# Injectable Cell-based Tissue Engineered Bone Formulations

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# Injectable Cell-based Tissue Engineered Bone Formulations

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## 2010

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Submitted for the degree of Doctor of Philosophy,
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# ABSTRACT

Current golden standard therapy for bone repair and regeneration involves the use of autografts. Nevertheless, there are many drawbacks associated with autografts including donor site morbidity, requirement for an invasive surgery, post-operative pain and infection. The use of injectable tissue engineered bone is an attractive alternative, providing a minimally invasive approach to regenerate bone. It offers faster healing, less pain and exact conformation to irregular defects. The present work is designed to achieve injectable formulations of tissue engineered bone that fulfil the requirements needed. It involves investigation of potential polymeric binders that are biocompatible, biodegradable and allow bone formation when combined with cells.

Chitosan binders were tested for biocompatibility, biodegradability, gelation, angiogenic potential and osteogenic differentiation and bone formation when mixed with goat and human bone marrow derived mesenchymal stem cells (gMSCs, hMSCs). An *in vivo* bone formation study was performed to investigate the bone formation ability of gMSCs in contact with chitosan binder. Chick chorioallantoic membrane assay was carried out to examine the angiogenic potential of the chitosan binder combined with/without hMSCs. Furthermore, MC3T3-E1 cells were employed to assess the osteogenic potential of cells exposed to chitosan polymeric systems.

Chitosan binder was proved to be an attractive polymer to carry cell-scaffold combination. hMSCs were able to survive and differentiate along the osteogenic lineage when encapsulated with 1.5% (w/v) chitosan-15% (w/v) glycerol phosphate (GP)-0.18% (w/v) hydroxyethyl cellulose (HEC) in a 14-day study. Furthermore, chitosan-GP-HEC solutions demonstrated fast gelation at 37°C. Chitosan was biodegradable following 42 days in the presence/absence of lysozyme. Moreover, gMSCs combined with chitosan binder produced 24.6 ± 13.7% bone comparable to the control group after a 6-week implantation in mice. Chitosan was shown to be non-angiogenic unlike hMSCs which showed angiogenic potential. Also, chitosan was found to be osteogenic at 2 and 0.05 mg/ml concentrations.

# ACKNOWLEDGEMENTS

In the name of the one and only Lord, the most compassionate, the merciful. He heard me when I cried and comforted me when I was down. At the time of need and despair, he was my only help, for which I am ever so thankful.

I would like to thank my supervisor, Professor Joost de Bruijn for his kind guidance and support. Without his constructive efforts and patience, this thesis would not have been completed.

I would also like to thank my parents, Naser and Susan Ahmadi, my brother Ali Ahmadi and my sister Elham Ahmadi for their love, inspiration and support all these years.

Finally, a very special thanks goes to my colleagues and friends at Queen Mary University of London. Many thanks especially to Anil Ramlogan, Kruba Shankar and Girish Pattappa.

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# ABBREVIATIONS

| | |
|---|---|
| ALP | alkaline phosphatase |
| AsAP | L-ascorbic acid 2-phosphate |
| BCP | biphasic calcium phosphate |
| BMPs | bone morphogenic proteins |
| BMSC | bone marrow-derived stromal cells |
| CAM | chick chorioallantoic membrane |
| DBM | demineralized bone matrix |
| DDA | degree of deacetylation |
| gMSCs | goat bone marrow derived mesenchymal stem cells |
| GP | $\beta$-glycerol phosphate disodium salt |
| HA | hydroxyapatite |
| HEC | hydroxyethyl cellulose |
| hMSCs | human bone marrow derived mesenchymal stem cells |
| MC3T3-E1 | Murine preosteoblast cell line |
| PBS | phosphate buffered saline |
| TCP | tricalcium phosphate |

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network extending over the surface of the yolk (b); the window through the shell, used to gain access to the CAM, is sealed with clear tape then the egg is returned to the incubator (c).

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# CHAPTER I


## INTRODUCTION

# 1.1 GENERAL INTRODUCTION

The bone graft and bone graft substitute market is continuously growing mostly due to an increase in the population of the elderly. Presently in the UK, the population of over 65 age group is more than under 16 age group (Datamonitor-Publications, 2002). In addition, there is an increase in the number of osteodegenerative diseases in the rapidly aging population present in the developed countries (Heng *et al.*, 2004). This has made bone to be the second most commonly transplanted tissue after blood (Angermann and Jepsen, 1991). The current golden standard therapy for bone repair and regeneration is autograft (Yaszemski *et al.*, 1996-a), however, there are certain disadvantages associated with their use such as post-operative pain, infection, nerve injury and donor-site morbidity (Damien and Parsons, 1991; Gitelis and Saiz, 2002; Lane *et al.*, 1999; Younger and Chapman, 1989). Therefore, a new efficient approach is needed to create living autologous equivalent which combines cells (osteogenic component), scaffold (to provide volume and attachment sites for cells) and binder (for better handling properties) in order to replace autografts. Mesenchymal stem cells are attracting much attention due to their differentiation potential to many lineages including the osteogenic lineage (Haynesworth *et al.*, 1992; Prockop, 1997). Stem cells together with calcium phosphate scaffolds hold great potential to create suitable regeneration models which aim at regenerating bone tissue. This thesis investigates a cellular therapy approach to repair and regenerate bone through the use of stem cells, calcium phosphate scaffolds and an injectable binder, all of which will be explained in more detail in this chapter along with the background behind this thesis.

# 1.2 THE STRUCTURE AND FUNCTION OF BONE

Bone is a connective tissue which provides an internal support system in vertebrates therefore functioning as a structural frame supporting the body. It also offers attachment points for muscles and protects the vital organs such as heart, lung, spinal cord and brain. It holds the blood forming elements of bone marrow. Furthermore, it contains 99% of the body's calcium, 85% of the body's phosphorous and 65% of the

body's sodium and magnesium. It participates in maintaining homeostasis of calcium and phosphorous in the blood and other body fluids such as extracellular fluid (Fawcett and Raviola, 1994; Rosenberg, 1999). Bone is a complex tissue which unites the elastic properties of collagen fibers together with the compressive strength of hydroxyapatite crystals.

There are two structural forms associated with bone, cancellous and cortical bone. Typically, 80% of the adult skeleton is composed of cortical (compact) bone and the remaining 20% is in fact cancellous (trabecular) bone (Buckwalter *et al.*, 1995). Cortical bone is stiff, dense, smooth and continuous. It forms around the loosely organized porous cancellous bones as well as the shaft that surrounds the marrow cavity of long bones (Athanasiou *et al.*, 2000). Cortical bone holds significant mechanical and protective roles while cancellous bone is more metabolically active (Table 1.1).

**Table 1.1-** Tensile/compressive strength and tensile/compressive modulus of cortical and trabecular bones (Mistry and Mikos, 2005; Yaszemski *et al.*, 1996-a).

| Type of Bone | Tensile Strength | Compressive Strength | Tensile/Compressive Modulus |
|---|---|---|---|
| **Cortical Bone** | 79-151 MPa | 131-224 MPa | 17-20 GPa |
| **Trabecular Bone** | 5-10 MPa* | 5-10 MPa* | 50-100 MPa* |

\* The values vary with density but are reported to be within the mentioned range.

Both the cortical and cancellous bones contain an extracellular matrix composed of an organic component – about 90-95% collagen type I while the rest is comprised of non-collagenous proteins (osteocalcin, osteopontin, osteonectin, bone sialoprotein) – and an inorganic component. The inorganic component contains deposits of crystals of calcium phosphate and significant amount of carbonate ions and citrate as well as traces of sodium, magnesium, silicone and fluoride. Biochemically, organic matrix forms 35% and inorganic matrix forms 65% of the bone dry weight (Fawcett and Raviola, 1994; Rosenberg, 1999). The bone cells are surrounded by extracellular matrix. Bone cellular composition is explained below in more detail.

## 1.2.1 Cellular Composition

Bone tissue is composed of four major cell types:

1. Osteoblasts – derived from mesenchymal stem cells and situated on the internal and external surfaces of bone where there is active bone formation (Jilka, 2003). They produce collagenous and non-collagenous proteins that form the organic matrix of bone called the osteoid (Jilka, 2003).

2. Bone lining cells – known as inactive osteoblasts. They line bone surfaces appearing flatten and are involved in the regulation of passage of calcium in and out of bone (Junqueira *et al.*, 1998).

3. Osteocytes – mature osteoblasts surrounded by osteoid or mineralized bone. They stop secreting this matrix and are involved in bone mechanotransduction since mechanical loading leads to enhancement of osteocyte metabolic activity (Sikavitsas *et al.*, 2001).

4. Osteoclasts – derived from hematopoietic cells of the bone marrow and secrete acids and proteolytic enzymes which dissolve mineral salts and digest bone's organic matrix (Buckwalter *et al.*, 1995; Jilka, 2003). They are involved in bone remodelling process that takes place on the surface and within the bone (Buckwalter *et al.*, 1995). The bone remodelling process is performed by both osteoclasts and osteoblasts through an interaction referred to as coupling which is important for keeping a balance between the rate of bone resorption and formation.

All of these cell types are important in the bone formation process which is described below in more detail.


## 1.2.2 Bone Formation

Bone is a dynamic living tissue which is constantly being renewed and reconstructed throughout the individual's lifetime. Generally bone forms by two mechanisms, endochondral and intramembranous ossification. Endochondral bone formation occurs by replacement of the pre-existing cartilage whereas intramembranous bone formation takes place by direct replacement of the primitive connective tissue (de

Vernejoul, 1996; Fawcett and Raviola, 1994; Rosenberg, 1999). Intermembranous ossification is involved in the formation of flat bones from the skull and the addition of bone on the periosteal surfaces of long bones while endochondral ossification is associated with the formation of long bones and fracture repair (Junqueira *et al.*, 1998; Yaszemski *et al.*, 1996-a).

There are three key steps associated with bone formation:

1. Extracellular matrix production
2. Matrix mineralization
3. Remodelling of bone by resorption and reformation

Osteoblasts, osteocytes and osteoclasts are all important in the process of bone formation. On the surface of the existing matrix, there are osteoblasts which secrete osteoid (Figure 1.1). Osteoblasts are also involved in the mineralization of osteoid and while osteoid is changing into a hard matrix through apatite crystal deposition, the trapped osteoblasts become osteocytes. Osteocyte cellular processes occupy microscopic channels (canaliculi). Since the metabolites cannot diffuse through bone calcified matrix, canaliculi have an important role in signal transduction and exchanges of nutrients between osteocytes and blood vessels because they perforate the bone matrix. Bone is constantly going through dynamic remodelling which is essential for bone mechanical function and skeletal growth. This is a joined process between osteoclasts and osteoblasts which operate in a balanced manner therefore through resorption and reformation of bone, bone remodelling is taken place.

In order to combine the natural healing response of the body in case of fracture with cellular therapy and to benefit from both, a deep knowledge of the healing events that occur at the fracture site is crucial. The following section explains in more detail about the fracture healing events that take place inside the body.

Osteoclast    Mesenchyme    Newly formed matrix (osteoid)

Osteoblast    Osteocyte    Bone matrix
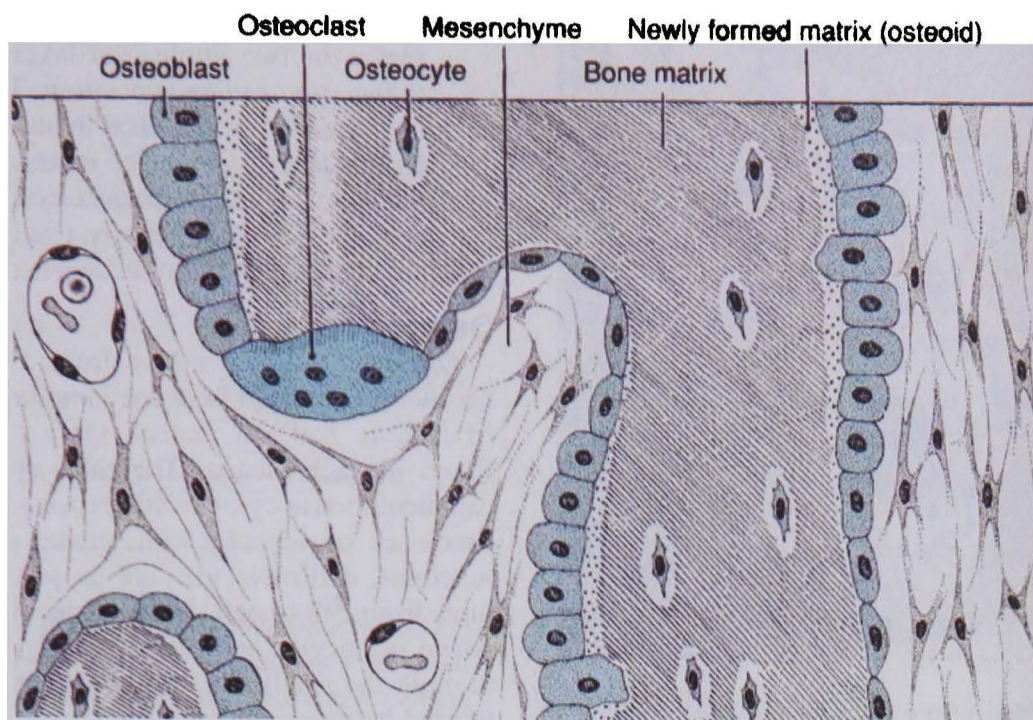
**Figure 1.1-** Bone formation events from (Junqueira *et al.*, 1998).

## 1.2.3 Fracture Healing

A fracture causes a discontinuity within bone as well as causing function loss and tissue damage (i.e. blood vessels). Due to the injury, a cascade of healing events takes place to start the repair process which sum up some of the steps of embryonic bone formation (Yaszemski *et al.*, 1996-a). There are three stages involved in fracture healing:

- Inflammation
- Repair
- Remodelling

In the inflammatory phase, a hematoma is formed where blood vessels are damaged. Neutrophil granulocytes and macrophages arrive and ingest the cellular debris of necrosis as well as secreting cytokines and growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), tumour necrosis factor (TNFα) and interleukin (IL) (Braddock *et al.*, 2001). These biochemical signals result in migration, differentiation and activation of mesenchymal stem cells from neighbouring bone, marrow, endosteum and periosteum. Mesenchymal stem cells

produce bone morphogenic proteins (BMPs) which induce proliferation and differentiation of these cells into bone-producing cells (Braddock *et al.*, 2001).

A fibrovascular granulation tissue is formed at the site of the injury by capillary growth and fibroblast activity. With progenitor cells accumulating and differentiating into osteoblasts, a repair blastema is produced. After the relatively short inflammatory stage, repair phase begins when osteoblasts rapidly secrete osteoid forming woven bone at the injury site. Slowly, the remodelling stage starts during which collagen fibers are reorganized resulting in mechanically strong lamellar bone. Also, days following fracture, periosteal cells close to the fracture site develop to chondroblasts and produce hyaline cartilage. This is then followed by endochondral ossification. In terms of time scale for bone healing events, initial inflammatory reaction takes place within minutes to hours, chemotaxis and mitosis last hours to days, and osteoid production, remodelling and angiogenesis last days to weeks (Braddock *et al.*, 2001).

Although, the remodelling phase may take up to a year in case of severe fractures, it will return bone back to its original strength before fracture (Yaszemski *et al.*, 1996-a). Nevertheless, if the response of the body to the injury is such that not enough active cells are provided or in case a defect is too large for the natural healing response of the body, non-union will take place at the site of the fracture (Bancroft and Mikos, 2001). Other causes of non-union fractures include infection, insufficient blood supply to the bone and inadequate fracture stabilization.

## 1.2.4 Bone Related Medical Conditions

In the field of orthopaedic and oral-maxillofacial surgery, treatment of bone related clinical conditions is a significant challenge in case of critical size defects. Critical size defects are the type of defects that will not heal during the lifetime of the patient through natural healing response of the body (Schmitz and Hollinger, 1986). Infections, tumour, trauma and wear are clinical situations that indicate the need for augmentation of fracture healing and reconstruction of bone defects.

In 2000, approximately 2.2 million bone graft surgeries were carried out throughout the world (Lewandrowski et al., 2000). Every year, bone procedures have increased by 7-10% since 2001 (Vilquin and Rosset, 2006). In the US, spinal fusion counts for more than 50% of the total bone graft procedures (Datamonitor-Publications, 2002) (Table 1.2). This is followed by cranio-maxillofacial procedures and non-union fractures.

**Table 1.2-** Bone graft Procedures performed in the US in year 2001 (Datamonitor-Publications, 2002).

| Type of Procedure | % of Bone Graft Procedures |
|---|---|
| Spinal Fusion | 51 |
| Cranio-maxillofacial | 12 |
| Non-union Fractures | 8 |
| Bone Void Filling | 6 |
| Hip Fractures | 6 |
| Total Hip Revisions | 6 |
| Tibial Plateau Fractures | 5 |
| Trauma | 5 |
| Other | 1 |
| Total | 100 |

Furthermore, there is an increase in the number of osteodegenerative diseases in the rapidly aging populations present in the developed countries (Heng et al., 2004). According to South-Paul (2001) and Verzijl et al. (2003), osteoarthritis and osteoporosis are major public health issues since they affect quite high proportion of the elderly population (South-Paul, 2001; Verzijl et al., 2003). Arthritis is comprised of more than 100 different diseases of the joints. Nearly 500 million or one in six people worldwide will be affected by arthritis in their life time (Knowledge-Enterprises, 2002). Osteoarthritis and rheumatoid arthritis are the most frequent types of the disease. Arthritis is associated with inflammation, stiffness and degeneration of the joints. Osteoarthritis leads to the breakdown of the joint cartilage which functions as cushion for the ends of the bones. Hence, bones start contacting each other with

subsequent pain and possibly mobility loss. Rheumatoid arthritis is a chronic systemic disease causing inflammation of the synovium of articular joints as a result of abnormal functioning of the immune system. The patient's own antibodies attack the joints which finally lead to reduced mobility, pain and the eventual cartilage, tendon and ligament destruction. Approximately 17 million people from all age groups suffer from rheumatoid arthritis whereas osteoarthritis counts for 190 million people throughout the world, most of whom are over 60 years old (Knowledge-Enterprises, 2002).

More than 70% of all primary knee and hip replacement procedures takes place in osteoarthritis patients (Knowledge-Enterprises, 1998). In 2001, more than 1.5 million joint replacement procedures were carried out throughout the world (Knowledge-Enterprises, 2002). The cost of total hip replacement surgeries are about £250 million per year however, with the growing trend of the aging population, this figure could rise by 50% by 2026 (Birrell *et al.*, 1999; Bolland *et al.*, 2007). Bone grafts are potentially used in hip and knee replacement procedures. This is because there is an unmet medical need with respect to cartilage repair in patients with osteoarthritis and therefore the entire joint should ultimately be replaced. Therefore, such joint replacement procedures especially in revision surgeries could possibly require bone grafts due to the low quality and quantity of the remaining bone.

Degenerative disc disease is characterized by progressive intervertebral disc compression and affects virtually 50% of the US population between forty and sixty years of age and about 90% of the population older than sixty years of age (Datamonitor-Publications, 2002). In 2001, approximately 2 million spine procedures were performed worldwide which include fusion, fracture repair, disectomy and laminectomy (Knowledge-Enterprises, 2002). Back pain conditions are responsible for more hospitalizations that other musculoskeletal conditions with more than $100 billion spent annually throughout the world for the treatment of such conditions (Knowledge-Enterprises, 2002). Spinal fusion is the largest indication for bone graft substitutes as most of these operations need extra bone to confirm the stability of the fusion site. Bone grafts alone or in combination with metal implants are used in spinal fusion. Presently, the bone graft used for patients requiring spinal fusion is often

harvested from the iliac crest which offers many disadvantages as explained in section 1.2.5.5. Therefore, there is a great need for more suitable alternatives for spinal fusion.

Approximately 500,000 of the orthopaedic fractures that take place every year in the US will ultimately progress to delayed union or non-union fractures (Datamonitor-Publications, 2002). These delayed unions or non-unions count for about 10% of all fractures (Cattermole *et al.*, 1996). This is especially the case in ageing population where basic fractures could potentially become more serious. These non-unions in critical size defects stop function restoration to the damaged bone (Holy *et al.*, 2000).

For all the above mentioned medical conditions, bone regeneration is required in order to fill in the defect and therefore restore the structure and function of the injured tissue.

## 1.2.5 Current Treatments

Generally, bone related injuries can be treated by external and internal fixation devices as well as bone growth stimulators (Figure 1.2). Other than fixation devices, cell-based therapies, bone growth factors and natural and synthetic bone grafts could be used to treat bone defects (Figure 1.3).

**Bone Growth Stimulators**

- DC Electrical Stimulation
- Ultrasound
- Electromagnetic fields

**External Fixation**

- Bars/Rods
- Screws/Clamps
- Wires/Pins

**Internal Fixation**

- Pins/Wires/Staples
- Plates/Screws
- IM Nails
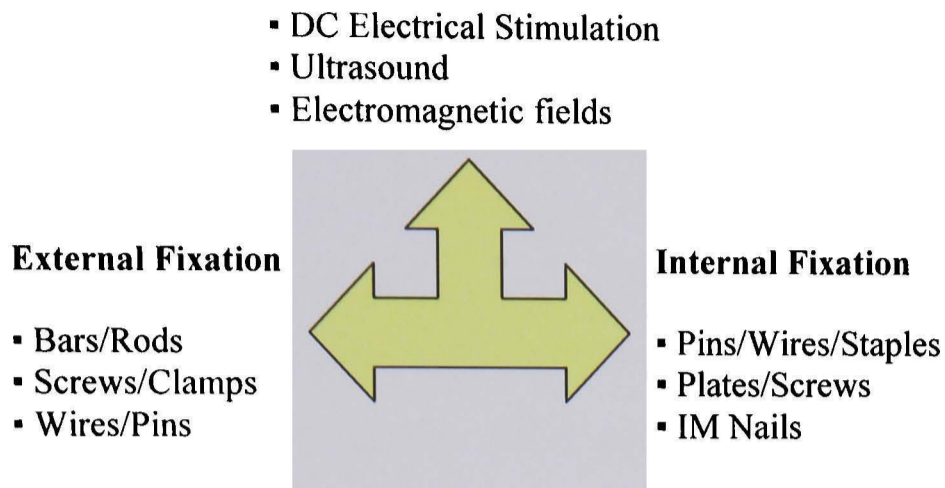
**Figure 1.2-** Overview of methods to treat bone defects using bone growth stimulators and internal and external fixation devices.

**Bone Growth Factors**

- Bone Morphogenic Proteins (BMPs)
- Transforming Growth Factors (TGFs)
- Platelet concentration systems

**Cell-based Therapies**

- Tissue Engineering
- Stem Cells
- Bone Marrow

**Natural Bone Grafts**

- Autografts
- Allografts
- Demineralized Bone Matrix (DBM)
- Xenografts

**Synthetic Bone Graft Substitutes**

- Metals
- Ceramics
- Polymers
- Composites
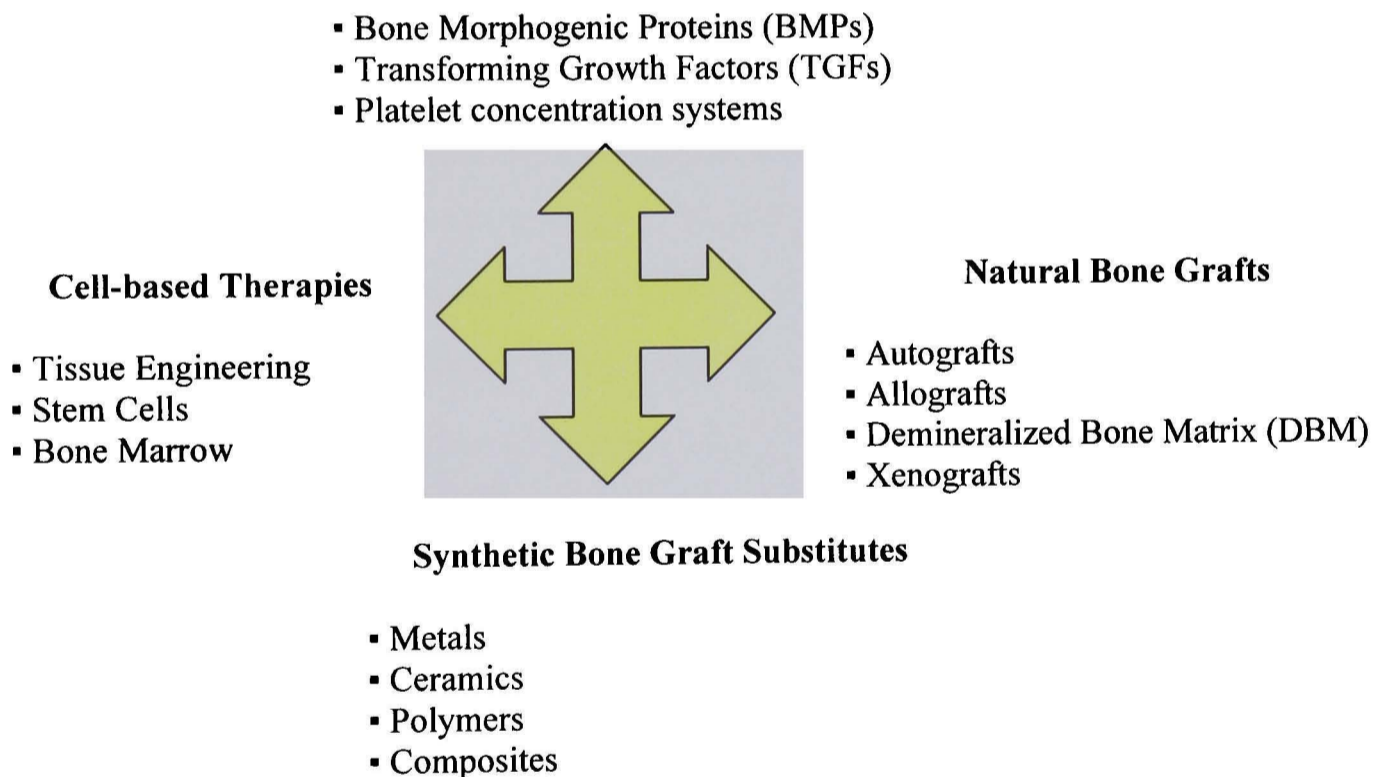
**Figure 1.3-** Overview of methods to treat bone defects using cell-based therapies, bone growth factors and natural and synthetic bone grafts.

These methods are further discussed in the following sections together with the shortcomings and advantages associated with them.

## 1.2.5.1 Internal Fixation

If a fracture is not healed efficiently using closed techniques, it becomes essential to invasively open the fracture site using surgery and reposition the bones. The bones are then held in place through the use of internal fixation devices. Internal fixation devices could consist of rods, screws, wires, nails and plates. Internal fixation is a comparatively inexpensive and efficient way to manage a fracture nevertheless, there is often a need for a secondary procedure to remove the implanted metalwork. However, this could be solved by the development of internal fixation devices that have the ability to be slowly absorbed by the body without any adverse tissue response.

## 1.2.5.2 External Fixation

External fixation devices (e.g. Ilizarov) function as fracture stabilizers with the use of pins and rods on the outer surface of the limb. The fixator is attached to the bone through metal pins that are drilled into the bone. External fixation is in fact less invasive than internal fixation and is mainly used to treat long bone fractures. With regard to the advantages of external fixation, the appliance can be regulated throughout the healing process to confirm perfect alignment. When healing is complete, the external fixation device can be removed easily without a secondary invasive operation. However, infections in the area where pins penetrate the skin (percutaneous infection) and also where they enter the bone is the major down side to the use of external fixators. Also external fixators can be burdensome as they stick out from the body.

Despite the use of internal and external fixation devices to heal bone fractures, about 10% of all the fractures lead to delayed unions or non-unions (Cattermole *et al.*, 1996). This is because natural bone healing using mechanical fixation can generally result in sufficient repair of only minor fractures over time (Mistry and Mikos, 2005). These non-unions will not heal even when immobilization period is increased. In these situations, natural bone grafts can be used in order to bridge the fracture gap and to enhance bone formation (Bauer and Muschler, 2000).

## 1.2.5.3 Bone Growth Stimulators

Bone growth stimulators are an emerging treatment technology. These devices produce ultrasonic waves or electromagnetic fields to stimulate bone growth. They can either be implanted inside the body or worn externally. For instance, electromagnetic fields such as constant direct current (DC) are known to enhance bone formation (Baranowski *et al.*, 1983; Brighton *et al.*, 1976; Brighton and Friedenberg, 1974; Friedenberg and Brighton, 1974). More specifically, Friedenberg and Brighton (1974) showed the repair of non-union fractures using 20 μA current when applied at the non-union site for 14 weeks (Friedenberg and Brighton, 1974). Moreover, epiphyseal plate has also been shown to enhance its growth through the use of an electrical field of 1500 volts/cm (Brighton *et al.*, 1976), nevertheless, such growth was observed *in vitro* and its effect *in vivo* is subject to question.

## 1.2.5.4 Bone Growth Factors

Urist and Strates (1971) observed a distinct protein in demineralized bone matrix (DBM) which induced bone formation. This protein was referred to as Bone Morphogenic Protein (BMP) (Urist and Strates, 1971). BMPs belong to the transforming growth factor β superfamily which can stimulate mesenchymal stem cell differentiation into chondro and osteogenic lineages (Lane *et al.*, 1999). These proteins produce bone when implanted in animals such as rats and baboons (Ripamonti *et al.*, 2001; Whang *et al.*, 1998). BMPs demonstrated that they have a growing potential to become the new golden standard therapy with sales of approximately $500 m (Datamonitor-Publications, 2002). Nevertheless, the success of BMPs in bone regeneration *in vivo* depends on the addition of a suitable carrier which permits a controlled release (Mendes, 2002). Carriers like collagen (Balk *et al.*, 1997; Wikesjo *et al.*, 2001), hyaluronan (Brekke and Toth, 1998), demineralised bone matrix (Wikesjo *et al.*, 2001) have been used for the delivery of BMPs. More specifically, freeze-dried Poly (lactide-co-glycolide) (PLG) scaffolds when used along with BMP-2 result in controlled release and ectopic bone formation (Ripamonti *et al.*, 2001; Whang *et al.*, 1998). This confirms the bone formation ability of BMP-2 and the suitability of PLG as a career in an animal model. Also, Osteogenic protein-1 (OP-1) which is a morphogenic protein when placed in collagen scaffold and implanted into the site of tissue formation result in significantly more bone formation

in rabbit lumbar fusion study after 6 weeks in comparison with the control groups (Qian et al., 2009). This further demonstrates the suitability of collagen scaffolds for OP-1 delivery and bone formation.

## 1.2.5.5 Natural Bone Grafts: Autologous grafts

Autologous bone grafts are defined as the grafts taken from one site (i.e. iliac crest) and transferred to the defect site within the same individual. Autologous grafting is considered as the golden standard therapy for bone repair and regeneration (Yaszemski et al., 1996-a). However, there are certain advantages and disadvantages associated with their use.

Autografts offer a source of tissue that is biologically active as they deliver progenitor stem cells in the bone marrow for osteogenesis (Lane et al., 1999). They are osteoinductive due to the presence of non-collagenous bone matrix proteins and osteoconductive because of the existence of bone mineral and collagen (Damien and Parsons, 1991; Lane et al., 1999). The most commonly used donor site for autologous bone harvest is the iliac crest as bone obtained from this site has demonstrated the highest osteogenic potential (Prolo and Rodrigo, 1985).

Despite the advantages mentioned, autografts are less efficient in defects with irregular shapes and may even be resorbed before complete healing occurs (Mistry and Mikos, 2005). Autografts require an invasive surgical procedure to harvest bone from the iliac crest of the hip which is associated with complication rates as high as 45% consisting of post-operative pain, infection, fracture, nerve injury, paresthesia, permanent gait disturbances, cosmetic disability and donor-site morbidity (Damien and Parsons, 1991; Gitelis and Saiz, 2002; Lane et al., 1999; Younger and Chapman, 1989). The operation and recovery time is also longer when autologous bone graft procedure is performed.

Limited availability of harvest site without causing loss of function is another concern associated with autografts (Brown and Cruess, 1982; Enneking et al., 1980). This is especially the case for young patients with limited donor sites or in situations where large quantities of bone are required. All these drawbacks indicate the necessity to

find a suitable alternative to autografts. An alternative which offers the advantages of autografts without the disadvantages associated with them is therefore preferred.

## 1.2.5.6 Natural Bone Grafts: Allogenic grafts

Allogenic bone grafts are the grafts harvested from a donor of the same species. These grafts can resolve some of the drawbacks associated with autografts but offer their own limitations when applied. Allografts are more readily available compared to autografts and the secondary harvest procedure and the amount of tissue available are not concerns for patients anymore. Also, due to the elimination of the harvest procedure, the operation time is reduced. Nevertheless, they carry the risk of immune rejection and disease transmission (Strong *et al.*, 1996) as well as having minimal osteoinductivity (Bauer and Muschler, 2000), therefore, they cannot replace autografts for bone regeneration purposes.

## 1.2.5.7 Natural Bone Grafts: Demineralized Bone Matrix

Processed allograft is known as demineralized bone matrix (DBM). DBM is obtained using cortical allograft bone which has surface lipids removed followed by dehydration with ethanol and ethyl ether. The bone is further treated with hydrochloric acid to remove the mineralized component but leaving behind proteins, collagen and growth factors. DBM could be available in different forms such as putties, granules, gels and blocks and could be used alone or in combination with autologous and allogenic bone grafts. Also, it is believed that the acid demineralization process eliminates cellular components present in the graft that express transplantation antigens. As a result, the demineralization process may assist in reducing the likelihood of implant rejection. DBM is widely used to repair skeletal defects since *in vivo* osteoinductivity of DBM has been demonstrated by various researchers (Bernick *et al.*, 1989; Bolander and Balian, 1986). However, the inductivity between various DBMs vary and is reduced by the treatments due to growth factor inactivation (Lane and Sandhu, 1987).

## 1.2.5.8 Natural Bone Grafts: Xenogenic grafts

Xenografts are the bone grafts derived from different species commonly having porcine or bovine origin however, the most frequent xenograft material is processed

bovine collagen. Unfortunately, xenografts offer the possibility of disease transfer and immunologic rejection (Erbe *et al.*, 2001). Partial deproteination can reduce severe antigenic response related to these implants but this process eliminates osteoinductive proteins (Lane and Sandhu, 1987). Moreover, when implanted into soft or hard tissues, xenografts do not induce bone formation (Damien and Parsons, 1991), hence, they cannot substitute autografts for bone repair.

### 1.2.5.9 Synthetic Bone Graft Substitutes: Synthetic Biomaterials

The problems related to the application of autologous bone grafts led to the investigation of several products in order to find an alternative without the disadvantages. There are various synthetic biomaterials currently available. Synthetic biomaterials offer the advantage of increased storage time and lack of disease transmission.

Two of the most important requirements for *in vivo* application of biomaterials are biocompatibility and biofunctionality. Biocompatibility is referred to as the non-toxic effects of a material on biological systems and biofunctionality is the ability of a biomaterial to carry out the purpose for which it was designated for. There are four groups of synthetic biomaterials concerning bone reconstruction applications:

- Metals like stainless steel, cobalt chrome alloys (Co-Cr), titanium alloys (Ti-6Al-4V) and ceramics such as zirconia ($ZrO_2$) and alumina ($Al_2O_3$)
- Calcium phosphate ceramics and bioglass
- Polymers such as poly(methyl methacrylate) (PMMA), polyethylene and polylactide
- Composites such as polymers filled with hydroxyapatite

Metals have been used successfully for hip and knee replacement procedures nevertheless, since they demonstrate poor integration with bone, they are often coated with a layer of calcium phosphate in order to promote integration (de Groot *et al.*, 1987). Mechanical properties of metals are considerably higher when compared to natural bone, thus resulting in stress-shielding effect by absorbing much of the mechanical stimuli required for bone re-growth (Bobyn *et al.*, 1992). This in fact leads to bone resorption around the implant which may ultimately necessitate whole implant removal (Mistry and Mikos, 2005).

Ceramic materials especially calcium phosphates such as hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP) have been investigated for applications as bone graft substitutes since they have similar composition to bone and teeth mineral (Damien and Parsons, 1991; Jarcho, 1981; Yuan *et al.*, 2000). However, calcium phosphate ceramics are too brittle with respect to providing structural support to load-bearing bones (Mistry and Mikos, 2005). Ceramic bone grafts contain cements and biphasic mixtures with calcium phosphate content. It is interesting to note the relation between the Ca/P ratio, acidity and solubility of the mixture such that the lower the Ca/P ratio, the higher the acidity and solubility of the mixture. Ca/P <1 shows high acidity and solubility while Ca/P ratios close to 1.67 demonstrate considerable reduction in acidity and solubility. Table 1.3 illustrates various calcium phosphate ceramics based on their Ca/P ratio.

**Table 1.3-** Showing different calcium phosphate ceramics with their corresponding Ca/P atomic ratios (Vallet-Regi and Gonzalez-Calbet, 2004).

| Ca/P | Ceramic name | Formula | Acronym |
|------|--------------|---------|---------|
| 2.00 | Tetracalcium phosphate | $Ca_4O(PO_4)_2$ | TetCP |
| 1.67 | Hydroxyapatite | $Ca_{10}(PO_4)_6(OH)_2$ | HAp |
| 1.50 | Tricalcium phosphate ($\alpha,\beta,\gamma$) | $Ca_3(PO_4)_2$ | TCP |
| 1.33 | Octacalcium phosphate | $Ca_8H_2(PO_4)_6.5H_2O$ | OCP |
| 1.00 | Dicalcium phosphate dihydrate | $CaHPO_4.2H_2O$ | DCPD |
| 1.00 | Dicalcium phosphate | $CaHPO_4$ | DCPA |
| 1.00 | Calcium pyrophosphate ($\alpha,\beta,\gamma$) | $Ca_2P_2O_7$ | CPP |
| 1.00 | Calcium pyrophosphate dihydrate | $Ca_2P_2O_7.2H_2O$ | CPPD |
| 0.70 | Heptacalcium phosphate | $Ca_7(P_5O_{16})_2$ | HCP |
| 0.67 | Tetracalcium dihydrogen phosphate | $Ca_4H_2P_6O_{20}$ | TDHP |
| 0.50 | Monocalcium phosphate monohydrate | $Ca(H_2PO_4)_2.H_2O$ | MCPM |
| 0.50 | Calcium metaphosphate ($\alpha,\beta,\gamma$) | $Ca(PO_3)_2$ | CMP |

The *in vitro* bioactivity of bone replacement materials like calcium phosphates is defined as their ability to form an apatite-like layer when soaking the materials in a simulated body fluid (SBF) (Filgueiras *et al.*, 1993). Bioactive ceramics such as sintered hydroxyapatite bond to living bone through a biologically active bone like apatite layer (Hench and Wilson, 1984; Jarcho, 1981; Jarcho *et al.*, 1977). This apatite layer is produced on the surface of the materials in the body (Ohtsuki *et al.*, 1991) through which they bond to living bone (Kokubo, 1991). Apatite layer formation on bioactive ceramics which occurs inside the body was demonstrated to be reproducible in acellular simulated body fluid having ion concentrations about equal to that of human blood plasma (Filgueiras *et al.*, 1993). It should be noted that bioactive materials such as calcium phosphate ceramics and glass ceramics demonstrate bonding osteogenesis which is bone formation initiating at the implant surface and progressing away from the surface (de Bruijn, 1993).

Several polymeric materials have been used as bone graft substitutes however, amongst them, non-biodegradable polymers such as ultra high molecular weight polyethylene (UHMWPE) and poly (methyl methacrylate) (PMMA) have been widely applied in bone reconstruction (Mendes, 2002; Oreffo and Triffitt, 1999).

UHMWPE has been used to produce acetabular cups in total hip prostheses whereas PMMA has been applied as bone cement and dental prosthesis (Oreffo and Triffitt, 1999). With respect to degradable polymers, poly(hydroxy esters) such as poly(glycolic acid) (PGA) and poly(L-lactic acid) (PLLA) have been extensively studied (Mistry and Mikos, 2005). Since degradation rates can vary based on the amorphous and crystalline parts of the polymer, at the time of *in vivo* degradation, the presence of non-degraded polymer particles might cause foreign body inflammatory reactions (Bostman, 1991; Bostman *et al.*, 1992; Temenoff *et al.*, 2000).

An overview of the presently used bone graft and bone graft substitutes together with their associated advantages and disadvantages are demonstrated below in Table 1.4. Autografts which are the golden standard therapy having osteoconductive, osteoinductive and osteogenic properties (presence of cells that can ultimately produce bone), are still associated with serious drawbacks (Damien and Parsons, 1991; Lane *et al.*, 1999). On the other hand, none of the other bone graft and bone graft substitutes offer the advantages of autografts therefore, there is a need for autograft equivalents without their inherent drawbacks. There is thus an unmet medical need which needs to be solved.

**Table 1.4-** An overview of the currently used bone substitutes and their related advantages and disadvantages.

| Factors | Bone grafts and bone graft substitutes | | | | | |
|---------|-----------|-----------|---------------------------|-----------|------|------------------|
| | Autografts | Allografts | Synthetic Biomaterials | Xenografts | DBMs | Growth factors |
| Availability | X | ✓ | ✓ | ✓ | ✓ | ✓ |
| Osteogenesis (i.e. presence of cells) | ✓ | X | X | X | X | X |
| Osteoconductivity | ✓ | ✓ | * | ✓ | X | X |
| Osteoinductivity | ✓ | X | * | X | ✓ | ✓ |
| Donor site morbidity | ✓ | X | X | X | X | X |
| Immune reaction | X | ✓ | X | ✓ | § | § |

* varies depending on the material used. § immune reaction is possible depending on the type of carrier used.

## 1.2.6 Unmet Medical Need

All procedures that repair missing tissue need some kind of replacement structure for the defect or injury region (Vacanti and Langer, 1999). The unmet medical need in the field of orthopaedics and bone related conditions with regard to large bone defects require an alternative which lacks all the drawbacks associated with autografts. There is therefore a need for a new golden standard. Bone tissue engineering provides a promising treatment solution by enhancing the natural healing response of the body through the combination of bone forming cells, growth factors and three-dimensional scaffolds (Figure 1.4) (Langer and Vacanti, 1993). The tissue engineered bone constructs have the potential to replace the existing bone graft technologies.
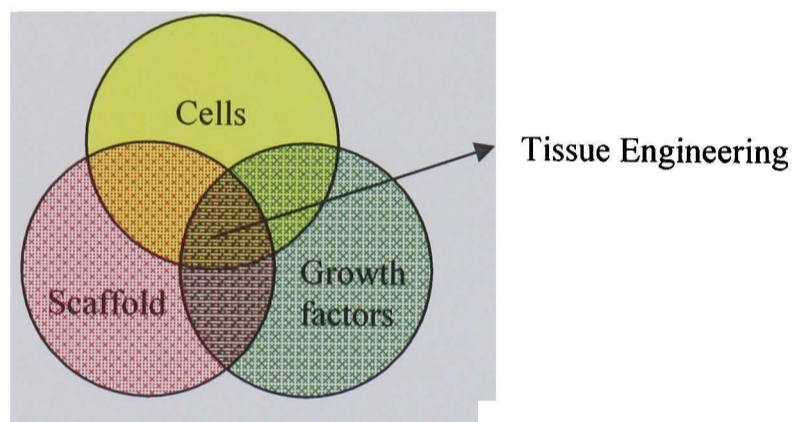


**Figure 1.4-** Tissue engineering approach using cells, scaffolds and growth factors combination.

Ideally, a treatment solution should mimic or improve the natural healing response of the body to bone injury by using cells and bioactive growth factors which will eventually result in natural bone with correct mechanical properties (Mistry and Mikos, 2005). Therefore, knowledge of the fracture healing events -section 1.2.3- that takes place inside the body is necessary to appropriately design therapeutic solutions to repair the injured bone.

## 1.3 TISSUE ENGINEERING

Tissue engineering could become a potential alternative to autografts. It is applied to replace living tissue with living tissue that is designed for the needs of each individual patient (Vacanti and Langer, 1999). It is defined as "an interdisciplinary

field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function" (Langer and Vacanti, 1993). Tissue engineering has broadened the search for less invasive and better treatment approaches for many disease processes.

Living tissue equivalents have the potential to regenerate bone tissue at a defect using cells which provide dynamics (osteogenic potential to produce bone) and scaffolds which offer volume (surface for cells to attach). Therefore, most research in the field of tissue engineering utilizes cell/scaffold combination to form new tissue (Vacanti and Langer, 1999). Addition of a cellular element within a scaffold may help to repair tissue at a more enhanced rate and to repair larger defects (Elisseeff et al., 2005). Ideal tissue engineered bone substitutes should possess osteogenic properties (cells to form bone) and porous structure (to permit vascularization and bone ingrowth) as well as being biocompatible, osteoinductive and osteoconductive (Bancroft and Mikos, 2001; Goldstein, 2002; Temenoff and Mikos, 2000).

Bone tissue engineering substitutes containing cell (tissue)-scaffold combination could be prepared in different ways. The cells can either be seeded onto the scaffolds just prior to implantation or they can be cultured on the scaffolds before implantation to allow extracellular matrix formation and differentiation of cells (Mankani et al., 2001; Ohgushi and Caplan, 1999; Yoshikawa et al., 2000). Several comparative studies have demonstrated the benefits of cell culturing before implantation (Kruyt et al., 2004-b; Kruyt et al., 2006; Mendes et al., 2002-a). More specifically, Yoshikawa et al. (1996), observed faster bone formation in rat marrow stromal cell/HA scaffolds which were triggered to osteogenic differentiation before subcutaneous implantation in rats compared with fresh bone marrow or undifferentiated mesenchymal stem cells (Yoshikawa et al., 1996). Differentiated mesenchymal stem cells can start building new osteoid immediately after arrival at the defect site. Despite the advantages associated with the cell cultured scaffolds, this technique takes longer because of the cell culture needed and requires more strategic planning with regard to clinical applications. Figure 1.5 demonstrates a possible bone tissue engineering approach where in stage 1 bone marrow cells are harvested from a patient followed by culture expansion in vitro. Following proliferation, the cells are cultured on biomaterial

scaffolds and triggered to differentiate into the osteogenic lineage by particular stimulatory factors like dexamethasone, ascorbic acid and β-glycerophosphate (Maniatopoulos *et al.*, 1988). In stage 4 the resulting tissue engineered construct is then implanted in the bone defect of the same patient.

It should be noted that different cell types from both autologous and allogeneic sources could be used for bone tissue engineering nonetheless, the most commonly used source of osteogenic cells is the bone marrow (Johansson and Persson, 2003). The procedure taken to obtain the bone marrow from the iliac crest or other parts of the skeleton is less invasive when compared to the harvest procedure associated with the collection of autologous bone. Cell sources for bone tissue engineering will be discussed further in section 1.6 of this chapter.
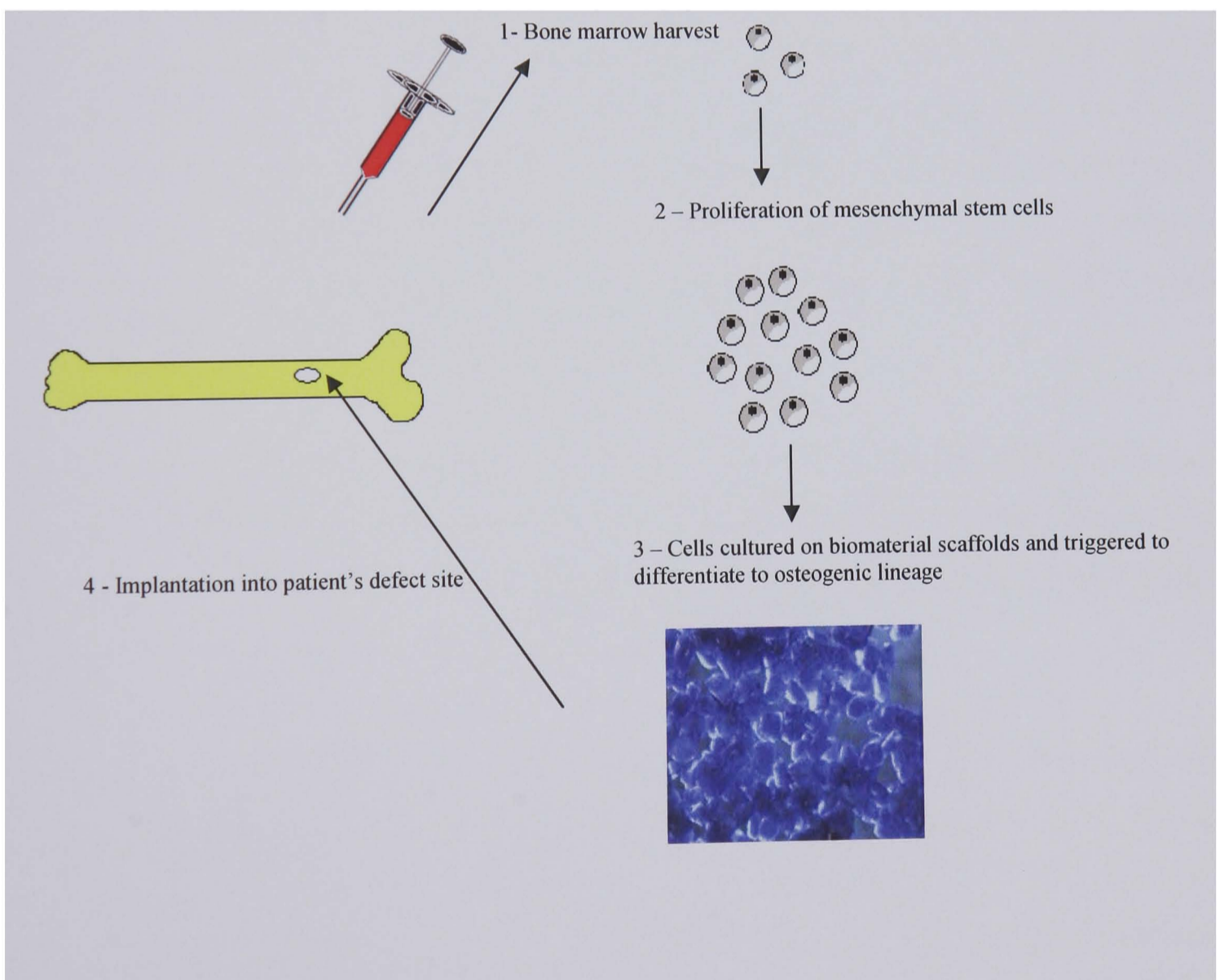
**Figure 1.5-** Bone Tissue Engineering using cells and scaffolds.

The cell (tissue)-scaffold combination described requires the addition of an injectable binder so that it could be applied to the defect site in a minimally invasive manner. Injectable formulations of tissue engineered bone provide a step forward in technology compared to non-injectable functional tissue engineered bone. Minimally invasive application of tissue engineered bone is the focus of this thesis which will be discussed in the following section.

