglycan, decorin, fibromodulin and lumican are also present in lesser amounts within articular cartilage [23]. While the function of biglycan remains unclear, decorin, fibromodulin and lumican appear to play a role in the control of collagen fibril growth.

The fluid phase represents between 60 and 80% of the wet weight of articular cartilage and contains small signalling molecules, as well as a high concentration of cations to balance the fixed negative charges of proteoglycans in the extracellular matrix [24]. This phenomenon – known as the Donnan effect – leads to increased osmolarity of the tissue and influences the water content and the associated compressive stiffness of articular cartilage [25]. A fraction of the fluid phase that is closer to the joint surface can
move freely in and out of cartilage following the application of mechanical stresses, providing the tissue with flow-dependent viscoelastic properties [24]. The fibrillar collagen network counteracts the expansion of proteoglycans [24]. Collagen fibrils also resist shear and tensile stresses applied to the tissue. Hence, the mechanical properties of articular cartilage are highly dependent on the ratio of collagen-to-proteoglycan content and the collagen fibrillar network organization.

1.1.3 Articular cartilage zonal organization

The composition of adult articular cartilage is anisotropic as the tissue is characterized by a complex depth-dependant zonal organization. It is generally accepted that articular cartilage is divided into four zones; from superior to inferior of the joint surface: the superficial, mid and deep zone, as well as the ZCC. With the exception of the ZCC, demarcations between these different regions are not clearly defined.

The superficial zone is the thin layer covering the joint surface [20,24]. It consists of a sheet of collagen fibrils oriented parallel to the joint surface covering a layer of ellipsoid-shaped chondrocytes. The collagen content of this zone is the highest of any zone in articular cartilage, while the aggrecan content is low. However, the small size of collagen fibrils in this zone results in the greatest water content. The high collagen content of this zone provides the tissue with adequate strength to withstand the high shear and tensile forces generated by gliding of the joint surfaces. It is also suggested that the dense collagen meshwork acts to limit the entry and exit of molecules within the tissue.

The mid-zone of articular cartilage is composed of larger collagen fibrils with a random orientation [25]. The concentration of proteoglycans is higher than in the
superficial zone, providing it with the ability to withstand and absorb the compressive forces transmitted to the joint [20]. Chondrocytes in the mid-zone exhibit a round shape and appear more metabolically active than the cells of the superficial zone [25].

The deep zone of articular cartilage is composed of large spherical chondrocytes, which synthesize the largest collagen fibrils leading to the lowest water content of any of the zones of articular cartilage, despite the highest proteoglycan content and the lowest collagen content of any zone [24]. These large collagen fibrils are oriented perpendicular to the joint surface. Deep-zone chondrocytes have similarities with hypertrophic chondrocytes of the growth plate including the expression of proteins such as collagen type X and tissue non-specific alkaline phosphatase (TNAP), which have been linked to cartilage calcification [26,27]. However, as discussed in a previous subsection, the two tissues are distinct in many ways.

The ZCC is the highly mineralized region of the deep zone of articular cartilage that interfaces and anchors hyaline cartilage with subchondral bone. The mineral content of the ZCC is significantly higher than that of subchondral bone [16]. Furthermore, the mineral crystals in both tissues are nanocrystalline particles of carbonated apatite of similar sizes [28,29]. Nevertheless, the mechanical properties of calcified cartilage have been reported as one order of magnitude lower than that of subchondral bone [30]. This can be explained by the underlying collagen matrix, which is very different between both tissues and is believed to play a major role in transferring shear stresses between mineral platelets [31]. The lower degree of orientation of mineral crystals in cartilage compared to bone caused by the large void areas in cartilage due to the high proteoglycan content of the tissue that could foster mineral deposition may also contribute to the inferior
mechanical properties [16]. Thus, the ZCC may act as a zone of intermediate mechanical properties at the interface between hyaline cartilage and subchondral bone, which helps diffuse the high shear stresses generated at the interface due to joint loading. However, it should be noted that two studies have reported similar stiffness for the ZCC and subchondral bone with a nanoindentation technique that requires substantial sample preparation including a dehydration step, which may have affected the measurements [32,33].

At the interface between the hyaline and calcified zones of cartilage is the tidemark. The integrity of this smooth boundary is maintained by collagen fibrils organized perpendicular to the joint surface that bridge the junction between the two tissues [33]. A gradient of mineral content is present at the tidemark that may act to diffuse the stresses applied to this interface through an associated gradient of tissue stiffness. Studies of tetracycline incorporation have demonstrated that the tidemark is metabolically active, as it advances slowly in the hyaline cartilage [34]. In some cases, more than one tidemark can be identified in a single tissue section, suggesting that cells up to a certain distance within the ZCC are metabolically active and contribute to the calcification process. As the thickness of the ZCC remains relatively constant through life, a control mechanism that is not yet understood is required to ensure that the tidemark advancement occurs at the same rate as replacement of cartilage by bone through endochondral ossification [35]. The mechanical environment in the joint seems to play a role in this mechanism, as modifications of the stresses and strains applied to the joint due to hyaline cartilage damage or changes in the joint biomechanics resulting from
trauma lead to thickening of the ZCC and concentration of those forces within a thinner layer of articular cartilage [36,37].

The cement line is the interface between the ZCC and the subchondral bone. While collagen fibrils have been observed to cross that interface, the penetration seems limited to approximately 1µm, as the collagen types of bone and cartilage are different [38]. Thus, the integrity of this boundary is ensured through the high level of interdigitation of the two tissues rather than collagen fibrils reinforcement [39].

The extracellular matrix of articular cartilage is also organized differently with respect to its distance from chondrocytes. The pericellular matrix surrounding each chondrocyte has a high proteoglycan content and may play an important role in protecting the cell against mechanical loading, as well as in matrix turnover [24]. The territorial matrix is composed mainly of thin collagen fibrils also arranged around chondrocytes to resist loading and deformation, whereas the interterritorial matrix constitutes the bulk of the tissue with larger diameter fibrils oriented with respect to the joint surface, rather than around individual cells [24].

### 1.1.4 Age-related changes in articular cartilage

There is a direct correlation between the incidence of osteoarthritis (OA) and age [40]. While it is often proposed that articular cartilage slowly wears away due to mechanical loading, this is no longer accepted as the only mechanism leading to OA. For example, although contact sports at an elite level are associated with an increased prevalence of OA [41], recreational sports activities including repetitive high impact activities such as long distance running are not strongly associated with the development of OA [42].
However, articular cartilage changes resulting from aging are generally distinct to those associated with OA and may cause the tissue to be more vulnerable to damage.

With aging, chondrocytes undergo changes including telomere shortening that lead to senescence [43]. Because of the limited mitogenic activity of adult articular chondrocytes in healthy articular cartilage, it is improbable that replicative senescence is solely responsible for telomere shortening. Stress-induced senescence caused by increased levels of reactive oxygen species and associated DNA damage leading to telomere erosion has been proposed in adult chondrocytes. In addition to a decreased proliferative capability, which is essential to cartilage repair, senescent chondrocytes may lose their ability to maintain homeostasis between anabolic and catabolic pathways, which is essential to the maintenance of healthy tissue as reviewed by Loeser (2009) [43].

Along with chondrocyte senescence, aging causes changes to the cartilage matrix. Notably, the aggrecan size and glycosaminoglycan content of the tissue are reduced with aging [44]. Similarly, advanced glycation end-products accumulate within the tissue and cause increased cross-linking of the collagen network [45,46]. These changes lead to decreased water content [47] and decreased biomechanical properties of the tissue [48]. Another important change is the increased number of tidemarks present in the ZCC resulting in a thinner layer of hyaline cartilage [43,49].

1.2 Cartilage damage and treatment

Under normal conditions, the integrity of articular cartilage can be maintained throughout life despite age-related changes and the high cyclic loading it sustains. However, cartilage erosion can occur following abnormal loading of a joint caused by trauma directly or
leading to modification of its biomechanics [50,51]. Alternatively, cartilage damage may arise under normal loading through the contribution of genetic, environmental and dietetic factors on the quality of the tissue [50,51].

1.2.1 Cartilage defects and regeneration

More than 250 years ago, Hunter (1742) observed that cartilage “when destroyed, it is never recovered” [52]. In fact, partial thickness defects confined within the cartilage layer over a certain size generally do not show signs of healing [53]. This lack of regenerative capabilities of articular cartilage has been partly attributed to the limited migration and mitogenic potential of chondrocytes entrapped within the cartilage extracellular matrix, as well as the avascular nature of the tissue, which prevents access to blood-borne progenitor cells and the formation of a fibrin clot on which these cells can deposit their matrix [54]. It has recently been shown that mesenchymal progenitor cells present in the synovial membrane and the superficial zone of articular cartilage can migrate to the defect site [55]. However, the ability of these cells to populate the defect space was limited by the presence of small proteoglycans at the cartilage defect surfaces that prevent cell adhesion, the low mitogenic activity of these progenitor cells and the absence of a fibrin clot. Efforts to overcome these limitations via the enzymatic degradation of proteoglycans at the defect surface, the administration of mitogenic growth factors and the deposition of a fibrin matrix were helpful in inducing the formation of repair tissue, albeit not cartilage. This was overcome by the controlled release of chondrogenic differentiation factors [56].
In contrast, full thickness defects extend across the osteochondral interface allowing for the formation of a fibrin clot within the defect that can then be invaded by inflammatory cells. These cells are eventually replaced by mesenchymal progenitors with the ability to differentiate into chondrocytes [53]. The resulting repair tissue is characterized as fibrocartilagenous with a lower proteoglycan content than surrounding native hyaline cartilage and eventually exhibits degenerative changes occasionally including exposed subchondral bone [57,58]. Furthermore, this repair tissue is generally poorly integrated with the surrounding hyaline cartilage.

1.2.2 Osteoarthritis

If critical size cartilage defects remain untreated, they may lead to degenerative changes in the joint and the eventual development of OA [59,60]. OA is the most common joint disorder and a major cause of disability and decreased quality of life for a majority of the world population aged 65 years or older [61]. While it is characterized by the progressive degeneration of the articular cartilage, it is not limited to that tissue as changes are observed in the other tissues of an affected joint including the subchondral bone and the synovium [62]. The pathophysiological sequence of the disease is not yet fully resolved; however, early signs of OA include the fibrillation of the superficial zone of articular cartilage and changes in the synovial membrane including thickening and infiltration with inflammatory cells [63]. At the molecular level, an imbalance between anabolic and catabolic processes in the tissue occurs despite extensive evidence suggesting an increased anabolic response in the early stages of the disease, which even contributes to a temporary increase in cartilage thickness [64]. Nevertheless, these changes cause a
mostly gradual loss of proteoglycan, as well as damage to the collagen network [50], thereby negatively impacting the stiffness of the articular cartilage [65]. The resulting decrease in mechanical properties of the tissue may further contribute to abnormal loading and additional damage. Other changes include remodelling of the subchondral plate and an increased thickness of the ZCC [66]. This observation suggests a loss of control of the cartilage mineralization mechanisms and eventually leads to a decreased ratio of hyaline to calcified articular cartilage. This change may also contribute to increased mechanical stresses within the diseased articular cartilage. Additional changes associated with OA include the formation of osteophytes and bone cysts, as well as the vascular invasion of the ZCC [51]. Eventually, the diseased cartilage is replaced by fibrocartilagenous tissue [67] or shows signs of complete breakdown including exposure of the subchondral bone [68].

1.2.3 Current treatment options for articular cartilage damage

While most cartilage lesions do not cause clinical symptoms, some defects may lead to pain, swelling and locking of the joint before the degenerative changes of OA become apparent [59,69]. Currently no pharmaceutical treatments are clinically available to slow or reverse the progression of articular cartilage degeneration. Hence, non-surgical treatments including the administration of acetaminophen and non-steroidal anti-inflammatory drugs aim to reduce the symptoms associated with cartilage defects and degeneration [70]. Along with lifestyle changes including increased physical activity and weight loss, such pharmaceutical treatments are often sufficient to temporarily alleviate the symptoms of patients with cartilage lesions or early articular cartilage degenerative
changes. Another non-surgical intervention employed to reduce the symptoms of cartilage damage is the injection of viscosupplementation such as hyaluronic acid to the joint capsule [71]. Additionally, a number of potential pharmacological options are currently being investigated on the basis that they target pathways involved in articular cartilage homeostasis [70]. One such growth factor of interest in the treatment of OA is fibroblast growth factor-18 (FGF-18), which was shown to reduce cartilage degeneration scores in a rat meniscal tear OA model via intra-articular injections [72].

A range of different surgical procedures is available to attempt the repair of cartilage defects in cases when pharmacological approaches have proven ineffective. The most commonly used surgical approaches consist of marrow stimulation techniques including drilling, abrasion and microfracture [69,73]. These are based on the premise that full thickness cartilage defects are naturally replaced by fibrocartilage repair tissue through the formation of a fibrin clot that can be infiltrated by blood-borne progenitor cells. While this approach involves a single intervention, the resulting fibrocartilage repair tissue is mechanically inferior to the native articular cartilage and is prone to deterioration within a few years, a process that occurs faster in athletes [57,69]. Alternatively, periosteal and perichondrial grafts have been transplanted at cartilage defect sites to provide a source of progenitor cells. Some encouraging results have been obtained with this method but complete regeneration of the damaged tissue has not been achieved. Notably, the fixation of the graft to the surrounding tissues has proved difficult and long-term survival of the repair tissue still remains to be assessed [53,74-77]. Periosteal and perichondrial grafts are best used in combination with other repair strategies as will be discussed later. Mosaicplasty is another common surgical procedure
employed to repair small cartilage defects. This procedure consists of harvesting small cylindrical osteochondral samples from a low weight-bearing location of the joint and transplanting these into the defect. Encouraging clinical results have been obtained for this technique with the clinical outcome of 92% of patients deemed good to excellent at 3 to 6 years follow-up but issues related to donor site morbidity, poor integration of the autografts to surrounding tissues, as well as limitations in the treatable defect size have been identified [78-80].

In the case of patients with advanced OA, when other treatment options have failed or are inadequate, the best treatment approach remains the partial or total replacement of the diseased joint with a synthetic prosthesis. Total joint arthroplasty is generally very successful at reducing pain and improving the quality of life of patients [81]. Nevertheless, failure of orthopaedic implants due to infection, fatigue failure or implant loosening due to wear debris induced osteolysis amongst other causes requires technically challenging revision surgeries, rendering this approach less suitable for the treatment of young patients [82,83].

1.2.4 Tissue engineering approaches

As was emphasized in the previous subsection, current clinical treatment strategies for the repair of articular cartilage defects or degenerative joint changes have been successful at relieving pain and improving the quality of life of patients. However, these approaches have shortcomings that limit their application and/or their long-term outcome. These limitations of available treatment options have prompted the development of an impressive number of tissue engineering strategies to attempt to promote the regeneration
of damaged articular cartilage. Langer and Vacanti (1993) defined tissue engineering as “an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ” [84]. The avascular and aneural nature of articular cartilage has simplified the design of such strategies in comparison to other tissue but many challenges remain to be overcome to successfully treat articular cartilage defects with tissue engineering constructs.

Autologous chondrocyte implantation (ACI) was the first cell-based approach for the treatment of cartilage defects tested clinically [85]. This methodology involves the excision of autologous cartilage from areas surrounding the defect site in a first surgical intervention, followed by the enzymatic release of chondrocytes from their matrix and their amplification \textit{in vitro}. The chondrocytes are then implanted back into the cartilage defect under a periosteal graft in a second surgery. The long-term clinical outcome for ACI with a mean follow-up of 12.8 years has been positive with 92% of patients responding that they were satisfied with the surgery, while a failure rate of 16% was observed at a 7.4 years mean follow-up [86,87]. A number of clinical studies have compared ACI to microfracture and found no significant differences in clinical and functional scores between the two treatments at 2 and 5 years [88,89]. However, it was suggested that ACI may exhibit a slower recovery than microfracture at time points up to a year [89]. A comparison of microfracture and ACI treatments in a canine model demonstrated that defect filling was more complete with microfracture, but a larger percentage of the repair tissue was hyaline-like following ACI treatment [90]. These results clearly indicate some of the important research concerns of cartilage tissue
engineering. Notably, difficulties associated with retaining the cells at the defect site have become apparent because of the hypertrophy and/or detachment of the periosteal patch, as well as the need for two surgical interventions compounded with a loss of the chondrocytic phenotype of the cells following in vitro amplification have stressed the need for an adequate cell source and the development of other approaches [70].

A variety of three-dimensional scaffolds have been designed for cartilage tissue engineering applications [91]. These porous scaffolds are tailored to act as support matrices for the delivery and retention of cells with chondrogenic potential or cartilaginous tissue matured in vitro. Alternatively, scaffolds may provide a three-dimensional network to direct the migration of endogenous cells obtained from marrow stimulation. These scaffolds must be biocompatible, biodegradable, allow the cells to adhere and maintain their phenotype, have sufficient mechanical integrity to sustain the cyclic loads applied on the targeted joint and allow for the fixation of the tissue engineering constructs within the defect site [91]. Two classes of biomaterials are generally used for cartilage tissue engineering applications: natural and synthetic polymers. Natural polymers include collagens [92-94], fibrin [95] and hyaluronan [96] and have been used extensively because they provide an environment on which cells can adhere and proliferate [91]. The most commonly used synthetic polymers are polylactic acid (PLA) [97], polyglycolic acid (PGA) [98] and their copolymer poly(lactic-co-glycolic) acid (PLGA) [99]. Synthetic polymers are easily processed with tailored properties in a reproducible manner compared to natural polymers [91]. Nevertheless, their biocompatibility is limited as degradation products may elicit an inflammatory response and they lack natural adhesion sites [91]. Recent advances have focussed on the
design of hybrid scaffolds incorporating both natural and synthetic polymers to overcome the limitations of each category of biomaterials [100,101].

To address the technical difficulties associated with obtaining a large number of primary chondrocytes with an adequate phenotype following amplification, a number of alternative cell sources have been investigated. Embryonic stem cells and adult progenitor cells with chondrogenic potential from a number of different tissue sources have been investigated [102]. However, stem cells are as of yet limited in their applications by the development of efficient differentiation protocols. Additionally, embryonic stem cells cause immunological and ethical concerns. However, these limitations of embryonic stem cells could eventually be overcome by the use of induced pluripotent stem cells [103,104]. Alternatively, protocols have also been devised to redifferentiate chondrocytes that have undergone dedifferentiation during in vitro amplification [105-108]. An extensive body of work has been produced to optimize the effects of growth factor combinations, doses and timing on the differentiation, maintenance or improvement of the chondrogenic potential of cells seeded in tissue engineering constructs [91,109].

The long-term integration of bioengineered cartilage to the native articular cartilage and subchondral bone following implantation has been identified as a major requirement for the success of these constructs and one that has been difficult to attain [110,111]. One approach to achieve tissue integration consists of designing biphasic constructs incorporating chondrocytes or cartilage tissue formed in vitro on the intended articulation surface of a bone substitute to mimic the osteochondral architecture of the joint [112]. The rationale for this approach lies in part in the ability to integrate bone
more easily than cartilage, thus stabilizing the engineered construct rapidly after its implantation and potentially providing an environment that favours the integration of the \textit{in vitro}-formed tissue to native cartilage. Biphasic constructs for which the cartilage phase is generated \textit{in vivo} by incorporating cells with chondrogenic potential or chondrocytes in scaffolds placed onto bone substitutes have had limited success in regenerating a continuous layer of hyaline cartilage properly integrated with the surrounding tissues [113-118]. On the other hand, promising results were achieved with biphasic constructs for which the formation of the articular cartilage phase was allowed to take place \textit{in vitro} prior to implantation [119-122]. This is supported by work in a canine microfracture model, which demonstrates that the \textit{in vitro} incubation of chondrocytes seeded and cultured in a collagen type II scaffold for 4 weeks improves both the percent filling of the defect with repair tissue and the amount of repair tissue with hyaline-like characteristics compared to a short 24 hours incubation [92,123].

1.2.5 Biphasic constructs with a porous calcium polyphosphate (CPP) bone substitute

The potential of CPP as a material for bone substitute applications was previously established [124,125]. Our group developed a procedure to produce a porous CPP bone substitute tailored for applications as a substrate on which to form articular cartilage intended for joint resurfacing [126]. The porous CPP substrate is produced by gravity sintering of dried amorphous calcium polyphosphate powder ranging from 75 to 150 µm in size. The resulting substrate is polycrystalline in nature and characterized by an interconnected porosity of 35 to 40% with pores ranging from 40 to 140 µm in diameter.
The as-made tensile strength of porous CPP is comparable to that of trabecular bone. As was stated by Pilliar et al. (2001) the chain-like structure of CPP made of phosphoanhydride bonds represents locations for hydrolysis to occur, leading to the eventual degradation of the substrate in aqueous solutions and associated decrease in tensile strength [126]. The in vivo degradation behaviour suggested an increased degradation rate of the CPP following interactions with cells [127]. This study also demonstrated that the CPP degradation rate is inversely proportional to the size of particle with which the substrate is fabricated. Further work showed that the sintering temperature is proportional to the crystallinity of the CPP and influences the degradation rate of the substrate [128]. The degradation profile of CPP indicated a fast initial rate of degradation, which was stabilized after a few days.

Porous CPP was found to be osteoconductive, as bone ingrowth was observed within the porous structure and in direct contact with CPP particles [127]. A methodology was devised to form articular cartilage-like tissue in vitro on the top of and integrated with the subsurface of cylindrical porous CPP substrates by seeding and culturing primary chondrocytes isolated from the full thickness of bovine articular cartilage, thereby generating biphasic constructs [129]. The resulting cartilaginous tissue was anchored to the substrate through tissue interdigitations within the porous structure. The cartilaginous tissue cultured in vitro for 8 weeks contained large proteoglycans and collagen type II. However, the collagen content of in vitro-formed tissue was deficient compared to that of native articular cartilage, leading to much inferior tissue stiffness. A number of approaches have been investigated to improve the quality of in vitro-formed cartilaginous tissue formed on CPP with moderate success [130-135]. Nevertheless,
animal studies in sheep demonstrated that small cylindrical biphasic constructs (diameter of 4 mm) can integrate with the surrounding host cartilage and subchondral bone and are suitable for the repair of focal osteochondral defects [120,136]. Furthermore, the stiffness of the in vitro-formed cartilaginous tissue was improved in vivo suggesting there was a maturation phase.

Because the interfacial shear properties of these in vitro-formed constructs are 1 to 2 orders of magnitude lower than that of the native cartilage-subchondral bone interface, their application is potentially limited to the repair of small, contained cartilage defects [137]. A methodology has been established in our group to generate cartilage tissue incorporating a ZCC directly apposed to the membrane insert used as a substrate in vitro by selecting for chondrocytes from the deep zone of articular cartilage and supplementing the culture medium with a high calcium concentration and a source of organic phosphate (β-glycerophosphate; β-GP) during tissue formation [138,139]. The mineral that forms within the cartilage is poorly crystalline apatite similar to that of native calcified cartilage in its crystal length and orientation with regards to the collagen network of the extracellular matrix [28]. This methodology was applied to attempt to improve the mechanical integrity of the interface between in vitro-formed cartilaginous tissue and the porous CPP substrate. [137]. While a ZCC was successfully generated, it was located slightly above the tissue-CPP interface. Further efforts are required to understand the mechanism by which the location of the ZCC is shifted away from the interface on CPP substrates and overcome this limitation of the system.
1.3 Cartilage Biomineralization

The formation of poorly crystalline apatite crystals within articular cartilage is a complex process that is not yet fully understood. To gain insight into the mechanisms involved in the formation of the ZCC at the interface between articular cartilage and subchondral bone, the physical principles governing the crystallization process will be reviewed, as will the processes by which chondrocytes initiate and mediate the mineralization process.

1.3.1 Crystal nucleation and growth

Crystallization is essentially a phase transition from a solution to a solid crystal, which occurs if the free energy of the initial solution is greater than the sum of free energies of the crystal and the final solution (i.e. the free energy change is negative) [140].

The first step of this process is the nucleation of ions to form an initial crystal referred to as a critical nucleus [141]. In a supersaturated solution, clusters of ions constantly form and dissolve. Because ions at the surface of these clusters are not as strongly bound to surrounding molecules as ions in the bulk of these clusters, the free energy change associated with molecules at the surface has a positive contribution to the free energy change of the clusters [140]. Conversely, molecules in the bulk phase of a cluster contribute negatively to its free energy change. Hence, small clusters with a high surface area-to-volume ratio have a positive free energy change and are not stable. However, if a cluster of sufficient size is formed, the negative free energy change associated with its bulk overcomes the positive free energy change associated with its surface and a stable nucleus is formed. Parameters controlling the critical nucleus size and the associated probability for nucleation include the level of supersaturation, the
interfacial free energy (i.e. the difference between the free energy per molecule of the bulk and of the surface) and temperature [140]. In vertebrate mineralization, modulation of the first two parameters leads to control over the location and orientation of crystal deposition. Because molecules can form stronger bonds with a substrate than those of solvation, such a substrate can reduce the interfacial free energy and increase the probability for nucleation [140,142]. This effect is greater for surfaces with arrangements that complement a plane of the crystal lattice and that include functional groups to promote strong bonds [140].

Following nucleation, crystal growth occurs via the addition of ions to the crystal lattice as a function of the associated negative free energy change [140]. This addition of ions to a crystal surface occurs preferentially at thermodynamically favoured locations. In fact, a crystal surface contains flat surfaces referred to as terraces with raised surfaces known as steps, which in turn contain kinks leaving vacancies within the steps [140,143]. Because a molecule that binds to a kink site can make more bonds with surrounding ions of the lattice, kinks are thermodynamically favoured growth site. Hence, the growth rate of crystals is related to its kink density. Because crystals are not static entities, kinks are always being formed and occupied at rates dependent on the strength of bonds at specific locations. The rate and direction of crystal growth can be controlled by blocking kink sites to effectively reduce their density or roughening steps, possibly by increasing the supersaturation of the surrounding solution [140]. Besides the thermodynamically unfavoured formation of steps by addition of ions to terraces, dislocations in the crystal lattice may also give rise to steps [140].
1.3.2 Role of matrix vesicles in cartilage calcification

Matrix vesicles have been proposed as potential locations for the initial nucleation of apatite crystals in a number of mineralizing vertebrate tissues including growth plate cartilage, as they have been closely associated with apatite crystals [144,145]. These vesicles have also been identified in normal and OA articular cartilage [146]. They are small spherical bodies budding from the plasma membrane of cells in mineralizing tissues and containing high levels of proteins known to have a role in the mineralization process, such as TNAP and annexin V [147].

It has been suggested that matrix vesicles are a location for the accumulation of high concentrations of calcium and phosphate aided by enzymatic activity to facilitate crystal nucleation [148]. Annexin V can bind to collagen fibrils within the extracellular matrix to mediate the influx of calcium into matrix vesicles. Within the vesicles, it creates complexes with calcium ions, phosphate and phosphatidylserine to maintain a low apparent ion concentration and permit a continuous ion influx [149,150]. Furthermore, this complex acts as the location for crystal nucleation within matrix vesicles [149]. The action of phospholipases eventually leads to the formation of lysophospholipids that cause the breakdown of matrix vesicles and the exposure of mineral crystals to the extracellular environment [151].

While the extent of the role played by matrix vesicles in tissue mineralization is contentious, strong evidence suggests that they are also involved in regulating the extracellular levels of pyrophosphate produced by cellular ectonucleotide pyrophosphatase/phosphodiesterase family member 1 (ENPP-1) and transported to the extracellular environment by progressive ankylosis (ANK) [147]. In fact, membrane-
bound TNAP cleaves pyrophosphate, an inhibitor of tissue mineralization, thereby releasing orthophosphate that can be used for apatite crystals formation locally.

### 1.3.3 Role of extracellular matrix components in cartilage calcification

It is well accepted that components of the extracellular matrix are essential in mediating tissue calcification [152]. In fact, the extracellular matrix of mineralizing tissues is generally reorganized by cells prior to the initiation of mineralization to provide an oriented support for mineral deposition. In both growth plate and articular cartilage, the calcification is limited to the hypertrophic and deep zone, respectively. This suggests a high level of control of the crystallization process. Similar to matrix vesicles, little information is available on the role of the extracellular matrix in the control of healthy articular cartilage mineralization in the ZCC, as most of the data summarized below are derived from experiments with growth plate cartilage and osteoarthritic cartilage. Hence, this information must be interpreted with caution due to the observed differences between the mineral phases of these tissues.

As was specified previously, the orientation of mineral crystals in calcified articular and growth plate cartilage is not controlled to the same extent as in bone [16]. Additionally, mineral crystals do not appear to be associated with the collagen type II fibrils in articular and growth plate cartilage [28,29]. This suggests that collagen type II may not have a critical role in cartilage mineralization compared to the importance of collagen type I in bone mineralization. However, collagen type II has been shown to bind to matrix vesicles through membrane bound annexin V, leading to accelerated calcium influx inside the vesicles [153]. Additionally, the C-propeptide of collagen type II also
know as chondrocalcin appears to be concentrated in the hypertrophic zone of growth plate cartilage. Similarly, its levels are raised in the synovial fluid of osteoarthritic articular cartilage [154]. This peptide has been shown to bind strongly to hydroxyapatite in solution [155] and has been associated with increased mineralization [156,157]. Nevertheless, purified chondrocalcin was shown to have no nucleating or inhibiting effect on the formation of hydroxyapatite in solution [158].

Collagen type X is localized exclusively in the hypertrophic zone of growth plate cartilage [159] and the deep zone of articular cartilage [160]. It is also expressed by chondrocytes from the upper layer of articular cartilage undergoing osteoarthritic changes [160,161]. These observations have lead to the suggestion that it plays a role in cartilage matrix mineralization. Collagen type X has been shown to exhibit matrix vesicle binding abilities similar to those of collagen type II [162]. Due to the ability of collagen type X to bind collagen type II, it has been hypothesized that collagen X may have a role in maintaining collagen fibril organization during the matrix remodelling process that precedes mineral deposition [163]. Its ability to bind to proteoglycans could help to keep these macromolecules in their proper location in the tissue, as indicated via the investigation of a collagen type X knockout mouse model [164]. This knockout mouse also exhibited an increased number of matrix vesicles in the resting and proliferative zones of the growth plate compared to wild-type animals. Other changes observed in collagen type X knockout mice include decreased trabecular bone content at day 2 followed by an increased content at 4 weeks.

Proteoglycans have been proposed as sites of crystal nucleation in cartilaginous tissue because they are highly negatively charged and attract a large concentration of
positively charged calcium ions [152]. However, studies performed with purified proteoglycans in vitro demonstrated that they can also act as inhibitors of hydroxyapatite growth [165]. Others have suggested that proteoglycans must exhibit altered properties for calcification to occur [166]. Hence, the role of proteoglycans in cartilage mineralization still remains unclear.

Transglutaminases are protein cross-linking enzymes [167]. Two of these enzymes, transglutaminase 2 and coagulation factor X IIIA, are present in hypertrophic cartilage [168,169] and highly expressed in osteoarthritic cartilage [170]. Knockout models for either protein did not exhibit changes in the mineralization patterns [171,172]. However, it was shown that mineralization in a co-culture system comprised of hypertrophic chondrocytes and preosteoblastic cells from the non-mineralized section of the bone collar extracted from chicken growth plates could be modulated by changes in the transglutaminase levels [173].

