A Mechanical and Biological Evaluation of Calcium Polyphosphate as a Structural Bone Scaffold in Revision Total Hip Replacement

by

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B.Sc., The University of British Columbia, 2005

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF APPLIED SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Materials Engineering)

THE UNIVERSITY OF BRITISH COLUMBIA
September 2007

Abstract

Revision total hip replacement (THR) commonly uses the addition of morsellized cancellous bone (MCB) for filling bone defects and to encourage new bone growth, however problems associated with allograft include limited availability, disease transmission and implant migration. The use of synthetic scaffolds as a substitute material is attractive due to its availability and ease of sterilization, however due to the high loads associated with the hip and the need to promote bone growth the number of suitable materials is limited. The novel ceramic calcium polyphosphate (CPP) has been shown to have good mechanical and biological properties and may be a suitable scaffold material for revision THR.

In the first part of this study particulate CPP is tested in compression and shear and its mechanical properties are compared to that of the gold standard graft of MCB. In addition, compression and shear tests are carried out on a number of spherical particles of varying size and material in order to determine a relationship between the particle and graft bed properties. The results show that CPP has similar mechanical properties to that of MCB in both compression and shear, making it a suitable substitute graft material. Furthermore a relationship between the particle modulus and graft bed modulus show minimal gain in bed stiffness with increased particle stiffness.

The second aspect of the study is to determine the effect of substrate material on MSC expansion and differentiation. CPP, allogeneic bone and hydroxyapatite/tricalcium phosphate (HA/TCP) are seeded with marrow stromal cells (MSCs) and cultured in expansion conditions. At 0, 3, 7, 14 and 21 days cell numbers and gene expressions are evaluated. After an initial drop in cell numbers, CPP and bone supported an increase in

proliferation activity and show no up-regulation of the mature bone markers on CPP and Bone, which suggests that these substrates support MSC expansion rather than differentiation. In contrast, MSC number on HA/TCP decreased with time and it showed a down regulation of early osteogenic markers. This along with a substantial increase in mature markers indicate that HA/TCP favours MSC differentiation and maturation along the osteogenic lineage.

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List of Abbreviations

THR	Total hip replacement
UHMWPE	Ultra high molecular weight polyethylene
MCB	Morsellized cancellous bone
HA	Hydroxyapatite
TCP	Tricalcium Phosphate
CPP	Calcium polyphosphate
E_c	Modulus of the particulate constructs
E_p	Modulus of particle
MSCs	Marrow stromal cells
RUNX-2	Runt related transcription factor 2
ALP	Alkaline phosphatase
COL-I	Collagen I
ON	Osteonectin
OP	Osteopontin
BSP	Bone sialoprotein
OC	Osteocalcin
SOX 9	SRY-box containing gene 9
COL II	Collagen II
AGG	Aggrecan
PPARγ	Peroxisome proliferator-activated receptor gamma
LPL	Lipoprotein Lipase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
BMPs	Bone morphogenic proteins
FGF	Fibroblasts growth factor
TC	Tissue culture plastic
PBS	Phosphate buffered saline
EM	Expansion media
OM	Osteogenic media
TC/EM	Tissue culture plastic in expansion media
TC/OM	Tissue culture plastic in osteogenic media
MTT	3-(4, 5-dimethylthiazol-2yl)-2, 5diphenyltetrazolium
DMSO	Dimethyl sulfoxide
BrdU	5-bromo-2-deoxyuridine
SEM	Scanning election microscope

Acknowledgements

Special thanks to my supervior, Dr Göran Fernlund, who has given a great deal of support and guidance through the development and completion of my masters.

I would also like to thank Dr Hanspeter Frei, who spent many hours advising and teaching me in the field of cell biology.

I would also like to acknowledge the help I received from Dr Fabio Rossi and the members of his group at the Biomedical Research Center

To my wife Mariko.

1 Introduction

1.1 Background

A total hip replacement (THR) is a highly effective procedure for restoring the hip function of patients suffering from ailments such as osteoarthritis or a fracture of the hip due to trauma. The total hip replacement originally developed in the 1950's by Sir John Charnley consists of a metallic ball joint which is fixed in the medullary canal of the femur and an ultra high molecular weight polyethylene (UHMWPE) cup secured into the socket of the pelvis[1]. Due to an aging population and improvements to the procedure the incidence of total hip replacements is increasing, and in Canada has jumped from 16,500 in 1994 to over 25,000 in 2004[2]. Although complications over the past two decades have decreased significantly there are still problems associated with THR. One major concern is femoral osteolysis following surgery causing excessive loosening of the implant. Osteolysis is a degeneration or dissolution of bone due to a number of reasons, including aging, stress shielding and implant wear particles[3]. In all cases a revision surgery is required to correct the problem and accounts for between 10 and 20% of all hip surgeries each year[4]. In severe cases a revision total hip replacement may be required.

A revision THR involves the removal of the loose implant and the source of wear particles; the joint is then prepared and fitted with a new implant. In the case where severe osteolysis has occurred, a long-stem prosthesis is cemented into an impacted allograft. Impaction allografting is a method first made popular by Gie in which

morsellized cancellous bone (MCB) from a donor is impacted in the medullary canal of femur while leaving a small canal for the new prosthesis to be inserted [3,5]. Acrylic bone cement is then injected into the graft under pressure followed by the insertion of the new prosthesis, Figure 1.1. This cemented allograft construct has the benefit of providing initial mechanical support of the implant as well as being a scaffold for bone ingrowth from surrounding bone[6]. Although impaction allografting is the only method that has been shown to reverse the loss of bone stock caused by osteolysis[6] there are a number of problems associated with this technique. Allograft material has been known to transmit pathogens such as hepatitis C and HIV[7,8] . The impaction allografting procedure has also been associated with early implant subsidence of the femoral component as well as a high incidence of intra- and postoperative fracture[9]. Furthermore, due to the increased use of allograft, the bone bank supply of cancellous bone may not be sufficient to meet the increasing demand in the future.

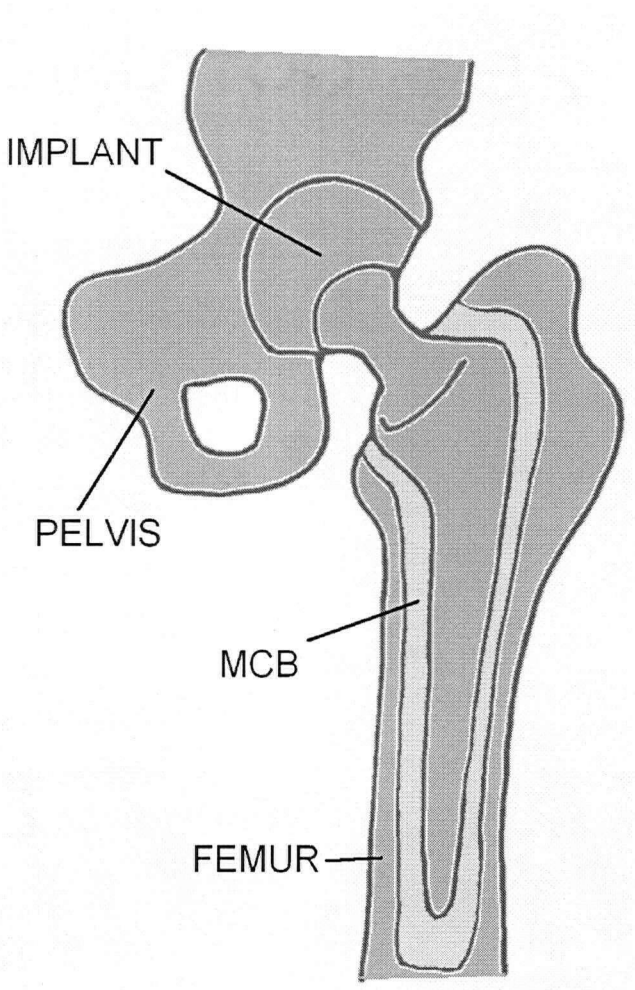

Figure 1.1 Schematic of revision total hip replacement

A number of synthetic materials are now being investigated for the use as bone graft substitutes. When using a biomaterial for bone tissue engineering a number of factors must be considered. The material must be biocompatible in that they should not elicit an inflammatory response or exhibit any cytotoxicity when placed in the body. They should maintain structural support and integrity until the newly formed bone is able to take over the mechanical role. The scaffold material should also encourage the formation of bone and have enough void space to allow bone ingrowth as well as vascularization for

maintenance of the newly formed tissue. And finally they must be easy to use, such that they can be easily sterilized and handled during surgery[10]. Among the more popular materials being studied that meet many of these design requirements are ceramics of calcium phosphate, a principal inorganic constituent of natural bone. Hydroxyapatite (HA), tricalcium phosphate (TCP) and a biphasic calcium phosphate (HA/TCP) are the most widely used and have shown to form tight bonds with host bone tissue[11,12]. Hydroxyapatite has a chemical composition of $Ca_{10}(PO_4)_6(OH)_2$ or $3Ca_3(PO_4)_3 \cdot Ca(OH)_2$ and spatially it is a triangular structure made up of 3 $Ca_3(PO_4)_3$ surrounding a central $Ca(OH)_2$[13]. TCP has a chemical composition of $Ca_3(PO_4)_2$ that has three polymorphs β, α, and ά. β-TCP is the most commonly used for tissue engineering applications and has a structure made up of two different columnar crystals. The first column (A) has the form PO_4 CaO_3 CaO_6 PO4 and the second column (B) has the form PO_4 CaO_7 CaO_8 CaO_8 PO_4. Each A column is surrounded by 6 B columns while each B is surrounded by 2 A and 4 B columns[14]. However due to there brittle nature, these ceramics in porous form have low strength and toughness, which has hindered their use in clinical applications.

A novel ceramic, calcium polyphosphate (CPP), currently being investigated for the regeneration of cartilage has been shown to be biocompatible and to have good mechanical properties and may prove to be a suitable graft material for the hip[15]. CPP $[Ca(PO_4)_2]_n$ is an inorganic polymer whose structure of CPP is comprised of 2 element units of a circular chain made up of eight PO_4 tetrahedrons. The circle chain units are thought to be connected through the O atom[16]. Some of the features that make CPP particularly appealing are that CPP can be produced with an interconnected porous

network of approximately 100um in suitable for ingrowth of bone[17]. Porous CPP is reported to have a tensile strength up to 24.1 MPa which is similar to the properties of bone (5-10 MPa cancellous, 80-150 MPa cortical)[10,15]. The degradation product of CPP is calcium orthophosphate which is naturally occurring and easily metabolized in the body[15]. Furthermore CPP can be made into particles of varying size which can easily form to any defect site in the bone.

1.2 Scaffold Requirements

One of the main requirements of a scaffold being used as a replacement for MCB in revision THR is that it has adequate mechanical properties to prevent micromotion and subsidence of the implant in the hip. In impaction allografting a particulate bed of bone surrounds the implant and is confined by the cylindrical shell of the cortical bone. The load from the weight of the body is transmitted from the pelvis down through the implant to the graft bed then outwards to the surrounding cortical bone. Thus in order to prevent subsidence of the implant in the femur the graft bed must effectively transfer the load from the implant to the surrounding cortical bone without significant movement or failure.

The mechanical properties of MCB have been investigated thoroughly in an effort to improve its stability in the hip. A number of different techniques have been used to improve the graft bed quality including washing and drying of the MCB, using different impaction protocols, and the addition of extender particles[18,19,20]. In these studies the

metrics used to measure any improvements to the graft bed during these procedures are compressive modulus and the shear strength[18,21,22]. The compressive modulus, related to the packing density and contact between particles, is thought to be beneficial for initial implant stability as well as limiting the intermittent motion of the graft allowing for easier integration of surrounding bone[23,24]. The shear strength of the graft bed, which is influenced by the internal friction and interlocking of the graft particles, is thought to be important in the prevention of stem subsidence in the femur[18,21]. Thus in order to be suitable for the use in revision THR, the graft bed of a scaffold material must have a compressive modulus and shear strength similar or better than that of MCB.

Another important requirement of a scaffold material being used in revision hip surgeries is that it must provide a suitable environment for bone growth. The process of bone growth is a complex development of osteogenic cells comprised of three major stages: cell colonization and multilayering, initial intercellular matrix production, and matrix mineralization[25,26]. In the initial colonization stage there is a large recruitment of osteogenic cells from the surrounding bone. This group of cells is a heterogeneous population of cells in the osteogenic lineage ranging from undifferentiated mesenchymal stem cells to fully committed osteoblasts. Each cell type in the osteogenic lineage has a distinct functionality and all are required for the continued growth and maintenance of new bone. Osteoblasts are the primary bone forming cells and during new bone development they arrange themselves in layers located close to the bone surface. These cells lay down a collagen matrix that they in turn mineralize. Once osteoblasts are surrounded by mineralized bone they become osteocytes which remain in the bone and

regulate the transportation of nutrients and waste. Cells that are close to the osteoblast on the side away from the newly forming bone front are called preosteoblasts. These cells are unable to produce bone but they are capable of cell division and therefore are the supporting cells of the osteoblasts and will differentiate into osteoblasts as the bone front moves toward them[27]. The preosteoblasts are in turn supported by a class of cells known as osteoprogenitors. These cells are highly proliferative and are the earliest cells committed to the osteogenic lineage which can be induced to differentiate into preosteoblasts[25,28]. However, none of the cells in the osteogenic lineage have the ability for self renewal. This is a characteristic reserved for a class of cells called stem cells. This ability for stem cells to self renew makes them an important component in maintaining tissues throughout the lifetime. Mesenchymal stem cells in particular, are responsible for the maintenance of mesenchymal tissues including bone and give rise to committed cells such as osteoprogenitors.