# 1.4 INJECTABLE TISSUE ENGINEERED BONE

Tissue engineered formulations that can be injected straight into a bone defect in a minimally invasive manner provide a major advancement in the field of bone tissue engineering. Injectable formulations of tissue engineered bone could be used for variety of applications such as spinal fusion which indicates about 50% of the total bone graft procedures (Datamonitor-Publications, 2002). Using this technique, the surgeon will leave out the invasive bone harvest procedure required for obtaining autologous bone grafts since the osteogenic cells are obtained through minimal invasive procedure from bone marrow.

Injectable tissue engineered bone formulations offer less invasive surgery at implantation therefore resulting in less pain for patients, faster healing, reduced post-operative complications and surgery time. In addition, they conform to the shape of the defect and provide easy handling properties for physicians.

Injectable formulations consist of osteogenic cells combined with biomaterial scaffolds like most bone tissue engineering techniques but they also need a binder to hold the components together (Figure 1.6). The major functions of the binder are the provision of injectability and fixation. Injectability is the ability to inject the tissue engineered cell/particle combination while fixation refers to the ability of the binder to fix the cell/particle mixture in the bone defect such that they would remain in the injection site until the extracellular matrix formation. To provide for injectability, the scaffolds should be sub-millimetre size particles. Many researchers demonstrated that

the shape and size of the microparticles affect bone formation *in vivo* (Malard *et al.*, 1999; Mankani *et al.*, 2001). Mankani *et al.* (2001) showed that microparticles with 100-250 μm diameter when mixed with human bone marrow cells and implanted ectopically in nude mice produce abundant bone formation. Moreover, Fischer *et al.* (2003) investigated the influence of size (212-300 μm versus 500-706 μm microparticles) and microporosity (dense versus microporous) on bone formation for injectable formations of tissue engineered bone (Fischer *et al.*, 2003). Goat bone marrow cells were cultured on hydroxyapatite microparticles of different sizes and implanted subcutaneously in nude mice for 4 weeks. Abundant bone formation was observed in the 212-300 μm diameter particle range in both dense and microporous microparticles. The results of these studies suggest that the optimal particle size for bone formation lies in the range of 100-300 μm. Therefore, the microparticles used in this thesis were in the size range of 212-300 μm.
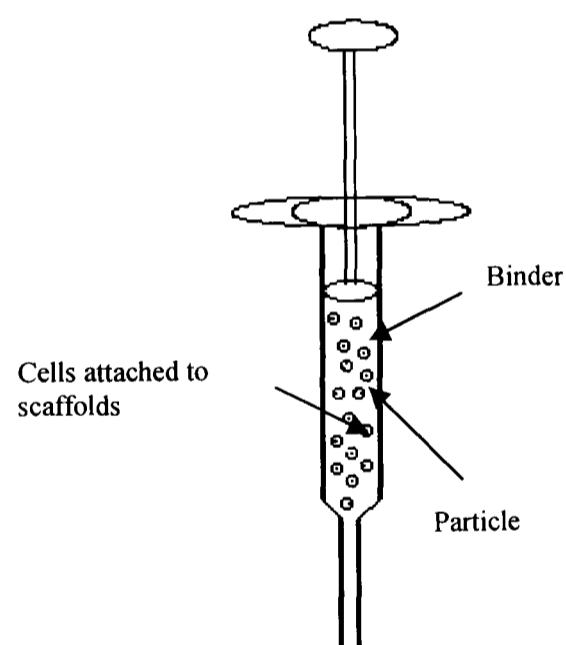


**Figure 1.6-** Injection device for injectable tissue engineered bone.

Polymers which undergo *in situ* polymerization via thermal cross-linking offer a major advantage for injectable formulations. These polymers can be added to cell/scaffold mixture and then injected through minimal invasive surgery to the injury site. These polymers should be biocompatible and degrade in a controlled manner to allow bone ingrowth into the defect. Moreover, they should gel at 37°C when applied to the defect site so that cell-scaffold combination would remain in the defect.

Various polymeric binders have been used previously in combination with cells. For instance, Weinand *et al.* (2006) combined collagen type I, alginate, fibrin glue and pluronic F127 with differentiated mesenchymal stem cells and $\beta$-tricalcium phosphate and examined bone formation through gene expression and histological analysis (Weinand *et al.*, 2006). The result of this study showed that collagen type I and fibrin produce the highest bone formation and are therefore suitable binders to carry cell-scaffold mixtures. In a different study, platelet rich plasma was used together with mesenchymal stem cells and applied to 10 mm diameter bone defect in dogs (Yamada *et al.*, 2004). Histological observations showed mature bone formation in cell-platelet rich plasma group after 2 and 4 weeks. Eight weeks following implantation, there was significantly more bone formation in cell-platelet rich plasma group compared to the defect only and platelet-rich plasma controls. This study indicates the potential of platelet-rich plasma to encapsulate stem cells. The potential binder materials for bone tissue engineering will be discussed further in section 1.8 of this chapter.

It should be noted that the studies in this thesis focus on the biological and material aspects of injectable bone tissue engineering as opposed to the mechanical aspects. It is of paramount significance that the cells inside the injectable tissue engineered bone constructs survive and produce bone when implanted in defects. In line with this requirement, the biological and material aspects of such constructs were the subject of investigation in this thesis. Therefore, the potential use for these constructs would be non load-bearing applications such as spinal fusion and skull defects. The current golden standard therapy for bone repair and regeneration (autografts) are applied to the defects in the form of bone chips without having high tensile strength and fracture toughness. Even though these bone chips lack sufficient mechanical properties, they are still the best solution available for bone regeneration. It is the subject of discussion how relevant mechanical properties are with regard to bone defects. Nonetheless, the ideal solution is when tissue engineered bone constructs perform well both biologically and mechanically.

Injectable tissue engineered bone formulations in this thesis contained three elements which include:

- Bone marrow derived mesenchymal stem cells
- Sub-millimetre (microparticle) size calcium phosphate scaffolds
- An injectable binder

In this thesis, mesenchymal stem cells attached to calcium phosphate microparticles will be combined with a suitable binder to create a tissue engineered bone formulation. Various studies with regard to suitability of the potential binders and more generally the performance of cell-scaffold-binder combination *in vitro* and *in vivo* will be performed. All these elements (cells, scaffolds, binder) are influential in injectable tissue engineered bone formulations and are discussed further in sections 1.6, 1.7 and 1.8 of this chapter.

# 1.5  CELL SURVIVAL PROBLEM

Clinical use of tissue engineering techniques is the ultimate aim in the field of regenerative medicine. With this in mind, Meijer *et al.* (2008) performed a study in which the potential of bone tissue engineering to treat jaw defects was examined in 6 patients (Meijer *et al.*, 2008). Mesenchymal stem cells were seeded onto hydroxyapatite particles and cultured for 7 days in osteogenic medium. Following this, the tissue engineered bone substitutes were implanted in each patient. Also, the tissue engineered bone constructs were subcutaneously implanted in mice to confirm their osteogenic potential. The results showed bone formation in mice however, tissue engineered bone constructs were able to produce bone in only 1 patient. In other words, tissue engineered bone constructs formed bone in ectopic site but not in orthotopic location. This study shows that clinical success of tissue engineered bone in osseous defects is still a major challenge. This is mainly due to the cell survival problem in such defects since the diffusion limit of oxygen is around 100-200 μm and therefore, formation of new blood vessels is essential for the tissue to grow further than this limit (Awwad *et al.*, 1986; Carmeliet and Jain, 2000). Vascularization is the major problem in bone tissue engineered constructs that are primarily employed to regenerate large bone defects.

Addition of an angiogenic binder to cell-scaffold combination in order to enhance vascularization is a potential way to overcome the cell survival problem in injectable bone tissue engineering. This is further examined in chapter 6 of this thesis.

## 1.6 CELL SOURCES

Cell sources that have the potential to differentiate into the osteogenic lineage are necessary in a tissue engineered construct to restore the biological function of bone (Hee *et al.*, 2006). Stem cells provide a promising cell source for bone reconstruction purposes since they have the ability of dividing to form equal daughter cells (self-renewal) and (multi) lineage differentiation with the provision of appropriate cues (Shamblott *et al.*, 1998; Smith, 2001; Thomson *et al.*, 1998; Weissman, 2000). More specifically, stem cells from mesenchymal and embryonic origins have been used for therapeutic purposes in tissue engineering (Czyz *et al.*, 2003). These cells provide a potential source for bone regeneration which will be discussed in more detail in the following section.

### 1.6.1 Embryonic Stem Cells

Embryonic stem cells are pluripotent cells that when placed in clusters and left to differentiate form embryoid bodies (EBs) that possess cell types from all three germ layers (Figure 1.7, Figure 1.8) (Smith, 1998; Smith, 2001; Weiss and Orkin, 1996). This resulted in a growing interest in their use (Reubinoff *et al.*, 2000; Thomson *et al.*, 1998) as a supply of differentiated cell types for cell transplantation therapy and drug discovery applications despite controversial ethical issues surrounding them (Smith, 1998; Solter and Gearhart, 1999). These cells are isolated from the inner cell mass of a blastocyst or from the primordial gonadal ridge of the fetus (Shamblott *et al.*, 1998; Thomson *et al.*, 1998). They can be multiplied infinitely in culture while still maintaining their ability for multilineage differentiation (Smith, 2001). Despite the fact that these cells have the potential of indefinite expansion in an undifferentiated status and differentiate into all tissues inside the body, isolation of homogenous cell populations from embryonic stem cells needs selection strategies so that a pure cell type is selected. Moreover, due to the plasticity of these cells, the

purity of any isolated cell population is subject to question since embryonic stem cells are at different stages of differentiation (Elisseeff *et al.*, 2005).

Inner cell mass

Mesoderm    Endoderm    Ectoderm

liver, lung, gut

muscle, connective tissue, bone, blood vessels            brian, spinal cord, skin
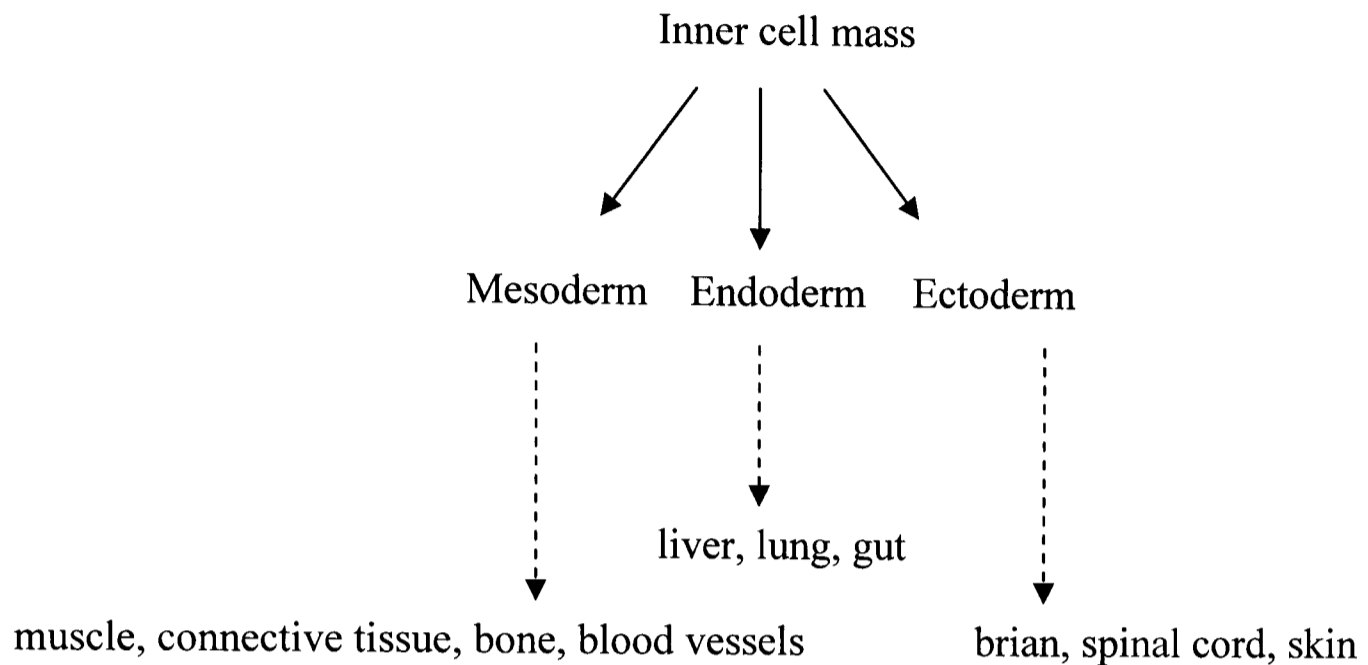
**Figure 1.7-** Schematic presentation of embryonic stem cells differentiating into three germ layers (Thomson *et al.*, 1998).

Embryonic stem cells can be genetically modified to reduce their immunogenicity as immunosuppressive drugs are associated with many unwanted side-effects. The genetic modification can take place in different ways such as removing immunoactive proteins such as B7 antigens and inserting immunosuppressive molecules like Fas ligand (Harlan and Kirk, 1999; Walker *et al.*, 1997). Also, foreign major histocompatibility complex (MHC) genes can be eliminated and genes coding for the recipient's MHC can be inserted (Hardy and Malissen, 1998; Westphal and Leder, 1997). The major challenge in the field of embryonic stem cells is in fact efficient and reliable control of the differentiation process into specific cell types. Some investigations performed on embryonic stem cells contain manipulation of the culture conditions such as addition of biochemical factors or coculture with specific cells or tissues (Gepstein, 2002; Soria *et al.*, 2001). Buttery *et al.* (2001) showed differentiation of murine embryonic stem cells into osteoblasts using both coculture with fetal murine osteoblasts and osteogenic differentiation medium containing dexamethasone, ascorbic acid and β-glycerophosphate (Buttery *et al.*, 2001). Furthermore, Kawaguchi *et al.* (2005) derived osteogenic and chondrogenic cells

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from mouse embryonic stem cells treated with bone morphogenic protein (BMP-4) and transforming growth factor (TGF-β) respectively (Kawaguchi *et al.*, 2005). In a different study, Jukes *et al.* (2008) examined the differentiation of embryonic stem cells into osteoblasts through the process of endochondral bone formation (Jukes *et al.*, 2008). Murine derived embryonic stem cells were seeded on ceramic particles of 2-3 mm and cultured in serum-free chondrogenic differentiation medium for a period of 21 days. Following the culture period, the tissue engineered constructs were implanted in nude mice for 21 days. The results showed bone formation in all constructs which were differentiated *in vitro* into chondrogenic as opposed to osteogenic lineage. This study indicates that a cartilage matrix is necessary for bone production by embryonic stem cells. Moreover, it demonstrates new facts with regard to the way in which bone tissue engineering is performed since most current tissue engineering strategies for bone repair and regeneration involve direct osteogenic differentiation of stem cells. Nevertheless, the natural healing response of the body in case of fracture is also through endochondral bone formation and as discussed above, Jukes *et al.* (2008) observed bone tissue formation through endochondral ossification (Jukes *et al.*, 2008). It is therefore reasonable to combine tissue engineering techniques with natural healing response of the body (endochondral bone formation) when devising strategies to regenerate bone defects in future.
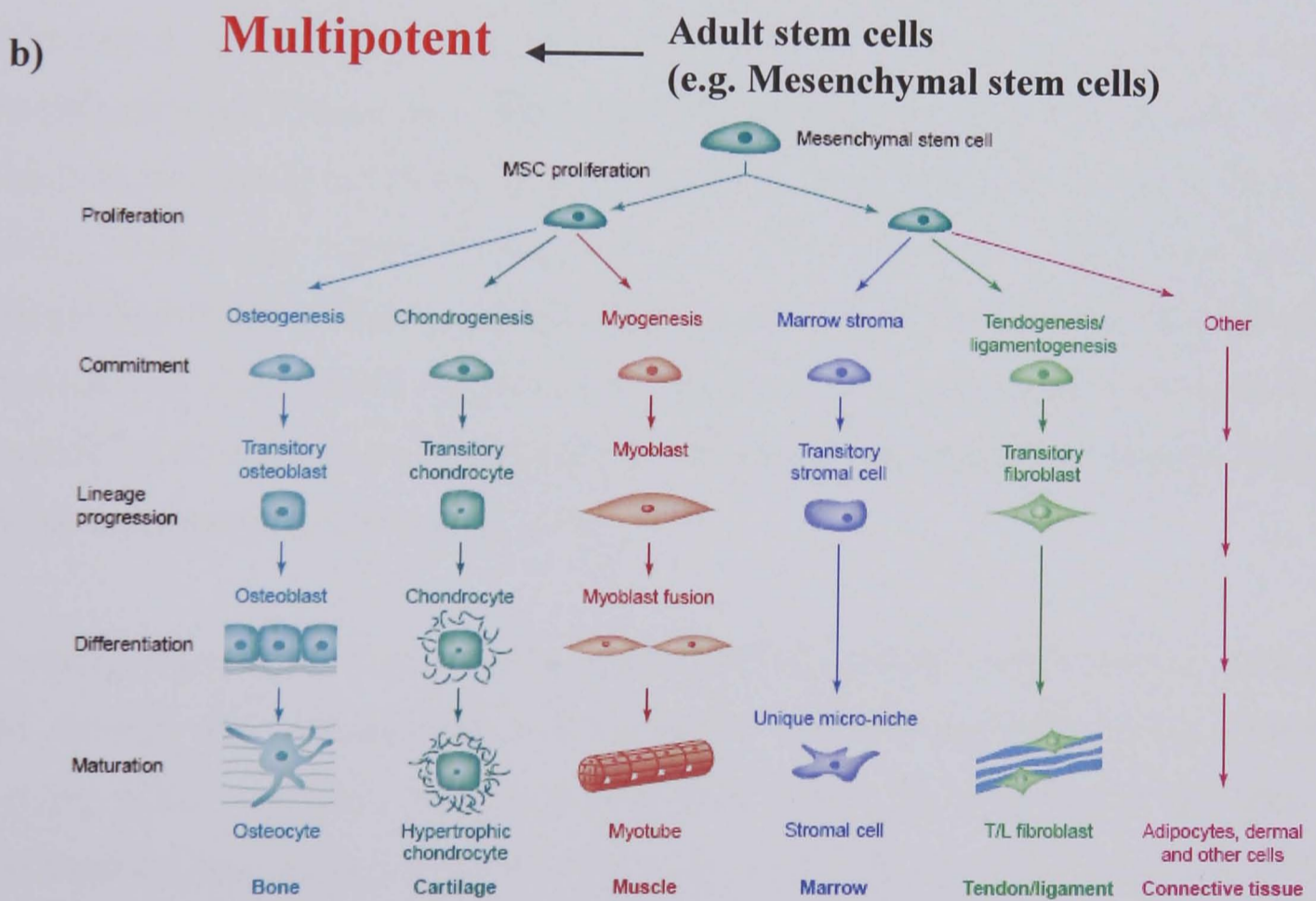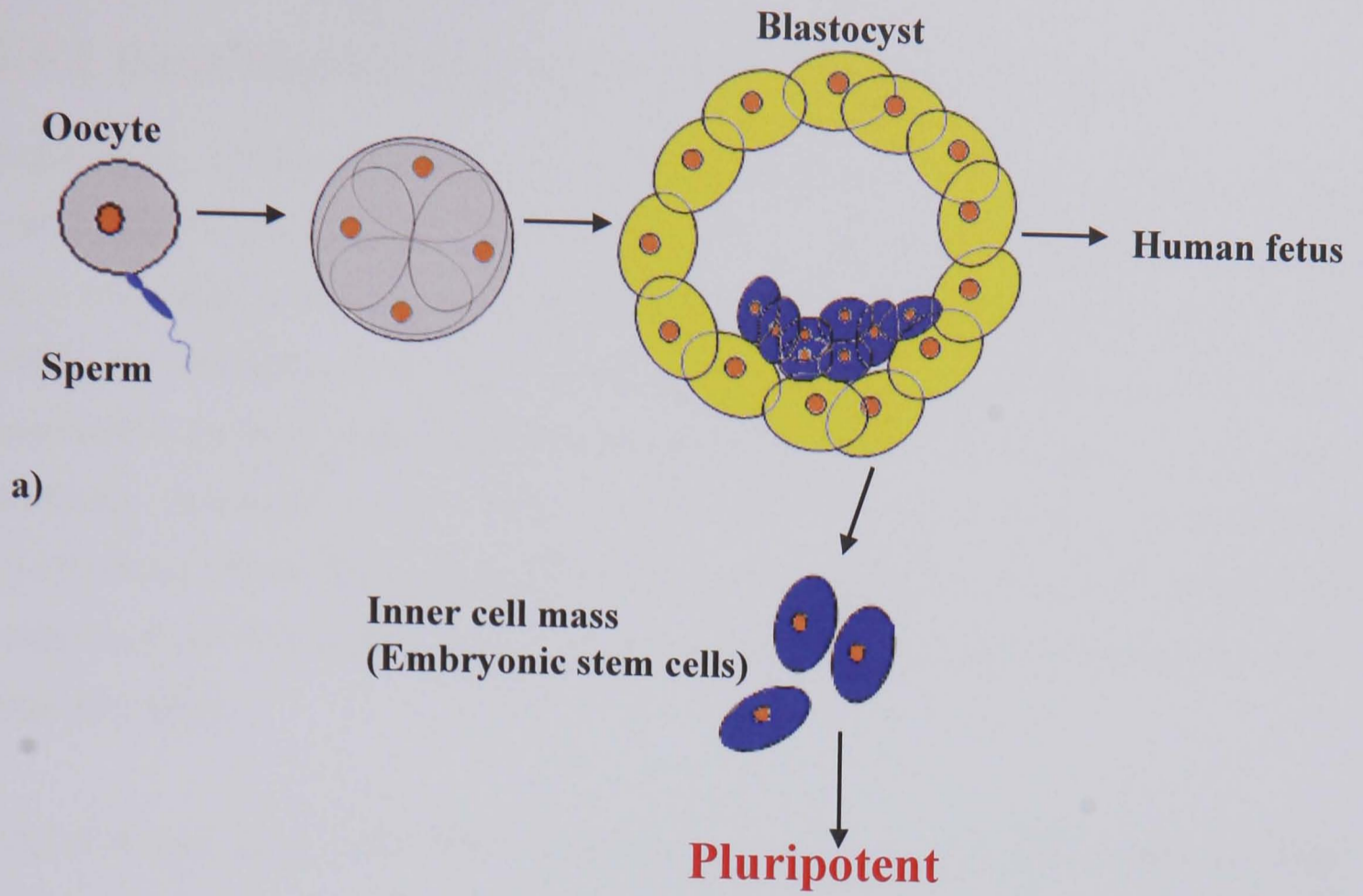
**Figure 1.8-** Schematic presentation of pluripotent embryonic stem cells versus multipotent adult stem cells (e.g. mesenchymal stem cells from (Caplan and Bruder, 2001)).

## 1.6.2 Bone Marrow Derived Mesenchymal Stem Cells

Considering a different cell source for bone production, osteoblasts, which are the native bone producing cells, are difficult to isolate and expand *in vitro* thus they result in a non-ideal source for bone tissue engineering applications (Notingher *et al.*, 2004). An alternative cell type is therefore required for bone formation. Cell-based approaches to bone tissue engineering need a great number of cells to seed onto scaffolds (Elisseeff *et al.*, 2005). Hence cells must be capable of extensive proliferation while still retaining their differentiation ability as well as their ability to maintain tissue forming activities such as secretion of extracellular matrix (ECM) and mineralization.

Mesenchymal stem cells from mesodermal germ layer were discovered in 1966 (Friedenstein *et al.*, 1966). Following their discovery, Friedenstein *et al.* (1970) showed the presence of colony-forming unit fibroblasts (CFU-F) within adult bone marrow which had the ability to differentiate to bone forming cells (Friedenstein *et al.*, 1970). Further studies demonstrated that mesenchymal stem/progenitor cell populations are able to differentiate into multiple mesenchymal lineages which include cartilage (Yoo *et al.*, 1998), bone (Haynesworth *et al.*, 1992; Prockop, 1997), ligament (Altman *et al.*, 2002), adipose (Beresford *et al.*, 1992), muscle (Ferrari *et al.*, 1998), tendon and stroma (Pittenger *et al.*, 1999) (Figure 1.8). This multi-lineage differentiation is particularly interesting since these cells could be used for variety of applications. Also, MSCs in the body are employed for repairing injured tissues thus making these cells good candidates for cell-based applications in musculoskeletal tissue engineering (Musaro *et al.*, 2004).

Postnatal mammalian bone marrow consists of two distinct developmental systems: the stromal and hematopoietic cell networks. The hematopoietic system includes mainly CD34 - positive stem and progenitor cell populations which are able to differentiate into all the mature blood cell phenotypes of an adult (Civin *et al.*, 1996; Ema *et al.*, 1991; Spangrude *et al.*, 1988; Uchida and Weissman, 1992). The stromal cell network predominantly consists of vascular endothelial and mesenchymal stem/progenitor cell (MSC) populations (Prockop, 1997; Seshi *et al.*, 2000; Strobel *et al.*, 1986). Despite the fact that different cell types from various tissues and locations

have been studied for bone tissue engineering applications, the most commonly used source of osteogenic cells is the bone marrow (Johansson and Persson, 2003). Practically, bone marrow seems to be an appropriate source of osteogenic cells since it can be obtained through a fairly simple aspiration technique (Elisseeff *et al.*, 2005; Johansson and Persson, 2003; Tuli *et al.*, 2003). Apart from the bone marrow, recent studies showed that MSCs are also present in subcutaneous fat (Zuk *et al.*, 2002), umbilical cord blood (Lee *et al.*, 2004), amniotic fluid (In 't Anker *et al.*, 2003), synovial membrane of the knee (De Bari *et al.*, 2001) and foetal liver (Gotherstrom *et al.*, 2003). MSCs for cell-based therapies could be either obtained autologously from patient's own bone marrow aspirates or other locations as explained above, or even allogenically from a cell bank (Elisseeff *et al.*, 2005). Interestingly, it was demonstrated that these cells exhibit immuno-privileged status which gives new hopes for their allogenic applications since both differentiated and undifferentiated MSCs do not cause alloreactive lymphocyte response (Le Blanc *et al.*, 2003).

In terms of drawbacks associated with MSCs, it should be noted that there is no single exclusive marker available for these cells however, using flow cytometry, antigens on MSCs could be identified. Human MSCs are positive for SH2, SH3, CD29, CD44, CD71, CD90, CD106 and CD120a markers and negative for CD14, CD34 and CD45 markers (Pittenger *et al.*, 1999). In addition, the concentration of mesenchymal stem cells in the adult bone marrow aspirates are less than 1 in 100,000-500,000 nucleated cells (Caplan, 2005) which is quite low and therefore leads to possible concerns regarding clinical application of these cells.

## 1.6.3 Multipotent Adult Progenitor Cells (MAPCs)

Multipotent Adult Progenitor Cells (MAPCs) form a new class of stem cells that are derived from adult tissues such as bone marrow, brain and muscle (Jiang *et al.*, 2002-a; Jiang *et al.*, 2002-b; Reyes and Verfaillie, 2001; Schwartz *et al.*, 2002). They are pluripotent stem cells with a wide differentiation potential such that when a single MAPC is injected into a blastocyst, it contributes to all tissues from three germ layers including skeletal muscles, liver, lung, cardiac muscle, skin, spleen, blood, marrow, central nervous system and intestine (Jiang *et al.*, 2002-a; Reyes and Verfaillie,

2001). Nonetheless, not much is known regarding the phenotype and genotype of these cells, their function *in vivo* or their differentiation potential following transplantation (Reyes and Verfaillie, 2001).

The cell type used in this thesis for majority of the studies involved mesenchymal stem cells. Individual results chapters (chapters 3-7) in this thesis explain more about the use of these cells in different experimental studies.

# 1.7 BIOMATERIAL SCAFFOLDS FOR BONE TISSUE REGENERATION

Early challenges to produce potential alternatives to traditional allografts and xenografts led to the creation of synthetic bone replacement materials (Elisseeff *et al.*, 2005). In an attempt to find synthetic bone replacement materials, various research groups throughout the world performed studies which resulted in a list of biomaterials with osteoconductive properties such as porous hydroxyapatite and calcium phosphates (Eggli *et al.*, 1988), bioglass (Hench and Wilson, 1984; Oonishi *et al.*, 1997) and titanium (Vehof *et al.*, 2002).

Synthetic bone replacement materials have also been used as scaffolds in tissue engineering applications. For instance, polymers such as poly(α-hydroxy acids) and collagen (Burg *et al.*, 2000; Middleton and Tipton, 2000), and osteoconductive ceramics like synthetic calcium phosphates (Frayssinet *et al.*, 1993; Yuan *et al.*, 2001) have previously been used for bone regeneration. The scaffold is designed to generate a three-dimensional environment which enhances tissue development from the cells that were seeded on or within the scaffold (Elisseeff *et al.*, 2005). The following sections explain about different polymers and ceramics that have been used as scaffold material for bone formation.

## 1.7.1 Poly(α-hydroxy esters)

Poly(α-hydroxy esters), such as poly(L-lactic acid) (PLLA), Poly(glycolic acid) (PGA) and poly(DL-lactic-co-glycolic acid) (PLGA) have been frequently used in

bone tissue engineering (Mistry and Mikos, 2005). This is because PLLA, PGA and their copolymer, PLGA are FDA- approved for certain applications. Previously, PGA meshes were employed to engineer bone tissue successfully *in vitro* and *in vivo* (Freed *et al.*, 1994; Vunjak-Novakovic *et al.*, 1999). Montjovent *et al.* (2005) made three-dimensional bioresorbable foams of PLLA alone or filled with hydroxyapatite or β-tricalcium phosphate (β-TCP) and studied the effect of alkaline phosphatase activity, *in vitro* mineralization and osteocalcin synthesis on human primary osteoblasts (Montjovent *et al.*, 2005). They observed that the cells adhere, proliferate and differentiate towards osteoblasts. The cells also formed a mineralized extracellular matrix which indicates osteoblastic differentiation. Moreover, Ishaug *et al.* (1996) reported that osteoblasts have the ability to migrate on PLGA films which indicates that PLGA is a promising scaffold material for bone tissue regeneration (Ishaug *et al.*, 1996).

Altering the proportions of lactic and glycolic acid in the copolymer formulation can change the hydrophobicity and crystallinity of the system, therefore the degradation time for the scaffold will vary as a result (Temenoff *et al.*, 2000). It has been reported that a modified copolymer of poly(lactic acid) and poly(lactic acid-co-lysine) could give attachment sites for specific peptides such as Arg-Gly-Asp (RGD) sequences in order to control adhesion and growth of the cells (Cook *et al.*, 1997). Interestingly, inflammatory reactions and poor incorporation in bone were observed in some cases when PLA and PGA were applied *in vivo* (Bostman, 1991; Bostman *et al.*, 1992), however the inflammatory reactions observed depended on the amount of material used. Therefore, it is not correct to generally assume that these polymers cause immune reactions. Nonetheless, it should be noted that polymeric materials are not osteoconductive which provides a major drawback to their use as scaffolds in bone tissue engineering.

## 1.7.2 Poly(propylene fumarate)

Poly(propylene fumarate) (PPF) scaffolds have previously shown to enhance proliferation and differentiation of primary rat stromal osteoblasts when cultured *in vitro* for four weeks (Peter *et al.*, 2000). Furthermore, when PPF scaffolds were

implanted in rat's tibial defects, the outer margins of the material was replaced by bone in 5 weeks (Yaszemski *et al.*, 1995). These studies indicate that PPF scaffolds are promising for bone tissue engineering applications.

## 1.7.3 Collagen

Collagen scaffolds have been used with marrow stromal cells (Farrell *et al.*, 2007; Glowacki *et al.*, 1998) and osteoblasts (Mueller *et al.*, 1999) and were demonstrated to interact positively within 3-dimentional collagen lattices. Contact dependent cells such as mesenchymal stem cells and osteoblasts migrate along the fibers and result in a uniform distribution all over the collagen lattice structure (Glowacki *et al.*, 1998; Mueller *et al.*, 1999). Purified type I collagen has been applied in different forms for three-dimensional growth of osteoblast-like cells *ex vivo* (Mueller and Glowacki, 2000). They therefore provide an interesting scaffold material for bone tissue engineering.

## 1.7.4 Calcium Phosphates

Many different scaffold materials have been used for bone tissue engineering applications. However, one commonly used scaffold material is calcium phosphate ceramic because of its biocompatibility and osteoconductivity (Hench and Wilson, 1984; Jarcho, 1981). Calcium phosphate materials containing $\beta$-tricalcium phosphate have been able to enhance bone formation when implanted with (Dong *et al.*, 2002; Fredericks *et al.*, 2004; Knabe *et al.*, 2000; Liu *et al.*, 2008; Wang *et al.*, 2004) or without cells (Frayssinet *et al.*, 1993; Yuan *et al.*, 2001).

Depending on the application, calcium phosphate scaffolds could be made degradable or non-degradable. It was observed that different types of calcium phosphate scaffolds such as hydroxyapatites and tricalcium phosphates have different characteristics *in vivo*. Generally, hydroxyapatites were known to have a slower degradation rate whereas tricalcium phosphates were found to degrade much faster (Daculsi *et al.*, 1989; Klein *et al.*, 1983). Combination of hydroxyapatites and tricalcium phosphate results in biphasic calcium phosphates with a wide range of

resorption rates that can be tailored to the specific application (Daculsi, 1998; Klein *et al.*, 1989). It is possible to inject or use these materials as coatings or in the bulk form to fill bone defects (Grimandi *et al.*, 1998).

## 1.7.4.1 Calcium phosphate scaffolds

Biodegradable calcium phosphate (CaP) ceramics have been used in orthopaedic surgery as bone replacement materials (Jarcho, 1981; Jarcho, 1986). A biphasic calcium phosphate (BCP) composition containing 85% hydroxyapatite (HA) and 15% $\beta$-tricalcium phosphate ($\beta$-TCP) has been shown to enhance new bone formation once applied in periodontal osseous defects (Nery *et al.*, 1992). Furthermore, porous HA scaffolds combined with rat marrow stromal cells and cultured with osteogenic supplements produced rapid bone formation following subcutaneous implantation in rats (Yoshikawa *et al.*, 1996). In a different study, bone tissue engineering was evaluated clinically using bone marrow derived mesenchymal stem cells seeded on porous HA scaffolds which were custom made based on the shape and size of the critical size bone defect in patients (Marcacci *et al.*, 2007). Follow-up studies showed a complete fusion between the host bone and the implant after 5 to 7 months without any pain, swelling or infection. Moreover, following 6-7 years, good implant integration was observed. This study is promising in terms of the clinical application of bone tissue engineering using calcium phosphate scaffolds.

When BCP implants are used in bulk or granule form, invasive surgery becomes necessary to implant them in patients. However, sub-millimetre size CaP scaffolds together with an injectable binder is needed to develop minimally invasive formulations. Previously, a system composed of biphasic calcium phosphate particles mixed with 2% methyl cellulose carrier gel was produced such that the system was easily injectable (Gauthier *et al.*, 1999-a). Moreover, i*n vivo* studies demonstrated higher bone ingrowth in BCP particles/cellulose groups than that of the BCP blocks/particles alone (Dupraz *et al.*, 1998; Gauthier *et al.*, 1999-a). This could be due to the cellulose which is spacing the BCP particles apart therefore creating a ceramic scaffold with free space in between (macropores) that support the formation of bone and blood vessels (Gauthier *et al.*, 1999-b; Grimandi *et al.*, 1998).

In this thesis, biphasic calcium phosphate microparticles were used. When powder form materials are used for bone filling instead of granules, one of the key advantages is their superior adaptability to the shape of the defect. However, such materials are hard to place and secure in the defect region because of the movement of these particles. The solution is to mix these powder-like particles with a suitable injectable binder (Dupraz *et al.*, 1999). Injectable binders and potential materials that could serve as injectable binders are discussed in more detail in section 1.8.

# 1.8 DELIVERY SYSTEMS: BINDERS

Polymeric binders are necessary for injectable formulations of tissue engineered bone. They hold the cell/microparticle scaffolds together as well as providing injectability and fixation. They also provide better handling properties. Binders should be biocompatible and allow bone ingrowth. Furthermore, they should be biodegradable and gel quickly at 37°C (i.e. in a few minutes).

Researchers have previously used binders to carry stem cells into the defect area. For instance, Richards *et al.* (1999) cultured mesenchymal stem cells in a collagen gel carrier and injected the cell-collagen combination into distraction gaps of rat femora (Richards *et al.*, 1999). A significantly higher amount of bone was observed in gaps which were treated with mesenchymal stem cell injections compared with the gaps with cell-free injections. Also, Collett *et al.* (2007) used thermally responsive poly(N-isopropyl acrylamide) (polyNIPAM) in combination with chondrocytes and showed that chondrocytes attach and survive (Collett *et al.*, 2007). In addition, it was possible to release the chondrocytes by just lowering the temperature. PolyNIPAM could be used with polyelectrolyte microcapsules while retaining its thermally responsive behaviour (Prevot *et al.*, 2006). This combination could be useful for targeted drug delivery applications.

## 1.8.1 Hydrogels

An ideal division of polymeric materials are hydrogels which have several applications in the biomedical field. They only include small amounts of polymeric

material typically in the scale of 1-30 % weight in aqueous solvent, have high molecular and oxygen permeability and possess mechanical properties similar to physiological soft tissues (Ratner and Hoffman, 1976). Basically, hydrogels are cross-linked polymeric systems that absorb large amounts of aqueous solution (Elisseeff *et al.*, 2005).

## 1.8.2 Thermosensitive Polymers

Thermosensitive polymers are certain group of polymers which demonstrate temperature dependent sol-gel transition. These polymeric solutions can be injected whilst kept below or above their transition temperature and form a gel *in situ* upon reaching a particular temperature for instance 37°C (body temperature).

The following pages explain about different binders that have been used previously with the potential to carry cell/scaffold combination for bone tissue engineering purposes. Table 1.5 describes some of the potential binders for injectable tissue engineered bone and their inherent drawbacks/benefits. As polymers that are mentioned in Table 1.5 are associated with drawbacks such as non-degradability, low gelation temperatures, high erosion rates and high polymer concentrations, they therefore provide non-optimal binders for injectable tissue engineered bone applications.

**Table 1.5-** Showing the advantages/disadvantages associated with some potential binders for injectable tissue engineered bone.

| Binders | Authors | Advantages | Disadvantages |
|---|---|---|---|
| Poloxamers | (Johnston et al., 1992); (Pec et al., 1992); (Johnston and Miller, 1989); (Ruel-Gariepy et al., 2000); (Desai and Blanchard, 1998); (Bhardwaj and Blanchard, 1996); (Jarry et al., 2001); (Palmer et al., 1998) | * One of the mostly studied thermo-sensitive polymers; * Present in the form of a viscous liquid at low temperatures but forms a rigid semisolid gel upon temperature increase | * Gelation at 15°C limits room temperature handling; * High polymer concentration needed e.g. 20-30% (w/v); * Rapidly erodable, * Non- biodegradable |
| N-Isopropylacrylamide | (Dinarvand and D'Emanuel, 1995; Okano et al., 1990) | * Gelation at 37°C | * Non-biodegradable; * Limited toxicity data |
| Poly(Ethylene Oxide) and poly(Lactic Acid) | (Jeong et al., 1997) | * Gelation at 37°C; * Biodegradable; * Biocompatible | * Need to heat the system to 45°C to get a liquid phase before incorporating drugs or cells |
| Xyloglucan | (Miyazaki et al., 1998) | * Gelation at low concentrations e.g. 1-2% (w/w) with temperature above 22-27°C | * Low transition temperature limits room temperature handling |

With respect to the *in vitro/in vivo* performance of the materials, natural binders such as alginate, plasma, fibrin glue, hyaluronic acid and chitosan/glycerol phosphate are promising candidates for use as injectable binder. These polymeric binders are explained below in more detail.

## 1.8.3 Chitosan

Chitosan, an aminopolysaccharide derived from alkaline deacetylation of chitin, is a natural polymer which is present in fungal cell walls and exoskeletons of arthropods such as shrimps and crabs (Khor and Lim, 2003; Montembault et al., 2005; Pelletier et al., 1990). Chitosan is a cationic copolymer composed of glucosamine and N-acetyl-glucosamine (Figure 1.9) (Crompton et al., 2005; Jarry et al., 2001). The

properties of chitosan are mainly governed by the degree of deacetylation, DDA, which is based on the relative amount of acetyl and amine groups and also its molecular weight (Mw) (Crompton *et al.*, 2005). These factors are determined depending on the conditions selected during the preparation process; however additional modification at a later stage is still possible. For instance, the DDA can be lowered through reacetylation (Sorlier *et al.*, 2001).
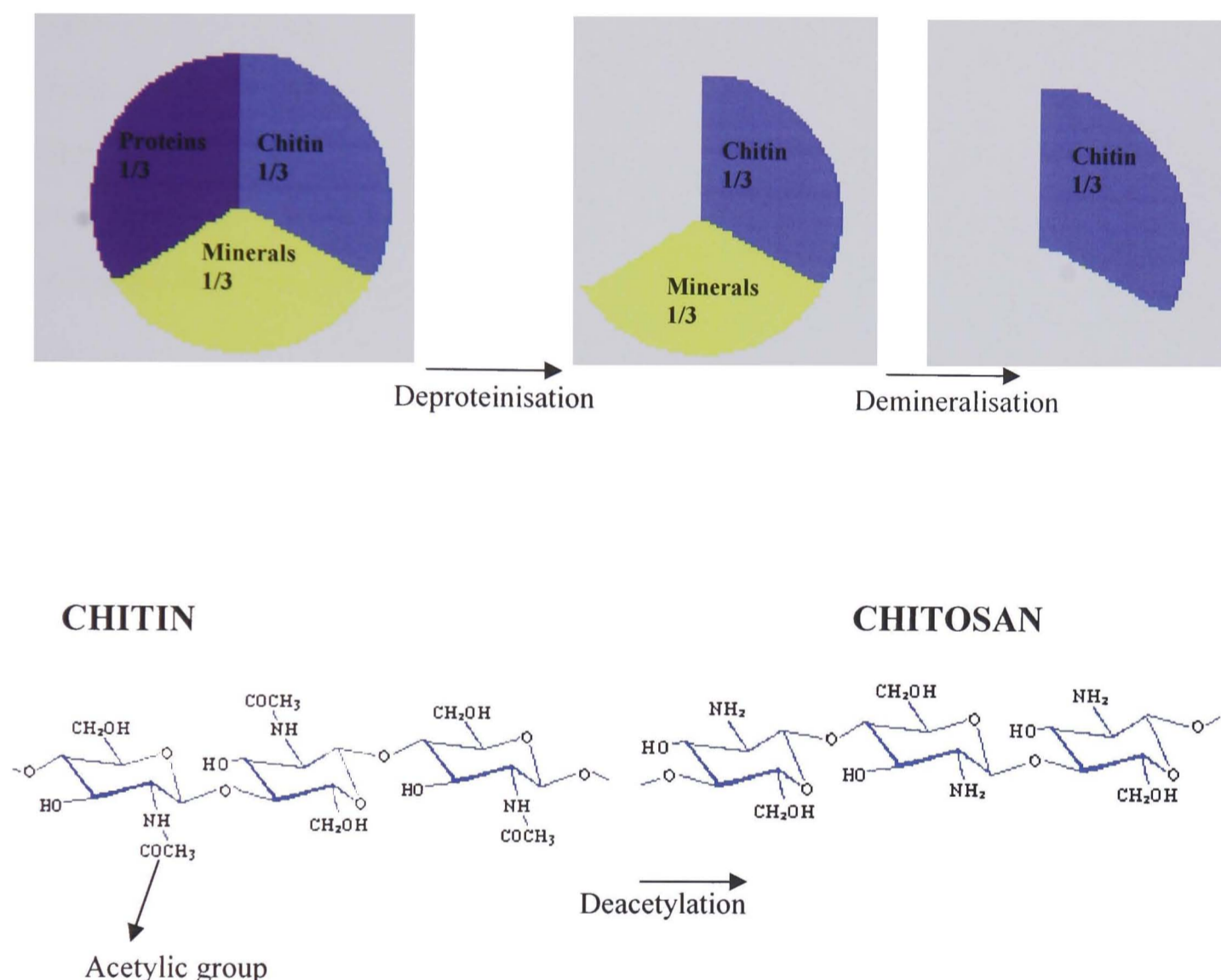




**Figure 1.9-** Illustrating the derivation process of chitosan. After deproteinisation and deminiralisation of shrimp's exoskeleton, chitin is obtained which is then deacetylated to result in chitosan.

Chitosan is attracting a great deal of attention due to its abundance in nature, its low cost production and its interesting intrinsic properties (Berger *et al.*, 2004). Indeed, chitosan is a biocompatible (Chandy and Sharma, 1990; Hirano and Noishiki, 1985; Park *et al.*, 2005) and biodegradable (Muzzarelli, 1997) biopolymer playing an important role in biomedical and pharmaceutical applications (Sandford, 1989).

Biocompatibility of chitosan resulted in its use in numerous medical applications such as topical ocular application (Felt *et al.*, 1999), injection (Song *et al.*, 2001) and implantation (Patashnik *et al.*, 1997). Chitosan can be metabolized by certain enzymes within the human body such as lysozyme thus giving it biodegradability (Muzzarelli, 1997). It possesses a positive charge at the physiological pH therefore making it bioadhesive which enhances retention at the site of application (Calvo *et al.*, 1997). One of the main advantages of applying chitosan as a carrier is that chitosan gel is formed at very low polymer concentration (e.g. < 2% w/v) therefore high aqueous contents of the gel increases its biocompatibility (Jarry *et al.*, 2001). It has been reported by Ueno *et al.* (2001-a) that chitosan encourages and supports wound-healing (Ueno *et al.*, 2001-a). In addition, chitosan possesses bacteriostatic effects (Felt *et al.*, 2000).

Apart from the interesting properties mentioned, chitosan possesses other useful properties. With respect to the angiogenic potential of chitosan, there have been suggestions that chitosan is angiogenic (Biagini *et al.*, 1989; de Castro-Bernas, 2003). De Castro-Bernas (2003) reported chick chorioallantoic membrane (CAM) assay results showing that chitosan stimulated neovascularization in a dose-dependent manner at even higher levels than the known angiogenic growth factor b-FGF (basic fibroblastic growth factor). In a study by Biagini *et al.* (1989), N-Carboxymethyl chitosan placed into the cornea of 10 adult rabbits induced neovascularisation in 7 rabbits after 30 days whereas control rabbits did not show any new vasculature. In their paper, Biagini *et al.* (1989) referred to the biochemical effects of chitosan or the foreign body reaction caused by chitosan as potential reasons behind chitosan's induced neoangiogenesis.

Also, chitosan has been reported to stimulate bone healing in different animal models (Klokkevold *et al.*, 1996; Malette *et al.*, 1986; Muzzarelli *et al.*, 1994; Pang *et al.*, 2005). In an *in vivo* study, Muzzarelli *et al.* (1994) reported the use of chitosan containing imidazolyl groups in a 7 mm diameter defect in the femoral condyle of sheep. Following 40 days there was more bone formation in the defects treated with chitosan whereas the control group demonstrated no indication of bone formation.

Chitosan polymers have an average molecular weight of 50 kDa ≤ Mw ≤ 2000 kDa and the degree of deacetylation of 40% < DDA < 98% (Chenite *et al.*, 2001). Chitosan is not soluble in water and at neutral and alkaline pH but chitosan solutions are made in acidic aqueous media that protonate chitosan amino groups, therefore making the polymer positively charged (Chenite *et al.*, 2001; Ruel-Gariepy *et al.*, 2000). With the addition of a strong base like NaOH, chitosan is in the solution form up to a pH in the vicinity of 6.2 however, extra basification to pH of > 6.2 causes hydrated gel-like precipitates to form which is due to chitosan phase-separation (Chenite *et al.*, 2001). This gel is formed because of the neutralization of the amine groups of chitosan and subsequent elimination of repulsive interchain electrostatic forces that permit massive hydrogen bond formation and hydrophobic interactions between the chains (Chenite *et al.*, 2001). Nevertheless, upon the addition of a polyol counterionic salt like glycerol phosphate (GP) to chitosan solution, the pH is changed to neutral without phase-separation (Chenite *et al.*, 2001; Chenite *et al.*, 2000). The liquid can maintain its sol state for a long time at or below room temperature (Ruel-Gariepy *et al.*, 2000). This system turns into thermally sensitive thus creating a gel above a particular temperature (37°C), therefore it is only the temperature of the milieu that decides the sol or gel state (Chenite *et al.*, 2000; Crompton *et al.*, 2005).

Chitosan/GP system has shown great potential for drug delivery and cell encapsulation (Chenite *et al.*, 2000; Ruel-Gariepy *et al.*, 2000). Chondrocytes encapsulated within chitosan-GP were capable of maintaining more than 80% viability over an extended period of time *in vitro* (Chenite *et al.*, 2000). Moreover, chitosan-GP systems have also been applied *in vivo* (Molinaro *et al.*, 2002). For instance, Molinaro *et al.* (2002) implanted 50 µl of 1.8% (w/w) chitosan - 45% (w/w) GP in the hindpaw of the rat and observed an acute inflammatory response.

All the information mentioned indicates that chitosan is an interesting hydrogel for injectable applications of tissue engineered bone.

# 1.8.4 Alginate

Sodium alginate has been used in various biotechnology applications mostly to encapsulate and immobilize several cell types for immunoisolatory and biochemical processing applications since cells maintain their viability when in contact with the cross-linked gel (Park and Chang, 2000; Trivedi *et al.*, 2001). The term alginate refers to a family of polyanionic copolymers which are derived from brown sea algae and are composed of $\beta$-D-mannuronic (M) and $\alpha$-L-guluronic (G) residues in altering proportions.

Sodium alginate is soluble in aqueous solutions and produces stable gels that can be processed into any shape at room temperature with the aid of non-cytotoxic divalent cations such as $Ca^{2+}$ and $Ba^{2+}$ through the ionic interaction between the guluronic acid groups (Wang *et al.*, 2003-b). This leads to the formation of three-dimensional structures which can be loaded with viable cells. Calcium cross-linked sodium alginates are therefore able to immobilize cells and bioactive factors (Augst *et al.*, 2006). Moreover, alginate can be uncross-linked with the use of mild chelating agents that can result in the release of entrapped cells (Lindenhayn *et al.*, 1999). However, large variations exists in degradation rates of calcium cross-linked sodium alginates which could be a possible disadvantage (Ishikawa *et al.*, 1999).

Although Wang *et al.* (2003-b) demonstrated that rat bone marrow stromal cells (rMSCs) attach, proliferate and differentiate on unmodified calcium cross-linked sodium alginate gels (Wang *et al.*, 2003-b), Lawson *et al.* (2004) observed little or no attachment with human bone marrow derived stromal cells (hMSCs) (Lawson *et al.*, 2004). Nevertheless, when calcium cross-linked sodium alginate gels were modified through the addition of collagen type I and $\beta$-tricalcium phosphate, which are materials similar to bone extracellular matrix, successful attachment and proliferation of hMSCs were obtained (Lawson *et al.*, 2004). This shows that sodium alginate gels might need modification before hMSCs attach and proliferate within the gel.