Evidence from knockout studies suggests that the regulation of the location of tissue mineralization within a number of soft tissues including articular cartilage is dependent on the action of a number of mineralization inhibitors [174]. As was discussed in some detail previously, one such subset of proteins includes ENPP-1, ANK and TNAP involved in the metabolism of inorganic pyrophosphate, a potent inhibitor of tissue mineralization [175,176]. Interestingly, abnormally high levels of pyrophosphate in articular cartilage can lead to the formation of the calcium pyrophosphate dihydrate (CPPD) crystals that are often present in osteoarthritic tissues [177]. Osteopontin is found in healthy and osteoarthritic cartilage [178] and can act as an inhibitor or an activator of mineralization [174]. Interestingly, a deficiency in extracellular pyrophosphate in
osteoblast cultures is associated with decreased osteopontin levels [176]. This synergy between pyrophosphate and osteopontin may explain the formation of CPPD crystals in osteoarthritic tissues as a consequence of elevated levels of osteopontin [179]. Another extracellular protein present in cartilage and that acts to inhibit its mineralization is apatite- and calcium-binding matrix GLA protein (MGP) [180]. MGP was also shown to enhance arterial mineralization under specific conditions [181]. A number of other mineralization inhibitors present in cartilage have been reviewed [174]. Inorganic polyphosphates are potential novel mineralization regulators in articular cartilage and will be discussed in detail in a subsequent section.

1.3.4 Role of apoptosis in cartilage calcification

Terminally differentiated growth plate chondrocytes undergo apoptosis and this process has been associated with matrix mineralization [177,182]. Similarly, signs of apoptosis have been observed in chondrocytes of the upper layer of articular cartilage undergoing late osteoarthritic changes and have also been associated with mineralization [183]. The formation of apoptotic bodies containing phosphatidyserine associated with this process is thought to play a role in apoptosis-induced mineralization, as phosphatidyserine binds calcium [184]. Interestingly, high phosphate concentrations have been shown to trigger chondrocyte apoptosis in culture [185]. However, recent reports have demonstrated that apoptosis only contributes partially to cell-mediated cartilage mineralization and that this effect may be species-specific [186,187].
1.3.5 Initiation of mineralization in vitro with β-glycerophosphate

Robison and Rosenheim (1934) first demonstrated that β-GP could be added to in vitro calcifying cartilage cultures to increase the rate of mineralization [188]. The same approach was also employed successfully to facilitate osteogenesis and calcium phosphate crystal deposition in chick periosteal cultures [189,190]. The authors suggested that the use of an organic source of phosphate would initiate mineralization specifically in locations where phosphatases are present. These studies led to the routine use of β-GP as a phosphate donor for the study of mineralizing culture systems. However, the mechanism by which β-GP induces tissue mineralization remains poorly understood and the wide range of culture systems in which its effects have been investigated has resulted in contradictory reports. It is generally accepted that the action of β-GP is closely linked to the high phosphatase activity of cells with calcifying potential, leading to increased concentrations of phosphate in the tissue. However, consistent reports of reduced alkaline phosphatase activity following treatment with organic phosphate have been puzzling in that regard [191-193]. To explain these results, Tenenbaum et al. (1989) suggested that the presence of other phosphatases with increased affinity for the substrate may replace alkaline phosphatase [193]. Conflicting reports have also been published on the ability of β-GP to impact metabolic processes and to produce the same type of apatite crystals as in native tissues [191-196]. In addition to the stimulation of mineralization within in vitro cell cultures with calcifying potential, β-GP has been associated with other changes including a possible interference with the metabolism of thymidine [193], in addition to affecting the activity of phosphoprotein phosphatases and casein kinase II [197,198]. Nevertheless, gene expression changes associated with the addition of β-GP to deep zone
chondrocytes have been attributed to the mineralization process rather than these other pathways. This was demonstrated through the observation of similar gene expression changes following initiation of mineralization by treatment with phosphoethanolamine and by the absence of gene expression changes following β-GP treatment of superficial zone chondrocytes that do not have the potential to form calcified tissue [192].

1.4 **Inorganic Polyphosphates**

Inorganic polyphosphates are linear polymers of orthophosphate residues linked by high-energy phosphoanhydride bonds. Cyclic inorganic polyphosphates are referred to as metaphosphates or cyclophosphates and have been identified in biological systems but do not have well defined roles and will not be discussed in this work [199]. Similarly, branched inorganic polyphosphates, also know as ultraphosphates, will not be discussed as they have yet to be observed in biological systems [200]. Inorganic polyphosphates vary in the length of their chain from two to several hundreds of orthophosphate units. The shortest inorganic polyphosphate consisting of a chain of two orthophosphates is referred to as inorganic pyrophosphate in the literature and has been studied extensively, notably with regards to its role in the modulation of matrix calcification [201]. As will be briefly discussed subsequently, a number of enzymes involved in the metabolism of condensed phosphates are specific to inorganic pyrophosphate or longer chain inorganic polyphosphates suggesting different biological roles. Hence, the term inorganic polyphosphates will be used in this text to refer to phosphate polymers consisting of three or more orthophosphate residues.
1.4.1 Natural occurrence of inorganic polyphosphates

Biological inorganic polyphosphates were first identified by Wiame (1947) in yeast [202]. Since then, the ubiquitous occurrence of these phosphate polymers has been established as they have been found in members of the three domains of the phylogenetic tree of life: archaea, bacteria and eukaryotes [200]. Of interest for this text, the presence of inorganic polyphosphates has been demonstrated in a number of vertebrate cells and tissues [203-207].

Inorganic polyphosphates found in vertebrate cells are generally characterized by a bimodal chain length distribution, whereby the phosphate polymers can be ascribed to short chain and long chain pools with somewhat narrow ranges of chain lengths [204,207,208]. Interestingly, the ranges of inorganic polyphosphate chain lengths appear to be cell type-specific. Perhaps more importantly, the distribution of condensed phosphate polymer lengths is dependent on the metabolic state of cells, as evidenced by Lorenz et al. (1997), who have shown that the induction of apoptosis in HL60 human premyelocytic leukemia cells is associated with the loss of the pool of long chain inorganic polyphosphates [208]. The same report also demonstrates age-dependent changes in the content of long chain inorganic polyphosphates in rat brain and liver tissues without equivalent effects on the pool of short chain phosphate polymers. Taken together, these results suggest that the chain length of inorganic polyphosphates may influence their biological functions.

Inorganic polyphosphates are also distributed non-randomly within vertebrate cells and tissues. For example, reports have shown that condensed phosphates are particularly concentrated in the cell nuclei of rat livers [204,205]. Inorganic
polyphosphates have also been found in the plasma membrane [205,209], mitochondria [205,210,211] and lysosomes [207]. Of interest, inorganic polyphosphates were recently identified in the extracellular matrix of hypertrophic growth plate cartilage [212].

It has been suggested that condensed phosphates may have played a role in the transition from the prebiotic earth to the RNA world and eventually the DNA and protein world [213,214]. The presence of inorganic polyphosphates in the prebiotic world is highly probable because polymers of orthophosphate can be formed from phosphate-containing rocks by dehydration at elevated temperature and have been identified in volcanic gases [214]. However, the validity of such a theory on the origin of life has been questioned because of the absence of known mechanisms for the formation of high yields of condensed phosphates and processes to concentrate them [215,216].

1.4.2 Metabolism of inorganic polyphosphates

Despite the fact that inorganic polyphosphates are ubiquitously conserved in biological organisms, the functions of inorganic polyphosphates are relatively poorly understood as will be discussed in subsequent subsections. An understanding of the enzymes involved in the metabolism of condensed phosphates is essential to the determination of their functions and a better comprehension of the pathways modulating their levels.

1.4.2.1 Enzymes of inorganic polyphosphates synthesis

The most studied enzyme involved in the synthesis of inorganic polyphosphates is polyphosphate kinase 1 (PPK1). This enzyme was first isolated from Escherichia coli and catalyzes the transfer of the terminal phosphate of adenosine triphosphate (ATP) to an
inorganic polyphosphate chain in a reversible reaction [217,218]. The genetic disruption of the ppk1 gene was shown to considerably reduce the inorganic polyphosphate levels in *E. coli*, while its overexpression led to increased levels [219]. Homologues of PPK1 have been identified and studied in a large number of prokaryotes as recently reviewed by Rao et al. (2009) [220]. In eukaryotes, such a homologue has been observed and investigated in *Dictyostelium discoideum* (*DdPPK1*) [221]. Other eukaryote PPK1 homologues have been proposed based on bioinformatics analyses but no protein with significant amino acid sequence identity was found in yeast and higher eukaryotes including vertebrates [222]. Interestingly, the synthesis of inorganic polyphosphates in vertebrate cells was proposed to take place via ATP-independent processes, suggesting the involvement of other polyphosphate kinases [205,211]. In addition, PPK1 produces long chains of inorganic polyphosphate containing approximately 750 orthophosphate units and does not produce chains of intermediate length [223]. This is in disagreement with observations in vertebrate cells which incorporate $^{32}$P in culture within the short chain inorganic polyphosphates fraction prior to the long chain fraction [208].

Another polyphosphate kinase which is well conserved within prokaryotes has been identified in *Pseudomonas aeruginosa*. *PaPPK2* (also referred to as PPK2) synthesizes guanosine triphosphate (GTP) from the transfer of a phosphate residue from inorganic polyphosphate to guanosine diphosphate (GDP), as well as the reverse reaction, albeit at a considerably slower rate [224]. PPK2 also uses ATP as a donor and acceptor of phosphate. Interestingly, a homologue of PPK2 in *Corynebacterium glutamicum* favours the synthesis of inorganic polyphosphates [225]. Nevertheless, a PPK2 homologue has yet to be identified in vertebrates.
A third class of enzymes with polyphosphate kinase activity was identified in D. discoideum. DdPPK2 (also known as PPK3) is a complex of three actin-related proteins (Arp) which synthesizes long chains of inorganic polyphosphate upon polymerization into actin-like filaments in the presence of ATP [226]. Interestingly, this protein complex composed of ArpA, ArpB and Actin 28 shares 60%, 72% and 62% amino acid identity with human Arp1, Arp2 and Actin, respectively [222]. Additionally, Ca\(^{2+}\)-ATPase extracted from the plasma membrane of human erythrocytes and forming a complex with poly(3-hydroxybutyrate) (PHB) and inorganic polyphosphates was shown to transfer phosphate from ATP to short chain inorganic polyphosphate in a reversible reaction [227]. While these complexes are potential candidates for the synthesis of inorganic polyphosphates in vertebrates, an understanding of the enzymes involved in this process remains elusive.

1.4.2.2 Enzymes of inorganic polyphosphates degradation

Enzymes responsible for the hydrolysis of inorganic polyphosphates are referred to as polyphosphatases. More specifically, exopolyphosphatases catalyze the hydrolysis of inorganic polyphosphate molecules at the end of the chain to release an orthophosphate residue, while endopolyphosphatases facilitate hydrolysis in the middle of the chain. A third category of polyphosphatases utilize inorganic polyphosphate as a phosphate donor for other molecules, as exemplified with PPK1 and PPK2.

Of the enzymes involved in the metabolism of inorganic polyphosphates, exopolyphosphatases have been better characterized in eukaryotic cells. While a number of these enzymes have been isolated or studied in prokaryotes [228], the available reports
in yeast and vertebrate tissues provide more relevant information and will be the focus of the following discussion. In *Saccharomyces cerevisiae*, the gene sequence for only one enzyme with exopolyphosphatase activity has been sequenced thus far [229,230]. This enzyme found in cell cytosol and referred to as *scPPX1* hydrolyzes inorganic polyphosphates over a wide range of chain lengths with a preference for molecules with chains averaging 250 orthophosphate residues but does not cleave pyrophosphate, ATP or trimetaphosphate. However, a number of other enzymes with exopolyphosphatase activity have been purified and studied in *S. cerevisiae* as was reviewed by Lichko et al. (2003) [231]. These enzymes, some of which are not encoded in the *ppx1* gene, differ in their substrate affinity based on chain length, their metal cations requirements, their optimal pH and the cellular location, with specific enzymes present in the cell membrane, nucleus, mitochondria, vacuoles and cytosol. These observations further suggest an impact of inorganic polyphosphates chain length and cellular location on their biological roles.

Exopolyphosphatase activity has been identified in mammalian cell extracts, as well as in blood plasma and synovial fluid [206,208,232,233]. Interestingly, the levels of exopolyphosphatase activity measured in brain and liver tissues in rat are age-dependent, with levels in the brain seemingly inversely proportional to the inorganic polyphosphate content [208]. Thus far, four enzymes of mammalian origin were found to exhibit exopolyphosphatase activity. In a first report, it was shown that the calf intestinal isoform of alkaline phosphatase (CIAP) hydrolyzes inorganic polyphosphates with chain lengths up to 800 orthophosphate residues in addition to inorganic pyrophosphate [234]. Orthophosphate cleavage occurs according to a processive mechanism and at an optimal
pH of 9.5. Unlike many exopolyphosphatases studied in other organisms, CIAP hydrolyses inorganic polyphosphates in the absence of divalent metal cations, which act as inhibitors. Under these experimental conditions the placental and tissue non-specific isoforms of alkaline phosphatases did not exhibit exopolyphosphatase activity. The addition of divalent metal cations resulted in enzymatic activity in the placental isoform but not in TNAP. A recent report has demonstrated the exopolyphosphatase activity of TNAP for inorganic polyphosphates with an average chain length of 28 orthophosphate residues at a pH of 9.0 and in the absence of divalent metal cations [212]. Another exopolyphosphatase, h-prune, was recently reported [235]. In contrast to alkaline phosphatases, h-prune does not hydrolyse inorganic pyrophosphate or ATP and has a preference for short chain inorganic polyphosphates (tripolyphosphate and tetrapolyphosphate) over longer chain polyphosphates. The reaction requires a divalent metal cation and is optimal at near neutral pH.

Enzymes with endopolyphosphatase activity have been scarcely studied. In *Saccharomyces cerevisiae*, the gene sequence for one such enzyme, PPN1, has been sequenced [236]. It was found that few eubacteria exhibit endopolyphosphatase activity, while archaea are characterized by moderate levels of activity and all eukaryotes investigated exhibit activity [237]. In *S. cerevisiae*, PPN1 must be activated through post-translational protease cleavage and acts in a non-processive way to form a range of shorter chain inorganic polyphosphates with orthophosphate and tripolyphosphate as final digestion products [238]. Endopolyphosphatase activity measured in rat tissues was notably high in the brain and appeared to yield a comparable digestion product distribution to PPN1 [237].
A number of enzymes catalyzing the transfer of phosphates from inorganic polyphosphates to other molecules have been studied in lower organisms, but these have not been studied in eukaryotes. They include a polyphosphate:adenosine monophosphate phosphotransferase, a nicotinamine adenine dinucleotide kinase and a glucokinase and have recently been reviewed by Rao et al. (2009) [220].

1.4.3 Inorganic polyphosphates modulate vertebrate biomineralization

A wealth of reports has been published on the involvement of inorganic polyphosphates in biomineralization. As will be discussed in the following subsection, these reports support an involvement of condensed phosphates in the modulation of this process within vertebrate tissues.

1.4.3.1 Inorganic polyphosphates inhibit apatite crystal nucleation, growth and solubility

Fleisch and Neuman (1961) demonstrated that the addition of inorganic polyphosphates to solutions of collagen with nucleating properties significantly elevated the minimum ion product (calcium × phosphate) required for spontaneous apatite precipitation to occur [239]. This observation suggests that condensed phosphates act as inhibitors of apatite formation. It was also shown that inorganic polyphosphates exhibit stronger inhibitory effects than pyrophosphates at equivalent concentrations. Because concentrations of inorganic polyphosphates as low as $10^{-7}\text{M}$ were sufficient to inhibit the precipitation process, it was suggested that this effect could not be explained by calcium chelation. Interestingly, plasma ultrafiltrate and urine were both shown to cause increases in the minimum ion product required for spontaneous apatite precipitation and this effect was
reversible upon treatment with intestinal alkaline phosphatase. Based on these results, the authors proposed that the local presence of phosphatases to remove inhibitors such as pyrophosphate and inorganic polyphosphates may be a prerequisite to tissue mineralization.

It was also shown that the growth of hydroxyapatite crystals is inhibited by the presence of inorganic polyphosphates [240] and pyrophosphate [240,241]. It was demonstrated that this inhibitory effect of condensed phosphates on apatite crystal growth occurs in conjunction with the adsorption of the phosphate polymers on apatite growth sites [212,241,242]. This effect appears to be transient, as the incubation of apatite-bound condensed phosphates leads to their hydrolysis and an associated reversal of inhibition [241,242]. Francis (1968) argued that the rapid loss of condensed phosphate inhibition due to the action of phosphatases prevents their use as a treatment of anomalous tissue calcification [240]. It should be specified that the inorganic polyphosphate selected for this study had a short chain length of three orthophosphate residues rendering it potentially easier to hydrolyse than longer phosphate polymers.

Apatite bound condensed phosphates have also been shown to reduce the acid solubility of the mineral [242,243]. This effect appears to be chain length dependent with polyphosphates characterized by longer chain lengths exhibiting an increased effect.

1.4.3.2 Exogenous administration of inorganic polyphosphates modulates vertebrate tissue mineralization

Direct evidence of the inhibitory effects of inorganic polyphosphates and pyrophosphate on tissue mineralization has been demonstrated in numerous animal models and cell
culture experiments. Fleisch et al. (1966) showed that embryonic chicken femur maintained in vitro in medium containing high doses of inorganic polyphosphates (4 and 16 µg of phosphorus per ml) did not affect the extent of bone tissue formation but acted to significantly reduce the amount of mineral deposited in bone [244]. Interestingly, low doses of the phosphate polymers (1 µg of phosphorus per ml) resulted in slightly increased mineralization. This study also specified that polyphosphates did not affect the hydroxyproline content of the tissue.

Subcutaneous injections of condensed phosphates were also shown to inhibit calcification in rat aorta induced by treatment of the animals with large amounts of vitamin D [245, 246]. Another study by the same group showed the ability to inhibit calciphylactic skin reactions induced by administration of dihydrotachysterol, through subcutaneous treatment with inorganic polyphosphates [247]. It was noted that the effective dose for the skin treatment was insufficient to affect the normal bone calcification process.

More recently, mineral deposition by SaOS-2 human osteosarcoma cells induced by β-GP was shown to be inhibited by treatment with inorganic polyphosphates [233]. Conversely, a similar treatment with condensed phosphates on MC3T3-E1 pre-osteoblasts was shown to induce the calcification of the cell monolayer cultured in the absence of β-GP or any other source of phosphate [248, 249]. The extent of calcification was more important than in cultures treated with an equivalent concentration of sodium orthophosphate or in typical calcification conditions including ascorbic acid and β-GP. Similarly, the treatment of human mesenchymal stem cells and human dental pulp cells with inorganic polyphosphates was shown to cause changes suggestive of osteoblastic
differentiation and to increase calcification [250,251]. The same group has also demonstrated a stimulatory effect of phosphate polymers on bone formation in a rat alveolar bone regeneration model, whereby inorganic polyphosphate was injected in a surgically created bone defect [252]. Bone defects created in rabbit femurs were also filled with a porous hydroxyapatite scaffold on which inorganic polyphosphate had been adsorbed with similar success [253,254]. However, the experimental methodology employed for these last three studies does not permit comment on the extent of mineralization of the newly formed bone tissue.

The seemingly conflicting observations with regards to the effects of exogenous inorganic polyphosphate treatment on tissue mineralization may be attributed to the release of orthophosphate ions that can participate in the mineralization process upon action of exopolyphosphatases on the phosphate polymers. Hence, it is probable that the ratio of inorganic polyphosphate-to-exopolyphosphatase contents will dictate the inhibitory or promoting potential of condensed phosphates on mineralization.

1.4.3.3 Mechanism of calcification inhibition by inorganic polyphosphates

As was discussed in a previous subsection, condensed phosphates bind to apatite crystals and inhibit their growth. These observations support the idea that the inhibition of tissue calcification by inorganic polyphosphates occurs through their adsorption to growth sites of the calcification nuclei [240,241]. This process is associated by the release of approximately one orthophosphate per molecule of condensed phosphate bound to the apatite crystal [242].
Irving et al. (1966) reported that changes to the extracellular matrix of aorta associated with tissue calcification did not occur in the presence of inorganic polyphosphates and suggested that these matrix changes may arise secondarily as a consequence of calcification [246]. More recently, it was shown that inorganic polyphosphates directly interact with pre-osteoblastic cells and cause upregulation of differentiation markers such as osteopontin, osteocalcin and bone sialoprotein [248,249,255]. Furthermore, inorganic polyphosphates were shown to induce the osteoblastic differentiation of human dental pulp cells [250] and mesenchymal stem cells [251], as demonstrated by the expression of a number of osteoblastic markers. These studies illustrate the ability of polyphosphates to affect cellular processes and suggest the possibility of a biological mechanism of action for the inhibition of tissue calcification along with its physicochemical interactions with apatite crystals.

1.4.3.4 Endogenous inorganic polyphosphates modulate vertebrate tissue mineralization

As was stated previously, inorganic polyphosphates are ubiquitous in biological systems including vertebrate cells and tissues. Of particular interest, they are present in high concentration in osteoblasts compared to other cell types in humans [206]. Inorganic polyphosphates were also identified in bone resorption pits and in growth plate cartilage with what appears to be a higher concentration in the hypertrophic zone just above the calcifying cartilage [212]. Omelon and Grynpas (2008) have also revisited reports of the presence of electron-dense granules, as well as positive staining that have since been associated with inorganic polyphosphates in mineralizing tissues including bone, growth
plate cartilage and heart valve tissue [256]. These observations are suggestive of a role for endogenous inorganic polyphosphates in the modulation of tissue mineralization.

In support of these observations, inorganic polyphosphate levels in primary human osteoblasts cultured in vitro were considerably reduced by the combined treatment with dexamethasone, β-GP, epidermal growth factor and ascorbic acid composing a cocktail known to cause increased proliferation and differentiation of osteoblasts [206]. Additional treatment with 1α,25-dihydroxyvitamine D₃, a compound that is known to increase the expression of genes associated with the mineralization process, further reduced the cellular levels of the phosphate polymers. These changes were accompanied by variations in exopolyphosphatase activity [206].

There is increasing evidence to suggest that inorganic polyphosphates are involved in the mechanisms by which vertebrate tissue mineralization is controlled. A better understanding of the enzymes involved in the metabolism of phosphate polymers in mammalian tissues is also being achieved. However, nothing is currently known of the mechanisms by which the activity of these enzymes and the associated levels of inorganic polyphosphates are controlled within mineralizing tissues or the role of these pathways in tissue homeostasis and disease. Nevertheless, applicable information on the pathways involved in the biological activity of long chain condensed phosphates can be gained from studies of their other functions within vertebrate organisms.

1.4.4 Other biological roles of inorganic polyphosphates and associated pathways

Potential biological functions for inorganic polyphosphates have mostly been studied in prokaryotes and lower eukaryotes. While it is difficult to conclusively establish the roles
played by phosphate polymers in microorganisms, a number of functions have been proposed and reviewed extensively [200,228,257]. These include roles as an alternative source of energy to ATP, a phosphate reserve, a phosphate donor, a chelator of cations, a component of cell membrane channels and a regulator of stress and survival. Inorganic polyphosphates have also been shown to interact with DNA-histone complexes, as well as to impact gene expression and protein activity. These roles are generally dependant on the species and the location within cells. This subsection will focus on studies of the functions of inorganic polyphosphates within vertebrates other than the modulation of the mineralization process discussed previously.

One such function of inorganic polyphosphates is the modulation of mammalian cell proliferation [258,259]. In fact, inorganic polyphosphate treatment was shown to enhance the mitogenic activity of acidic and basic fibroblast growth factors (FGF-1 and FGF-2, respectively) in normal human fibroblasts [258]. FGF-2 has been studied in more detail. The FGF-2 effect appears to be caused by a physical interaction between the growth factor and the phosphate polymer resulting in the physical and functional stabilization of the protein. Additionally, binding of FGF-2 to cell surface receptors appears to be facilitated by the presence of the phosphate polymers, perhaps in the way it is presented to the receptor. Similarly, the proliferation of human dental pulp cells was enhanced by inorganic polyphosphates in conjunction with increased stability of receptor bound FGF-2 and increased activation of the ERK pathway [250]. Inorganic polyphosphates with chain lengths of at least 15 orthophosphate residues also stimulate the activity of mammalian target of rapamycin (mTor), a protein involved in sensing the
growth factor, energy and nutrient levels in the environment in order to control MCF-7 mammary cancer cell proliferation [259].

Inorganic polyphosphates were also shown to block the development of pulmonary metastasis after B16BL6 melanoma cells were injected intravenously in mice by inhibiting angiogenesis [260]. Interestingly, condensed phosphate did not affect B16BL6 \(^{3}\)H-thymidine incorporation \textit{in vitro} and blocked FGF-2 induced cell proliferation, ERK and p38 MAPK activation. These effects were attributed to the decreased ability of FGF-2 to bind to fibroblast growth factor receptor 1 (FGFR-1) in the presence of inorganic polyphosphates. These results are in contradiction with the study by Shiba et al. (2003) discussed previously and suggest cell type-specific effects and/or the possibility that culture conditions including inorganic polyphosphate concentration, chain length and the presence of serum can affect cell response to inorganic polyphosphate [258,260].

Similar to their roles in lower organisms, inorganic polyphosphates have also been shown to regulate calcium channel functions via the formation of complexes with the channel protein and PHB [261,262] and to act as a source of energy [211] in vertebrate cells. They also exhibit antibacterial and antiviral activity [232,263]. Multiple studies have demonstrated that inorganic polyphosphates accumulating within platelets are released upon platelet activation and act as procoagulant and proinflammatory agents while increasing the thickness of the fibrin clot [264-268]. Interestingly, the chain length of inorganic polyphosphate is a critical parameter in the activation of the different pathways involved in coagulation [268]. In fact, this study demonstrates that phosphate polymers with chains of at least 500 units are optimal for the activation of the contact
pathway, while chains of approximately 100 units are optimal for the activation of factor V. Inorganic polyphosphates were also shown to induce apoptosis in B lymphoid cell lines [269]. In this study, phosphate polymers had to be at least 75 orthophosphate residues for a significant effect to be observed. These last studies clearly emphasize the specificity of inorganic polyphosphate chain length on its biological effects.

1.4.5 Fibroblast growth factor signaling, condensed phosphates and cartilage mineralization

A number of FGF signaling pathways are essential to the coordination of growth plate differentiation towards endochondral ossification [9]. Specifically, FGF-2, -18 and at lower levels, FGF-7 and -22 are expressed in the postnatal growth plate in rats [270]. Of relevance to this work, FGF-2 and -18 have both been implicated in maintaining articular cartilage homeostasis [271]. In articular cartilage, FGF-2 signals preferentially via FGFR-1 to stimulate chondrocyte proliferation [271,272]. FGF-2 has been proposed as an anabolic agent in articular cartilage but this view is controversial [271]. In fact, a number of studies have demonstrated that the mitogenic effects of FGF-2 were not associated with increased matrix accumulation [271,273,274]. Inactivation of FGF-2 in mice has resulted in decreased trabecular bone volume, mineral apposition and bone formation rate. Bone marrow stromal cells extracted from FGF-2 knockout animals exhibited significantly decreased mineralization potential compared to cells extracted from wild type animals in culture [275]. However, overexpression of FGF-2 resulted in similar observations as for the knockout model in mice [276]. The contradictory results were recently explained as it was demonstrated that FGF-2 up-regulates ENPP-1 and ANK,
which generate pyrophosphate from ATP and transport it to the extracellular environment respectively, while down-regulating TNAP, which is responsible for its degradation. The subsequent release of orthophosphate may be contributing to the mineralization process [277]. On the other hand, FGF-18 is accepted as an anabolic agent in articular cartilage working via FGFR-3 signaling but its effects on proliferation remain controversial [72,271,278-281]. Similar to FGF-2, FGF-18 has exhibited contradictory effects on the mineralization process. Knockout studies exhibited skeletal development abnormalities including delayed ossification and suture closure [280,281]. In addition, exogenous FGF-18 was shown to inhibit mineral deposition by murine primary osteoblasts [282], while a murine mesenchymal stem cell line over-expressing FGF-18 was shown to form an increased number of mineral nodules [283]. Despite these puzzling results, the effects of FGF-2 and FGF-18 on inorganic polyphosphate metabolism have yet to be investigated.

1.5 Hypothesis

The integration of bioengineered cartilage to surrounding tissues upon implantation in the site of a cartilage defect in a synovial joint is a major challenge of cartilage tissue engineering. In an attempt to address this issue, an approach was developed to generate biphasic constructs incorporating a ZCC at the tissue-substrate interface, thereby mimicking the native architecture of the osteochondral junction. However, mineralization was inhibited at the interface between \emph{in vitro}-formed deep zone cartilage and a porous CPP bone substitute [137]. It is hypothesized that inorganic polyphosphates released from the substrate and accumulating in the tissue inhibit the formation of the ZCC at the tissue-substrate interface.
1.6 Specific Aims

i. Demonstrate the inhibitory role of inorganic polyphosphate in articular cartilage calcification.

ii. Determine the impact of a properly located ZCC on the mechanical integrity of the \textit{in vitro}-formed cartilage-CPP biphasic constructs interface.

iii. Investigate the signaling pathway by which endogenous inorganic polyphosphate levels are modulated to control cartilage calcification.

iv. Establish the anabolic effects of inorganic polyphosphate on \textit{in vitro}-formed cartilage matrix accumulation.

1.7 References


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CHAPTER 2: CALCIFICATION OF CARTILAGE FORMED IN VITRO ON CALCIUM POLYPHOSPHATE BONE SUBSTITUTES IS REGULATED BY INORGANIC POLYPHOSPHATE

Jean-Philippe St-Pierre¹, Robert M. Pilliar¹,²,³, Marc D. Grynpas¹,³, Rita A. Kandel¹,³.
¹Department of Materials Science and Engineering and Institute of Biomaterials and Biomedical Engineering, University of Toronto. ²Faculty of Dentistry, University of Toronto. ³CIHR-BioEngineering of Skeletal Tissue Team, Mount Sinai Hospital, Toronto.

A version of this chapter appears in Acta Biomaterialia. 2010. Aug;6(8):3302-9 with permission from Elsevier. As first author of this multi-author contribution, I designed and conducted most of the experiments. Histological sections were prepared by others. I also analyzed the results and wrote the manuscript.
2.1 Introduction

Articular cartilage possesses a limited intrinsic capacity for self-repair following damage caused by trauma and/or disease, partly because of its avascular nature and the inability of chondrocytes to migrate to the injury site [1,2]. Current treatment strategies for diseased joint surfaces, including surgical methods to enhance natural repair of articular cartilage, osteochondral transplantation and prosthetic joint replacement, have shortcomings that limit their applications and benefits [3,4]. This has prompted the development of tissue engineering strategies to promote the regeneration or repair of focal cartilage lesions. While the use of bioengineered cartilage holds promise for the treatment of damaged articular cartilage, there are still many challenges that remain to be addressed before it can be used clinically.

The integration of engineered cartilage to the native articular cartilage and subchondral bone following implantation has been identified as a major requirement for the success of such an intervention [5,6]. One approach to achieve tissue integration consists of designing biphasic constructs incorporating chondrocytes or cartilage tissue formed on the intended articulation surface of a bone substitute to mimic the osteochondral structure of the joint [7]. The rationale for this approach lies in part in the ability to integrate bone more easily than cartilage, thus stabilizing the engineered construct rapidly after the implantation and potentially providing an environment that favours cartilage integration. Biphasic systems for which the cartilage phase is generated in vivo by incorporating cells with chondrogenic potential or chondrocytes in various polymeric scaffolds or fibrin glue placed onto bone substitutes have had limited success in regenerating a continuous layer of hyaline cartilage properly integrated with the
surrounding tissues [8-13]. However, promising results were achieved with biphasic constructs for which the formation of the articular cartilage phase was allowed to take place in vitro prior to implantation [14-17].