A common technique being used in tissue engineering is to seed a heterogeneous cell population (acquired from bone marrow) containing mesenchymal stem cells and osteogenic cells onto scaffolds in order to enhance the bone growth on the scaffolds[12,29,30]. Signals received from both the substrate they are attached to and the surrounding culture medium, regulate the expansion and differentiation behaviour of a cell population[11]. As these cells differentiate down the osteogenic lineage there are characteristic changes in the genes they express[31]. By monitoring the gene expression along with the cell numbers, investigators can determine the osteogenic potential of a

substrate. It order to be a suitable substrate for bone growth a material must be osteogenic in that it allows the growth and differentiation of osteogenic cells[32].

1.3 Thesis Objectives

The objectives of this study are to investigate the mechanical and biological feasibility of using calcium polyphosphate as a replacement scaffold for morsellized cancellous bone in revision hip surgery. In the mechanical testing we will use confined compression and direct shear tests to compare the compressive modulus and the shear strength of CPP to that of MCB. Furthermore we will investigate how the properties of the constitute particles such as size, shape and modulus influence the properties of the entire graft bed. In the biological testing we will investigate the effect of substrate material on the growth and differentiation of bone marrow cells, through cell population and gene expression assays.

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2 Mechanical properties of particulate bone scaffolds in THR: a comparison of CPP and MCB[1]

2.1 Introduction

Impaction allografting is a technique used in revision total hip replacement in which morsellized cancellous bone (MCB) is impacted into the medullary canal of the femur in order to fill bone defects, support a new implant and provide a matrix for bone regeneration [1,2]. This technique has been shown to have positive results in stabilizing the implant [2,3,4,5], however clinical problems such as intra- and post-operative fracture of the femur and stem subsidence have been observed [2,6,7,8]. Additional problems associated with the use of allograft in revision surgeries, such as disease transmission and availability [9,10], have prompted the development of substitute synthetic bone grafts.

Synthetic calcium phosphates, such as hydroxyapatite (HA) and tricalcium phosphate (TCP) have been extensively studied for the use as bone scaffolds [11,12,13]. Calcium phosphate materials are promising because they are readily available and, being similar to the mineral component of bone, they have shown good osteoconductivity[13,14]. Furthermore the porosity and degradation rates of these bioceramics can be tailored in the manufacturing process. One of the major drawbacks however is their brittleness as well as their poor strength when processed with pore sizes suitable for bone incorporation. Calcium polyphosphate (CPP) is novel calcium phosphate ceramic currently being investigated for the use in cartilage repair [15,16,17]. CPP has good mechanical properties as

[1] A version of this chapter will be submitted for publication.

shown in a study by Pilliar *et al*, where CPP rods of 30-45% porosity were measured to have tensile strengths of up to 24.1 MPa [15]. The biocompatibility of CPP was examined in a later experiment by Grynpas *et al*, where similar rods were surgically implanted in the distal femurs of New Zealand white rabbits and after 1 year had bone ingrowth up to 25% of available pore area and degradation of 59%[16]. CPP has been shown to have suitable biocompatibility and with better mechanical properties than many other calcium phosphate materials it may provide a suitable bone graft for revision hip surgeries.

In addition to the material properties, the structure of the graft is important in determining the mechanical characteristics of the graft bed. The use of particulate materials as bone grafts has a number of attractive attributes. Particles are easy to use during surgery, they readily conform to defect sites, and when packed together they inherently form a porous network (important for bone ingrowth and vascularization). However particulate graft beds are susceptible to shear failure and shear deformation, and in impaction allografting this has been suggested as a major cause for migration of the implant in femur [18,19,20].

In soil mechanics, the Mohr-Coulomb failure law is used to describe the shear strength, τ_f, of an aggregate given by

$$\tau_f = c + \sigma \tan\phi \qquad (2.1)$$

Where σ is the applied normal stress, c is the cohesion of the particles, and ϕ is the internal friction between the particles [21]. Many studies use the Mohr-Coulomb criteria as

a measure of the graft bed shear capability [18,19,20]. Graft bed compressive stiffness or modulus has also been suggested to be of primary importance for implant stability, progressive vascularization, and replacement by host bone [22,23]. We believe that by measuring both the shear strength and compressive modulus of the graft beds we can get an overall measure of the graft bed load bearing capability.

Our first objective is to determine the mechanical properties of a calcium polyphosphate particulate and compare it to the mechanical properties of the current gold standard for impaction allografting, MCB. The stiffness of the constructs is compared through testing in confined compression and the shear strengths of the materials are compared through direct shear tests.

A number of studies have tried to improve the properties of allograft beds through different impaction techniques or adding extender particles such as bioglass or HA/TCP but no one has attempted to determine the maximum properties attainable[18,24,25]. Thus, our second objective is to establish a relationship between the properties of the particles and the properties of the particulate construct. This is achieved by characterizing the mechanical properties of ideal particulate constructs comprised of spherical balls of varying material and size.

2.2 Materials and Methods

2.2.1 Materials

The CPP particles used in this study were provided by Dr. Pilliar (University of Toronto), and were formed by calcining precursor powders of calcium phosphate monobasic monohydrate in a platinum crucible [15]. These powders were then gravity sintered in cylindrical tubes which were later crushed into angular particles of 1-3mm in diameter (Figure 2.1a). The resulting particles have 30-45% internal porosity with interconnected pores in the 100μm range (Figure 2.1b). The compressive modulus of the particles is reported to be approximately 5 GPa and tensile strengths in the range of 5-24MPa [15].

The MCB used for comparison to CPP was not tested in this study but were tested using the same experimental setup by Albert *et al* [26]. Briefly, thirty-nine femoral heads were morsellized with a Lere bone mill (DePuy, Warsaw, IN, USA). The cortical bone from the femoral neck and cartilage were removed prior to morsellization. The graft was pooled together and rinsed in a saline solution to remove fat and marrow tissue. The graft particles ranged in size between 0.6 and 8mm, with 50% finer than 2.4mm[26].

The spherical balls (Cincotta Industrial Componets Inc.) used for the second objective of this study are comprised of three different materials; steel, glass and nylon of approximate modulus 200, 70 and 2 GPa respectively [27]. The steel balls consisted of three different sizes (1.19, 1.54 and, 2.38mm in diameter) while glass and nylon balls were 1.54mm in diameter (Figure 2.1a). All balls had a dimensional tolerance of

±0.127mm. The geometry and mechanical properties of all particles used in this study are shown in Table 2.1.

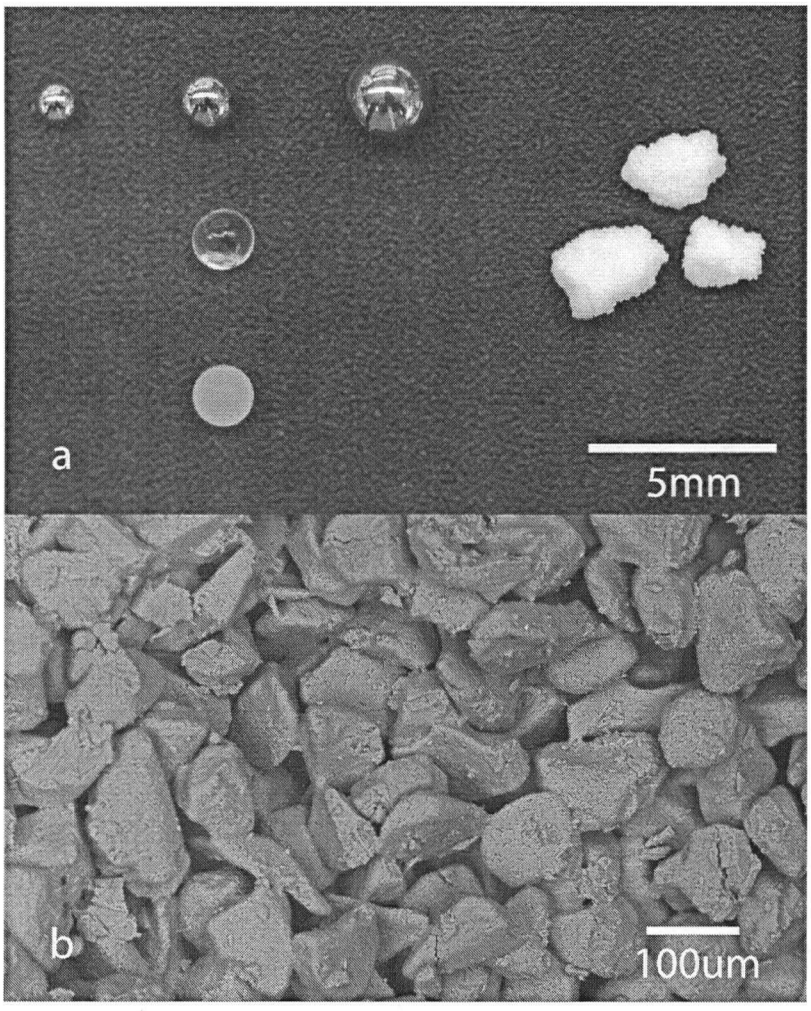

Figure 2.1: a) Particles used in testing: steel, glass, nylon and CPP b) Surface of CPP particles

Material	Particle Diameter (mm)	Particle Shape	Internal Pore Size (μm)	Young's Modulus E_p (Gpa)
Steel	1.19, 1.54, 2.38	Sphere	N/A	200 [a]
Glass	1.54	Sphere	N/A	70 [a]
Nylon	1.54	Sphere	N/A	2 [a]
CPP	1-3	Irregular	~100	5 [b]
MCB	0.6-8	Irregular	~500[c]	0.1-1 [c]

Table 2.1: *Material properties of particles used in testing*
[a] *From CRC Material Science and Engineering Handbook*[27]
[b] *From Pilliar et al* [15]
[c] *From Gibson and Ashby* [35]

2.2.2 Methods

The CPP and spherical particles were tested in confined compression using a servohydrolic testing machine (Instron Model 8874, Instron, Canton, Massachusetts). A stainless steel cylinder of 19mm internal diameter, wall thickness of 6mm was filled to a height of ~35mm. The samples were then loaded via a piston at a stress rate of 400 kPa per minute to a maximum stress of 2000 kPa (Figure 2.2a). The stress σ is calculated as the applied normal force F_{normal} divided by the cross-sectional area of the graft bed. The compressive strain ε is calculated as $\varepsilon = 1\text{-}H/H_0$ where H_0 is the initial height of the graft bed and H is the instantaneous height. A total of six samples (N=6) were tested for each particle type. The MCB was tested on the same testing equipment however due to its biphasic nature, the test cylinder was prepared with 36 radial holes of 1mm diameter to allow for fluid drainage. The time effects of the MCB also required a different compression test protocol described previously [26].

The particles were tested in confined shear in a custom designed shear box, which is similar to the confined compression setup. The shear box consists of two cylinders with internal diameter of 19mm and wall thickness 6mm. The top cylinder was rigidly fixed in place while the bottom cylinder was free to move along a linear guide. The samples were filled to a height of ~25mm, with half the sample on either side of the shear plane. A normal load was applied to the sample via the material testing machine (Instron Model 8874, Instron, Canton, Massachusetts), while a lateral shear load was applied to the bottom cylinder using a side mounted actuator (Instron Model A591-4, Instron, Canton, Massachusetts) (Figure 2.2b). Three normal stresses were used, 125, 250, 375 kPa, representing a range of stress seen by allograft material in a hip reconstruction [18]. The samples were sheared at a rate of 1.2mm/min up to a total of 6mm. A total of six samples (N = 6) were sheared for each particle type. The shear stress of the material was defined as shear force over the area of intersection of the two cylinders (Equation 2.2). This equation accounts for the decrease in cross sectional area as the cylinders slide past each other.

$$\tau(\delta) = \frac{F_{shear}}{(d/2)^2\left[2\cos^{-1}(\delta/d) - \sin(2\cos^{-1}(\delta/d))\right]} \qquad (2.2)$$

Where F_{shear} is the shearing force, d is the cylinder diameter and δ is the displacement of the bottom cylinder relative to the top. To allow for a direct comparison between CPP and the MCB, the CPP constructs were impacted prior to shear testing. The samples were impacted 20 times at 900N by the material testing machine at a rate of 4hz which represents the impaction procedure used clinically[28].