## 1.8.5 Fibrin Glue/Plasma

Fibrin glue is widely used as biological sealant for hemostasis and wound dressing applications but because of its biomimetic and physical properties, it is frequently used as a carrier for different cell types such as chondrocytes and osteoblasts (Kneser *et al.*, 2005; Sims *et al.*, 1998).

Plasma could also be used as binder in tissue engineering applications. It was shown to be biocompatible when used with hMSCs (Trombi *et al.*, 2008). In combination with chondrocytes, platelet-rich plasma demonstrated great potential as a carrier material since new cartilage formation was detected after 2 months subcutaneous injection in the dorsal tissue of rabbits (Wu *et al.*, 2007). Moreover, plasma can be obtained from the patients at the time of operation and centrifuged. This provides fresh autologous plasma thus eliminating possible immune reactions.

## 1.8.6 Hyaluronic Acid

Hyaluronic acid – also known as hyaluronan – is a non-sulfated glycosaminoglycan containing repeated disaccharide units of N-acetyl-glucosamine and D-glucuronic acid and is found in the extracellular matrix and the synovial fluid (Luo *et al.*, 2001). Hyaluronan is associated with water homeostasis of tissues as well as being the lubricating molecule of the musculoskeletal system (Fraser *et al.*, 1997). It plays an important role in cartilage matrix stabilization (Fraser *et al.*, 1997) and the regulation of motility and cell adhesion (Collis *et al.*, 1998; Hardwick *et al.*, 1992). Hyaluronic acid receptors were observed on the cell surfaces since CD44 which is a broadly distributed cell surface glycoprotein, belongs to hyaluronan cell surface receptors (Johansson and Persson, 2003; Luo *et al.*, 2001).

Hyaluronic acid has great potential for drug delivery and tissue engineering applications since it is biocompatible (Luo *et al.*, 2001). When used with mesenchymal stem cells and chondrocytes, hyaluronan based scaffolds demonstrated positive tissue formation (Solchaga *et al.*, 1999; Solchaga *et al.*, 2002). However, the molecular weight of hyaluronan which is the crucial factor in physical properties can

vary when obtained from different sources (Luo *et al.*, 2001). This could result in different physical properties depending on the source of hyaluronan.

# 1.9 AIMS AND OBJECTIVES OF THE THESIS

The drawbacks associated with the use of the current golden standard therapy for bone repair and regeneration (autografts) and the potential of tissue engineering principles to enhance bone repair has been discussed in this chapter. The addition of an injectable angiogenic binder which holds the cell-scaffold combination could have the additional advantage of repairing bone defects in a minimally invasive fashion. Therefore, the general aim of this project is to contribute towards better understanding of the use of mesenchymal stem cells in combination with calcium phosphate ceramic microparticles for injectable bone tissue engineering application. With this in mind, this thesis concentrates on the use of chitosan binders in combination with cell-scaffold mixture as well as analysing various aspects of injectable tissue engineered bone construct. This aim will be achieved by realising the following objectives:

- Investigating the biocompatibility and gelation of chitosan-GP hydrogels

- Analysing the effect of degradation, gelation, cell survival, growth and osteogenic differentiation when chitosan-based hydrogels are used

- Assessing the *in vivo* bone formation ability of cultured mesenchymal stem cells on calcium phosphate ceramic microparticles when combined with chitosan-based hydrogels

- Examining the angiogenic potential of chitosan-based matrices with/without mesenchymal stem cells using chick chorioallantoic membrane assay

- Measuring the osteogenesis by MC3T3-E1 cells through chitosan-based hydrogels

# CHAPTER II

## GENERAL CELL CULTURE TECHNIQUES

# 2.1 INTRODUCTION

This chapter is devised to explain the materials and methods regularly used during the experimental part of this thesis. Materials and methods for specific experimental works are described within the materials and method section of the relevant chapters.

# 2.2 CELL CULTURE TECHNIQUES

## 2.2.1 Monolayer Culture of Cells

Human mesenchymal stem cells (hMSCs) (from female, age 36 (iliac crest); male, age 44 (acetabulum); female, age 55 (iliac crest); Xpand Biotechnology BV, The Netherlands), MC3T3-E1 cells (Murine cell line, Department of Tissue Regeneration, Twente University, The Netherlands) and goat mesenchymal stem cells (gMSCs) (from iliac crest of adult Dutch milk goats, 2-4 years old, Xpand Biotechnology BV, The Netherlands) were expanded in a monolayer culture in α-MEM (Gibco Invitrogen, UK) supplemented with different concentrations of fetal bovine serum (FBS, Gibco Invitrogen, UK) as shown in Table 2.1. More specific supplements added to the culture media are explained in individual chapters. Cell suspensions were added to the culture media in relevant sized polystyrene culture flasks and expanded in monolayer at different seeding densities as demonstrated in Table 2.1. Based on the number of cells to be seeded, culture flasks of different sizes were employed (Table 2.2). The cells were then incubated at 37°C, 5% $CO_2$ and 95% humidity. The FBS was chosen through batch-testing according to the maximum cell growth and alkaline phosphatase (ALP) activity levels. This is because cell proliferation and osteogenic differentiation (ALP activity) were involved in most studies in this thesis and hence a serum that enhances proliferation of cells and their osteogenic differentiation is the most suitable.

The colourimetric indicator Alamar Blue (SeroTec, Oxford, UK) was employed to evaluate cell proliferation at days 1, 3, 7, 10 and 14 for the batch-testing. The medium was composed of α-MEM (Gibco Invitrogen, UK) supplemented with 10% v/v fetal bovine serum from different batches (FBS, Gibco Invitrogen, UK), 100 U/ml

penicillin/100 µg/ml streptomycin, 1 ng/ml basic fibroblast growth factor (b-FGF, SeroTec, Oxford, UK), 2 mM L-glutamine and 0.2 mM AsAP. At days 1, 3, 7, 10 and 14, hMSCs were washed three times with pre-warmed PBS solution. Consequently, 1 ml of a 5% Alamar Blue solution in culture medium (Lawson *et al.*, 2004) was added to each well-plate. The plates were then incubated for 3 hours in a humidified atmosphere at 37°C/5% $CO_2$. Fluorescence of 100 µl aliquots of the extracted dye from each sample was determined using a Fluorimeter (FLUOstar Galaxy Fluorimeter, JENCONS-PLS, Germany) at excitation and emission wavelengths of 544 nm and 590 nm respectively. A standard calibration curve according to known numbers of hMSCs reacting with the Alamar Blue solution was used in order to quantify the number of cells present in each sample (Lawson *et al.*, 2004; Li *et al.*, 2005).

The osteogenic differentiation was measured through the alkaline phosphatase (ALP) activity and protein content at days 1, 3, 7, 10 and 14 for the batch-testing. For this, the human mesenchymal stem cells were cultured in the basic medium which was composed of α-MEM (Gibco Invitrogen, UK) supplemented with 10% v/v fetal bovine serum from different batches (FBS, Gibco Invitrogen, UK), 100 U/ml penicillin/100 µg/ml streptomycin, 2 mM L-glutamine and 0.2 mM AsAP as well as the osteogenic medium which was composed of the basic medium supplemented with 10 mM β-glycerophosphate and $10^{-8}$ M dexamethasone. In brief, the samples were washed with pre-warmed PBS and stored at -80°C freezer (New Brunswick Scientific, USA). The samples from all time points were defrosted all at once, lysed by the addition of 1 ml of 0.2% Triton X-100 followed by sonification (Decon Ultrasonics, UK). The ALP activity in the supernatant was measured as the release of p-nitrophenol from p-nitrophenylphosphate substrate over a period of 1 hour (AP 307 kit, Randox Laboratories, UK) by assessing the absorbance at 405 nm wavelength on a ELx808 Ultra Microplate Reader (Bio-Tek Instruments, USA). A standard calibration curve based on the absorbance measurements of known concentrations of p-nitrophenol standard solution were used to compare the values obtained. The total protein content was evaluated with bicinchoninic acid (BCA) protein assay kit (product no: 23227, Pierce, USA) following manufacturer's instructions to normalize the ALP activity levels. This assay applies a reactive solution of bicinchoninic acid

(BCA) and $CuSO_4$. Proteins of the cells reduce $Cu^{2+}$ ions into $Cu^{+1}$ ions, which make a complex with BCA. The absorbance was measured at 562 nm wavelength on a ELx808 Ultra Microplate Reader. A series of bovine serum albumin (BSA) was used as standards. The ALP activity levels were normalized to the total protein content at the end of the experiment (expressed as nmol P-nitrophenol/mg protein/hr).

**Table 2.1-** Summary of FBS concentration and seeding density for different cell types.

| Cell type | FBS concentration (v/v) | Seeding density (cells/cm$^2$) |
|---|---|---|
| hMSCs | 10% | 5000 |
| gMSCs | 15% | 2000 |
| MC3T3-E1 cells | 10% | 2000 |

**Table 2.2-** Summary of culture flasks used and relevant growth medium, Trypsin and PBS volumes.

| Culture flask/Reagent | Surface area of flasks | | | |
|---|---|---|---|---|
| | 25 cm$^2$ | 80 cm$^2$ | 175 cm$^2$ | 225 cm$^2$ |
| Flask manufacturer | Corning | Nunclon | Nunclon | Corning |
| Neck | Angled | Angled | Straight | Angled |
| Cap | Filtered | Filtered | Filtered | Filtered |
| Volume of growth medium | 6 ml | 14 ml | 35 ml | 50 ml |
| Volume of PBS wash | 4 ml | 7 ml | 10 ml | 12 ml |
| Volume of Trypsin used | 1 ml | 3 ml | 5 ml | 5 ml |

## 2.2.2 Passaging Monolayer Cell Cultures

When cells reached approximately 95% confluency, as detected by light microscopy, the growth medium was removed and the cells were washed with pre-warmed phosphate buffered saline (PBS) such that the surface of the culture flask was covered. This was performed to remove any medium (calcium) from the flask since

medium (calcium) neutralizes the action of Trypsin. After the removal of PBS, an appropriate amount of Trypsin-EDTA (Invitrogen, UK) was added to the culture flasks to detach the cells from the flasks such that they can be replated (Table 2.2). The culture flasks were then transferred to the incubator (Heraeus, Germany) for about 10 minutes. Growth medium containing α-MEM + FBS at least twice the volume of Trypsin was added to each culture flask to neutralize the action of Trypsin. The cell suspension was then centrifuged at 300 × g (Centrifuge ALC 4218, Italy). The medium containing Trypsin was then removed and the cell pellet was mixed with a known volume of medium to count the number of cells.

## 2.2.3 Cell Quantification and Determination of Viability by Trypan-blue Exclusion Assay

Trypan-blue staining assay (Sigma-Aldrich, UK) was used along with a haemocytometer (Burker-Turk, Germany) to determine the number of viable cells. Ruptured membrane causes Trypan-blue stain to enter the dead cells thus allowing to distinguish between the dead cells and live transparent cells. For this, well mixed cell suspension (10 μl) was combined with Trypan blue (10 μl) and then mixed thoroughly. Cell-Trypan blue combination (20 μl) was then added to the haemocytometer. Haemocytometer is a glass chamber which holds known volume of fluid ($1 \times 10^{-4}$ ml) having a grid structure. Cells were counted from different grid areas (Figure 2.1) and then incorporated into the following formula to obtain the total number of viable cells:

$$N = M \times D \times 10^4 \times V$$

N = Total number of viable cells in original cell suspension

M = Mean cell number counted using haemocytometer

D = Dilution factor for Trypan-blue

V = Volume of original cell suspension
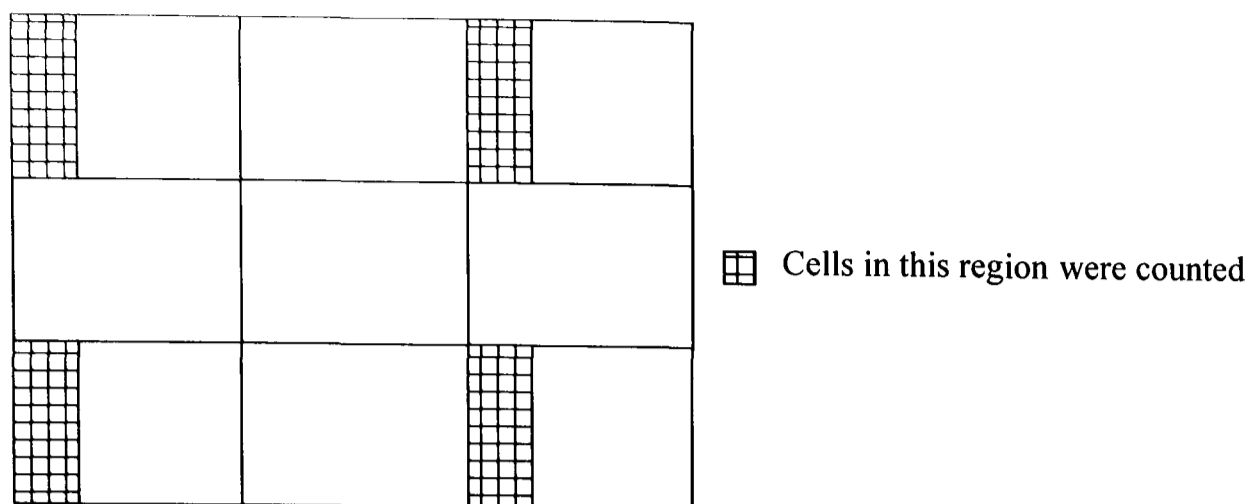
**Figure 2.1-** Schematic presentation of a haemocytometer. Cells were counted from 4 different grid areas shown above.

## 2.2.4 Cryo-preservation and Resuscitation of Cell Suspensions

After obtaining cell suspension from monolayer cultures, the cells were centrifuged at 300 × g and suspended in 1 ml of α-MEM + 20% FBS + 10% Dimethyl sulphoxide (DMSO, Sigma-Aldrich, UK) which is a cryo-protective agent. The number of cells per vial was 2 million for hMSCs and 10 million for gMSCs and MC3T3-E1 cells. Cryo-vials (Nalgene, USA) were used for long term storage of cell suspensions in a liquid nitrogen cryo-bank. Nalgene cryo-freezing container (Nalgene, USA) was used to achieve a cooling rate of approximately -1°C/min in -80°C freezer (New Brunswick scientific, USA) overnight. The samples were then stored in liquid nitrogen.

To defrost the cells, the cryo-preserved samples were thawed in a 37°C waterbath (GFL 1092, Germany). When all the ice was thawed, the cell suspension was mixed with pre-warmed α-MEM + FBS followed by centrifugation at 300 × g. Total number of viable cells was assessed (2.2.3) before seeding the cells in culture flasks (2.2.1).

# CHAPTER III

# BIOCOMPATIBILITY AND GELATION OF CHITOSAN-BASED HYDROGELS

# 3.1 INTRODUCTION

Thermo-sensitive binders have attracted great deal of interest in cell encapsulation, drug delivery, and tissue engineering (Chen and Hoffman, 1995; Jeong and Gutowska, 2002). These polymers can be added to cells, drugs, scaffolds, or a combination thereof and then injected through minimal invasive surgery to the injury site. The mixture can then set *in situ* upon change in temperature. Development of a tissue engineered formulation that can be injected straight into the injury site such as a bone defect is a major advancement in the field of tissue engineering. The injectable formulations offer several advantages compared to the conventional solid implants such as lower cost, easy handling by the physicians, reduced scarring and healing period, less pain to the patient, minimized surgery time, less complications, and exact conformation to irregular defects.

Polymers like poloxamers which are poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) block copolymers have been widely investigated as temperature dependent reversible polymers (Johnston and Miller, 1989; Johnston *et al.*, 1992; Pec *et al.*, 1992). Some reported disadvantage of poloxamer gels is that they have demonstrated rapid erosion (Bhardwaj and Blanchard, 1996). Bhardwaj and Blanchard (1996) have reported that a 25 wt% poloxamer 407 gel is completely dissolved in 4 h at 37°C, which would limit its potential as an injectable system that would have to remain for days, not hours (Bhardwaj and Blanchard, 1996).

Chitosan is an aminopolysaccharide derived from alkaline deacetylation of chitin; a natural polymer material which is present in fungal cell walls and exoskeletons of arthropods like shrimps and crabs (Khor and Lim, 2003; Montembault *et al.*, 2005; Pelletier *et al.*, 1990). Due to its biocompatibility (Hirano and Noishiki, 1985), biodegradation properties (Muzzarelli, 1997), wound healing support (Ueno *et al.*, 2001-a) and bacteriostatic effects (Felt *et al.*, 2000), the natural biopolymer chitosan has been used in the biomedical field for a long time. Chitosan provides a biologically renewable non-toxic polymeric source (Li *et al.*, 2005) offering a hydrophilic surface which encourages proliferation, differentiation, and adhesion of cells (Hutmacher *et al.*, 2001; Suh and Matthew, 2000). In combination with glycerol phosphate (GP –

disodium salt), this cationic polyelectrolyte becomes thermo-sensitive in diluted acids and can undergo gelation around body temperature (Chenite *et al.*, 2000). These properties make chitosan-based materials promising for a variety of applications such as drug delivery, cell encapsulation, and injectable tissue engineering.

When GP is added to chitosan solution, the pH of the solution increases as a result of the neutralizing phosphate groups. Interestingly, chitosan-GP solutions can maintain their liquid state at physiological pH and then gel when heated at 37°C, also it was suggested that hydrogen bonding, hydrophobic and electrostatic interactions are the three forces which are potentially involved in the gelation process (Chenite *et al.*, 2000).

Chitosan/Glycerol phosphate (GP) systems have shown potential to be used for drug delivery and cell encapsulation (Chenite *et al.*, 2000; Ruel-Gariepy *et al.*, 2000). The addition of 2% (w/v) chitosan-5.6% (w/v) GP to chondrocytes has been evaluated before by Chenite *et al.* (2000). Chondrocytes encapsulated within chitosan-GP were capable of maintaining more than 80% cell viability over an extended period of time *in vitro*. Cartilage formation was observed within chondrocytes-chitosan-GP gels after three weeks *in vitro* and *in vivo*. Furthermore, chitosan-GP systems have been also applied *in vivo* (Molinaro *et al.*, 2002). According to an study performed by Molinaro *et al.* (2002), 50 µl of 1.8% (w/w) chitosan with deacetylation degrees in the range of 84-95% mixed with 45% (w/w) GP resulted in an acute inflammatory response when injected in the hindpaw of the rat. Solutions containing chitosan of higher deacetylation degrees, however, cause a lesser inflammatory response.

Interestingly, increasing GP concentration speeds up the gelation process (Cho *et al.*, 2006). As reported by Zan *et al.* (2006), the higher the pH of the chitosan-GP solutions, the faster the gelation (Zan *et al.*, 2006). Higher GP content will result in higher pH. An increase in GP amount causes more chitosan amino groups to combine with GP by ionic interaction, resulting in the weakening of electrostatic repulsive force between amino groups that lead to easier aggregation of polymer chains and therefore faster gelation (Wu *et al.*, 2006). Higher GP concentration in chitosan-GP

systems which results in faster *in situ* gelation is desirable provided that it does not affect cell viability.

## 3.1.1 Aims and Objectives

In this study, sixteen different combinations of 0.5-2% (w/v) chitosan mixed with 5-20% (w/v) GP were prepared. To our knowledge, nothing is known with regard to the biocompatibility and reaction of chitosan-GP to mesenchymal stem cells. Therefore, the aim of this study was to evaluate the cytotoxicity of the different combinations of chitosan-GP gels by monitoring the growth rate of goat bone marrow derived mesenchymal stem cells (gMSCs). In addition, the osmolality and pH of the extracts from different combinations of chitosan-GP and the effect of extracts from 0.5-2% (w/v) chitosan without GP on gMSC proliferation was assessed in order to understand the response of gMSCs towards the extracts. Furthermore, the pH and the gelation times of all chitosan-GP combinations were monitored.

# 3.2 MATERIALS AND METHODS

All chemicals were ordered from Sigma-Aldrich, UK unless otherwise indicated. The experiments were repeated twice and all the measurements were performed in triplicates.

## 3.2.1 Preparation of Chitosan/GP Solutions

Chitosan powder (Bright Moon Seaweed Industrial Co. Limited, Qingdao, China) with an 80% degree of deacetylation (DDA) and a molecular weight (Mw) of 1000 kDa was sterilized by autoclaving at 126°C for 20 minutes. Sterile chitosan powders with quantity of 0.075g and 0.15g were dissolved in 8 ml of 0.1 M hydrochloric acid while 9 ml of 0.1 M hydrochloric acid was used to dissolve 0.225g and 0.3g of sterile chitosan powders. Subsequently, 0.75g, 1.5g, 2.25g, and 3g of β-glycerol phosphate disodium salt (GP) was dissolved in deionized water in order to make the total hydrochloric acid and deionized water volume in the solutions to 15 ml. The GP-deionized water mixtures were then sterilized using a 0.2 μm filter (Triple Red Laboratory, UK). Solutions of chitosan-hydrochloric acid and GP-deionized water were chilled in an ice bath for 15 minutes in order to avoid gelation when mixed together. The ice cold GP-deionized water was then added drop-wise to the ice cold chitosan solution with continuous stirring to form a clear solution. Sixteen different combinations of chitosan-GP were prepared. The final concentration of chitosan in the solutions was 0.5%, 1%, 1.5%, and 2% (w/v) while the concentration of GP was 5%, 10%, 15%, and 20% (w/v) (Table 3.1).

**Table 3.1-** Sixteen different concentrations of chitosan-GP solutions were made. Concentrations are based on percentage weight/volume.

| Chitosan (w/v) + GP (w/v) | | | |
|---|---|---|---|
| 0.5% + 5% | 1.0% + 5% | 1.5% + 5% | 2.0% + 5% |
| 0.5% + 10% | 1.0% + 10% | 1.5% + 10% | 2.0% + 10% |
| 0.5% + 15% | 1.0% + 15% | 1.5% + 15% | 2.0% + 15% |
| 0.5% + 20% | 1.0% + 20% | 1.5% + 20% | 2.0% + 20% |

## 3.2.2 Cytotoxicity Tests

After preparing 16 different combinations of chitosan-GP solutions, 0.5 ml of each solution was placed in sterile 12 well-plates (Falcon, VWR international, UK). The well-plates were then left at 37°C/5% $CO_2$ incubator overnight in order to obtain gel films of more than 1 mm thickness. Cytotoxicity of the gels was evaluated by an extraction test according to ISO10993-5. In brief, the chitosan-GP gels were immersed in 2.5 ml of culture medium composed of α-MEM (Gibco Invitrogen, UK) supplemented with 15% v/v fetal bovine serum (FBS, Gibco Invitrogen, UK), 100 U/ml penicillin/100 μg/ml streptomycin (Sigma Aldrich, UK) and 0.2 mM L-ascorbic acid 2-phosphate (AsAP) (Sigma Aldrich, UK) and placed for 24 hours at 37°C/5% $CO_2$. The ratio of culture medium to gel surface area was set at 1ml/1.25cm². The culture medium added to empty well-plates served as control. Passage 2-3 goat bone marrow derived mesenchymal stem cells (gMSCs) (Kruyt *et al.*, 2004-b) (from iliac crest of adult Dutch milk goats, 2-4 years old, Xpand Biotechnology BV, The Netherlands) were seeded at a density of 40,000 cells/cm² in sterile 12 well-plates and kept in a humid atmosphere at 37°C/5% $CO_2$ for 24 hours until the cells reached 80-90% confluency. Before the addition of 0.5 ml of extraction fluids from different mixtures of chitosan-GP gels and the control medium to 80-90% confluent layer of gMSCs, the extraction fluids were passed through an sterile 0.2 μm filter (Triple Red Laboratory, UK). Cell proliferation was measured using the colourimetric indicator Alamar Blue assay (SeroTec, Oxford, UK). After 48 hours of incubation, extraction medium was removed from the well-plates and gMSCs were washed three times with pre-warmed

phosphate buffered saline solution. Subsequently, 1 ml of a 5% Alamar Blue solution in culture medium (Lawson *et al.*, 2004) was added to each well-plate and the plates were incubated for 3 hours in a humidified atmosphere at 37°C/5% $CO_2$. Fluorescence of 100 µl aliquots of the extracted dye from each sample was determined using a Fluorimeter (FLUOstar Galaxy Fluorimeter, JENCONS-PLS, Germany) at excitation and emission wavelengths of 544 nm and 590 nm respectively. A standard calibration curve based on known numbers of gMSCs reacting with the Alamar Blue solution was employed to quantify the number of cells present in each well (Lawson *et al.*, 2004; Li *et al.*, 2005).

## 3.2.3 Gelation Time of Chitosan-GP

The gelation time of 16 different mixtures of chitosan-GP solutions was measured using an AR 2000 Rheometer (Advanced Rheometer AR 2000, TA Instruments, UK) fitted with a plate-plate tool. The diameter of the plates was 25 mm. The elastic modulus (G') and the viscous modulus (G"), as a function of time at 37°C, were evaluated from the oscillatory measurements at a frequency of 1 Hz. The gelation time was determined at the intersection of G' and G" (Luginbuehl *et al.*, 2005; Tung and Dynes, 1982).

## 3.2.4 pH Measurements

The pH of the ice cold chitosan/GP solutions and extraction medium were measured using an electronic pH meter (Fisherbrand Hydrus 300, Orin Research Incorporation, USA).

## 3.2.5 Osmolality of Extraction Medium from Chitosan-GP

From all sixteen different combinations of chitosan-GP solutions, 0.5 ml of each type was placed in sterile 12 well-plates (VWR international, UK). The well-plates were then left at 37°C/5% $CO_2$ incubator overnight in order to get gel films of more than 1 mm thickness. The chitosan-GP gels were immersed in 2.5 ml of culture medium with the same composition as before and placed for 24 hours at 37°C/5% $CO_2$.

Osmolality of 200 μl aliquots of the filter-sterilized extraction medium from different combinations of chitosan-GP gels was measured by the Advanced Osmometer (Model 3250, Advanced Instruments Inc., USA), in order to assess the concentration of leaching ions in the extraction fluids. Culture medium in empty well-plates was used as control. The osmolality of the extraction media from 0.5-2% (w/v) chitosan solutions without GP with pH neutralized with 1 M NaOH as described earlier were also evaluated in the same manner.

## 3.2.6 Effect of Chitosan Degradation on Proliferation of gMSCs

Chitosan powder with quantities of 0.075g, 0.15g, 0.225g and 0.3g were sterilized through autoclaving at 126°C for 20 minutes before being dissolved in 8 ml (the first two concentrations) and 9 ml (the last two concentrations) of 0.1 M hydrochloric acid. The pH was neutralized to 7.4 with the addition of 1 M NaOH solution followed by the addition of deionized water to make the final solution volume to 15 ml. The final concentration of chitosan in the solution was 0.5%, 1%, 1.5%, and 2% (w/v). Extraction cytotoxicity of the gels was evaluated in exactly the same way as described earlier.

## 3.2.7 Statistical Analysis

Results were analysed using two sample two-tail Student's $t$-test and one-way analysis of variance (ANOVA) followed by a post hoc least significant difference (LSD) test for multiple comparisons. All measurements were performed in triplicate. Data are presented in mean ± standard deviation. A $p$ value of $<0.05$ indicated statistical significance.

# 3.3 RESULTS

## 3.3.1 Gelation of Chitosan-GP Hydrogels

The chitosan-GP is solution at room temperature i.e. 22°C, however the solution sets at 37°C (Figure 3.1 a, b). The colour of the solution changes from transparent to opaque upon gelation at 37°C.

Different chitosan concentrations (0.5-2% (w/v)), with 5-20% (w/v) GP all gel around physiological temperature but in different time periods (Figure 3.2). Chitosan in combination with 15-20% (w/v) GP gels in less than 2 minutes whereas 5-10% (w/v) GP leads to longer gelation times of around 10 minutes. The gelation time decreases with increasing GP concentration. Chitosan concentration also influences the gelation time such that higher chitosan concentrations lead to reduced gelation time when combined with the same quantity of GP. Statistically, within one group, the gelation times of different GP concentrations are significantly different from one another ($p<0.05$). At 15-20% (w/v) GP, chitosan concentrations of 1-2% (w/v) have similar gelation rates. Nevertheless, 0.5 % (w/v) chitosan mixed with 15-20% (w/v) GP illustrates longer gelation time with respect to 1-2% (w/v) chitosan mixed with 15% or 20% (w/v) GP.
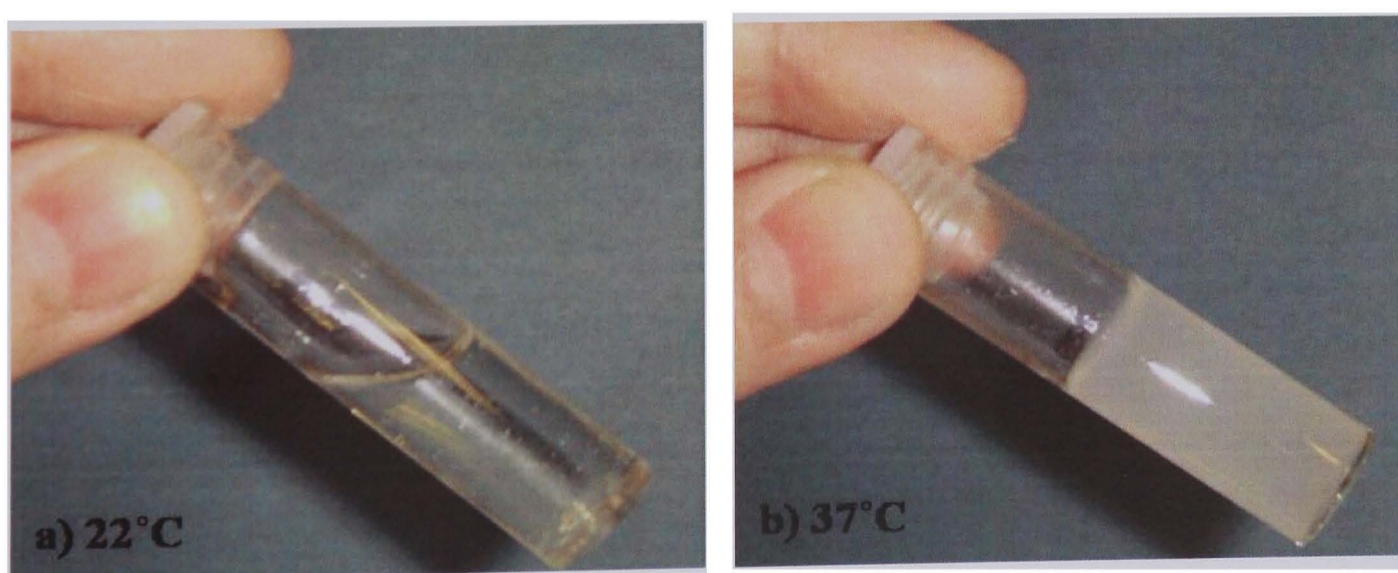


**Figure 3.1-** Chitosan (1% w/v)-GP (20% w/v) solution before gelation (a) and after gelation (b).
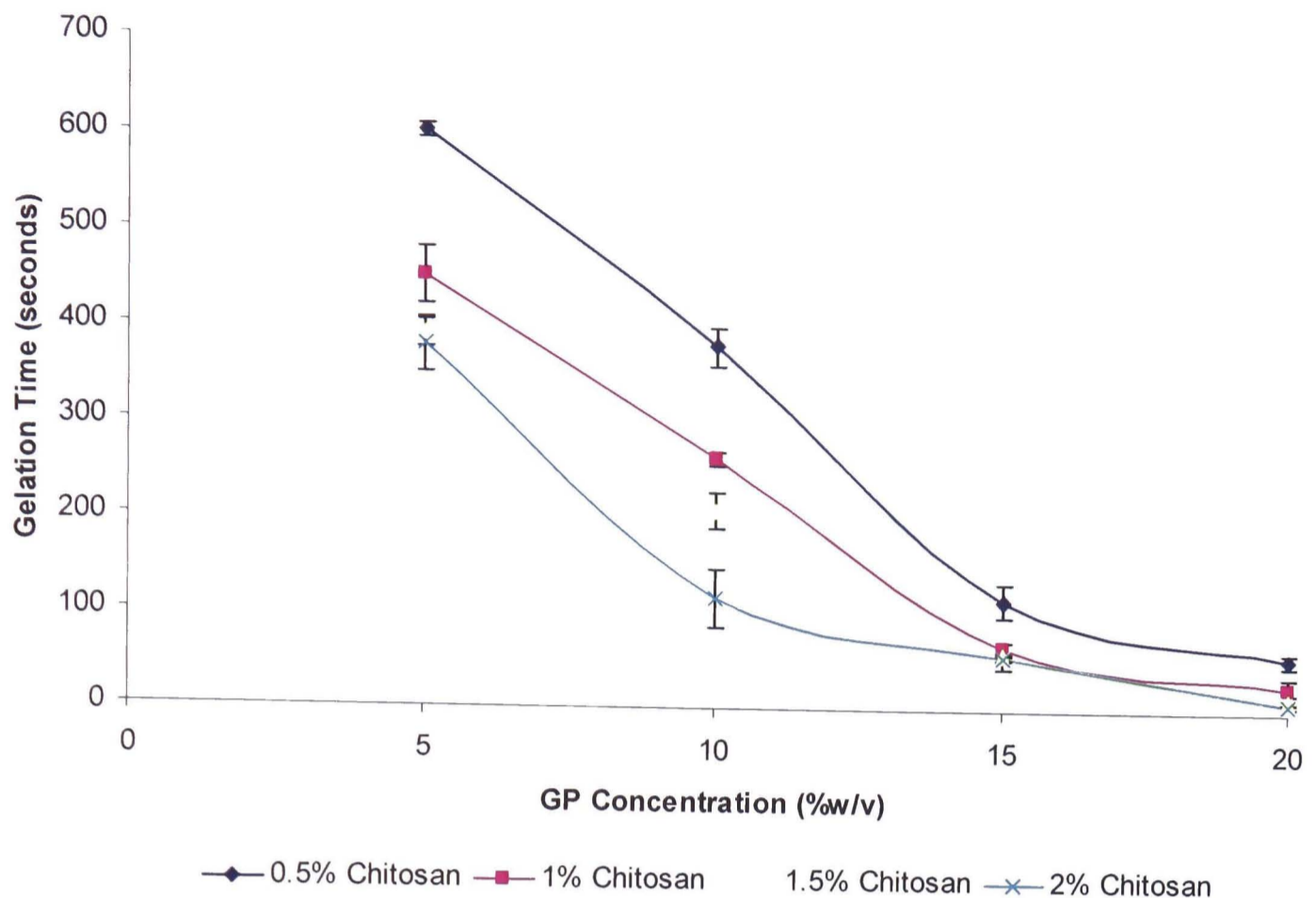
**Figure 3.2-** Gelation time (time needed for the solution to set) in relation to GP concentration.

## 3.3.2 Cytotoxicity of Chitosan-GP Hydrogel Extracts

Extracts of 0.5-2% (w/v) chitosan mixed with 5% GP (w/v) enhance gMSC proliferation compared to the control (Figure 3.3). Extracts of 2% (w/v) chitosan combined with 10% (w/v) GP also result in an improved cell proliferation relative to the control while the rest of the extracts from other chitosan concentrations mixed with 10% (w/v) GP result in reduced cell growth compared to the control. Extracts from all chitosan concentrations mixed with 20% (w/v) GP lead to 100% cell death except for 2% (w/v) chitosan for which a less significant toxic response was observed. Extracts from 0.5-1% (w/v) chitosan mixed with 15% (w/v) GP caused around 93% cell death but that of 1.5-2% (w/v) chitosan produced a lower toxic response. The highest proliferation belongs to extracts of 2% (w/v) chitosan-5% (w/v) GP. This combination results in up to 34% more cells relative to the control. Generally, the cytotoxicity of the extracts from chitosan mixed with more than 10% (w/v) GP increases, while in case of 2% chitosan this phenomenon holds true when the quantity of GP is higher than 15% (w/v) (Figure 3.3). Considering the extracts of 0.5-2% (w/v) chitosan combined with 5% (w/v) GP, the higher the concentration of

chitosan, the higher the percentage cell growth relative to control. Similarly, when combined with the same quantity of GP, higher concentrations of chitosan create lower extract cytotoxicity response.

Taking 1.5% (w/v) chitosan experimental group as an example, the cytotoxic response of the extracts from this chitosan concentration mixed with 5% and 20% (w/v) GP is demonstrated in Figure 3.4. Clearly, an improved cell proliferation for the extracts from 1.5% (w/v) chitosan-5% (w/v) GP is observed when compared to the control (Figure 3.4 a and b respectively). Considerable cell death with a GP concentration of 20% (w/v) is visible where the cells appear to have shrunk (Figure 3.4 c).
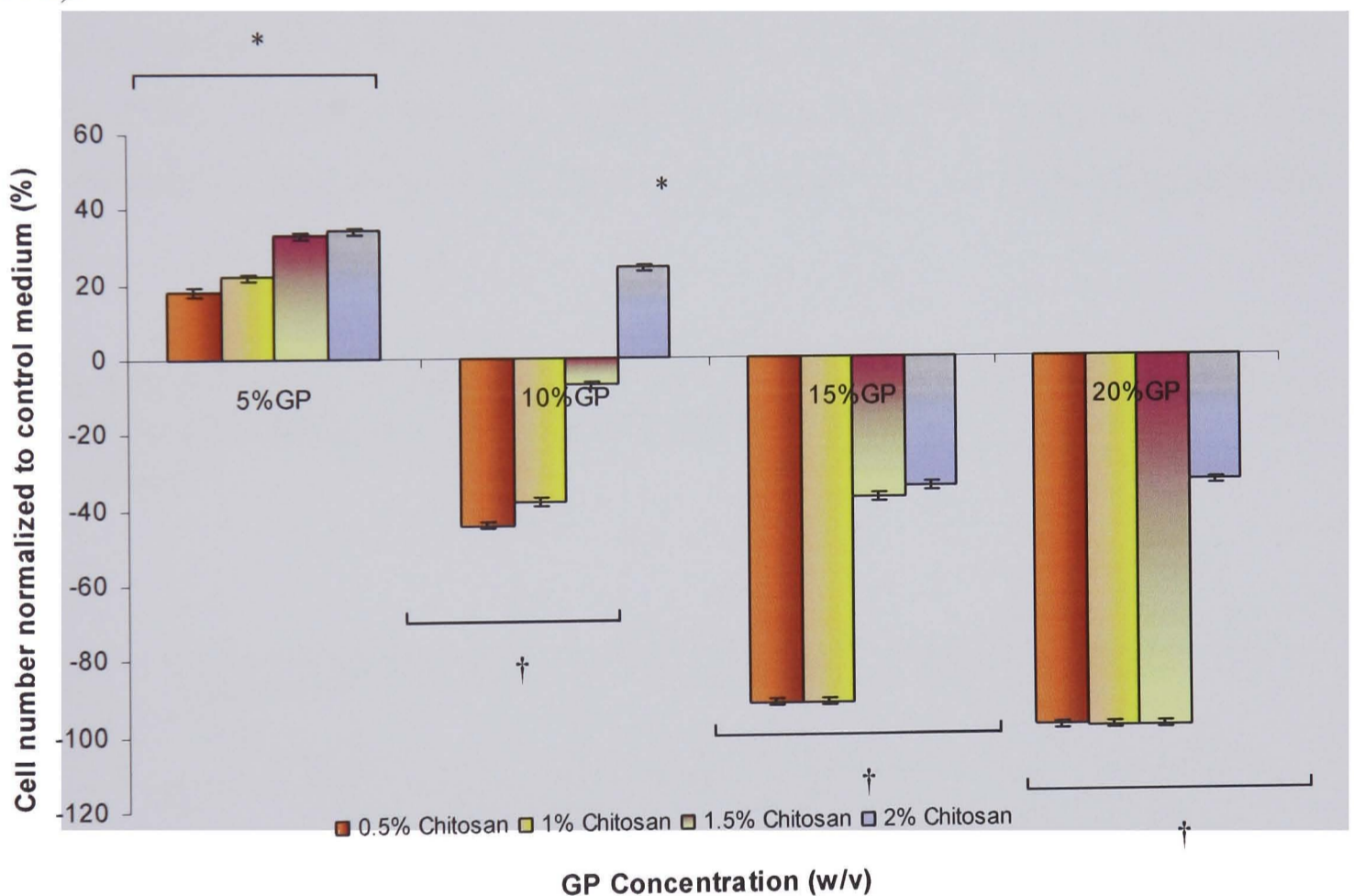


**Figure 3.3-** gMSC proliferation with different chitosan-GP extracts, normalized to control medium. (percentage of cells compared to control). * Statistically higher compared to control ($P<0.05$). † Statistically lower compared to control ($P<0.05$).

**Figure 3.4-** gMSCs after incubation with a) extraction medium from 1.5% (w/v) chitosan-5% (w/v) GP; b) control medium; c) extraction medium from 1.5% (w/v) chitosan-20% (w/v) GP.

## 3.3.3 pH Measurements

The pH of all different combinations of chitosan-GP solutions and the extraction media are around physiological range (Table 3.2). There is a general decrease in pH values of the extraction media compared to the chitosan-GP solutions. For each concentration of chitosan solution, the pH values increase with increasing quantities of GP.

**Table 3.2-** pH values of chitosan-GP solutions (bold) and of chitosan-GP extraction medium (italic). Based on an average of three measurements. The standard deviation is less than 0.1.

| GP (w/v) \ Chitosan (w/v) | 0.5% | | 1.0% | | 1.5% | | 2.0% | |
|---|---|---|---|---|---|---|---|---|
| 5% | 7.1 | *7.1* | 6.9 | *7.0* | 6.9 | *7.2* | 6.9 | *7.1* |
| 10% | 7.4 | *7.0* | 7.3 | *7.2* | 7.1 | *7.2* | 7.3 | *7.0* |
| 15% | 7.6 | *7.1* | 7.4 | *7.1* | 7.3 | *7.1* | 7.4 | *7.0* |
| 20% | 7.7 | *7.1* | 7.6 | *7.0* | 7.5 | *7.1* | 7.6 | *7.1* |

## 3.3.4 Osmolality of Extraction Medium from Chitosan-GP

Increasing the GP concentration of the gels mixed with any of the chitosan concentrations results in a linear increase in osmolality of the extraction media relative to the control as illustrated in Figure 3.5. However, there is not a significant difference in the osmolality of the extraction media obtained from different chitosan concentrations combined with the same GP concentration.

The osmolality of the extracts from 0.5-2% (w/v) chitosan solutions increase steadily with an increase in chitosan concentration compared with the control indicating the contribution of the chitosan degradation products towards increased osmolality (Figure 3.6). Statistically, there is a significant difference between the osmolality of the extracts from 2% (w/v) chitosan and that of 0.5 and 1% (w/v) chitosan ($p<0.05$). Extracts of 1%, 1.5%, and 2% chitosan are statistically significantly higher relative to the control ($p<0.05$).
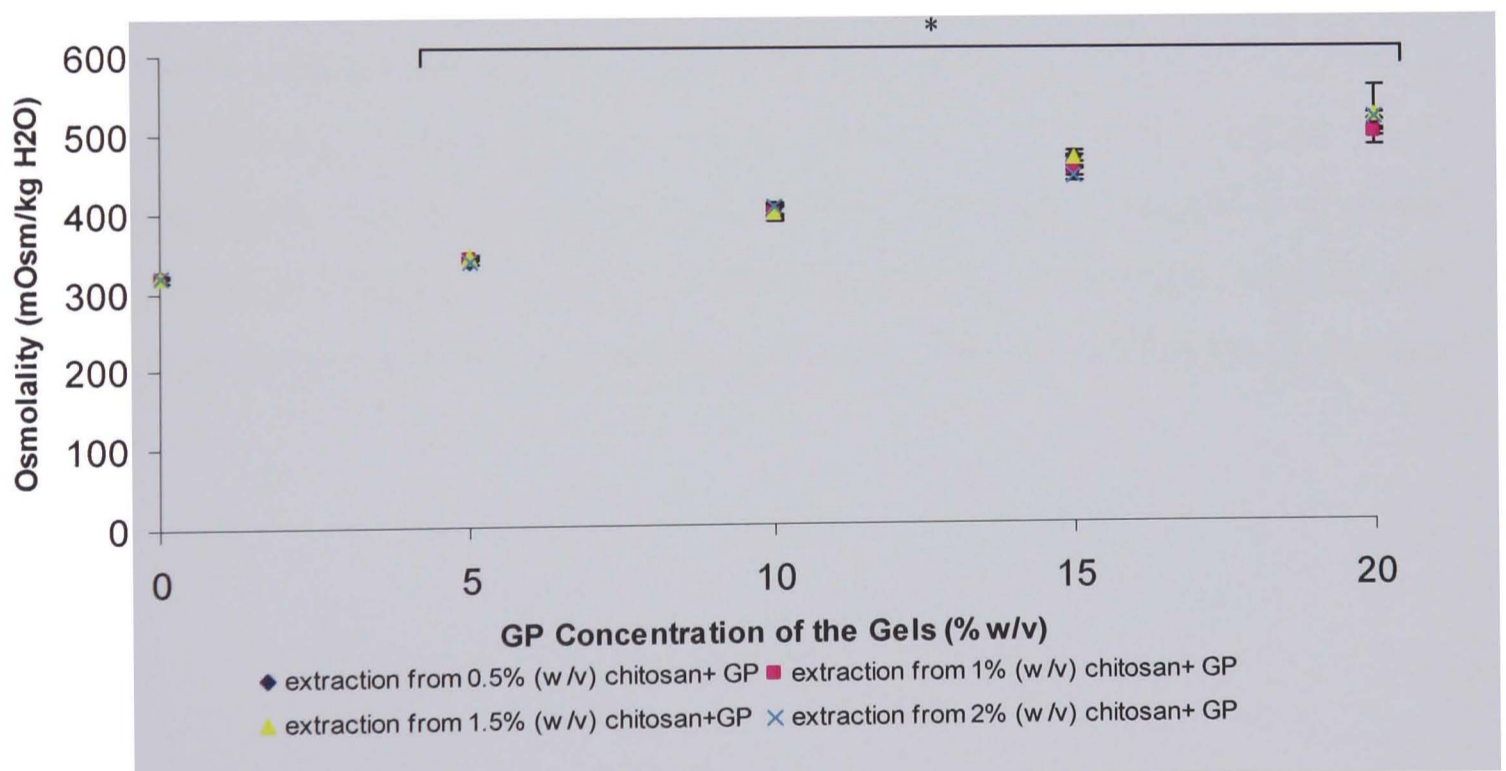


**Figure 3.5-** Osmolality of the extraction media obtained from different combinations of chitosan-GP hydrogels. 0% GP (w/v) refers to the osmolality of the control group.* statistically significantly higher relative to control ($p<0.05$).
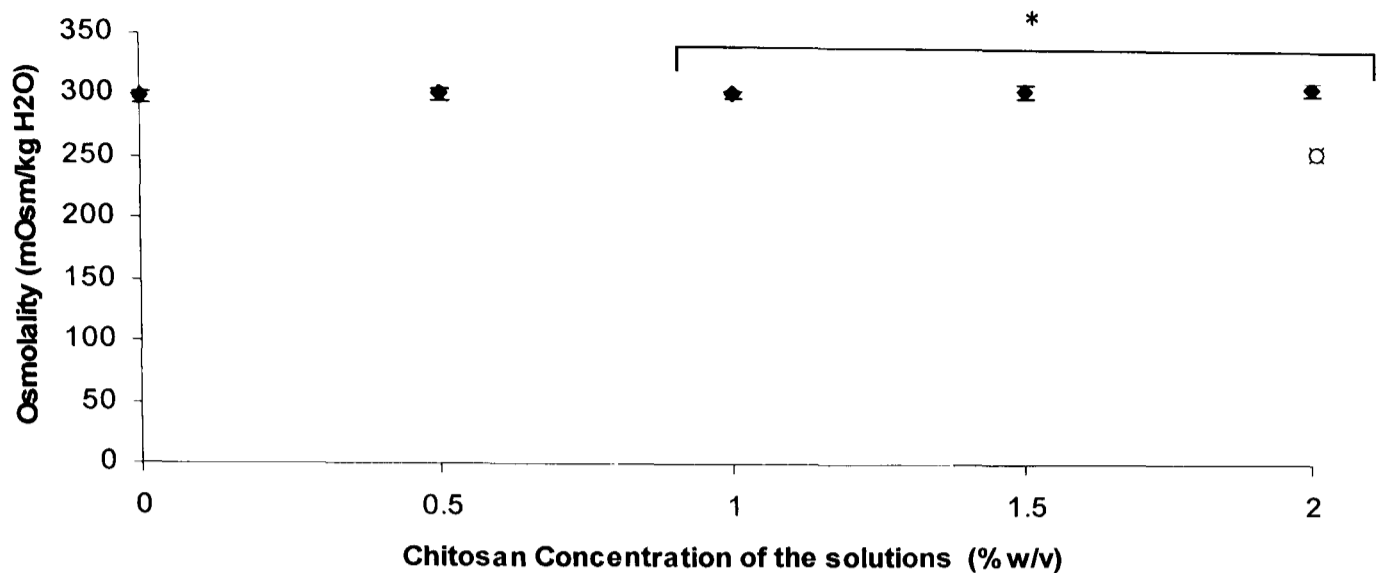
**Figure 3.6-** Osmolality of the extraction media obtained from 0.5-2% (w/v) chitosan solutions without GP. The pH of the chitosan solutions were neutralized with 1 M NaOH. 0% GP (w/v) refers to the osmolality of the control group.* Statistically significantly higher relative to control ($p<0.05$). ¤ Statistically significantly higher compared to extracts of 0.5% and 1% (w/v) chitosan ($p<0.05$).