We have developed one such approach, whereby cartilaginous tissue is formed in vitro by chondrocytes seeded on the top surface of a porous calcium polyphosphate (CPP) bone substitute [18]. The porous structure of the CPP permits anchorage of the cartilage on its top surface through formation of cartilage within the sub-surface pores, while the remaining pores of the unoccupied surfaces are available for bone ingrowth following implantation. Animal studies in sheep demonstrated that these biphasic constructs can integrate with the surrounding host cartilage and subchondral bone and are suitable for the repair of focal osteochondral defects [14,19]. However, the interfacial shear properties of these in vitro-formed constructs are 1 to 2 orders of magnitude lower than that of the native cartilage-subchondral bone interface potentially limiting their application to small, contained cartilage defects [20].

In native cartilage, a zone of calcified cartilage (ZCC) is present at the interface between hyaline cartilage and subchondral bone forming an effective transition zone [21]. While the mineral crystals in the ZCC and bone are carbonated hydroxyapatite crystals of similar sizes, the mineral content of the ZCC is higher than that of subchondral bone [22-25]. Nevertheless, the stiffness of the ZCC has been reported to be intermediate to that of hyaline cartilage and the adjacent subchondral bone with which it interfaces when tested on bulk tissue specimens [21]. Using nanoindentation, the ZCC and subchondral bone were reported to have comparable stiffness values but this technique required substantial sample preparation including a dehydration step [22,25]. It is suggested that the distinct
mechanical properties of the ZCC are the result of the different collagen matrices and the lower degree of crystal orientation in the ZCC compared to bone [24,26]. The structure of the ZCC contributes to preventing the generation of high shear stresses at the cartilage-subchondral bone interface, thus permitting the integration of the two tissues despite the high mechanical loads experienced in the joint [21,24,27].

As a step towards the design of biphasic constructs suitable for the treatment of damaged articular cartilage, a procedure was developed to improve the interfacial shear properties of in vitro-formed cartilage constructs through the generation of a ZCC. This is achieved by selecting for chondrocytes from the deep-zone of articular cartilage and supplementing the culture medium with a source of organic phosphate (β-glycerophosphate; β-GP) during tissue formation [28,29]. The mineral that forms within the cartilage is poorly crystalline hydroxyapatite similar to that of in vivo calcified cartilage [20]. However, the resulting ZCC is located slightly above the cartilage-CPP interface, in contrast to cartilage formed in vitro on Millicell-CM® membrane inserts in which calcification occurs at the tissue-substrate interface.

Previous reports have demonstrated that inorganic polyphosphate inhibits hydroxyapatite crystal nucleation and growth in solution [30,31]. This phenomenon is physicochemical in nature and results from binding of condensed phosphate to hydroxyapatite crystals resulting in the effective poisoning of the crystals [32]. Polyphosphate has also been shown to prevent tissue calcification in different animal models, although the mechanism of action may be more complex than in inorganic solutions [33-35]. The degradation of CPP results in the release of inorganic polyphosphates [36]. Thus, it is hypothesized that the absence of cartilage calcification at
the tissue-CPP interface is a consequence of the accumulation in the tissue of polyphosphate released from the CPP bone substitute as it degrades. The purpose of this study is to determine if polyphosphate does accumulate at the cartilage-CPP interface in biphasic constructs and to investigate the ability of polyphosphate to inhibit cartilage calcification in tissues formed on membrane inserts. The membrane insert culture was selected in order to be able to control the amount of polyphosphate administered to the deep-zone chondrocytes. An improved understanding of biomaterial-cartilage interactions is essential to the development of successful cartilage tissue engineering therapies.

2.2 Materials and Methods

2.2.1 Substrates

Porous CPP bone substitute rods were produced by gravity sintering of 75 to 106 μm calcium polyphosphate powder at 950ºC as described previously [37]. The resulting rods characterized by a 35 volume percent interconnected porosity were cut to form discs 2 mm in height and 4 mm in diameter. To prevent cell leakage from the CPP substrates, the discs were inserted into silicone tubing (Tygon 3350, Saint-Gobain Performance Plastics Corp., Aurora, OH, USA), forming a culture well around the disc. The CPP constructs were sterilized by gamma-irradiation (2.5MRad) and soaked in phosphate buffered saline (PBS) for a week prior to cell culture. Membrane inserts (Millicell-CM®8, Millipore Corp., Bedford, MA, USA) were coated with type II collagen (0.5mg/ml in 0.1N acetic acid; Sigma Chemical Co., St.Louis, MO, USA) and dried overnight. The membrane inserts were UV sterilized for 30 minutes and soaked in Ham’s F12 for 30 minutes prior to cell culturing.
2.2.2 Chondrocytes Culture

Cartilage was aseptically excised from the deep zone (bottom 30%) of metacarpal-phalangeal articular cartilage from 9 to 12 months old calves within 24 hours of death as previously described [29]. Cartilage from several animals was pooled to obtain a sufficient number of cells for each experiment. Chondrocytes were harvested from the tissue by sequential enzymatic digestion (0.5% protease (Sigma Chemical Co.) for 2 hours followed by 0.1% collagenase (Roche Diagnostics GmbH, Mannheim, Germany) overnight). The cells were then seeded on top of membrane inserts or CPP substrates (1X10^6 cells per construct) in Ham’s F-12 supplemented with 5% fetal bovine serum (FBS) (HyClone, Logan, UT, USA) and incubated at 37°C in an atmosphere characterized by 95% relative humidity and 5% CO₂. On day 5, the medium was changed to Dulbecco’s modified Eagle medium (DMEM) supplemented with 20% FBS and ascorbic acid (100µg/ml, Sigma Chemical Co.). At this time, calcification was initiated in selected cultures by the addition of 10mM β-GP (Sigma Chemical Co.) (mineralization medium) and cultures were incubated in the presence or absence of various concentrations of sodium phosphate glasses (inorganic polyphosphate; Sigma Chemical Co.), calcium chloride (Sigma Chemical Co.), or sodium phosphate buffer. The concentrations of inorganic polyphosphate utilized for each experiment are based on the phosphate content. Cultures were grown for up to 4 weeks following placement into mineralization medium. The culture medium was changed every 2 days.
2.2.3 Histological Evaluation

*In vitro*-formed cartilage constructs were harvested and washed twice in PBS. The tissues were removed from the CPP, fixed in 10% formalin and embedded in paraffin. Cartilage formed on membrane inserts was processed with the membrane insert. Five micron sections were cut, stained with toluidine blue and von Kossa and examined by light microscopy. To visualize the presence of polyphosphate in the tissues, sections were cut, dewaxed in xylene and stained with 4'-6-diamidino-2-phenylindole (DAPI) (5 to 15 µg/ml, Pierce Biotechnology, Inc., Rockford, IL, USA). The fluorescence was visualized with a Zeiss Axioplan epifluorescence microscope using a wide pass DAPI filter. Inorganic polyphosphate specifically shifts the emission peak of DAPI from 456nm to 526nm, permitting its visualization in the yellow-green spectrum rather than the blue spectrum associated with nucleic acids or glycosaminoglycans [38,39].

2.2.4 Dry Weight of Cartilaginous Tissue

Cartilage tissues formed *in vitro* on membrane inserts were harvested, washed twice in PBS and removed from their substrate. The tissues were then lyophilized overnight and weighed using an electrical balance (Explorer, Ohaus Corp., Florham Park, NJ, USA).

2.2.5 Mineral Content in the Cartilaginous Tissues

To measure the mineral content of *in vitro*-formed cartilage, the lyophilized tissues were wet-ashed as described previously [20]. Briefly, the tissue was placed in an HNO$_3$/HClO$_4$ (4:1 vol/vol) acid mixture and heated at 150°C for 90 minutes in a closed Teflon beaker, the mixture was evaporated to a small drop (approximately 100µl) by continued heating
following removal of the cover and diluted with dH₂O (2ml). The pH of the solutions was then adjusted to 2.5-2.9 and distilled deionized water added to a final volume of 3ml.

Calcium content was measured by mixing an aliquot of wet-ashed solution with 0.01% cresolphthalein complexone (Sigma Chemical Co.) buffered with 0.25M sodium borate in a 1:4 vol/vol ratio and the resulting color quantified spectrophotometrically at 570nm. The standard curve was generated using a calcium chloride solution.

Phosphate content was measured by reaction of an aliquot of wet ashed solution with a solution containing 1:6 vol/vol ratio of 10% ascorbic acid and 0.42% ammonium molybdate (Sigma Chemical Co.) in 1N H₂SO₄ (1:3 vol/vol ratio) at 37°C for 1 hour and spectrophotometrical analysis at 620nm. The standard curve was generated using a sodium phosphate dibasic solution (pH=7.5).

2.2.6 Exopolyphosphatase Activity of Cartilaginous Tissues

*In vitro*-formed cartilage constructs were harvested and washed twice in PBS. Proteins were extracted and assessed for exopolyphosphatase activity according to a protocol modified from Lorenz et al. [40]. Briefly, cartilage tissues were freeze-thawed three times in 50mM Tris-HCl buffer (pH 7.5) containing 10mM MgCl₂, 0.5mM EDTA, 150mM NaCl and 0.2% Triton X-100 (homogenization buffer; 150µl per construct) and mechanically homogenized. The resulting solutions were centrifuged (14,000 X g for 15 min) and the protein content of the supernatant was measured using a Pierce BCA protein assay. Reaction mixtures were prepared for each sample containing 25µg of protein and 0.4µmol of sodium phosphate glass (50mM Tris-HCl buffer (pH 7.5), 10mM MgCl₂, 0.5mM EDTA and 150mM NaCl) to a final volume of 200µl. To correct for the
phosphate content of protein extracts and hydrolysis of the condensed phosphate, mixtures containing only the protein extract (for each sample) or inorganic polyphosphate were also prepared. The reaction mixtures were incubated at 37°C for 24 hours (enzymatic phosphate release from polyphosphate is linear between 1 and 48 hour). The phosphate produced by enzymes with exopolyphosphatase activity was measured spectrophotometrically at 620nm. The phosphate standard curve was prepared using the homogenization buffer and bovine serum albumin at protein levels equivalent to that of samples.

2.2.7 Statistical Analysis

All experiments were done in triplicate and repeated 3 times with cells from different extractions. Results are expressed as the mean ± standard error of the mean (SEM) of 3 experiments (unless specified otherwise) and analyzed using a one-way ANOVA (for more than 2 conditions) or Student’s t-test (between groups). Tukey’s test post hoc analysis was performed. P values ≤ 0.05 were considered statistically significant.

2.3 Results

2.3.1 Polyphosphate released from CPP accumulates in mineralizing cartilage

Light microscopical examination showed that the deep-zone chondrocytes formed cartilage with a ZCC that did not extend to the tissue-substrate interface when grown in vitro on CPP for up to 3 weeks in the presence of mineralization medium (Figure 2.1A). In contrast, deep-zone chondrocytes cultured for the same period on membrane inserts formed a ZCC directly apposed to the substrate (data not shown).
Epifluorescence imaging of DAPI stained tissue sections showed polyphosphate in cartilage formed on CPP substrates after 3 weeks in culture (Figure 2.1B). The intensity of the fluorescence was highest at the tissue-substrate interface and appeared concentrated in the pericellular region. Little fluorescence was detected above this interface.

2.3.2 Inorganic polyphosphate inhibits cartilage calcification

Deep-zone chondrocytes were grown *in vitro* on membrane inserts for 4 weeks in mineralization medium supplemented with different concentrations of inorganic polyphosphate (concentrations calculated based on the phosphate content; chain length of 45 phosphate units). As demonstrated in Figure 2.2A, treatment of deep-zone cartilage with polyphosphate at a concentration of 0.1mM or higher resulted in a significant decrease in deposition of calcium and phosphate in the tissues compared to cartilage formed in medium that was not supplemented with polyphosphate. The inhibition of cartilage calcification was concentration dependent and complete at a polyphosphate concentration of 0.5mM. The inhibition of cartilage calcification by polyphosphate was verified by histological evaluation of tissues (Figure 2.2B-C).

Supplementation of mineralization medium with similar concentrations of sodium phosphate to those used for polyphosphate did not significantly alter the calcium and phosphate contents in these tissues as compared to cartilage formed in non-supplemented mineralization medium only (Table 2.1). This result suggests that the effect of polyphosphate on cartilage calcification is specific to condensed phosphate rather than its degradation product, orthophosphate.
Figure 2.1: Histological appearance of cartilage formed by deep-zone chondrocytes on CPP. Tissues were cultured for 3 weeks. (A) Tissue visualized by light microscopy (toluidine blue and von Kossa, mag. x50). The arrowhead indicates the zone of calcified cartilage. (B) Tissue visualized by epifluorescence microscopy (DAPI). The arrowhead indicates the polyphosphate. * indicates where the CPP substrate was located.
Figure 2.2: Effect of inorganic polyphosphate on in vitro-formed cartilage calcification. (A) Calcium and phosphate contents of deep-zone cartilage formed *in vitro* on membrane inserts after 4 weeks in mineralization medium supplemented with different concentrations of polyphosphate (concentrations calculated based on the phosphate content; average chain length of 45 phosphate units). The data were normalized to tissue dry weight. Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean. * indicates a significantly lower mineral content compared to non-treated tissues. (B,C) Histological appearance of cartilage formed after 4 weeks of culture from deep-zone chondrocytes on membrane inserts in mineralization medium alone (B) or supplemented with 1mM polyphosphate (C). Tissues were visualized by light microscopy (toluidine blue and von Kossa). * indicates the ZCC.
Table 2.1: Calcium and Phosphate Contents of Cartilage

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Calcium Content (% of dry weight)</th>
<th>Phosphate Content (% of dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6.31 ± 0.65</td>
<td>11.23 ± 1.26</td>
</tr>
<tr>
<td>Phosphate</td>
<td>6.75 ± 1.46</td>
<td>13.35 ± 1.24</td>
</tr>
<tr>
<td>Polyphosphate</td>
<td>0.08 ± 0.05*</td>
<td>0.44 ± 0.07*</td>
</tr>
</tbody>
</table>

Chondrocytes were grown in mineralization medium alone or in the presence of phosphate (1mM) or polyphosphate (1mM based on the phosphate content; chain length of 45 phosphate units). The calcium and phosphate contents were determined after 4 weeks in culture. The data are from one representative experiment repeated three times and expressed as mean ± standard deviation. * indicates a significantly lower mineral content than untreated tissues (p<0.001).

2.3.3 Range of polyphosphate chain lengths inhibits articular cartilage calcification

Chondrocytes were grown *in vitro* on membrane inserts for 4 weeks in the presence of mineralization medium supplemented with polyphosphates (0.1mM) of various average chain lengths (5, 45 and 75 phosphate units). The polyphosphate concentration in this experiment was selected to cause an incomplete inhibition of cartilage calcification and permit the comparison of mineral contents between treatments. As shown in Figure 2.3A, polyphosphates of the three chain lengths significantly inhibit cartilage calcification. Treatment with polyphosphate with an average chain length of 45 phosphate units resulted in a significantly lower mineral deposition than with polyphosphate of shorter chain length (5 phosphate units). The mineral content of cartilage treated with polyphosphate with a chain length of 75 phosphate units was intermediate to that of the other two conditions. Based on these results, all further studies were performed using polyphosphates with an average chain length of 45 phosphate units.
To further understand the effect of polyphosphate chain length on the inhibition of cartilage calcification, the exopolyphosphatase activity in 3 day old *in vitro*-formed cartilage was quantified by incubation of the protein extracts with polyphosphate of the three chain lengths investigated. The exopolyphosphatase activity was significantly higher for short chain polyphosphate (5 phosphate units) than longer chain polyphosphate (45 and 75 phosphate units) (Figure 2.3B). The exopolyphosphatase activity was not significantly different between the two longer chain length polyphosphates. This experiment was repeated with polyphosphate concentrations measured based on the polyphosphate rather than the phosphate content and similar trends were observed (data not shown).

2.3.4 Inhibition of cartilage calcification requires the continuous presence of polyphosphate

Deep-zone chondrocytes were grown *in vitro* on membrane inserts for 4 weeks. The mineralization medium was supplemented with 1mM polyphosphate for either the first 2 days, 1, 2 or 3 weeks of culture or for the entire 4 weeks period (control). Upon removal of the polyphosphate treatment, cartilage calcification resumed. The amount of mineral deposited was only slightly lower than that anticipated from the proportion of the incubation period that was performed in the absence of polyphosphate treatment, indicating that polyphosphates must be present continually in the culture medium to maintain the inhibition of cartilage calcification (Figure 2.4A).
Figure 2.3: Effects of inorganic polyphosphate chain length on in vitro-formed cartilage calcification and exopolyphosphatase activity. (A) Calcium and phosphate contents of deep-zone cartilage formed in vitro on membrane inserts in mineralization medium supplemented with polyphosphate (0.1mM, calculated based on the phosphate content) of different chain lengths for 4 weeks. The data were normalized to tissue dry weight and expressed relative to untreated control. Each condition was done in triplicate and the experiment was repeated 3 times. * indicates a significantly lower mineral content than that of non-treated controls. # indicates a significant difference between tissues treated with polyphosphates of average chain lengths composed of 5 and 45 phosphates. (B) Exopolyphosphatase activity of deep-zone cartilage formed in vitro on membrane inserts for 3 days. Polyphosphates of different chain length (concentrations normalized for the phosphate content) were incubated with protein extract and the resulting phosphate quantified. * indicates a significantly higher activity than that of other polyphosphate chain lengths. Each condition was done with pooled tissues from three samples and the experiment was repeated 3 times. The results were combined and expressed as mean ± standard error of the mean.
Epifluorescence imaging of DAPI stained tissue sections demonstrated that polyphosphate accumulates predominately around the chondrocytes within in vitro-formed cartilage treated with condensed phosphate for a period of 4 weeks (Figure 2.4B). In contrast, non-treated cartilage exhibits lower levels of polyphosphate (Figure 2.4C). However, protein extracts obtained from treated cartilage tissues also exhibited higher exopolypophosphatase activity compared to protein extracts from non-treated tissues (p=0.067; Figure 2.4D).

2.3.5 Polyphosphate inhibits ongoing cartilage calcification

To determine if polyphosphate can inhibit ongoing calcification, deep-zone chondrocytes were grown on membrane inserts for 2 weeks in mineralization medium followed by 2 weeks in the same medium supplemented with 1mM polyphosphate (4w PP). As shown in Figure 2.5, the calcium content of tissues grown in the presence of polyphosphate for the last 2 weeks of culture was similar to that of 2 weeks old in vitro-formed cartilage (2w) and significantly lower than that of cartilage formed over a 4 weeks period (4w).

2.3.6 Inhibition of cartilage calcification is partially recovered by calcium

Deep-zone chondrocytes were grown in vitro for 1 week in the presence of mineralization media alone or supplemented with 1mM inorganic polyphosphate. Selected tissues treated with polyphosphate were also treated with various amounts of calcium chloride (0.5, 1.0 or 2.0mM), to attempt to reverse the cartilage calcification inhibited by polyphosphate. The lowest calcium concentration used corresponds to the theoretical
Figure A: Bar graph showing the calcium content (% of Total Culture Period) in the culture period without polyphosphate (% of Total Culture Period).

Figure B: Image showing a close-up view with a scale of 200µm.

Figure C: Image showing a broader view with a scale of 200µm.
Figure 2.4: Effect of discontinuous inorganic polyphosphate treatment on in vitro-formed cartilage calcification and exopolyphosphatase activity. (A) Calcium content of cartilage formed in vitro on membrane inserts in mineralization medium for 4 weeks and supplemented with 1mM polyphosphate (concentration calculated based on the phosphate content; average chain length of 45 phosphate units) for various time periods up to 4 weeks. The result was normalized to tissue dry weight. Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean. (B, C) Histological appearance of cartilage formed after 4 weeks of culture from deep-zone chondrocytes on membrane inserts in mineralization medium in the presence (B) or absence (C) of 1mM polyphosphate and stained with DAPI. Tissues were visualized by epifluorescence microscopy. The arrowhead indicates the polyphosphate. * indicates the ZCC. (D) Exopolyphosphatase activity of deep-zone cartilage formed in vitro on membrane inserts in the presence or absence of 1mM polyphosphate for 4 weeks. The data were normalized to the time of reaction and expressed per membrane insert. Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean.
Figure 2.5: Effects of inorganic polyphosphate on ongoing in vitro-formed cartilage calcification and exopolyphosphatase activity. Calcium and phosphate contents of deep-zone cartilage formed *in vitro* on membrane inserts in mineralization medium for 2 (2w) or 4 weeks (4w) or for 2 weeks followed by 2 weeks in media supplemented with polyphosphate (4w PP; 1mM based on the phosphate content; average chain length of 45 phosphate units). The data represent content per membrane insert. Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean. * indicates a significantly higher mineral content compared to other conditions.
chelation potential of 1mM inorganic polyphosphate. As shown in Figure 2.6, treatment of deep-zone cartilage (formed in the presence of inorganic polyphosphate) with 0.5 or 1.0mM calcium chloride results in a slight (not significant) increase in calcium deposition compared to cartilage treated only with inorganic polyphosphate. This suggests that chelation is not the major mechanism by which polyphosphate inhibits cartilage calcification in vitro and that crystal poisoning may be more important. Treatment with 2.0mM calcium chloride resulted in a higher level of calcium deposition.

2.4 Discussion

This study demonstrates that inorganic polyphosphate released from CPP bone substitutes during the in vitro maturation of cartilage accumulates at the tissue-CPP interface. Inorganic polyphosphate was shown to inhibit cartilage calcification in a concentration and chain length dependent manner. The inhibition of cartilage calcification was dependent on the continuous presence of polyphosphate in culture, as calcification resumed upon interruption of the polyphosphate treatment. Furthermore, addition of polyphosphate inhibits ongoing mineralization. These results suggest that the problem encountered in generating biphasic constructs with a properly localized ZCC is likely caused by the release of polyphosphate from the CPP bone substitute.

The inhibitory effect of inorganic polyphosphate on articular cartilage calcification demonstrated in this study is in agreement with observations by Fleisch and Neuman [30], as well as Francis [31] who showed that condensed phosphates inhibit hydroxyapatite crystal nucleation and growth in inorganic solutions of calcium and phosphate. Similar observations have been reported in biological systems. The in vitro
Figure 2.6: Recovery of inorganic polyphosphate-induced inhibition of cartilage calcification with calcium. Calcium content of deep-zone cartilage formed in vitro on membrane inserts in mineralization medium alone or supplemented with 1mM polyphosphate (concentration calculated based on the phosphate content; average chain length of 45 phosphate units) for 1 week. Selected tissues were also treated with various concentrations of calcium chloride. The data were normalized to tissue dry weight. Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean. * indicates a significantly higher mineral content compared to other conditions.
culture of chick embryonic femurs in the presence of polyphosphate was characterized by reduced bone tissue mineralization [41]. Polyphosphate was also shown to prevent skin calciphylaxis induced by dihydrotachysterol and calcification of the aorta caused by administration of vitamin D$_3$ in a rat model [33-35]. However, Kawazoe et al. [42] demonstrated an increase in mineralization following the treatment of pre-osteoblasts maintained in vitro with polyphosphate in concentrations similar to those investigated in the present study. This discrepancy can be attributed to the differences between the models utilized, notably the use of primary deep-zone chondrocytes forming a three-dimensional tissue versus a pre-osteoblastic cell line grown in monolayer. Furthermore, levels of exopolyphosphatase activity measured for deep-zone chondrocytes in this study are one order of magnitude lower than that reported for osteoblasts, suggesting that cell types may be differentially responsive to polyphosphate signaling. It is also possible that the addition of a second source of phosphate (β-GP) to the culture medium in this study could contribute to the different observations reported.

An important design criterion for bone substitutes is their ability to biodegrade, as they must be replaced by bone following implantation. In vitro studies have identified that the CPP degradation products are polyphosphate, calcium and phosphate [36,37]. These are likely generated by hydrolysis of the polyphosphate chains forming the bone substitute; however, cell-regulated dissolution is also possible. Thus, it is possible that the presence of chondrocytes on the top surface of CPP contributes to its degradation in vitro. In the present study, DAPI staining of tissue sections showed that polyphosphate released from the CPP bone substitute accumulates in the in vitro-formed articular cartilage. The distribution of staining intensity suggests that the highest concentration of polyphosphate
is present directly above the tissue-substrate interface, correlating with the zone of the cartilage that does not mineralize. The observation of a ZCC above this interface suggests that the polyphosphate concentration at this height in the tissue has reached a level at which the chondrocytes can degrade the polyphosphate through hydrolysis and/or exopolyphosphatase activity, thereby permitting matrix calcification.

The inhibition of cartilage calcification by polyphosphate was observed for the three chain lengths of phosphate polymers investigated ranging from 5 to 75 phosphate units. The range of chain lengths investigated is representative of the range of polyphosphate released from CPP by *in vitro* degradation [36]. Polyphosphate with 45 phosphate units appeared to be the optimal chain length for the inhibition of cartilage calcification. The reduced ability of short chain polyphosphate (5 phosphate units) to inhibit cartilage calcification compared to longer chain polyphosphate is in agreement with observations reported by Schibler and Fleisch [35] and Fleisch et al. [33], who demonstrated that long chain polyphosphates (~20 phosphate units) were more potent calcification inhibitors than pyrophosphate (2 phosphate units). Furthermore, Fleisch and Neuman [30] reported that longer chain polyphosphates were more effective at inhibiting the formation of hydroxyapatite crystals from saturated calcium and phosphate solutions. This observation may be explained by the significant increase in the exopolyphosphatase activity of chondrocytes protein extracts for short chain polyphosphate resulting in the faster removal of the calcification inhibitor from culture. The discrepancy between the inhibitory capabilities of polyphosphates with chain lengths of 45 and 75 phosphate units and their associated exopolyphosphatase activity is attributed to the normalization of polyphosphate concentrations on a phosphate basis resulting in a lower effective
condensed phosphate concentration for longer chain polyphosphates. Further studies are required to confirm this.

The mechanisms whereby inorganic polyphosphate inhibits mineralization are likely multifactorial. Early work has demonstrated that condensed phosphates bind to hydroxyapatite, supporting the notion that inhibition of calcification by polyphosphate may occur through adsorption of the inorganic polymer to growth sites of the crystals [31,32]. It was proposed that calcium chelation by polyphosphate does not contribute considerably to its inhibitory properties because of the low concentrations of condensed phosphate required for inhibition of hydroxyapatite crystal formation in solution [30]. Our results also suggest that calcium chelation is not the main mechanism by which inorganic polyphosphate inhibits calcification, as a high concentration of calcium is required to recover cartilage calcification relative to the chelation potential of polyphosphate. High concentrations of calcium probably result in the generation of more nucleation sites than can be poisoned by the polyphosphate present in culture. Alternatively, polyphosphate may prevent matrix changes that favor mineralization as Irving et al. [34] reported that changes to the extracellular matrix of aorta associated with tissue calcification did not occur in the presence of calcification inhibiting concentrations of polyphosphate. However, the authors suggested that the matrix changes were secondary and arose as a consequence of calcification rather than being the cause. Another mechanism by which polyphosphate may influence mineralization involves the modulation of cell signaling. Polyphosphate is present in various mammalian tissues including growth plate cartilage [43] and accumulates in particularly high concentrations in osteoblasts [44]. It has been shown that polyphosphate induced gene expression of
differentiation markers such as osteopontin and osteocalcin in pre-osteoblastic cells [42]. Phosphate polymers have also been shown to accelerate blood clot formation through activation of the contact pathway [45-47], to activate fibroblast growth factor signaling [48,49] and to stimulate the protein kinase mammalian target of rapamycin [50]. Interestingly, it has been reported that pyrophosphate is cleaved by different enzymes than longer chain polyphosphates [51]. This suggests that the cellular responses to polyphosphate may be different than that of the better understood pyrophosphate. However, more studies are required to determine if biological responses are activated by polyphosphate in chondrocytes and are involved in the inhibition of cartilage calcification in addition to its ability to poison mineral crystals.

Deep-zone cartilage calcification promptly resumes upon interruption of the polyphosphate treatment, suggesting that a continuous exposure to polyphosphate is necessary for inhibition of cartilage calcification to occur. Other groups have demonstrated that the effects of polyphosphate are quickly lost due to hydrolysis of the phosphate polymers [30,31]. The loss of polyphosphate inhibitory effects in biological systems is enhanced by the presence of proteins with exopolyphosphatase activity, i.e. proteins which have the ability to cleave the phosphoanhydride bonds of the phosphate polymers [44]. Hence, this result can be explained by the high level of exopolyphosphatase activity of in vitro-formed deep-zone cartilage, which is further increased by treatment with polyphosphate.
2.5 Conclusions

In summary, this study demonstrates that the polyphosphate released by the CPP substrate may prevent calcification directly at the cartilage-bone substitute interface in a biphasic construct. As it was shown that mineral deposits are not removed by polyphosphate treatment, preventing or reducing the release of degradation products from CPP during in vitro formation of cartilaginous tissue may allow the formation of the ZCC directly at the tissue-substrate interface, thereby substantially improving the interfacial shear properties of the bioengineered cartilage construct. Studies are ongoing to address this issue and allow the generation of bioengineered cartilage with a zonal architecture that mimics native cartilage.

2.6 References


CHAPTER 3: THE INCORPORATION OF A ZONE OF CALCIFIED CARTILAGE INFLUENCES THE INTERFACIAL SHEAR STRENGTH OF BIOENGINEERED CARTILAGE TO THE UNDERLYING SUBSTRATE

Jean-Philippe St-Pierre¹, Lu Gan², Jian Wang², Robert M. Pilliar¹,²,³, Marc D. Grynpas¹,², Rita A. Kandel¹,².
¹Department of Materials Science and Engineering and Institute of Biomaterials and Biomedical Engineering, University of Toronto. ²CIHR-BioEngineering of Skeletal Tissue Team, Mount Sinai Hospital, Toronto. ³Faculty of Dentistry, University of Toronto.

As first author of this multi-author contribution, I designed and conducted all of the experiments. The histological sections were prepared by others. I also analyzed the results and wrote the manuscript.
3.1 Introduction

Articular cartilage is a connective tissue that forms the gliding surfaces of synovial joints, while also transferring and distributing the loads applied through the joint to the subchondral bone [1]. Cartilage damage due to disease or trauma often progresses to clinical signs of osteoarthritis because of the limited capacity for self-repair of this tissue [2,3]. Current repair strategies including mosaicplasty, marrow stimulation and autologous chondrocytes implantation have had limited long-term clinical success ultimately leading to the need for total joint arthroplasty [4].

Numerous cartilage tissue engineering approaches have been developed to improve the quality of repair tissue and the functional outcome compared to currently available surgical interventions to treat cartilage defects [5]. However, only recently have efforts to generate functionally relevant interfaces between the bioengineered cartilage and surrounding tissues in the joint received attention [6]. Because of the essential role of the cartilage-subchondral bone interface in the transfer and distribution of loads between these two tissues, it has been speculated that the long-term success of bioengineered cartilage depends on its proper integration to the subchondral bone upon implantation [7-9]. Efforts to achieve this have focused mainly on the design of biphasic constructs that mimic the osteochondral architecture and are reviewed in detail by Yang and Temenoff (2009) [6]. A number of these bioengineered osteochondral constructs have developed a zone of calcified cartilage (ZCC) following their implantation in place of cartilage defects [10-12]. Our group and others have attempted to generate biphasic constructs incorporating a calcified interface during an in vitro maturation period [13-15].
The ZCC is the highly mineralized region of the deep zone of articular cartilage that interfaces and anchors the hyaline cartilage with the underlying subchondral bone. The tidemark at the interface between the hyaline and calcified cartilages maintains its mechanical integrity through collagen fibrils arranged perpendicular to the joint surface that bridge the junction between the two tissues [16]. A mineral density gradient at the tidemark may contribute to diffuse the stresses applied to this interface through changes in tissue stiffness [16]. The integrity of the cement line at the interface between the ZCC and subchondral bone is ensured by the high level of interdigitation between the two tissues [17]. While the mineral content of the ZCC is significantly higher than that of bone [18], its compression modulus is one order of magnitude lower [19]. Two studies have reported similar stiffness for the ZCC and subchondral bone but a nanoindentation technique that requires substantial sample preparation including a dehydration step was used [16,20]. Hence, it is widely proposed that the ZCC acts as a zone of intermediate stiffness to diffuse stresses resulting from joint loading and to prevent lateral strains that could compromise the integrity of the cartilage-subchondral bone interface.