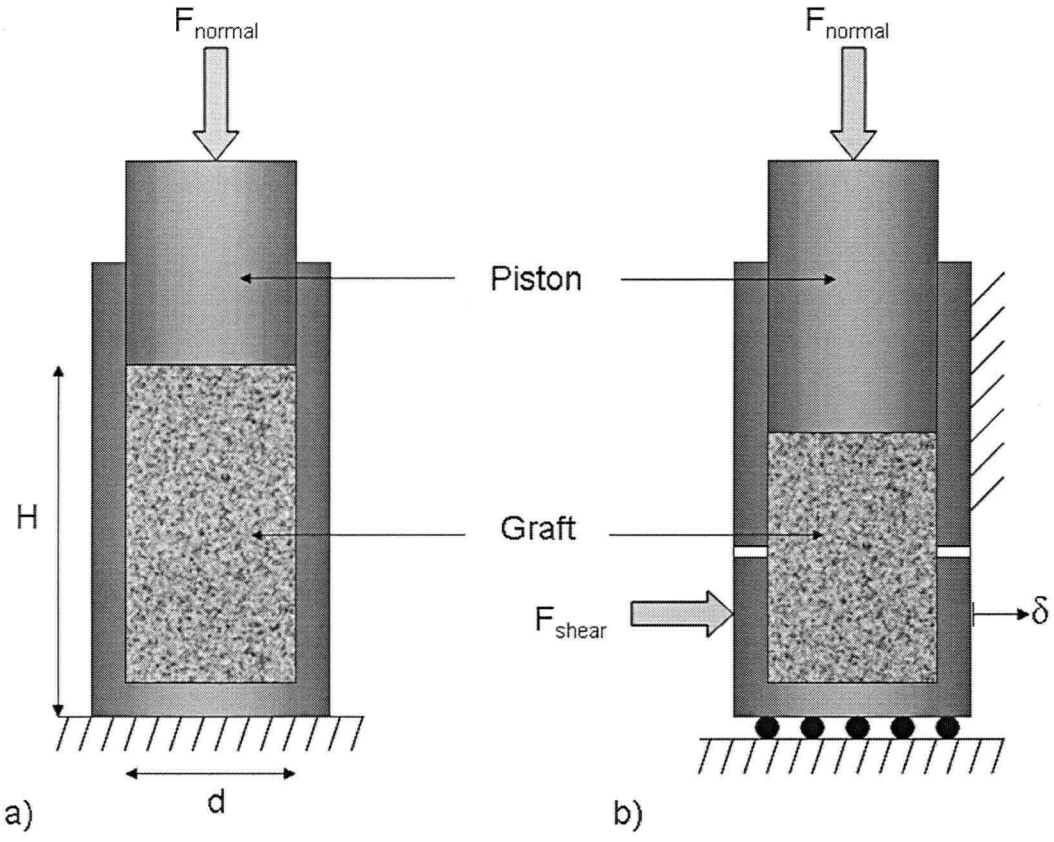

Figure 2.2: Experimental setup: a) confined compression and b) direct shear.

The volume fraction of particles in the graft bed of the CPP and spherical particles was found by measuring the amount of water displaced by the sample in a cylinder with an internal diameter of 19mm and at a height of 25mm. The volume fraction of MCB was measured from histology by sectioning the graft bed and measuring the percent of the cross-sectional area occupied by bone [26]. This measurement technique counts internal pores as being unoccupied space and will therefore result in a lower volume fraction than the technique used for the CPP and spherical particles which do not account for internal pores.

2.2.3 Statistical Analysis

Pearson correlations were used to determine the linear correlation, R, of the construct modulus E_c with the particle size. Compression results were analyzed with a student T-Test. Shear strength was compared between the materials with a 2-way ANOVA (material, normal stress). Student-Newman Keuls tests were used for post-hoc analyses, and a significance level of 0.05 was used.

2.3 Results

The results of the compression tests are shown in Figure 2.3 as the average compressive stress σ of the samples (N=6) as a function of strain ε for each particulate (CPP and spherical). Due to the non linear nature of the stress-strain curves the modulus of the particulate constructs, E_c, was quantified as the secant modulus at a compressive stress σ of 1100 kPa. The secant modulus represents the effective stiffness of the construct as it is used clinically. The resulting E_c for all materials are presented in Table 2.2, along with the moduli of the particles themselves, E_p. Figure 2.3 and Table 2.2 show that E_c increases with E_p and if linear correlations are performed E_c is also shown to increase linearly with the particle diameter d for the steel particles (R = 0.82). Table 2.2 shows that the construct modulus E_c is much smaller than the particle modulus E_p.

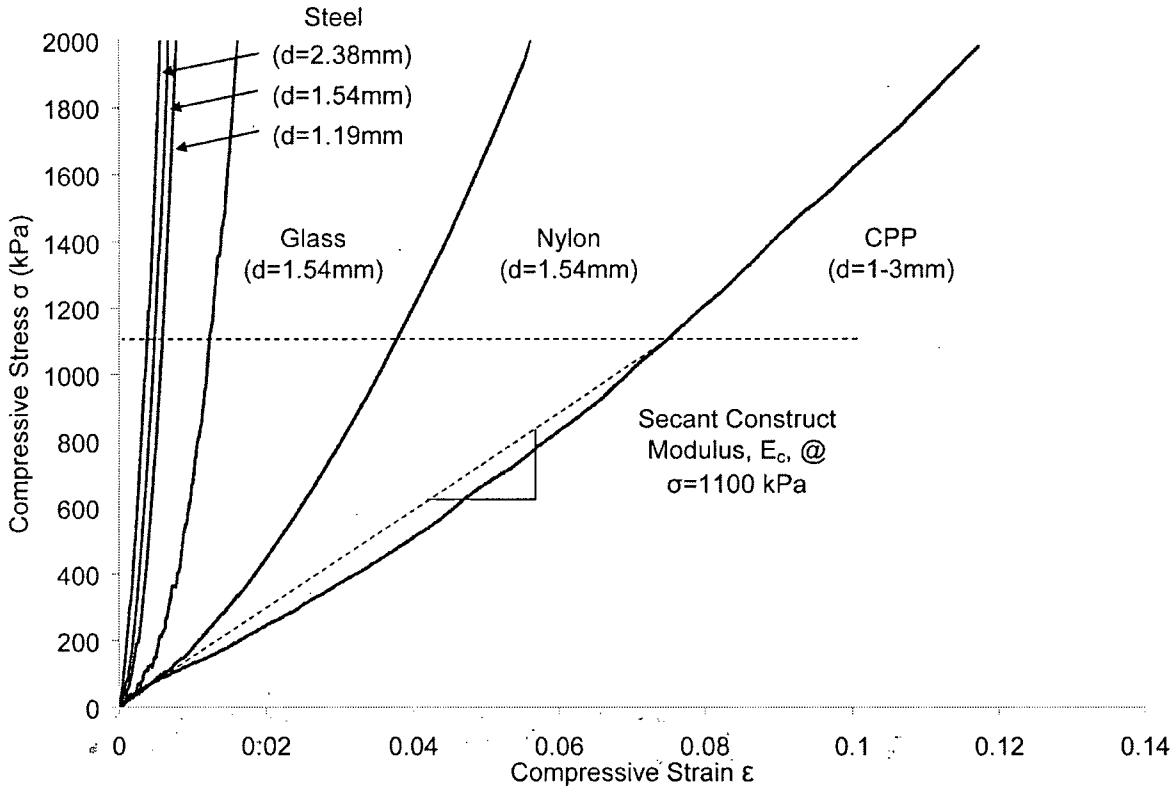

Figure 2.3: Confined compression results – stress as a function of strain - for spherical and CPP particles. The lines represent an average of six individual tests. Construct modulus E_c defined as secant modulus at a compressive stress σ = 1100 kPa.

Material	Size (mm)	E_p (GPa)	E_c (GPa)	V_f (%)	E_p/E_c
Steel	Large (d=2.38)	200	0.310	60.1 ± 0.2	645
	Medium (d=1.54)	200	0.237	58.1 ± 0.2	844
	Small (d=1.19)	200	0.198	59.5 ± 1.0	1008
Glass	Medium (d=1.54)	70	0.093	59.3 ± 0.9	751
Nylon	Medium (d=1.54)	2	0.031	59.0 ± 0.1	64.5
CPP	(d=1-3)	5	0.0151	48.9 ± 0.9	331
MCB	(d=0.6-8)	0.1-1	0.0149	38 (33-45)[a]	6.71-67.1

Table2.2: *Construct modulus E_c measured as the secant modulus of the stress strain curve from confined compression tests at σ = 1100 kPa. Volume fraction of particles in the graft bed is the V_f(%).*
[a] Measured from histology as the graft density by Albert [26]. Reported as median (range)

Measurements showed significant difference between CPP and spherical particle volume fractions, 48.9 ± 0.9%, and ~59% respectively. The volume fraction of the MCB graft is reported to be approximately 38% [26].

The results of the shear tests for the CPP particles are shown in Figure 2.4 as the average shear stress τ of the tests (N=6) at each of the different normal stresses σ (125kPa, 250kPa, and 375kPa). The shear strength, τ_f, is commonly defined as the value of the shear stress corresponding to the "plateau" of the shear displacement curve. Since there is no clear plateau for the CPP tests, and to allow for direct comparison with the shear results of MCB, the shear strength τ_f is taken to be the shear stress at a displacement of δ=1mm (approximately 5% cylinder width). For the spherical particles (graphs not shown) the shear strengths were taken to be at displacement equal to particle diameter, which was found to correspond with the plateau of the shear stress. There was an increase in the shear strength with increasing normal stress σ for all materials ($p<0.01$). The results of shear tests are presented in terms of the Mohr-Coulomb parameters cohesion intercept (c) and friction angle (φ) in Table 2.3.

Mohr-Coulomb Data ($\tau_f = c + \sigma \tan\Phi$)			
Material	**Size**	**Cohesion c (kPa)**	**Friction Angle Φ (Degrees)**
Steel	Large	25.9	39.7
	Medium	16.6	37.4
	Small	20.3	29.9
Glass	Medium	14.5	38.5
Nylon	Medium	28.5	33.9
CPP		380	45.6
MCB		510	29.2

Table 2.3: *Results of shear tests reported as Mohr-Coulomb parameters.*

Figure 2.5 shows the results of the shear strength τ_f as a function of the compressive normal stress σ. These results show that there is no significant difference in the shear

strengths of CPP and MCB particulates ($p \geq 0.3$), there is a significant increase of the shear strength of CPP and MCB compared to the spherical balls ($p<0.01$). Furthermore, the spherical ball data yielded no difference in shear strength between different materials of the same size (steel, glass, and nylon of 1.54mm diameter)($p \geq 0.1$), but the steel particles of different sizes (1.18mm, 1.54mm and 2.36mm diameter) showed a significant difference between large and small particles at normal stresses of 250 kPa and 375 kPa ($p<0.05$).

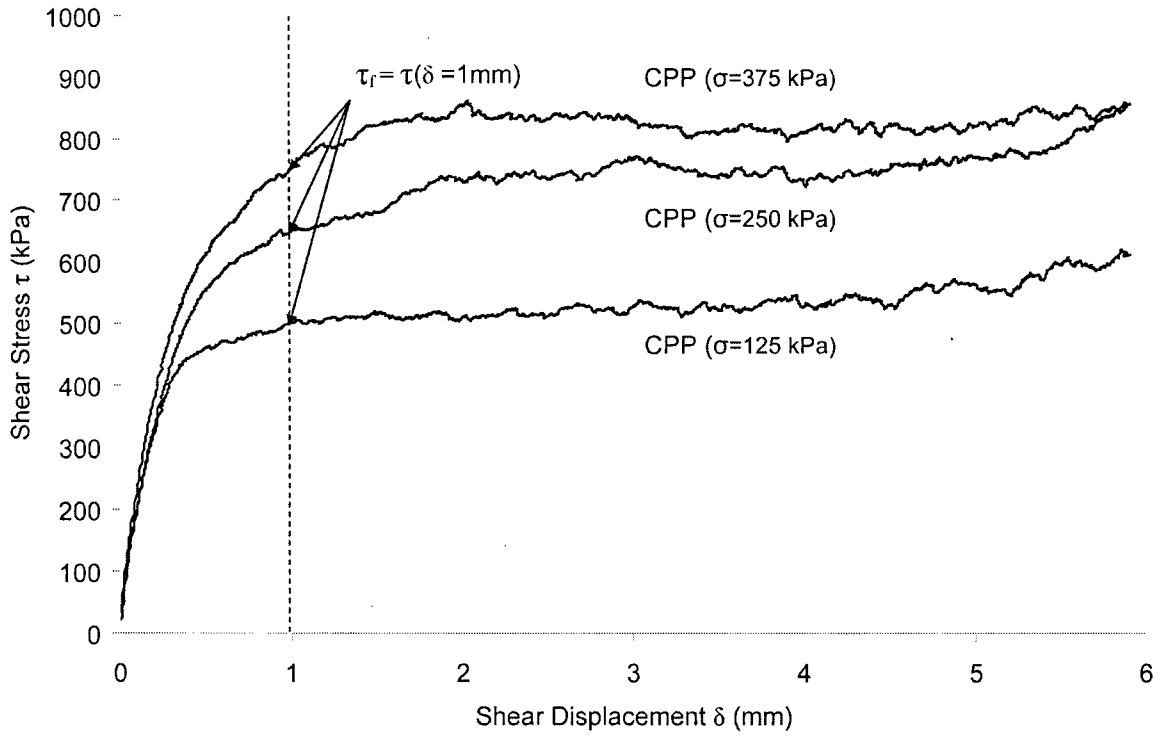

Figure 2.4: Results of shear test for CPP particles for normal stress: 125, 250 375 kPa. The lines represent an average of six individual tests. Shear strength, τ_f, defined as shear stress at a displacement of 1 mm.