## 3.3.5 Effect of Chitosan Degradation on Proliferation of gMSCs

Extracts from chitosan solutions without GP demonstrate up to 17% increase in gMSC proliferation relative to the control (Figure 3.7). All the groups show significantly higher cell growth as compared with the control. Cell growth obtained from exposure of extracts of 1.5-2% (w/v) chitosan are statistically significantly higher in comparison with the cell proliferation observed with 0.5-1% (w/v) chitosan ($p<0.05$).
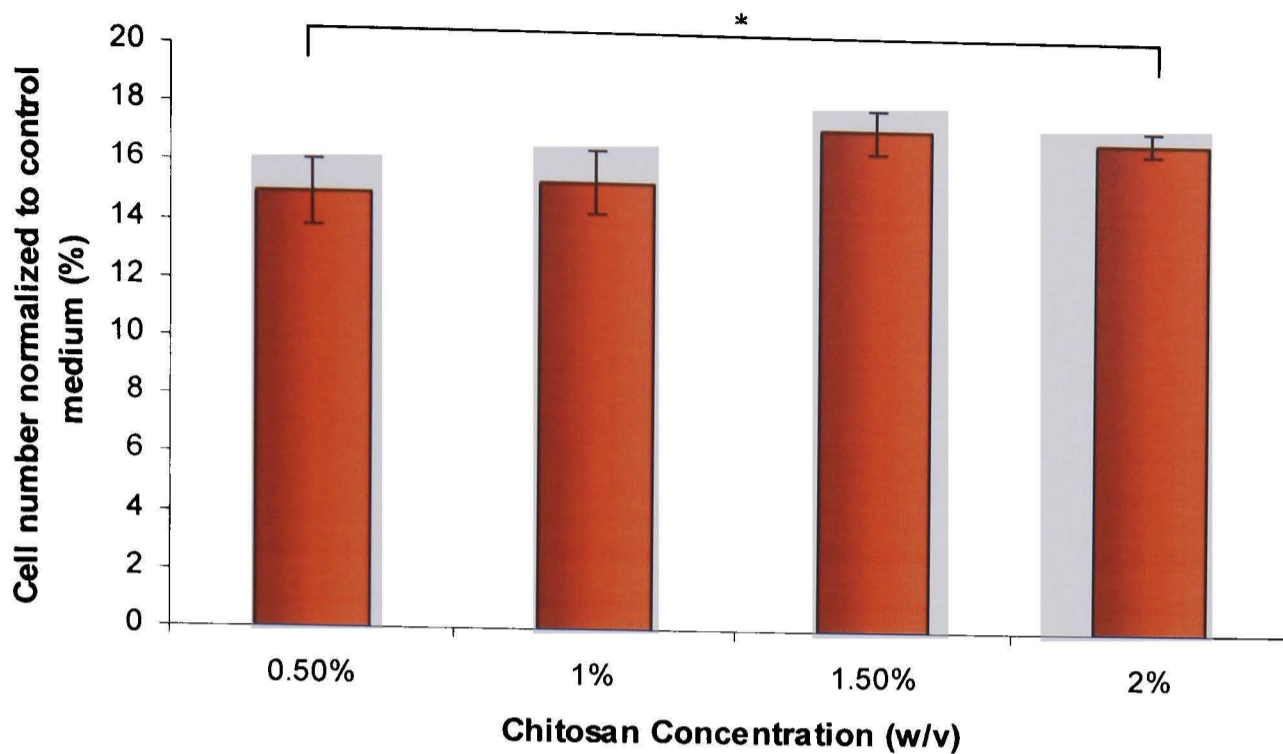
**Figure 3.7-** gMSC proliferation with different chitosan extracts, normalized to control medium. (percentage of cells compared to control). * Statistically significantly higher compared to control ($^*P<0.05$).

## 3.4 DISCUSSION

The results of this study show that extracts from 0.5-2% (w/v) chitosan containing 5% (w/v) GP are not only biocompatible but lead to high gMSCs proliferation relative to the control. Extracts from 0.5-2% (w/v) chitosan mixed with 10-20% (w/v) GP cause reduced cell growth compared to the control except for the extract of 2% (w/v) chitosan combined with 10% (w/v) GP for which an enhanced cell proliferation relative to the control is observed. The chitosan-GP is a solution at room temperature i.e. 22°C, but sets at 37°C (Figure 3.1). As shown in Figure 3.2, the gelation or setting time of the chitosan-GP solutions depend on GP concentration; the higher the concentration of GP, the lower the gelation time. The pH values of 0.5-2% (w/v) chitosan combined with 5-20% (w/v) GP are all in the physiological range. The extraction media also had pH around physiological range. For each concentration of chitosan solution, the pH values increase with increasing quantities of GP. This is due to neutralizing action of the phosphate groups in GP (Cho *et al.*, 2006). There is up to 17% increase in cell proliferation compared to the control when extracts from 0.5-2%

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(w/v) chitosan solutions without GP were used. Furthermore, a linear increase in osmolality of the extracts from chitosan-GP with increasing GP content is observed. The osmolality of the extracts from 0.5-2% (w/v) chitosan solutions raise gradually with an increase in chitosan concentration in comparison with the control.

The gelation time decreases with increasing GP concentration. This is in agreement with the observations performed by Ganji et al. (2007) however, in their paper, the gelation time was measured based on the inverted test tube method which is not as accurate as using rheometery for measuring gelation time (Ganji et al., 2007). When 15-20% (w/v) GP was added to 1-2% (w/v) chitosan, gelation time was reduced to less than 1 minute but lower GP concentrations led to increased gelation time. High GP concentrations of 15% and 20% (w/v) produce similar gelation rates when combined with 1-2% (w/v) GP however, 0.5% (w/v) chitosan combined with 15% and 20% (GP) demonstrates lower gelation rate relative to 1-2% (w/v) chitosan. Chitosan concentration also influences the gelation time such that higher chitosan concentrations lead to lower gelation time when mixed with the same quantity of GP. This trend was also observed by Ganji et al. (2007) whereby 2% (w/v) chitosan resulted in reduced gelation time compared to 1% (w/v) chitosan when combined with similar GP quantities. Increasing GP and chitosan concentration speeds up the gelation process due to an increase of intermolecular interactions and entanglements (Cho et al., 2006). This is in agreement with the findings in this study. Montembault et al. (2005) compared the effect of chitosan concentration on gelation time without any external cross-linking agents in acetic acid-water-propanediol solution and found that higher chitosan concentrations result in lower gelation time, similar to the results presented in this study (Montembault et al., 2005). It was then suggested that more entangled polymer chains are responsible for faster gelation in case of higher polymer concentrations.

Chenite et al. (2001), investigated the effect of gelation temperature as a function of pH and GP concentration on chitosan-GP solution and found that higher GP concentration results in higher pH which in turn leads to lower gelation temperature (Chenite et al., 2001). They suggested that the number of charged ammonium groups

on the chitosan chain is influential in terms of controlling the gelation. A decrease in charge density on chitosan chains causes reduction of interchain electrostatic repulsion therefore leading to smaller amount of thermal energy needed to initiate the gelation. This is again in accordance with the observation in the present study since higher GP concentration and thus higher pH leads to smaller addition of thermal energy required to start the gelation. This would result in faster gelation but chitosan solutions with lower GP concentrations need more thermal energy which can be achieved by increasing the gelation temperature or increasing the gelation time.

According to Chenite *et al.* (2000), when chondrocytes were added to 2% (w/v) chitosan-5.6% (w/v) GP, they were able to maintain more than 80% viability over an extended period of time *in vitro* and give rise to cartilage and proteoglycan formation (Chenite *et al.*, 2000). In this study, the response of gMSCs to extracts from various concentrations of chitosan-GP gels was evaluated as this was not performed in the literature before. Extracts of 0.5-2% (w/v) chitosan mixed with 5% GP (w/v) enhance gMSCs proliferation compared to the cells cultured in control medium. Improved cell proliferation relative to the control ranging from 18-34% was observed with the extracts from 0.5-2% (w/v) chitosan combined with 5% (w/v) GP (Figure 3.3). Chitosan-GP extracts with higher concentrations of chitosan result in more cell proliferation. For instance, in the case of extracts from 10% (w/v) GP mixed with chitosan, there is enhanced cell proliferation with 2% (w/v) chitosan and reduced cell proliferation with 0.5-1.5% (w/v) chitosan. This could potentially be due to two reasons. Firstly, it could be due to higher chitosan concentrations needing more GP to react with the amine group as the electrostatic interactions between the chitosan and the GP is one of the forces involved in gel formation (Chenite *et al.*, 2000). When more GP is reacting with the chitosan, there would therefore be less leaching to the outside medium i.e. extraction medium and thus better cell growth response. Secondly, higher chitosan concentrations lead to higher degradation products and thus stimulating higher cell growth based on the possible theories discussed later on in the discussion section. To investigate this further, the osmolality of the extraction media obtained from different concentrations of chitosan-GP hydrogels was studied as well as the osmolality of the extraction media exposed to chitosan solutions without GP.

The proliferation of the cells exposed to the extracts of chitosan solutions without GP was also examined.

As demonstrated in Figure 3.5, there is not a significant difference in the osmolality of the extraction media obtained from different chitosan concentrations combined with the same GP concentration. This rules out the first theory reason of getting more enhanced cell growth with higher chitosan concentrations. After gelation GP is diffusing out of the physically cross-linked network (Chenite et al., 2001). Ruel-Gariepy et al. (2000) also suggested that there is a significant leaching of excess GP which do not contribute to the physical cross-linking of the chitosan-GP gels (Ruel-Gariepy et al., 2000). But the extra GP is needed for raising the pH and in turn the gelation rate. But according to the osmolality results, even though more GP is needed to react with increased amine groups present in higher chitosan concentrations, the extra GP leaching out to the medium is similar for all conditions. Extracts from chitosan of 0.5-2% (w/v) without GP have shown an increase in gMSC proliferation compared to the control (Figure 3.7). This proves that GP is not responsible for the enhanced growth seen with certain extracts and it is in fact due to the degradation products of chitosan in the extraction media through the possible mechanisms described below. All the groups have shown significant difference relative to the control. Cell growth obtained from exposure of extracts of 1.5-2% (w/v) chitosan are statistically significantly higher in comparison with the cell proliferation observed with 0.5-1% (w/v) chitosan ($p < 0.05$). This result proves the second theory reason of getting more enhanced cell growth with higher chitosan concentrations, indicating that higher chitosan concentrations cause higher degradation products and therefore leading to higher cell proliferation. This is further evidenced by the fact that the osmolality of the extracts from 0.5-2% (w/v) chitosan solutions increase progressively with a raise in chitosan concentration compared with the control indicating more chitosan degradation products with higher chitosan concentrations (Figure 3.6). There is a significant difference between the osmolality of the extracts from 2% (w/v) chitosan and that of 0.5 and 1% (w/v) chitosan ($p < 0.05$). Extraction media from 1%, 1.5%, and 2% chitosan are significantly higher relative to the control ($p < 0.05$).

As shown in Figure 3.5, a linear increase in osmolality of the extracts is seen with higher levels of GP in gels containing 0.5-2% (w/v) chitosan in comparison with the control. This illustrates that there is more GP in the extracts exposed to higher GP content hydrogels. The osmolality for bioencapsulation should be ideally around 270-340 mOsm/kg H2O (Chenite *et al.*, 2002). Extracts from all chitosan concentrations mixed with 5% (w/v) GP all show osmolality less than 340 mOsm/kg H2O. Nevertheless, osmolality of the extraction media from all chitosan concentrations mixed with 10-20% (w/v) GP are more than 400 mOsm/kg H2O and since the physiological osmolality is around 285 mOsm/kg H2O (Davies *et al.*, 2001) this could be a possibility for resultant cell death obtained with extracts from 0.5-2% (w/v) chitosan mixed with 10-20% (w/v) GP causing the cells to shrink as they are trying to reach an isotonic environment with the extraction medium. Figure 3.4c illustrates the shrunk cells which are exposed to the extracts from 1.5% (w/v) chitosan-20% (w/v) GP. However, there is an increase of more than 20% relative to the control in the proliferation of the cells in contact with extracts from 2% (w/v) chitosan-10% (w/v) GP despite the known high osmolality of the extract. The extracts from 2% (w/v) chitosan-10% (w/v) GP possess higher chitosan degradation products compared to the extracts of 0.5-1.5% (w/v) chitosan-10% (w/v) GP. The relatively high polymer degradation in extracts exposed to 2% chitosan overrules the high osmolality resulting in increased cell growth compared to the control.

Chitosan has been observed to cause improved cell proliferation response as Howling *et al.* (2001), investigated the effect of chitin and chitosan on the proliferation of human skin fibroblasts and keratinocytes (Howling *et al.*, 2001). They observed about 50% increase in the proliferation rate of fibroblasts over the control group when they were treated with an initial chitosan concentration of 50 μg/ml in culture media. The mechanism by which chitosan stimulates cell growth is unknown however, chitosan may indirectly enhance fibroblast proliferation through the formation of poly-electrolyte complexes with serum components like heparin (Mori *et al.*, 1997), or potentiating growth factors like platelet derived growth factor (Inui *et al.*, 1995). According to Howling *et al.* (2001) the effect of chitosan on the growth of fibroblasts depended on the presence of serum in the medium. It was shown by Inui *et al.* (1995), that chitosan oligosaccharides interact with a receptor present on the surface of

vascular smooth muscle cells (VSMC). Based on their observations, four or more D-glucosamine units were needed for activation. They also pointed out that chitosan oligosaccharides play a role as progression factor in mitogenesis induced by platelet derived growth factor in vascular smooth muscle cells. Platelet derived growth factor is known as a competence factor that needs the existence of progression factors like insulin in order to induce cell proliferation (Howling *et al.*, 2001). As shown by Inui *et al.* (1995), platelet derived growth factor induced cell proliferation was in fact stimulated by chitosan and insulin therefore chitosan may imitate insulin in terms of action as progression factor.

As reported by Howling *et al.* (2001), chitosan might bind with serum components thus activating them. This suggests that chitosan by binding to serum components may present the components to the cells in an activated form. As mentioned before, only extracts from certain concentrations of chitosan-GP resulted in improved cell growth relative to control despite the presence of chitosan in all groups. This could potentially be due to high GP concentrations leaching to the extraction medium leading to high osmolality and the subsequent toxicity.

All the above information suggests that chitosan may activate the serum components present in goat bone marrow derived mesenchymal stem cell medium used in this study by binding/interacting with the serum components thus the extraction medium was able to produce higher proliferation rates compared to the control.

## 3.5 CONCLUSION

Extracts of all chitosan concentrations mixed with less than 10% GP (w/v) are biocompatible and result in enhanced cell growth compared to the control group. Extracts from other chitosan-GP combinations lead to reduced cell proliferation in comparison with the control.

Chitosan 0.5-2% (w/v) containing 5-20% (w/v) GP all gel around physiological temperature but in different time periods.

The gelation time decreases with increasing GP concentration. Higher chitosan concentrations result in faster gelation time. The pH values of 0.5-2% (w/v) chitosan combined with 5-20% (w/v) GP are all in the physiological range. Extracts from chitosan solutions without GP demonstrate up to 17% increase in gMSC proliferation relative to the control. A linear increase in osmolality of extraction media with increasing GP content was also observable. Chitosan-GP can act as a potential future vehicle for cell encapsulation and injectable tissue engineering applications.

# CHAPTER IV

## CHITOSAN-BASED HYDROGELS: GEL COMPOSITION, PHYSICAL PROPERTIES AND BIOLOGICAL EVALUATION

# 4.1 INTRODUCTION

Presently, autologous bone grafts are regarded as the golden standard therapy to repair and regenerate bone (Yaszemski *et al.*, 1996-a). However, autograft harvesting leads to many complications including donor site morbidity (Banwart *et al.*, 1995) therefore, a substitute technology is needed. Bone tissue engineering which combines osteoprogenitor cells with a suitable scaffold to form bone holds the promise to be a possible solution (Kruyt *et al.*, 2006). This is because osteoprogenitor cells attached to scaffolds and derived from the patient could produce bone with no immunological issues (Johansson and Persson, 2003).

Minimal invasive surgery is today's advancement in the field of medicine. To improve the bone tissue engineering technique even further and to apply the minimal invasive surgery in bone repair and regeneration, a method that reduces intrusion and scar formation is required. Minimally invasive injectable tissue engineered bone is therefore a promising solution since it provides faster healing, easier handling for physicians and less pain.

In previous chapter, cytotoxicity studies on chitosan-glycerol phosphate (GP) extracts were performed using goat bone marrow derived mesenchymal stem cells (gMSCs). The results showed that chitosan-GP extracts were biocompatible at certain concentrations. In addition, gelation of chitosan-GP was carried out which demonstrated that chitosan-GP was able to gel within minutes. Based on the work done by Roughley *et al.* (2006), it was shown that chitosan-GP has a weak structure (Roughley *et al.*, 2006). The weak structure by chitosan-GP is problematic since BCP microparticles are relatively heavy and they will be likely to settle at the bottom of the gel causing inhomogeneity within the chitosan-GP-microparticle structure. Therefore, there is a need to enhance the integrity of the structure in order to prevent this from happening. Addition of hydroxyethyl cellulose (HEC) allows cross-linking of chitosan and thus enhancing gel rigidity (Hoemann *et al.*, 2002; Hoemann *et al.*, 2005; Roughley *et al.*, 2006). With chitosan-GP-HEC hydrogel having a firmer composition, BCP microparticles can be mixed with the gel and remain homogeneously distributed within the gel structure. Therefore, in the current study,

HEC was added to chitosan-GP and the effect of gelation and degradation of chitosan-GP-HEC hydrogels was examined. Additionally, the survival and growth of human bone marrow derived mesenchymal stem cells (hMSCs) encapsulated with chitosan-GP-HEC was investigated together with their osteogenic differentiation since in previous chapter only extract cytotoxicity test was carried out. It should be mentioned that hMSCs were used in this study as opposed to gMSCs in previous chapter since hMSCs are closer to the final application of the injectable tissue engineered bone. Furthermore, in terms of osteogenic differentiation, hMSCs will produce higher ALP activity in comparison with control when in contact with osteogenic medium (Mendes *et al.*, 2004) therefore making it easy to examine osteogenic tendency. This is further explained in the discussion section of this chapter.

To allow injectability, sub-millimetre size particles should be used. Calcium phosphate ceramics are commonly used as scaffold material due to their biocompatibility and osteoconductivity (Hench and Wilson, 1984; Jarcho, 1981). Many researchers demonstrated that the size of ceramic particles affect bone formation *in vivo* with the optimal diameter size lying in the range of 100-300 μm (Fischer *et al.*, 2003; Malard *et al.*, 1999; Mankani *et al.*, 2001). This has been related to the three-dimensional microenvironment produced by the optimal size microparticles such that the macroporosity created (the free space created in between the microparticles) allowed entry of blood vessels, body fluids and nutrients whereas bigger microparticles produced a different macroporosity unsuitable for bone formation (Fischer *et al.*, 2003). The created macroporosity should be such that there is some empty space for blood vessel ingrowth without fibrous tissue development. Mankani *et al.* (2001) and Kruyt *et al.* (2006) have successfully used biphasic calcium phosphate (BCP) microparticles consisting of hydroxyapatite (HA) and tricalcium phosphate (TCP) in bone tissue engineering (Kruyt *et al.*, 2006; Mankani *et al.*, 2001). Therefore, in the study described herein, biphasic calcium phosphate microparticles of 212-300 μm diameter were used. In general, two different approaches have been used regarding injectable bone tissue engineering which consists of osteoprogenitor cells, ceramic microparticles and an injectable binder. The cells can either be seeded onto the scaffolds just prior to implantation or they can be

cultured on the scaffolds for a few days before implantation to allow extracellular matrix formation and differentiation of cells (Kruyt *et al.*, 2004-a; Mankani *et al.*, 2001; Ohgushi and Caplan, 1999; Yoshikawa *et al.*, 2000). With respect to bone formation, cell culturing on the scaffolds has shown to be more beneficial compared to the cells seeded onto scaffolds prior to implantation (Kruyt *et al.*, 2004-b; Kruyt *et al.*, 2006; Mendes, 2002). More specifically, Yoshikawa *et al.* (1996), observed faster bone formation in rat marrow stromal cell/HA scaffolds which were triggered to osteogenic differentiation before subcutaneous implantation in rats compared with fresh bone marrow or undifferentiated mesenchymal stem cells (Yoshikawa *et al.*, 1996). Differentiated mesenchymal stem cells can start building new osteoid immediately after arrival at the defect site. Moreover, bone marrow-derived stromal cells (BMSCs) have been successfully used with ceramic particles to produce osteogenic constructs through the cell culturing approach (Fischer *et al.*, 2003; Mankani *et al.*, 2001). Therefore, the cell culturing approach was used in the present study together with BMSCs.

With regard to the application of minimal invasive surgery to patients, methods to easily apply tissue engineered bone constructs such as injection are required. A binder is therefore needed to offer injectability and fixation. Fixation of the cell-ceramic microparticles at the defect site can be achieved by gelation of the carrier binder at body temperature. As discussed in previous chapter, chitosan has great potential to be used as injectable binder mainly due to its biocompatibility (Hirano and Noishiki, 1985; VandeVord *et al.*, 2002), biodegradability (Muzzarelli, 1997) and thermo-sensitivity in combination with glycerol phosphate (GP) at 37°C (Chenite *et al.*, 2000). The use of chitosan-GP in drug delivery and cell encapsulations has been demonstrated before (Chenite *et al.*, 2000; Cho *et al.*, 2008; Ruel-Gariepy *et al.*, 2000). Cho *et al.* (2008) studied the attachment and proliferation of rat bone marrow mesenchymal stem cells when suspended in 1.8% (w/v) chitosan mixed with 20% GP (w/v) (Cho *et al.*, 2008). Immunohistochemical visualization confirmed that the cells remained viable in the gel for at least 28 days following implantation. As explained in the preceding chapter, chitosan-GP was found to be biocompatible at certain concentrations when used in combination with gMSCs. Nevertheless, it is important to examine the biocompatibility of chitosan-GP in the presence of hMSCs in order to

use this hydrogel system for injectable bone tissue engineering involving hMSCs. Nothing is known with regard to biocompatibility and reaction of chitosan-GP to hMSCs and even though chitosan-GP has been shown to be biocompatible at certain concentrations when used with gMSCs (previous chapter) however, it is more appropriate to confirm the biocompatibility of chitosan-GP when in contact with hMSCs.

Several investigators have applied chitosan-GP-HEC for tissue engineering and cell encapsulation purposes (Hoemann et al., 2007; Hoemann et al., 2005; Roughley et al., 2006). Hoemann et al. (2005) encapsulated chondrocytes in 1.5% (w/v) chitosan-3.3% (w/v) GP-2.5% (w/v) HEC-18 mM glucosamine followed by subcutaneous injection in nude mice for 3, 6 and 9 weeks. The results showed that chitosan gel support chondrocyte viability, phenotype and cartilage matrix accumulation *in vivo*. Moreover, HEK293 cells (a transformed epithelial cell line derived from human embryonic kidney) remained viable after 4 days of culture in 1.6% (w/v) chitosan-2.9% (w/v) GP-0.25% (w/v) HEC (Hoemann et al., 2007). In another study, Roughley et al. (2006) examined the behaviour of intervertebral disc cells consisting of nucleus pulposus and annulus fibrosus encapsulated within 1.5% (w/v) chitosan-2.9% (w/v) GP-0.36% (w/v) HEC for 20 days. Chitosan-GP-HEC hydrogels were able to retain around 70% of the proteoglycan produced by nucleus pulposus cells. Furthermore, nucleus pulposus and annulus fibrosus cells survived and continued to proliferate during the 20 day culture period when cultured with fetal calf serum and TGF-β. For the final application of injectable tissue engineered bone, it is necessary to assess the viability, growth and osteogenic differentiation of hMSCs encapsulated within chitosan-GP-HEC.

Regarding chitosan-GP-HEC gelation, it is known that increasing concentration of HEC leads to enhanced chitosan-GP-HEC gelation (Hoemann et al., 2007; Li and Xu, 2002). According to Hoemann et al. (2007), 1.5% (w/v) chitosan- 2.9% (w/v) GP mixed with HEC concentrations of 0.1%-0.5% (w/v) demonstrated faster gelation with higher HEC concentrations. Furthermore, Li and Xu (2002) examined the influence of HEC content on the gelation rate and elastic strength of chitosan-GP hydrogels. Hydrogels with higher concentrations of HEC had considerably higher

storage modulus (strength) compared to those with lower levels. Also, increasing HEC content enhanced the gelation rate. It was then suggested that higher number of hydrogen bonding in the hydrogel network leads to enhanced hydrogel strength. Higher storage modulus (strength) corresponding to stiffer hydrogels is more suited for applications in bone defects compared to hydrogels with lower storage modulus. As explained in previous chapter, higher GP contents result in faster gelation. Higher concentration of GP and HEC which corresponds to faster gelation is therefore preferable in the chitosan-GP-HEC hydrogel system without compromising cell viability. For injectable bone tissue engineering purposes, the gelation time in the range of a few minutes should be adequate to permit rapid hardening inside the body.

Other than having rapid gelation properties, an injectable binder should be degradable in order to provide space for bone formation. However, injectable binders should not have rapid degradation since they have to remain for days and not hours to offer stability to the cell (tissue)-scaffold combination in the defect site until the cells start producing extracellular matrix and forming bone. Chitosan (2% w/v)-GP (5.6% w/v) was shown to be degradable with about 60% of the dry weight remaining after 72 hours (Wu et al., 2006). Moreover, chitosan-GP/blood implants remained for about 3 weeks after implantation in chondral defects (3.5×4.5 mm) in rabbits (Chevrier et al., 2007). These studies demonstrate that chitosan-GP does not have rapid degradation rate, which is useful for injectable applications of tissue engineered bone. However, addition of HEC could possibly affect the degradation rate of chitosan-GP, which will be investigated in the current study.

## 4.1.1 Aims and Objectives

In previous chapter, chitosan-GP extract cytotoxicity and gelation studies were successfully performed. Nonetheless, due to the weak structure of chitosan-GP (Roughley et al., 2006), it was necessary to enhance the gel structure such that BCP microparticles could be homogenously distributed within the gel network. Therefore, the aim of the present study was to evaluate the optimal chitosan binder composition for injectable tissue engineered bone purposes. For this, HEC was added to chitosan-GP hydrogels. The biocompatibility and pH of various concentrations of chitosan-GP

mixed with and without HEC when in contact with hMSCs were assessed by means of biochemical assays. A temporal assessment of hMSCs survival, growth and osteogenic differentiation when encapsulated with chitosan-GP-HEC using live/dead staining and biochemical assay techniques were performed. Moreover, gelation and degradation properties of different compositions of chitosan-GP-HEC were examined.

# 4.2 MATERIALS AND METHODS

All chemicals were ordered from Sigma-Aldrich, UK unless otherwise indicated. The experiments were repeated three times and all the measurements were performed in triplicates unless otherwise stated.

## 4.2.1 Preparation of Chitosan-GP Solutions

Chitosan powder (Kitomer, Marinard Biotech, Quebec, Canada) with a 94% degree of deacetylation (DDA) and a molecular weight (Mw) of 679 kDa was sterilized by autoclaving at 126°C for 20 minutes. Sterile chitosan powder (0.225g) was dissolved in 9 ml of 0.18 M hydrochloric acid. Subsequently, 0.75g, 1.5g, 2.25g and 3g of β-glycerol phosphate disodium salt (GP) was dissolved in deionized water in order to bring the total hydrochloric acid and deionized water volume in the solutions to 15 ml. A 0.2 μm filter (Triple Red Laboratory, UK) was used to sterilize the GP-deionized water mixtures. Chitosan-hydrochloric acid and GP-deionized water solutions were placed in an ice bath to chill for 15 minutes to avoid gelation when combined together. The ice cold GP-deionized water was then added drop by drop to the ice cold chitosan solution with constant stirring in order to create a clear solution. Four different combinations of chitosan-GP were prepared. The final concentration of chitosan in the solutions was 1.5% (w/v) while the concentration of GP was 5%, 10%, 15%, and 20% (w/v).

## 4.2.2 Cytotoxicity Tests

After preparing 1.5% (w/v) chitosan solution combined with 5-20% (w/v) GP, 0.5 ml of each solution was added to sterile 12 well-plates (Falcon, VWR international, UK). The well-plates were then kept at 37°C/5% $CO_2$ incubator overnight to obtain gel films of more than 1 mm thickness. Cytotoxicity of the gels was assessed by an extraction test based on ISO10993-5. Briefly, the chitosan-GP gels were immersed in 2.5 ml of proliferation culture medium composed of α-MEM (Gibco Invitrogen, UK) supplemented with 10% v/v fetal bovine serum (FBS, Gibco Invitrogen, UK), 100 U/ml penicillin/100 μg/ml streptomycin, 1 ng/ml basic fibroblast growth factor (b-

FGF, SeroTec, Oxford, UK), 2 mM L-glutamine, and 0.2 mM AsAP and kept for 24 hours at 37°C/5% $CO_2$. The ratio of culture medium to gel surface area was 1ml/1.25cm². The culture medium in empty well-plates was used as control. Passage 2 hMSCs (from female, age 36 (iliac crest); male, age 44 (acetabulum); female, age 55 (iliac crest); Xpand Biotechnology BV, The Netherlands) were seeded at a density of 50.000 cells/cm² in sterile 12 well-plates (Falcon, VWR international, UK) and placed in a humid atmosphere at 37°C/5% $CO_2$ for 24 hours until the cells reached 80-90% confluency. The extraction fluids from different mixtures of chitosan-GP gels were sterilized using an sterile 0.2 µm filter (Triple Red Laboratory, UK). This was then followed by the addition of 0.5 ml of such extraction fluids to hMSCs with 80-90% confluency. The control medium was also added to hMSCs in a similar way.

The colourimetric indicator Alamar Blue (SeroTec, Oxford, UK) was employed to evaluate cell proliferation. The extraction medium was removed from the well-plates following 48 hours of incubation, and hMSCs were washed three times with pre-warmed PBS solution. Consequently, 1 ml of a 5% Alamar Blue solution in culture medium (Lawson *et al.*, 2004) was added to each well-plate. The plates were then incubated for 3 hours in a humidified atmosphere at 37°C/5% $CO_2$. Fluorescence of 100 µl aliquots of the extracted dye from each sample was determined using a Fluorimeter (FLUOstar Galaxy Fluorimeter, JENCONS-PLS, Germany) at excitation and emission wavelengths of 544 nm and 590 nm respectively. A standard calibration curve according to known numbers of hMSCs reacting with the Alamar Blue solution was used in order to quantify the number of cells present in each sample (Lawson *et al.*, 2004; Li *et al.*, 2005).

## 4.2.3 Preparation of Chitosan-GP-HEC Solutions

HEC was added to α-MEM (Gibco Invitrogen, UK) at the concentration of 0.0125, 0.025, and 0.05 g/ml (Roughley *et al.*, 2006). The HEC solutions were sterilized using a 0.2 µm filter and then added to the chitosan-GP solution at the ratio of 0.8 ml to 4.8 ml respectively. The final concentration of chitosan and GP in the solutions was 1.5% (w/v) and 15% (w/v) respectively while the concentration of HEC was 0.18%, 0.36%, and 0.72% (w/v).

## 4.2.4 Direct Contact Biocompatibility Tests

Passage 2 hMSCs (from female, age 36 (iliac crest); male, age 44 (acetabulum); female, age 55 (iliac crest); Xpand Biotechnology BV, The Netherlands) were seeded at a density of 20,000 cells/cm$^2$ in sterile 24 well-plates (Falcon, VWR international, UK) after obtaining ethical approval and kept in a humid atmosphere at 37°C/5% CO$_2$ for 24 hours until a subconfluent monolayer is observed. Direct contact biocompatibility tests were performed based on ISO10993-5. A one mm-thick layer of 1.5% (w/v) chitosan-15% (w/v) GP mixed with HEC concentrations of 0%, 0.18% (w/v), 0.36% (w/v), and 0.72% (w/v) was placed on top of a subconfluent layer of hMSCs. hMSCs exposed to tissue culture plastic were used as control. The proliferation culture medium mentioned above was used to feed the cells and was refreshed every two days. Following 1, 3, and 7 days of incubation, the DNA contents of the cells were evaluated using the cyquant assay kit (Molecular Probes, USA) following manufacturer's instructions and a Fluorimeter (FLUOstar Galaxy Fluorimeter, JENCONS-PLS, Germany). To perform the analysis, the samples were washed with pre-warmed PBS and stored at -80°C freezer (New Brunswick Scientific, USA). The samples from all time points were defrosted at once, lysed by the addition of 1 ml of 0.2% Triton X-100 followed by sonification (Decon Ultrasonics, UK). The supernatants were then used to carry out the assay. The DNA solution obtained was spectrometrically analysed using emissions measured at 485 and 520 nm by a Hitachi U- 2000 Spectrophotometer. A standard calibration curve based on the DNA measurements of known numbers of hMSCs was used in order to quantify the number of cells present.

## 4.2.5 pH Measurements

The pH of the ice cold chitosan-GP solutions and extraction medium from the cytotoxicity tests above were assessed using an electronic pH meter (Fisherbrand Hydrus 300, Orin Research Incorporation, USA). Also, the pH of 1.5% (w/v) chitosan-15% (w/v) GP combined with HEC concentrations of 0% (w/v), 0.18% (w/v), 0.36% (w/v) and 0.72% (w/v) were measured.

# 4.2.6 hMSCs Survival, Growth and Osteogenic Differentiation within Chitosan-GP-HEC

## 4.2.6.1 hMSCs Survival and Growth Study

Passage 2 hMSCs (from female, age 36 (iliac crest); male, age 44 (acetabulum); female, age 55 (iliac crest); Xpand Biotechnology BV, The Netherlands) with a seeding density of 30,000 cells/cm$^2$ were added to 212-300 µm BCP microparticles that were sintered at 1150°C and were composed of 80% ± 3 HA and 20% ± 3 β-TCP (Figure 4.1, Siemens D5000 x-ray diffractometer, Germany) with a porosity of 70.8 % (Xpand Biotechnology BV, The Netherlands). The seeding density of 30,000 cells/cm$^2$ was used since after 7 days, 85-95% cell coverage around the BCP microparticles were observed based on previous experiments. The medium used was the proliferation culture medium and was refreshed every two days. hMSCs attached to BCP microparticles were cultured for 7 days in non-tissue culture (cells do not attach) 25 square well-plates (Greiner Bio-one, Germany) at 37°C/5% $CO_2$ incubator after which 1.5% (w/v) chitosan-15% (w/v) GP-0.18% HEC was added such that the final BCP/gel ratio was 20% (w/v). The optimal BCP/gel ratio was 20% (w/v) as it involves the maximum amount of BCP microparticles while the binder is still injectable. The cell/BCP/Chitosan-GP-HEC mixture was then cultured for another 7 days. hMSCs attached to BCP microparticles without chitosan-GP-HEC and hMSCs in contact with tissue culture plastic were the control groups.

The viability of the cells was evaluated before and after gel addition at days 1, 3, 7, 8, 10, and 14 through live/dead viability staining using Calcein AM and Ethidium homodimer-2 (Molecular Probes, USA). Briefly, the samples were washed with pre-warmed PBS. Subsequently, 2.5 µl of both stains were mixed with 1 ml of α-MEM and applied to the samples in 250 µl volume such that the samples were covered by the staining solution. Following this, the samples were kept at 37°C/5% $CO_2$ incubator for 15 minutes after which they were washed three times with pre-warmed α-MEM and viewed under Nikon Eclipse fluorescent microscope (Nikon Eclipse TE200, Japan) equipped with an UltraPix camera (UltraPix, model: 4100V/002, PerkinElmer, UK). In the live/dead viability assay, metabolically active cells, covert

Calcein AM into green fluorescent Calcein by intracellular esterases, whereas Ethidium homodimer enters the dead cells and binds to deoxyribonucleic acid (DNA) through damaged membranes. Viable and dead cells are presented as green and red respectively. The DNA contents of the samples were also measured at days 1, 3, 7, 8, 10, and 14 using the supernatant of the samples obtained as explained before. The cyquant assay kit was employed to measure the DNA contents following manufacturer's instructions and using a Fluorimeter. The DNA solution obtained was spectrometrically analysed using emissions measured at 485 and 520 nm by a Hitachi U- 2000 Spectrophotometer. A standard calibration curve based on the DNA measurements of known numbers of hMSCs was used for quantification of the number of cells present.
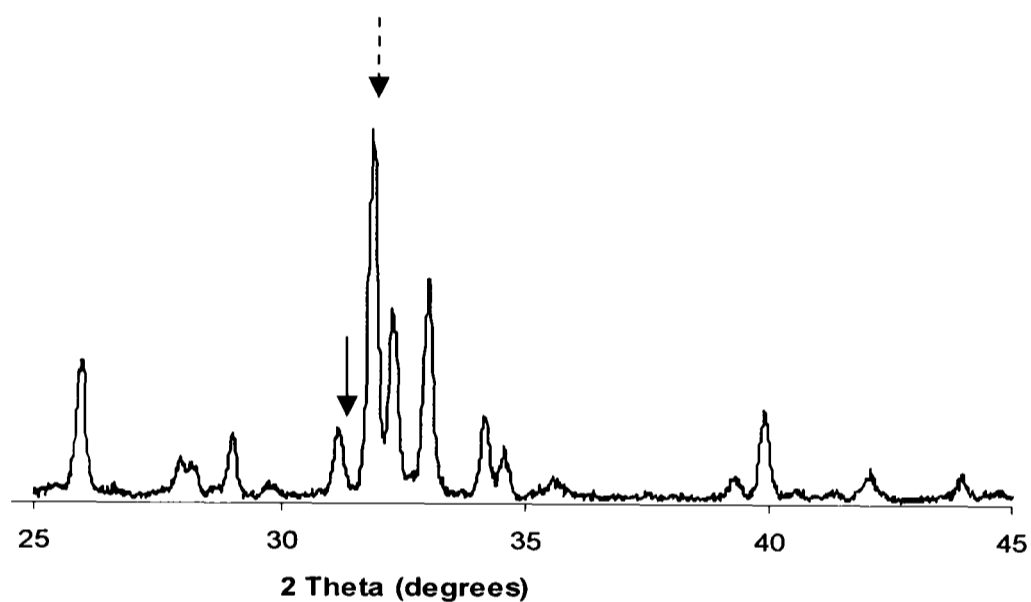


**Figure 4.1-** X-ray diffraction (XRD) spectra of BCP microparticles. Arrow indicates the main β-TCP peak while dashed arrow shows the main HA peak.

## 4.2.6.2 hMSCs Osteogenic Differentiation Study

The alkaline phosphatase (ALP) activity and protein content were measured at days 1, 3, 7, 8, 10, and 14. For this, the cells were cultured in the basic medium which was composed of the proliferation culture medium without b-FGF as well as the osteogenic medium which was composed of the basic medium supplemented with 10 mM β-glycerophosphate and $10^{-8}$ M dexamethasone. In brief, the samples were washed with pre-warmed PBS and stored at -80°C freezer (New Brunswick Scientific, USA). The samples from all time points were defrosted at once, lysed by the addition of 1 ml of 0.2% Triton X-100 followed by sonification (Decon Ultrasonics, UK). The ALP activity in the supernatant was measured as the release of p-nitrophenol from p-nitrophenylphosphate substrate over a period of 1 hour (AP 307

kit, Randox Laboratories, UK) by assessing the absorbance at 405 nm wavelength on a ELx808 Ultra Microplate Reader (Bio-Tek Instruments, USA). A standard calibration curve based on the absorbance measurements of known concentrations of p-nitrophenol standard solution were used to compare the values obtained. The total protein content was evaluated with bicinchoninic acid (BCA) protein assay kit (product no: 23227, Pierce, USA) following manufacturer's instructions to normalize the ALP activity levels. This assay applies a reactive solution of bicinchoninic acid (BCA) and $CuSO_4$. Proteins of the cells reduce $Cu^{2+}$ ions into $Cu^{+1}$ ions, which make a complex with BCA. The absorbance was measured at 562 nm wavelength on a ELx808 Ultra Microplate Reader. A series of bovine serum albumin (BSA) was used as standards. The ALP activity levels were normalized to the total protein content at the end of the experiment (expressed as nmol P-nitrophenol/mg protein/hr). The sample size for all the measurements in this experiment was 6.

## 4.2.7 Gelation Time and Storage Modulus of Chitosan-GP-HEC

The gelation time of 1.5% (w/v) chitosan-15% (w/v) GP combined with HEC concentrations of 0% (w/v), 0.18% (w/v), 0.36% (w/v) and 0.72% (w/v) mixed with and without 20% (w/v) BCP microparticles was examined using an AR 2000 Rheometer (Advanced Rheometer AR 2000, TA Instruments, UK) fitted with a plate-plate tool. The diameter of the plates was 25 mm. The elastic (G′) and viscous modulus (G″) as a function of time at 37°C, were measured from the oscillatory measurements at a frequency of 1 Hz. The intersection of G′ and G″ was known as the gelation time (Luginbuehl *et al.*, 2005; Tung and Dynes, 1982). In addition, the elastic modulus (G′) at complete gelation was assessed for all the above mentioned gel compositions. Complete gelation is reached when G′ levels off.

## 4.2.8 Chitosan-GP-HEC Degradation

Chitosan 1.5% (w/v)-15% (w/v) GP solutions combined with 0% (w/v), 0.18% (w/v), 0.36% (w/v) and 0.72% (w/v) HEC were prepared and kept at 37°C incubator to gel overnight. Subsequently, 10 ml of phosphate buffered saline (PBS) with or without 10 mg/L of lysozyme (50,000 U/mg form chicken egg white) were added to 2 ml of

the gels. This concentration of lysozyme was used to imitate lysozyme levels in plasma which is around 4-13 mg/L (Henry, 1991). The degradation behaviour of the gels were examined in terms of wet and dry weight measurements at days 1, 7, 14, 21, 28, 35, and 42 and compared to day 0 measurements and expressed as % dry/wet weight remaining. For dry weight measurements, the samples were washed three times with distilled water, snap freezed in liquid nitrogen at -196°C for 2 minutes and then dried using a freeze dryer (Biopharma process systems, UK) for 24 hours. For wet weight measurements, the gels were washed with distilled water and gently blotted. The PBS with or without lysozyme was changed weekly until the end of the experiment. The samples from 1.5% (w/v) chitosan-15% (w/v) GP mixed with 0% and 0.18% (w/v) HEC following exposure to PBS with or without lysozyme were prepared for scanning electron microscopy after 1, 7, 14, 21, 28, 35 and 42 days. The samples prepared for dry weight measurements were coated with a layer of gold (Agar Auto Sputter Coater, UK) and placed into the JEOL JSM 6300 Scanning Electron Microscope (JEOL, JSM-6300F, Japan).

## 4.2.9 Statistical Analysis

Results were analyzed using two sample two-tail Student's $t$-test and one-way analysis of variance (ANOVA) followed by a post hoc least significant difference (LSD) test for multiple comparisons. All measurements were performed in triplicate unless otherwise stated. Data are presented in mean ± standard deviation. A $p$ value of <0.05 indicated statistical significance.

# 4.3 RESULTS

## 4.3.1 Cytotoxicity Tests

Extracts of 1.5% (w/v) chitosan combined with 5-15% (w/v) GP improve hMSCs proliferation compared to the control (Figure 4.2). They can therefore be regarded as not only biocompatible but also a stimulatory material for hMSCs proliferation. There are however no significant differences in terms of hMSCs percentage normalized to the control medium when exposed to extracts of 1.5% (w/v) chitosan in combination

with 5-15% (w/v) GP. Chitosan concentration of 1.5% (w/v) mixed with 15% (w/v) GP was chosen as the preferred composition for future studies since based on the results from previous chapter, higher GP concentrations demonstrate higher gelation rates.
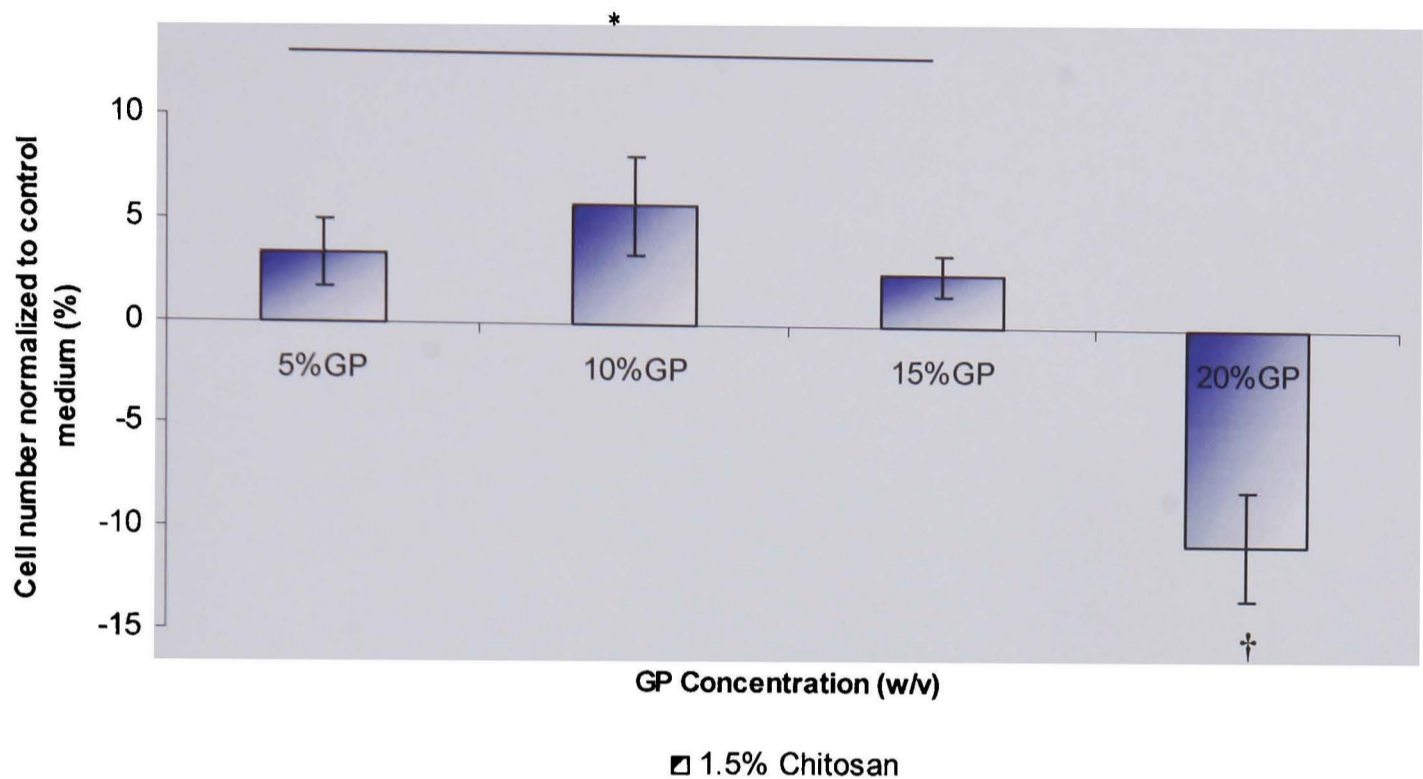


**Figure 4.2-** hMSCs proliferation after 48 h of exposure to extraction medium from 1.5% (w/v) chitosan mixed with 5-20% (w/v) GP, normalized to control medium. (Percentage of cells compared to control). * Statistically higher compared to control ($P<0.05$). † Statistically lower compared to control ($P<0.05$).

## 4.3.2 Direct Contact Biocompatibility Tests

hMSCs were exposed to various gel compositions for a period of 7 days. Initially at day 1, there are no significant differences between different groups (Figure 4.3). hMSCs continue to grow between days 1 and 3 in all the groups and the differences in cell number are significantly different. Nevertheless, after 3 days the number of cells in 1.5% (w/v) chitosan-15% (w/v) GP-0.72% (w/v) HEC group is significantly lower relative to all the other groups. After 7 days of exposure, the cells in the control group as well as the groups containing 1.5% (w/v) chitosan-15% (w/v) GP combined with 0 and 0.18% (w/v) HEC have grown significantly in number compared to day 3 and there are no significant differences between any of these groups at day 7 which demonstrates that the mentioned concentrations of chitosan-GP-HEC are biocompatible relative to the control. Interestingly, cells exposed to higher

concentrations of HEC are not growing as well as the control and the groups containing 0 and 0.18% (w/v) HEC as observed by the significantly lower number of cells in these groups (Figure 4.3). Chitosan concentration of 1.5% (w/v) mixed with 15% (w/v) GP and 0.18% (w/v) HEC was selected as the ideal composition for hMSCs survival, growth and osteogenic differentiation studies since it is biocompatible.
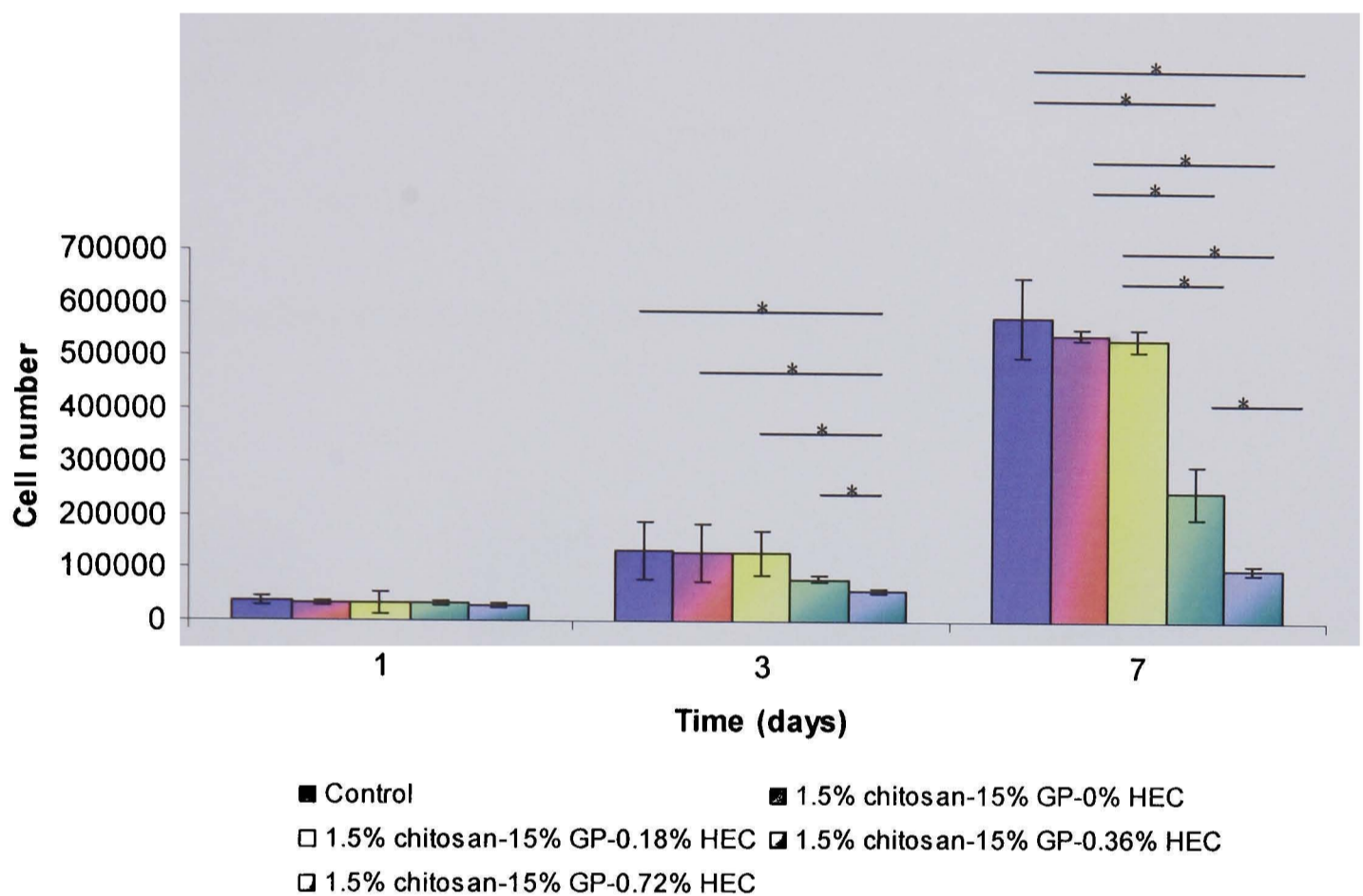


**Figure 4.3-** hMSCs in direct contact with different gel compositions. * Statistically significantly different (*P*<0.05).