Our group has developed a biphasic construct, whereby cartilaginous tissue is formed in vitro by primary chondrocytes seeded on the top surface of a porous calcium polyphosphate (CPP) bone substitute [21]. The porous structure of the CPP permits anchorage of the hyaline-like cartilage layer on its top surface through interdigitation similar to that in native tissues. The remaining pores of the unoccupied surfaces of CPP are available for bone ingrowth following implantation into bone. Porous CPP substrates have favourable characteristics for applications as bone substitutes in osteochondral biphasic constructs. These properties include its osteoconductivity resulting in rapid bone
ingrowth to stabilize the substrate in the joint upon implantation, a comparable tensile strength to bone and its biodegradability [22,23]. While these constructs were shown to integrate well to surrounding host cartilage and subchondral bone in sheep studies [24,25], the tissue-substrate interfacial shear strength is one order of magnitude lower than that of the native cartilage-subchondral bone interface potentially limiting their application to small, contained cartilage defects [14].

A methodology was devised to induce the formation of a ZCC within bioengineered cartilage during the *in vitro* maturation phase. This is accomplished by seeding primary chondrocytes from the deep zone of articular cartilage on the substrate and supplementing the culture medium with a source of organic phosphate (β-glycerophosphate; β-GP) [26,27]. The mineral deposited within the bioengineered cartilage matrix was characterized as poorly crystalline apatite of similar crystal length and morphology to that present in native articular cartilage [14,26]. However, the ZCC is formed slightly above the cartilage-CPP interface because the degradation of CPP results in the accumulation of inorganic polyphosphate with the ability to inhibit mineralization in the tissue directly above that interface [28]. In this study, it was hypothesized that the formation of a ZCC directly located at the interface between *in vitro*-formed cartilage tissue and a CPP bone substitute would improve the mechanical integrity of the interface of biphasic constructs. To accomplish this, a calcium phosphate (CaP) coating was applied to the surfaces of porous CPP and its effect on cartilage composition, mineral characteristics and the location of the ZCC was determined. The interfacial shear properties of the mineralized interface were also established.
3.2 Materials and Methods

3.2.1 Substrates

Porous CPP rods were produced by gravity sintering of 75 to 150 µm calcium polyphosphate powder at 950ºC as described previously [23]. The resulting rods characterized by a 35 volume percent interconnected porosity were cut to form discs 2 mm in height and 4 mm in diameter. The CPP constructs were soaked in phosphate buffered saline (PBS) for a week prior to further processing. The soaking solution was replaced every 2-3 days.

CaP thin films were applied to the porous CPP discs according to an inorganic sol-gel deposition method as described previously [29]. Briefly, a sol-gel solution was prepared by mixing calcium nitrate tetrahydrate (Sigma Chemical Co., St.Louis, MO, USA) and ammonium dihydrogen phosphate (Fischer Scientific, Fair Lawn, NJ, USA) at a molar ratio of calcium-to-phosphorus of 1.67. The pH of the sol-gel solution was adjusted to 12 with concentrated ammonium hydroxide (Fischer Scientific). The sol-gel solution was diluted with deionized water to adjust the particle density of the solution and permit optimized penetration within the pores of the CPP. Up to 8 separate coating layers were applied to CPP discs at a withdrawal rate of 30 cm/min. A drying step was performed between each application at 210°C for 15 min. Following the deposition of the last layer, samples were annealed at 500°C for 60 min in air to form the final coating structure and furnace cooled to room temperature. Uncoated CPP discs were submitted to the same drying and annealing steps as coated CPP.

To prevent cell leakage from the CPP substrates, the uncoated and CaP coated discs were inserted into silicone tubing (Tygon 3350, Saint-Gobain Performance Plastics
Corp., Aurora, OH, USA) to form a culture well around each disc. The CPP constructs were sterilized by gamma-irradiation (2.5MRad) prior to cell culture.

3.2.2 CaP Film Characterization Techniques

3.2.2.1 Scanning Electron Microscopy

The top and fracture surfaces of uncoated and CaP coated CPP substrates were sputter coated with gold (Desk II, Denton Vacuum, Moorestown, NJ, USA). The surface coverage and microstructure of CaP films were visualised by secondary electron imaging under a scanning electron microscope (SEM) (XL30, FEI, Portland, OR, USA). Fracture surface micrographs were used to measure the thickness of CaP films. Measurements were obtained from micrographs acquired for both surface and subsurface CPP particles.

3.2.2.2 Transmission Electron Microscopy, Electron Diffraction and Energy Dispersive X-ray Spectroscopy

CPP particles dislodged from the surface of CaP coated CPP were embedded in Spurr epoxy resin and sections were cut (RMC MT6000 ultramicrotome, Leica, Toronto, ON, Canada) and analyzed using a transmission electron microscope (TEM) (Tecnai 20, FEI) at 200kV. The crystal structure of the coating was determined using selected area electron diffraction (SAED) and compared to that of CPP. SAED patterns were generated using a 200 µm selected-area aperture and an 890 mm camera length and were calibrated by comparison with a gold diffraction standard under the same conditions. Ring diameters were measured and d-spacing calculated and compared to that of hydroxyapatite and octacalcium phosphate.
CaP coated CPP particles were also analysed for elemental analysis of the thin film and compared to CPP particles. Energy dispersive x-ray spectroscopy (EDX) (Phoenix, EDAX, Tilburg, Netherlands) spectra were obtained. Semi-quantitative atomic percent calcium-to-phosphorus ratios were determined from the area under spectrum peaks.

3.2.2.3  **CaP Film Degradation**

The stability of the CaP films deposited on the surfaces of porous CPP was determined by soaking coated CPP substrates in Ham’s F12 for 2 days followed by Dulbecco’s modified Eagle medium (DMEM) for up to 4 weeks. Similarly, samples were soaked in Ham’s F12 supplemented with 5% fetal bovine serum (FBS) followed by DMEM supplemented with 20% FBS. The samples were incubated at 37°C in an atmosphere characterized by 95% relative humidity and 5% CO₂. The solution was changed every 2-3 days. These conditions mimic the cell culture protocol used in this study. Following soaking in Ham’s F12, as well as after 1, 2 and 4 weeks in DMEM, substrates were washed twice in dH₂O, lyophilized overnight and analyzed for evidence of degradation of the CaP film by SEM.

3.2.3  **Cartilage Tissues**

3.2.3.1  **Chondrocytes Culture**

Cartilage was aseptically excised from the deep zone (bottom 30%) of metacarpal-phalangeal articular cartilage from 9 to 12 months old calves within 24 hours of death as previously described [27]. Cartilage from several animals was pooled to obtain a sufficient number of cells for each experiment. Chondrocytes were harvested from the
tissue by sequential enzymatic digestion (0.5% protease (Sigma Chemical Co.) for 2 hours followed by 0.1% collagenase (Roche Diagnostics GmbH, Mannheim, Germany) overnight). The cells were then seeded on top of CaP coated and uncoated CPP substrates (1X10^6 cells per construct) in Ham’s F-12 supplemented with 5% FBS (HyClone, Logan, UT, USA) and incubated at 37°C in an atmosphere characterized by 95% relative humidity and 5% CO₂. On day 2, the medium was changed to DMEM supplemented with 20% FBS and ascorbic acid (100µg/ml, Sigma Chemical Co.) (non-mineralizing medium). At this time, calcification was initiated in selected cultures by the addition of 10mM β-GP (Sigma Chemical Co.) (mineralization medium). Cultures were grown for up to 4 weeks following the change to DMEM. The culture medium was changed every 2 to 3 days.

3.2.3.2 Native Tissue Samples

Native full thickness and deep zone articular cartilage were excised and shaped as 4 mm diameter discs using a biopsy punch to serve as standards for the biochemical analysis of in vitro-formed cartilage. Similarly, native full thickness articular cartilage was excised to include a portion of the ZCC to serve as standard for the characterization of mineral within the ZCC of bioengineered cartilage. Native osteochondral samples (4 mm diameter by 6 mm height) were trephined to serve as standard for interfacial shear properties.
3.2.4 Cartilage Characterization Techniques

3.2.4.1 Histological Evaluation

To examine the ZCC of *in vitro*-formed cartilage, tissues were harvested and washed twice in PBS. The tissues were removed from the CPP substrate, fixed in 10% formalin and embedded in paraffin. Five micron sections were cut, stained with toluidine blue or von Kossa and examined by light microscopy. To visualize the presence of polyphosphate in the tissues, sections were cut, dewaxed in xylene and stained with 4’-6-diamidino-2-phenylindole (DAPI) (5 µg/ml, Pierce Biotechnology, Inc., Rockford, IL, USA). The fluorescence was visualized with a Zeiss Axioplan epifluorescence microscope using a wide pass DAPI filter. Inorganic polyphosphate specifically shifts the emission peak of DAPI from 456nm to 526nm, permitting its visualization in the yellow-green spectrum rather than the blue spectrum associated with staining of nucleic acids and glycosaminoglycans (GAG) [30,31].

3.2.4.2 Biochemical Analysis

*In vitro*-formed cartilage tissues and native cartilage samples were washed twice in PBS (*in vitro*-formed tissues were removed from their substrate) and wet weighed for water content calculations using an electrical balance (Explorer, Ohaus Corp.). The tissues were then lyophilized overnight and dry weighed. The lyophilized tissues were digested by papain (40µg·ml⁻¹; Sigma Chemical Co.) in digestion buffer (20mM ammonium acetate, 1mM EDTA and 2mM DTT) for 48 hours at 65°C. Papain digests were then stored at -20°C until further analysis.
The DNA content of papain digests was assessed using the Hoechst 33258 dye (Polysciences Inc., Washington, PA, USA) binding assay and fluorometry (emission wavelength: 365nm; excitation wavelength: 458nm) [32]. The standard curve was generated with calf thymus DNA (Sigma Chemical Co.).

The proteoglycan and collagen contents of in vitro-formed and native cartilage tissues were also measured from aliquots of the papain digest. The proteoglycan content was estimated by quantifying the amount of sulphated glycosaminoglycans using the dimethylmethylene blue dye (Polysciences Inc., Washington, PA, USA) binding assay and quantifying the color spectrophotometrically at 525nm [33]. The standard curve was generated with bovine trachea chondroitin sulphate A (Sigma Chemical Co.). The collagen content was estimated by quantifying the hydroxyproline. Papain digest aliquots were hydrolyzed in 6N HCl at 110°C for 18 hours. The hydroxyproline of the hydrolysate was determined using the choramine-T/Ehrlich’s reagent assay and the color change quantified spectrophotometrically at 560nm [34]. The standard curve was generated with L-hydroxyproline (Sigma Chemical Co.) and it was assumed that hydroxyproline comprises 10% of the weight of collagen.

3.2.4.3 Analysis of gene expression

Total RNA was extracted from in vitro-formed cartilage washed twice with PBS using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA (0.5µg) was reverse transcribed into cDNA using Superscript II and Random Hexamers (Invitrogen, Carlsbad, CA, USA) according to the protocol provided by the manufacturer. The resulting cDNA was diluted 5x and subjected to relative quantitative polymerase chain reaction (qPCR)
with a Realplex² Master Cycler (Eppendorf, Hamburg, Germany). Briefly, 4µl of cDNA was amplified in 10µl total volume with 0.6µM of each primer suspended in Express SYBR Green ER (Invitrogen, Carlsbad, CA, USA). Reaction mixtures were subjected to an initial 10 min incubation at 95°C to activate the enzyme followed by 15 s denaturation at 95°C and 30 s annealing at 60°C (annealing temperature for collagen type X was 59°C). Amplification data were collected for 40 cycles. Mean relative quantification (RQ) values were calculated with 18S rRNA as endogenous control and tissues formed on uncoated CPP as calibrator. The efficiency of each primer pair was verified. Only primer pairs with an efficiency superior to 90% were used. Primer sequences have previously been published [35].

3.2.4.4 Mineral Content

Cartilage tissues formed in vitro were harvested, washed twice in PBS and removed from their substrate. The tissues were then lyophilized overnight and weighed using an electrical balance (Explorer, Ohaus Corp., Florham Park, NJ, USA). The lyophilized tissues were then wet-ashed as described previously [14]. Briefly, the tissue was placed in an HNO₃/HClO₄ (4:1 vol/vol) acid mixture and heated at 150°C for 90 minutes in a closed Teflon beaker, the mixture was evaporated to a small drop (approximately 100µl) by continued heating following removal of the cover and diluted with dH₂O (2ml). The pH of the solutions was then adjusted to 2.5-2.9 and distilled deionized water added to a final volume of 3ml.

Calcium content was measured by mixing an aliquot of wet-ashed solution with 0.01% cresolphthalein complexone (Sigma Chemical Co.) buffered with 0.25M sodium
borate in a 1:4 vol/vol ratio and the resulting color quantified spectrophotometrically at 570nm. The standard curve was generated using a calcium chloride solution.

Phosphate content was measured by reaction of an aliquot of wet ashed solution with a solution containing 1:6 vol/vol ratio of 10% ascorbic acid and 0.42% ammonium molybdate (Sigma Chemical Co.) in 1N H₂SO₄ (1:3 vol/vol ratio) at 37°C for 1 hour and spectrophotometrical analysis at 620nm. The standard curve was generated using a sodium phosphate dibasic solution (pH=7.5).

3.2.4.5 Transmission Electron Microscopy and Electron Diffraction

*In vitro*-formed cartilage tissues and native cartilage samples were washed twice in PBS (bioengineered cartilage were removed from their substrate), fixed in 2% glutaraldehyde in 0.1M Sorensen phosphate buffer and post-fixed in 0.1% osmium tetroxide. Tissues were embedded and sections cut and analyzed by TEM as described in section 3.2.2.2.

The crystal structure of the mineral deposited within *in vitro*-formed cartilage and native articular cartilage was determined by SAED as described in section 3.2.2.2. Dark field images were generated by placing a 10 µm objective aperture over the (002) diffraction ring of the SAED pattern of minerals within the ZCC of *in vitro*-formed cartilage and native articular cartilage. The length of crystals with their (002) plane aligned parallel to the surface plane of the section (the crystals appearing the largest) was measured.
3.2.4.6 Interfacial Shear Testing

*In vitro*-formed cartilage tissues and native osteochondral plugs were washed and kept in PBS until testing to ensure that the tissues remained fully hydrated. The interfacial shear properties of the bioengineered biphasic constructs and native articular osteochondral tissues were determined by applying a force at the interface region of these samples using a specifically designed jig attached to an Instron universal testing machine [14,36]. The specimens were placed in a tightly fitting copper sleeve in a manner that adjusted the edge of the copper sleeve with the cartilage-bone substitute (or subchondral bone) interface. This allowed positioning of the sample so that its interface is approximately 100 µm away from the area of contact with the metal shear plate. This gap ensured that the plate would not contact with the irregular surface of the sintered CPP or subchondral bone at the interface. However, the moment arm resulting from this approach prevents the determination of absolute interface shear strength for specimens. All tests were performed at a cross-head speed of 1 mm/min. The peak load at failure was defined as the highest point of the first perceived peak of the force-displacement curve. The shear stiffness was calculated as the slope of the steepest and most linear portion of the force-displacement curve prior to failure. The energy to failure was calculated as the area under the force-displacement curve from the point of contact to the point of failure by trapezoid rule estimation.

3.2.4.7 Histological Evaluation of Interface Failure

To examine the mode of failure of cartilage-CPP constructs, tissues tested for interfacial shear strength were fixed in 10% formalin, infiltrated with Spurr resin and processed
undecalcified. Two hundred micron thick sections were cut and ground to an approximate thickness of 40 µm. The sections were stained with toluidine blue and examined by light microscopy.

3.2.5 Statistical Analysis

All experiments were performed in triplicate unless specified otherwise. Experiments involving the use of bovine chondrocytes were repeated 3 times with cells from different extractions. Results are expressed as the mean ± standard error of the mean and analyzed using a one-way ANOVA (for more than 2 conditions) or Student’s t-test (between groups). Tukey’s test post hoc analysis was performed. P values ≤ 0.05 were considered statistically significant.

3.3 Results

3.3.1 CaP coating optimization

The particle density of the sol-gel solution used for the application of a thin CaP film to the surfaces of porous CPP was optimized to minimize pore occlusion and crack formation within the coating. As shown in SEM micrographs, the surface porosity of uncoated CPP (Figure 3.1A-B) appeared substantially occluded by the deposition of the coating using an undiluted sol-gel solution (Figure 3.1C-D). Large cracks were also observed in these coatings, exposing the underlying CPP. Diluting the sol-gel solution minimized pore occlusions and cracks but surface features of the CPP were visible suggesting a thinner film (Figure 3.1E-F). The optimal sol-gel solution particle density was established at 75% sol-gel solution mixed with 25% distilled water (Figure 3.1G-H).
To ensure the deposition of a uniform film throughout the CPP surfaces and to cause the generation of a ZCC directly at the interface between the *in vitro*-formed cartilage and the bone substitute, the number of coating layers required was evaluated. SEM micrographs clearly showed that an important proportion of the top surface of porous CPP remained uncoated after the application of 2 layers of the sol-gel solution (Figure 3.2A). Most of the top surface of porous CPP was covered by the application of 4 layers of sol-gel solution but small CPP areas remained exposed (Figure 3.2B). The application of 6 (Figure 3.2C) and 8 (Figure 3.2D) layers of sol-gel solution resulted in a completely coated CPP surface. A micrograph of uncoated CPP is provided for comparison purposes (Figure 3.2E).

A histological evaluation of *in vitro*-formed cartilage following the incubation of deep zone chondrocytes in mineralization medium for 2 weeks was performed. This culture time was selected to facilitate processing of undecalcified tissues. Tissues were formed on CPP substrates coated with 2 (Figure 3.2F), 4 (Figure 3.2G), 6 (Figure 3.2H) or 8 (Figure 3.2I) layers of sol-gel solution and compared to that on uncoated CPP (Figure 3.2J). The localization of the ZCC with regards to the tissue-substrate interface was influenced by the number of sol-gel layers applied to the CPP, with tissues formed on CPP coated with 6 and 8 layers of sol-gel solution exhibiting a ZCC directly apposed to the interface in most of the construct cross-section. However, the interface was not uniformly mineralized. Based on these results, all further experiments were carried out with CPP coated with an 8 layer calcium phosphate film.
Figure 3.1: Effect of sol-gel solution particle density on CPP morphology. Scanning electron micrographs of the top surface of (A-B) uncoated CPP bone substitutes as well as substrates coated with 8 applications of (C-D) 100%, (E-F) 50% and (G-H) 75% sol-gel solution. Dilution of the sol-gel solution was done with dH₂O to control the particle density of the solution. Each condition was performed in triplicate. Arrowheads indicate cracks in the coating.
Figure 3.2: Effect of the number of coating layers on CPP morphology and ZCC localization within in vitro-formed cartilage. (A-E) Scanning electron micrographs of the top surface of CPP bone substitutes coated with (A) 2, (B) 4, (C) 6 or (D) 8 applications of a sol-gel solution diluted to 75% with dH$_2$O and compared to (E) uncoated substrates. Each condition was performed in triplicate. The arrowheads indicate zones where the CPP is exposed. (F-J) Histological appearance of cartilage formed by deep-zone chondrocytes on CPP bone substitutes coated with (F) 2, (G) 4, (H) 6 or (I) 8 applications of a sol-gel solution diluted to 75% with dH$_2$O and compared to (J) uncoated substrates after 2 weeks of culture. The tissues were visualized by light microscopy (toluidine blue and von Kossa). The arrows indicate the zone of calcified cartilage. The scale bar in histological images indicates a distance of 100 µm.
3.3.2 CaP coating characterization

SEM examination of the fracture surface of CaP coated CPP showed that the sol-gel solution penetrated within the porous structure of CPP as a thin film (1µm thick) on most surfaces throughout the cross-section of porous CPP (Figure 3.3A). CaP film (coating) was evident between CPP particles and suggesting that there was occlusion of some of the pores within the center of the substrate. These blocked pores may have caused some enclosed surfaces to be poorly coated. With the exception of these uncoated areas, the coating was significantly thicker within the center of the CPP than at its surface (core: 1.11µm; surface: 0.85µm). Sintered neck regions were also characterized by increased coating thickness. TEM examination of the cross-section of CaP-coated CPP particles demonstrates that the coating was porous and composed of nanoscale particles (Figure 3.3B).

Elemental analysis of the cross section of CaP coated CPP particles by EDX indicated that the coating was characterized by a calcium-to-phosphorus ratio of 1.50 (Figure 3.3C) compared to 0.45 for the CPP particles (Figure 3.3D). A representative SAED pattern acquired from the thin CaP films on the surface of CPP particles removed from CPP substrates is presented in Figure 3.3E. The diffraction pattern is consistent with either hydroxyapatite or octacalcium phosphate crystal structure. The d-spacing measurements of the 4 rings of highest intensity support this observation (Table 3.1).

3.3.3 CaP coating degradation

SEM examination of coated CPP soaked for 2 days in Ham’s F12 and up to 28 days in DMEM was performed to investigate the extent of the thin film degradation under similar
Figure 3.3: Characterization of the CaP coating on CPP. (A) Scanning electron micrograph of the fracture surface of CPP bone substitutes coated with 8 applications of a sol-gel solution diluted to 75% with dH₂O. The micrograph was taken in the center of the substrate. * indicates a coating build-up between CPP particles (occlusion). Arrowheads indicate locations where the underlying CPP is exposed. Arrows indicate the fracture surface of the CaP coating. (B) Transmission electron micrograph of the calcium phosphate coating at the surface of a CPP particle. (C-D) Representative energy dispersive x-ray analyses of (C) calcium phosphate thin film applied by sol-gel processing and (D) a CPP particle. (E) Selected area electron diffraction pattern obtained from the calcium phosphate thin film applied on the surfaces of CPP.
conditions as those used for cartilage culture in this study. Compared to as-made coated CPP (Figure 3.4A), the thin film of coated CPP soaked for 2 days in Ham’s F12 followed by 28 days in DMEM both in the absence (Figure 3.4B) and presence (Figure 3.4C) of FBS showed signs of degradation as the surfaces appeared rough. However, the observed degradation was relatively minor and did not expose the underlying CPP.

<table>
<thead>
<tr>
<th>CaP Coating</th>
<th>HA Reference</th>
<th>OCP Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>d&lt;sub&gt;hkl&lt;/sub&gt; (Å)</td>
<td>d&lt;sub&gt;hkl&lt;/sub&gt; (Å)</td>
<td>I/I&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>3.41</td>
<td>002</td>
<td>3.44</td>
</tr>
<tr>
<td>3.11</td>
<td>002</td>
<td>3.42</td>
</tr>
<tr>
<td>2.78</td>
<td>250</td>
<td>3.21</td>
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<tr>
<td>2.26</td>
<td>260</td>
<td>2.83</td>
</tr>
<tr>
<td>2.74</td>
<td>241</td>
<td>2.82</td>
</tr>
<tr>
<td>2.22</td>
<td>331</td>
<td>2.74</td>
</tr>
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</table>

The diameters of specific rings associated with distinct crystallographic planes of selected area electron diffraction patterns were measured and the associated d-spacing (d<sub>hkl</sub>) values calculated using a camera constant obtained from a gold standard. These values are compared to reference d-spacing values for hydroxyapatite (HA) and octacalcium phosphate (OCP). The proximity and intensity of specific rings means that a single measurement was made for groups of crystallographic planes (hkl). I/I<sub>1</sub> represent the intensity of a ring as a percent of the most intense ring in the pattern. The calculated d-spacing are averages of diffraction patterns obtained from three calcium phosphate films obtained from different samples.

### 3.3.4 Effect of CaP film on the histological appearance of bioengineered cartilage formed on CPP

Deep-zone chondrocytes were cultured for 2 weeks on both coated and uncoated CPP. No histological difference was observed between tissues formed on the two different conditions.
Figure 3.4: Degradation of the CaP coating on CPP in vitro. Scanning electron micrographs of the top surface of CPP bone substitutes coated with a calcium phosphate film. Micrograph taken (A) prior to incubation in culture media, (B) following incubation for 2 days in Ham’s F12 followed by 28 days in DMEM, or (C) following incubation in the same in the media supplemented with FBS (5% in Ham’s F12 and 20% in DMEM). Arrowheads indicate coating roughness.
substrates in non-mineralizing medium (Figure 3.5A-B). On both substrates, the cartilaginous tissues exhibited a zonal organization similar to that of native articular cartilage with superficial, mid and deep zone chondrocyte morphologies and more intense proteoglycan staining in the deep zone directly above the substrate.

Epifluorescence imaging of DAPI stained tissue sections of cartilaginous tissues cultured for 1 week on CaP coated and uncoated CPP in non-mineralizing medium showed weaker polyphosphate staining intensity (yellow-green fluorescence) at the cartilage-CPP interface of coated CPP (Figure 3.5C) compared to uncoated CPP (Figure 3.5D). This suggests that the application of a thin CaP film on the surface of porous CPP prevents the accumulation of inorganic polyphosphate within \textit{in vitro}-formed cartilage. Interestingly, yellow-green fluorescence (indicative of polyphosphate) was also seen in the superficial aspect of the cartilage in greater amount in the uncoated CPP.

3.3.5 Effect of CaP film on the extracellular matrix of bioengineered cartilage formed on CPP

Deep-zone chondrocytes were allowed to form cartilage on coated and uncoated CPP for 4 weeks in either non-mineralizing or mineralization media. The presence of a thin CaP film on the surfaces of the CPP did not affect matrix accumulation as the proteoglycan and collagen contents were comparable within bioengineered tissues formed on coated and uncoated CPP (Table 3.2). Similarly, the DNA and water contents of \textit{in vitro}-formed cartilage were not affected. However, water content was significantly lowered in calcified \textit{in vitro}-formed cartilage compared to non-mineralized tissues, likely because of the contribution of the mineral to wet and dry weight measurements.
Table 3.2: Biochemical properties of cartilage tissues

<table>
<thead>
<tr>
<th></th>
<th>In Vitro NC/NM</th>
<th>In Vitro NC/M</th>
<th>In Vitro C/NM</th>
<th>In Vitro C/M</th>
</tr>
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<tbody>
<tr>
<td>Water Content (%)</td>
<td>90.5 ± 0.3a</td>
<td>87.1 ± 0.5</td>
<td>90.5 ± 0.3a</td>
<td>87.9 ± 0.7</td>
</tr>
<tr>
<td>DNA Content (µg/mg dry wt.)</td>
<td>3.67 ± 0.63</td>
<td>2.29 ± 0.38</td>
<td>3.79 ± 0.51</td>
<td>2.54 ± 0.37</td>
</tr>
<tr>
<td>GAG Content (µg/µg DNA)</td>
<td>78.4 ± 11.4</td>
<td>76.7 ± 15.8</td>
<td>76.9 ± 11.7</td>
<td>74.2 ± 10.6</td>
</tr>
<tr>
<td>Collagen Content (µg/µg DNA)</td>
<td>58.4 ± 9.3</td>
<td>55.4 ± 10.2</td>
<td>56.8 ± 12.8</td>
<td>59.2 ± 12.6</td>
</tr>
<tr>
<td>Collagen : GAG</td>
<td>0.75 ± 0.05</td>
<td>0.74 ± 0.08</td>
<td>0.72 ± 0.05</td>
<td>0.79 ± 0.08</td>
</tr>
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</table>

Deep-zone chondrocytes were grown in vitro on calcium phosphate coated (C) or uncoated (NC) CPP in non-mineralizing (NM) or mineralization medium (M). The biochemical properties of the cartilaginous tissues were determined after 4 weeks in culture. The data are from three experiments performed in triplicate with chondrocytes from different extractions and expressed as mean ± standard error of the mean. a indicates a significant difference between bioengineered cartilage formed in mineralization medium and non-mineralizing medium for a specific substrate (p<0.05).

3.3.6 The presence of CaP film does not affect chondrocyte-related gene expression

Deep-zone chondrocytes were cultured for 5 days and the expression of cartilage-related genes for tissues formed on coated and uncoated CPP were compared (Figure 3.6). Collagens type II and type X, as well as sox9 expression levels were not significantly affected by the CaP coating of the CPP in either non-mineralizing or mineralization medium. Interestingly, collagen type I expression was significantly higher in chondrocytes cultured on coated CPP than on uncoated CPP.

3.3.7 Characterization of the mineral within the ZCC of bioengineered cartilage formed on CaP coated CPP

Deep-zone cartilage tissues formed after 4 weeks of culture on coated and uncoated CPP in either non-mineralizing or mineralization media were analyzed. There were
Figure 3.5: Effect of CaP coating on the histological appearance of cartilage formed in vitro on CPP. (A-B) Histological appearance of cartilage formed by deep-zone chondrocytes on (A) calcium phosphate coated and (B) uncoated CPP after 2 weeks of culture in non-mineralizing conditions (toluidine blue). (C-D) Histological appearance of cartilage formed by deep-zone chondrocytes on (C) calcium phosphate coated and (D) uncoated CPP after 1 week of culture in non-mineralizing and visualized by epifluorescence microscopy (DAPI). * indicates where the CPP substrate was located. Arrowheads indicate polyphosphate staining.
Figure 3.6: Effect of CaP coating on the expression of cartilage-specific genes by chondrocytes cultured in vitro on CPP. Differential gene expression of cartilage-related genes as determined by the ∆∆Ct method after normalization to 18S rRNA in chondrocytes grown on calcium phosphate coated CPP after 4 weeks in culture relative to chondrocytes grown on uncoated CPP (calibrator). This experiment was performed in the presence of non-mineralizing and mineralization media. The data of three separate experiments were pooled and expressed as the mean ± SEM. * indicates a significant difference compared to the calibrator (p<0.05).
significantly higher levels of both calcium and phosphate within the mineralized tissues (Table 3.3). In calcified tissues, the presence of the CaP film resulted in decreased calcium (not significant; p<0.06) and phosphate (significant; p<0.05) contents compared to tissues formed on the uncoated CPP. In both cartilaginous tissues incorporating a ZCC the atomic calcium-to-phosphorus ration was slightly lower than the theoretical value of 1.67 for hydroxyapatite.