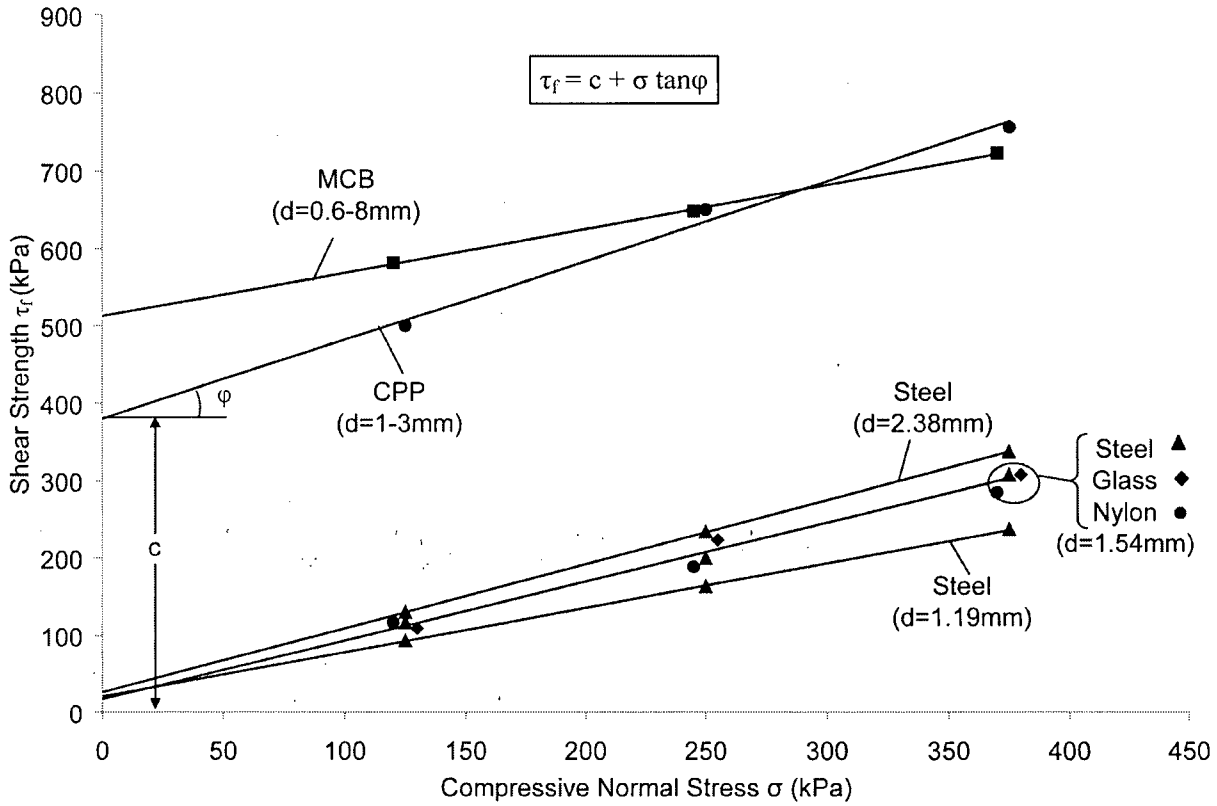

Figure 2.5: Results of shear test – shear strength as a function of normal stress σ (125, 250, 375 kPa) . Mohr-Coulomb parameters are defined as shown. Results were offset on the abscissa for clarity. MCB data from Albert [26].

2.4 Discussion

In the confined compression results of Figure 2.3 a non linear stress-strain relationship is observed in all materials. This effect can be explained by contact mechanics models such as the Hertz contact model and the de Gennes soft crust model which describe the stress-strain relationship between two particles being forced together [29]. Both models give a relationship of the form

$$\sigma \propto E_p \varepsilon^n \qquad (2.3)$$

Where σ is the applied compressive stress, E_p is the modulus of the particle, ε is the compressive strain, and n is a model parameter. In the Hertz model of two homogenous spheres the term *n* is equal to 3/2 whereas the de Gennes model which accounts for a softer surface due to irregularities of oxidation results in an *n* value of 2. Both these models indicate that particles with higher moduli will result in stiffer constructs and that the secant modulus E_c increases with increasing particle modulus E_p and strain as

$$E_c = \frac{\sigma}{\varepsilon} \propto E_p \varepsilon^{n-1} \qquad (2.4)$$

This result is consistent with our compression results for the spherical particles but not if we compare the CPP results with the nylon ball results. This is most likely explained by the difference in shape of the two particles. It has been shown that irregularity hinders particle motion and their ability to reach dense packing configurations (Table 2.2), resulting in fewer contact points between particles [30]. Duran suggests that a particulate construct becomes more rigid with increasing load through a proliferation of contact points (which further explains the non-linearity of the stress-strain curves in Figure 2.3) [29]. Therefore CPP having a lower packing density than nylon has fewer contact points and results in a lower construct stiffness. Another effect of the particle shape is a difference in deformation of the particles. Irregular particles are more deformable than round particles, because of more localized deformation, which is seen by comparing cone-to-plane and sphere-to-plane contact in a Hertzian contact model [31].

Because spherical particles represent an ideal particulate it is useful to use the confined compression data of the spherical particles to gain an understanding of the effect of particle modulus E_p on the construct modulus E_c. The E_c and E_p data of Table 2.2 plotted on a log-log graph (Figure 2.6) shows a relationship between the construct modulus and particle modulus. The medium ball data is approximately described by the straight line fit

$$E_c \approx C(E_p)^{0.4} \qquad (2.5)$$

where the constant term C = 0.02 and E_c and E_p are in units of GPa. This non linear result is consistent with the Hertz contact model, and the de Gennes soft crust model which can be shown to have a similar relationship with power terms of ⅔ and ½ respectively.

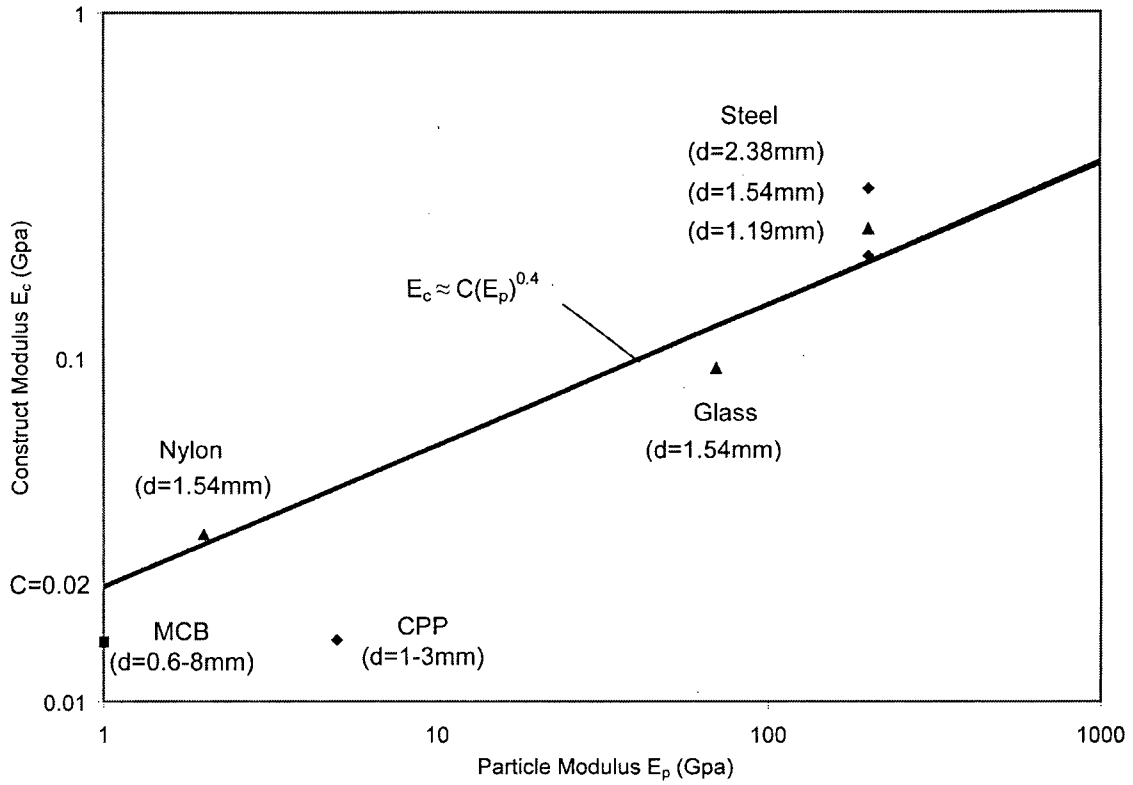

Figure 2.6: Experimental construct modulus E_c as a function of particle modulus E_p. The points represent an average of six individual tests. Straight line fit to medium ball data.

A notable outcome of Equation 2.5 is the constant term (C =0.02), which suggest that the construct modulus E_c of a spherical particulate will always be a small fraction of the particle modulus. This result is similar to that of by Abel-Ghani *et al* who showed a construct modulus of glass ballotini being 0.007-0.03 of the particle modulus [32].

Equation 2.5 can be further refined by defining the construct modulus E_c as a function of the applied stress σ. A family of curves can be used to show

$$E_c \approx C\sigma^{1/3}(E_p)^{0.4} \qquad (2.6)$$

where C = 0.002 and E_c and E_p units of GPa and σ is in units of kPa. Equation 2.6 was found to closely describe current experimental data with an applied stress between 1000 and 2000 kPa. One of the requirements of materials used for bone scaffolds is that they have an interconnected pore structure to allow bone ingrowth and vascularization. Therefore it is useful to extend Equation 2.6 to include the effects of internal porosity in the particles. A number of studies have shown a decrease in modulus with increased porosity [33,34]. For example the Gibson-Ashby model [35] is given by

$$\overline{E}_p \approx E_s(1-p)^2 \qquad (2.7)$$

Where $\overline{E}_p$ the effective particle modulus, p is the porosity of the particle and E_s is Young's modulus of the solid material. Combining Equations 2.6 and 2.7 results in a relationship for the modulus of a construct of porous particles

$$E_c \approx C\sigma^{1/3}\left(E_s(1-p)^2\right)^{0.4} \qquad (2.8)$$

As this relationship is based on a model for spherical particles it does not account for the two important features of irregular particles: increased deformation and decreased packing density. The constant (C = 0.002) in Equation 2.8 assumes a random packing density of approximately 60% which is in between the loose stack limit for spheres of 56% and the theoretical maximum random packing of spheres of 64% [36]. Since irregular particles have lower packing density and increased deformation they will inevitable have a lower construct modulus as is evidenced by the CPP particles. Equation 2.8 predicts

that CPP particles of 40% porosity and Young's modulus of 100% dense CPP of 48 GPa [37] to have a construct modulus of 0.066 GPa, significantly higher than the measured E_c of 0.0151 GPa. Hence we believe Equation 2.8 represents an approximate upper bound for the construct modulus of porous particles.