## 4.3.3 pH Measurements

The pH of all the chitosan-GP solutions and the extraction media are around the physiological levels (Figure 4.4). For the chitosan concentration of 1.5% (w/v) used in this study, the pH values increase with increasing quantities of GP. Statistically, there are no significant differences between the pH values of the chitosan-GP solutions and that of the extraction media for all four groups (*P*<0.05). Also, the pH values of 1.5% (w/v) chitosan-15% (w/v) GP combined with HEC concentrations of 0% (w/v), 0.18% (w/v), 0.36% (w/v) and 0.72% (w/v) are around the physiological range (Figure 4.5).
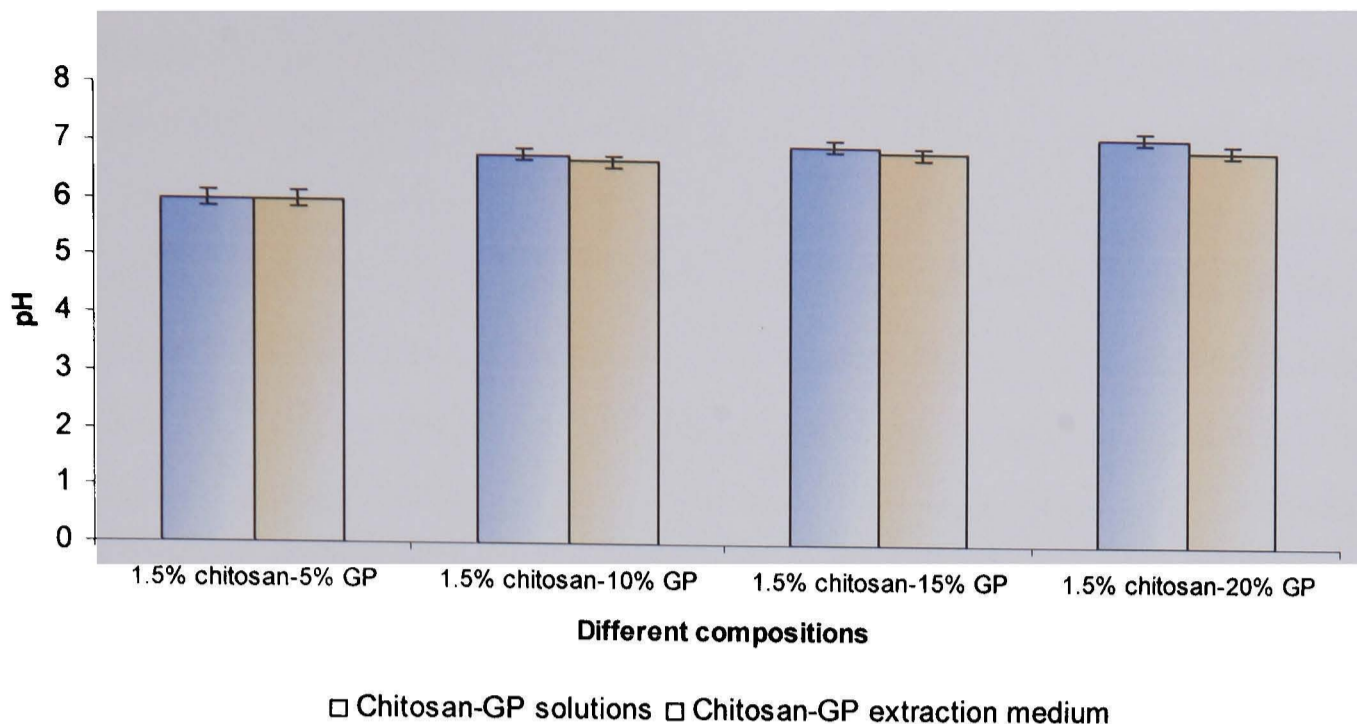
**Figure 4.4-** pH values of chitosan-GP solutions and extraction medium.
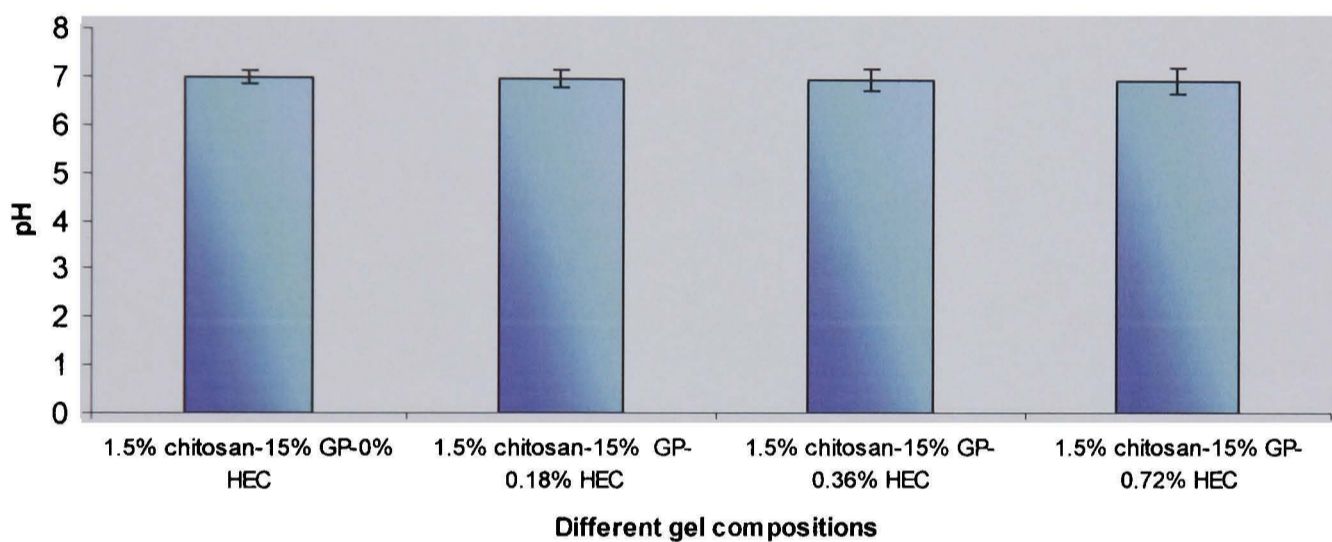


**Figure 4.5-** pH values of different gel compositions.

## 4.3.4 hMSCs Survival, Growth and Osteogenic Differentiation within Chitosan-GP-HEC

### 4.3.4.1 hMSCs Survival Study

Viable and dead cells are presented as green and red respectively when the cells are stained with Calcein AM and Ethidium homodimer-2. During the first 7 days of culture, the number of viable cells increases in both tissue culture plastic control as well as the BCP microparticles groups (Figure 4.6). There are no dead cells in any of

these groups in the first 7 days. After 7 days of culture, the BCP microparticles are almost covered with cells and a confluent layer of cells is also observed in tissue culture plastic control (Figure 4.6). Following 7 days of incubation, 1.5% (w/v) chitosan-15% (w/v) GP-0.18% HEC is added to hMSCs attached to BCP microparticles. hMSCs attached to BCP microparticles exposed to 1.5% chitosan-15% GP-0.18% HEC proliferate and remain viable after 7 days (Figure 4.7). There is only limited number of dead cells observed when the cells are surrounded by chitosan-GP-HEC. Similarly, cells attached to BCP microparticles without chitosan-GP-HEC continue to grow and expand with only a few dead cells as shown in Figure 4.7.
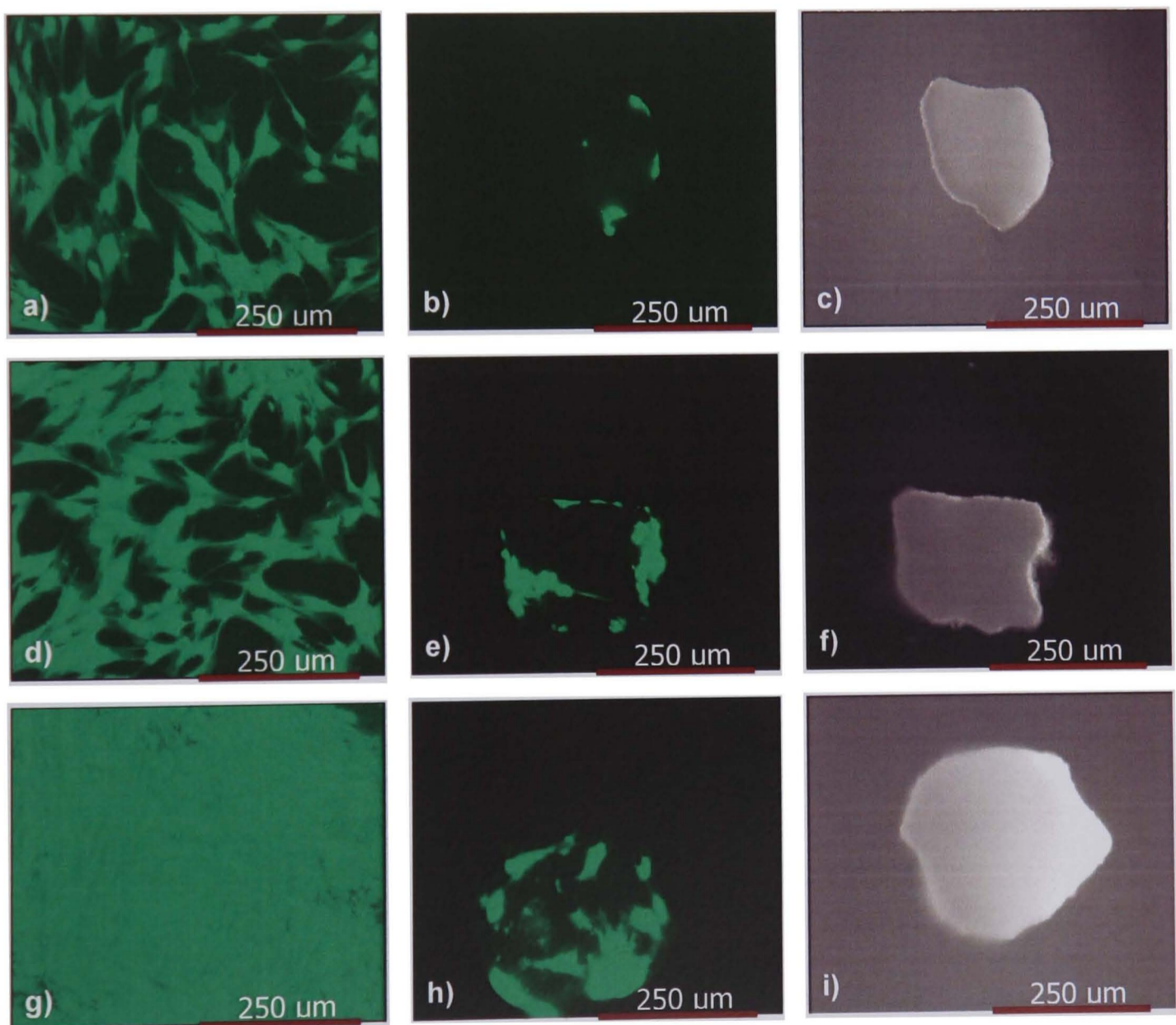


**Figure 4.6-** hMSCs on tissue culture plastic at a) day 1, d) day 3, g) day 7; hMSCs attached to BCP microparticles at b) day 1, e) day 3, h) day 7; BCP microparticle only (i.e. non-fluorescent image) at c) day 1, f) day 3, i) day 7.
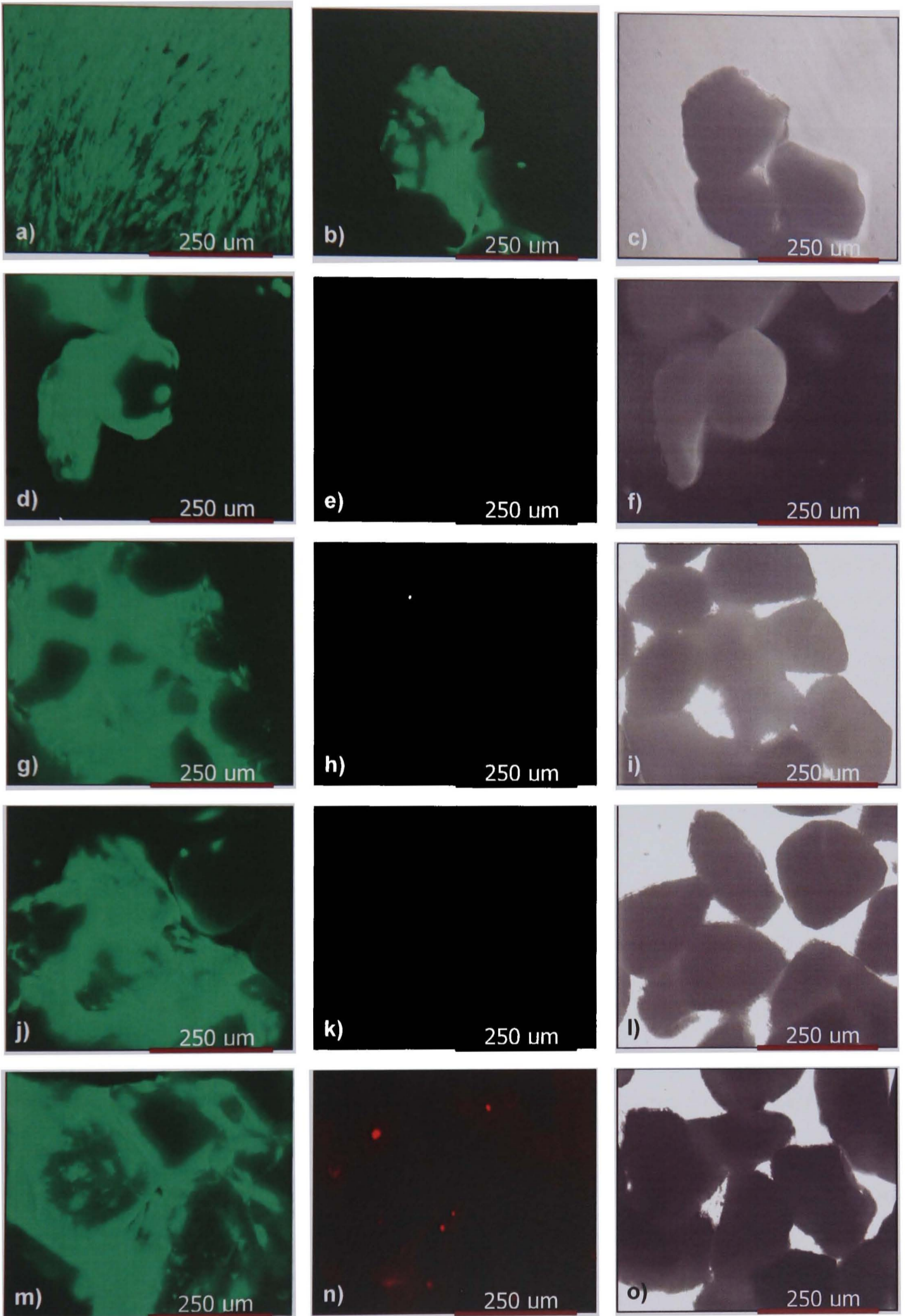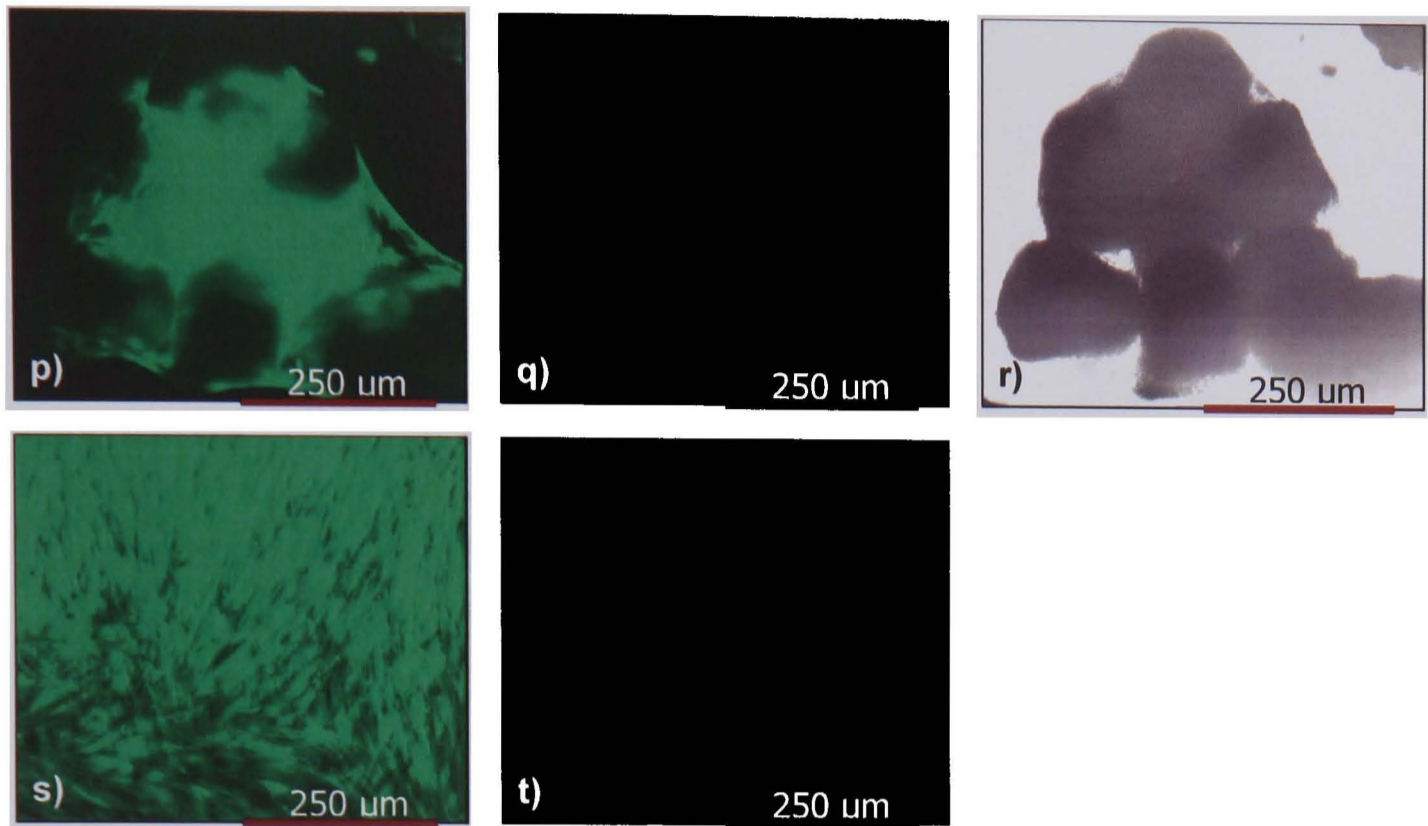
**Figure 4.7-** hMSCs on tissue culture plastic at a) day 8, s) day 14; dead hMSCs on tissue culture plastic at t) day 14; hMSCs attached to BCP microparticles at b) day 8, g) day 10, m) day 14; dead hMSCs attached to BCP microparticles at h) day 10, n) day 14; BCP microparticles only for the hMSCs combined with BCP groups (i.e. non-fluorescent image) at c) day 8, i) day 10, o) day 14; hMSCs attached to BCP microparticles and surrounded by chitosan-GP-HEC at d) day 8, j) day 10, p) day 14; dead hMSCs attached to BCP microparticles and surrounded by chitosan-GP-HEC at e) day 8, k) day 10, q) day 14; BCP microparticles only for the hMSCs combined with BCP and chitosan-GP-HEC groups (i.e. non-fluorescent image) at f) day 8, l) day 10, r) day 14.

## 4.3.4.2 hMSCs Growth Study

The number of cells exposed to different conditions was quantified (Figure 4.8). The cells grow in tissue culture plastic and BCP microparticles groups mixed with or without chitosan-GP-HEC. After day 7, the cells in all groups were proliferating at slower rate since they were reaching confluency. There are significant differences between cells attached to tissue culture plastic and BCP microparticles at days 7 and 8. At days 8, 10, and 14, there was no significant differences between the number of hMSCs attached to BCP in the presence or absence of chitosan-GP-HEC (Figure 4.8). At days 10 and 14, the cells continue to proliferate but there are no significant differences between any of the groups indicating that hMSCs in contact with chitosan-GP-HEC are viable and proliferating at rates similar to both control groups.

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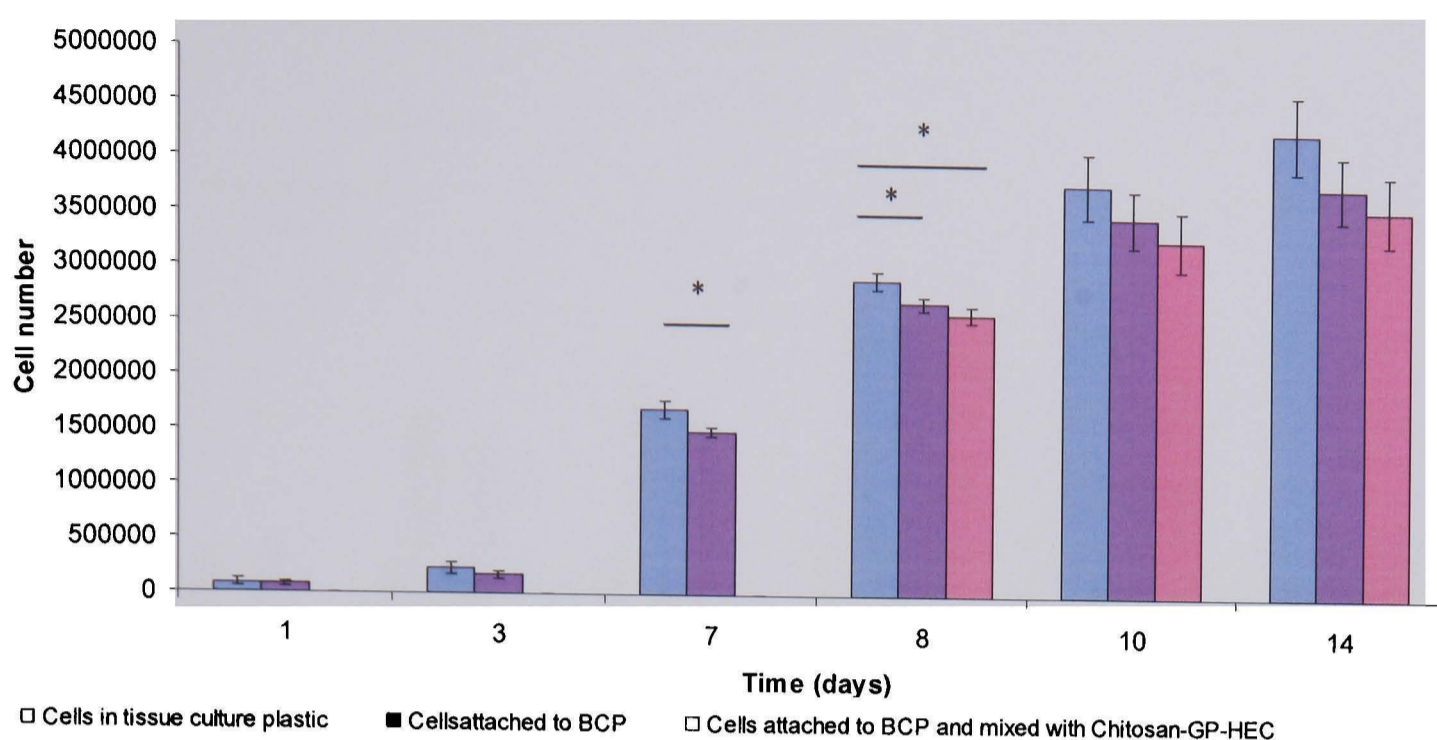
**Figure 4.8-** hMSCs proliferation in different groups. * Statistically significantly different ($P<0.05$).

## 4.3.4.3 hMSCs Osteogenic Differentiation Study

With respect to the osteogenic differentiation of hMSCs attached to tissue culture plastic and BCP microparticles, addition of the osteogenic medium results in significantly higher ALP activity compared to the basic medium for all different time points as shown in Figure 4.9 ($P<0.05$). Similarly, hMSCs attached to BCP microparticles surrounded by chitosan-GP-HEC when exposed to osteogenic medium produce significantly higher ALP activity relative to the basic medium at all time points (Figure 4.9). More importantly, the presence of chitosan-GP-HEC does not negatively influence osteogenic differentiation of hMSCs as the ALP activities are similar to when the cells are attached to tissue culture plastic and BCP microparticles. There are no significant differences between the ALP activities of the cells in any of the three different groups at each individual time point after the first 7 days of culture (i.e. days 8, 10, and 14) whether the cells are in contact with basic or osteogenic medium. When the cells are in contact with the osteogenic medium, the ALP activity reaches its peak between days 8 and 10 followed by a reduction at day 14. Furthermore, addition of chitosan-GP-HEC does not change this pattern of ALP activity over time.
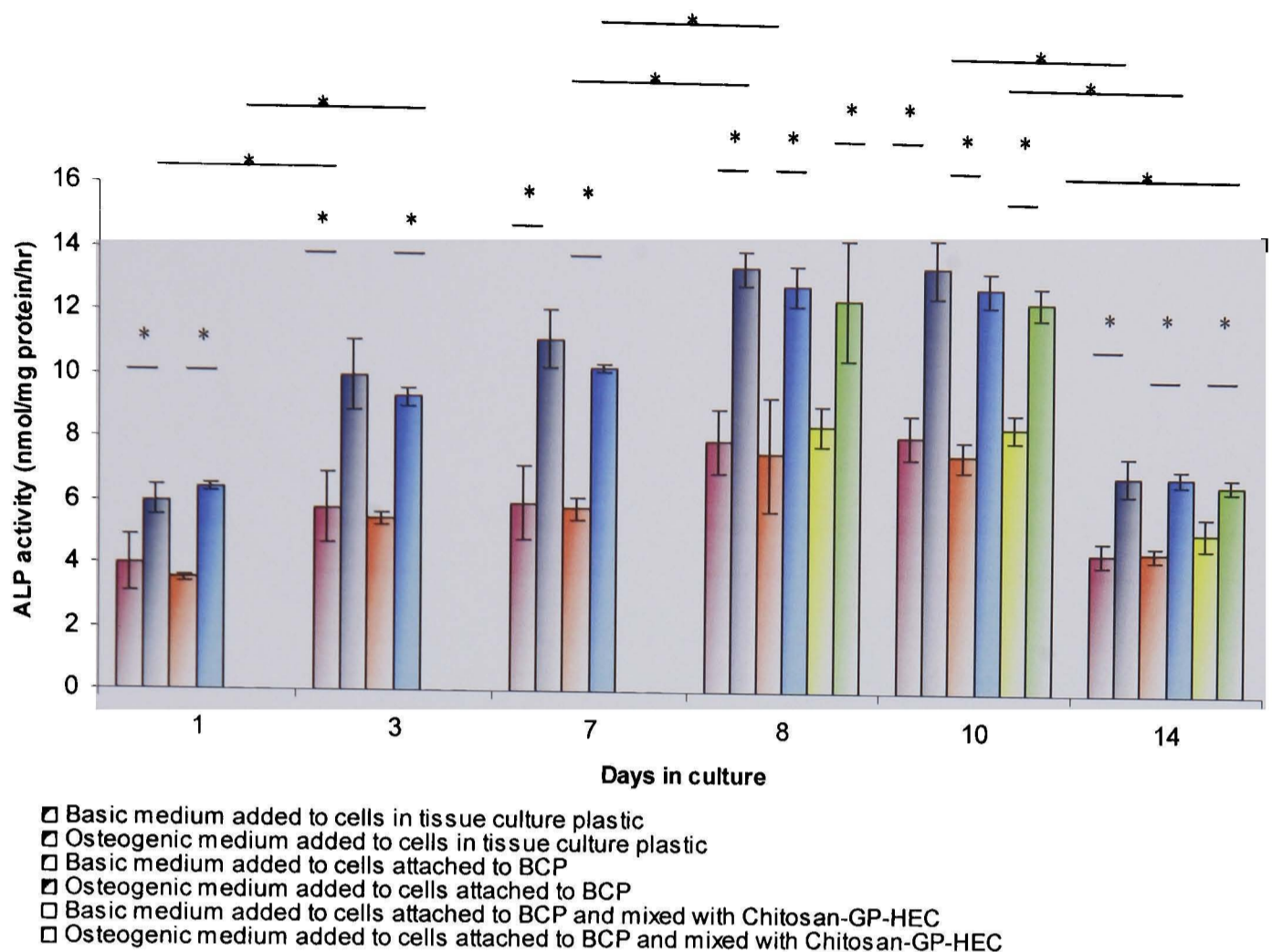
**Figure 4.9-** Osteogenic differentiation of hMSCs assessed by the measurement of ALP activity per protein content per hour. * Statistically significantly different (*P*<0.05).

## 4.3.5 Gelation Time and Storage Modulus of Chitosan-GP-HEC

Chitosan concentration of 1.5% (w/v) combined with 15% (w/v) GP and 0-0.72% (w/v) HEC all gel at 37°C however at different time points (Figure 4.10). The gelation time decreases significantly with increasing concentrations of HEC. Nevertheless, there is not much difference in the gelation rate when the HEC concentration is increased above 0.36% (w/v). Furthermore, in the presence of BCP microparticles, the gelation is even more enhanced however, for HEC concentration of 0.36% (w/v) and 0.72% (w/v) the gelation time is the same in the absence or presence of BCP microparticles. For chitosan-GP without HEC, there is no significant different in the gelation rate in the absence or presence of BCP.

Additionally, the storage modulus of different gel compositions at complete gelation was measured (Figure 4.11). Once more, the storage modulus increases with

increasing HEC concentrations. The storage modulus increases significantly when BCP microparticles are mixed with the chitosan-GP-HEC hydrogels indicating that the hydrogel is becoming stiffer.
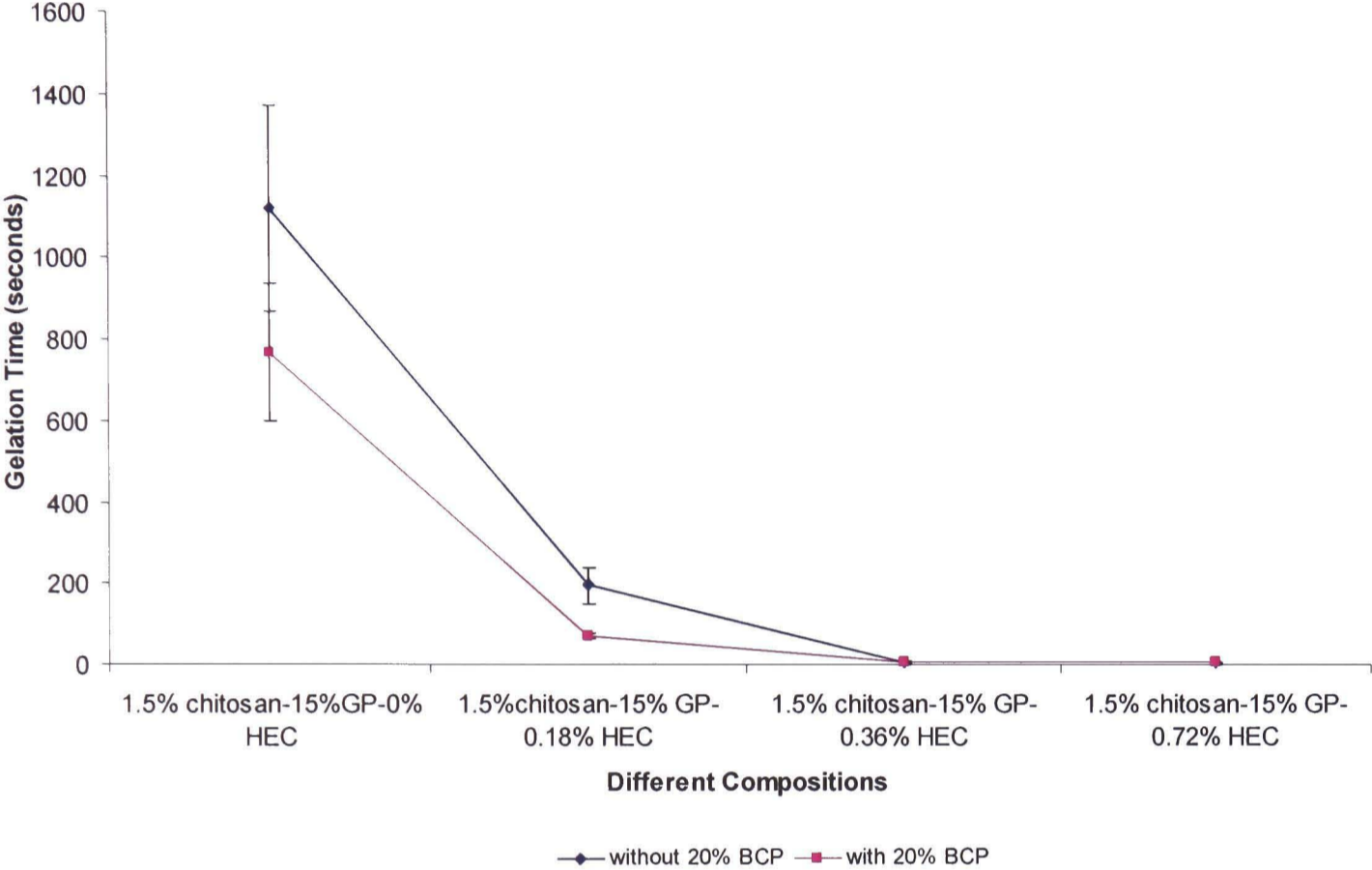


**Figure 4.10-** Gelation time of different compositions of chitosan-GP-HEC ± BCP microparticles.
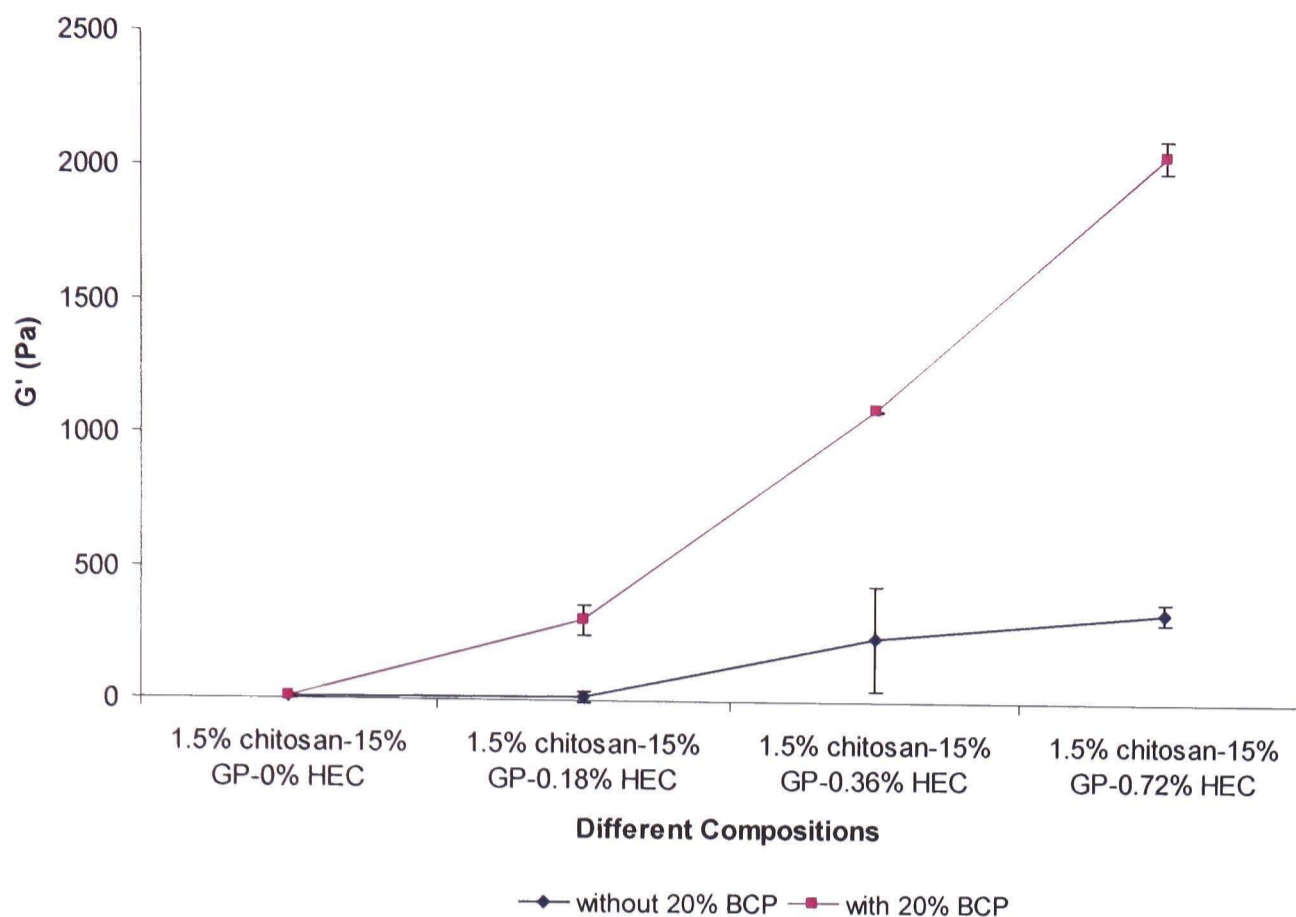
**Figure 4.11-** Elastic modulus (storage modulus) (G′) at complete gelation.

## 4.3.6 Chitosan-GP-HEC Degradation

The degradation rate of chitosan-GP-HEC is faster with lower HEC concentrations whether exposed to PBS or PBS mixed with lysozyme (Figures 4.12 and 4.13). Generally, the degradation rate is higher when the gels are in contact with lysozyme compared to PBS (Figures 4.12, 4.13 and 4.14). The dry and wet weight measurements show similar degradation profiles for different gel compositions. The percentage dry and wet weight remaining for all different gel compositions decreases throughout the 42 day period of this experiment (Figures 4.12 and 4.13). This is further evidenced by scanning electron microscopy images showing the external morphology of the gels over the 42 days (Figure 4.14). When the gels are degrading, the structure becomes more porous. Chitosan-GP mixed with 0-0.72% (w/v) HEC exposed to lysozyme demonstrate significantly faster degradation rates initially in the first 7 days followed by a steady reduction of percentage dry and wet weight remaining throughout the experiment. Nevertheless, with regard to the gels in PBS solution, there is almost similar rate of weight loss all through the experiment although the weight loss becomes insignificant towards the end. As shown in Figures

4.14 i and l, after 42 days of contact with lysozyme, the hydrogels are degraded however, chitosan-GP has a faster degradation rate compared to chitosan-GP-HEC as evidenced by higher removal of the gel structure. When chitosan-GP is exposed to PBS after 42 days, the degradation is faster compared to chitosan-GP-HEC since the gel is having a slightly more open and porous morphology in comparison with chitosan-GP-HEC (Figures 4.14 c and f).

It should be noted that dry weight measurements presented in this chapter were obtained after freeze drying under vacuum however since before measuring the weight at each time point, the lid in which the gels were kept was opened, the moisture from the air could have had an effect on the dry weight measurements. Nevertheless, such change in weight is not expected to make a significant effect on dry weight measurements. Also, wet weight measurements were obtained by blotting the samples and measuring the weight however, the weight of the water is measured in fact as well which could introduce a variable in the measurements. Nonetheless, the samples were treated consistently throughout the experiment which should reduce such an effect. Moreover, wet weight measurements were sensitive enough to show reduction as a result of chitosan degradation (Figure 4.12) despite the possible inconsistency from the water component.

Such dry/wet weight measurements performed in this chapter is a basic approach to degradation which is commonly used however, it gives just an indication on the degradation behaviour of chitosan-GP-HEC *in vivo*. In fact, *in vivo* degradation will be significantly different to the *in vitro* degradation observed in this chapter and it will most likely be faster.
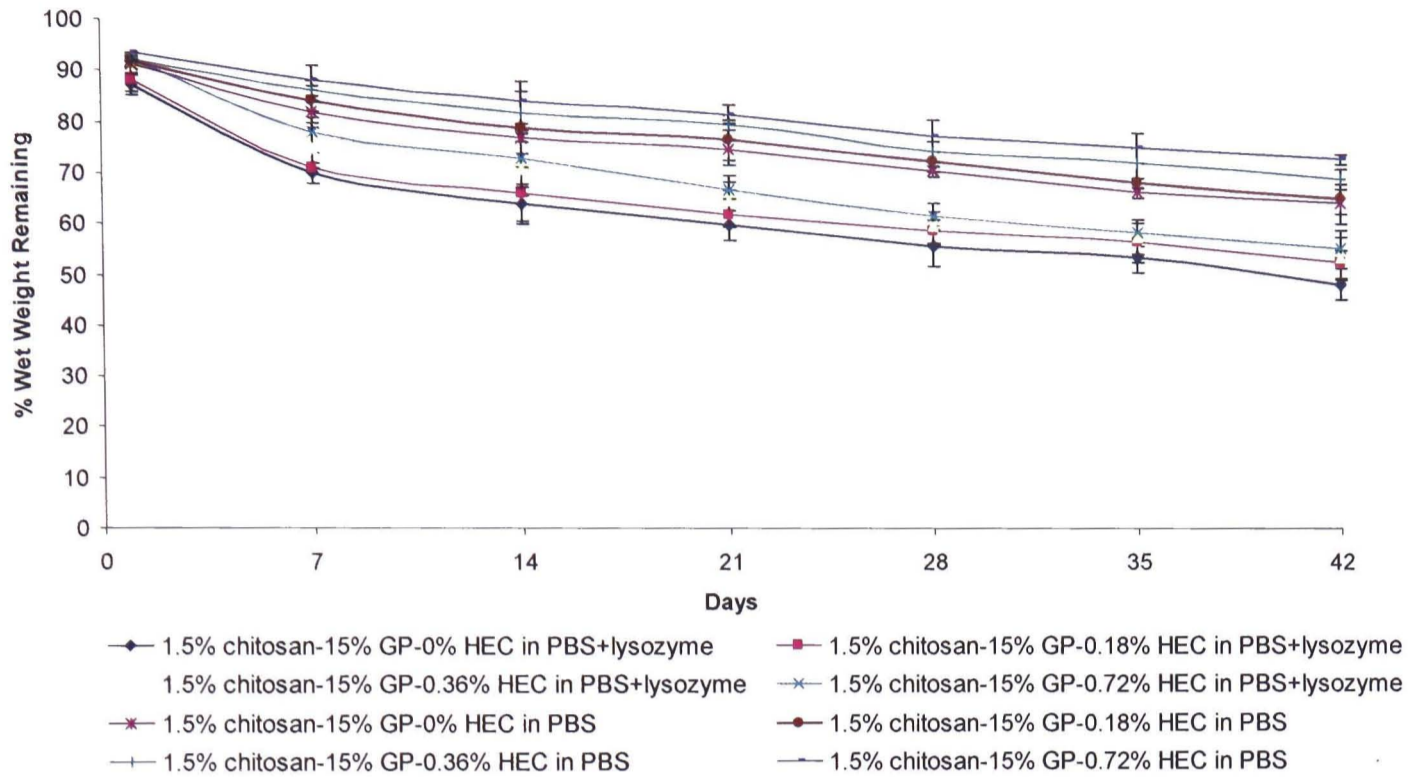
**Figure 4.12-** The percentage wet weight remaining of different gel compositions.
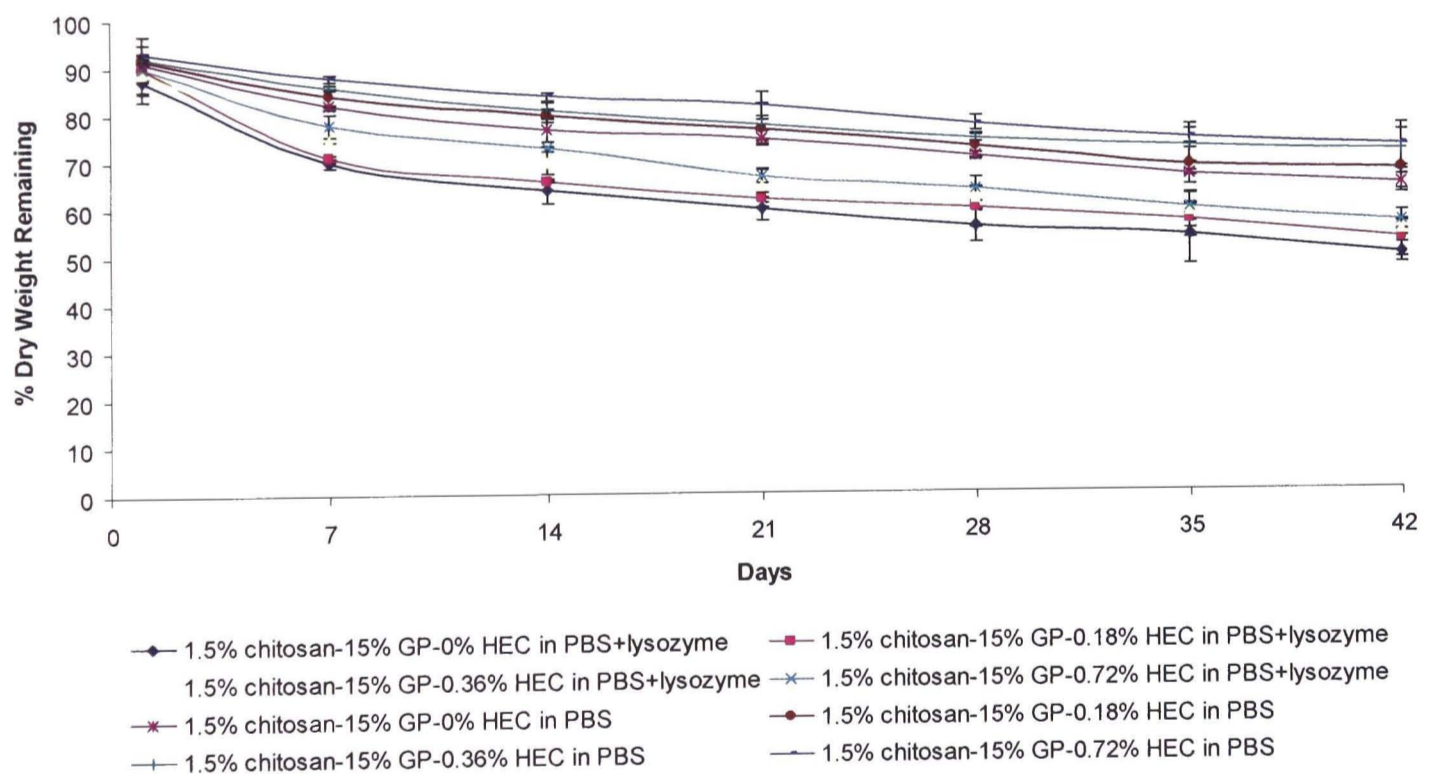


**Figure 4.13-** The percentage dry weight remaining of different gel compositions.
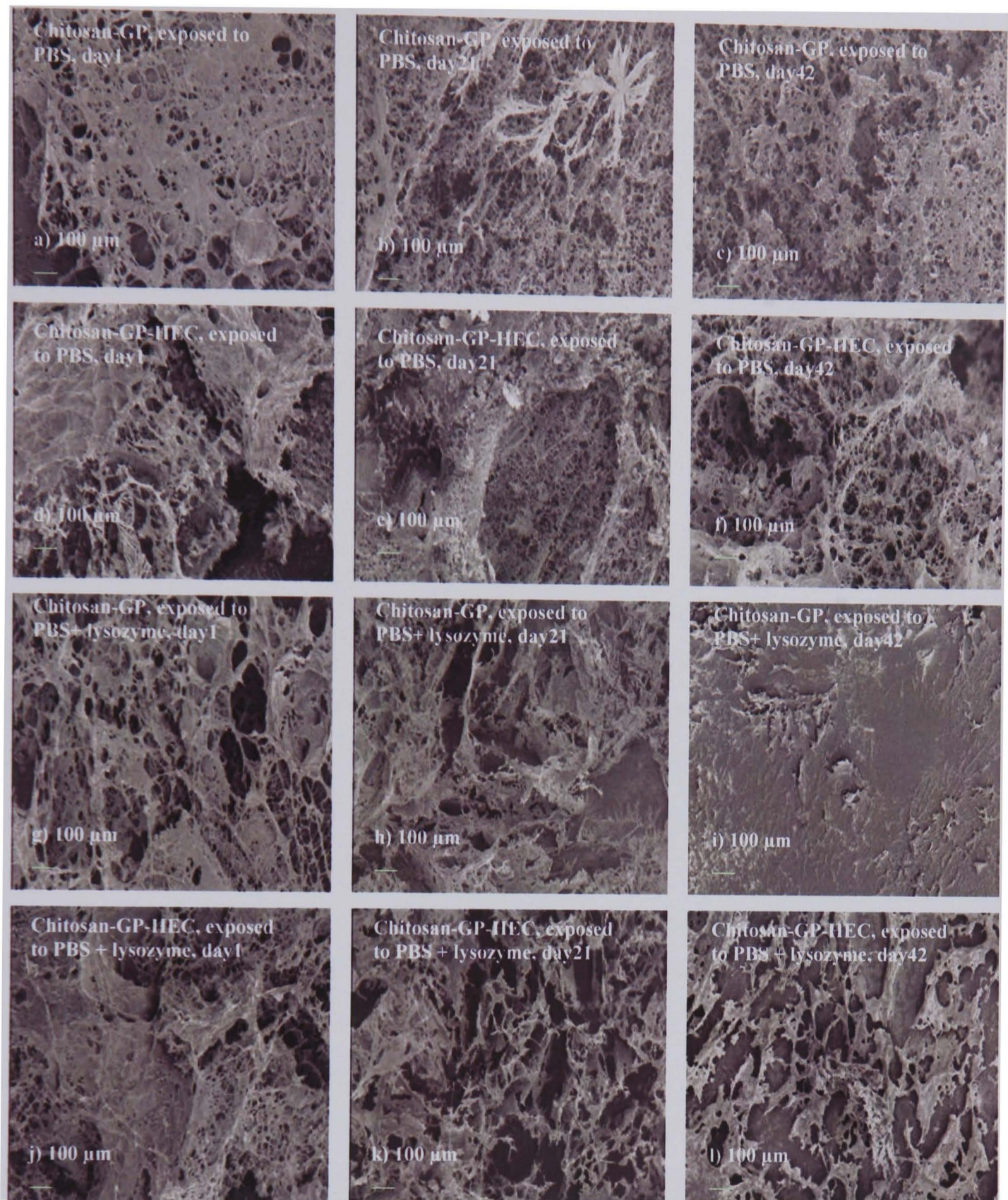
**Figure 4.14-** Scanning electron microscopy images of different gel compositions subject to degradation. Chitosan 1.5% (w/v) - GP 15% (w/v) in contact with PBS at a) day1, b) day 21, c) day 42; chitosan 1.5% (w/v) - GP 15% (w/v) - HEC 0.18% (w/v) in contact with PBS at d) day 1, e) day 21, f) day 42; chitosan 1.5% (w/v) - GP 15% (w/v) in contact with PBS + lysozyme at g) day 1, h) day 21, i) day 42; chitosan 1.5% (w/v) - GP 15% (w/v) - HEC 0.18% (w/v) in contact with PBS + lysozyme at j) day 1, k) day 21, l) day 42.