<table>
<thead>
<tr>
<th></th>
<th>In Vitro NC/NM</th>
<th>In Vitro NC/M</th>
<th>In Vitro C/NM</th>
<th>In Vitro C/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (% dry weight)</td>
<td>1.22 ± 0.25</td>
<td>8.45 ± 0.68</td>
<td>1.32 ± 0.16</td>
<td>6.07 ± 0.80(^a)</td>
</tr>
<tr>
<td>Phosphate (% dry weight)</td>
<td>1.80 ± 0.29</td>
<td>12.58 ± 0.78</td>
<td>1.51 ± 0.11</td>
<td>9.24 ± 0.98(^b)</td>
</tr>
<tr>
<td>Calcium : Phosphorus</td>
<td>NA</td>
<td>1.59 ± 0.03</td>
<td>NA</td>
<td>1.54 ± 0.05</td>
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</table>

Deep-zone chondrocytes were grown in vitro on calcium phosphate coated (C) or non-coated (NC) CPP in non-mineralizing (NM) or mineralization medium (M). The calcium and phosphate contents of the cartilaginous tissues were determined after 4 weeks in culture. The data are from three experiments performed in triplicates with chondrocytes from different extractions and expressed as mean ± standard error of the mean. \(^a\) indicates that the difference between the calcium contents of cartilaginous tissues formed on calcium phosphate coated and uncoated CPP is not significant (p<0.06). \(^b\) indicates a significant difference between the phosphate contents of cartilaginous tissues formed on calcium phosphate coated and uncoated CPP (p<0.05).

TEM examination of the mineral crystals in the ZCC of bioengineered cartilage formed on coated (Figure 3.7A) and on uncoated CPP (data not shown) have a similar morphology to crystals in the ZCC of native articular cartilage (Figure 3.7B). The crystal structure of mineral in the ZCC of bioengineered cartilage formed on coated CPP (Figure 3.7C) and uncoated CPP (data not shown) as determined from SAED patterns was comparable to that formed in the ZCC of native articular cartilage (Figure 3.7D). Additionally, the average length of crystals in the ZCC of tissues formed on coated CPP
as measured from selected area dark field images (17.6 ± 0.8nm; Figure 3.7E) was not significantly different than that in tissues formed on uncoated CPP (16.7 ± 0.5nm) and in native articular cartilage (17.2 ± 0.5nm; Figure 3.7F).

3.3.8 The ZCC affects the interfacial shear properties of bioengineered cartilage

Interfacial shear tests performed on biphasic constructs cultured in non-mineralizing culture conditions after 4 weeks indicate that the CaP coating on the CPP causes decreased interfacial shear properties (C-NM versus NC-NM). In fact, the peak load at failure, shear stiffness and energy to failure are reduced to less than 45%, 60% and 35% of that for uncoated CPP, respectively (Table 3.4; non-significant differences). The generation of a ZCC directly apposed to the interface between the cartilaginous tissue and the CaP coated CPP bone substitute (C-M) resulted in 3.2-fold, 3.8-fold and 2.6-fold increases in peak load, energy to failure and shear stiffness respectively compared to non-mineralized cartilaginous tissues formed on the same substrate (C-NM) (Table 3.4). Only the difference in energy to failure was significant, likely as a result of experimental variability. Following normalization to cartilaginous biphasic constructs formed in non-mineralizing medium on uncoated CPP (NC-NM), a significant difference was observed between the peak loads measured for C-M and C-NM (data not shown). Conversely, the generation of a ZCC away from the interface between the cartilaginous tissue and the uncoated CPP bone substitute (NC-M) resulted in non-significant 1.2-fold, 1.4-fold and 1.1-fold increases in peak load, energy to failure and shear stiffness respectively compared to non-mineralized cartilaginous tissues formed on the same substrate (NC-NM) (Table 3.4).
Figure 3.7: Effect of CaP coating on the microstructural analysis of the mineral crystals in the ZCC of cartilage formed in vitro on CPP. (A, C, E) Cartilage formed by deep zone chondrocytes on calcium phosphate coated CPP after 4 weeks in culture in mineralization medium and (B, D, F) native articular cartilage. (A-B) Transmission electron micrographs. (C-D) Selected area electron diffraction patterns. (E-F) Selected area dark field images. Arrowheads indicate single crystals in dark field images.
Table 3.4: Interfacial shear properties of cartilage tissues.

<table>
<thead>
<tr>
<th></th>
<th>In Vitro NC/NM</th>
<th>In Vitro NC/M</th>
<th>In Vitro C/NM</th>
<th>In Vitro C/M</th>
<th>Ex Vivo OC Plugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak Load (N)</td>
<td>3.8 ± 0.5</td>
<td>4.5 ± 0.8</td>
<td>1.7 ± 0.5</td>
<td>5.4 ± 1.6</td>
<td>84.7 ± 22.3</td>
</tr>
<tr>
<td>Shear Stiffness (N/mm)</td>
<td>9.8 ± 1.5</td>
<td>11.0 ± 2.1</td>
<td>5.8 ± 2.3</td>
<td>14.8 ± 5.3</td>
<td>87.4 ± 16.6</td>
</tr>
<tr>
<td>Energy to Failure (kJ)</td>
<td>1.0 ± 0.1</td>
<td>1.4 ± 0.3</td>
<td>0.4 ± 0.1</td>
<td>1.5 ± 0.3a</td>
<td>58.3 ± 17.7b</td>
</tr>
</tbody>
</table>

Deep-zone chondrocytes were grown in vitro on calcium phosphate coated (C) or uncoated (NC) CPP in non-mineralizing (NM) or mineralization medium (M). The interfacial shear properties of the biphasic constructs were determined after 4 weeks in culture and compared with ex vivo osteochondral (OC) plugs. The data are from three experiments performed with 4-8 replicates using chondrocytes from different extractions and expressed as mean ± standard error of the mean. a indicates a significant difference between bioengineered cartilage formed in mineralization medium versus non-mineralizing medium for a specific substrate (p<0.05). b indicates a significant difference between ex vivo tissues and bioengineered constructs (p<0.05).

Representative biphasic constructs were processed for histological evaluation in order to evaluate the sheared interface (Figure 3.8). All constructs without a ZCC (NC-NM and NC-M) failed directly at the tissue-substrate interface. Of the five constructs incorporating a ZCC displaced from the tissue-substrate interface (NC-M) evaluated, one failed at the interface, one failed above the ZCC and three failed at both locations. Of the seven constructs with a ZCC directly apposed to the tissue-substrate interface (C-M), two failed at the interface, three failed above the ZCC and two failed at both locations. For constructs incorporating a ZCC, there was no connection between the peak load at failure and the location of the failure in the tissue.

3.4 Discussion

This study reports the formation of osteochondral-like biphasic constructs incorporating a ZCC at the interface between in vitro-formed cartilage and a porous CPP bone substitute.
Figure 3.8: Histological appearance of failed cartilage-CPP constructs following interfacial shear testing. Deep zone cartilage was formed in vitro on uncoated (NC) or calcium phosphate coated (C) CPP for 4 weeks in non-mineralizing (NM) or mineralization (M) media (toluidine blue). Arrows indicate the fracture line. Arrowheads indicate the location of the zone of calcified cartilage.
This was achieved via the application of a thin CaP film to the surfaces of porous CPP which prevented the accumulation of polyphosphate released from the CPP within the \textit{in vitro}-formed cartilage layer. The presence of the CaP coating did not affect the overall histological appearance or the biochemical composition of bioengineered cartilage. Expression of cartilage-related genes collagen type II, collagen type X and Sox 9 was not affected but there was increased collagen type I gene expression in the cells. Similarly, the application of CaP coating to CPP did not alter the appearance, structure or length of the mineral crystals contained in the \textit{in vitro}-formed ZCC. Of importance, the incorporation of a ZCC directly apposed to the tissue-substrate interface in biphasic constructs did result in a 220\% increase in the peak load at failure measured in an interfacial shear test compared to a 20\% increase for a ZCC is apposed away from the tissue-substrate interface.

This study establishes a methodology to prevent the inhibitory effects of CPP degradation products (polyphosphate) on cartilage calcification resulting in a ZCC directly apposed to the tissue-substrate interface, while maintaining the favourable properties of the CPP substrate via the application of a thin CaP film to the surfaces of porous CPP. This coating decreased the accumulation of polyphosphate within the \textit{in vitro}-formed cartilage as visualized by DAPI staining. The mechanism by which the thin film prevents this accumulation remains unresolved. While the coating may act as a barrier to the release of substrate degradation products including polyphosphate, its nanometer scale porous structure and associated high surface area may cause it to act as a polyphosphate sink as phosphate polymers are known to bind to hydroxyapatite [37].
The coating technique selected for this study was an inorganic route of the sol-gel process. This non-line-of-site approach was shown to deposit a calcium phosphate film with characteristics in keeping with that shown previously [29]. Taken together, prior work from our group to establish the inorganic sol-gel coating procedure, as well as the XRD pattern and EDX elemental analysis performed on the CaP coating in this study suggest that the CaP film consists of poorly crystallized calcium-deficient hydroxyapatite [29]. However, it is possible that the film was composed of octacalcium phosphate and further study is required to fully characterize this film. Independent of the composition of this film, the shift of the zone of calcified cartilage contiguous to the substrate, was achieved. Because the osteoconductivity of thin films produced by this approach has been demonstrated, such treatment should not negatively impact the fixation of biphasic constructs within the joint by bone ingrowth [38]. Furthermore, it has been shown by others that the CaP material formed by this process are resorbed by osteoclasts upon implantation in bone confirming the biodegradability of this coating [39]. Taken together, this observation and the modest *in vitro* degradation observed in this study suggest that the long-term biodegradability of porous CPP will not be compromised by the application of a thin CaP coating.

The comparison of shear properties of the interface of non-mineralized biphasic constructs (NC-NM versus C-NM) indicates that they tend to be impacted negatively by the application of a CaP coating to CPP. A number of factors could contribute to this observation. The SEM examination of the top and fracture surfaces of coated and uncoated CPP has demonstrated that some pores of the substrate are blocked by the application of a coating. It is possible that a decreased number and depth of cartilage
interdigitations are formed within the porous subsurface of coated CPP, thereby compromising the mechanical integrity of the tissue-substrate interface. Alternatively, differences in the tissue composition and/or structure at the interface with the CPP may exist. As was demonstrated, the CaP coating of CPP does not affect the quality of the \textit{in vitro}-formed cartilage tissue as observed histologically or biochemically. However, a three-fold increase in collagen type I gene expression was observed in tissues formed on coated CPP. Increased collagen type I gene expression is often associated with chondrocyte dedifferentiation [40,41], or a fibrocartilagenous phenotype [42]. However, previous analysis of the collagen types present in cartilage tissue formed \textit{in vitro} on porous CPP did not detect collagen type I within the extracellular matrix [21]. Hence, even if this three-fold increase in collagen type I gene expression translates into increased protein levels, it would still represent a modest proportion of the total collagen content of the tissues. Nevertheless, this observation emphasizes the possibility that changes in the expression levels of other important constituents of the extracellular matrix can not be discounted. The cause of this gene expression change still remains unclear. It is possible that changes to the surface chemistry are impacting cellular responses. Alternatively, the decreased accumulation of inorganic polyphosphate at the tissue-substrate interface of biphasic constructs using coated CPP could cause local changes. It is also possible that the occlusion of some pores of the CPP by the CaP coating may be affecting cell function. It was previously shown that substrate porosity influences the response of chondrocytes through fluid-induced shear-stresses [43,44].

The generation of a ZCC at the tissue-substrate interface in biphasic constructs (C-M) resulted in substantial increases in the peak load at failure (218%), shear stiffness
(155%) and energy to failure (275%; significant difference) measured by an interfacial shear test compared to biphasic constructs prepared without a ZCC on the same substrate (C-NM). Comparatively, the generation of a ZCC above the tissue-substrate interface in biphasic constructs (NC-M) only resulted in a modest (non-significant) increase in these mechanical parameters (18% for the peak load at failure; 12% for the shear stiffness and 40% for the energy to failure) compared to biphasic constructs prepared without a ZCC on the same substrate (NC-NM). This is the first demonstration of the benefits of designing bioengineered cartilage incorporating a properly located ZCC on the mechanical integrity of the tissue-substrate interface. Three studies reported the results of interfacial shear tests performed on both native immature bovine osteochondral specimens that do not incorporate a ZCC and mature osteochondral explants with a ZCC [45-47]. In these studies, the presence of a ZCC did not positively impact the peak load causing failure. This discrepancy with the impact of the ZCC reported in this study may be attributed to differences in the composition, structural organization and biomechanical properties of immature and mature native cartilage rather than the ZCC [48]. Important set-to-set variations were observed for interfacial shear properties of samples incorporating a ZCC directly apposed to the interface (C-M). This variation is attributed to differences in the primary chondrocyte populations used for each experiments, which can influence the extent and amount of the ZCC formed at the time point examined [14].

Despite the mechanical effect associated with the formation of a ZCC at the tissue-substrate interface of cartilage biphasic constructs, the interfacial shear properties remain significantly inferior to those of the native osteochondral junction. The histological examination of biphasic construct incorporating a ZCC directly apposed to
the tissue-substrate interface (C-M) following interfacial shear tests provides insight as to potential reasons for this difference. In fact, constructs fail either within the tissue above the ZCC or within the mineralized tissue. Others have demonstrated that mature native articular cartilage failure following the application of a sufficient shear force at the cartilage-subchondral bone interface occurs at the tidemark between the hyaline and calcified cartilage [45,46]. Comparatively, failure occurs at the cement line between hyaline cartilage and subchondral bone in immature cartilage which does not have a ZCC or when the ZCC has not completely developed [46].

Hence, the results presented in this study suggest that some constructs failed in a similar manner to mature osteochondral tissues while others exhibited a mode of failure typical of tissues with an immature ZCC. This is consistent with histological observations of the mineralized cartilage formed in vitro on coated CPP (C-M), which exhibit a discontinuous ZCC without a proper tidemark. A longer in vitro incubation phase could contribute to mechanical improvements of the interface by causing increased mineral deposition within the ZCC. Similarly, identification of culture conditions that would allow a more uniform mineral deposition in the tissue and the formation of a tidemark could be beneficial. Also, many samples have failed within the unmineralized matrix in both experimental conditions incorporating a ZCC (C-M; NC-M). These results indicate that the interfacial shear test performed in this study underestimates the peak shear load of the cartilage-CPP interface in some biphasic constructs incorporating a ZCC as the interface remains intact and failure occurs within the tissue. This suggests that a strategy combining the formation of a ZCC with mechanical stimulation that has been shown to enhance cartilage matrix accumulation might further increase the stiffness of the in vitro-
formed matrix and further improve the mechanical integrity of osteochondral biphasic constructs.

Interestingly, the results of the interfacial shear test for biphasic constructs formed on uncoated CPP are superior to those previously published by our group [14]. A number of changes made to the CPP fabrication process may have contributed to this effect. Additionally, differences in the protocols in these two studies for in vitro tissue formation may partly explain this difference. The source of FBS, the number of chondrocytes seeded per constructs, the duration of the in vitro culture period and the use of sodium bicarbonate have all been changed for this study.

The interfacial shear test used in this study provides valuable information. However, a number of issues influence the quantification of the interfacial shear strength of the biphasic constructs. For example, the tissue-substrate interface of biphasic constructs and osteochondral tissues is irregular. To prevent contact between the shear plate and CPP or subchondral bone protrusions, the shear force is applied 100µm away from the interface resulting in a moment arm that impacts the evaluation of the shear strength. The tests were also performed under unconfined conditions. This permits fluid exudation during the test and this behavior is an integral part of the mechanical behaviour and properties of articular cartilage [49]. It may also lead to out-of-plane deformations of the cartilage and subsequent contribution of tensile opening mode to failure [47]. In addition, the cylindrical shape of samples makes it very difficult to accurately determine the shearing area. This is complicated by the fact that two different failure types have been observed in the specimens tested. Further, we are not able to take into account in the data analysis that the area of failure at the tissue-substrate interface of unmineralized
Cartilage is increased by the tissue interdigitations within the porous CPP compared to a smoother failure area in cartilage incorporating a ZCC.

3.5 Conclusions

In summary, this study demonstrates the formation of osteochondral biphasic constructs formed in vitro that incorporate a ZCC directly apposed to the cartilage-CPP substrate interface. This work is the first report to demonstrate the advantages associated with the design of biphasic cartilage constructs that mimic the native tissue organization of the cartilage-subchondral bone interface on the mechanical integrity of that interface. Nevertheless, the peak force and energy required to cause the failure of the interface remain significantly lower than for the native articular cartilage-subchondral bone interface. Further work is needed to improve the quality of bioengineered cartilage including the ZCC, notably through the development of a methodology to apply a coating on porous CPP which overcomes the limitations of the sol-gel approach.

3.6 References


CHAPTER 4: MINERALIZATION WITHIN THE ZONE OF CALCIFIED CARTILAGE IS MODULATED BY THE EFFECTS OF FIBROBLAST GROWTH FACTOR 18 ON INORGANIC POLYPHOSPHATE METABOLISM

Jean-Philippe St-Pierre\textsuperscript{1}, Robert M. Pilliar\textsuperscript{1,2,3}, Marc D. Grynpas\textsuperscript{1,3}, Rita A. Kandel\textsuperscript{1,3}.

\textsuperscript{1}Department of Materials Science and Engineering and Institute of Biomaterials and Biomedical Engineering, University of Toronto. \textsuperscript{2}Faculty of Dentistry, University of Toronto. \textsuperscript{3}CIHR-BioEngineering of Skeletal Tissue Team, Mount Sinai Hospital, Toronto.

As first author of this multi-author contribution, I designed and conducted most of the experiments. Histological sections were prepared by others. I also analyzed the results and wrote the manuscript.
4.1 Introduction

Osteoarthritis (OA) is the most common joint disorder, as well as a major cause of disability and decreased quality of life for a majority of the world population aged 65 years or older [1]. Current therapeutic approaches for OA aim at pain relief and recovery of the range of motion in the affected joints but do not reverse the progression of the disease, eventually leading to the need for surgical interventions, including total joint arthroplasty [2,3]. An improved understanding of the biological mechanisms by which articular cartilage maintains homeostasis is essential to the development of treatments that can arrest the disease process, as the balance between anabolic and catabolic processes in articular cartilage is lost during the progression of OA. Other distinctive changes take place within the articular cartilage during OA, including the duplication of the tidemark and advancement of the zone of calcified cartilage (ZCC) within hyaline cartilage [4,5]. This increased metabolic activity at the tidemark occurs at the expense of hyaline cartilage thickness and leads to increased stresses within the unmineralized tissue, possibly contributing to cartilage degeneration [6]. Nevertheless, the mechanism(s) by which ZCC mineralization is modulated remains poorly understood.

Inorganic polyphosphates are polymers of orthophosphate residues linked by energy-rich phosphoanhydride bonds and are ubiquitously present in biological systems [7]. These condensed phosphates have been identified in various vertebrate cell types and tissues and are present in particularly high concentrations in osteoblasts [8-10]. However, little is known about the enzymes and cellular pathways involved in their metabolism in vertebrates. For example, enzymes involved in polyphosphate synthesis have yet to be identified in vertebrates. Nevertheless, three human actin-like proteins were found to
exhibit significant sequence identity with those of the polyphosphate kinase complex responsible for the synthesis of polyphosphate in *Dictyostelium discoideum* [11]. Enzymes involved in polyphosphate degradation include exopolyphosphatases (cleavage of orthophosphates from the end of the chains) and endopolyphosphatases (cleavage in the middle of the chains) and their activities have been demonstrated in vertebrate cells [9,12]. While tissue non-specific alkaline phosphatase (TNAP), intestinal alkaline phosphatase and h-prune have been shown to exhibit exopolyphosphatase activity [13-15] in vertebrates, enzymes with endopolyphosphatase activity remain to be identified.

It is well established that condensed phosphates bind to apatite crystals to effectively inhibit their growth [16,17]. Interestingly, subcutaneous administration of these phosphate polymers was shown to prevent skin calciphylaxis induced by dihydrotachysterol [18] and calcification of the aorta caused by administration of vitamin D$_3$ in rat models [19,20]. Additionally, our group has shown that polyphosphate inhibits mineral deposition in cultures of bovine chondrocytes [21], while others have demonstrated a similar effect in human osteoblasts [14]. Conversely, polyphosphate was shown to induce mineralization in murine pre-osteoblast cultures [22]. Taken together, these reports suggest an important role for phosphate polymers as mineralization inhibitors endogenously produced by polyphosphate kinases and that can be enzymatically cleaved by exopolyphosphatases to release orthophosphate and promote tissue mineralization [13,14,16].

Shiba et al. (2003) have demonstrated that polyphosphate physically and functionally stabilizes basic fibroblast growth factors (FGF-2), thereby stimulating the mitogenic activity of human fibroblasts [23]. Another study by Han et al. (2007) showed
that the phosphate polymers inhibit FGF-2 signaling, thereby impeding the proliferation of endothelial cells and angiogenesis [24]. These studies suggest that FGFs may be involved in regulating the effects of polyphosphate. Interestingly, mice with a selective inactivation of the fgf2 gene were shown to exhibit decreased trabecular bone volume, mineral apposition and bone formation rate, while cultured bone marrow stromal cells from these knockout mice had decreased mineralization potential compared to cells from wild-type animals [25]. Mice over-expressing fgf2 exhibited a similar skeletal phenotype as knockout animals [26]. A number of \textit{in vitro} studies have shown that FGF-2 can act to inhibit [27-31] or stimulate [32-34] cell-mediated mineralization. These seemingly contradictory effects of FGF-2 on tissue mineralization were reconciled by the demonstration that this growth factor up-regulates ectonucleotide pyrophosphatase phosphodiesterase 1 (ENPP-1) and progressive ankylosis (ANK) in murine pre-osteoblasts, which respectively synthesize pyrophosphate from ATP and transport it to the extracellular environment, where it acts as a mineralization inhibitor [35]. FGF-2 was also shown to down-regulate TNAP, which is notably responsible for the degradation of pyrophosphate to release orthophosphate that can contribute to the mineralization process. Abnormally high levels of pyrophosphate can lead to the deposition of pathological calcium pyrophosphate dihydrate (CPPD) crystals [36]. Alternatively, FGF-2 was also shown to decrease pyrophosphate release by murine endochondral chondrocytes [37].

Inactivation of the fgf18 gene in mice was also shown to exhibit skeletal development abnormalities including delayed ossification and suture closure [38,39]. As with FGF-2, conflicting results have been reported regarding the effects of FGF-18 on the
mineralization process. Exogenous FGF-18 was shown to inhibit mineral deposition in murine primary osteoblasts [31], while a murine mesenchymal stem cell line overexpressing FGF-18 was shown to form increased numbers of bone nodules [40].

Our group has developed a methodology to form three-dimensional articular cartilage tissue incorporating a ZCC in vitro by culturing deep zone articular chondrocytes on a porous membrane insert and supplementing the culture medium with a source of organic phosphate (β-glycerophosphate; β-GP) [41,42]. The mineral formed within the ZCC is similar in composition (poorly crystalline apatite) and crystal length as that which occurs in native articular cartilage and is limited to the deeper portion of the tissue, thus making it a good model to study the mechanisms regulating cartilage mineralization. As polyphosphate and FGF-18 signaling can modulate mineralization, the purpose of this study was to determine the role of FGF-18 in modulating polyphosphate inhibition of mineralization in cartilage formed in vitro. It is hypothesized that this regulation occurs at the level of polyphosphate degradation by exopolyphosphatases. The mechanisms of modulation of articular cartilage calcification remain poorly understood and require further attention.

4.2 Materials and Methods

4.2.1 Substrates

Membrane inserts (Millicell-CM; Millipore Corp., Bedford, MA, USA) were coated with type II collagen (0.5mg/ml in 0.1N acetic acid; Sigma Chemical Co., St.Louis, MO, USA) and dried overnight. The membrane inserts as well as glass coverslips used in
immunostaining studies were UV sterilized for 30 minutes and soaked in Ham’s F12 for 30 minutes prior to cell culturing.

4.2.2 Tissue Culture

Cartilage was aseptically excised from either the full thickness or from the superficial zone (top 10%), the mid-zone (middle 60%) or the deep zone (bottom 30%) of metacarpal-phalangeal articular cartilage of 9 to 12 months old calves within 24 hours of death as previously described [42]. For *in vitro* calcification experiments, deep zone cartilage from several animals was pooled to obtain a sufficient number of cells for each experiment and cells were harvested by sequential enzymatic digestion (0.5% protease (Sigma Chemical Co.) for 2 hours followed by 0.1% collagenase (Roche Diagnostics GmbH, Mannheim, Germany) overnight). The cells were then seeded on top of membrane inserts (1X10^6 cells per construct) or glass cover slips (5X10^5 cells per well) in Ham’s F-12 supplemented with 5% fetal bovine serum (FBS; HyClone, Logan, UT, USA) and incubated at 37°C in an atmosphere characterized by 95% relative humidity and 5% CO₂. On day 5, the medium was changed to Dulbecco’s modified Eagle medium (DMEM) supplemented with 20% FBS, ascorbic acid (100µg/ml; Sigma Chemical Co.) and 10mM β-GP (Sigma Chemical Co.) to initiate cartilage mineralization (mineralization medium). For non-mineralizing culture conditions, β-GP was omitted. The cells were treated at the same time with different factors including human recombinant FGF-1, FGF-2 or FGF-18 (PeproTech Inc., Rocky Hill, NJ, USA), a pharmacological inhibitor of FGFR signaling, PD173074 (EMD Biosciences Inc., San Diego, CA, USA), sodium phosphate glasses with average chain lengths of 5, 45 and 75
phosphate units (inorganic polyphosphates; Sigma Chemical Co.), sodium pyrophosphate tetrabasic (inorganic pyrophosphate; Sigma Chemical Co.) or phosphate buffer prepared with sodium phosphate dibasic anhydrous and sodium phosphate monobasic monohydrate (pH 7.0; Sigma Chemical Co.). The concentrations of condensed phosphate species were calculated based on their phosphate content. Cultures were grown for up to 2 weeks following the initiation of mineralization. The culture medium was changed every 2 to 3 days.

4.2.3 Histological Evaluation

In vitro-formed cartilage constructs and full thickness native articular cartilage were harvested and washed twice in phosphate buffered saline (PBS). Tissues were fixed in 10% formalin and embedded in paraffin. Five micron sections were cut, stained with toluidine blue and von Kossa and examined by light microscopy. To visualize the presence of inorganic polyphosphate in the tissues, sections were cut, dewaxed in xylene and stained with 4′-6-diamidino-2-phenylindole (DAPI) (5 to 20 µg/ml, Pierce Biotechnology, Inc., Rockford, IL, USA). The fluorescence was visualized with a Zeiss Axioplan epifluorescence microscope using a wide pass DAPI filter. Inorganic polyphosphate shifts the emission peak of DAPI from 456nm to 526nm, permitting its visualization in the yellow-green spectrum rather than the blue spectrum associated with nucleic acids and glycosaminoglycans [43,44].
4.2.4 Analysis of gene expression

*In vitro*-formed cartilage constructs and native articular cartilage from the full thickness as well as the superficial, mid and deep zone were harvested and washed twice in PBS. Native tissues were disrupted by mortar and pestle in liquid nitrogen. Total RNA was extracted from tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA (0.5µg) was reverse transcribed into cDNA using Superscript II and Random Hexamers (Invitrogen) according to the protocol provided by the manufacturer. The resulting cDNA was diluted 5x and subjected to relative quantitative polymerase chain reaction (qPCR) with a Realplex² Master Cycler (Eppendorf, Hamburg, Germany). Briefly, 4µl of cDNA was amplified in a 10µl total volume with 0.6µM of each primer (0.4 µM for h-prune) suspended in Express SYBR Green ER (Invitrogen). Reaction mixtures were subjected to a 10 min enzyme activation step at 95°C followed by a 15 sec denaturation step at 95°C and a 30 sec annealing step at 60°C (61°C for h-prune) (primer sequences are provided in Table 4.1). Amplification data were collected for 40 cycles. Mean relative quantification (RQ) values were calculated with 18S rRNA as endogenous control. Calibrators were specific for each experiment. The efficiency of each primer pair was verified. Only primer pairs with efficiency between 90% and 105% were used.

4.2.5 Immunostaining

Deep zone chondrocytes monolayers cultured on glass coverslips in mineralization medium alone or supplemented with 1mM orthophosphate, pyrophosphate or polyphosphate were washed once in PBS and fixed in methanol for 10 minutes at -20°C. Samples were then blocked with 10% horse serum in PBS for 30 minutes at room
temperature and incubated with an antibody reactive to FGF-18 diluted in 3% horse serum in PBS (1:50; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at room temperature for 1 hour. Immunoreactivity was visualized using a secondary antibody conjugated to fluorescein isothiocyanate in 3% horse serum in PBS (1:100; anti-goat IgG; Santa Cruz Biotechnology Inc.) at room temperature for 1 hour. Cells were counterstained with propidium iodide in 2x saline sodium citrate buffer (1:3000; Sigma Chemical Co.). Cells were visualized with a Leica TCS SP2 confocal microscope (Leica Microsystems, Mannheim, Germany). Image analysis was performed with ImageJ. A total of 12 images from triplicates were taken per condition for each experiment, FGF-18 staining was quantified and the background subtracted. The staining intensity was normalized by the number of cells counted in the image.

**Table 4.1: Gene-specific primer sequences.**

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<tr>
<th>Gene</th>
<th>Primer Sequence</th>
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<td></td>
<td>rev: 5’-CAGCACCACAACAGAT TTC-3’</td>
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<tr>
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<td></td>
<td>rev: 5’-CCATCCAATCGTAGATGC-3’</td>
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4.2.6 Mineral Content

Cartilage tissues formed in vitro were harvested, washed twice in PBS and removed from the membrane insert. The tissues were then lyophilized overnight and weighed using an electrical balance (Explorer, Ohaus Corp., Florham Park, NJ, USA). The lyophilized tissues were then wet-ashed as described previously [45]. Briefly, each tissue was placed in an HNO$_3$/HClO$_4$ (4:1 v/v) acid mixture and heated at 150°C for 90 minutes in a closed Teflon beaker. The mixture was then evaporated to a small drop (approximately 100µl) by continued heating following removal of the cover and diluted with dH$_2$O (2ml). The pH of the resulting solutions was adjusted to 2.5-2.9 and distilled deionized water added to a final volume of 3ml.

Calcium content was measured by mixing an aliquot of wet-ashed solution with 0.01% cresolphthalein complexone (Sigma Chemical Co.) buffered with 0.25M sodium borate in a 1:4 v/v ratio and the resulting color quantified spectrophotometrically at 570nm. The standard curve was generated using a calcium chloride solution. Phosphate content was measured by reaction of an aliquot of wet ashed solution with a solution containing 1:6 v/v ratio of 10% ascorbic acid and 0.42% ammonium molybdate (Sigma Chemical Co.) in 1N H$_2$SO$_4$ (1:3 v/v ratio) at 37°C for 1 hour and the resulting color quantified by spectrophotometrical analysis at 620nm. The standard curve was generated using a sodium phosphate dibasic solution (pH=7.5). Calcium-to-phosphorus ratios were calculated from the resulting data.
4.2.7 Exopolyphosphatase Activity

*In vitro*-formed cartilage constructs were harvested and washed twice in PBS. Proteins were extracted and assessed for exopolyphosphatase activity according to a modified protocol by Lorenz et al. [46]. Briefly, cartilage tissues were freeze-thawed three times in 50mM Tris-HCl buffer (pH 7.5) containing 10mM MgCl₂, 0.5mM EDTA, 150mM NaCl and 0.2% Triton X-100 (exopolyphosphatase homogenization buffer; 150µl per construct) and mechanically homogenized. The resulting solutions were centrifuged (14,000 X g for 15 min) and the protein content of the supernatant was measured using a Pierce BCA protein assay. Reaction mixtures were prepared for each sample containing 25µg of protein and 0.4µmol of sodium phosphate glasses in 50mM Tris-HCl buffer (pH 7.5 containing 10mM MgCl₂, 0.5mM EDTA and 150mM NaCl to a final volume of 200µl. To correct for the phosphate content of the protein extracts and resulting from the hydrolysis of the condensed phosphates, mixtures containing only the protein extract (for each sample) or inorganic polyphosphates were also prepared. The reaction mixtures were incubated at 37°C for 24 hours (enzymatic phosphate release from polyphosphate is linear between 1 and 48 hour). The phosphate produced by enzymes with exopolyphosphatase activity was measured spectrophotometrically at 620nm as with samples prepared for quantification of the mineral content. The phosphate standard curve was prepared in the homogenization buffer and with bovine serum albumin at protein levels equivalent to that of the samples.
4.2.8 Statistical Analysis

Experiments were done in triplicate except for histological and gene expression analysis which were done in duplicate and repeated 3 times with cells from different extractions unless specified otherwise. Results are expressed as the mean ± standard error of the mean (SEM) of 3 experiments and analyzed using a one-way ANOVA (for more than 2 conditions) or Student’s t-test (between groups). Tukey’s test post hoc analysis was performed. P values ≤ 0.05 were considered statistically significant.