The shear results shown in Figure 2.5 give insight into the effects of particle modulus, size, and shape on shear strength. The shear data of medium sized balls (d = 1.54mm) shows that particle modulus does not significantly affect the shear strength of the construct. This observation is in agreement with soil mechanics theories where it is generally agreed that frictional forces and cohesion between particles are the primary mechanism in developing shear strength of a particulate construct [21]. There are two types of frictional forces that resist motion in a soil, sliding friction and translational or interlocking friction. Sliding friction develops through the interlocking of microscopic aspirates on the particle surface. Interlocking friction arises from particles being required to move from their original position and ride over adjacent particles. The effect of these frictional forces is reflected in the Mohr-Coulomb term of internal friction ϕ. In the shear data for the steel particles of all three sizes there is an increase in the internal friction angle with increasing particle size. Since there is no difference in the surface morphology of the particles this effect is likely due to the difference in interlocking friction between particle sizes. The breakdown of interlocking is associated with in increase in volume as the particles ride over one another [21]. For larger particles this volume change is greater and thus requires a higher shear force, which could explain the observed particle size effect. One distinct result from the shear data in Figure 2.5 is the

difference in shear strength of CPP and MCB compared to that of the spherical particles. This difference is mainly due to the cohesion (c) of the particles described by the vertical intercept in Figure 2.5, which represents the shear strength under zero applied normal stress. The cohesion is likely dependent on the particle shape since there is no significant difference in the cohesion of the spherical particles. Spherical particles have point-like contacts that are mechanically instable, which means local slipping and rotation can easily occur [30]. Irregular particles will have more stable face-to-face contacts which is superior at resisting sliding and rotation [30]. The effect of cohesion far outweighs any gain through internal friction as even a large steel spherical particle construct under a normal load of 375 kPa will not match the shear strength of an angular CPP construct with no normal load (Figure 2.5). Another interesting point is that even though the spherical particles had a significantly higher volume fraction (V_f = 59%) to that of CPP (V_f = 48%), their shear strengths were much lower. This suggests that in shear the number of contact points is not as important as the type of contact points. This is consistent with the work of Dunlop, who found no significant difference in the shear strength of MCB with varying amounts of particle grading [18].

When interpreting the results of this study it is important to note the limitations of the tests. Both the confined compression and shear tests were performed in testing chambers which are small relative to the ASTM standards [38]. By using a small size cylinder the edge effects may be significant, but it was felt that a cylinder of this size more closely represented the clinical situation of a medullary canal. Furthermore, shear testing was performed in a direct shear box which forces a material to shear along a prescribed plane

rather than allowing the material to shear along the weakest plane as in a triaxial shear test. This may lead to artificially high shear strength. However, many studies have used this method of testing and it is felt to be adequate for the purpose of comparing different constructs [18,19,20]. Finally, the measurement of shear strength is difficult to define. The ASTM standards define shear failure occurring in the between 10-20% shear displacement however there is no indication of what this represents [38]. The shear strength of CPP was taken at δ = 1mm to allow for direct comparison with previous results for MCB, whereas the shear strength of the spherical balls was taken to be at a displacement equal to their diameter. Physically this represents the horizontal displacement one ball must travel in order to move from beside an adjacent ball to the top of it.

There are a couple main implications of this study. First, even if a particulate construct is comprised of very stiff particles, it will have a relatively low compressive construct modulus. For example, using the upper bound solution in Figure 2.6 we can see that even a construct of alumina particles ($E_p \sim 400$ GPa) would result in a compressive construct modulus no higher than 0.5 GPa. This is much lower than the modulus of PMMA (~3 GPa) currently used as a bone cement to strengthen and stiffen allograft in revision surgery. This indicates that bone graft made of particles may need to be supplemented with a binder material in order to achieve an adequate stiffness. Second, shear strength is significantly increased for irregular shaped particles, however, irregular shaped particles give a much lower packing density which negatively affects the compressive construct modulus. Therefore, when making a particle construct, a balance between packing

density and shape must found in order to optimize both the construct modulus and shear strength.

2.5 Conclusion

This study showed that calcium polyphosphate particulate graft beds had similar mechanical properties both in terms of stiffness in confined compression and shear strength to that of morsellized bone. This suggests that CPP, from a mechanical point of view, is a suitable bone graft material for the use in revision surgery. Confined compression tests showed that the stiffness of the construct increased with particle stiffness and applied stress. It was also observed that irregular shaped particles had a lower packing density and resulted in lower a construct modulus. Shear tests showed that shear strength increased with increasing normal load and was much higher for irregular shaped particles than spherical particles. The shear strength of the construct was not affected by the particle modulus, and only was weakly affected by the size of the particles.

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3 Effect of bone graft substitute on marrow stromal cell proliferation and differentiation[2]

3.1 Introduction

Marrow stromal cells (MSCs) have been well established as a source of progenitor cells for various lineages of the musculoskeletal system capable of differentiating into osteoblasts, adipocytes, chondrocytes, and myoblasts [1,2,3,4]. MSCs can be readily isolated from bone marrow aspirates by virtue of their adherence to plastic and expanded a billion-fold in culture while maintaining their proliferation and differentiation potential [5,6]. This makes MSCs ideally suited for tissue engineering and regenerative medicine approaches to repair musculoskeletal tissue since they will not only contribute to the regeneration but also maintain the repaired tissue [7,8,9].

MSCs are believed to contain a small population of mulitpotential mesenchymal stem cells. Although their "stemness" is still debatable they are thought to have the capacity for asymmetric division and give rise to committed progenitors of the different mesenchymal tissues including bone [10]. The commitment of mesenchymal stem cells towards the osteoblast linage can be characterized by different maturation stages starting with a multipotential stem cell, and progressing through osteoprogenitor, preosteoblast, osteoblast and finally to osteocyte or bone lining cell [11,12,13]. These differentiation or maturation stages are associated with changes in expression levels of osteoblast-

[2] A version of this chapter will be submitted for publication.

associated molecules including cbfa1 (RUNX-2), alkaline phosphatase (ALP), collagen I (Col-I), osteonectin (ON), osteopontin (OP), bone sialoprotein (BSP), and osteocalcin (OC)[11,12]. Differentiation of MSC towards the ostogenic lineage can be induced by soluble factors including dexamethasone, ascorbic acid and β-glycerphosphate or with growth factors such as bone morphogenic proteins (BMPs) and fibroblasts growth factor (FGF) on a number of substrates[14,15,16]. Cell differentiation and proliferation can also be induced by signals directly received from the substrate through trans-membrane or cytoplasmic receptors[17]. It has been shown recently that differences in substrate elasticity and surface microstructure can lead to different lineage commitment[18,19].

However it remains unclear from these investigations whether different substrates affect proliferation and differentiation of MSCs under expansion conditions. Therefore the aim of this study was to determine the effect of different substrates, currently used for bone regeneration and tissue engineering, on the proliferation and differentiation of MSCs under expansion conditions.

The four substrates evaluated in this study were bone, calcium polyphosphate (CPP), biphasic hydroxyapatite/tricalcium phosphate (HA/TCP) and tissue culture plastic (TC). Bone is currently one of the most used bone graft materials, with a natural composition and structure it makes an ideal scaffold for bone healing and remodelling[20,21]. CPP is a novel ceramic currently being investigated for its use in cartilage repair due to its good biocompatibility and mechanical properties[22]. HA/TCP is a ceramic that has been studied extensively and has been shown to be suitable for bone ingrowth *in vitro* and *in vivo*[23].

The objectives of this study were to observe the effect of the different materials on MSC proliferation and determine their linage commitment through gene expression profile.

3.2 Materials and Methods

3.2.1 Scaffold Materials

The bone particles were harvested from the femora and tibiae of seven rats (Figure 3.1a). The soft tissue was scraped from the bones which were then crushed into particles of 1-7mm in size. Particles containing cartilaginous tissue were removed and the remaining particles were washed several times in 70% ethanol for sterilization and to remove any remaining bone marrow.

The CPP scaffold material was provided by Dr. Pilliar (University of Toronto) and was formed by calcining precursor powders of calcium phosphate monobasic monohydrate in a platinum crucible (Figure 3.1b)[24]. These powders were then gravity sintered in cylindrical tubes which were later crushed into angular particles of 1-3mm in diameter. The resulting particles have an interconnected porous network (30-45% porosity) with pore diameter in the range of 100μm[25].

The hydroxyapatite/tricalcium phosphate (HA/TCP) scaffold material was purchased from Berkeley Advanced Biomaterials Inc (Figure 3.1c). The ceramic was manufactured with a composition of 20% HA and 80% TCP, pores of approximately 250μm, and a

particle size of 1-3mm. Both CPP and HA/TCP particles were rinsed several times in 70 % ethanol.

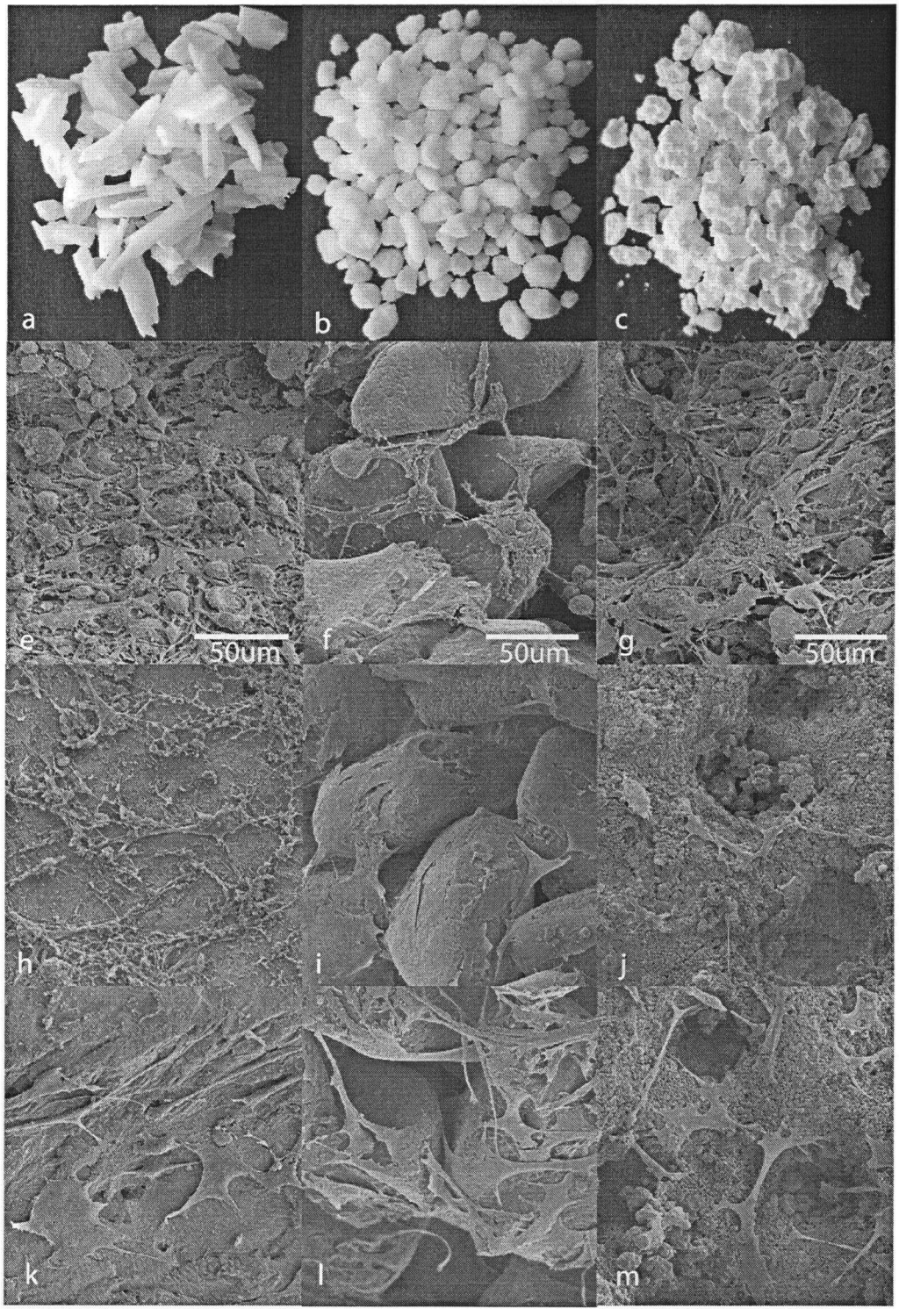

Figure 3.1 Particulates of substrate materials a) Bone b) CPP c) HA/TCP. SEM images of MSC seeded on bone (e)(h)(k) at days 0, 7, 21 respectively, on CPP (f)(i)(l) at days 0, 7, 21 respectively and on HA/TCP (g)(j)(m) as days 0, 7, 21 respectively.

3.2.2 Cell isolation, seeding and culture

MSCs were isolated from the femora and tibiae of a 6 weeks old transgenic GFP Sprague-Dawley rat (NBRP, Japan). The bones were crushed and the bone marrow was removed through several washes with phosphate buffered saline (PBS). The bone marrow was then plated on to 15cm fibronectin coated tissue culture dish in expansion media (EM) containing Mesencult basal medium (Cat# 05401 Stem Cell Technologies, Vancouver) supplemented with 15% fetal bovine serum (Cat# 06471 Stem Cell Technologies, Vancouver) and 100 U/ml penicillin-streptomycin. After 4 days, the non-adherent cells were removed and the attached cells were expanded to approximately 80% confluence. The cells were further expanded to passage two then cryopreserved until they were used. Before seeding the cryopreserved MSC were thawed and further expanded to passage five in EM.