# 4.4 DISCUSSION

In this study, the effect of different chitosan-GP ± HEC compositions on hMSCs proliferation was evaluated. Moreover, survival, growth and osteogenic differentiation of hMSCs in contact with chitosan-GP-HEC hydrogel was assessed in order to determine if chitosan-GP-HEC allows cell survival, growth and osteogenic differentiation. In addition, pH, gelation and degradation behaviour of chitosan-GP-HEC binders was examined to verify if such binders gel fast at body temperature and degrade to create free space for bone formation.

The results of this study demonstrated that extracts of 1.5% (w/v) chitosan containing 5-15% (w/v) GP have physiological pH levels and enhance hMSCs proliferation relative to the control. They are therefore not only biocompatible but also stimulate hMSCs proliferation. Stimulation of cell proliferation was also observed in previous chapter using gMSCs which was followed by discussion of potential reasons behind this observation. In previous chapter, 0.5-2% (w/v) chitosan mixed with 15-20% (w/v) GP led to reduced gMSCs proliferation and cell death. In this study, the same cytotoxic response was observed although only extracts from GP concentration of 20% (w/v) were associated with cell death and the rest resulted in improved cell growth. It should be noted that chitosan with a 94% DDA was used in this study compared to 80% in previous study. This was due to the fact that chitosan with higher DDA has proved to be more biocompatible (Molinaro *et al.*, 2002). Chitosan of higher DDA need more concentrated hydrochloric acid to solubilise because of the more amine groups present in the chitosan chains that need to be protonated in acidic solutions (Chenite *et al.*, 2002). This leads to chitosan requiring more GP to interact with the amine groups in case of 94% DDA as compared to 80% DDA since the electrostatic interactions between the GP and the chitosan is one of the forces responsible for gel formation (Chenite *et al.*, 2000). As discussed in previous chapter, when more GP is reacting with the chitosan, there would be less leaching to the outside extraction medium therefore leading to higher cell growth when tested for cytotoxicity. As a result, when a similar GP concentration is added to both 80% and 94% DDA chitosan, there is less GP leaching to the medium in contact with the cells in case of 94% DDA chitosan compared to 80% DDA. Therefore, 15% (w/v) GP

mixed with 94% DDA chitosan is not toxic while the same GP concentration combined with 80% DDA chitosan was shown to be toxic in previous chapter. For all the remaining experiments in the present study, chitosan-GP concentration of 1.5% and 15% (w/v) respectively was chosen as the preferred composition. This was based on the results from previous chapter indicating that higher GP concentration leads to higher gelation rate which is preferable as long as it does not compromise cell survival.

Despite the fact that chitosan-GP is promising as a binder for injectable tissue engineered bone applications, it is difficult to obtain homogenous distribution of BCP microparticles in chitosan-GP hydrogels due to mass differences. To overcome this, an agent was introduced into the chitosan-GP gel structure to increase gel strength so that the BCP microparticles can be approximately uniformly distributed inside the gel. HEC is a non-ionic macromolecule that can be added to chitosan-GP in order to increase gel strength (Hoemann et al., 2002; Hoemann et al., 2005; Li and Xu, 2002; Roughley et al., 2006).

With regard to addition of HEC, chitosan 1.5% (w/v)-15% (w/v) GP containing 0 and 0.18% (w/v) HEC has physiological pH levels and is biocompatible based on hMSCs proliferation when in direct contact with the materials for 7 days. The rate of cell growth is similar to the control group. Chitosan-GP mixed with HEC concentrations higher than 0.18% (w/v) are not biocompatible and cause significant cell death when exposed to hMSCs. As suggested by Hoemann et al. (2007), HEC contains glyoxal. Glyoxal is a toxic by-product of advanced glycation endproducts (AGE) (Shangari et al., 2003) which has caused apoptosis following extended contact with lung epithelial cells at 0.1 mM concentration (Kasper et al., 2000). In a study, Hoemann et al. (2007) encapsulated HEK293 cells in chitosan-GP hydrogels containing different concentrations of glyoxal (Hoemann et al., 2007). Glyoxal concentration of 0.6 mM demonstrated the highest toxicity response whereas 0.15 mM concentration was associated with the highest cell viability. This observation is in agreement with the direct contact biocompatibility results obtained in this chapter since higher HEC concentrations therefore having higher glyoxal levels give rise to higher cytotoxicity response and thus cell death. This is the reason for the dose-dependent reduction in

hMSCs number observed in this study when exposed to HEC concentrations of 0.36 and 0.72% (w/v). Chitosan concentration of 1.5% (w/v) in combination with 15% (w/v) GP and 0.18% (w/v) HEC was selected as the optimal binder composition for hMSCs survival, growth and osteogenic differentiation studies performed in this chapter since it is biocompatible.

With respect to survival and growth study, hMSCs encapsulated within 1.5% (w/v) chitosan-15% (w/v) GP-0.18% HEC remain viable and proliferate at levels similar to the controls with minimum number of dead cells present based on quantification of the number of cells as well as live/dead staining using Calcein AM and Ethidium homodimer-2. This observation further confirms the results obtained from the direct contact biocompatibility tests with regard to suitability of 1.5% (w/v) chitosan-15% (w/v) GP-0.18% HEC to carry cells for the purpose of injectable bone tissue engineering. Scanning electron microscopy images obtained in this study prove the existence of a porous structure that permits penetration of biological nutrients through the hydrogel network, therefore justifying the viability and growth of the cells.

In order to examine osteogenic differentiation of hMSCs in the present study, the ALP activity was measured. ALP is the most commonly known osteogenic marker (Cheng et al., 1994; Jaiswal et al., 1997; Martin et al., 1997; Weinreb et al., 1990). Pre-osteoblasts in bone as well as osteogenic cells in culture are associated with high ALP activity levels (Weinreb et al., 1990). The osteogenic medium that was used in the current study contained $10^{-8}$ M dexamethasone and 10 mM β-glycerophosphate since based on previous studies, osteogenic differentiation in vitro has been enhanced with these two components present (Maniatopoulos et al., 1988). As demonstrated in the present study, osteogenic medium gives rise to significantly higher ALP actively relative to the basic medium for all different groups at all time points. It should be noted that the existence of chitosan-GP-HEC does not negatively affect hMSCs osteogenic differentiation, since the ALP activities are similar to when hMSCs are in contact with tissue culture plastic and BCP microparticles. For hMSCs exposed to osteogenic medium in the presence/absence of chitosan-GP-HEC, the ALP activity reaches its peak between days 8 and 10 followed by a decrease at day 14. Mendes et al. (2004) also observed the same pattern of ALP activity over time since the relative

amount of cells positive for ALP increased followed by a peak and then a decrease (Mendes *et al.*, 2004).

hMSCs unlike gMSCs were used in this study owning to their clinical relevance. Also, in terms of osteogenic differentiation, hMSCs will produce higher ALP activity in comparison with control when in contact with osteogenic medium (Mendes *et al.*, 2004) therefore making it easy to examine osteogenic tendency. However, we previously observed that gMSCs do not respond to osteogenic differentiation medium by enhancing ALP activity (unpublished). Furthermore, after 1 and 2 weeks of culture in osteogenic differentiation medium only 10% and 12% of gMSCs were stained positive for ALP respectively (Vermonden *et al.*, 2008) indicating that the marker ALP is not a reliable marker to assess osteogenic differentiation of gMSCs. Therefore, using ALP activity measurement to evaluate osteogenic differentiation, hMSCs are a more suitable cell type to study osteogenic potential.

Regarding the gelation of chitosan-GP-HEC hydrogels, it was demonstrated in the present study that chitosan concentration of 1.5% (w/v) in combination with 15% (w/v) GP and 0-0.72% (w/v) HEC all gel at 37°C providing the necessary fixation. This gelation temperature is ideal for injection into the body. Moreover, the gelation time decreases with increasing concentrations of HEC. This is in accordance with the studies carried out by Hoemann *et al.* (2007) and Li and Xu (2002). The interaction of negatively charged GP with highly protonated cationic chitosan through electrostatic attractions is one of the forces responsible for the gelation (Chenite *et al.*, 2000). As suggested by Li and Xu (2002), this system is composed of molecular aggregates of neutral origin presented as colloidal precipitates leading to weak structure and low storage modulus. The neutral molecule HEC is able to bond with chitosan through hydrogen bonding therefore resulting in bridge formation between chitosan molecules (Li and Xu, 2002). Higher HEC content causes stronger hydrogels mainly due to higher number of hydrogen bonding in the hydrogel structure (Li and Xu, 2002). Faster gelation rates resulting from higher HEC content within the gels could also be attributed to enhanced hydrogen bonding inside the gel.

The presence of BCP microparticles in chitosan-GP-HEC solutions results in more improved gelation. In a study by Couto *et al.* (2009), rheological properties of chitosan 2% (w/v)-GP 16 % (w/v) hydrogels combined with 0-50% (w/w) bioactive glass nanoparticles of 40-100 nm diameter showed that the gelation temperature decreased with increasing concentrations of bioactive glass (Couto *et al.*, 2009). This in fact shows improved gelation with increasing concentrations of bioactive glass component, which is in agreement with the results obtained in the present study in which BCP microparticles improved the gelation of chitosan-GP-HEC hydrogels. As was suggested by Couto *et al.* (2009), release of ions into the hydrogel solution by bioactive glass nanoparticles could be the potential reason behind the improved gelation observed since these ions affect the hydrophilic interactions between chitosan chains. Similarly, in the current study, release of calcium phosphate ions into the chitosan-GPHEC solution could be the possible reason for the enhanced gelation observed in the presence of BCP microparticles. For injectable bone tissue engineering applications, the gelation time of a few minutes should be sufficient to allow hardening inside the body as mentioned previously. Chitosan 1.5% (w/v)-GP 15% (w/v) in combination with 0.18% (w/v) HEC fits into this range therefore proving to be the best possible binder composition.

With regard to degradation properties, chitosan-GP-HEC was demonstrated to be degradable in this study. An injectable binder needs to be degradable in order to create space for new bone formation when injected in to the body. Faster degradation is observed when the hydrogels are exposed to PBS with lysozyme in comparison with PBS alone. The reason why lysozyme was used in this study was that various studies demonstrated chitosan degradability in contact with lysozyme (Huang *et al.*, 2005-a; Moshfeghian *et al.*, 2006). More importantly, lysozyme is present in human plasma at around 4-13 mg/L (Henry, 1991). Therefore, in order to simulate physiological conditions, lysozyme (10 mg/L) was used in degradation studies. Furthermore, lysozyme is well-known to digest chitosan based on the amount and distribution of N-acetyl groups (Aiba, 1992). According to Aiba (1992), lysozyme identified N-acetyl-D-glucosamine sequences however, it could not operate on D-glucosamine sequences and small portions of N-acetyl-D-glucosamine residues distributed at random. Lysozyme through enzymatic hydrolysis cleaves the glycosidic

bonds in chitosan leading to degradation products such as chitoligomers, N-acetyl-D-glucosamine residues, and lower molecular weight chitosans (Ren *et al.*, 2005). It should also be mentioned that higher DDA chitosan has lower rate of degradation (Aiba, 1992; Ren *et al.*, 2005).

Concerning HEC concentration and degradation properties, it was shown in the present study that lower HEC content leads to faster degradation when in contact with PBS, with or without lysozyme. Further confirmation is seen in scanning electron microscopy images in which chitosan-GP is degrading at a faster rate compared to chitosan-GP- 0.18% (w/v) HEC following 42 days of exposure with lysozyme. This could be attributed to the lower number of hydrogen bonds bridging chitosan molecules (Li and Xu, 2002) and therefore resulting in relatively weaker network more prone to degradation. Also, the existence of another polymer (HEC) could cause reduced chitosan degradation mainly due to limitation of lysozymal transport (Huang *et al.*, 2005-a). Therefore, higher concentrations of the second polymer (HEC) lead to lower degradation rates as observed in this study.

Regarding the scanning electron microscopy images, it was demonstrated that such images validate the loss of weight both dry and wet during the course of the experiment. The structure gets more porous as the gels degrade. Chitosan-GP combined with 0-0.72% (w/v) HEC in contact with lysozyme exhibit significantly faster degradation rates in the first 7 days which is then followed by a steady decrease of percentage dry and wet weight remaining. Towards the end of the experiment, the degradation becomes almost negligible. This could be related to absence of acetyl groups needed for binding to lysozyme (Varum *et al.*, 1996). On the other hand, there is approximately similar rate of weight loss throughout the experiment when hydrogels are in PBS solution although the weight loss becomes insignificant near the end. In an study performed by Ruel-Gariepy *et al.* (2000), chitosan-GP exposed to PBS had a rapid weight loss in the first 4 hours of the experiment primarily because of excess GP leaching out but there was no significant weight loss afterwards during the 24 hour time course of the experiment (Ruel-Gariepy *et al.*, 2000). Furthermore, scanning electron microscopy images after 24 hours did not show a considerable

change in the structure in that study. The weight loss of chitosan-GP-HEC exposed to PBS seen in this study could also be related to the GP leaching out of the network.

All the degradation characteristics of chitosan-GP-HEC strongly suggest that chitosan-GP-HEC is biodegradable over time without having rapid degradation therefore satisfying the degradability requirement of the injectable binder for bone tissue engineering.

## 4.5 CONCLUSION

Chitosan-GP-HEC is a promising binder material as it supports hMSCs viability, proliferation and osteogenic differentiation. Moreover, chitosan-GP-HEC hydrogels possess physiological pH levels. Chitosan-GP-HEC hydrogels are biodegradable and demonstrate fast gelation properties at body temperature. All the results obtained in this chapter indicate that chitosan-GP-HEC hydrogel is a promising binder for injectable tissue engineered bone application. Future studies will concentrate on the bone formation abilities of cells in contact with chitosan-GP-HEC hydrogels *in vivo* in order to evaluate whether chitosan-GP-HEC binder will interfere with bone formation *in vivo*. This is examined in the next chapter.

# CHAPTER V

## *IN VIVO* BONE FORMATION BY MESENCHYMAL STEM CELLS ENCAPSULATED WITH CHITOSAN-BASED HYDROGELS

# 5.1 INTRODUCTION

Successful *in vivo* bone formation involving cells, ceramic scaffolds and binder depends on several factors. Binder biocompatibility, biodegradability and gelation, as well as survival and osteogenic differentiation of cells encapsulated within the binder are all important factors to allow *in vivo* bone formation. As mentioned in previous chapters, the *in vitro* results were particularly promising in terms of biocompatibility, biodegradability and gelation of chitosan-glycerol phosphate (GP)-hydroxyethyl cellulose (HEC) binder. Furthermore, cell survival and osteogenic differentiation within the binder were all adequately achieved. Nevertheless, it is necessary to evaluate the *in vivo* bone formation ability of cell (tissue)-ceramic scaffold system combined with chitosan-GP-HEC binder in order to understand the *in vivo* effectiveness of such a system. Therefore, in this study, following from previous studies (chapter 4), cells seeded on calcium phosphate ceramics and cultured for 7 days, will be mixed with chitosan-GP-HEC hydrogel followed by *in vivo* implantation.

The initial *in vivo* experiment is usually concerned with ectopic implantation within a small animal such as mice. This is because ectopic implantation in a place such as underneath the skin (subcutaneous) for bone formation removes any factor affecting the formation of bone and any bone formation observed is purely derived from the implanted cells attached to scaffolds. The choice of cells for *in vivo* studies is another crucial issue to take into account. Bone marrow derived mesenchymal stem cells from several species have produced bone after *in vitro* expansion when transplanted into nude mice (Ashton *et al.*, 1984; Friedenstein *et al.*, 1974; Gundle *et al.*, 1995; Krebsbach *et al.*, 1997; Ohgushi and Okumura, 1990). However, donor variations associated with hMSCs would limit their applications in *in vivo* experiments (D'Ippolito *et al.*, 1999; Evans *et al.*, 1990; Mendes *et al.*, 2002-b). This is because in case of lack of bone formation, it would be difficult to know whether it was due to the specific donor or the failure of tissue engineering technique. Interestingly, goat bone marrow derived mesenchymal stem cells (gMSCs) have been frequently used for *in vivo* evaluation of bone formation (Fischer *et al.*, 2003; Kruyt *et al.*, 2003; Kruyt *et al.*, 2006). This is due to the fact that they are relatively easy to expand. In addition,

since the future studies in large animals following the current study are likely to take place in goats using gMSCs, therefore gMSCs are the ideal choice for the present study.

## 5.1.1 Aims and Objectives

In previous chapters, biocompatibility, gelation, and biodegradability of chitosan-GP-HEC were established. Cell survival, growth and osteogenic differentiation in combination with chitosan-GP-HEC binder showed encouraging results. Therefore, as the next step, the aim of the present study was to evaluate the *in vivo* bone formation ability of gMSCs attached to BCP ceramic microparticles in combination with chitosan-GP-HEC binder in order to examine whether chitosan-GP-HEC binder will interfere with bone formation *in vivo*.

# 5.2 MATERIALS AND METHODS

All chemicals were ordered from Sigma-Aldrich, UK unless otherwise indicated.

## 5.2.1 Experimental Design

The control and experimental groups are shown in Table 5.1. Goat bone marrow derived mesenchymal stem cells (gMSCs) (from iliac crest of adult Dutch milk goats, 2-4 years old) were seeded on 212-300 μm biphasic calcium phosphate (BCP) microparticles that were sintered at 1150°C and were composed of 80% HA and 20% β-TCP with a porosity of 70.8 % (Xpand Biotechnology BV, The Netherlands). Following this, gMSCs-BCP combination were implanted after one week with or without chitosan-GP-HEC binder to serve as experimental and control groups respectively. BCP microparticles mixed with/without chitosan-GP-HEC were also implanted as control groups. All four conditions within the study group were implanted subcutaneously in 13 nude mice based on power analysis (n=13). All the samples were explanted 6 weeks following implantation. The samples were analysed by histology and histomorphometry.

## 5.2.2 Preparation of Chitosan-GP-HEC Solutions

Chitosan powder (Kitomer, Marinard Biotech, Quebec, Canada) with a 94% degree of deacetylation (DDA) and a molecular weight (Mw) of 679 kDa was sterilized by autoclaving at 126°C for 20 minutes. Sterile chitosan powder (0.225g) was dissolved in 9 ml of 0.18 M hydrochloric acid. Subsequently, 1.5 g of glycerol phosphate (GP) was dissolved in deionized water to bring the total volume to 15 ml. A 0.2 μm filter (Triple Red Laboratory, UK) was used to sterilize the GP-deionized water mixtures. Chitosan-hydrochloric acid and GP-deionized water solutions were placed in an ice bath to chill for 15 minutes to avoid gelation when combined together. The ice cold GP-deionized water was then added drop by drop to the ice cold chitosan solution with constant stirring in order to create a clear chitosan-GP solution.

Hydroxyethyl cellulose (HEC) was prepared (Gibco Invitrogen, UK) at the concentration of 0.0125 g/ml in α-MEM (Roughley *et al.*, 2006). In order to prepare chitosan-GP-HEC solutions, the HEC solutions were sterilized using a 0.2 μm filter and then added to the chitosan-GP solution at the ratio of 0.8 ml to 4.8 ml respectively. The final concentration of chitosan and GP in the solutions was 1.5% (w/v) and 10% (w/v) respectively while the concentration of HEC was 0.18% (w/v).

## 5.2.3 Cell Culture and Seeding Conditions

Passage 2 gMSCs ($1 \times 10^5$ cells) with a seeding density of about 15,000 cells/cm$^2$ (Fischer *et al.*, 2003; Kruyt *et al.*, 2006) were added to 100 mg of 212-300 μm BCP microparticles. The medium used was osteogenic differentiation culture medium which was composed of α-MEM (Gibco Invitrogen, The Netherlands) supplemented with 15% (v/v) fetal bovine serum (Cambrex, The Netherlands), 0.2 mM L-ascorbic acid 2-phosphate (AsAP), $10^{-8}$ M dexamethasone, 10 mM β-glycerophosphate, 2 mM L-glutamine (Lonza, The Netherlands), 100 U/ml Penicillin and 100 μg/ml Streptomycin (Invitrogen, The Netherlands). The medium was refreshed every two days. gMSCs attached to BCP microparticles were cultured for 7 days in non-tissue culture (cells do not attach) 25 square well-plates (Greiner Bio-one, Germany) at 37°C/5% $CO_2$ incubator after which 1.5% (w/v) chitosan-10% (w/v) GP-0.18% HEC was added such that the final BCP/gel ratio was 20% (w/v). This ratio was determined according to previously performed experiments in this thesis and is the optimal BCP/gel ratio as it involves the maximum amount of BCP microparticles while the binder is still injectable.

gMSCs attached to BCP microparticles and surrounded by chitosan-GP-HEC was referred to as the experimental group. The control groups consisted of gMSCs attached to BCP microparticles without chitosan-GP-HEC, BCP mixed with chitosan-GP-HEC, and BCP alone. A summary of different groups (control and experimental) is shown in Table 5.1. All the groups were implanted subcutaneously in nude mice and a number of samples from BCP microparticles with attached gMSCs were used for construct characterization *in vitro*.

**Table 5.1-** Different groups (experimental and control) implanted subcutaneously in mice.

| Type of sample | Sample description |
|---|---|
| Experimental | gMSCs + BCP + chitosan-GP-HEC |
| Control | gMSCs + BCP |
| Control | BCP + chitosan-GP-HEC |
| Control | BCP |

## 5.2.4 Construct Characterization *In Vitro*

The number of gMSCs on BCP microparticles after 1 week of culture was evaluated by the colourimetric indicator Alamar Blue (SeroTec, Oxford, UK) before subcutaneous implantation (n=6). Constructs composed of gMSCs attached to BCP microparticles were washed three times with pre-warmed PBS solution. Consequently, 1 ml of a 5% Alamar Blue solution in culture medium (Lawson *et al.*, 2004) was added to each well-plate. The plates were then incubated for 3 hours in a humidified atmosphere at 37°C/5% $CO_2$. Fluorescence of 100 µl aliquots of the extracted dye from each sample was determined using a Fluorimeter (Zenyth 3100 Multimode Detector, Anthos Labtec Instruments GmbH, Austria) at excitation and emission wavelengths of 544 nm and 590 nm respectively. A standard calibration curve according to known numbers of gMSCs reacting with the Alamar Blue solution was used in order to quantify the number of cells present in each sample (Lawson *et al.*, 2004; Li *et al.*, 2005).

Scanning electron microscopy images were obtained for gMSCs seeded on BCP microparticles after 1 week of culture (n=6). Samples of BCP microparticles with attached gMSCs were fixated in 10% (v/v) formalin solution for 15 minutes. Following fixation, the samples were exposed to increasing concentrations of ethanol (70%, 80%, 90%, 100%) (BDH laboratory supplies, UK). The ethanol solutions consisted of x% ethanol and (100-x) % distilled water. Ultimately, the drying agent,

hexamethyl-disilazane was added to the samples. The samples were then coated with a layer of gold (Agar Auto Sputter Coater, UK) and placed into the FEI INSPECT F Scanning Electron Microscope (The Netherlands).

Moreover, methylene blue staining on cell-microparticle constructs was performed following 1 week of culture in order to analyse the attachment and distribution of gMSCs on BCP microparticles (n=6). The BCP microparticles with attached gMSCs were washed in pre-warmed PBS solution. The samples were then fixated using 10% (v/v) formalin solution for 15 minutes. Methylene blue (1% w/v) solution was then added to the samples and left for 1-2 minutes after which the samples were rinsed with distilled water and viewed under Leica stereomicroscope (Leica MZ6, UK).

## 5.2.5 Subcutaneous Implantation

Based on power analysis, a total of thirteen 6 to 8 week-old nude mice (hsd-cpb NMRI-nu, male, Harlan, The Netherlands) were used after obtaining approval by the local animal care committee (n=13). The mice were anaesthetized by inhalation of isoflurane, oxygen and carbon dioxide through a small cap over the nose. Before creation of subcutaneous pockets on either side of the spine, the skin was disinfected with 70% ethanol.

A small cut was made on the back of the mice using scissors. A pair of scissors with a blunt tip was used to create pockets underneath the skin (Figure 5.2 a). A total of 4 pockets per mouse were created as shown in Figure 5.1. Each animal received each condition presented in Table 5.1 (Figure 5.2 b). Sample implantation was based on randomized scheme. After implantation, the pockets were closed for 6 weeks until explantation (Figure 5.2 c and d).
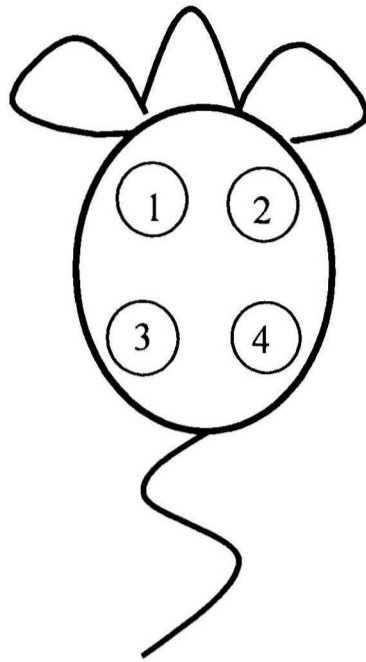
**Figure 5.1-** Schematic picture of a mouse. Numbers correspond to implant positions.
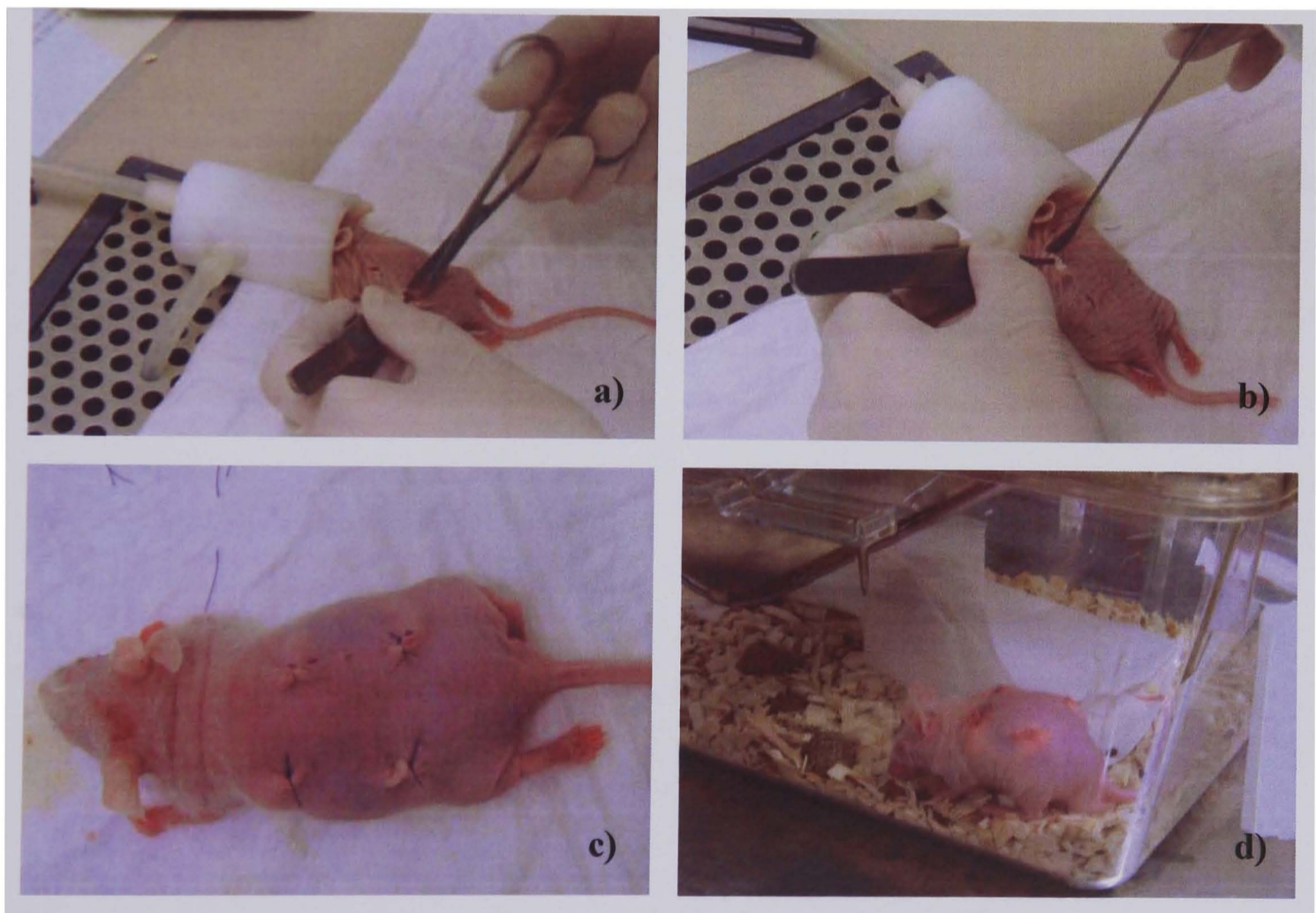


**Figure 5.2-** Implantation images. a) Creation of subcutaneous pockets, b) Subcutaneous implantation, c) Animal after implantation of all 4 groups, d) Animal back in the cage for 6 weeks until explantation.

## 5.2.6 Explantation, Histology, and Histomorphometry

Six weeks following implantation, the mice were sacrificed by $CO_2$ inhalation. The skin was opened using scissors and samples were removed and cleared from adhering tissues. The samples were fixated in 1.5% (v/v) glutaraldehyde in 0.14 M cacodylate buffer for 2 days at 4° C. Samples were dehydrated through increasing concentrations of ethanol (70%, 80%, 90%, 96%, 100%) and embedded in polymethylmethacrylate (MMA, K-Plast, LTI, The Netherlands).

Bone formation was assessed through histology and histomorphometry. Using histological diamond saw (Leica SP1600, Leica Microsystems, Germany), three semi-thin sections of 10 μm were obtained from each sample specimen. Methylene blue (1% w/v) and 0.3% (w/v) basic fuchsin were used to stain the sections. Histological sections were examined by a light microscope (Leica DMI 4000B, Leica, UK) attached to Leica digital camera (Leica DFC300 FX, Leica, UK). Histomorphometry was performed to quantify the amount of bone formation using Adobe Photoshop 6.0 software. The samples were blinded before the analysis. Bone formation was determined as the percentage of newly formed bone (B) inside the available space (Figure 5.3). The available space was calculated as the total implant area - the total BCP scaffold area (S) (Figure 5.3).
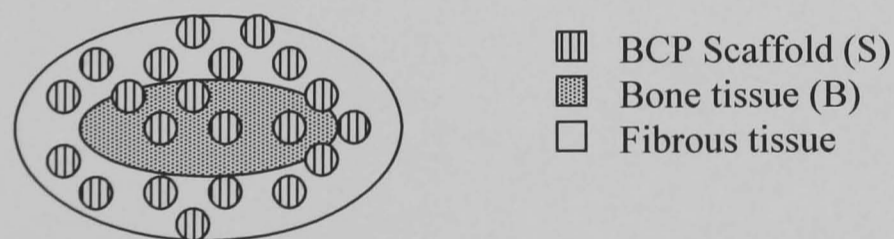


**Figure 5.3-** Schematic picture of a histological section after staining. The scaffold area (S) and the bone area (B) are shown. The total implant area is comprised of the scaffold area (S), the bone area (B), and the fibrous tissue area.

## 5.2.7 Statistical Analysis

Results were analyzed using two sample two-tail Student's *t*-test and one-way analysis of variance (ANOVA) followed by a post hoc least significant difference (LSD) test for multiple comparisons. Data are presented in mean ± standard deviation. A *p* value of <0.05 indicated statistical significance.

# 5.3 RESULTS

## 5.3.1 Construct Characterization *In Vitro*

The number of cells on BCP microparticles after 7 days of culture is $2.3 \pm 0.4 \times 10^5$ cells per construct. Scanning electron microscopy images demonstrate that gMSCs are spread on the surface of BCP microparticles forming a layer of cells following 7 days of culture (Figure 5.4a). Furthermore, extracellular matrix deposition on the surface of ceramic microparticles is apparent after 1 week of culture (Figure 5.4b).
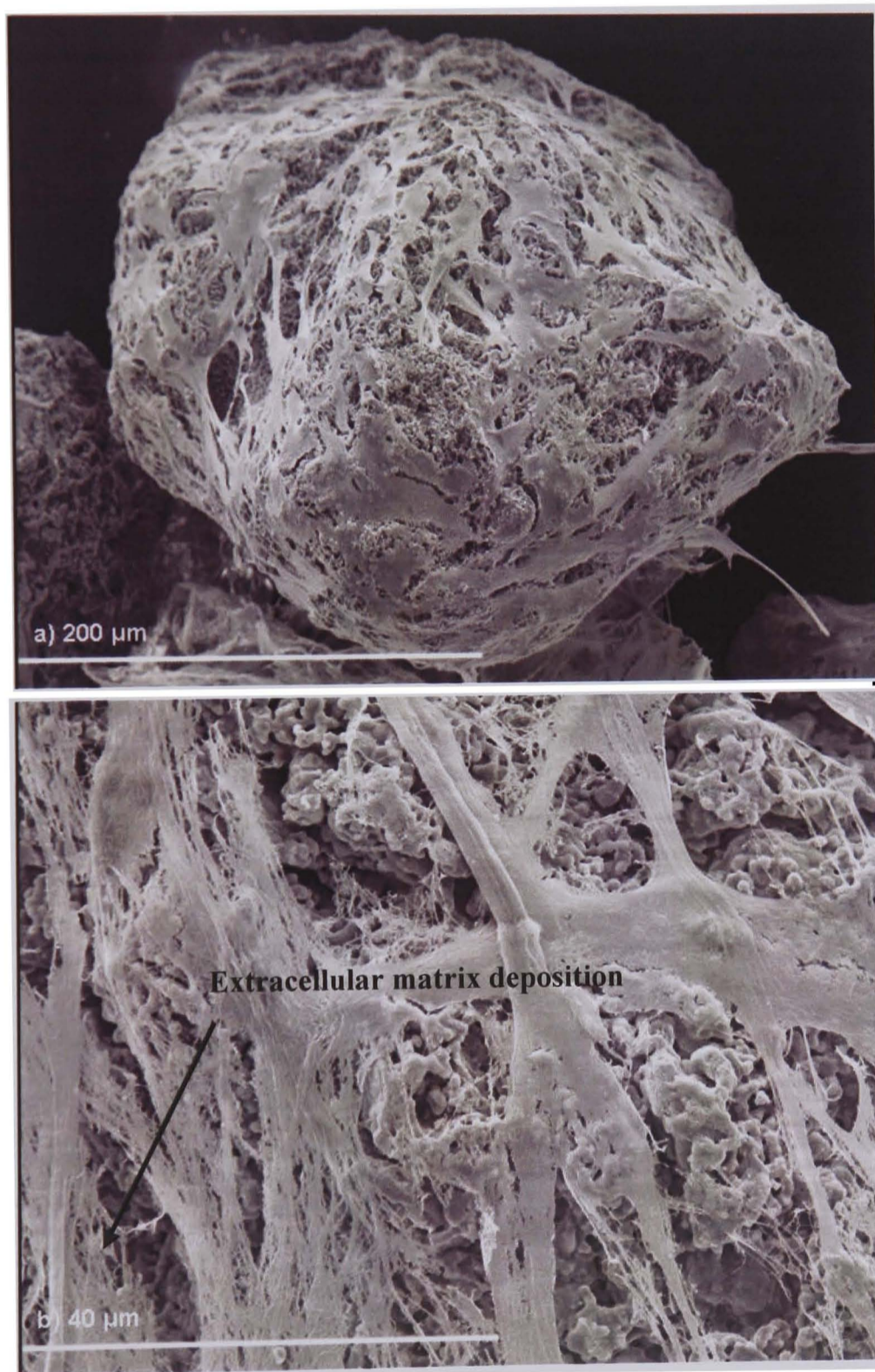


**Figure 5.4-** Scanning electron microscopy images of 7-days cultured BCP microparticles showing a) almost full cell coverage around the particle and b) flattened cells together with extracellular matrix. Extracellular matrix deposition is shown.

Methylene blue staining of gMSCs attached to BCP microparticles after 1 week of culture demonstrates almost full coverage around the microparticles with some particle bridging which is a mask of cells attaching BCP microparticles together (Figure 5.5). Moreover, stereomicroscopy shows homogeneous cell attachment and distribution all over the BCP microparticles before implantation in mice (Figure 5.5).
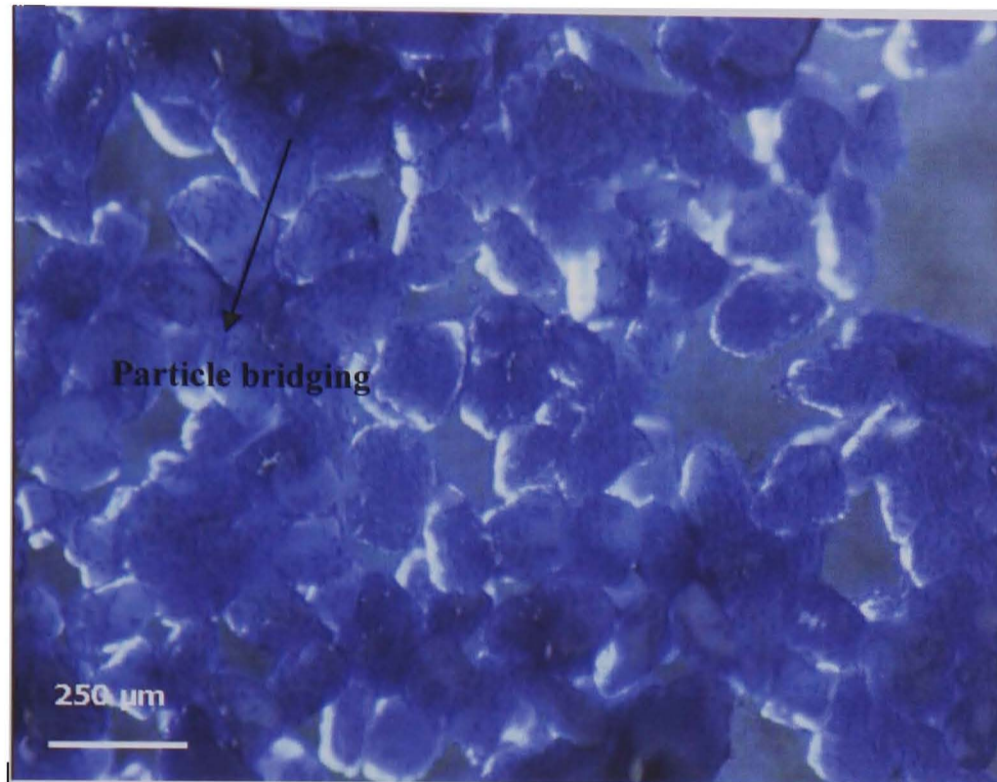


**Figure 5.5-** Stereomicroscopy of 7-days cultured BCP microparticles stained with methylene blue. Cells are attached to BCP microparticles connecting one particle to another (i.e. particle bridging is shown).

## 5.3.2 Histology and Histomorphometry

All the implants were implanted without any surgical complications. In addition, all 13 animals survived the 6-week *in vivo* implantation phase. All the samples were seen by macroscopic examination before explantation. Blood vessels were visible macroscopically around the samples when explanted after 6 weeks.

Histology was performed on all the explanted samples. Ectopic bone formation was consistently observed throughout BCP microparticles in groups containing cells (gMSCs + BCP + chitosan-GP-HEC and gMSCs + BCP) (Figure 5.6 and 5.7). Bone was formed on the surface of ceramic microparticles. Both mineralized (dark red) and non-mineralized (light red/pink) bone was seen in both groups containing gMSCs. Bone marrow was also detected in all samples containing bone. Interestingly, bone

bridging between ceramic microparticles was detected on the interior and exterior of the samples (Figure 5.6 and 5.7). Furthermore, samples without cells (BCP mixed with/without chitosan-GP-HEC) did not show any sign of bone formation in all implanted animals instead they were filled with fibrous tissue (Figure 5.8 and 5.9).
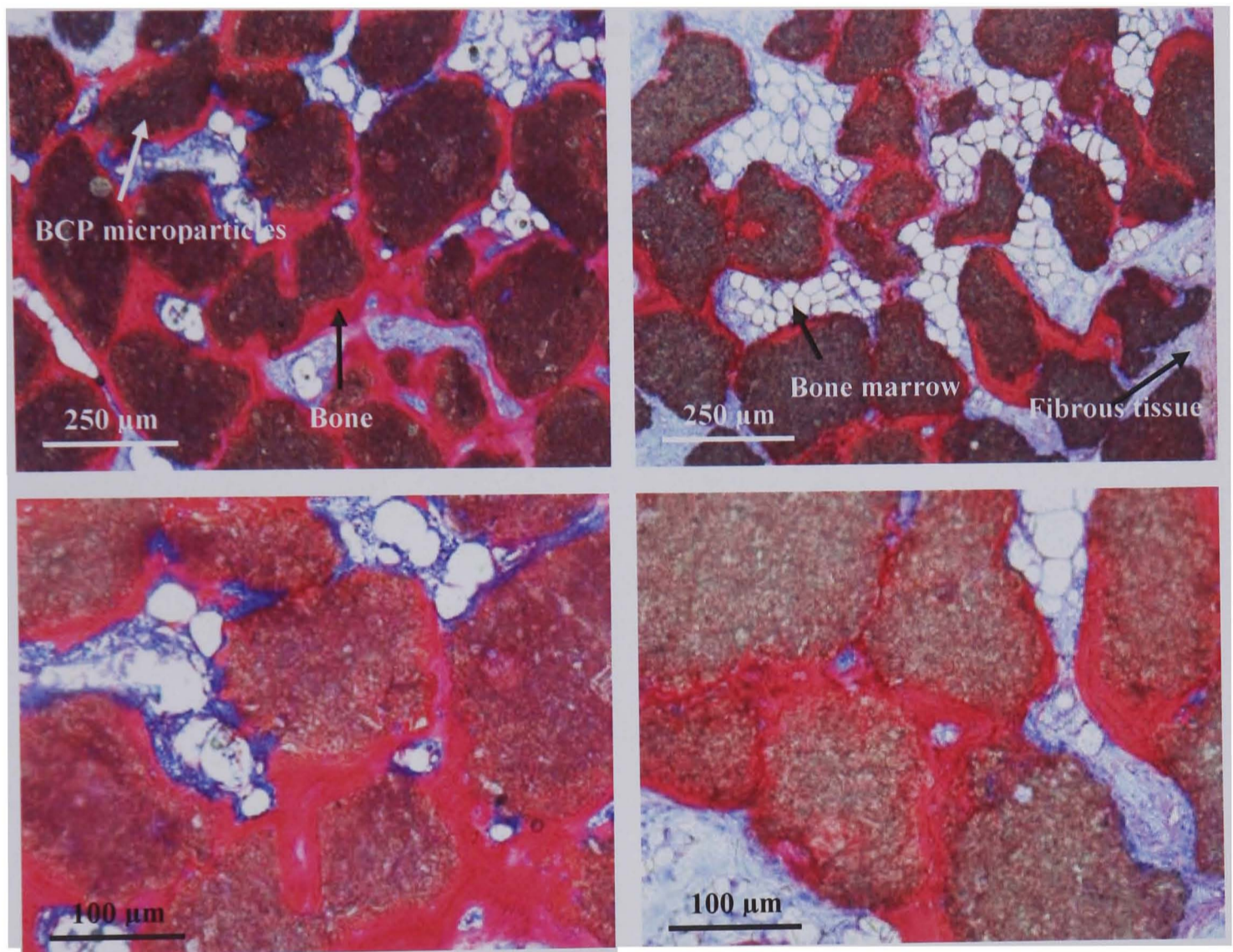


**Figure 5.6-** Bone formation between BCP microparticles in gMSCs + BCP + chitosan-GP-HEC group after 6 week *in vivo* implantation. Bone is coloured as dark red and osteoid is coloured as light red/pink. BCP microparticles, Bone, Bone marrow, and fibrous tissue are shown.
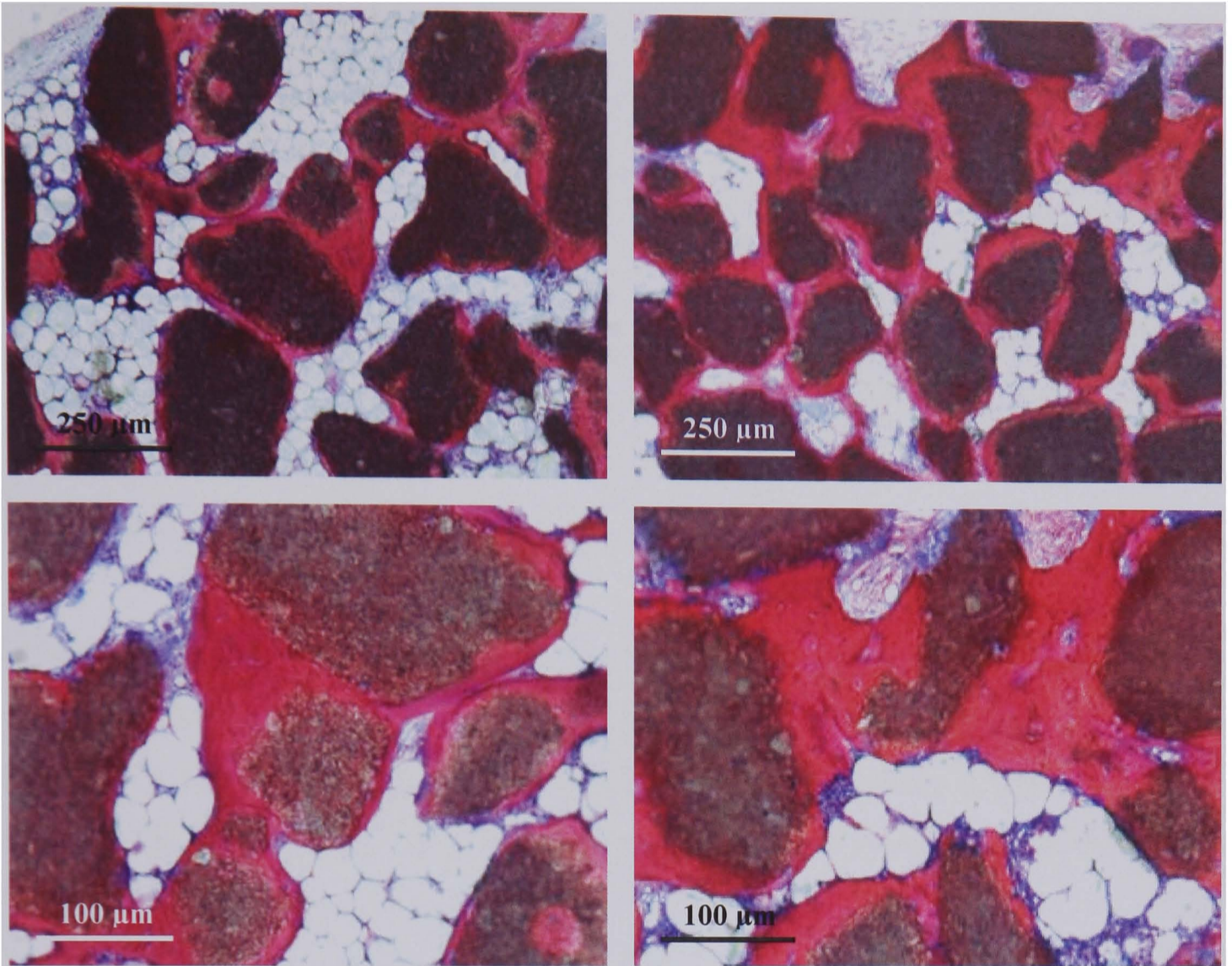
**Figure 5.7-** Bone formation between BCP microparticles in gMSCs + BCP group after 6 week *in vivo* implantation. Bone is coloured as dark red and osteoid is coloured as light red/pink.
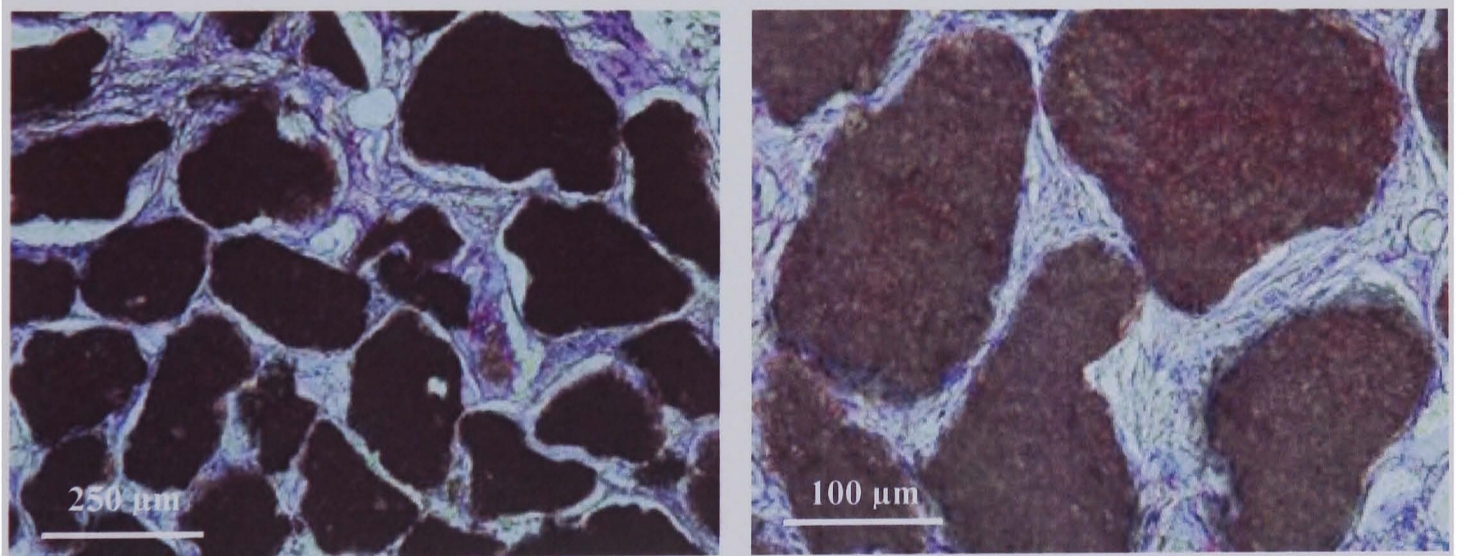


**Figure 5.8-** BCP + chitosan-GP-HEC group after 6 weeks, no bone formation was detected.

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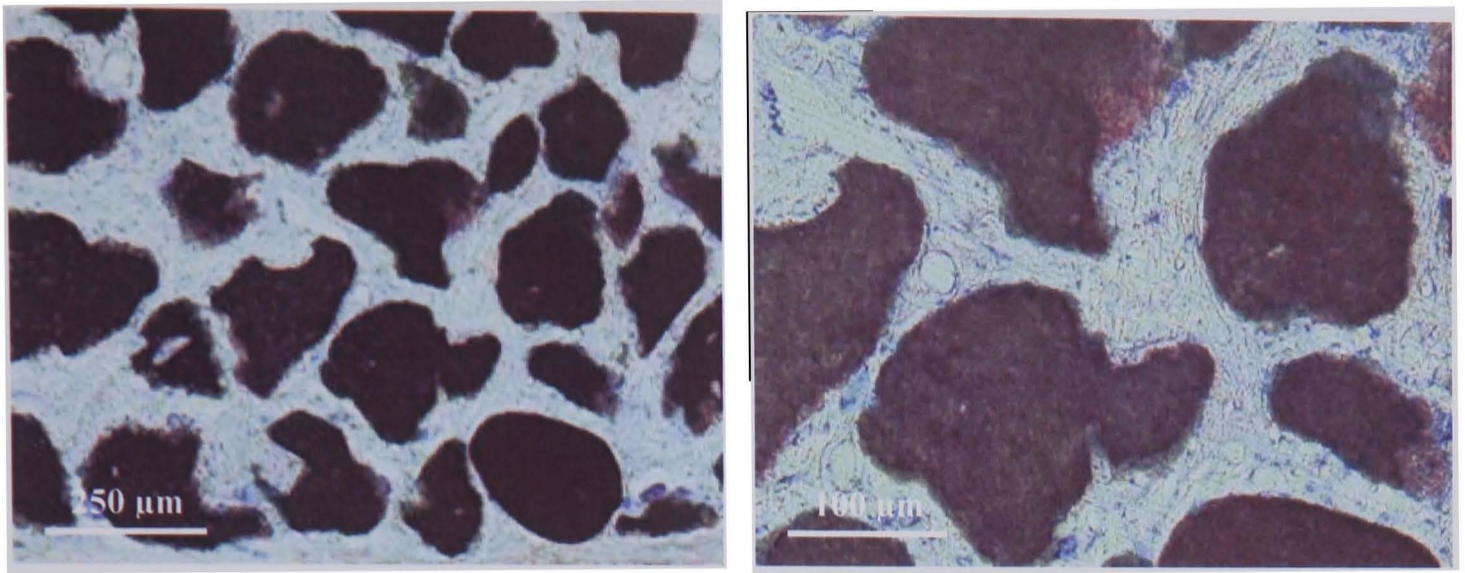
**Figure 5.9-** BCP group after 6 weeks, no bone formation was seen.

gMSCs attached to BCP and combined with/without chitosan-GP-HEC show high levels of bone formation (24.6 ± 13.7% and 34.5 ± 10.8% respectively) in all 13 samples and statistically there is no significant difference between these two groups (Figure 5.10). Moreover, gMSCs deficient groups fail to produce bone in any of the 13 implanted mice (Figure 5.10).
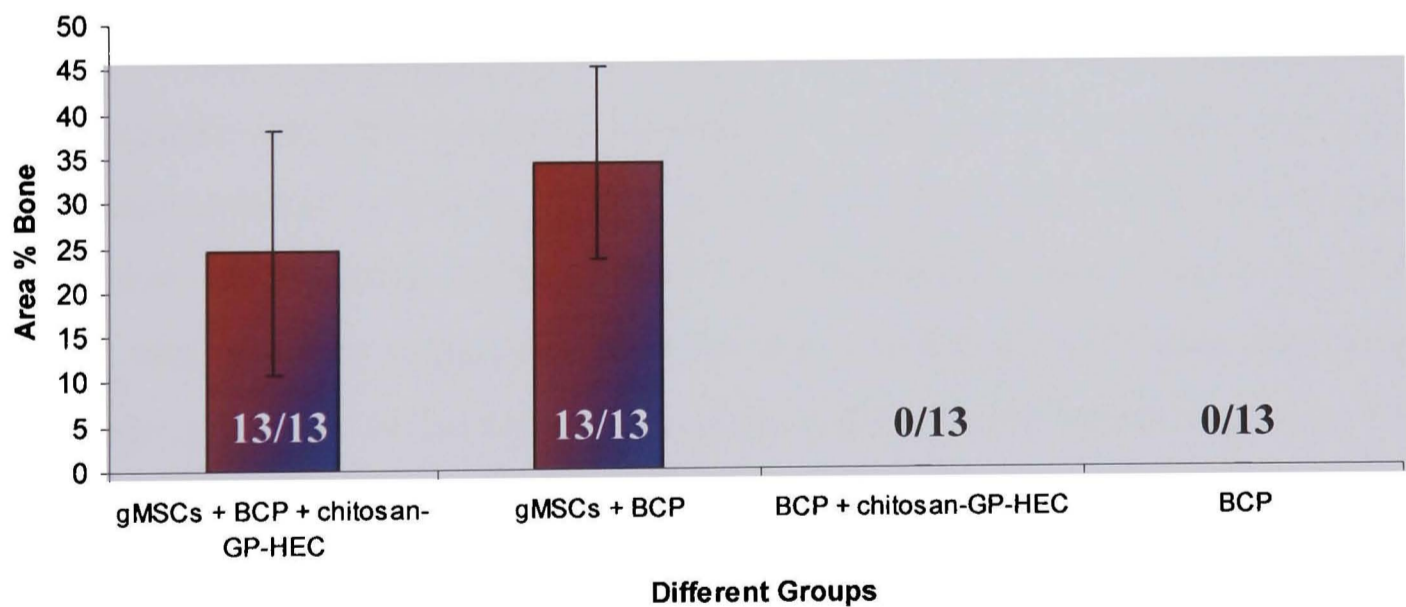


**Figure 5.10-** Histomorphometry of bone in terms of percentage of bone area inside the available space within samples implanted *in vivo* for 6 weeks (n=13). The incidence of bone formation in each group is shown on the graph.