4.3 Results

4.3.1 Fibroblast growth factor 18 expression is modulated by polyphosphate

To determine if FGF-18 signaling was involved in the inhibitory effects of polyphosphate on cartilage calcification, in vitro-formed deep zone articular cartilage cultured in mineralization medium was treated with two concentrations of polyphosphate (average chain length of 45 phosphate units) for 24 hours. A significant up-regulation of FGF-18 gene expression was observed at the high concentration (1mM) compared to untreated tissues (Figure 4.1A). Orthophosphate, pyrophosphate and short chain polyphosphate (average chain length of 5 phosphate units) did not impact FGF-18 gene expression at 24 hours (Figure 4.1B). However, long chain polyphosphate (average chain length of 45 and 75 phosphate units) caused a significant increase in FGF-18 gene expression compared to untreated tissues. Following the administration of mineralization medium (time 0 hours), FGF-18 gene expression levels were significantly down-regulated to 39% of their initial levels by 48 hours (Figure 4.1C). By 72 hours, this down-regulation was no longer significant. Treatment with polyphosphate prevented this down-regulation.
FGF-18 protein was detected in deep zone chondrocytes cultured in a monolayer in mineralization medium for 48 hours (Figure 4.1D). Treatment with 1mM polyphosphate caused an increase in FGF-18 staining per cell compared to untreated controls (p<0.17). This effect was significant compared to chondrocytes treated with equivalent concentrations of orthophosphate or pyrophosphate (p<0.05). The number of cells per image was lower in samples treated with polyphosphate (20 ± 4) than in untreated (49 ± 5), orthophosphate-treated (68 ± 9) and pyrophosphate-treated (65 ± 8) specimens. The lower chondrocyte count in polyphosphate-treated cultures may be the result of cell detachment during processing, suggesting a decreased adhesion strength to glass coverslips. Alternatively, cell death through apoptosis or necrosis due to the polyphosphate treatment may cause this effect.

4.3.2 Fibroblast growth factor 18 inhibits deep zone chondrocytes mineralization

Similar to polyphosphate treatment [47], the administration of exogenous human recombinant FGF-18 to in vitro-formed deep zone cartilage cultured in mineralization medium for 2 weeks caused a concentration-dependant decrease in mineral deposition within tissues as demonstrated by significantly reduced calcium and phosphate contents measured in cultures treated with FGF-18 concentrations of 10 ng/ml or higher (Figure 4.2A). As shown in Figure 4.2B, the calcium-to-phosphorus ratio in cultures remained unchanged until the cells were treated with higher FGF-18 doses (20 and 40 ng/ml) when it was reduced to 77% and 58% of that of untreated controls, respectively (non-significant differences).
Figure A: Effect of inorganic polyphosphate concentration on RQ value. The y-axis represents log10 of RQ value, and the x-axis represents inorganic polyphosphate concentration (mM).

Figure B: Effect of chain length on RQ value. The y-axis represents log10 of RQ value, and the x-axis represents chain length (phosphate units).

Figure C: Time course of RQ value with and without PP45. The y-axis represents log10 of RQ value, and the x-axis represents time (hrs).

Figure D: Images showing the distribution of MM, Pi, PPi, and PP45. The images show the cellular localization of these compounds under different conditions.
Figure 4.1: Polyphosphate stimulates fibroblast growth factor 18 expression. (A-B) Differential gene expression of FGF-18 as determined by the $\Delta\Delta$Ct method. Deep zone chondrocytes were cultured for 24 hours in mineralization medium supplemented with (A) two concentrations of polyphosphate (average chain length of 45 phosphate units) or (B) orthophosphate or condensed phosphates with different chain lengths (1.0mM). Levels are expressed relative to untreated cells (calibrator). (C) Time course of FGF-18 gene expression in chondrocytes cultured in mineralization medium alone or supplemented with 1.0mM inorganic polyphosphate (average chain length of 45 phosphate units). Results are expressed relative to levels in cartilage at time 0 (calibrator). Each condition was done in duplicates and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean. * indicates a significant difference compared to the calibrator ($p<0.05$). # indicates a significant difference between inorganic polyphosphates-treated and untreated conditions ($p<0.05$). (D) Deep zone chondrocytes cultured on glass cover slips for 48 hours in mineralization medium alone (MM) or supplemented with 1.0mM phosphate buffer (Pi), pyrophosphate (PPi) or polyphosphates (average chain length of 45 phosphate units) and immunostained for FGF-18 (green fluorescence). Cells were counter-stained with propidium iodide (red fluorescence) and visualized by confocal microscopy. Confocal images were taken at a single representative plane. FGF-18 specific fluorescence intensity was measured and normalized by the number of cells in cultures from each condition. Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean.* indicates a significant difference compared to the Pi- and PPi-treated chondrocytes ($p<0.05$). Condensed phosphate concentrations were calculated based on the phosphate content.
Figure 4.2: Fibroblast growth factor 18 inhibits cartilage calcification. (A) Calcium and phosphate contents and (B) calcium-to-phosphorus ratio of deep-zone cartilage formed in vitro on membrane inserts after 2 weeks in mineralization medium supplemented with different concentrations of FGF-18. The data were normalized to tissue dry weight. Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean. * indicates a significantly lower mineral content compared to non-treated tissues.
4.3.3 Fibroblast growth factor 2 expression is also modulated by polyphosphate and inhibits cartilage calcification

To verify if the effects described previously are specific to FGF-18, FGF-1 and FGF-2 gene expression were also investigated in cultures submitted to the aforementioned treatments. Similar to FGF-18, polyphosphate (average chain length of 45 phosphate residues) elicited a dose-dependent effect on FGF-2 gene expression at 24 hours with a significant up-regulation noted at 1mM compared to untreated controls (Figure 4.3A). Conversely, FGF-1 gene expression was not significantly up-regulated by polyphosphate treatment. Orthophosphate, pyrophosphate, and polyphosphates with chain lengths of 5 or 75 phosphate residues did not cause significant FGF-1 or FGF-2 gene expression changes (Figure 4.3B). However, the expression of both genes was significantly reduced by 24 hours following initiation of the mineralization process and remained significantly reduced compared to their initial levels up to 72 hours (Figure 4.3C-D).

Treatment with exogenous human recombinant FGF-2 also led to a significant decrease in mineral deposition within in vitro-formed deep zone cartilage compared to untreated samples, whereas the effect of FGF-1 on mineral deposition was not significant (Figure 4.3E). Although the inhibitory effects of FGF-2 on mineral deposition appeared greater than FGF-18, this difference was not significant.

4.3.4 Fibroblast growth factor receptor signaling modulates cartilage calcification

To confirm the involvement of FGF signaling in cartilage calcification, in vitro-formed deep zone cartilage cultured in mineralization medium was treated with an
Figure 4.3: Effect of polyphosphate on other fibroblast growth factors and their implication in cartilage calcification. (A-B) Differential gene expression of FGF-1 and FGF-2 as determined by the ΔΔCt method. Deep zone chondrocytes were cultured for 24 hours in mineralization medium (MM) supplemented with (A) two concentrations of polyphosphate (average chain length of 45 phosphate units) or (B) orthophosphate or condensed phosphates with different chain lengths (1.0mM). Levels are expressed relative to untreated conditions (calibrator). (C-D) Time course of FGF-1 and FGF-2 gene expression in deep zone chondrocytes cultured in MM alone or supplemented with 1.0mM polyphosphate (average chain length of 45 phosphate units). Results are expressed relative to levels in cartilage at the initiation of the mineralization process (time 0).
0; calibrator). Each condition was done in duplicates and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean. Condensed phosphate concentrations were calculated based on the phosphate content. (E) Calcium and phosphate contents of deep-zone cartilage formed \textit{in vitro} after 2 weeks in MM alone or supplemented with different FGFs (40 ng/ml). NM indicates non-mineralizing culture conditions. The data were normalized to tissue dry weight. Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean. * indicates a significantly difference compared to control tissues (MM).

inhibitor of fibroblast growth factor receptor (FGFR) signaling, PD173074, for 2 weeks. This treatment did not cause a significant increase in mineral deposition in the absence of polyphosphate treatment (Figure 4.4). However, in tissues grown in the presence of a low concentration of polyphosphate (0.1mM), treatment with PD173074 led to significantly increased calcium and phosphate contents, while tissues treated with a high concentration of polyphosphate (1.0mM) did not mineralize in the presence or absence of PD173074. The low polyphosphate concentration was selected because it does not fully inhibit mineral deposition, while the high concentration fully inhibits cartilage calcification.

4.3.5 Combined effects of polyphosphate and fibroblast growth factor 18 on cartilage calcification

Others have demonstrated that polyphosphates physically and functionally stabilize FGF-2 [23]. To determine if a similar effect is taking place in this system, the effect of combined treatment with low concentrations of polyphosphate (0.05mM) and FGF-18 (10ng/ml) on \textit{in vitro}-formed deep zone cartilage cultured in mineralization medium for 2 weeks was investigated. The inhibition effect of the combined treatment was additive and was not further enhanced suggesting that growth factor stabilization is not occurring in this system (Figure 4.5).
Figure 4.4: Effect of fibroblast growth factor receptor signaling inhibitor on cartilage calcification. Calcium and phosphate contents of cartilage formed in vitro in mineralization medium alone or supplemented with 0.1 or 1.0mM polyphosphate (PP45; concentrations calculated based on the phosphate content; average chain length of 45 phosphate units) for 2 weeks. For each concentration of polyphosphate, cultures were maintained in the absence or presence of 30nM PD173074, an inhibitor of fibroblast growth factor receptor signaling. The data were normalized to tissue dry weight and expressed relative to untreated control. Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean. * indicates a significant difference between PD173074-treated and untreated samples at a given inorganic polyphosphate concentration.
Figure 4.5: Effect of fibroblast growth factor 18 and polyphosphate co-treatment on cartilage calcification. Percent inhibition in the calcium and phosphate deposition within cartilage formed in vitro in mineralization medium (MM) for 2 weeks and treated with fibroblast growth factor 18 (FGF-18; 10 ng/ml), polyphosphate (PP45; concentration calculated based on the phosphate content; 0.05mM), or co-treatment (FGF-18 and PP45) compared to untreated controls in MM. The result was normalized to tissue dry weight. Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean.
4.3.6 Effect of fibroblast growth factor signaling on polyphosphate metabolism

The effect of FGF-18 signaling on polyphosphate levels within in vitro-formed cartilage cultured in mineralization medium for 1 week was investigated (Figure 4.6). Compared to untreated controls (Figure 4.6A), tissues treated with FGF-18 exhibited increased DAPI staining in the yellow-green range suggesting increased polyphosphate accumulation within the tissues (Figure 4.6B). Inhibition of FGFR signaling with PD173074 appeared to cause a slight decrease in DAPI staining within the tissue (Figure 4.6C) compared to untreated controls. Samples cultured with polyphosphate (Figure 4.6D) or in non-mineralizing conditions (Figure 4.6E) exhibited intense DAPI staining.

Because FGF-18 signaling was shown to influence polyphosphate levels within in vitro-formed cartilage, its effect on exopolyphosphatases was investigated. Treatment of deep zone chondrocytes cultured in mineralization medium with FGF-18 for 24 hours resulted in a significant decrease in TNAP gene expression compared to untreated controls, whereas treatment with PD173074 did not significantly alter gene expression (Figure 4.7A). Treatment with polyphosphate also caused a concentration-dependent increase in TNAP gene expression with a significant difference detected at 1mM compared to untreated control (Figure 4.7B). Orthophosphate and pyrophosphate did not alter TNAP gene expression. However, TNAP gene expression was significantly higher within chondrocytes cultured in non-mineralizing conditions compared to mineralization medium. Conversely, h-prune gene expression was not significantly modulated by these treatments (Figure 4.7A-B). The same trends were observed for exopolyphosphatase activity measurements on cartilage tissue extracts after 2 weeks in culture as for TNAP gene expression (Figure 4.7C-D).
Figure 4.6: Polyphosphate accumulation within in vitro-formed deep zone cartilage. Polyphosphate distribution within cartilage formed after 1 week of culture in mineralization medium alone (A) or treated with (B) 20 ng/ml fibroblast growth factor 18, (C) 30nM PD173074, (D) 1.0mM inorganic polyphosphates (based on the phosphate content), or (E) in non-mineralizing culture conditions as visualized by epifluorescence microscopy of histological sections stained with DAPI (yellow-green staining). * indicates the membrane insert. The arrowheads indicate mineral deposits.
Figure 4.7: Effect of polyphosphate and fibroblast growth factor 18 on exopolyphosphatases. (A-B) Expression level of exopolyphosphatase genes in deep zone cartilage formed in vitro on membrane inserts in mineralization medium (MM) and treated with (A) FGF-18, or PD173074, or (B) phosphate species for 24 hours. Differential gene expression was determined by the ΔΔCt method after normalization to 18S rRNA. Cultures were compared to levels in cartilage formed in untreated conditions (calibrator). Each condition was done in duplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean. (C-D) Exopolyphosphatase activity of cartilage cultured for 2 weeks in MM and treated with (C) FGF-18, or PD173074, or (D) phosphate species. The data were normalized to untreated samples in MM alone. Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean. * indicates a significant difference compared to untreated controls (p<0.05). Condensed phosphate concentrations were calculated based on the phosphate content. NM refers to non-mineralizing culture conditions; PP45 refers to inorganic polyphosphates; Pi refers to phosphate buffer; PPi refers to inorganic pyrophosphate
4.3.7 Zonal distribution of polyphosphate, exopolyphosphatases and fibroblast growth factors in native articular cartilage

To gain insight on the interactions between FGF-18 signaling and polyphosphate metabolism in native articular cartilage, the zonal distribution of these molecules was investigated. DAPI staining of native articular cartilage demonstrates that polyphosphate is concentrated in the pericellular region of deep zone chondrocytes above the ZCC (Figure 4.8A). Some staining is also apparent in the superficial zone, whereas the mid-zone of articular cartilage and the territorial matrix of the deep zone seem to have low concentrations of the phosphate polymers.

There appears to be a correlation between the zonal distribution of polyphosphate and the associated exopolyphosphatase activity of protein extracts as the deep zone exhibits the highest activity followed by the superficial zone and mid-zone (Figure 4.8B). Gene expression of chondrocytes from the different zones of native articular cartilage is presented in Figure 4.8C. Both FGF-2 and FGF-18 were significantly upregulated in the superficial zone compared to the other zones. FGF-1 expression was higher in the mid-zone compared to the other zones (not significant). FGFR3c is the preferential receptor for FGF-18 and was expressed diffusely throughout the three zones. Another receptor for FGF-18, FGFR2c was highly expressed in the superficial zone (significant compared to other zones). The exopolyphosphatase h-prune did not show significant differences in zonal expression in native cartilage, whereas TNAP gene expression was significantly increased in the deep zone compared to the other two zones.
Exopolyphosphatase Activity (% of full thickness cartilage)

A

SZ

MZ

DZ

100µm

B

Exopolyphosphatase Activity (% of full thickness cartilage)

SZ

MZ

DZ

*
Figure 4.8: Zonal distribution of polyphosphate, exopolyphosphatases and fibroblasts growth factors in native articular cartilage. (A) Histological section of native articular cartilage stained with DAPI. The tissue was visualized by epifluorescence microscopy. (B) Exopolyphosphatase activity of the superficial, mid and deep zones of native articular cartilage. The data were normalized to full thickness cartilage. Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean. (C) Expression level of fibroblast growth factor signaling and exopolyphosphatase genes in chondrocytes of the superficial, mid and deep zone of native articular cartilage. Differential gene expression was determined by the ∆∆Ct method after normalization to 18S rRNA. Cultures were compared to levels in full thickness cartilage (calibrator). Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean. SZ = superficial zone; MZ = mid-zone; DZ = deep zone. * indicates a significant difference compared to other zones (p<0.05).
4.4 Discussion

Our group has previously demonstrated that polyphosphate inhibits cartilage calcification in a three-dimensional tissue culture system [47]. In this study, it is demonstrated that this inhibition is accompanied by an up-regulation of FGF-18 gene expression and protein levels within deep zone chondrocytes. This effect is specific to polyphosphate because phosphate, the degradation product of condensed phosphates by exopolyphosphatases (or hydrolysis), did not significantly impact FGF-18 gene and protein levels. To the best of our knowledge, this is the first report demonstrating that polyphosphate modulates the expression of FGF-18. Interestingly, pyrophosphate and short chain polyphosphate with an average chain length of 5 phosphate residues did not up-regulate FGF-18 gene expression, suggesting that this effect is specific to a range of condensed phosphate chain lengths. Chain length specificity in modulating cellular pathways has been demonstrated by others [48,49].

The data also suggest that FGF-18 causes increased polyphosphate accumulation in an in vitro cartilage model by decreasing the exopolyphosphatase activity of the tissue. This function of FGF-18 could explain its inhibitory effects on cartilage calcification. It also appears that the signaling pathway regulating in vitro calcification of cartilage includes a feedback mechanism, whereby high polyphosphate concentrations lead to increased exopolyphosphatase activity. As the deep zone of native articular cartilage exhibits strong polyphosphate staining, the mechanism described above may contribute to the regulation of the orthophosphate-to-condensed phosphates ratio and participate in the control of tissue calcification.
The study of native articular cartilage suggests that the findings from the in vitro cartilage calcification model are likely not an artefact of in vitro culture. In fact, FGF-18 gene expression occurs preferentially in the superficial zone, whereas FGFR3c is expressed in all zones of the tissue and exopolyphosphatase activity is predominantly in the deep zone. From these results, it is proposed that a gradient of FGF-18 may exist across the depth of articular cartilage that confines the gene expression of TNAP to the deep zone. While it was not the focus of this study, the mechanism(s) by which polyphosphate is synthesized in articular cartilage is also essential to a complete understanding of the role played by polyphosphate in the modulation of the mineralization process.

Along with its inhibitory effect on cartilage calcification, treatment with high doses of FGF-18 resulted in lower calcium-to-phosphorus ratios compared to untreated tissues. This suggests that treatment with FGF-18 leads to a dose dependant accumulation of phosphate species within the tissue, possibly condensed phosphates. This is supported by the increased polyphosphate staining observed in tissues treated with FGF-18 compared to untreated cartilage. Interestingly, FGF-18 expression is down-regulated following initiation of the mineralization process, suggesting that either decreased FGF-18 levels are necessary for mineral deposition to take place under the experimental conditions utilized in this study or that the decreased polyphosphate levels associated with the onset of mineralization lead to lower levels of FGF-18 expression. Treatment of mineralizing cartilage with a pharmacological inhibitor of FGFR signaling, PD173074, did not cause increased mineral deposition within the tissue. This was not surprising given the decrease in FGF-1, FGF-2 and FGF-18 expression levels that occurred
following the initiation of the mineralization process. However, treatment with PD173074 in the presence of a low concentration of polyphosphate (0.1mM) resulted in a significant recovery of the mineral deposition. This supports a role for FGF signaling in polyphosphate regulation of tissue mineralization. Treatment with PD173074 in the presence of a high concentration of polyphosphate (1mM) did not exhibit this effect, likely because the system is overwhelmed by polyphosphate, which inhibits mineral deposition via physicochemical interactions with crystal nuclei [50].

An alternative mechanism to explain how FGF-18 and polyphosphate may regulate mineralization is the possibility that polyphosphate and FGF-18 form a complex that increases the half-life of the growth factor and facilitates binding to its receptor. This was demonstrated by Shiba et al. (2003) for FGF-2 in fibroblasts [23]. However, this possibility was ruled out, as no functional stabilization was observed for FGF-18 administered with polyphosphate and their effects were additive under the experimental conditions investigated.

FGF-18 appears to cause increased accumulation of polyphosphate within cartilage cultured in mineralization medium. This is accompanied by significant decreases in the exopolyphosphatase activity and mineral content. Taken together, these results suggest that the endogenous levels of polyphosphate in cartilage are modulated in part by the effect of FGF-18 on exopolyphosphatase activity and can regulate tissue mineralization. When cartilaginous tissues were cultured in mineralization medium in the presence of a high concentration of polyphosphate (1mM), increased FGF-18 levels were observed. This would have been anticipated to result in, a reduction in exopolyphosphatase activity. Instead, the opposite effect was observed. Similarly, in non-
mineralizing culture conditions, there is intense polyphosphate accumulation in the tissues together with elevated exopolyphosphatase activity. These results suggest that a yet undefined signaling pathway acts to elevate the exopolyphosphatase activity in the presence of high concentrations of polyphosphate. Such a feedback mechanism may be essential to closely regulate the endogenous levels of polyphosphate and orthophosphate in conjunction with FGF-18 signaling, thereby controlling the mineralization process. A schematic diagram of the mechanism proposed based on the results presented in this study is shown in Figure 4.9.

The effects of polyphosphate and FGF-18 on the gene expression of two enzymes with known exopolyphosphatase activity, TNAP [13] and h-prune [15], were investigated. TNAP gene expression was significantly down-regulated by FGF-18 treatment, while the administration of PD173074 resulted in a non-significant up-regulation, correlating with exopolyphosphatase activity. TNAP gene expression was also significantly up-regulated both in non-mineralizing culture conditions as well as in the presence of a high polyphosphate concentration (1mM) similar to the effects on exopolyphosphatase activity. Conversely, h-prune gene expression was not affected by these treatments. This result was not entirely unexpected, as it has been shown that h-prune preferentially cleaves short polyphosphate chains (tripolyphosphate and tetrapolyphosphate) and its activity is inhibited by longer chain phosphate polymers, [15]. These results suggest that TNAP is involved in the metabolism of polyphosphate within deep zone cartilage. However, further studies are required to confirm this. The physiological factors that may affect the polyphosphate levels in articular cartilage
Figure 4.9: Proposed mechanism for the modulation of inorganic polyphosphate levels and mineralization within in vitro-formed articular cartilage with a ZCC. In this mechanism, it is suggested that the ratio of polyphosphate-to-orthophosphate in cartilage will dictate if tissue mineralization will take place with a high polyphosphate level resulting in calcification inhibition. Data presented in this study suggest that the levels of polyphosphate are modulated in part by the inhibitory effect of fibroblast growth factor 18 on the activity of exopolyphosphatases with the ability to cleave polyphosphate, thereby releasing orthophosphate. It was also observed that high polyphosphate concentrations result in increased exopolyphosphatase activity, possibly indicating a feedback mechanism. PP refers to polyphosphate, Pi refers to orthophosphate and exoPPase refers to exopolyphosphatase.
remain unknown but could include the oxygen tension and the tissue pH, which are properties susceptible to change across the depth of articular cartilage.

Other FGF family members have been implicated in regulating tissue mineralization and the current study also found that FGF-2 may be involved in cartilage calcification. FGF-2 (but not FGF-1) was also shown to significantly inhibit mineral deposition within in vitro-formed cartilage. The temporal profiles of FGF-1 and FGF-2 expression were similar to that of FGF-18, exhibiting a significant down-regulation following the initiation of the mineralization process. However, polyphosphate treatment did not cause an up-regulation of their expression to initial levels. This would suggest that polyphosphate does not regulate the expression of these growth factors relevantly. Interestingly, others have shown that FGF-2 is involved in the metabolism of pyrophosphate [35]. The synthesis of pyrophosphate occurs via ATP-dependant reactions [51], while a number of studies have demonstrated that the synthesis of polyphosphate in vertebrates is independent of ATP [10,52]. This suggests that different signaling pathways regulate the synthesis of pyrophosphate and polyphosphate. However, our data suggest that TNAP may be involved in the degradation of polyphosphate, indicating a potential overlap between the pathways regulating polyphosphate and pyrophosphate degradation. Hence, it is possible that FGF-2 also plays a role in the modulation of polyphosphate levels through the expression of TNAP. Further investigations will be required to clarify these observations.

The predominant expression of FGF-18 in the superficial zone of native articular cartilage and the diffuse expression of its preferential receptor, FGFR3c, may indicate the presence of a FGF-18 gradient modulating exopolyphosphatase activity and the
orthophosphate-to-polyphosphate ratio in the tissue. Interestingly, preliminary work has indicated that superficial zone chondrocytes co-cultured with in vitro-formed deep zone cartilage inhibit its calcification. This effect is accompanied by increased polyphosphate staining and exopolyphosphatase activity within the deep zone cultures (Appendix 1). Further work is under way to establish the role of superficial zone chondrocytes in polyphosphate metabolism. Others have previously demonstrated that the co-culture of superficial and deep zone chondrocytes in a system allowing for cell-to-cell contact between the two chondrocytes sub-populations led to decreased alkaline phosphatase activity and inhibition of the mineralization process [53]. This study also indicated that parathyroid hormone related peptide (PTHrP) signaling plays a role in the mechanism by which superficial zone chondrocytes inhibit articular cartilage mineralization. Interestingly, a number of studies have linked FGF-2 and FGFR3 signaling to the expression of PTHrP [37,54,55]. Additional work will be required to verify the involvement of PTHrP in the control of polyphosphate metabolism. A better understanding of the mechanisms by which cartilage calcification is modulated is essential to the formation of bioengineered cartilage incorporating a ZCC closely mimicking the native tissue.

4.5 Conclusion

In summary, this study demonstrates that cartilage mineralization is modulated by the inhibitory action of FGF-18 on exopolyphosphatases, which controls polyphosphate levels within the tissue. Polyphosphates also exhibit stimulatory effects on exopolyphosphatases possibly indicating a feedback control mechanism. TNAP may be
the exopolyphosphatase involved in articular cartilage calcification. The mechanisms by which polyphosphate levels control tissue mineralization remain poorly understood and require a better understanding of the pathways and enzymes by which polyphosphate synthesis is regulated.

4.6 References


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CHAPTER 5: INORGANIC POLYPHOSPHATE STIMULATES CARTILAGE MATRIX DEPOSITION

Jean-Philippe St-Pierre\textsuperscript{1}, Quishan Wang\textsuperscript{2}, Shu Qiu Li\textsuperscript{2}, Robert M. Pilliar\textsuperscript{1,2,3}, Rita A. Kandel\textsuperscript{1,2}.
\textsuperscript{1}Department of Materials Science and Engineering and Institute of Biomaterials and Biomedical Engineering, University of Toronto. \textsuperscript{2}CIHR-BioEngineering of Skeletal Tissue Team, Mount Sinai Hospital, Toronto. \textsuperscript{3}Faculty of Dentistry, University of Toronto.

As first author of this multi-author contribution, I designed and conducted most of the experiments. Histological sections were prepared by others. I also analyzed the results and wrote the manuscript.
5.1 Introduction

Articular cartilage is a connective tissue that covers the articulating surfaces of bones in synovial joints and permits low friction gliding as well as the transmission and distribution of applied forces to the subchondral bone. The avascular nature of articular cartilage contributes to its limited intrinsic capacity for self-repair following partial thickness damage caused by trauma and/or disease [1,2]. Full thickness defects are temporarily replaced by fibrocartilagenous tissue of inferior mechanical quality due to the disruption of the vascularised subchondral bone [1]. Hence, cartilage tissue damage often leads to the progressive development of osteoarthritis with resultant pain and loss of range of motion.

Current surgical interventions to enhance the repair of cartilage such as marrow stimulation techniques and autologous chondrocyte implantation (ACI) have yielded limited long-term clinical success, partly because of the fibrocartilagenous nature of the resulting repair tissue [1]. Efforts to improve the functional outcome of cell-based techniques such as ACI have resulted in the development of numerous tissue engineering approaches to deliver chondrogenic cells or in vitro-formed hyaline-like cartilage to the injury site. However, tissue engineered cartilage is often characterized by low contents of extracellular matrix components compared to native articular cartilage which results in inferior tissue mechanical properties and limited long-term in vivo survival [3,4].

Various approaches have been investigated to improve the quality of tissue engineered cartilage including the stimulation of tissue formation with growth factors during the in vitro maturation period and/or following implantation in vivo. Growth factors with demonstrated anabolic effects on cartilage include IGF-1, FGF-2, FGF-18,
TGF-β1, BMP-2, BMP-4, BMP-5 and BMP-7 and have been reviewed in detail [5]. Our group and others have shown that mechanical stimulation of \textit{in vitro}-formed cartilage increases extracellular matrix accumulation and yields improved tissue mechanical properties [3,6-9]. Other techniques such as the application of ultrasound [10,11], laser stimulation [12] and changes in the culture conditions such as oxygen tension [13,14] and temperature [15] have all resulted in modest improvements in matrix accumulation. Despite these efforts, \textit{in vitro}-formed cartilage remains qualitatively and functionally inferior to native articular cartilage and our understanding of the signals that stimulate extracellular matrix deposition by chondrocytes is still lacking.

Inorganic polyphosphate is a linear polymer of orthophosphate residues linked by energy-rich phosphoanhydride bonds that is ubiquitous in biological systems as it has been identified in bacteria, fungi, algae, insects, plants and animals [16,17]. While polyphosphate has been extensively investigated in lower organisms, its functions in mammalian cells and tissues have only recently started to be uncovered. Treatment with the phosphate polymer has been shown to modulate mineralization in different tissue types through direct physico-chemical interactions with nucleating mineral crystals, as well as biological responses [18-23]. Polyphosphate also exhibits other biological effects including the ability to direct mesenchymal stem cell differentiation towards the osteoblastic phenotype [26,27], to act as a pro-coagulant and pro-inflammatory mediator [28-30], to modulate the activity of ion channels [31] to block tumour metastasis [32] and to enhance the mitogenic activity of different cell types through stabilization of growth factors such as basic FGF-2 and activation of the mTor pathway [24,25].
Our group has demonstrated that exogenously administered polyphosphate reversibly inhibits the formation of a zone of calcified cartilage within in vitro-formed hyaline cartilage [33]. It was also observed that cartilage tissues treated with condensed phosphate are characterized by an increased thickness compared to untreated controls (unpublished data). Furthermore, polyphosphate has been shown to functionally stabilize FGF-2, a growth factor which has been suggested to exhibit an anabolic effect on chondrocytes [24]. Thus, it is hypothesized that the exogenous administration of polyphosphate to three dimensional in vitro cultures of chondrocytes will enhance cartilage tissue accumulation. In this study, the effect of polyphosphates of average chain lengths ranging from 5 to 75 phosphate units on matrix accumulation within in vitro-formed cartilage was examined along with the ability of chondrocytes to metabolise condensed phosphate through enzymes with exopolyphosphatase activity. The responsiveness of different articular chondrocytes zonal subpopulations cultured in vitro and of native cartilage samples cultured ex vivo to polyphosphate was also investigated. Identifying molecules that positively impact matrix accumulation in cartilage will facilitate successful engineering of cartilage for joint repair.