The multipotential nature of the MSC population was confirmed by differentiating the cells along osteogenic, chondrogenic and adiopogenic lineages (Figure 3.2). Prior to seeding all scaffold materials were repeatedly washed in 70% ethanol and PBS then incubated in EM for 48hrs. The particles were placed in 48-well non-tissue culture plates with an equal mass of particles in each well. The MSCs were seeded on the scaffolds with 800µl of a cell suspension (1.0×10^6 cell/ml). After an initial incubation period of 5 min at 37°C the plates were centrifuged for 6min (1,000g, 4°C) and incubated for another 6hrs at 37°C [26]. Following the 6hr incubation period the supernatant was removed and the scaffolds were washed with PBS to remove any non-adherent cells. The cells in the supernatant and wash were collected and counted as a measure of the seeding efficiency.

The scaffolds were transferred to 24-well non-tissue culture plates and incubated at 37°C in 2ml of culture medium for up to 21 days, with the medium changed every two days. In addition to the three particulate substrates, MSCs were plated on two controls of tissue culture plastic at density of 10,000 cells/cm^2. The plastic controls (TC/EM and TC/OM) were cultured in EM and in osteogenic media (OM) consisting of Mesencult, 15% FBS (Stem Cell Technologies, Cat# 06473) 0.01μM dexamethasone, 50μg/ml ascorbic acid and 5mM β-Glycerophosphate. All experiments were performed in triplicates at time periods of 0 (directly following 6hr seeding period), 3, 7, 14 and 21 days.

3.2.3 Cellular Proliferation

Cell numbers at the different time intervals were determined by measuring the metabolic activity of the MSCs with a MTT (3-(4, 5-dimethylthiazol-2yl)-2, 5diphenyltetrazolium bromide) assay. The particles were transferred from the 24 well plates to 96 well plates and a volume of 200μl of MTT solution (5mg/ml in PBS) was added and incubated at 37°C for 3 ½hrs. The MTT solution was aspirated from the wells and the formazan crystals were dissolved in 160μl of dimethyl sulfoxide (DMSO) for 20 min. Aliquots of 80μl the DMSO solution were then transferred to new wells in the plate and the absorbance was read on a microplate reader (Spectra Max 190, Molecular Devices, Union City, CA) at 570nm.

Cellular proliferation was measured through BrdU (5-bromo-2-deoxyuridine) incorporation into the newly synthesized DNA of replicating cells. BrdU was added to

the media at a concentration of 10μM and cultured for 24hrs. At the different time intervals the MSCs were fixed with 2% paraformaldehyde, permeabilized with 10% saponin and stained with a BrdU antibody. To facilitate counting of BrdU positive MSCs the nuclei were co-stained with Hoechst. Three arbitrary locations on the samples were used to quantify the percent BrdU positive cells.

3.2.4 Cellular Differentiation

The expression levels of osteogenic, chondrogenic and adiopogenic markers were determined with qRT-PCR using Taqman probes (Applied Biosystems, Foster City, CA, Tabel 3.1). Following the culture period (0, 3, 7, 14, 21d) total cellular RNA was extracted using TRIZOL (Invitrogen) and reverse transcribed using superscript III (Invitrogen). Target gene expression was normalized relative to the house keeping gene GAPDH.

Gene Name	Symbol	Rat Genebank #	Taqman #
Osteogenic			
Runt related transcription factor 2	RUNX 2	XM_346016	Mm03003491_m1
Collagen I	COL I	XM_213440	Rn00801649_g1
Alkaline Phosphatase	ALP	NM_013059	Rn00564931_m1
Osteonectin	ON	NM_012656	Rn00561955_m1
Osteopontin	OP	NM_012881	Rn01449972_m1
Bone Sialoprotein	BSP	NM_012587	Rn00561414_m1
Osteocalcin	OC	NM_013414	Rn00566386_g1
Chondrogenic			
SRY-box containing gene 9	SOX 9	XM_343981	Rn01751069_mH
Collagen II	COL II	NM_012929	Mm00491926_g1
Aggrecan	AGG	NM_022190	Rn00573424_m1
Adipogenic			
Peroxisome proliferator-activated receptor gamma	PPARγ	NM_013124	Mm00440945_m1
Lipoprotein Lipase	LPL	NM_012598	Rn00561482_m1
Housekeeping			
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	NM_002046	Hs02758991_g1

Table 3.1 *Gene names, symbols and reference numbers. The probes for RUNX2, COL II, PPARγ and GAPDH are cross-reactive for Mouse and Rat.*

3.2.5 Scanning election microscope (SEM)

The scaffold materials were prepared for SEM imagining by being fixed in 4% paraformaldehyde and then were dehydrated in series of graded ethanol washings followed by critical point drying. The dried samples were sputter coated with gold and imaged using a scanning electron microscope (S-2600 VP SEM, Hitachi).

3.2.6 Statistics

The effect of substrate and time on cell number, BrdU incorporation and mRNA expression was determined with a 2-way ANOVA with the substrate and time as factors.

Student-Newman Keuls tests were used for post-hoc analyses at a significance level of 0.05.

3.3 Results

3.3.1 Seeding

There were no significant differences of initial MSC attachment to the substrates ($p>0.07$). The seeding efficiencies, given as the number of cells seeded minus the cells in effluent wash solution, were 90.1% ±4.4, 87.3% ±5.1, 83.2% ±6.9 for Bone, CPP and HA/TCP respectively. No MSCs were detected in the effluent wash of the plastic controls and therefore we assume a seeding efficiency of approximately 100%.

3.3.2 Cellular proliferation

The MTT assay shows a significant initial decrease in cell numbers on CPP and Bone over the first three days with a recovery by Day 21 ($p<0.047$) (Figure 3.3). In contrast HA/TCP showed a gradually decreasing trend in cell numbers over the entire 21 days. BrdU incorporation increased with time for all scaffolds and was the highest (>30%) in Bone and CPP ($p<0.001$) (Figure 3.4). Bone and HA/TCP had little or no BrdU incorporation on Days 3 and 7, but showed a significant increase at Day 14 ($p<0.003$) (Figure 3.4). The plastic controls (TC/EM and TC/OM) showed constant increase in cell numbers over the entire 21 day culture period (not shown).

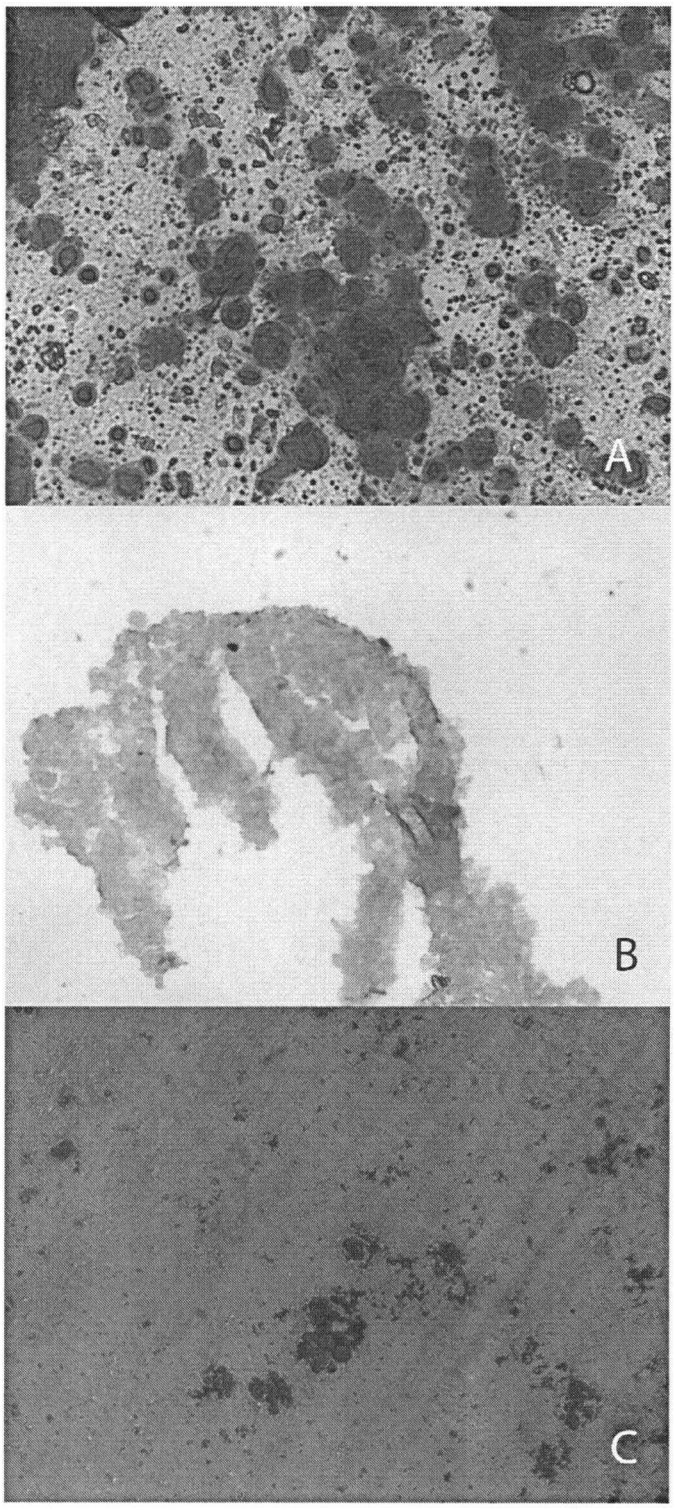

Figure 3.2 Osteogenic, adiopogenic and chondrogenic differentiation of MSCs. For the osteogenic and adiopogenic differentiation, 5000 and 50,000 MSC respectively were plated in 96 well plates. The osteogenic differentiation media consisted of Mesencult, 15% FBS (Stem Cell Technologies, Cat# 06473) 0.01μM dexamethasone, 50μg/ml ascorbic acid and 5mM β-Glycerophosphate and the plates were stained with Alizarin Red S after 4 weeks in culture. The adipogenic medium consisted of EM supplemented with 0.1μM dexamethasone and 6ng/ml insulin and stained with Oil Red O after 3 weeks in culture. For

chondrogenic differentiation 500,000 MSCs were pelleted and grown for 3 weeks in Mesencult and 10% FBS (Stem Cell Technologies Cat# 06471) supplemented with 0.01μM dexamethasone, 50μg/ml ascorbic acid and 10ng/ml TGF-B1. Cryosection of the pellet was stained with Alcian blue.

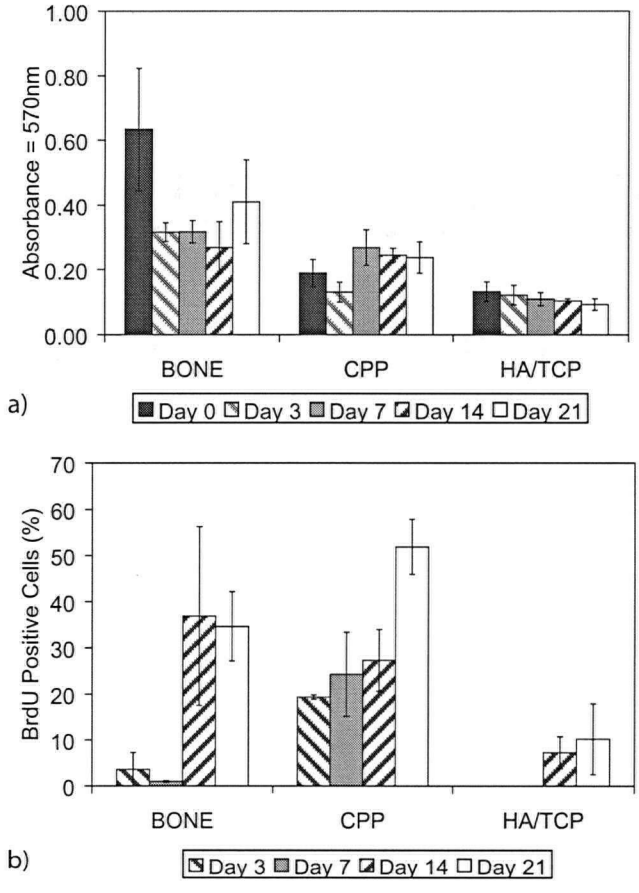

Figure 3.3 (a) Cell numbers were determined by an MTT assay for the 21 day culture period. (b) The percent BrdU incorporation into the cells over the 21 days of culture on the different substrates.

3.3.3 Cellular differentiation

The results of the qRT-PCR for the osteogenic, chondrogenic and adipogenic markers are shown in Figures 3.5, 3.6 and 3.7 respectively. There was a significant decrease in Col-I and ALP expression within the first 3 days on all scaffold materials ($p<0.0002$). After the initial decline, Col-1 remained unchanged until Day 14 and increased significantly on Bone and CPP ($p<0.02$) by Day 21. Col-1 expression was significantly higher on bone and CPP compared with HA/TCP ($p<0.002$). Similarly, ON initially decreased on all

particles within the first 3 days and then gradually increased until day 21. On the plastic control, TC/EM, there were significant increases on ALP, Col-1 and ON from Day 0 to Day 21 ($p<0.0002$) with significantly higher expression than TC/OM by Day 21 ($p<0.0002$). RUNX 2 showed no significant change on the Bone, CPP or TC/EM, however there was a significant decrease in expression on HA/TCP ($p<0.001$) and increase in expression on TC/OM by day 21 ($p<0.005$). Late stage osteogenic markers such as OC and BSP were expressed at very low levels close to the detection limit on all substrates, except in osteogenic medium where OC and BSP expression constantly increased from Day 0 to Day 21. The expression of OP remains constant on Bone, CPP and TC/EM, however shows a significant increase by Day 7 on both HA/TCP and TC/OM ($p<0.0002$).