# 5.4 DISCUSSION

The results of this study showed that gMSCs seeded onto BCP scaffolds prior to implantation, forms significant amount of bone *in vivo*. In addition, the presence of chitosan-GP-HEC in gMSCs-BCP constructs does not interfere with the bone formation process. Although the overall quantity of bone formed after 6 weeks is lower in the chitosan-GP-HEC group compared to the control group (gMSCs attached to BCP microparticles), this difference is not statistically significant. It should be noted that chitosan-GP-HEC was fully degraded after 6 weeks of *in vivo* implantation creating available space for bone formation.

In this study, gMSCs were seeded onto BCP scaffolds and cultured for a week in medium containing $10^{-8}$ M dexamethasone and 10 mM β-glycerophosphate in order to promote osteogenic differentiation (Maniatopoulos *et al.*, 1988). This was then followed by *in vivo* implantation. Previous work has shown the advantage of cell culture on the constructs before *in vivo* implantation since the *in vitro* culturing period allows extracellular matrix formation and differentiation of cells (Mankani *et al.*, 2001; Ohgushi and Caplan, 1999; Yoshikawa *et al.*, 2000). Nevertheless, Kruyt *et al.* (2004-a) demonstrated that cells seeded onto BCP scaffolds containing 80% hydroxyapatite and 20% tricalcium phosphate (cubes of 7×7×7 mm) just before implantation produce just as much bone as the pre-culture group (cells cultured for a week before implantation) in intramuscular implantation in goats (Kruyt *et al.*, 2004-a). They attributed this to the osteoinductive nature of the scaffolds since the superior osteogenic properties of the pre-culture group became inappropriate regarding bone formation. Despite the fact that similar bone formation was observed in both groups, the added benefit of pre-culture technique was clear for non-osteoinductive scaffolds. In a different study, Mendes *et al.* (2002-a) seeded rat bone marrow cells at a density of $1 \times 10^5$ cells on HA particle of $3 \times 2 \times 2$ mm$^3$ dimension followed by culturing for 5 days *in vitro* before *in vivo* implantation (Mendes *et al.*, 2002-a). The control groups consisted of $7.5 \times 10^5$ (equivalent to the number of cells on HA particle seeded with $1 \times 10^5$ cells following 5 days of *in vitro* culture) and $1 \times 10^5$ cells seeded for 16 hours on each HA particle. All the groups were implanted in rats subcutaneously for 2, 4, 7, 9, and 12 days. The results demonstrated that after 4 days of subcutaneous

implantation there was statistically significantly higher quantity of bone formation in the cell cultured group in comparison to the cell seeded groups therefore further confirming the efficiency of cell culturing prior to implantation. Moreover, bone formation was observed earlier at day 2 of implantation in the cell cultured group whereas it was identified in the cell seeded groups only after 4 days of implantation thus demonstrating faster bone formation in the pre-cultured group.

In this study, bone formation was observed in between ceramic microparticles within the gMSCs containing samples both on the interior and exterior of the samples. This is in contrast to the results achieved by Kruyt *et al.* (2006) who never observed bone on the exterior of the samples. Bone distribution on the interior and exterior of the samples could be due to the fact that gMSCs were homogeneously attached and distributed before implantation as evidenced by stereomicroscopy image of 7-days cultured BCP microparticles stained with methylene blue (Figure 5.5). Although homogenous attachment and distribution of cells on microparticles was also achieved in the study performed by Kruyt *et al.* (2006), it is not clear why bone was not formed on the exterior of the samples.

With regard to chitosan's ability to support bone formation, Kim *et al.* (2008) performed an study in which rat muscle-derived stem cells surrounded by 1.8% (w/v) chitosan-20% (w/v) GP, produced mineralized bone deposits after subcutaneous implantation in rats for 4 weeks (Kim *et al.*, 2008). This is in agreement with the results obtained in the current study since chitosan-GP-HEC was demonstrated to support bone formation *in vivo* when in contact with gMSCs.

## 5.5 CONCLUSION

The results obtained in this study demonstrate the osteogenic potential and bone formation ability of cells encapsulated in chitosan-GP-HEC hydrogel. Chitosan-GP-HEC hydrogel supports bone formation and provides a minimally invasive option for bone tissue engineering.

# CHAPTER VI

## CHITOSAN-BASED HYDROGELS UNLIKE HUMAN BONE MARROW DERIVED MESENCHYMAL STEM CELLS DO NOT INDUCE ANGIOGENESIS

# 6.1 INTRODUCTION

Angiogenesis is the process in which new blood vessels grow from the endothelium of pre-existing vasculature (Folkman and Shing, 1992). It plays an important role for success in bone tissue engineering approaches since bone is a highly vascularized tissue that needs blood vessels to supply oxygen and nutrients to the cells. Since the diffusion limit of oxygen is around 100-200 μm, formation of new blood vessels is necessary for the tissue to grow further than this limit (Awwad et al., 1986; Carmeliet and Jain, 2000). This can result in major cell survival problems with regard to bone tissue engineered constructs that are principally used to regenerate large bony defects.

Chitosan-based matrices have gained increasing interest in regenerative medicine as they can be used to prepare injectable formulations of cells combined with micron sized biomaterial scaffolds. The approach to injectable tissue engineered bone that was pursued in this study was to combine bone producing cells attached to biphasic calcium phosphate (BCP) microparticles surrounded by an injectable binder as explained in previous chapters. Advantages of this approach is that it can be applied in a minimally invasive manner that offers many benefits such as reduced cost, pain, complications, surgery time, scarring and healing period. In the current study, chitosan combined with glycerol phosphate (GP) and hydroxyethyl cellulose (HEC) is used as the injectable binder. Chitosan is biocompatible (Hirano and Noishiki, 1985) and biodegradable (Muzzarelli, 1997) and when combined with GP, it becomes thermo-sensitive in diluted acids and gels around body temperature (Chenite et al., 2000). Thermo-sensitivity is needed for minimally invasive applications of the injectable tissue engineered bone since it allows injection to the defect site followed by the gelation. Heavy weighted BCP microparticles tend to sink to the bottom of the chitosan-GP rather than remain homogeneously distributed within the gel structure. However, HEC improves chitosan-GP rigidity (Hoemann et al., 2002; Hoemann et al., 2005; Roughley et al., 2006) which can be useful for future incorporation of the BCP microparticles.

With regard to the angiogenic potential of chitosan, there have been suggestions that chitosan is angiogenic (Biagini et al., 1989; de Castro-Bernas, 2003). De Castro-

Bernas (2003) reported chick chorioallantoic membrane (CAM) assay results demonstrating that chitosan stimulated neovascularization in a dose-dependent manner at even higher levels than the known angiogenic growth factor b-FGF (basic fibroblastic growth factor). Nevertheless, the study failed to mention more detailed information including the chitosan concentration used. In a study by Biagini *et al.* (1989), N-Carboxymethyl chitosan placed into the cornea of 10 adult rabbits induced neovascularisation in 7 rabbits after 30 days whereas control rabbits did not demonstrate any new vasculature. In their paper, Biagini *et al.* (1989) referred to the biochemical effects of chitosan or the foreign body reaction caused by chitosan as possible reasons behind chitosan's induced neoangiogenesis. According to Biagini *et al.* (1989), foreign body reaction initiated by chitosan led to the presence of leukocytes and macrophages. It is known that angiogenesis is related to inflammation and inflammatory cells can function as indirect mediators of angiogenesis (Benelli *et al.*, 2002). In addition, inflammatory cells possess proteolytic enzymes able to digest the basal membrane which is the initial step involved in the formation of new blood vessels (Biagini *et al.*, 1989; Guo *et al.*, 2001). Moreover, presence of angiogenic stimuli leads to endothelial cell proliferation (Silver *et al.*, 2007). All these mechanisms could potentially lead to the angiogenesis observed in relation to chitosan.

Interestingly, it has been reported that mesenchymal stem cells produce angiogenic cytokines including vascular endothelial growth factor (VEGF), b-FGF, hepatocyte growth factor, insulin-like growth factor 1, MCP-2 and MCP-3 (Chen *et al.*, 2003; Kamihata *et al.*, 2001; Kinnaird *et al.*, 2004; Nagaya *et al.*, 2004; Tang *et al.*, 2005). Chitosan combined with human bone marrow derived mesenchymal stem cells (hMSCs) could have synergistic effects on angiogenesis and as a result enhance the overall angiogenic response in orthotopic defects.

A range of *in vitro* and *in vivo* angiogenesis assays have been used in order to elucidate the effects of different agents on endothelial cell proliferation and blood vessel growth (Auerbach *et al.*, 2003). Most of these assays were performed on isolated cell preparations. However, such assays do not demonstrate the complicated host components and interactions that take place in the *in vivo* environment (Miller *et*

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*al.*, 2004). Nevertheless, *in vivo* small animal models are a closer estimate to the processes observed in humans but it should be noted that these experiments are expensive and time consuming (Miller *et al.*, 2004).

The CAM assay is an alternative to small animal models and is one of the most commonly used *in vivo* assay systems to study angiogenesis (Ribatti *et al.*, 2000; Staton *et al.*, 2004). The chick egg possesses a natural environment of growing blood vessels as well as all the components of the complex host interactions, and many angiogenic factors have been tested by this assay (Olivo *et al.*, 1992; Ribatti *et al.*, 2000; Wilting *et al.*, 1993; Yang and Moses, 1990). The CAM is characterized by a dense microvascular network which first emerges at day 3 of incubation and thereafter quickly develops (Ribatti *et al.*, 2001). It is formed when the adjacent mesodermal layers of the chorion and the allantois fuse and it functions as a transient gas exchange surface similar to the lung until the time of hatching (Dimitropoulou *et al.*, 1998; Laschke and Menger, 2007; Ribatti *et al.*, 2001). When the CAM is used as an assay for angiogenesis, an increased vessel density around the implant in which the vessels radially converge towards the centre resembling spokes in a wheel is associated with an angiogenic response (Ribatti *et al.*, 1995). The CAM assay has many advantages such as low cost in comparison with *in vivo* animal models and the simplicity of the preparation of the CAM vascular network. Moreover, xenografts from mammalian species implanted onto the CAM do not show rejection since the early chicken embryo does not have a complete immune system (Laschke and Menger, 2007). It should also be mentioned that the CAM assay has some drawbacks such as the limitation of its use to a time period of about 10 days, difficulty in detailed quantification of angiogenesis by microscopy since smaller blood vessels (capillaries) are not easy to visualize, and also, the CAM is composed of embryonic tissue that is characterized by a different growth factor profile compared to adult tissue (Laschke and Menger, 2007).

## 6.1.1 Aims and Objectives

The aim of this study was to evaluate the effect of added hMSCs on the angiogenic potential of chitosan binder. The angiogenic response of chitosan-GP-HEC combined with/without hMSCs was therefore assessed using the CAM assay. For this purpose, hMSCs were cultured on BCP microparticles since the cells require scaffolds for attachment and subsequent bone deposition.

# 6.2 MATERIALS AND METHODS

All chemicals were ordered from Sigma-Aldrich, UK unless otherwise indicated. The experiments were repeated twice.

## 6.2.1 Preparation of Chitosan-GP-HEC Solutions

Chitosan powder (Kitomer, Marinard Biotech, Quebec, Canada) with a 94% degree of deacetylation (DDA) and a molecular weight (Mw) of 679 kDa was sterilized by autoclaving at 126°C for 20 minutes. Sterile chitosan powder with a quantity of 0.225g was dissolved in 9 ml of 0.18 M hydrochloric acid. Consequently, 2.25g of GP was dissolved in deionized water in order to bring the total hydrochloric acid and deionized water volume in the solutions to 15 ml.

The GP-deionized water was filter-sterilized using a 0.2 μm filter (Triple Red Laboratory, UK). Solutions of chitosan-hydrochloric acid and GP-deionized water were placed in an ice bath to chill for 15 minutes to avoid gelation when mixed together. The ice cold GP-deionized water was then added drop by drop to the ice cold chitosan solution with constant stirring to create a clear solution. HEC was added to α-MEM (Gibco Invitrogen, UK) at the concentration of 0.0125 g/ml (Roughley et al., 2006). The HEC solution was filter-sterilized and then added to the chitosan-GP solution at the ratio of 0.8 ml to 4.8 ml respectively. The final concentrations of chitosan, GP, and HEC in the solutions were 1.5% (w/v), 15% (w/v), and 0.18% (w/v) respectively.

## 6.2.2 Culture of Human Mesenchymal Stem Cells on Biphasic Calcium Phosphate Microparticles

Passage 2 hMSCs (from female, age 36 (iliac crest, donor 1); male, age 44 (acetabulum, donor 2); female, age 55 (iliac crest, donor 3), Xpand Biotechnology BV, The Netherlands) were seeded at a density of 30,000 cells/cm$^2$ on 212-300 μm BCP that were sintered at 1150°C and were composed of 80% hydroxyapatite and 20% β-tricalcium phosphate with a porosity of 70.8 % (Xpand Biotechnology BV,

The Netherlands). The BCP microparticles with cells were kept in a humid atmosphere at 37°C/5% $CO_2$ for 7 days until they were 85-90% covered with cells. The medium was composed of α-MEM (Gibco Invitrogen, UK) supplemented with 10% v/v fetal bovine serum (FBS, Gibco Invitrogen, UK), 100 U/ml penicillin/100 µg/ml streptomycin, 2 mM L-glutamine, 10 mM β-glycerophosphate (BGP), $10^{-8}$ M dexamethasone, and 0.2 mM L-ascorbic acid 2-phosphate (AsAP) and was refreshed every two days. After 7 days, chitosan-GP-HEC solution was added to hMSCs attached to BCP microparticles such that the final BCP/gel ratio was 20% (w/v). This ratio was determined according to previously performed experiments and is the optimal BCP/gel ratio as it involves the maximum amount of BCP microparticles while the binder is still injectable. Chitosan-GP-HEC solution was also added to the BCP microparticles without hMSCs to serve as the control for the experimental group for the following CAM assay.

Cell proliferation was assessed using the colourimetric indicator Alamar Blue assay (SeroTec, Oxford, UK). To determine the total number of cells attached to BCP microparticles at the time of placement on the CAM, hMSCs were washed three times with pre-warmed phosphate buffered saline solution (PBS). Consequently, 1 ml of a 5% Alamar Blue solution in culture medium (Lawson *et al.*, 2004) was added to hMSCs/BCP combination in the well-plate and then the plates were incubated for 3 hours in a humidified atmosphere at 37°C/5% $CO_2$. Fluorescence of 100 µl aliquots of the extracted dye from each sample was determined using a Fluorimeter (FLUOstar Galaxy Fluorimeter, JENCONS-PLS, Germany) at excitation and emission wavelengths of 544 nm and 590 nm respectively. A standard calibration curve according to known numbers of hMSCs reacting with the Alamar Blue solution was used in order to quantify the number of cells present in each sample (Lawson *et al.*, 2004; Li *et al.*, 2005).

## 6.2.3 CAM Assay

Ethical approvals needed for this study were obtained prior to the experiments. Fertilized chicken eggs (Henry Stewart and Co, UK) were placed in an incubator at 37°C. After 3 days a hole was created in the pointed end of the egg and 2 ml of

albumin was removed using a 21 G needle (Appleton Woods, UK) (Figure 6.1a). A window was then made on the upper surface of the egg by removing a small 2 cm oval of shell, thus allowing access to the CAM. The viability of the eggs was assessed then the windows were sealed with adhesive tape and the eggs returned to the incubator (Figure 6.1c). Figure 6.1b shows a day 3 chicken embryo with the beginnings of the blood vessel network.
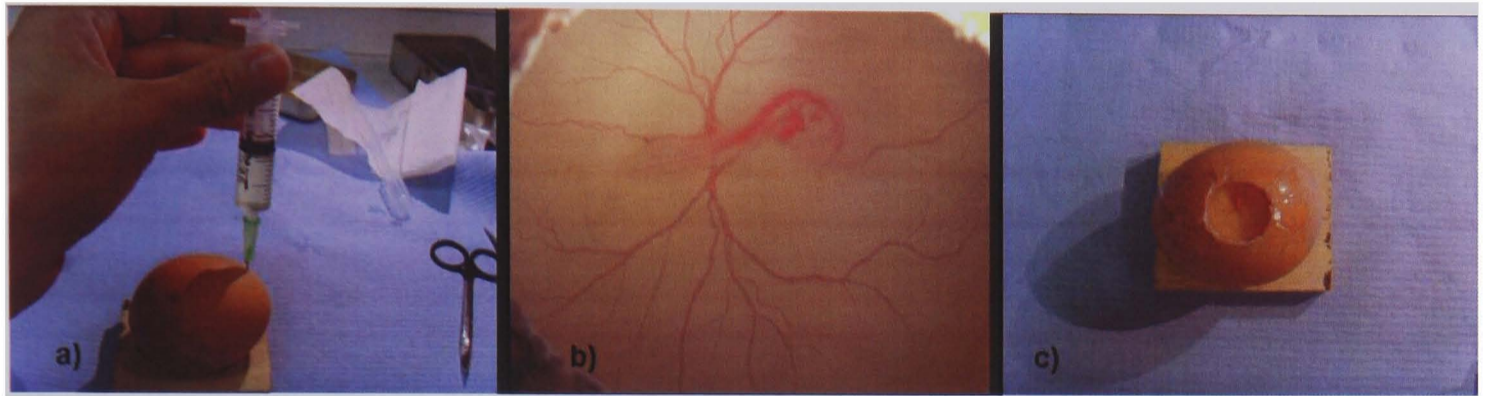


**Figure 6.1-** Procedure for exposing and accessing the chick chorioallantoic membrane. Two mls of albumin is removed from 3 day old chick eggs using a 21 G needle and syringe (a); 3 day old chicken embryo showing the fine blood vessel network extending over the surface of the yolk (b); the window through the shell, used to gain access to the CAM, is sealed with clear tape then the egg is returned to the incubator (c).

Filter paper discs with a diameter of 2 mm (Whatman, UK) were prepared and autoclaved at 126°C for 20 minutes. The filters were then impregnated with PBS or 200 ng of basic fibroblastic growth factor in 10 µl of PBS (Marks et al., 2002) (b-FGF, SeroTec, Oxford, UK) which served as negative and positive controls respectively. The experimental groups consisted of filter paper discs saturated with chitosan-GP-HEC solutions, hMSCs attached to BCP microparticles surrounded by chitosan-GP-HEC solution, and BCP microparticles surrounded by chitosan-GP-HEC solution all prepared as explained above. The experimental groups were referred to as chitosan-GP-HEC, chitosan-GP-HEC/hMSCs/BCP, and chitosan-GP-HEC/BCP respectively throughout this study.

At day 7, control and experimental groups (10 µl each) were placed on the CAM (Oates et al., 2007) based on the n numbers shown in Table 6.1. The windows were then sealed again with adhesive tape and returned to the incubator. At day 10, the images of the CAM with filter paper discs were obtained (Oates et al., 2007) using a Leica DC500 camera (Leica Microsystem, Milton Keynes, UK) connected to Adobe

Photoshop 6.0 software and attached to a Leica MZ FLIII microscope (Leica Microsystem, Milton Keynes, UK). Quantification of the results was performed based on the total number of vessels converging towards the centre of the implant in a spoke-wheel pattern traversing a 4mm diameter circle from the centre of the filter paper disc. For quantification purposes, the magnification of the images was kept constant. Blood vessels were quantified blindly by 3 different observers.

**Table 6.1-** Table showing the n numbers for the different control and experimental groups in the study.

| Control and experimental groups | n number |
|---|---|
| Negative control | 9 |
| Positive control | 9 |
| Chitosan-GP-HEC | 9 |
| Chitosan-GP-HEC/hMSCs/BCP | 3 for each donor |
| Chitosan-GP-HEC/BCP | 9 |

## 6.2.4 Statistical Analysis

Results were analysed using two sample two-tail Student's $t$-test and one-way analysis of variance (ANOVA) followed by a post hoc least significant difference (LSD) test for multiple comparisons. Data are presented as mean ± standard deviation. A $p$ value of $<0.05$ indicated statistical significance.

# 6.3 RESULTS

## 6.3.1 Culture of Human Mesenchymal Stem Cells on Biphasic Calcium Phosphate Microparticles

After 7 days of culture, the total number of hMSCs attached to BCP microparticles at the time of placement on the CAM is $1.6 \pm 0.1 \times 10^5$ for donor 1, $1.66 \pm 0.05 \times 10^5$ for donor 2, and $1.58 \pm 0.06 \times 10^5$ for donor 3. There is no significant difference between different donors in terms of the number of cells on BCP microparticles after

7 days of culture. The standard deviations within each donor do not vary significantly.

## 6.3.2 CAM Assay

An increased vessel density around the implant in a spoke-wheel pattern is observed in the b-FGF positive control (Figure 6.2). The same enhanced vessel density in a spoke-wheel manner is also detected around the chitosan-GP-HEC/hMSCs/BCP group (Figure 6.2). Angiogenesis is thus greatly stimulated when hMSCs are added to the BCP microparticles and then mixed with chitosan-GP-HEC. The BCP microparticles without hMSCs combined with chitosan-GP-HEC do not lead to an enhanced angiogenic response in the CAM assay. Therefore, the stimulation of angiogenesis can be attributed to the presence of hMSCs in the system. The b-FGF shows very strong angiogenesis as expected of a positive control, however, more interestingly, the strength of the angiogenic reaction is similar to the chitosan-GP-HEC/hMSCs/BCP group (Figure 6.2). The comparable level of angiogenic response between these two groups demonstrates the efficacy and the significance of hMSCs in the system in terms of angiogenesis.

There is no spoke wheel pattern observed for the chitosan-GP-HEC group (Figure 6.2) therefore, chitosan-GP-HEC does not enhance angiogenesis. Also, the negative control group which is composed of the filter paper impregnated with PBS does not result in a spoke-wheel pattern.

Quantitatively, significantly more converging blood vessels growing towards the centre of the implant are detected for the chitosan-GP-HEC/hMSCs/BCP group as compared to all other groups ($p < 0.05$), except for the b-FGF positive control (Figure 6.3). The difference between the number of converging blood vessels in the stem cell containing group and the rest of the groups excluding b-FGF positive control is large (maximum percentage difference of 61.5%). The number of blood vessels radiating from the centre of the implant does not show any significant difference between the b-FGF and the chitosan-GP-HEC/hMSCs/BCP group.

The number of blood vessels around the implants is slightly higher in the chitosan-GP-HEC and the chitosan-GP-HEC/BCP groups compared to the PBS negative control nevertheless, these differences are not statistically significant (Figure 6.3). Hence, chitosan-GP-HEC and chitosan-GP-HEC/BCP without hMSCs do not result in enhanced angiogenic response.
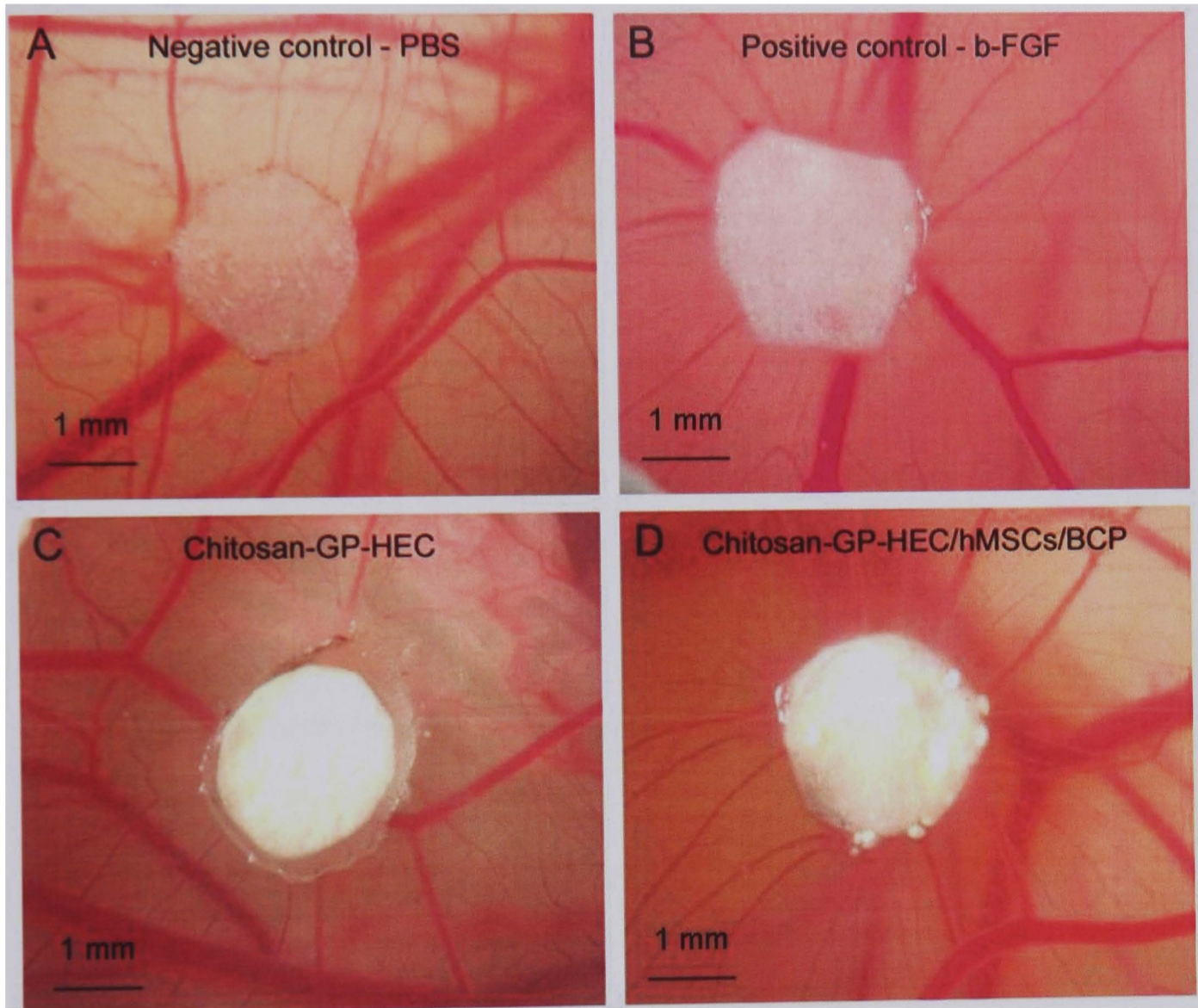


**Figure 6.2-** Extent of angiogenesis after 3 days in contact with the CAM, (a) Negative control, (b) Positive control, (c) Chitosan-GP-HEC, (d) Chitosan-GP-HEC/ hMSCs/BCP.
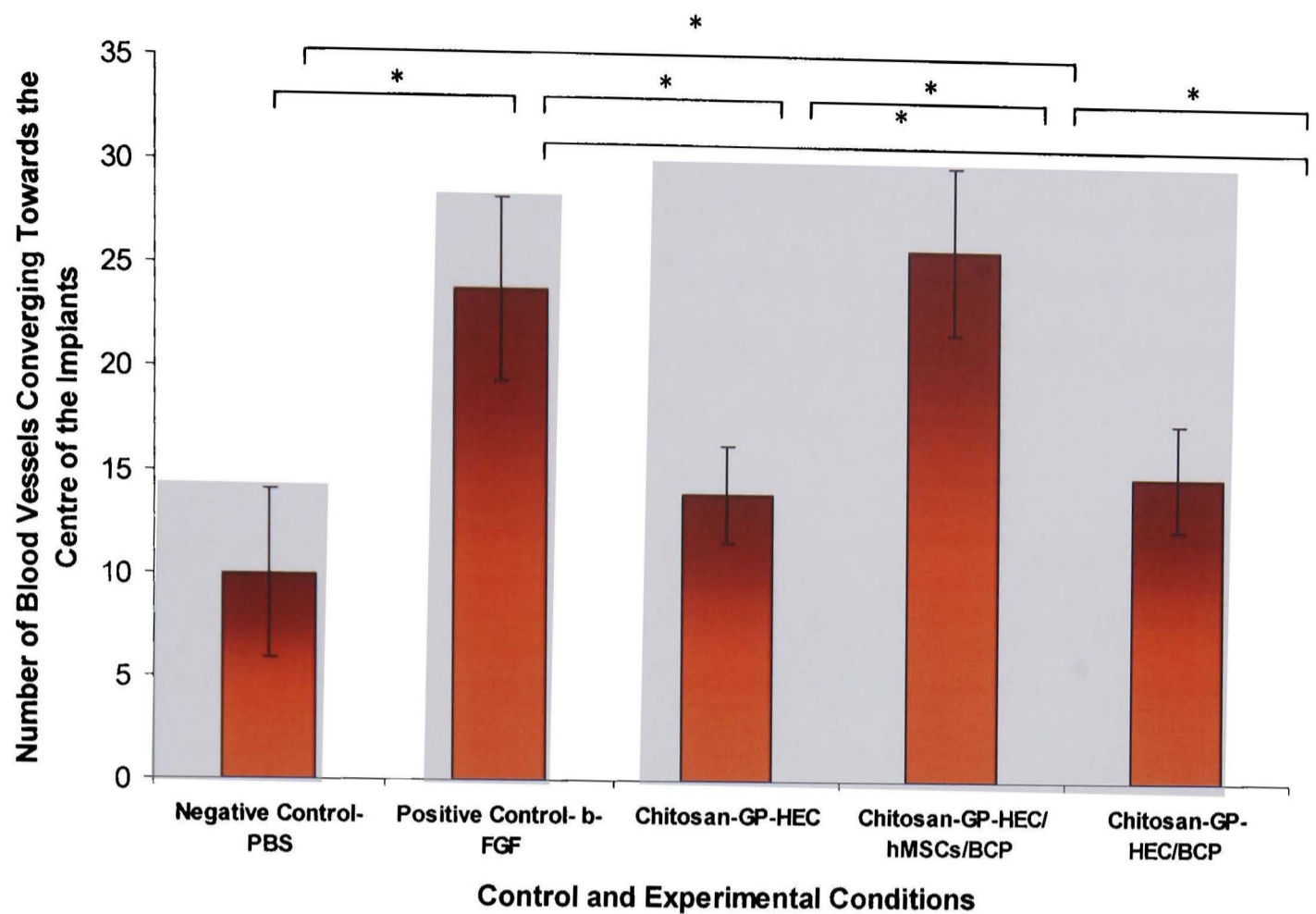
**Figure 6.3-** Bar graph showing the number of blood vessels converging towards the centre of the implants in the different conditions. *Statistically significant difference (*P*<0.05).

# 6.4 DISCUSSION

Angiogenesis is crucial for success in bone tissue engineering since cells within bone tissue engineering scaffolds must be alive after implantation in order to create a positive effect on the formation of bone (Kruyt *et al.*, 2003). Implanted cells *in vivo* depend on diffusion and vascularization for the nutrient and oxygen supply. As mentioned before, since the diffusion process can only supply the cells within a maximum range of 200 μm (Goldstein *et al.*, 2001) and the initial vascularization is not usually optimal, the cells in the middle of large constructs have often limited survival (Kneser *et al.*, 1999). Therefore in this study, the angiogenic potential of chitosan and hMSCs was examined in an attempt to increase the overall angiogenic response in orthotopic defects.

The results of this study demonstrated that hMSCs attached to BCP microparticles and combined with chitosan-GP-HEC lead to enhanced angiogenic effect in comparison with the negative control and chitosan-GP-HEC ± BCP microparticles when placed on the CAM for 3 days. Chitosan-GP-HEC does not enhance angiogenesis. The angiogenic response in this study was manifest as a spoke-wheel pattern. The spoke-wheel pattern is a well described indicator of angiogenesis, where an increased density of radially converging vessels occurs around the implanted tissue on the CAM (Oates *et al.*, 2007; Ribatti *et al.*, 1995).

The increased angiogenic potential of the chitosan-GP-HEC/hMSCs/BCP group observed in this study could be due to several factors relating to the presence of hMSCs. Based on previous work, hMSCs secrete VEGF which can stimulate recruitment and proliferation of endothelial cells as well as functioning as an angiogenic factor in bone healing (Furumatsu *et al.*, 2003; Kaigler *et al.*, 2003). As previously stated, mesenchymal stem cells produce angiogenic cytokines like b-FGF, hepatocyte growth factor, insulin-like growth factor 1, MCP-2 and MCP-3 (Chen *et al.*, 2003; Kamihata *et al.*, 2001; Kinnaird *et al.*, 2004; Nagaya *et al.*, 2004; Tang *et al.*, 2005). Factors such as b-FGF, a-FGF, and VEGF have direct mitogenic and chemotactic effects for endothelial cells and encourage tube formation *in vitro* (Rifkin and Moscatelli, 1989; Thomas, 1996). VEGF is produced by osteoblasts (Deckers *et al.*, 2000; Steinbrech *et al.*, 1999), mesenchymal progenitor cells with an osteogenic phenotype (Kaigler *et al.*, 2003), and undifferentiated mesenchymal progenitor cells (Furumatsu *et al.*, 2003). Also, undifferentiated mesenchymal progenitor cells can stimulate the sprouting of blood vessels *in vivo* (Al-Khaldi *et al.*, 2003; Furumatsu *et al.*, 2003).

According to the study performed by Potapova *et al.* (2007), concentrations of VEGF, b-FGF, angiogenin, and BMP-2 were raised from 5-20 times in hMSCs spheroid conditioned media (Potapova *et al.*, 2007). The hMSCs spheroids conditioned media stimulated umbilical vein endothelial cell proliferation, migration, and the invasion of the basement membrane. Moreover, the conditioned media encouraged the survival of endothelial cells *in vitro*. This study suggests that hMSCs produce angiogenic factors that can stimulate endothelial cell activity.

Considering the fact that there are still problems about the survival of mesenchymal stem cells orthotopically, further approaches and strategies could be used to improve vascularization inside tissue engineered constructs implanted in large bony defects. Besides blood vessel ingrowth from the host, strategies like improvement of scaffold architecture to enhance the ingrowth of blood vessels (Druecke *et al.*, 2004; Karageorgiou and Kaplan, 2005; Pinney *et al.*, 2000; Yang *et al.*, 2001), addition of angiogenic factors (Huang *et al.*, 2005-b; Leach *et al.*, 2006; Perets *et al.*, 2003; Richardson *et al.*, 2001), and *in vitro* (Choong *et al.*, 2006; Rouwkema *et al.*, 2006) or *in vivo* pre-vascularization have been studied (Akita *et al.*, 2004; Casabona *et al.*, 1998; Kim and Kim, 2005). These strategies could be applied to mesenchymal stem cells in large bony defects in order to enhance cell survival.

The results of the CAM assay regarding the angiogenic potential of chitosan presented in this study show a discrepancy with the work published by de Castro-Bernas (2003) and Biagini *et al.* (1989), since chitosan-GP-HEC was not found to be angiogenic. It should be noted that de Castro-Bernas (2003) failed to mention more specific details like the concentration of chitosan and the time point used in their study therefore making an exact comparison with this study more difficult. In terms of the study performed by Biagini *et al.* (1989), 0.8% N-Carboxymethyl chitosan was utilized. It is apparent that the concentration of chitosan used and the composition is different to the current study which could have an effect in the final outcome related to angiogenesis. It is also possible that longer time was needed in the CAM assay in order to observe angiogenic response with the chitosan although this is highly unlikely since 10 days was enough for hMSCs to demonstrate their angiogenic potential. Moreover, quantifying the CAM assay after 10 days is practised by many researchers. Interestingly on a different account, Mochizuki *et al.* (2007) performed a study in which the AG73 peptide covalently conjugated to a chitosan membrane was tested for angiogenesis using the CAM assay (Mochizuki *et al.*, 2007). The attractive point about this study was the results which showed that the chitosan membrane alone with the concentration of 1.67% (w/v) used as the control did not show angiogenic activity. This is in agreement with the results of this study regarding the angiogenic effects of chitosan. Also, the concentration of chitosan used by Mochizuki *et al.*

(2007) is similar to the chitosan concentration of 1.5% (w/v) used in this study. Based on the results from the present study, it can be concluded that chitosan with the concentration and composition used is not angiogenic.

The lack of angiogenic potential by chitosan is not expected to solve the cell survival problem in injectable bone tissue engineering. In case of the injectable tissue engineered bone applied orthotopically, it is likely that hMSCs would face survival problems despite the angiogenic potential of these cells. If chitosan was angiogenic then together with angiogenic potential of hMSCs, it might be possible to obtain an enhanced overall angiogenic response. Nevertheless, since this is not the case, the cell survival issue still remains to be solved.

# 6.5 CONCLUSION

Chitosan-GP-HEC gel does not demonstrate any angiogenic potential while the presence of hMSCs results in an increased angiogenic response following 3-day placement on the CAM. There are significantly more blood vessels formed for the group containing stem cells in comparison with other groups with the exception of b-FGF positive control. New approaches to solve the cell survival problem in injectable tissue engineered bone needs to be devised.

# CHAPTER VII

## OSTEOGENESIS BY MC3T3-E1 CELLS IS ENHANCED THROUGH CHITOSAN-BASED HYDROGELS

# 7.1 INTRODUCTION

In previous chapters, chitosan-based hydrogels were shown to be biocompatible when used with goat and human bone marrow derived mesenchymal stem cells (gMSCs, hMSCs). They provide a promising binder material for injectable bone tissue engineering applications and examining the osteogenic potential of cells exposed to these binders would be useful for future applications of these binders.

Various studies have shown a stimulatory effect of chitosan on bone formation (Klokkevold et al., 1996; Malette et al., 1986; Muzzarelli et al., 1994; Pang et al., 2005). Malette et al. (1986) demonstrated increased bone regeneration in radii of dogs following 6 weeks treatment with chitosan. Wounds were generated through the cortex right into the marrow cavity. The wounds treated with saline showed a osteoblastic-osteoclastic remodelling sequence with callus production but wounds in contact with chitosan produced direct cortical bone without callus formation. In a different study, Klokkevold et al. (1996) assessed the in vitro effect of chitosan on mesenchymal stem cells in terms of osteogenic differentiation and bone formation. Based on this study, 2 mg/ml chitosan created significantly higher numbers of bone nodules than the control group. However, as the cell population was not monitored, it was not clear whether chitosan stimulated proliferation of cells and subsequently nodule formation or if it directly enhanced nodule formation and therefore osteogenesis.

In addition, Pang et al. (2005) examined the effect of chitosan on bone formation in calvarial defects in rats and on proliferation and osteogenic differentiation of human periodontal ligament fibroblasts in vitro. They did not see a significant difference between the cell proliferation rates in the control group and the group containing 0.1 mg/ml chitosan. In contrast, they reported that 0.1 mg/ml chitosan enhanced the alkaline phosphatase (ALP) activity of the human periodontal ligament fibroblasts while the same concentration of chitosan gave rise to significantly higher amounts of bone after 8 weeks of implantation in 8 mm diameter calvarial defects. In another in vivo study, Muzzarelli et al. (1994) reported the use of chitosan containing imidazolyl groups in a 7 mm diameter defect in the femoral condyle of sheep. Following 40

days, there was more bone formation in the defects treated with chitosan compared to the control however, since quantitative analysis of bone formation was not included in the study; it was not possible to know if the difference was statistically significant.

In the current study, the effect of chitosan on undifferentiated preosteoblast cell line was evaluated in terms of osteogenic differentiation, cell proliferation and viability *in vitro* in order to examine whether chitosan has any effect on osteogenesis directly.

## 7.1.1 Aims and Objectives

The aim of the present study was to evaluate the effect of chitosan-based hydrogels on osteogenic properties of preosteoblast cell line (MC3T3-E1 cells). With this in mind, temporal assessment of MC3T3-E1 survival, growth and osteogenic differentiation when exposed to chitosan-containing media and control media was performed. pH and osmolality of different media containing chitosan in addition to control media was measured in order to examine whether presence of chitosan allows physiological pH and osmolality levels within the media.

# 7.2 MATERIALS AND METHODS

All chemicals were ordered from Sigma-Aldrich, UK unless otherwise indicated. The experiments were repeated three times. The n number for all the measurements was 6.

## 7.2.1 MC3T3-E1 Cell Culture and Seeding Conditions

Murine preosteoblast cell line, MC3T3-E1 cells (kindly provided by Department of Tissue Regeneration, Twente University, The Netherlands) were seeded at a density of $5 \times 10^3$ cells/cm$^2$ (Quarles *et al.*, 1992) on sterile 12 well-plates (Falcon, VWR international, UK). The same number of cells was exposed to both control and experimental groups. The control groups involved the cells exposed to the proliferation and differentiation medium. The proliferation medium was composed of α-MEM (Gibco Invitrogen, UK) supplemented with 10% (v/v) FBS (FBS, Gibco Invitrogen, UK), and 100 U/ml penicillin/100 μg/ml streptomycin and the differentiation medium consisted of α-MEM supplemented with 10% (v/v) FBS, 100 U/ml penicillin/100 μg/ml streptomycin, 10 mM β-glycerophosphate and 0.2 mM L-ascorbic acid 2-phosphate (AsAP) (Quarles *et al.*, 1992). The medium was refreshed every two days.

### 7.2.1.1 Preparation of Chitosan Samples

To prepare the experimental groups, chitosan (Kitomer, Marinard Biotech, Quebec, Canada) with a degree of deacetylation (DDA) of 94% and a molecular weight (Mw) of 679 kDa was sterilized by autoclaving at 126°C for 20 minutes. Sterile chitosan powder (0.225g) was dissolved in 15 ml of 0.18 M hydrochloric acid and diluted to the following concentrations: 2 mg chitosan/ml of proliferation medium, 0.05 mg chitosan/ml of proliferation medium, and 0.005 mg chitosan/ml of proliferation medium. The proliferation medium combined with various concentrations of chitosan known as the experimental groups were added to the cells in the 12 well plates. The medium was refreshed every two days.

## 7.2.2 MC3T3-E1 Survival, Growth and Osteogenic Differentiation Study

### 7.2.2.1 MC3T3-E1 Survival and Growth Study

The viability of the cells was evaluated at days 1, 3, 6, 10, and 15 through live/dead viability staining using Calcein AM and Ethidium homodimer-2 (Molecular Probes, USA). Briefly, the samples were washed with pre-warmed PBS. Subsequently, 2.5 μl of both stains were mixed with 1 ml of α-MEM and applied to the samples in 250 μl volume such that the samples were covered by the staining solution. Following this, the samples were kept at 37°C/5% $CO_2$ incubator for 15 minutes after which they were washed three times with pre-warmed α-MEM and viewed under Leica fluorescent microscope (Leica DMI 4000B, UK) equipped with a Leica DFC camera (Leica DFC 300 FX, UK). In the live/dead viability assay, metabolically active cells, covert Calcein AM into green fluorescent Calcein by intracellular esterases, whereas Ethidium homodimer enters the dead cells and binds to deoxyribonucleic acid (DNA) through damaged membranes. Viable and dead cells are presented as green and red respectively. The DNA contents of the samples were also measured at days 1, 3, 6, 10, and 15 using the supernatant of the samples obtained in order to evaluate the number of cells over time. The cyquant assay kit was employed to measure the DNA contents following manufacturer's instructions and using a Fluorimeter. The DNA solution obtained was spectrometrically analysed using emissions measured at 485 and 520 nm by a Hitachi U- 2000 Spectrophotometer. A standard calibration curve based on the DNA measurements of known numbers of hMSCs was used for quantification of the number of cells present.

### 7.2.2.2 MC3T3-E1 Osteogenic Differentiation Study

The ALP activity and protein content were measured at days 1, 3, 6, 10, and 15. The samples were washed with pre-warmed PBS and stored at -80°C freezer (New Brunswick Scientific, USA). The samples from all time points were defrosted at once, lysed by the addition of 1 ml of 0.2% Triton X-100 followed by sonication (Decon Ultrasonics, UK). The ALP activity in the supernatant was measured as the release of p-nitrophenol from p-nitrophenylphosphate substrate over a period of 1 hour (AP 307

kit, Randox Laboratories, UK) by assessing the absorbance at 405 nm wavelength on a ELx808 Ultra Microplate Reader (Bio-Tek Instruments, USA). A standard calibration curve based on the absorbance measurements of known concentrations of p-nitrophenol standard solution were used to compare the values obtained. The total protein content was evaluated with bicinchoninic acid (BCA) protein assay kit (product no: 23227, Pierce, USA) following manufacturer's instructions to normalize the ALP activity levels. This assay applies a reactive solution of bicinchoninic acid (BCA) and $CuSO_4$. Proteins of the cells reduce $Cu^{2+}$ ions into $Cu^{+1}$ ions, which make a complex with BCA. The absorbance was measured at 562 nm wavelength on a ELx808 Ultra Microplate Reader. A series of bovine serum albumin (BSA) was used as standards. The ALP activity levels were normalized to the total protein content at the end of the experiment (expressed as nmol P-nitrophenol/mg protein/hr).

## 7.2.3 pH Measurements

The pH of the control and experimental media were measured using an electronic pH meter (Fisherbrand Hydrus 300, Orin Research Incorporation, USA) to evaluate whether different concentrations of chitosan in the experimental groups have physiological pH levels.