5.2 Materials and Methods

5.2.1 Substrates

Membrane inserts (Millicell-CM<sup>R</sup>, Millipore Corp., Bedford, MA, USA) were coated with type II collagen (0.5mg/ml in 0.1N acetic acid; Sigma Chemical Co., St.Louis, MO, USA) and dried overnight. The membrane inserts were UV sterilized for 30 minutes and soaked in Ham’s F12 for 30 minutes prior to cell culturing.
5.2.2 Tissue Culture

Cartilage was aseptically excised from the full thickness of metacarpal-phalangeal articular cartilage from 9 to 12 months old calves within 24 hours of death as previously described [34]. Chondrocytes were isolated from the tissue by sequential enzymatic digestion (0.5% protease (Sigma Chemical Co.) for 2 hours followed by 0.1% collagenase (Roche Diagnostics GmbH, Mannheim, Germany) overnight). The cells were then seeded on top of membrane inserts (1X10^6 cells per membrane) in Ham’s F-12 supplemented with 5% fetal bovine serum (FBS) (HyClone, Logan, UT, USA) and incubated at 37°C in an atmosphere characterized by 95% relative humidity and 5% CO₂. On day 5, the serum concentration was increased to 20% and supplemented with ascorbic acid (100µg/ml, Sigma Chemical Co.). At this time, cultures were incubated in the presence or absence of various concentrations of sodium phosphate glasses (inorganic polyphosphate; Sigma Chemical Co.) or sodium phosphate buffer. The concentrations of polyphosphate utilized for each experiment are calculated based on the phosphate content. Cultures were grown for up to 4 weeks following initiation of the inorganic polyphosphate treatment. The culture medium was changed every 2-3 days.

For the experiments investigating the response of chondrocytes zonal subpopulations to polyphosphate, the upper 70% (superficial-mid zone) and the bottom 30% (deep zone) of the cartilage layer were harvested separately and processed as with full thickness chondrocytes.

To investigate the effect of polyphosphate on native cartilage, 4mm diameter cartilage samples were obtained from excised full thickness cartilage using a biopsy
punch and cultured directly in 24-well plates under the same conditions as \textit{in vitro}-formed cartilage.

\subsection*{5.2.3 Histological Evaluation}

\textit{In vitro} and \textit{ex vivo}-cultured cartilage tissues were harvested and washed twice in phosphate buffered saline (PBS). The tissues were fixed in 10\% formalin and embedded in paraffin. Five micron sections were cut, stained with toluidine blue and examined by light microscopy. To visualize the presence of polyphosphate in the tissues, sections were cut, dewaxed in xylene and stained with 4\'-6-diamidino-2-phenylindole (DAPI) (5 \( \mu \)g ml\(^{-1} \) in dH\(_2\)O, Pierce Biotechnology, Inc., Rockford, IL, USA). The fluorescence was visualized with a Zeiss Axioplan epifluorescence microscope using a wide pass DAPI filter. Inorganic polyphosphate specifically shifts the emission peak of DAPI from 456nm to 526nm, permitting its visualization in the yellow-green spectrum rather than the blue spectrum associated with nucleic acids or glycosaminoglycans (GAG) [35,36].

\subsection*{5.2.4 Dry Weight of Cartilaginous Tissue}

Cartilage tissues formed \textit{in vitro} on membrane inserts were harvested, washed twice in PBS and removed from membrane inserts. The tissues were then lyophilized overnight and weighed using an electrical balance (Explorer, Ohaus Corp., Florham Park, NJ, USA).
5.2.5 Determination of DNA Content

Cartilage tissues were digested with papain (40µg·ml⁻¹; Sigma Chemical Co.) in digestion buffer (20mM ammonium acetate, 1mM EDTA and 2mM DTT) for 48 hours at 65°C. Papain digests then stored at -20°C until further analysis. The DNA content was assessed using the Hoechst 33258 dye (Polysciences, Inc., Washington, PA, USA) binding assay and fluorometry (excitation wavelength: 365nm; emission wavelength: 458nm) [37]. The standard curve was generated with calf thymus DNA (Sigma Chemical Co.).

5.2.6 Determination of proteoglycan and collagen content

The proteoglycan and collagen contents of cartilage tissues were measured from aliquots of the papain digest. The proteoglycan content was estimated by quantifying the amount of sulphated GAG using the dimethylmethylene blue dye (Polysciences, Inc.) binding assay and quantifying the colour spectrophotometrically at 525nm [38]. The standard curve was generated with bovine trachea chondroitin sulphate A (Sigma Chemical Co.). The collagen content was estimated by quantifying hydroxyproline. Papain digest aliquots were hydrolyzed in 6N HCl at 110°C for 18 hours. The hydroxyproline of the hydrolysate was determined using the choramine-T/Ehrlich’s reagent assay and the colour change quantified spectrophotometrically at 560nm [39]. The standard curve was generated with L-hydroxyproline (Sigma Chemical Co.).

5.2.7 Analysis of gene expression

Total RNA was extracted from in vitro-formed cartilage after culture in the presence or absence of inorganic polyphosphate for various time points up to 2 weeks using Trizol
reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was reverse transcribed into cDNA using Superscript II and Random Hexamers (Invitrogen) according to the protocol provided by the manufacturer. The resulting cDNA was subjected to polymerase chain reaction (PCR) in an Eppendorf Mastercycler PCR using primers and conditions described in Table 5.1. PCR products were analyzed by electrophoresis on a 1.5% agarose gel and stained with SYBR Safe (Invitrogen).

Table 5.1: Gene-specific primer sequences and amplification conditions.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Product Size (bp)</th>
<th>PCR Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I collagen</td>
<td>fwd: 5'-TGCTGGCCAACTATGCCTCT-3' rev: 5'-TTGCACAATGCTCTGATC-3'</td>
<td>496</td>
<td>95°C 60s, 60°C 60s, 72°C 60s, 40 cycles</td>
</tr>
<tr>
<td>Type II collagen</td>
<td>fwd: 5'-CCACTGCAAGAACAGCATTG-3' rev: 5'-CCAGTTCAGGTCTCTTTAGAG-3'</td>
<td>463</td>
<td>95°C 60s, 60°C 60s, 72°C 60s, 26 cycles</td>
</tr>
<tr>
<td>Type X collagen</td>
<td>fwd: 5'-ATACACGTGAAAGGGACCATTGCT-3' rev: 5'-TTGGAGCCACCAAGAATCCTGAGA-3'</td>
<td>231</td>
<td>95°C 60s, 60°C 60s, 72°C 60s, 32 cycles</td>
</tr>
<tr>
<td>Aggrecan</td>
<td>fwd: 5'-CAGCTTACCCACCTCCCCT-3' rev: 5'-GACATCGTTCCACCAAGACTGCCCCT-3'</td>
<td>303</td>
<td>95°C 60s, 60°C 60s, 72°C 60s, 26 cycles</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>fwd: 5'-AAACGGCTACCACATCCAAG-3' rev: 5'-CCTCAATGGATCTGTTA-3'</td>
<td>150</td>
<td>94°C 60s, 56°C 60s, 72°C 60s, 26 cycles</td>
</tr>
</tbody>
</table>

5.2.8 Exopolyphosphatase Activity of Cartilaginous Tissues

In vitro-formed cartilage constructs were harvested and washed twice in PBS. Proteins were extracted and assessed for exopolyphosphatase activity according to a protocol modified from Lorenz et al. [40]. Briefly, cartilage tissues were freeze-thawed three times in 50mM Tris-HCl buffer (pH 7.5) containing 10mM MgCl2, 0.5mM EDTA, 150mM NaCl and 0.2% Triton X-100 (homogenization buffer; 150µl per construct) and mechanically homogenized. The resulting solutions were centrifuged (14,000 X g for 15
min) and the protein content of the supernatant was measured using a Pierce BCA protein assay. Reaction mixtures were prepared for each sample containing 25µg of protein and 0.4µmol of sodium phosphate glass (average chain length of 45 phosphate units) in reaction buffer (50mM Tris-HCl buffer (pH 7.5), 10mM MgCl₂, 0.5mM EDTA and 150mM NaCl) to a final volume of 200µl. To correct for the phosphate content of protein extracts and hydrolysis of the condensed phosphate, mixtures containing only the protein extract (for each sample) or inorganic polyphosphate were also prepared. The reaction mixtures were incubated at 37°C for 24 hours (enzymatic phosphate release from polyphosphate is linear between 1 and 48 hours). The phosphate released was measured spectrophotometrically at 620nm. The phosphate standard curve was prepared using the homogenization buffer and bovine serum albumin at protein levels equivalent to that of samples.

### 5.2.9 Statistical Analysis

All experiments were done in triplicate and repeated 3 times with cells from different extractions. Results are expressed as the mean ± standard error of the mean unless specified otherwise and analyzed using a one-way ANOVA (for more than 2 conditions) or Student’s t-test (between groups). Tukey’s test post hoc analysis was performed. P values ≤ 0.05 were considered statistically significant.
5.3 Results

5.3.1 Inorganic polyphosphate stimulates chondrocyte matrix accumulation

Chondrocytes obtained from full thickness cartilage were grown in vitro on membrane inserts for 2 weeks in media supplemented with different concentrations of inorganic polyphosphate (chain length of 45 phosphate units). Treatment with polyphosphate at a concentration of 1mM resulted in significant increases in GAG and collagen normalized to DNA compared to cartilage formed in medium that was not supplemented with polyphosphate (Figure 5.1A). Lower concentrations of polyphosphate resulted in more modest increases in matrix accumulation. The DNA content of in vitro-formed cartilage treated with 1mM polyphosphate was significantly lower than that in non-treated tissues (Figure 5.1B).

Supplementation of the medium with equivalent concentrations of sodium phosphate to those used for polyphosphate treatment did not significantly alter the DNA, GAG and collagen contents in these tissues compared to cartilage formed in non-supplemented medium (Table 5.2). This suggests that the stimulatory effect of polyphosphate on cartilage matrix accumulation is specific to condensed phosphate rather than its degradation product, orthophosphate.

5.3.2 Inorganic polyphosphate upregulates the expression of cartilage matrix genes

Chondrocytes were grown in vitro on membrane inserts for up to 1 week in media supplemented with polyphosphate or left untreated. Treated tissues exhibited slightly increased collagen type II and aggregcan gene expression levels compared to
Figure 5.1: Effect of inorganic polyphosphate on in vitro-formed cartilage matrix and DNA accumulation. (A) Glycosaminoglycan and collagen contents and (B) DNA content of full thickness cartilage formed in vitro on membrane inserts after 2 weeks in medium supplemented with different concentrations of polyphosphate (concentrations calculated based on the phosphate content; average chain length of 45 phosphate units). The data were normalized to DNA content and presented as percent change compared to non-treated control. Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean. * indicates a significant increase compared to non-treated tissues.
untreated samples at the same time point (Figure 5.2). Conversely, collagens type I and type X gene expression levels remained unchanged by the polyphosphate treatment.

### Table 5.2: Glycosaminoglycan and collagen contents of cartilage

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DNA Content (µg)</th>
<th>GAG Content (% of control)</th>
<th>Collagen Content (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>9.05 ± 0.25</td>
<td>100.0 ± 0.0</td>
<td>100.0 ± 0.0</td>
</tr>
<tr>
<td>Phosphate</td>
<td>8.48 ± 0.92</td>
<td>112.5 ± 14.8</td>
<td>105.3 ± 9.5</td>
</tr>
<tr>
<td>Polyphosphate</td>
<td>6.81 ± 0.71</td>
<td>178.9 ± 15.1*</td>
<td>147.6 ± 16.1*</td>
</tr>
</tbody>
</table>

Chondrocytes were grown in the presence of phosphate (1mM) or polyphosphate (1mM based on the phosphate content; chain length of 45 phosphate units). The DNA, GAG and Collagen contents were determined after 2 weeks in culture. The data are averaged from three experiments performed in triplicates and expressed as mean ± standard error of the mean. The matrix content is normalized to DNA content and matrix content of untreated controls. * indicates a significantly different content than untreated tissues (p<0.05).

#### 5.3.3 Effect of inorganic polyphosphate chain length on matrix accumulation

Chondrocytes were grown *in vitro* on membrane inserts for 2 weeks in medium supplemented with 1mM polyphosphates characterized by various average chain lengths (5, 45 and 75 phosphate units). Only polyphosphate with an average chain length of 45 phosphate units exhibited significant increases in both GAG and collagen contents in the tissues (Figure 5.3A). Based on these results, polyphosphate with an average chain length of 45 phosphate units was employed for all further studies.

To further understand the effect of polyphosphate chain length on cartilage matrix accumulation, the activity of exopolyphosphatases in protein extracts from 3 day old *in vitro*-formed full thickness cartilage was measured following reactions with polyphosphates of the 3 chain lengths investigated in this study. As shown in Figure
Figure 5.2: Effect of polyphosphate treatment on cartilage extracellular matrix molecules gene expression. Total RNA was extracted from *in vitro*-formed full thickness cartilage cultured in the presence of polyphosphate (1mM, calculated based on the phosphate content; average chain length of 45 phosphate units) or left untreated at different time points from 6 hours to 2 weeks. This is one representative agarose gel from an experiment repeated two times. Gene expression of 18S rRNA was used as a housekeeping gene.
5.3B, the exopolyphosphatases present in the *in vitro*-formed cartilage cleaved significantly more short chain polyphosphate (5 phosphate units) than longer chain polyphosphate (45 and 75 phosphate units). No significant difference was observed between the two longer chain length polyphosphates. This experiment was repeated with polyphosphate concentrations measured based on the polyphosphate rather than the phosphate content and similar trends were observed (data not shown).

### 5.3.4 Time course of inorganic polyphosphate effect on matrix accumulation

Chondrocytes were grown *in vitro* on membrane inserts for 0, 1, 2 or 4 weeks after initiation of the treatment with 1mM polyphosphate in selected samples. The DNA content of non-treated tissues increased by more than 125% in 4 weeks, while polyphosphate treated tissues exhibited a more modest proliferation rate of 75% (Figure 5.4A). The DNA content of treated tissues was significantly lower than that of controls at 4 weeks. After normalization by the DNA content, GAG (Figure 5.4B) and collagen (Figure 5.4C) accumulation within tissues formed in the absence of polyphosphate increases rapidly in the first week of culture and then remains stable up to 4 weeks. Matrix accumulation within tissues treated with polyphosphate increases up to 2 weeks of culture and remains stable until 4 weeks. The GAG content was significantly higher in tissues treated with polyphosphate compared to the untreated condition at 2 and 4 weeks. However, a significant difference between polyphosphate-treated and untreated tissues was only observed at 4 weeks for the collagen content.
Figure 5.3: Effects of inorganic polyphosphate chain length on in vitro-formed cartilage matrix accumulation and exopolyphosphatase activity. (A) Glycosaminoglycan and collagen contents of full thickness cartilage formed in vitro on membrane inserts in medium supplemented with polyphosphate (1mM, calculated based on the phosphate content) of different chain lengths for 2 weeks. The data were normalized to DNA content and presented as percent change compared to non-treated control. Each condition was done in triplicate and the experiment was repeated 3 times. * indicates a significant increase compared to non-treated tissues. (B) Exopolyphosphatase activity of full thickness cartilage formed in vitro on membrane inserts for 3 days. Polyphosphates of different chain length (concentrations normalized for the phosphate content) were incubated with protein extract and the resulting phosphate quantified. Each condition was done with pooled tissues from three samples and the experiment was repeated 3 times. The results were combined and expressed as mean ± SEM. * indicates a significantly higher activity than that of other polyphosphate chain lengths.
**A**

- **DNA Content (µg)**
  - No Treatment
  - PP-45

**B**

- **GAG Content (µg/µg DNA)**
  - No Treatment
  - PP-45
Figure 5.4: Time course of matrix and DNA accumulation within untreated and polyphosphate treated in vitro-formed cartilage. (A) DNA, (B) glycosaminoglycan and (C) collagen contents of full thickness cartilage formed in vitro on membrane inserts in medium with and without supplementation with 1mM polyphosphate (calculated based on the phosphate content; average chain length of 45 phosphate units) at 0, 1, 2 and 4 weeks following initiation of the treatment. The matrix content data were normalized to DNA content. Each condition was done in triplicate and the experiment was repeated 3 times. The results are from one representative experiment and expressed as mean ± standard deviation. * indicates a significant difference compared to non-treated tissues.
5.3.5 **The stimulatory effect of inorganic polyphosphat requires continuous administration**

Chondrocytes were grown *in vitro* on membrane inserts for 4 weeks. The medium was supplemented with 1mM polyphosphate for either the first 1 or 2 weeks of culture or for the entire 4-week period. Significant increases in GAG and collagen contents were detected only after 4 weeks of treatment suggesting that polyphosphate must be present continuously (Figure 5.5A).

Epifluorescence imaging of DAPI stained tissue sections demonstrated that the polyphosphate accumulates within *in vitro*-formed cartilage treated with condensed phosphate for a period of 4 weeks (Figure 5.5B). This accumulation was less obvious with short term treatment (2 or 1 weeks) (Figure 5.5C-D). In contrast, non-treated cartilage exhibits the lowest levels of polyphosphate (Figure 5.5E). Treatment of cartilage tissues with polyphosphate did not affect exopolyphosphatase activity levels (Figure 5.5F).

5.3.6 **Deep-zone chondrocytes appear more responsive than superficial-mid zone chondrocytes to inorganic polyphosphate**

Two chondrocyte subpopulations (superficial-mid zone and deep zone) were grown *in vitro* on membrane inserts for 2 weeks in medium supplemented with 1mM inorganic polyphosphates and compared to zonal sub-population tissues formed without supplementation and full thickness chondrocytes. Figure 5.6 shows the DNA, GAG and collagen contents of tissues formed by treatment of the different chondrocyte
A

Matrix Content (ug/ug of DNA)

GAG
Collagen

* * *

Treatment time (Weeks)

B

C

D

E

100μm 100μm 100μm 100μm
Figure 5.5: Effect of discontinuous inorganic polyphosphate treatment on in vitro-formed cartilage matrix accumulation and exopolyphosphatase activity. (A) Glycosaminoglycan and collagen contents of full thickness cartilage formed in vitro on membrane inserts for 4 weeks in medium supplemented with 1mM polyphosphate (average chain length of 45 phosphate units) for various time periods up to 4 weeks. The data were normalized to DNA content. Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean. * indicates a significant increase compared to non-treated tissues. (B-E) Histological appearance of cartilage formed after 4 weeks of culture from full thickness chondrocytes on membrane inserts in medium not supplemented (B) or supplemented for the first week (C), the first 2 weeks (D) or the entire 4 weeks (E) with 1mM polyphosphate and stained with DAPI. Tissues were visualized by epifluorescence microscopy. * indicates the location of the membrane insert. (F) Exopolyphosphatase activity of full thickness cartilage formed in vitro on membrane inserts for 4 weeks in medium supplemented with 1mM polyphosphate for various time periods up to 4 weeks. The data were normalized to the time of reaction and expressed per membrane insert. Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean. The inorganic polyphosphate concentration was calculated based on the phosphate content.
Figure 5.6: Effect of inorganic polyphosphate on the matrix content of different chondrocyte subpopulation cultures. DNA, glycosaminoglycan and collagen contents of cartilage formed by full thickness, superficial-mid zone or deep zone chondrocytes cultured in vitro on membrane inserts in medium with and without supplementation with 1mM polyphosphate (concentration calculated based on the phosphate content; average chain length of 45 phosphate units) at 2 weeks. The matrix content data were normalized to DNA content. All data from cultures treated with polyphosphate are expressed as a percentage of untreated cultures for each chondrocyte subpopulation. Each condition was done in triplicate and the experiment was repeated 3 times. The results are from one representative experiment and expressed as mean ± standard deviation. * indicates a significant difference compared to non-treated tissues.
subpopulations with 1mM polyphosphate as a percentage of the contents in untreated tissues. No significant difference was observed in the responsiveness of the different subpopulations to polyphosphate, but a trend towards a larger effect in deep zone cultures was observed for the three parameters measured (DNA, GAG and collagen contents).

5.3.7 Inorganic polyphosphate stimulates matrix accumulation in native cartilage

Native cartilage samples were obtained from full thickness cartilage and cultured ex vivo directly in tissue culture plates for 1 week and selected samples were treated with polyphosphate at concentrations of 1 or 2mM. This study was performed to verify that the anabolic effects of polyphosphate were not limited to immature in vitro-formed cartilage. As is shown in Figure 5.7A, the GAG content of native cartilage samples was significantly increased by approximately 45% following supplementation of the culture medium with 2mM polyphosphate. The collagen content was also increased by approximately 30% but this difference was not significant. The DNA content of cartilage samples was not significantly decreased by treatment with polyphosphate (Figure 5.7B).

A histological evaluation of ex vivo-cultured native cartilage samples stained with toluidine blue shows new matrix deposited around tissues stimulated with 2mM polyphosphate compared to non-treated cartilage (Figure 5.7C-E). This matrix was deposited predominantly at the deep-zone aspect of cartilage samples.

5.4 Discussion

This study demonstrates that polyphosphate administered to bioengineered cartilage during in vitro growth or to native articular cartilage cultured ex vivo promotes GAG and
A

Matrix Content (% of Control)

Polyphosphate Concentration (mM)

GAG
Collagen

B

DNA Content (µg/mg of tissue)

Polyphosphate Concentration (mM)
Figure 5.7: Effect of inorganic polyphosphate on articular cartilage cultured ex vivo. (A) Glycosaminoglycan and collagen contents and (B) DNA content of native articular cartilage cultured ex vivo on membrane inserts for 1 week in medium supplemented with 1mM or 2mM polyphosphate (average chain length of 45 phosphate units). The data were normalized to DNA content and presented as percent change compared to non-treated control. Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean. * indicates a significant increase compared to non-treated tissues. (C-E) Histological appearance of native articular cartilage cultured ex vivo for 1 week in medium not supplemented (C) or supplemented with 1mM (D) or 2mM (E) polyphosphate and stained with toluidine blue. Inorganic polyphosphate concentrations were calculated based on the phosphate content.
collagen accumulation in the extracellular matrix. This effect of polyphosphate was concentration and chain length dependent. Conversely, polyphosphate treatment resulted in a smaller increase in DNA content of bioengineered cartilage compared to untreated tissues. The continuous presence of condensed phosphate in the culture is essential to obtain the full stimulatory effects on matrix accumulation, as cartilage expresses exopolypolyposphatases that degrade polyphosphate. The effect of polyphosphate on matrix accumulation appears to be slightly more pronounced on deep-zone chondrocytes than on superficial-mid zone mixed-population chondrocytes. These results suggest that polyphosphate may be used to stimulate the production of cartilage extracellular matrix components in bioengineered cartilage. Alternatively, polyphosphate could prevent catabolic events that lead to extracellular matrix breakdown or positively impact the retention of extracellular matrix components within the tissue.

To the best of our knowledge, this is the first report of the stimulatory effect of polyphosphate on articular cartilage matrix accumulation. However, anabolic effects of polyphosphate have been observed in bone. Hacchou et al. (2007) demonstrated that polyphosphate treatment enhanced bone regeneration in alveolar bone defects [41]. Similarly, porous calcium hydroxyapatite scaffolds containing adsorbed inorganic polyphosphate induced increased new bone regeneration compared to uncoated scaffolds in a rabbit femoral defect model [42]. While the mechanism by which polyphosphate stimulates cartilage matrix accumulation has not yet been determined, it may be acting by stabilizing endogenous growth factors and perhaps presenting them to cell surface receptors in an appropriate conformation as has been shown to occur for FGF-2 [24]. Interestingly, treatment of in vitro-formed cartilage with polyphosphate leads to the
upregulation of cartilage matrix genes such as aggrecan and collagen type II, while not affecting the expression of collagen type X and type I, suggesting that polyphosphate stabilizes the chondrocytes phenotype.

While increases in GAG and collagen contents were observed in cartilage tissues treated with polyphosphates of average chain length of 5, 45 and 75 phosphate units, only polyphosphate with an average chain length of 45 phosphate units exhibited a significant increase in matrix accumulation over non-treated tissue. Similarly, our group has shown that polyphosphate with an average chain length of 45 phosphate units was optimal for inhibition of cartilage calcification [33]. Chain length specificity is not unexpected as other groups have shown that short chain polyphosphates including pyrophosphate are less potent inhibitors of mineralization than longer chain polyphosphates [18,23]. Short chain polyphosphates (5 phosphate units) did not stimulate mTor activity in mammary cancer cells, whereas polyphosphate chains with 15 or more phosphate units exhibited increased activity [25]. Our data suggest that there may be faster removal of short chain polyphosphates as significantly higher exopolyphosphatase activity was detected in protein extracts incubated with short chain polyphosphate (5 phosphate units) compared to longer chain polyphosphates (45 and 75 phosphate units). This could explain the weaker effects of short chain polyphosphate on cartilage matrix deposition. As for the discrepancy observed between the anabolic effects of polyphosphates with chain lengths of 45 and 75 phosphate units and their associated exopolyphosphatase activities, this may be attributed to the normalization of polyphosphate concentrations on a phosphate basis resulting in a lower effective condensed phosphate concentration for longer chain polyphosphates. Conversely, Hernandez-Ruiz et al. (2006) demonstrate that
polyphosphate with a chain length of 75 phosphate units lowers the survival rate of U266 myeloma cells significantly compared to treatment with polyphosphate characterized by 45 and 25 phosphate units [43]. Along with our observations, this suggests that polyphosphate function may be dependent on chain length.

Interestingly, treatment with inorganic polyphosphate resulted in an increase in DNA content of the in vitro-formed cartilage over time, but at a much slower rate than in non-treated tissues suggesting that polyphosphate inhibits chondrocyte proliferation in our system. However, polyphosphate has been shown to stimulate proliferation in human fibroblasts and a murine pre-osteoblastic cell line through the FGF-2 signaling pathway [24] as well as in mammary cancer cells through the mTor signalling pathway [25]. This discrepancy may be due to cell type specific effects. Alternatively, polyphosphate may have an effect on apoptosis of chondrocytes while the mitogenic activity remains unaffected. This observation requires further investigation.

The extent of cartilage matrix accumulation is directly related to the length of the culture period in which the bioengineered tissues are exposed to polyphosphate. Our group and others have demonstrated that the action of exopolyphosphatases, with the ability to cleave phosphoanhydride bonds of the condensed phosphates, along with hydrolysis can lead to a rapid loss of the effects of polyphosphate [33,44,45]. In this study, polyphosphate accumulation was much more prominent in tissues which were treated with the condensed polymers for the entire culture period than in tissues which received treatment for shorter time periods (1 or 2 weeks) corroborating these results. Surprisingly, the exopolyphosphatase activity of cartilage formed by full thickness chondrocytes is not significantly affected by treatment with polyphosphate. This is in
contrast with our observations of deep-zone chondrocytes which showed a significant increased exopolypophosphatase activity when treated with condensed phosphate [33].

Polyphosphate treatment of native cartilage samples ex vivo showed increased GAG and collagen contents compared to untreated controls, supporting results obtained with bioengineered cartilage. Histological evaluation of the native cartilage tissues cultured ex vivo showed that new matrix accumulation was deposited predominantly on the deep zone cartilage. This is in keeping with the possible increased responsiveness of deep zone chondrocytes to polyphosphate treatment in vitro compared to superficial-mid zone chondrocytes. The reduced effect of polyphosphate on superficial-mid zone tissue DNA content might be explained by a lower proliferation potential of these cells compared to deep zone chondrocytes, but the lower effect of polyphosphate on GAG and collagen accumulation within the superficial-mid zone cartilage suggests an increased responsiveness of deep zone chondrocytes to the treatment. This potential differential response of chondrocytes sub-populations to inorganic polyphosphate may provide a useful system to understand the pathways by which condensed phosphate stimulates cartilage matrix deposition.

5.5 Conclusion

In summary, this study demonstrates that inorganic polyphosphate stimulates cartilage matrix accumulation in both in vitro-formed cartilage and ex vivo cultured native cartilage. Based on these results, polyphosphate treatment may be useful to improve the quality of in vitro-formed bioengineered cartilage. Further studies are required to
examine the potential of polyphosphate as an anabolic agent for the treatment of articular cartilage defects in vivo.

5.6 References


The initial motivation for the studies presented in this thesis was to attempt to improve the mechanical integrity of the tissue-substrate interface in bioengineered cartilage biphasic constructs by inducing the formation of a cell-mediated zone of calcified cartilage (ZCC) at the tissue-biomaterial interface. The work performed towards that end provided insights into the mechanisms by which the mineralization process is controlled in articular cartilage and on the role played by inorganic polyphosphate in this process.

### 6.1 Development of cartilage biphasic constructs incorporating a ZCC

As was discussed previously, a methodology has been devised by our group to form cartilage incorporating a cell-mediated ZCC in vitro on membrane inserts [1,2]. The ZCC generated by this approach contains poorly crystalline apatite crystals similar in composition, shape, size and orientation to those present in the native ZCC of articular cartilage [3]. However, the application of this methodology to generate a ZCC at the interface between in vitro-formed cartilage and a porous calcium polyphosphate (CPP) bone substitute proved difficult. In fact, mineral was not deposited in the tissue directly above the tissue-substrate interface, thereby causing a shift in the location of the ZCC away from that interface [4].

In Chapter 2 of this thesis, experiments were carried out to verify the working hypothesis that polyphosphate released from the porous CPP bone substitutes accumulates in the cartilage layer formed on its top surface and inhibits cartilage calcification. The inhibitory effects of polyphosphate on apatite crystal formation in solution [5,6] and in tissues [7-10] are well established. However, this study was the first
to demonstrate its effects in articular cartilage. Of importance for the development of strategies to generate a ZCC apposed directly to the tissue-substrate interface of cartilage biphasic constructs, it was demonstrated that polyphosphate treatment does not cause the dissolution of mineral deposited within the tissue. It was also demonstrated that the addition of calcium to the culture medium may partially overcome the inhibitory effects of polyphosphate on mineralization. However, such a treatment would probably lead to incomplete tissue mineralization directly at the interface of cartilage and CPP and a high mineral density above that interface and was not pursued. Hence, a strategy to temporarily prevent the release of polyphosphate from porous CPP may allow for the development of a durable and properly positioned ZCC.

Porous CPP has a number of advantageous characteristics for applications as a bone substitute material. Aside from its biocompatibility, osteoconductivity and biodegradability, this porous substrate is characterized by a comparable tensile strength to cancellous bone [11,12]. Because of these advantageous properties a strategy to generate cartilage biphasic constructs incorporating a ZCC, while maintaining this biomaterial as the core substrate for biphasic constructs was sought. Of note, the selected approach must not compromise the beneficial properties of porous CPP as a bone substitute including its ability to support the formation of cartilaginous tissues \textit{in vitro}.

Chapter 3 of this thesis demonstrates an approach, whereby a thin calcium phosphate film is applied to the surfaces of porous CPP to reduce the accumulation of polyphosphate within the cartilage layer of biphasic constructs. The sol-gel process employed was optimized to minimize pore occlusions, as well as cracks within the coating and to obtain a maximal coverage for most of the surfaces of CPP, thereby
permitting the formation of a ZCC directly apposed to the tissue-substrate interface of cartilage biphasic constructs. Thin calcium phosphate films formed by the sol-gel route have been studied extensively and have been shown to be both osteoconductive and susceptible to biodegradation by osteoclasts [13,14]. The cartilage tissue generated in vitro following the seeding of deep zone chondrocytes on the top surface of the coated CPP exhibited no histological or biochemical differences compared to tissues formed on uncoated CPP and its ZCC was composed of mineral crystals with comparable morphology, structure and length to those contained in native articular cartilage.