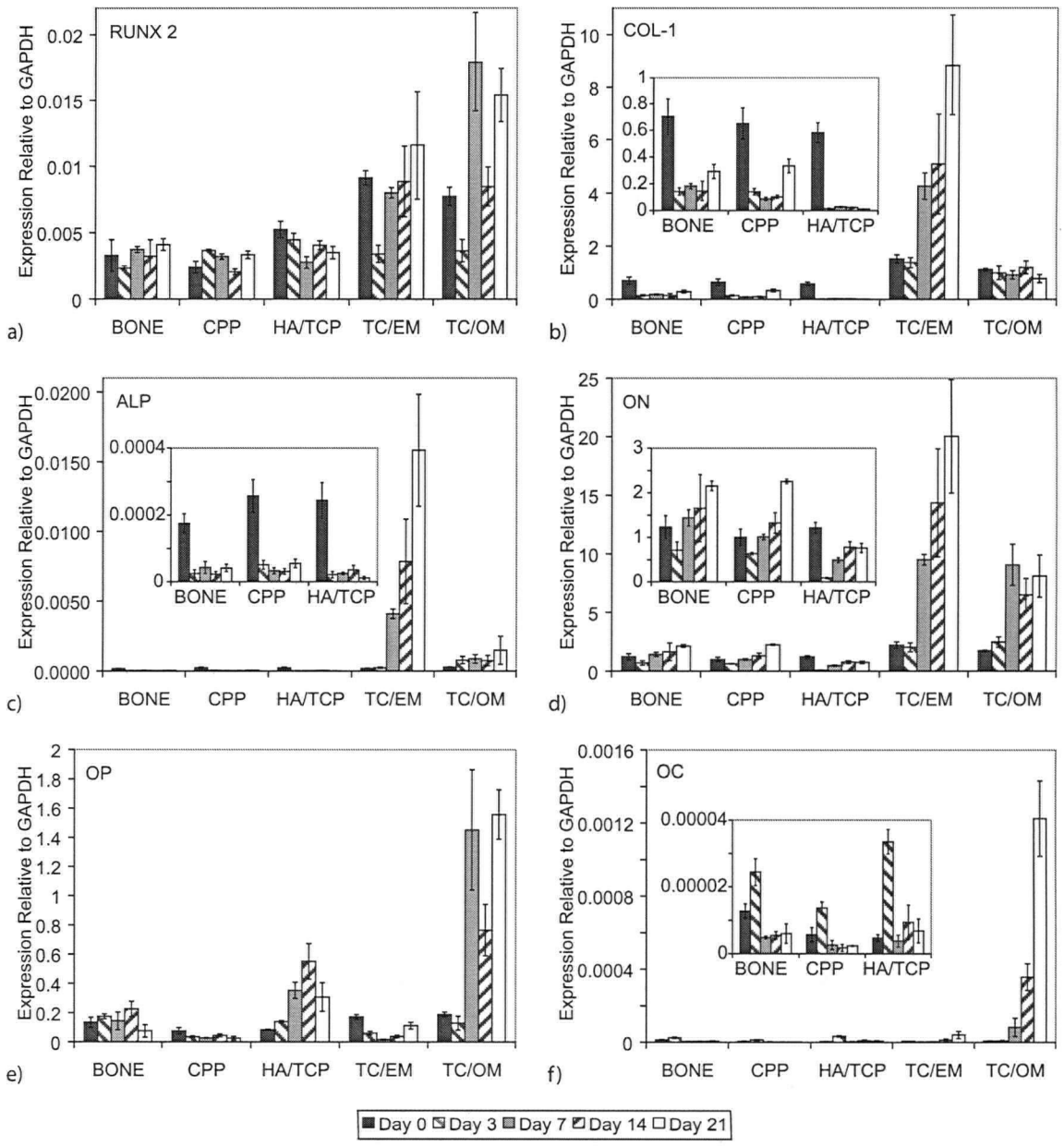

Figure 3.4 The temporal expression of osteogenic genes on the different substrates for 21 days. The osteogenic genes (a) RUNX-2, (b) COL I, (c) ALP, (d) ON, (e) OP, (f) OC are expressed relative to the house keeping gene GAPDH at time points of 0, 3, 7, 14 and 21 days, for all substrate materials. All values are presented as the means ± standard deviations of triplicates. Inset panels are magnified views of expression on scaffold materials, shown for clarity.

The highest expression of all chondrogenic markers with an increasing trend was observed on tissue culture plastic with expansion medium (TC/EM). There was a significant down regulation of AGG on all other substrates within the first 3 days

($p<0.001$). SOX-9 initially decreased and then was up regulated on Bone, CPP at Day 21 ($p<0.006$). Very low levels of Col-2 were expressed on and all substrates and culture conditions.

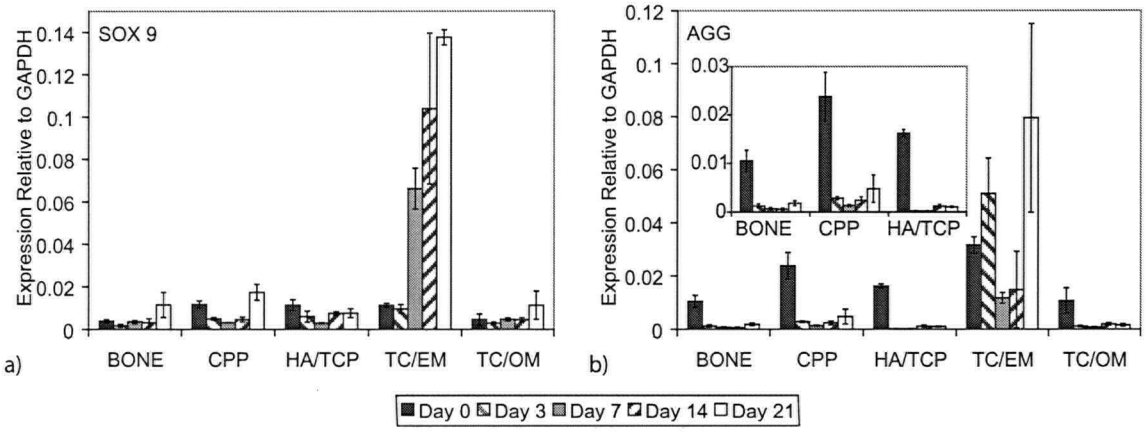

Figure 3.5 The temporal expression of chondrogenic genes on the different substrates for 21 days. The chondrogenic genes (a) SOX-9 and (b) AGG are expressed relative to the house keeping gene GAPDH at time points of 0, 3, 7, 14 and 21 days, for all substrate materials. All values are presented as the means ± standard deviations of triplicates. Inset panel is a magnified view of expression on scaffold materials, shown for clarity.

PPARγ expression constantly increased from Day 3 to 21 on HA and TC/OM, and was significant higher compared with Bone and CPP ($p<0.0002$). On Bone, CPP and TC/EM, PPARγ expression increased slightly and then decreased by Day 21. In contrast to PPARγ, LPL expression was high and increased significantly with time on Bone, CPP and TC/OM ($p<0.0003$), but not on HA and TC/EM which only slightly changed during the entire culture period.

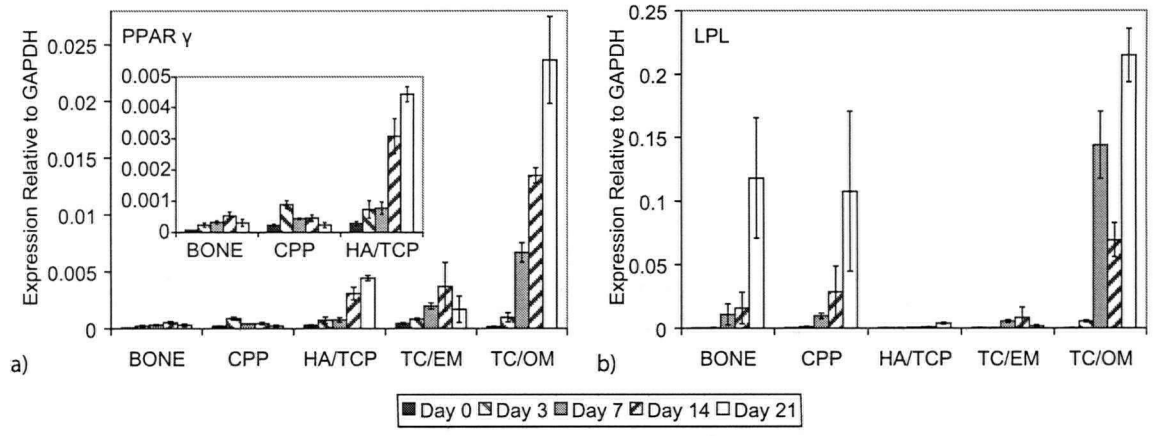

Figure 3.6 The temporal expression of adipogenic genes on the different substrates for 21 days. The adipogenic genes (a) PPARγ and (b) LLP are expressed relative to the house keeping gene GAPDH at time points of 0, 3, 7, 14 and 21 days, for all substrate materials. All values are presented as the means ± standard deviations of triplicates. Inset panel is a magnified view of expression on scaffold materials, shown for clarity.

3.3.4 SEM

The SEM images of the samples at Days 0, 7, and 21 are show in figure 3.1. These images show that the cells had sufficiently attached to the substrate after a 6 hour seeding period. The rounded cells in figures 3.1 d, e, f are indicative of dying or dead cells. There was also a noticeable decrease in cell numbers at day 7 on the bone and HA/TCP samples, with a recovery of cells by Day 21. In addition, all substrates had an increase in cell size by Day 21.

3.4 Discussion

This comprehensive analysis demonstrated a strong effect of the cell culture substrate on proliferation and differentiation of MSC in expansion conditions. MSCs proliferation and differentiation on bone, CPP and HA/TCP, which are commonly used for tissue

engineering applications, were compared to tissue culture plastic in both expansion and osteogenic differentiation culture conditions.

Using osteogenic, chondrogenic and adipogenic culture conditions we were able to induce MSCs differentiation into bone nodules, cartilage and fat respectively (Figure 3.1). This confirmed the tri-potency of the bone marrow derived heterogeneous cell population which were seeded and evaluated on the different substrates. By adapting the seeding method proposed by Dar et al we achieved high seeding efficiencies for bone, CPP and HA/TCP (90.1% ±4.4, 87.3% ±5.1, 83.2% ±6.9 respectively) ensuring that sufficient MSCs were attached to the substrates. Of the MSCs that were seeded on bone many died or migrated off the particles within the first 3 days. There was also a slight drop in cell numbers on the CPP particles. Most likely this initial decline in cell numbers is due to change of substrate since there was no drop in cell number observed on the tissue culture plastic (e.g. TC/EM and TC/OM). The corresponding down regulation in the expression of Col-1 and AGG in the first three days suggests that the deposition of collagen is no longer needed for the MSCs to adhere to the particulate substrates.

After an initial drop in cell numbers, CPP and bone supported an increase in cell numbers and BrdU incorporation at Day 14 and 21, while HA/TCP experienced a continued decline in cell numbers over the entire 21 day period with only minor BrdU incorporation. Since high proliferating cells are typically osteoprogenitors and lesser extent preosetoblast [11,12], the recovery of cell numbers on CPP and bone may indicate that

these two scaffolds maintained a immature MSC population which contain a larger number of osteoprogenitor or preosteoblasts compared with HA/TCP.

Along with the proliferation characteristics, the temporal gene expression helps to elucidate the extent of differentiation along the osteoblast lineage. A number of genes are known to be associated with the early stage of proliferation and matrix development. RUNX-2, a runt-related transcription factor, is crucial in controlling osteogenic commitment but is maintained during differentiation to other cell types such as adipocytes and chondrocytes[6,27]. Col-1 is expressed in the proliferative stage and during the early stages of matrix lay down[11,16,28], whereas ALP is expressed postproliferation during extracellular maturation[11,29]. ON is a non-collagenous protein that is expressed in preosteoblast all the way to osteocytes and is thought to give stability to extracellular matrix and provides a point of association between proteoglycans and collagen[30]. Late stage markers of osteogenesis, OP and BSP, are noncollagenous proteins associated with the end stages of matrix maturation and early stage of mineralization while OC is strongly linked with mineralization of the extracellular matrix[11,16,28].