Most importantly, the study presented in Chapter 3 proves the hypothesis that the generation of a ZCC at the interface between cartilaginous tissue and a bone substitute in osteochondral biphasic constructs improves the mechanical integrity of that interface. Since the first attempt from our group to generate biphasic constructs with a mineralized interface for cartilage tissue engineering applications [4], other groups have published studies establishing protocols to mineralize the tissue at that interface [15,16]. Abrahamsson et al. (2010) showed that human mesenchymal stem cells in a collagen type I gel seeded onto a three-dimensional woven poly(ε-caprolactone) scaffold and cultured in chondrogenic culture conditions formed a cartilage layer with a partially mineralized interface in the periphery of the construct [15]. Jiang et al. (2010) developed an approach whereby primary chondrocytes and osteoblasts are seeded in an agarose and a composite scaffold of poly(lactic-co-glycolic acid) and 45S5 bioactive glass microspheres, respectively. The interface region comprised of the scaffold penetrated with agarose was shown to partially mineralize in close proximity to the microspheres [16]. However, the report in this thesis is the first to demonstrate improved interfacial shear properties by
mimicking the native osteochondral architecture in vitro and generating a ZCC directly apposed to the cartilage-bone substitute interface of biphasic constructs. This result is significant as cartilage biphasic constructs are generally characterized by inferior interfacial shear strength [4], which may prevent the adequate integration of large constructs into cartilage defect in load-bearing joints.

The improved interfacial mechanical integrity associated with the generation of a ZCC in cartilage biphasic constructs demonstrated in this thesis is an important proof of concept. However, some limitations are associated with the approach selected and will have to be addressed. As described previously, the interfacial shear properties of non-mineralized cartilage formed on the top surface of coated CPP were largely inferior to those of tissues formed on top of uncoated CPP. Hence, the overall improvements obtained by the application of a ZCC at the tissue-substrate interface are not significant compared to that for tissues formed on uncoated CPP and remain significantly inferior to those of the native osteochondral junction. This may be the result of subtle pore occlusions at the CPP surface due to the coating and/or changes to the tissue composition and organization at the interface with the substrate. Additional work is required to establish the contribution of these two factors on the strength of the tissue-substrate interface. New strategies to generate the ZCC directly at that interface should be considered to overcome the limitations of the current system. For example, it may be possible to apply a thin calcium phosphate coating by soaking porous CPP in a simulated body fluid solution or an organic sol-gel solution. These solutions may minimize pore occlusions that likely occurred due to the elevated particle density of inorganic sol-gel solutions and this approach might allow the development of a cement line with similar
tissue interdigitations as for uncoated CPP [13,17]. Alternatively, modifications to the CPP fabrication parameters could lead to the formation of porous CPP with decreased rate of degradation and/or releasing polyphosphate with shorter chain lengths. Such a substrate design may permit the formation of a properly located ZCC within biphasic constructs without the need for a coating step. While the impact of a decreased degradation rate on the formation of a ZCC is straightforward, decreased chain length of the degradation products is also an interesting option as the rate of enzymatic degradation of polyphosphate with an average chain length of 5 phosphate units is 10- and 14-fold higher than for polyphosphates with average chain lengths of 45 and 75 phosphate residues, respectively (Appendix 1). Hence, conditions that would generate porous CPP with optimized degradation rate and degradation product size may be optimal bone substitutes for the application discussed in this work. Similarly, doping of the CPP with sodium phosphate or sodium carbonate can affect its degradation rate [18]. These alternatives may be essential for the formation of large cartilage biphasic constructs incorporating a ZCC as the application of sol-gel calcium phosphate coating on larger porous CPP substrates with a height of 6 mm was not sufficient to prevent a shift of the ZCC away from the tissue-substrate interface (Appendix 1).

The mode of failure of biphasic constructs incorporating a properly located ZCC following interfacial shear testing was deemed similar to that observed for adolescent osteochondral tissue incorporating an immature ZCC for a majority of constructs [19]. It is possible that an increased culture period prior to testing could lead to a denser and more uniform ZCC as it has been shown that there is increased mineral deposition over time in cartilage tissues formed on porous CPP [4], which could improve the mechanical
integrity of the interface. Similarly, a longer culture period has been shown to cause increased extracellular matrix accumulation within \textit{in vitro}-formed cartilage and could also contribute to an improved response to interfacial shear tests as failure also occurred within the matrix \cite{20}. In fact, the inferior collagen content and associated stiffness of bioengineered cartilage may be the limiting factor in improving the mechanical integrity of the interface of biphasic constructs. One way to improve the matrix composition and stiffness of \textit{in vitro}-formed cartilage involves mechanical stimulation of cartilage biphasic constructs during the \textit{in vitro} maturation phase \cite{21-24}. The combined generation of a ZCC and mechanical stimulation could prove beneficial in that regard. It should be noted that it may not be necessary to achieve interfacial shear properties that are comparable to those of the native osteochondral junction as the implantation of cartilage biphasic constructs in sheep joints were shown to result in further maturation of the bioengineered tissue \textit{in vivo} \cite{25}. In fact, the interfacial shear strength requirements of cartilage biphasic constructs for successful clinical applications remain unknown.

Other issues with the methodology described in these two studies must be addressed prior to the use of cartilage biphasic constructs incorporating a ZCC in clinical settings. Notably, an adequate cell source is required for the formation of cartilage incorporating a ZCC. In the work presented in this thesis, primary chondrocytes enriched in the deep zone subpopulation are employed. However, it is technically challenging to obtain a sufficient number of primary full thickness chondrocytes for the generation of bioengineered cartilage constructs and the need for a specific cell subpopulation makes this task more complicated. At least one study has shown that articular chondrocytes from the different subpopulations can be induced to recover some of the characteristics of their
specific phenotype following dedifferentiation by monolayer amplification [26]. Hence, a protocol could be developed to permit the redifferentiation of deep zone chondrocytes that mineralize their matrix following their amplification. Alternatively, mesenchymal progenitor cells could be used but differentiation protocols that direct the formation of cartilage tissue incorporating a ZCC will need to be developed. A feature of the ZCC generated in vitro is the absence of a tidemark. A recent paper by Jiang et al. (2008) has demonstrated that articular cartilage superficial zone chondrocytes modulate the mineralization deposited by deep zone chondrocytes [16]. These results have been corroborated in this thesis (Appendix 1). As stratified bioengineered cartilage constructs have previously been developed [27,28], it is hypothesized that a carefully optimized stratified cartilage biphasic construct could permit a strict control of the thickness of the ZCC to match that of the native tissue and would permit the formation of a tidemark. Finally, it will be essential to devise strategies to allow for the generation of a ZCC in cartilage biphasic constructs under serum-free culture conditions.

6.2 Role of endogenous polyphosphate in cartilage calcification

The methodology developed by our group to form cartilage incorporating a ZCC in vitro results in a ZCC in the deeper section of in vitro-formed cartilage despite the fact that the chondrocytes seeded on membrane inserts are cells from the deep zone subpopulation. This observation suggests that deep zone chondrocytes modify their phenotype according to their position in the maturing cartilaginous tissue. This observation has recently been verified experimentally by Hayes et al. (2007) using a similar culture system to the one developed by our group and full thickness chondrocytes [29]. Hence, the aforementioned
three-dimensional system can be used as an \textit{in vitro} model for the study of the mechanisms by which articular cartilage calcification is modulated in the ZCC and improve our understanding of this process towards the formation of biphasic cartilage constructs incorporating a ZCC that improves the biomechanical performance of bioengineered cartilage in loading conditions.

While studying the effects of polyphosphate released from porous CPP on \textit{in vitro}-formed cartilage, it was noted that chondrocytes accumulate endogenous phosphate polymers within their matrix and express enzymes with exopolyphosphatase activity (see Chapter 2). Polyphosphate has been proposed as an important factor in the modulation of vertebrate tissue calcification [30,31]. As the extent of its role in controlling tissue mineralization and the signaling pathways involved in regulating its accumulation within tissues are not known, further investigations were undertaken to elucidate this.

In Chapter 4 of this thesis, it is demonstrated that the polyphosphate levels within \textit{in vitro}-formed deep zone cartilage are modulated in response to culture conditions that impact tissue mineralization. In fact, high polyphosphate levels are present in tissues that do not mineralize, whereas low levels are associated with mineralizing cultures. This observation is in keeping with a statement made by Fleisch and Neuman (1961) to the effect that condensed phosphates must be removed from a tissue by phosphatases for mineralization to take place [5]. However, our data suggest that it is probable that the ratio of orthophosphate-to-condensed phosphates dictates the rate of mineral deposition rather than the need for a complete removal of the inhibitors [32].

More importantly, the work presented in this thesis demonstrates the involvement of FGF-18 in raising polyphosphate levels and inhibiting mineralization within \textit{in vitro}-
formed cartilage by decreasing the exopolyphosphatase activity of the tissue. Not surprisingly, initiation of the mineralization process was accompanied by a decreased FGF-18 expression, suggesting that either decreased FGF-18 levels are necessary for mineral deposition to take place or that the decreased polyphosphate levels associated with the onset of mineralization lead to lower levels of FGF-18 expression. While polyphosphate stimulated the expression of FGF-18, it also acted at high concentration to increase the exopolyphosphatase activity in the tissue. This suggests a feedback control system to tightly regulate the condensed phosphate levels within cartilage. Nevertheless, the extent of our understanding of this mechanism remains limited. In fact, the involvement of FGF-2 in the metabolism of pyrophosphate has previously been demonstrated [33,34]. The results presented in this study suggest an overlap between the pyrophosphate and polyphosphate metabolisms. Because FGF-2 was shown in this thesis to inhibit cartilage calcification, the possibility of its involvement regulating polyphosphate levels must also be investigated.

The signaling pathway(s) by which polyphosphate causes the biological effects reported in this thesis are still unknown. One way by which polyphosphate has been shown to influence cell signaling is through the physical and functional stabilizing FGF-2 [35]. It is possible that similar effects of polyphosphate on growth factors and/or signaling molecules take place in the system studied in this work. Alternatively, polyphosphate has been implicated with the formation and function of calcium channels, thereby modulating intracellular calcium levels [36-38]. Such a signaling cascade may be implicated in the modulation of chondrocytes phenotype. While no membrane transporter has been identified for polyphosphate yet, such a protein or complex may exists. This
could allow the entry of phosphate polymers within chondrocytes and subsequently permit its interactions with proteins involved in transcription [39].

The enzyme(s) responsible for the synthesis of polyphosphate in vertebrate tissues remain unknown. However, the polyphosphate levels in cartilage and the associated mineralization process are regulated by the balance between its synthesis and degradation. In Chapter 4, it is shown that a specific distribution pattern of the phosphate polymers exists in native articular cartilage. Relatively high concentrations are observed in the pericellular matrix of deep zone chondrocytes and diffuse staining is present in the superficial zone matrix. Given the up-regulation of FGF-18 expression noted in this thesis following treatment with polyphosphate, one role of superficial zone polyphosphate may be to regulate the level of FGF-18 in the tissue. This distribution suggests different roles for polyphosphate in the different zones of tissue. Interestingly, different exopolyphosphatases with specific chain length affinities are present in superficial and deep zones. These results stress the need to investigate the mechanisms by which polyphosphate synthesis is regulated in order to better understand their functions in tissues and apply this knowledge to the improved calcification of the interface of biphasic cartilage constructs.

In vitro models such as the one employed in this work for the study of mechanisms that control articular cartilage mineralization can be useful tools for the verification of research hypotheses. In this thesis, the information gathered from this model is directly applicable to the improvement of protocols for the calcification of biphasic cartilage constructs in vitro. However, articular cartilage interacts with a number of tissues of the joint [40]. Hence, the study of specific pathways in articular cartilage
alone using this *in vitro* model may not translate to the *in vivo* environment, where other tissues including the synovium and subchondral bone may participate in the modulation of these pathways. Similarly, the particular model employed in this work is formed from a specific deep zone chondrocyte subpopulation. While this tissue exhibits a zonal organization reminiscent of the native articular cartilage organization, the extent of the phenotype reorganization of deep zone chondrocytes within this system remains unresolved. Additionally, the culture conditions employed in this study do not fully recreate the joint environment. Notably, articular chondrocytes function under hypoxic conditions [41] and sustain complex mechanical forces during articulation [42] which were not replicated in the experiments described in this report. These differences stress the need to be careful in applying the information obtained from the *in vitro* model of articular cartilage calcification employed in this thesis to joint biology *in vivo*.

### 6.3 Effect of polyphosphate on cartilage matrix accumulation

Over the course of the studies presented in this thesis to investigate the effects of inorganic polyphosphate on cartilage calcification, it was noted that the exogenous administration of phosphate polymers to three-dimensional cartilage cultures affected the thickness of the resultant tissues (see Chapter 4). In Chapter 5, polyphosphate is shown to positively impact the accumulation of extracellular matrix in both *in vitro*-formed cartilage and *ex vivo* cultured articular cartilage explants. Deep zone chondrocytes appear to have a slightly more pronounced response to polyphosphate than the superficial-mid subpopulation. While this is the first report demonstrating the effect of polyphosphate on articular cartilage matrix accumulation, a number of studies have demonstrated increased
bone formation from similar treatments [43,44]. Treatment with inorganic polyphosphate also caused an increase in the DNA content of full thickness cartilage cultures, albeit at a much slower rate than in untreated cultures. This is interesting as it suggests that condensed phosphate inhibits chondrocyte proliferation. However, further studies will be required to rule out the possibility that the effect is caused by increased apoptosis. This observation is contrary to other reports which have noted increased mitogenic activities in fibroblasts and mammary cancer cells following treatment with polyphosphate [35,45].

Taken together, the effects of polyphosphate on cartilage matrix accumulation, as well as a potential role in reducing chondrocyte proliferation make it an interesting agent for the improvement of bioengineered cartilage properties. Given the inhibitory effects of polyphosphate on cartilage calcification, it may be difficult to generate biphasic constructs incorporating a ZCC, while taking advantage of these effects of polyphosphate. Because of the action of exopolyphosphatases in cartilage, it may be possible to divide the in vitro incubation period to permit the sequential cartilage calcification and increased matrix accumulation. Alternatively, these results suggest that polyphosphate could be a therapeutic candidate for the repair of cartilage defects in combination with other surgical procedures such as autologous chondrocyte implantation or marrow stimulation. Furthermore, treatment of in vitro-formed cartilage with phosphate polymers as been shown to up-regulate the expression of FGF-18 within deep zone chondrocytes. Moore et al. (2005) have demonstrated the anabolic effects of intraarticular administration of FGF-18 on cartilage in a rat model [46].
6.4 References


[18] Ue J. The Effect of Sodium Doping on Calcium Polyphosphate University of Toronto; 2009.


CHAPTER 7: CONCLUSIONS
1. Inorganic polyphosphate released from the degradation of porous calcium polyphosphate bone substitute accumulates within the \textit{in vitro}-formed cartilage layer in biphasic constructs and inhibits cartilage calcification, thereby possibly causing the observed shift in the localization of the zone of calcified cartilage away from cartilage-substrate interface.

2. The application of a thin calcium phosphate coating on the surfaces of porous calcium polyphosphate via an inorganic sol-gel methodology permits the formation of biphasic constructs incorporating a zone of calcified cartilage directly apposed to the cartilage-substrate interface.

3. In biphasic constructs formed on calcium phosphate-coated porous calcium polyphosphate, the incorporation of a zone of calcified cartilage directly apposed to the cartilage-substrate interface leads to substantial increases in the peak shear load at failure, shear stiffness and energy to failure compared to unmineralized constructs.

4. Deep zone chondrocytes in native articular cartilage and cultured \textit{in vitro} accumulate endogenous inorganic polyphosphate and express enzyme(s) with exopolyphosphatase activity.

5. Endogenous inorganic polyphosphate levels in deep zone chondrocytes cultured \textit{in vitro} are modulated in part through the effects of fibroblast growth factor 18 on exopolyphosphatase activity and may control cartilage calcification.

6. Inorganic polyphosphate leads to increased cartilage matrix accumulation in full thickness chondrocytes cultured \textit{in vitro}, as well as articular cartilage explants.
CHAPTER 8: FUTURE DIRECTIONS
The work presented in this thesis supports a number of hypotheses. However, many questions have also been raised that must be addressed to gain the necessary insight towards (i) the generation of clinically relevant cartilage biphasic constructs incorporating a ZCC, as well as (ii) the roles of polyphosphate in modulating the advancement of the ZCC within hyaline cartilage and (iii) maintaining articular cartilage homeostasis. These are enumerated below.

8.1 Towards clinically relevant cartilage biphasic constructs

- The work presented in this thesis demonstrates improvements to the mechanical integrity of the tissue-substrate interface associated with the incorporation of a ZCC in cartilage biphasic constructs. However, the limitations associated with the sol-gel coating method must be addressed. The incubation of porous CPP in simulated body fluid is proposed as an alternative method to apply a thin calcium phosphate film on the surfaces of the substrate, while reducing pore occlusions and the amount of labour associated with the current coating methodology. Alternatively, an improved understanding of the effects of fabrication parameters on the microstructure and degradation characteristics of CPP and/or doping may result in the fabrication of porous CPP with optimal degradation rate and inorganic polyphosphate chain length for rapid enzymatic removal from the cartilage.

- It appears that a major limitation with achieving an improved mechanical integrity of the tissue-substrate interface in biphasic constructs incorporating a ZCC is the maturation level of the ZCC. This is exemplified by the fact that the interface is not uniformly calcified and that failure occurs within the ZCC in some constructs.
undergoing interfacial shear testing. The duration of the in vitro maturation period should be optimized for the strength of the interface. Additionally, culture conditions should be adjusted to improve the uniformity of the ZCC. Notably, different tubing types could be investigated, as well as the effect of tubing removal during the maturation phase in vitro because it was observed that the cartilage at the CPP edges (in close proximity to the tubing) does not mineralize extensively.

- While the aforementioned changes may contribute to an improved mechanical integrity of the tissue-substrate interface of cartilage biphasic constructs, the limiting factor for the generation of constructs of comparable interfacial shear strength to that of the native osteochondral junction is probably the quality of the cartilage extracellular matrix. In fact, a number of constructs have failed in the matrix above the ZCC during interfacial shear tests. While an increased in vitro maturation period would undoubtly lead to an increased stiffness of the bioengineered cartilage, this effect may be insufficient. An investigation of the benefits of mechanical stimulation in calcified biphasic constructs could contribute to the desired outcome.

- The identification of an adequate cell source for the formation of bioengineered cartilage incorporating a ZCC is essential to the clinical application of these constructs. In our laboratory, a methodology has been developed to redifferentiate amplified full thickness chondrocytes. It may be possible to adapt this procedure to the amplification and subsequent redifferentiation of different chondrocyte subpopulations. However, the practicality of this approach for clinical applications depends on the feasibility of obtaining a sufficient number of autologous primary
chondrocytes from the different cartilage zones. If this is deemed improbable, focus should be aimed at the development of adult stem cell differentiation protocols to generate biphasic constructs incorporating a ZCC.

- The preparation of clinically relevant bioengineered cartilage constructs will require the optimization of serum-free culture conditions to reduce the immunoreactivity of the transplants. Hence, it will be essential to identify culture conditions inducing the calcification of bioengineered cartilage in the absence of serum.

8.2 Role of endogenous polyphosphate in cartilage calcification

8.2.1 Work to conclude the study in Chapter 4

- The current data on protein levels of FGF-18 were obtained by staining chondrocytes cultured in a monolayer. Under these experimental conditions, the extracellular accumulation of FGF-18 is not accounted for. Furthermore, a number of weakly adherent cells in the samples treated with polyphosphate are detached from the glass cover slip during processing. The FGF-18 protein levels in these detached cells are not known. For these reasons, methods to measure FGF-18 levels within tissues are being developed including western blot analysis and immunolocalization in tissue sections. Following the optimization of these methods, a more thorough investigation of the FGF-18 protein levels in the system will be undertaken.

- Pyrophosphate and orthophosphate treatments did not cause significant changes in exopolyphosphatase activity within in vitro-formed cartilage. However, their impact on inorganic polyphosphate levels must still be investigated. Similarly, FGF-2 is
implicated in the modulation of *in vitro*-formed cartilage mineralization. Its role in the control of exopolyphosphatase activity and polyphosphate levels must be established.

- It appears that polyphosphate and FGF-18 signaling (contrary to FGF-2 signaling) is independent of MAPK signaling (Appendix 2). An in depth investigation of the pathways involved in the modulation of cartilage calcification by polyphosphate via FGF-18 signaling should be performed. Notably, investigation of MAPK signaling at different time points following treatments with polyphosphate or FGF-18 and verification of the involvement of other pathways linked to FGF signaling should be performed.

- The results presented in this thesis suggest that TNAP may be one exopolyphosphatase responsible for the degradation of polyphosphate within cartilage, thereby controlling the mineralization process. This would indicate an overlap with the metabolism of pyrophosphate. However, this demonstration has yet to be done. The effects of the knockdown of TNAP within deep zone chondrocytes on their exopolyphosphatase activity and associated polyphosphate levels should be determined.

### 8.2.2 Potential future directions

- Adapting existing protocols for the extraction and quantification of polyphosphate contained within *in vitro*-formed and native cartilage would provide an important tool for the continuation of investigations on the mechanisms by which polyphosphate levels are regulated.
• If the implication of TNAP as an exopolyphosphatase in the mechanism described in this thesis is demonstrated, it may be interesting to adjust the reaction mixture pH for exopolyphosphatase activity measurements to 9.0, the optimal pH for the activity of TNAP, instead of 7.5. This may also help explain the gap in the extent of exopolyphosphatase activity and TNAP gene expression changes for any given treatment.

• The down-regulation of FGF-18 and FGF-2 gene expression levels was observed upon culturing of in vitro-formed deep zone cartilage in mineralization medium. A study of the factor(s) in mineralization media responsible for this effect could provide further insight into the regulation of cartilage calcification. Of interest are the roles played by calcium and orthophosphate in this mechanism.

• Preliminary results (see Appendix 2) have shown that superficial zone chondrocytes produce soluble factor(s) that cause the inhibition of deep zone cartilage calcification. This effect appears to involve inorganic polyphosphate. Further work is required to establish the involvement of FGF-18 or identify the soluble factor(s) responsible for this effect. PTHrP signaling was previously implicated with the effects of superficial zone chondrocytes on cartilage mineralization. The role of PTHrP in the metabolism of polyphosphate should also be investigated.

8.3 Effect of polyphosphate on cartilage matrix accumulation

• The suggestion that polyphosphate inhibits chondrocyte proliferation is intriguing in light of the fact that other cell types have an increased mitogenic activity in the
presence of polyphosphate. A verification of this suggestion, as well as a more in
depth study of the ability of polyphosphate to help maintain the chondrogenic
phenotype would provide valuable information.

• The effect of polyphosphate on cartilage matrix accumulation suggests that it acts as
an anabolic agent. However, this possibility remains to be demonstrated by
establishing the effect of polyphosphate treatment on the synthesis of cartilage matrix
components. Alternatively, the possibility that polyphosphate inhibits catabolic events
or influences the retention of synthesized matrix components should also be
investigated.

• The implication of FGF-18 in the modulation of polyphosphate levels within deep
zone chondrocytes was demonstrated in this thesis. Interestingly, FGF-18 is a known
anabolic agent for articular cartilage. Its implication in the modulation of the effects
of polyphosphate on cartilage matrix accumulation should be verified.

• The results from this thesis suggest that polyphosphate may exhibits anabolic effects
on chondrocytes and inhibit their mitogenic activity. An animal model should be
investigated to determine if these effects of polyphosphate on cartilage matrix
accumulation can be replicated in vivo.
APPENDIX 1: SUPPLEMENTARY DATA
Table A1.1: Biochemical properties of native articular cartilage.

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<th>Ex Vivo</th>
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<tr>
<td></td>
<td>DZ</td>
<td>FT</td>
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<tr>
<td>Water Content (%)</td>
<td>73.9 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>76.9 ± 0.7&lt;sup&gt;a,b&lt;/sup&gt;</td>
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<tr>
<td>DNA Content (µg/mg dry wt.)</td>
<td>1.53 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.08 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>GAG Content (µg/µg DNA)</td>
<td>244 ± 22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>125 ± 19&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Collagen Content (µg/µg DNA)</td>
<td>551 ± 45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>422 ± 24&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Collagen : GAG</td>
<td>2.32 ± 0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.49 ± 0.40&lt;sup&gt;a,b&lt;/sup&gt;</td>
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*Ex vivo* articular cartilage from the deep-zone (DZ) and the full thickness (FT) were harvested and the biochemical analysis performed. The data are from three experiments performed in triplicates with tissues from different extractions and expressed as mean ± standard error of the mean. <sup>a</sup> indicates a significant difference between *ex vivo* tissues and bioengineered tissues presented in Chapter 3 (p<0.05). <sup>b</sup> indicates a significant difference between deep-zone and full thickness *ex vivo* cartilage (p<0.05).
Figure A1.1: Effect of inorganic polyphosphate treatment on the time profile of alkaline phosphatase activity within in vitro-formed deep zone cartilage. Deep zone chondrocytes were cultured in mineralization medium alone or supplemented with 1mM polyphosphate (average chain length of 45 phosphate units) for different time points up to 28 days on membrane inserts. Tissues were digested collagenase and proteins extracted. The alkaline phosphatase activity of the resulting extracts was measured. The data were normalized to the DNA content. Each condition was done in triplicate and the experiment was repeated 3 times. The results were pooled and expressed as mean ± standard error of the mean. This figure consists of additional data from the study presented in Chapter 2.
Figure A1.2: Effects of inorganic polyphosphate chain length of concentrations normalized to the polyphosphate content on the exopolyphosphatase activity of full thickness and deep zone chondrocyte protein extracts. Full thickness and deep-zone cartilage were formed in vitro on membrane inserts for 3 days. Polyphosphates of different chain length were incubated with protein extract and the resulting phosphate quantified. The concentrations were normalized for the polyphosphate content. * indicates a significantly higher activity than that of other polyphosphate chain lengths. Each condition was done with pooled tissues from three samples and the experiment was repeated 3 times. The results were combined and expressed as mean ± standard error of the mean. This figure was discussed as data not shown in Chapters 2 and 5.
Figure A1.3: Microstructural analysis of cartilage extracellular matrix. Transmission electron micrographs of cartilage tissue formed by deep zone chondrocytes on calcium phosphate coated CPP after 4 weeks in cultures obtained (A) from the bulk and (B) interface zones with large collagen fibrils, as well as (C) native articular cartilage. Tissues processed for transmission electron microscopy were post-fixed in 0.1% osmium tetroxide. (D-E) Histological appearance of cartilage formed by deep-zone chondrocytes on (D) uncoated and (E) calcium phosphate coated CPP after 4 weeks of culture in non-mineralizing medium (toluidine blue). The arrowhead indicates the dark stained zones corresponding to areas with large diameter collagen fibrils. * indicates where the CPP substrate was located. This figure consists of additional data from the study in Chapter 3.
Figure A1.4: Peak load at failure normalized to biphasic constructs on non-coated CPP in non-mineralizing culture conditions. Deep zone cartilage was formed in vitro on non-coated (NC) and calcium phosphate coated (C) CPP for 4 weeks in non-mineralizing (NM) and mineralization (M) media. The data are from three experiments performed with 4-8 replicates with chondrocytes from different extractions, normalized to NC-NM and expressed as mean ± standard error of the mean. * indicates a significant difference between constructs incorporating a zone of calcified cartilage (M) and unmineralized constructs (NM). This figure was discussed as data not shown in Chapter 3.
Figure A1.5: Effect of calcium phosphate coated CPP thickness on the histological appearance of cartilage formed by deep-zone chondrocytes. Tissues were cultured for 2 weeks on (A) 4 mm thick and (B) 6 mm thick calcium phosphate coated CPP. Tissue visualized by light microscopy (toluidine blue and von Kossa, mag. x50). The arrowhead indicates the zone of calcified cartilage. * indicates where the CPP substrate was located. This figure was discussed in Chapter 6.
Figure A1.6: ERK-1/2 phosphorylation within in vitro-formed deep zone cartilage. (A) Representative western blot showing phospho-ERK-1/2 and ERK-1/2 in deep-zone cartilage formed in vitro on membrane inserts after 24 hours in mineralization medium alone (M) or supplemented with different fibroblast growth factors (40 ng/ml). (B) Representative western blot showing phospho-ERK-1/2 and ERK-1/2 in deep-zone cartilage formed in vitro on membrane inserts after 48 hours in mineralization medium supplemented with 0.1 or 1.0mM inorganic polyphosphates (PP45; average chain length of 45 phosphate units). For each concentration of inorganic polyphosphates, cultures were maintained in the absence or presence of 30nM PD173074, an inhibitor of fibroblast growth factor receptor signaling. Each condition was done in duplicates and the experiment was only performed 1 time. This figure represents preliminary data for the future work proposed for Chapter 4.
Figure 1: (A) Mineral content (% of dry weight) and (B) Exopolyphosphatase activity (nmol/min/mg) for DZ(DZ), DZ(MZ), and DZ(SZ) groups.
Figure A1.7: Effect of superficial zone chondrocytes co-culture on the calcification of deep zone chondrocytes. Deep zone cartilage was formed in vitro on membrane inserts for 1 week in mineralization medium and co-cultured with deep zone (DZ(DZ)), mid-zone (DZ(MZ)) or superficial zone (DZ(SZ)) chondrocytes. (A) Calcium and phosphate contents of the deep zone tissues. The data were normalized to tissue dry weight. Each condition was done in duplicates and the experiment was only performed once. The results were pooled and expressed as mean ± standard deviation. (B) Exopolyphosphatase activity of the deep zone tissues. The data were normalized to the time of reaction and the protein content. Each condition was done in triplicate and the experiment was only performed once. The results were pooled and expressed as mean ± standard deviation. (C) Histological appearance of deep zone cartilage visualized by epifluorescence microscopy (DAPI).
APPENDIX 2: OPTIMIZATION OF EXOPOLYPHOSPHATASE ACTIVITY ASSAY
Figure A2.1: Optimization of the protein extraction for the exopolyphosphatase activity assay. (A) Effect of the concentration of Triton X-100 in the homogenization buffer on the optical density (OD) measurement for a phosphate concentration of 0.4mM. This experiment was only performed once. (B) Effect of the concentration of Triton X-100 in the homogenization buffer on the exopolyphosphatase activity measurement in deep zone chondrocytes cultured for 3 days. Each condition was done in duplicates and the experiment was only performed once. The results were pooled and expressed as mean ± standard deviation. A Triton X-100 concentration of 0.2% was selected for all further experiments.
Figure A2.2: Optimization of the protein concentration in reaction mixtures for the exopolyphosphatase activity assay. (A) Effect of the protein content in reaction mixtures on the optical density (OD) measurement for a phosphate concentration of 0.6mM. This experiment was only performed once. The red data points represent protein contents for which evidence of protein precipitation was observed. A protein content of 25µg was selected for all further experiments.
Figure A2.3: Time course of phosphate production in an exopolyphosphatase activity reaction mixture. Phosphate content were measured up to 48 hours after initiation of the reaction at 37°C in a reaction mixture containing the protein extract and polyphosphate (blue data points) or only polyphosphate (red data points). The exopolyphosphatase produced phosphate content was calculated by subtracting the phosphate contents of reaction mixtures containing only polyphosphate and only protein extract to that of the reaction mixture with both the protein extract and polyphosphate (purple data points). This experiment was only performed once. The protein extract for this experiment was obtained by pooling 10 deep zone cartilage constructs cultured for 3 days. A reaction time of 24 hours was selected for all further experiments.
Figure A2.4: Effect of heat inactivation of the protein extract on the phosphate production in an exopolyphosphatase activity reaction mixture. Phosphate content were measured up to 24 hours after initiation of the reaction at 37°C in a reaction mixture containing the heat inactivated (red data point) or as extracted (blue data points) protein extract and polyphosphate. This experiment was only performed once. The protein extract for this experiment was obtained by pooling 10 deep zone cartilage constructs cultured for 3 days.