The expression of RUNX-2 on the all the substrates indicates the predisposition of the cells on all substrates to form bone. The significant decrease in RUNX2 on HA/TCP may suggest the cells are differentiating down the osteoblast lineage. It has been suggested however that RUNX2 is involved in osteogenesis in modulation of activity not through quantitative change in gene expression, which may explain the variability in expression in the TC/OM sample[31].

Comparing the TC/EM and TC/OM samples there is an obvious difference in expression levels of early stage markers and late stage markers. TC/EM shows increasing expression of early stage markers, Col-1, ALP and ON, over the 21 days whereas TC/OM shows no significant change in Col-1 or ALP after the first 3 days. The expression of ON for TC/OM does significantly increase by Day 7 however it is significantly lower than EM. In contrast the late stage marker show the opposite trend, with TC/OM showing significant increases in OP, BSP and OC over the 21 days while TC/EM has significantly lower expression levels of each gene. The gene expression on the two controls suggests that TC/EM is maintaining an immature cell population while TC/OM causes differentiation down the osteogenic lineage. This is in agreement with the fact that the cells expanded in TC/EM prior to seeding maintained their proliferation and differentiation ability while cell cultured in TC/OM formed bone nodules.

Similarly when comparing the different scaffold materials, allogeneic bone and CPP show and increase in Col-1 and ON from Day 3 to Day 21 while HA/TCP shows a decrease in levels over the culture period. After the initial drop the expression of ALP on bone and CPP remains unchanged during the culture period but show significantly higher levels than HA/TCP at Day 21. The late stage marker, OP, however showed a significant increase in expression on HA/TCP by day 14, and were substantial higher than bone and CPP. This suggest that bone and CPP like TC/EM had cell populations with a higher number of immature osteogenic cells while HA/TCP caused differentiation and osteoblast maturation of the MSCs. All three scaffold materials had very low expressions of the late

stage markers BSP and OC similar to TC/EM which suggest that there is not late stage osteoblast maturation or mineralization.

The similarity between bone and CPP extend into the expression of chondrogenic and adipogenic genes. Bone and CPP both have a significant increase of SOX9, the transcription factor for regulation of chondrogenic differentiation, at Day 21 while HA/TCP showed no change. Once more the expression of the scaffold material is paralleled in the controls with TC/EM showing an increase in SOX9 and TC/OM showing no change. Though this correspondence of scaffold material and plastic control is not seen in the markers for adipogenic differentiation the difference between the gene expression in bone and CPP to that of HA/TCP is apparent. Bone and CPP had increased expression of LPL and unchanged expression in PPARγ while HA/TCP showed the opposite with no change in LPL and significant increase of PPARγ. While it is difficult to infer whether the substrates are causing differentiation down the chondrogenic adipogenic lineages it is clear that the substrates have a major influence on gene expression.

Cell-substrate interaction is not fully understood, whether it is the substrate surface chemistry or morphology that is causing the proliferation of cells on bone and CPP and differentiation on HA/TCP is unclear. Preliminary SEM images (Figure 3.1) lead us to believe that the morphology of the substrate surfaces may impact cell attachment and expansion and that the smooth morphology of bone and CPP was beneficial for cell proliferation. Furthermore it is not clear whether the role of the matrix during bone

regeneration is to support proliferation or cause differentiation. However, this study suggests that in the absence of soluble induction factors bone supports cellular growth and not differentiation. CPP having very similar influence on cellular growth and differentiation makes it a promising candidate for a bone graft substitute. HA/TCP in contrast promotes differentiation and although it may aid in bone growth it does not provide a suitable environment for the maintenance of immature proliferating cells important for sustained bone regeneration.

3.5 Conclusion

In this study, mesenchymal stromal cells (MSCs) seeded on allogeneic bone and calcium polyphosphate showed similar proliferation characteristics and gene expression. They also showed a similar gene expression to that of MSC seeded on plastic and cultured in an expansion media TC/EM, suggesting that bone and CPP support immature proliferating MSCs. In contrast, MSCs seeded on biphasic hydroxyapatite/tricalcium phosphate showed similar gene expression to MSCs seeded on plastic in an osteogenic media TC/OM, suggesting HA/TCP induces differentiation of MSCs down the osteogenic lineage.

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4 Conclusion

4.1 Summary and Future Work

In this study, the preliminary evaluation of both the mechanical and biological characteristics of calcium polyphosphate (CPP) was conducted to determine its viability as a structural scaffold in revision total hip replacement (THR). Mechanical testing showed that CPP and MCB have similar mechanical properties in both confined compression and in shear. Further mechanical testing of ideal spherical particles showed that although increasing the particle modulus increases the particle construct modulus, but the value of the modulus is still very low compared to that of the acrylic bone cement currently used to aid in the structural support of the implant. The shear tests revealed that the particle modulus has little influence on the shear strength of the particle construct while the shape of the particle has a large effect. The biological testing showed that CPP had similar osteogenic properties to that of allogenic bone, both of which supported the growth of MSCs on their surface though did not cause a differentiation MSCs down the osteogenic lineage. In contrast HA/TCP promoted the differentiation down that osteogenic lineage but did not support cell proliferation. These results suggest CPP is a suitable substitute material for MCB in THR procedures, and would provide adequate structural support while allowing cellular proliferation to occur which is vital for sustained bone growth.

While preliminary testing yielded positive results for the use of CPP as a structural scaffold, further mechanical and biological testing is required to confirm its feasibility for

application in THR. The next stage of mechanical testing is to load the material dynamically in a geometry similar to that found in the hip. One of the main differences with CPP and MCB is that MCB is a biphasic visco-elasticplastic material that is subject to creep during sustained loading[1]. This behaviour is thought to allow for deformation of the graft post surgery and is a cause for implant subsidence[1,2]. Therefore it would be useful to measure the subsidence of the implant in a graft bed of both CPP and MCB under sustained cyclic loading to determine the stability of the graft over time. Further biological test would include the culturing of CPP seeded with MSCs in a media with osteogenic induction factors. Although it was shown that CPP was osteoconductive in that it allowed proliferation of cells on its surface it needs to be shown that it is possible to grow bone on the surface. Using a media with induction factors, the cells should have an environment that allows for both proliferation and differentiation, leading to sustained bone growth. Following this test an in vivo study in rats of the MSC growth on the various particles is needed to confirm the results of the in vitro tests.

4.2 Reference List

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Appendix A: Cell Culture Procedures

Plating MSC's

-Defrost vile of MSC in hand

-Immediately mix with 5ml of culture medium (CM)

-Centrifuge for 5 minutes at 1500 rpm

-Aspirate off solution

-Re-suspend in 10-20ml CM

-Split solution in half in separate 15cm tissue culture plates

-Add CM to bring total solution in each plate to 20ml

Wash particles

-Wash in 70% ETOH until no longer cloudy

-Leave in 70% ETOH over night

-3x PBS

-15% FBS CM for 48hrs

Splitting Cells

-Aspirate off solution from each plate

-Wash with 15ml PBS

-8 ml TE in each plate and incubate 5 min

-Add 15ml PBS and wash a couple of times

-Pipette contents into a Falcon tube

-Centrifuge

-Aspirate off solution

-Re-suspend in 10-20ml CM

-Split solution in half in separate 15cm tissue culture plates

-Add CM to bring total solution in each plate to 20ml

Seed MSCs on particles

-Aspirate off solution from each plate

-Wash with 15ml PBS

-8 ml TE in each plate and incubate 5 min

-Add 15ml PBS and wash a couple of times

-Pipette contents into a Falcon tube

-Centrifuge

-Aspirate off solution and add 1ml CM to each tube – Resuspend cells

-Pipette 10ul of solution and dilute with 90ul CM for each falcon tube

-Count cells

-Using tweezers sterilized with 70% ETOH place particles in 48 well plates (1/3 – 1/2 full)

-Seed ~800,000 on each sample in 48 well plates (non-tissue culture)

-incubate for 5 min

-centrifuged at 1000g for 6 min at 4°C

-Incubate cells for six hours

Seeding on plastic

-Seed 10,000 cells on 6 well tissue culture plates

Culturing Cells

-After 6 hour seeding period, pipette out CM (DO NOT ASPIRATE) count the cells in removed solution

-Wash particles in PBS (to remove any cells not attached) by pipetting, count cells in wash solution

-Transfer particles to new 24 well plate (non-tissue culture)

-Add 2ml of CM to each well

-Incubate and change CM every other day for the remainder of the test

MTT

-Aspirate solution off of all samples

-Wash particles/plastic with PBS (carefully with the plastic controls)

-Aspirate off PBS

-Transfer particles to 96 well plate

    -Each well from the 24 well gets split up into 2 wells

-Plastic samples (plastic and osteo) are left in their original plate

-Add 180ul MTT solution to each well (carefully on the plastic to be sure not to knock the cells off)

-Incubate for 3-4 hours

-After 3-4 hours aspirate off MTT solution (use a pipette for the plastic controls)

-Add 160 DMSO to plastic control wells and to the first well for each particle samples

-Incubate for 10 minutes

-After 10 minutes transfer 160ul DMSO from the first well to the second well for each particle sample

-Incubate for another 10 min

-Pipette the solution from the particle wells into a new well (this step prevents bubbles from being transferred to the final well)

-Pipette out 80ul from this well into the final well (these are what the absorbance are tested on)

-Test Absorbance at 570 nm

Lysis of Cells on particles

-Aspirate solution off of all samples

-Wash particles with PBS

-Aspirate off PBS

-Transfer particles to 1.2ml tubes

-Add 800ul Trizol, let stand at room temp for 30min

-Add 180ul chloroform

-Shake tubes by hand for 15 seconds then let stand for 2-3 min at room temp

-Centrifuge at samples @ 12000G for 15 min @4 degree

-Transfer aqueous phase to new tubes

-Add 400ul isopropyl and incubate in freezer for 2 hours

-Centrifuge @ 12000G for 10 min at 4 degrees

-Remove supernatant (carefully- leave a little solution in the bottom)

-Wash in 800ul 75% ethanol, mix by vortexing, then centrifuge at 7500G for 5min at 4 degrees

-aspirate off solution (**do the last bit with a pipette because the pellet doesn't always stick to the tube and can get sucked up if you try to aspirate all the solution off**) and air dry for 5 min

-redissolve in 30ul RNAse free water mix by pipetting

-incubate for 10 minutes in 55 degree water bath

Appendix B: Statistical Methods

Student t-test

The student T test was used to determine the probability that the difference between two normally distributed populations (of t distribution) are from chance. The normal distributions are described by the samples estimates (due to the small sample size) of standard deviation s, its mean η and number of data points. The student t-test is a null hypothesis and therefore returns a value of 0 if the distributions are from the same population. The t-test is given as follows:

$$t = \frac{\eta_1 - \eta_2}{SE(\eta_1 - \eta_2)} \quad where \quad SE(\eta_1 - \eta_2) = \sqrt{\frac{s_1^2}{n} + \frac{s_2^2}{n}}$$

Using the t value and the number of degrees of freedom one can determine the probability that the difference in the populations is not due to chance (in a table of significance). Typically a p value less that 0.05 is taken to mean the two distributions are from different populations.

ANOVA

Analysis of variance (ANOVA) used here was to test the effect of different treatments on a material or substrate. ANOVA tests the null hypothesis that all the population means are equal. It is calculated through the comparison of two estimates of the variance of the

entire population. The two estimates of variance are; the variance in within a treatment (mean square error or MSE) and the variance between the different treatments (mean square between or MSB) given by

$$MSE = \frac{\sum s_i^2}{n} \quad and \quad MSB = ns_m^2$$

Where s_i is the standard deviation of each population, s_m is the standard deviation of the means of each population relative to the total population mean, and n is the number of measurement in each population.

The ratio of MSB/MSE gives a value of 1 if the null hypothesis is true in which case all the population means are equal. If the ratio is much larger than 1 then the MSB is an inflated estimate of the variance and therefore the null hypothesis is false. Basically, when MSB is large then the variance between the sample means is large and therefore are likely not from the same population. Using the ratio know as an f value along with the degrees of freedom one can determine the probability that the difference in the populations is not due to chance (in a table of significance). Typically a p value less that 0.05 is taken to mean the two distributions are from different populations.

A two way ANOVA is an extension of a one way ANOVA in that it contains two independent factors (such as the substrates and the time of culture in this study). The two way ANOVA gives a null hypothesis if means of the first factor are the same, if the

means of the second factors are the same, or there is no interaction between the two samples.

Pearson's Correlation

The Pearson's correlation given as R is a measure of the linearity between the two variables (such as E_c and E_p). The value of R ranges from -1 to 1, with -1 representing and perfect inverse linear relationship, 1 representing a perfect linear relationship, and 0 representing no relationship.

The correlation is given as:

$$R = \frac{\sum XY - \frac{\sum X \sum Y}{N}}{\sqrt{\left(\sum X^2 - \frac{(\sum X)^2}{N}\right)\left(\sum Y^2 - \frac{(\sum Y)^2}{N}\right)}}$$