## 7.2.4 Osmolality of Various Media (Control and Experimental Groups)

The osmolality of 200 µl aliquots of the control and experimental media were measured by the Advanced Osmometer (Model 3250, Advanced Instruments Inc., USA), in order to assess whether addition of chitosan in the media exposed to the cells would result in physiological osmolality. Standard osmolality solutions were used to calibrate the machine before performing the measurements.

## 7.2.5 Statistical Analysis

Results were analyzed using two sample two-tail Student's *t*-test and one-way analysis of variance (ANOVA) followed by a post hoc least significant difference

(LSD) test for multiple comparisons. Data are presented in mean ± standard deviation. A $p$ value of <0.05 indicated statistical significance.

# 7.3 RESULTS

## 7.3.1 MC3T3-E1 Survival, Growth and Osteogenic Differentiation

### 7.3.1.1 MC3T3-E1 Survival

The number of cells increases in all groups during the first 6 days of culture leading to a confluent layer of cells (Figure 7.1). However, there are significantly more viable cells when MC3T3-E1 cells are exposed to proliferation and differentiation media (Figure 7.1i and 7.1j). MC3T3-E1 cells exposed to various types of media are viable with limited number of dead cells following 15 days of culture (Figure 7.2).
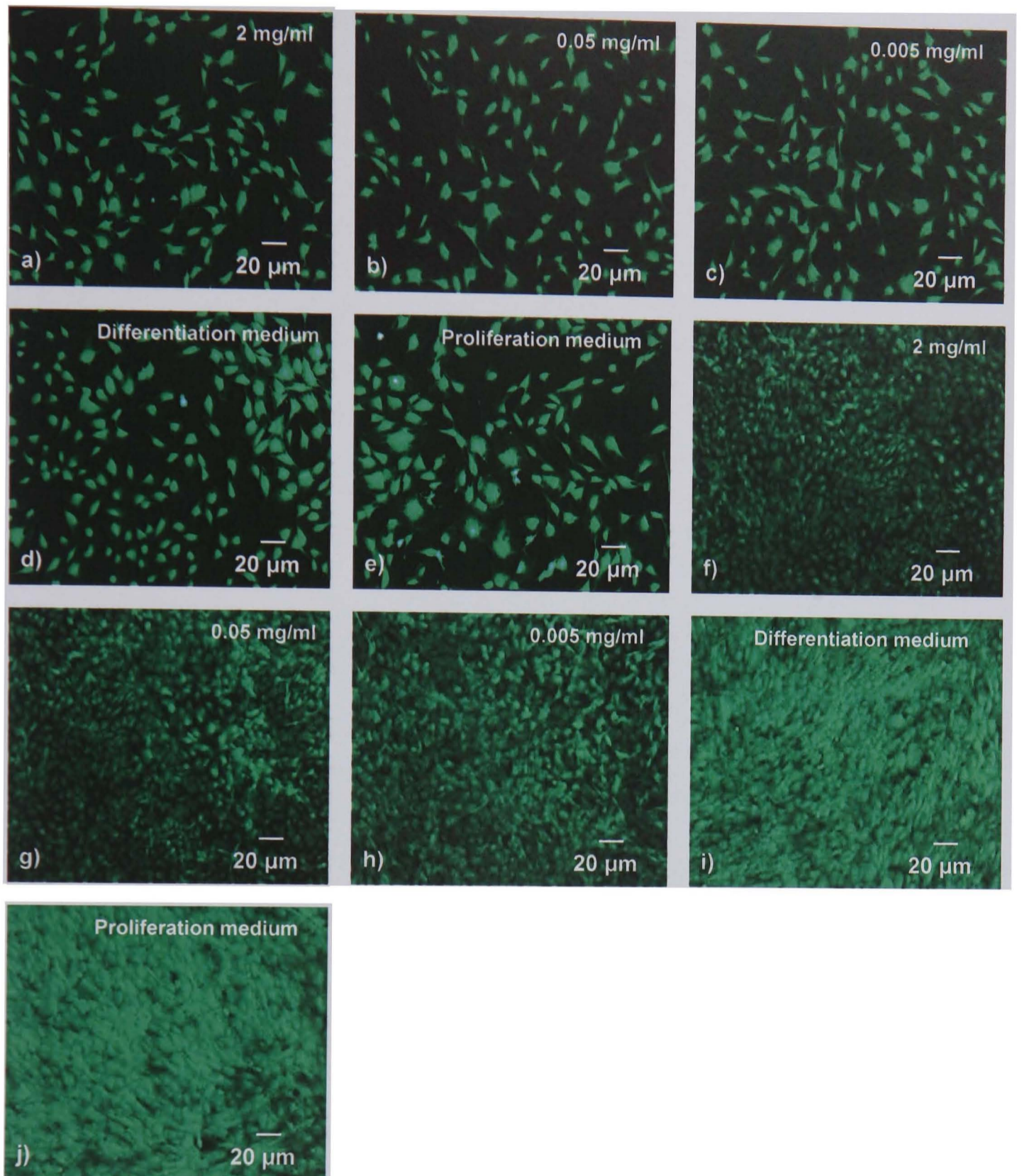
**Figure 7.1-** MC3T3-E1 cells on tissue culture plastic at day 1 in a) 2 mg/ml chitosan, b) 0.05 mg/ml chitosan, c) 0.005 mg/ml chitosan, d) differentiation medium, e) proliferation medium; MC3T3-E1 cells on tissue culture plastic at day 6 in f) 2 mg/ml chitosan, g) 0.05 mg/ml chitosan, h) 0.005 mg/ml chitosan, i) differentiation medium, j) proliferation medium.
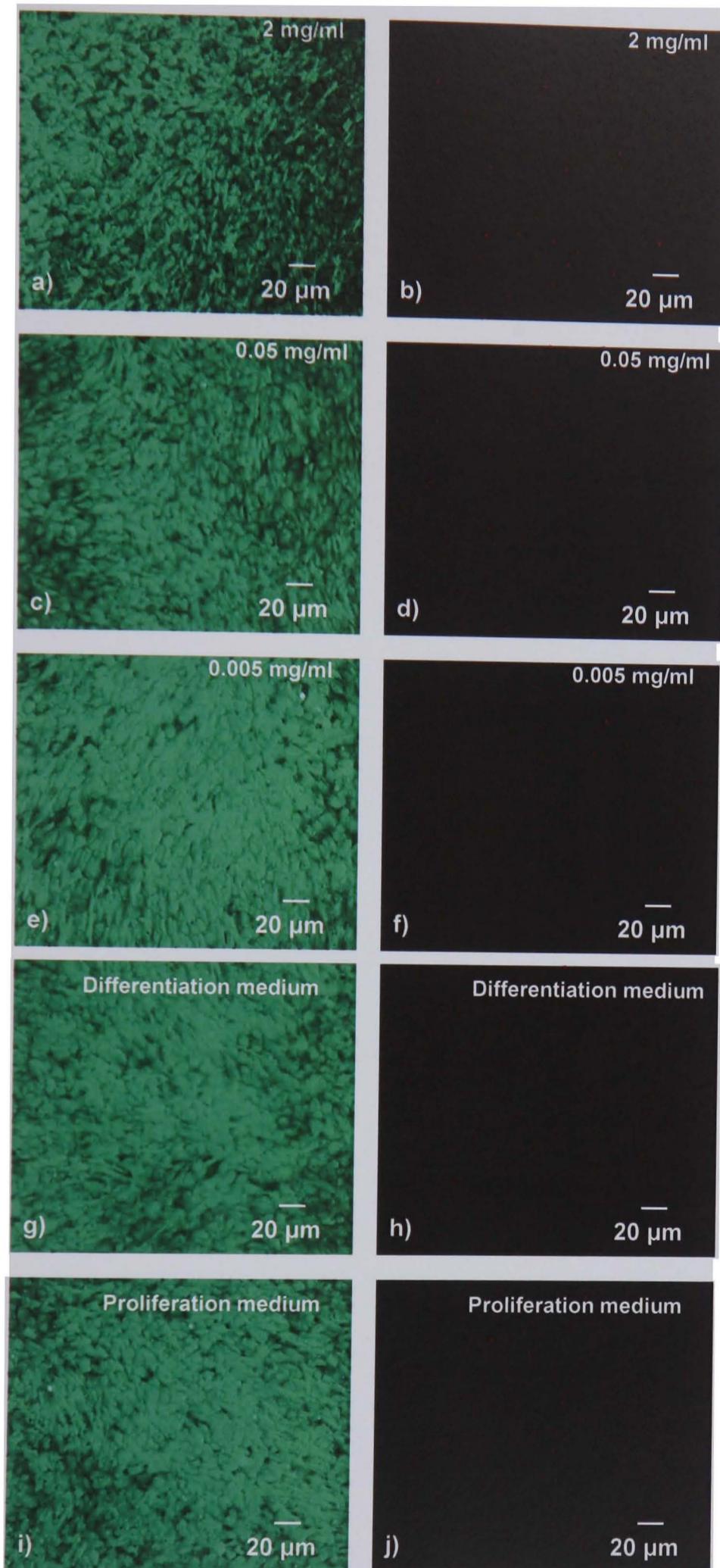
**Figure 7.2-** MC3T3-E1 cells on tissue culture plastic at day 15 in a) 2 mg/ml chitosan, c) 0.05 mg/ml chitosan, e) 0.005 mg/ml chitosan, g) differentiation medium, i) proliferation medium; dead MC3T3-E1 cells on tissue culture plastic at day 15 in b) 2 mg/ml chitosan, d) 0.05 mg/ml chitosan, f) 0.005 mg/ml chitosan, h) differentiation medium, j) proliferation medium.

## 7.3.1.2 MC3T3-E1 Growth

The number of cells exposed to different conditions was quantified based on DNA content measurement (Figure 7.3). The cells proliferate in all conditions throughout the experiment. After 6 days, cells exposed to the chitosan groups show a significantly reduced cell proliferation compared to the control groups. At later time points however, the cells demonstrate similar proliferation between different conditions (Figure 7.3). At days 10 and 15, the cells continue to proliferate but there are no significant differences between any of the groups. It should be noted that the x-axis of Figure 7.3 is not linear while the y-axis is linear.



■ 2 mg/ml chitosan  ■ 0.05 mg/ml chitosan  □ 0.005 mg/ml chitosan  □ Proliferation medium  ■ Differentiation medium
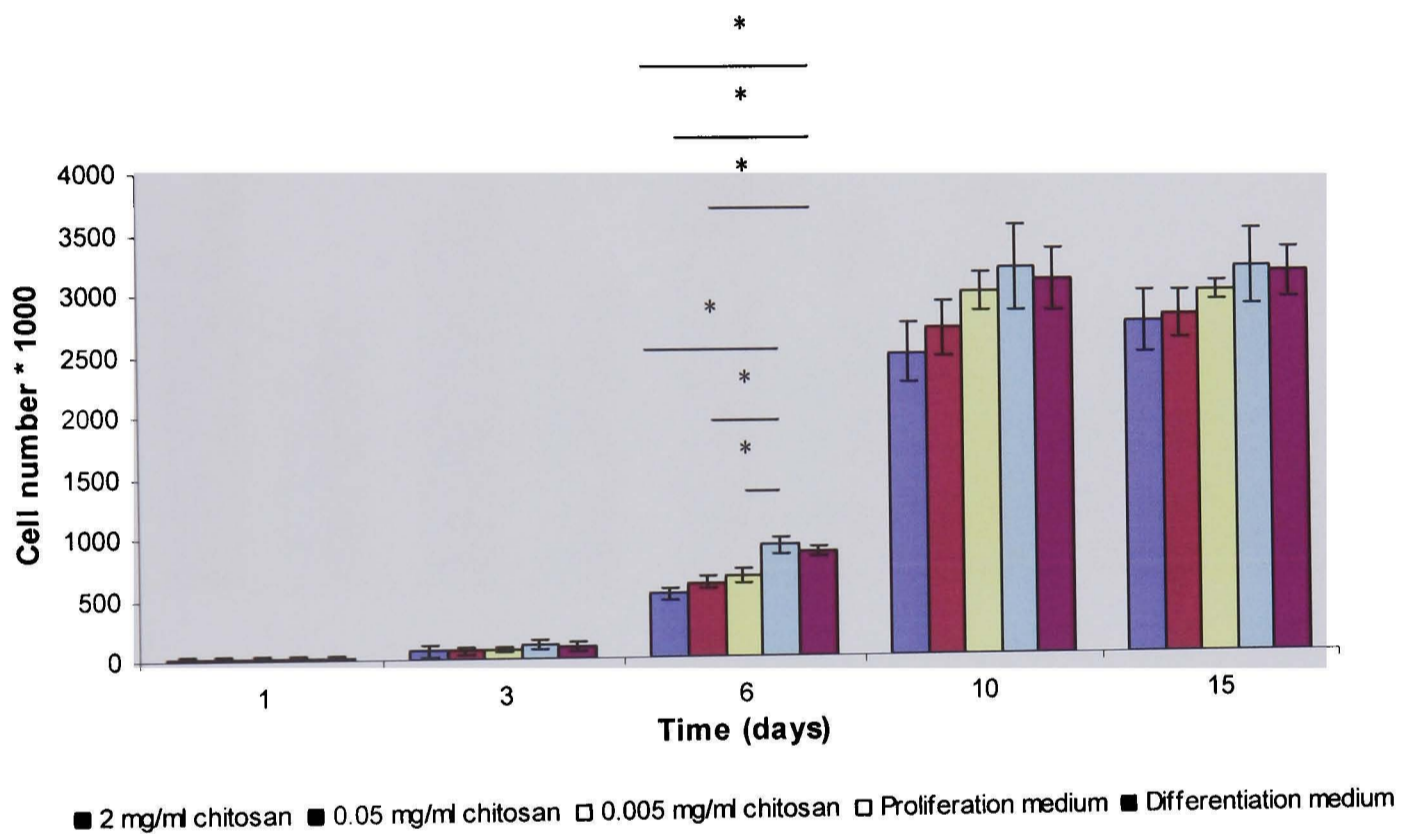
**Figure 7.3-** MC3T3-E1 cells proliferation in different groups. * Statistically significantly different ($P<0.05$).

## 7.3.1.3 MC3T3-E1 Osteogenic Differentiation

With respect to the osteogenic differentiation of MC3T3-E1 cells, addition of the osteogenic medium results in significantly higher ALP activity for all different time points as shown in Figure 7.4 ($P<0.05$). Chitosan concentrations of 2 mg/ml and 0.05 mg/ml result in significantly higher ALP activity compared to the differentiation medium. The peak of ALP activity is between days 6 and 10 followed by a reduction at day 15.
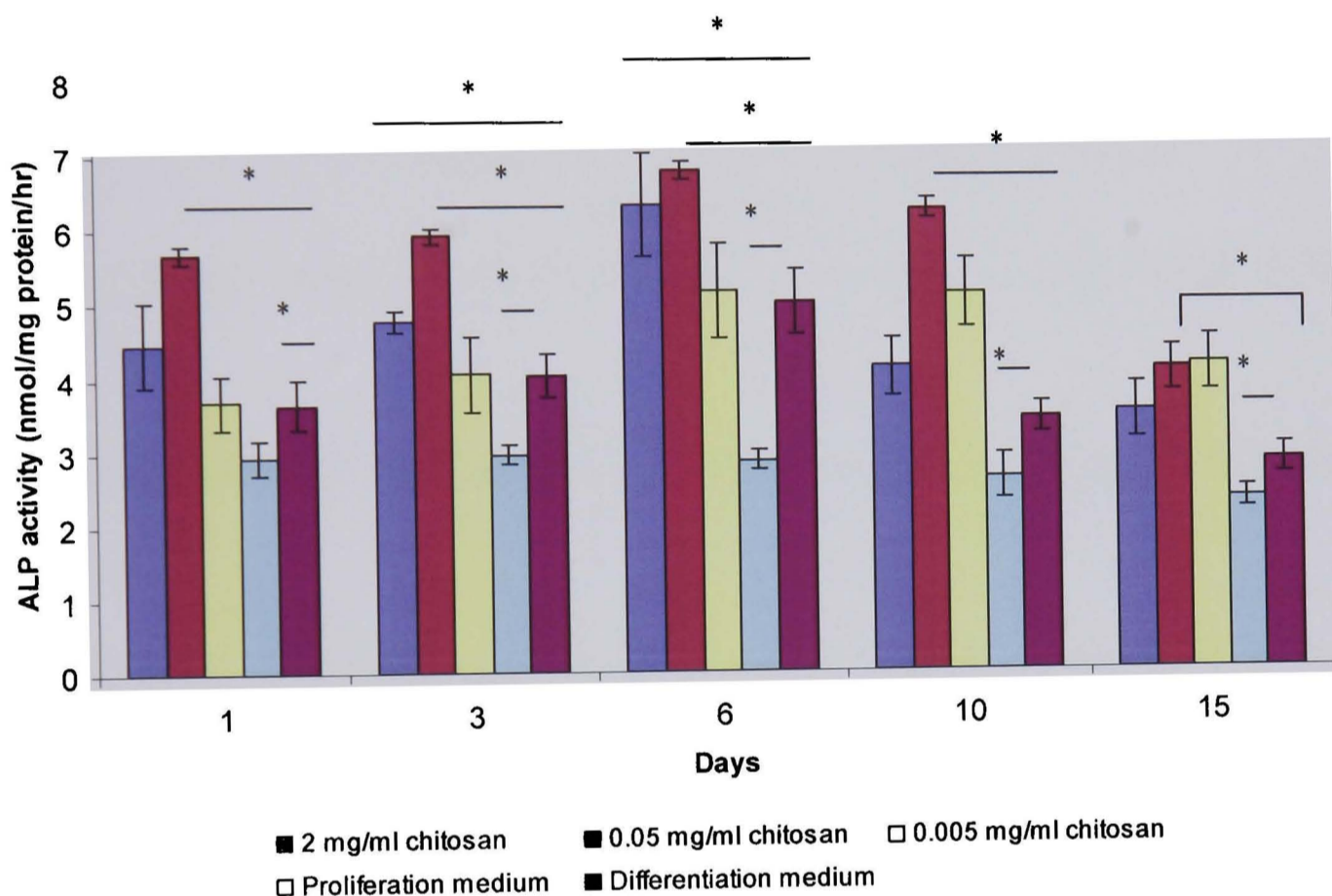


**Figure 7.4-** Osteogenic differentiation of MC3T3-E1 cells assessed by the measurement of ALP activity per protein content per hour. * Statistically significantly different ($P<0.05$).

## 7.3.2 pH Measurements

The pH of all media are around the physiological levels (i.e. pH 7.4) except for the 2 mg/ml chitosan group for which the pH level is 6.4 which is statistically significantly lower compared to the rest of the groups (Figure 7.5).
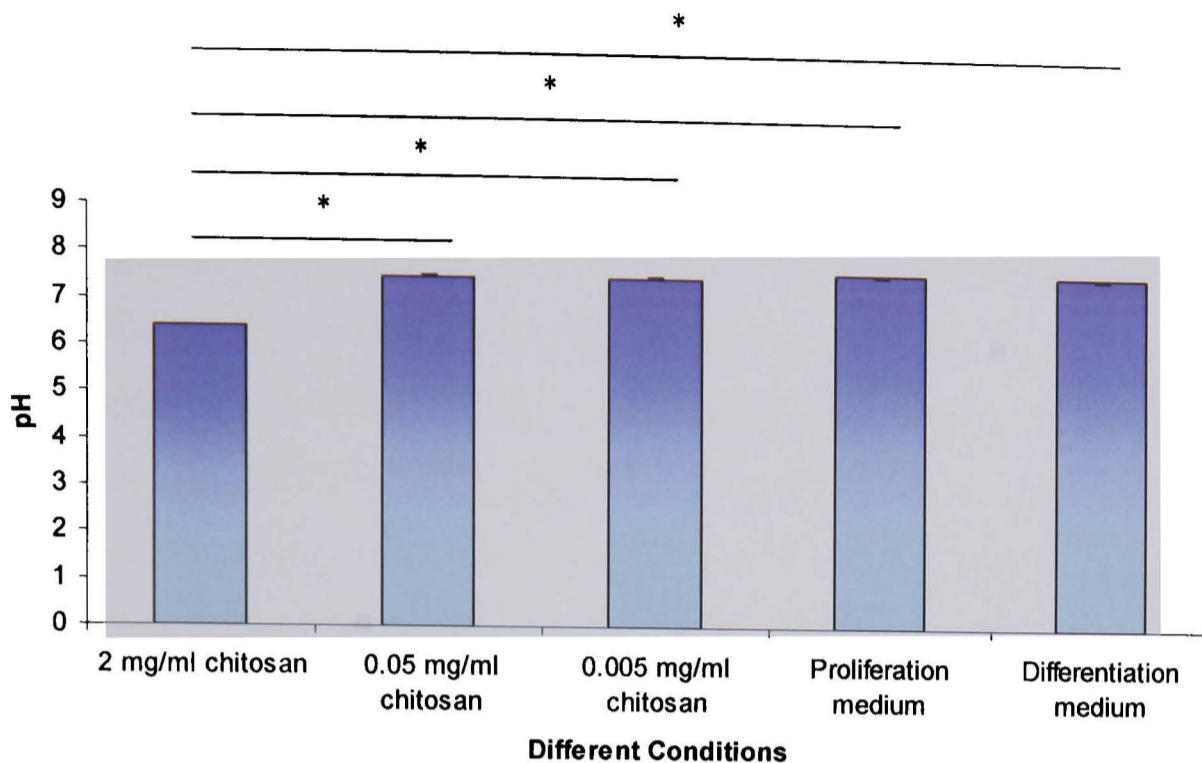
**Figure 7.5-** pH values of different media. * Statistically significantly different (*P*<0.05).

## 7.3.3 Osmolality of Various Media (Control and Experimental Groups)

The osmolality values are within the physiological range (i.e. around 285 mOsm/kg H2O (Davies *et al.*, 2001)) except for the 2 mg/ml chitosan group for which the osmolality is statistically significantly higher compared to all the other groups.
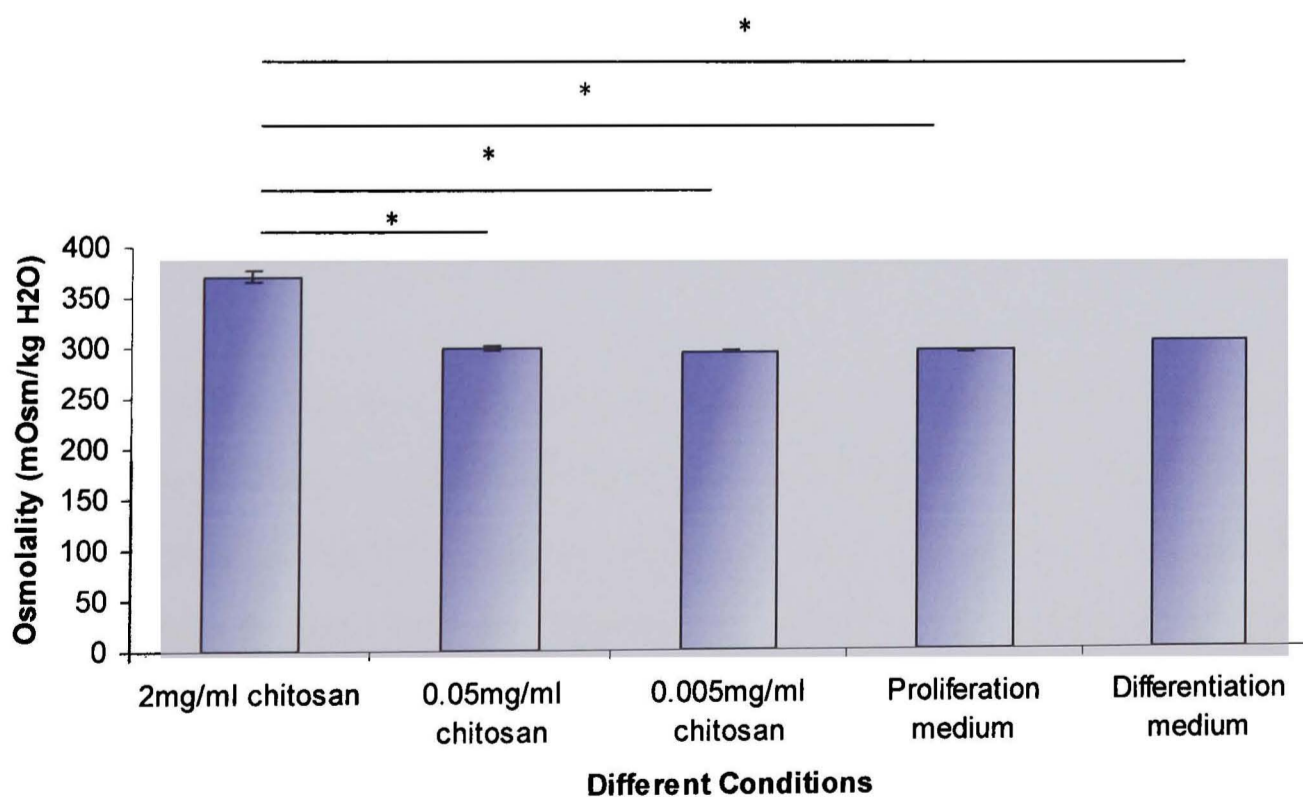


**Figure 7.6-** Osmolality of various media. * Statistically significantly different (*P*<0.05).

# 7.4 DISCUSSION

The results of this study showed that chitosan concentrations of 2 mg/ml and 0.05 mg/ml enhance the ALP activity levels of MC3T3-E1 cells compared to the control groups (proliferation and differentiation media). These findings support the positive effect of chitosan on osteogenic activity of MC3T3-E1 cells. The ALP activity trend in media containing different chitosan concentrations is similar to the differentiation medium. For MC3T3-E1 cells exposed to differentiation medium and media containing various chitosan concentrations, the ALP activity reached its peak between days 3 and 6 followed by a decrease at day 10. Previous studies also observed the same pattern of ALP activity over time (Kim *et al.*, 2005; Ueno *et al.*, 2001-b). MC3T3-E1 cells exposed to all groups continue to proliferate throughout the study with limited number of dead cells. The pH and osmolality of all the groups are around the physiological levels (i.e. pH 7.4 and osmolality around 285 mOsm/kg H2O (Davies *et al.*, 2001)) except for the 2 mg/ml chitosan group.

MC3T3-E1 cells are preosteoblastic cell line derived from neonatal mice calvaria which show a chronological expression of osteogenic features similar to bone formation *in vivo* (Quarles *et al.*, 1992). In other words, alkaline phosphatase (ALP) production, collagen synthesis, extracellular matrix deposition and mineralization of extracellular matrix which are associated with different phases of osteoblast development are all observed when MC3T3-E1 cells are cultured with appropriate stimuli (e.g. ascorbate and β-glycerophosphate) (Quarles *et al.*, 1992). Therefore, these cells provide an excellent model to study osteogenesis by an external factor.

Pang *et al.* (2005) studied the effect of chitosan on proliferation and osteogenic differentiation of human periodontal ligament fibroblasts *in vitro*. They reported that increased concentrations of chitosan were associated with reduced cell proliferation with 2 mg/ml chitosan showing significantly lower cell proliferation. This is in accordance with our results since as shown in Figure 7.3, a reduced cell proliferation was observed when MC3T3-E1 cells were exposed to increasing concentrations of chitosan although these differences were not significant except at day 6. These findings suggest that when MC3T3-E1 cells are in contact with different

concentrations of chitosan, the proliferation is slowed at initial time points (i.e. day 6) which corresponds to the highest ALP activity levels for these groups compared to the control groups because cells cease to proliferate when they start to differentiate (Conget and Minguell, 1999).

Chitosan has been shown to support and stimulate bone formation (Klokkevold et al., 1996; Malette et al., 1986; Muzzarelli et al., 1994; Pang et al., 2005). The results of this study support this since the ALP activity levels for 2 mg/ml chitosan and 0.05 mg/ml chitosan groups are higher as compared to the control groups at almost all time points. This demonstrates the stimulation of osteogenic differentiation of MC3T3-E1 cells when they are in contact with the above mentioned concentrations of chitosan.

Klokkevold et al. (1996) examined the effect of chitosan exposure on mesenchymal stem cells with regard to osteogenic differentiation and bone formation. Nevertheless, in their study, the number of cells was not counted and therefore it was not clear whether chitosan stimulated proliferation of cells and as a result more nodule formation or if it in fact increased nodule formation and consequently osteogenesis directly. However, the results of the current study indicate that chitosan enhances osteogenic differentiation of MC3T3-E1 cells. Moreover, freeze dried chitosan scaffolds have been observed to support osteoblast proliferation and differentiation (Seol et al., 2004). It should be noted that chitosan extracts were used in the present study however, both chitosan extracts and freeze dried chitosan (Seol et al., 2004) support osteoblast differentiation which suggests that the osteogenic stimulation takes place in both forms. Also, chitosan combined with hydroxyapatite offers an excellent scaffold for attachment, proliferation and differentiation of goat bone marrow derived stromal cells (Oliveira et al., 2006). All these information confirm the positive effects of chitosan on osteogenesis and cell attachment.

Comparing the results of chapter 4 with this chapter, it is noticed that the osteogenic differentiation of hMSCs is not enhanced when encapsulated with chitosan-GP-HEC which is contradictory to the results obtained when MC3T3-E1 cells are cultured with 2 and 0.05 mg/ml chitosan concentrations. However, the differences observed could be due to different set-up, different experimental conditions or the fact that chitosan

was mixed with GP, HEC and BCP when it was in contact with hMSCs. Nonetheless, since the results obtained with MC3T3-E1 cells are different to those of hMSCs with regard to osteogenic differentiation, this might suggest that the results obtained form MC3T3-E1 cells might not be directly relevant regarding the clinical application of injectable tissue engineered bone.

Chitosan was used in the range of 0.01-2 mg/ml in previous studies and was shown to be biocompatible when used within that range (Klokkevold *et al.*, 1996; Pang *et al.*, 2005). Therefore, in the preset study, chitosan concentrations of 0.05 and 2 mg/ml were used. Low chitosan concentration of 0.005 mg/ml was also used in this study to examine the osteogenic potential of cells exposed to such low concentration. It should be noted that 0.005 mg/ml chitosan shows lower ALP activity levels in comparison with 2 mg/ml and 0.05 mg/ml chitosan. This might be because of the lower concentrations of chitosan. Even though the chitosan concentration is the lowest in the 0.005 mg/ml group however, it still produces higher ALP activity than the negative control (proliferation medium) group. Chitosan concentration of 0.05 mg/ml is the most favourite concentration with respect to ALP activity and osteogenic differentiation, pH and osmolality levels in comparison with 2 mg/ml and 0.005 mg/ml. This is partly due to the fact that 2 mg/ml chitosan produces significantly lower pH levels compared to the rest of the groups as mentioned earlier. Also, the osmolality of the 2 mg/ml chitosan group is significantly higher relative to all the other groups however, the osmolality for bioencapsulation should be ideally around 270-340 mOsm/kg H2O (Chenite *et al.*, 2002).

The mechanism by which chitosan stimulates osteogenic differentiation is unknown. Due to its N-acetylglucosamine repeating units, chitosan has structural similarity to glycosaminoglycans especially hyaluronic acid (Sandford, 1989) and therefore may imitate their functional activities (Pang *et al.*, 2005). Hyaluronic acid facilitates progenitor cells migration thus assisting tissue regeneration (Zhu *et al.*, 2006). Also, chitosan has been suggested to increase the migration and differentiation of preosteoblastic progenitor cells (Klokkevold *et al.*, 1996; Malette *et al.*, 1986). Further studies are needed to understand chitosan's mechanism of action.

# 7.5 CONCLUSION

Chitosan concentrations of 2 mg/ml and 0.05 mg/ml enhance the ALP activity levels of MC3T3-E1 cells compared to the control groups. MC3T3-E1 cells exposed to all groups continue to proliferate throughout the study with limited number of dead cells. These results support the positive effect of chitosan on osteogenic activity of MC3T3-E1 cells.

# CHAPTER VIII

## GENERAL DISCUSSION

# 8.1 GENERAL DISCUSSION

This chapter reviews and discusses different *in vitro* and *in vivo* studies performed regarding the injectable tissue engineered bone formulations. Various studies described in this thesis (chapters 3-7) have shown that chitosan-glycerol phosphate (GP) is a biocompatible binder which undergoes sol-gel transition around body temperature. Moreover, extracts of all chitosan concentrations combined with or without 5% (w/v) GP and 2% (w/v) chitosan mixed with 10% (w/v) GP demonstrated up to 34% increase in proliferation rate of goat bone marrow derived mesenchymal stem cells (gMSCs) in comparison with the control medium. Addition of hydroxyethyl cellulose (HEC) to chitosan-GP hydrogels allowed cross-linking of the chitosan and thus enhancing gel rigidity (Hoemann *et al.*, 2002; Hoemann *et al.*, 2005; Roughley *et al.*, 2006). Furthermore, when HEC was added at 0.18% (w/v) concentration to 1.5% (w/v) chitosan-15% (w/v) GP, the cell survival, growth and osteogenic differentiation were not affected. Chitosan-GP-HEC was found to be degradable as well as having an increased gelation rate compared to chitosan-GP alone. With regard to the *in vivo* bone formation ability, gMSCs produced bone when surrounded by chitosan-GP-HEC after a 6-week subcutaneous implantation in nude mice which further confirmed the results obtained *in vitro* concerning the suitability of chitosan-GP-HEC as an injectable binder for bone formation. Additionally, chitosan-based binder was demonstrated to be non-angiogenic unlike human bone marrow derived mesenchymal stem cells (hMSCs) which showed angiogenic potential. Also, chitosan was found to enhance osteogenic activity of MC3T3-E1 cells at 2 and 0.05 mg/ml concentrations.

## 8.1.1 *In Vitro* Gelation and Degradation of Chitosan-GP ± HEC

Chitosan-GP solutions set at 37°C as expected therefore making them suitable to act as an injectable tissue engineered bone binder which needs to gel at body temperature when applied to the defect site (chapters 3-4). In addition, chitosan-GP demonstrated fast gelation properties such that a gelation time of less than 1 minute was seen when 15-20% (w/v) GP was added to 1-2% (w/v) chitosan. This is because increasing GP concentrations lead to reduced gelation time (chapter 3) which is in accordance with

the study performed by Ganji *et al.* (2007), nevertheless, in their study, an inverted test tube method was used to measure the gelation time as opposed to rheometry which is more accurate.

With regard to factors influencing the gelation time, it should be noted that chitosan concentration is influential such that higher chitosan concentrations result in decreased gelation time when mixed with the same amount of GP (chapter 3). This pattern was also detected by Ganji *et al.* (2007) whereby 2% (w/v) chitosan led to decreased gelation time in comparison with 1% (w/v) chitosan when mixed with similar GP quantities. In terms of the reasons behind this trend, it is known that increasing chitosan and GP concentration accelerates the gelation process owing to an increase of intermolecular interactions and entanglements (Cho *et al.*, 2006). Moreover, in a separate study, Montembault *et al.* (2005) evaluated the effect of chitosan concentration on gelation time with no external cross-linking agents in acetic acid-water-propanediol solution and demonstrated that higher chitosan concentrations result in lower gelation time. It was then suggested that more entangled polymer chains were responsible for faster gelation when higher polymer concentrations were involved.

To understand the influence of gelation temperature with regard to pH and GP concentration, Chenite *et al.* (2001) performed a study and observed that higher GP concentration which is coupled to higher pH, results in lower gelation temperature. They suggested that the number of charged ammonium groups on the chitosan chain is influential regarding the control of the gelation. A reduction in charge density on chitosan chains causes reduction of interchain electrostatic repulsion, thus leading to smaller amount of thermal energy required to start the gelation. The gelation results presented in chapter 3 are in agreement with this since higher GP concentration and thus higher pH, led to smaller addition of thermal energy necessary to initiate the gelation. However, chitosan solutions mixed with lower GP concentrations required more thermal energy which could be achieved by increasing the gelation time or gelation temperature.

In order to increase the rigidity of the chitosan-GP hydrogels such that biphasic calcium phosphate microparticles (BCP) could be homogeneously distributed with the gel network, HEC was added. The gelation time decreased significantly with increasing concentrations of HEC, in other words, presence of HEC increased the gelation rate (chapter 4). This is in agreement with the studies performed by Hoemann et al. (2007) and Li and Xu (2002). It should be noted that the interaction of negatively charged GP with highly protonated cationic chitosan through electrostatic attractions is one of the forces responsible for the gelation (Chenite et al., 2000). As suggested by Li and Xu (2002), this system is made of molecular aggregates of neutral origin presented as colloidal precipitates resulting in a weak structure and low storage modulus. The neutral molecule HEC, is capable of binding with chitosan through hydrogen bonding thus resulting in bridge formation between chitosan molecules (Li and Xu, 2002). Higher HEC content leads to stronger hydrogels primarily as a result of higher number of hydrogen bonding in the hydrogel structure (Li and Xu, 2002). Faster gelation rates which result from higher HEC content inside the gels could also be related to enhanced hydrogen bonding within the gel.

Regarding the gelation temperature, chitosan-GP-HEC solutions gel at 37°C similar to chitosan-GP solutions. Therefore, the presence of HEC in chitosan-GP hydrogels is beneficial in terms of increasing the integrity of the gel and decreasing the gelation time while still being able to undergo gelation at 37°C. Gelation time of a few minutes is suitable for injectable applications of tissue engineered bone which is achieved in this thesis (chapters 3-4).

Other than having the appropriate gelation times, chitosan-GP ± HEC hydrogels have physiological pH levels which is needed when cells are combined with such hydrogels as explained in section 8.1.2.

The other important characteristic of an injectable binder is the biodegradability, since an injectable binder for tissue engineered bone needs to degrade in a timely fashion after being placed in the defect in order to create space for bone formation. Nonetheless, it should not have rapid degradation since injectable binders have to

remain for days and not hours. The degradation behaviour of chitosan-GP ± HEC hydrogels was evaluated in chapter 4. Lysozyme was used in the degradation medium at 10 mg/L to imitate physiological conditions in which lysozyme is present in plasma at around 4-13 mg/L (Henry, 1991). The pH of the phosphate buffered saline (PBS) and PBS combined with lysozyme solutions in degradation studies was around the physiological level throughout the experiment demonstrating that the degradation products of chitosan-GP-HEC maintain the pH at physiological range. Lower HEC concentrations resulted in faster degradation rates when chitosan-GP-HEC hydrogels were exposed to PBS or PBS mixed with lysozyme. Moreover, additional confirmation was seen in scanning electron microscopy images in which chitosan-GP was degrading at a faster rate in comparison with chitosan-GP-0.18% (w/v) HEC after 42 days of exposure with lysozyme. This could be related to the lower number of hydrogen bonds bridging chitosan molecules (Li and Xu, 2002) and thus leading to relatively weaker network more subject to degradation. Also, the existence of another polymer i.e. HEC could cause reduced chitosan degradation primarily because of limitation of lysozymal transport (Huang *et al.*, 2005-a).

The degradation results in chapter 4 showed that presence of lysozyme lead to higher degradation rates. Lysozyme is well-known to digest chitosan according to the amount and distribution of N-acetyl groups (Aiba, 1992). In addition, several studies have shown degradability of chitosan in contact with lysozyme (Huang *et al.*, 2005-a; Moshfeghian *et al.*, 2006). As presented in chapter 4, the percentage dry and wet weight remaining for chitosan-GP ± HEC hydrogels decreased throughout the 42 day period (without having rapid degradation) which was further evidenced by scanning electron microscopy images showing the external morphology of the gels over the 42 days. Based on the results obtained in this thesis (chapter 4), chitosan-GP ± HEC binders meet the degradability requirement for application as a tissue engineered bone binder. Also, in the *in vivo* bone formation study, the behaviour of chitosan-GP-HEC binder was further evaluated in terms of *in vivo* degradation, which is discussed in section 8.1.3.

## 8.1.2 *In Vitro* Cell Survival, Growth and Osteogenic Differentiation of Mesenchymal Stem Cells Combined with Chitosan-GP-HEC

The biocompatibility of the injectable tissue engineered bone binder is of paramount importance since the binder is required to be in contact with the bone producing cells and hence it must not be toxic to the cells. Moreover, an ideal binder should allow cell survival, growth and differentiation towards the osteogenic lineage when surrounding the cells. All these features were investigated in chapters 3-4.

Initially, the biocompatibility of chitosan-GP was studied in chapters 3-4 followed by chitosan-GP-HEC. The results of chapter 3 showed improved cell proliferation relative to the control ranging from 18-34% with extracts from 0.5-2% (w/v) chitosan mixed with 5% (w/v) GP. Chitosan-GP extracts with higher concentrations of chitosan resulted in more cell proliferation. For example, in the case of extracts from 10% (w/v) GP combined with chitosan, there was improved cell proliferation with 2% (w/v) chitosan and decreased cell proliferation with 0.5-1.5% (w/v) chitosan. As previously discussed, this could possibly be due to two reasons. Firstly, it could be because of higher chitosan concentrations requiring more GP to react with the amine group as the electrostatic interactions between the chitosan and the GP is one of the forces involved in gel formation (Chenite *et al.*, 2000). When more GP is reacting with the chitosan, there would thus be less leaching to the outside medium which is the extraction medium and therefore better cell growth response. Secondly, higher chitosan concentrations result in higher degradation products and thus stimulating higher cell growth. Concerning the second point, chitosan has been shown to cause enhanced cell proliferation response as Howling *et al.* (2001) studied the effect of chitin and chitosan on the proliferation of human skin fibroblasts and keratinocytes. They observed about 50% increase in the proliferation rate of fibroblasts over the control group when they were treated with an initial chitosan concentration of 50 µg/ml in culture media. The mechanism by which chitosan stimulates cell growth is unknown nevertheless, chitosan may indirectly enhance fibroblast proliferation through the formation of poly-electrolyte complexes with serum components like

heparin (Mori *et al.*, 1997), or potentiating growth factors like platelet derived growth factor (Inui *et al.*, 1995).

After learning about the positive response of chitosan-GP hydrogels with regard to biocompatibility as discussed above, it was important to know the biocompatibility of the hydrogels when HEC was added. As explained before, HEC was added to chitosan-GP hydrogels to improve the rigidity of the gels so that BCP microparticles could be distributed homogenously within the gel network. Various tests were performed in chapter 4 to investigate cell survival, growth and osteogenic differentiation of mesenchymal stem cells when in contact with chitosan-GP-HEC hydrogels.

With respect to direct contact biocompatibility tests, cells exposed to higher concentrations of HEC were not growing as well as the control and the groups containing 0 and 0.18% (w/v) HEC as observed by the significantly lower number of cells in these groups (chapter 4). As suggested by Hoemann *et al.* (2007), HEC contains glyoxal. Glyoxal is a toxic by-product of advanced glycation endproducts (AGE) (Shangari *et al.*, 2003) which has caused apoptosis following extended contact with lung epithelial cells at 0.1 mM concentration (Kasper *et al.*, 2000). Higher HEC concentrations therefore having higher glyoxal levels give rise to higher cytotoxicity response and thus cell death which is the reason behind lower cell growth observed.

The pH values of 1.5% (w/v) chitosan-15% (w/v) GP combined with 0.18% (w/v) HEC was around the physiological range. Based on the biocompatibility and pH results, chitosan concentration of 1.5% (w/v) mixed with 15% (w/v) GP and 0.18% (w/v) HEC was chosen as the ideal composition for hMSCs survival, growth and osteogenic differentiation studies.

Live/dead staining using Calcein AM and Ethidium homodimer-2 proved that hMSCs attached to BCP microparticles exposed to 1.5% chitosan-15% GP-0.18% HEC proliferate and remain viable after 7 days of culture. In addition, only limited number of dead cells was detected when the cells were surrounded by chitosan-GP-HEC. This

was comparable to the control group which was composed of cells attached to BCP microparticles without chitosan-GP-HEC. The result of live/dead staining further confirmed the suitability of chitosan-GP-HEC hydrogels in terms of maintaining cell viability.

When the number of cells exposed to chitosan-GP-HEC was quantified, the results showed that the cells grow in BCP microparticles group mixed with or without chitosan-GP-HEC. With respect to the osteogenic differentiation, hMSCs produced significantly higher ALP activity when cultured in osteogenic medium as compared to the basic medium. In addition, chitosan-GP-HEC did not have any negative effect on hMSCs' osteogenic differentiation. These achievements are crucial for injectable tissue engineered bone binders since such binders should allow cell survival, growth and osteogenic differentiation when in contact with the cells.

## 8.1.3 *In Vivo* Bone Formation Ability of Mesenchymal Stem Cells Surrounded by Chitosan-GP-HEC

Following the successful survival and osteogenic differentiation of mesenchymal stem cells in contact with chitosan-GP-HEC hydrogels *in vitro*, the *in vivo* bone formation ability of mesenchymal stem cells surrounded by chitosan-GP-HEC was investigated. It is important to examine whether a certain tissue engineered product is functional *in vivo* since *in vivo* animal experiments are closer estimate of what actually happens inside the body as compared to *in vitro* experiments. In case of bone tissue engineering, the functionality of the product is assessed based on its bone formation ability *in vivo*.

gMSCs were cultured on BCP microparticles for 7 days before implantation. Cell seeding on the constructs *in vitro* before *in vivo* implantation allows extracellular matrix formation and differentiation of cells (Kruyt *et al.*, 2004-a; Mankani *et al.*, 2001; Ohgushi and Caplan, 1999; Yoshikawa *et al.*, 2000). This was indeed the case since gMSCs formed a layer of cells and produced extracellular matrix on the surface of BCP ceramic microparticles after 7 days of culture *in vitro* (chapter 5). Following implantation in nude mice, ectopic bone formation was consistently observed

throughout BCP microparticles in gMSCs containing groups in the presence and absence of chitosan-GP-HEC. Furthermore, control groups without cells did not show any sign of bone formation in all implanted animals instead they were filled with fibrous tissue. The results of this study showed that the presence of chitosan-GP-HEC in gMSCs-BCP constructs did not interfere with the bone formation process. Although the overall quantity of bone formed after 6 weeks was lower in the chitosan-GP-HEC group compared to the control group (gMSCs attached to BCP microparticles) however, this difference was not statistically significant. Also, chitosan-GP-HEC was degraded after 6 weeks, creating more space for bone formation. This further confirmed that chitosan-GP ± HEC binders have the degradation characteristics needed for minimally invasive applications of tissue engineered bone as previously discussed in section 8.1.1.

With respect to the ability of chitosan-GP to support osteogenic differentiation, Kim *et al.* (2008) reported an study in which rat muscle-derived stem cells surrounded by 1.8% (w/v) chitosan-20% (w/v) GP, produced mineralized bone deposits after subcutaneous implantation in rats for 4 weeks (Kim *et al.*, 2008). Similarly in chapter 5 study, chitosan-GP-HEC supported bone formation *in vivo* following the stimulation of gMSCs into the osteogenic lineage. Furthermore, according to Cho *et al.* (2008), rat bone marrow mesenchymal stem cells attached and proliferated when suspended in 1.8% (w/v) chitosan mixed with 20% GP (w/v) (Cho *et al.*, 2008). Immunohistochemical visualization verified that the cells remain viable in the gel for at least 28 days following implantation. This supports the fact that chitosan gel is biocompatible as proved in chapter 5 study by the bone formation that occurred in the presence of chitosan-GP-HEC.

This successful *in vivo* experiment further confirmed the results obtained *in vitro* concerning the biocompatibility of chitosan-GP-HEC hydrogels as well as the ability of such hydrogels to support osteogenic differentiation.

## 8.1.4 Angiogenic Potential of Chitosan-based Binders

Angiogenesis plays an important role for success in bone tissue engineering approaches since bone is a highly vascularized tissue that needs blood vessels to supply oxygen and nutrients to the cells. Since the diffusion limit of oxygen is around 100-200 μm, formation of new blood vessels is necessary for the tissue to grow further than this limit (Awwad et al., 1986; Carmeliet and Jain, 2000). This can result in major cell survival problems with regard to bone tissue engineered constructs that are principally used to regenerate large bony defects.

With regard to the angiogenic potential of chitosan, there have been suggestions that chitosan is angiogenic (Biagini et al., 1989; de Castro-Bernas, 2003). The angiogenic response of chitosan-GP-HEC combined with and without hMSCs was therefore examined in chapter 6 using the chick chorioallantoic membrane (CAM) assay. Chitosan-GP-HEC gel did not show any angiogenic potential whereas the presence of hMSCs gave rise to an enhanced angiogenic response when placed on the CAM for 3 days. Quantitatively, significantly more blood vessel formation was observed for the stem cell containing group as compared to all other groups (p<0.05), except for b-FGF positive control. The results indicated that the chitosan-GP-HEC binder did not contribute to enhanced angiogenesis and that the presence of hMSCs improved angiogenesis.

The absence of angiogenic potential by chitosan is not expected to solve the cell survival problem in injectable bone tissue engineering. If chitosan was angiogenic then in case of injectable tissue engineered bone applied orthotopically, it might have been possible to solve the cell survival problem. Nonetheless, since this was not the case, the cell survival issue still remains to be solved. Despite the fact that hMSCs support angiogenesis, when placed in a defect, the rate of blood vessel formation might not be enough to speed up vasculature inside the tissue engineered bone therefore leading to a possible problem with regard to cell survival. However, this needs to be verified by implantation of injectable tissue engineered bone formulations in critical size defects. Previous studies demonstrated that cells survive in critical size defects such as the study performed by Brodke et al. (2006). This study showed that demineralized bone chips combined with osteoprogenitor cells and implanted in

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critical size defects in canine model result in bone bridging across the defect in all animals after 16 weeks (Brodke *et al.*, 2006). Similar results were also obtained when autografts were implanted in the defects but in the control group (demineralized bone chips without cells) only 50% of the defects were healed. The interesting point about this study is that the cells seeded on the demineralized bone chips survived in critical size defects and led to bone bridging and complete healing. Even though this study shows that the cells survive in critical size defects in canine model nevertheless, Meijer *et al.* (2008) demonstrated that cell survival was an issue in the clinical study that was carried out which resulted in bone formation in only 1 patient (Meijer *et al.*, 2008). Having such contradictory results regarding cell survival, it is therefore necessary to implant injectable tissue engineered bone in critical size defects. If cell survival is found to be affected in critical size defects despite the angiogenic abilities of hMSCs, then further approaches and strategies could be used to improve vascularization inside tissue engineered constructs implanted in large bony defects. Besides blood vessel ingrowth from the host, strategies like improvement of scaffold architecture to enhance the ingrowth of blood vessels (Druecke *et al.*, 2004; Karageorgiou and Kaplan, 2005; Pinney *et al.*, 2000; Yang *et al.*, 2001), addition of angiogenic factors (Huang *et al.*, 2005-b; Leach *et al.*, 2006; Perets *et al.*, 2003; Richardson *et al.*, 2001), and *in vitro* (Choong *et al.*, 2006; Rouwkema *et al.*, 2006) or *in vivo* pre-vascularization have been studied (Akita *et al.*, 2004; Casabona *et al.*, 1998; Kim and Kim, 2005).

## 8.1.5 Osteogenic Potential of Cells Exposed to Chitosan-based Binders

With regard to the stimulatory effect of chitosan on bone formation, various studies have been performed (Klokkevold *et al.*, 1996; Malette *et al.*, 1986; Muzzarelli *et al.*, 1994; Pang *et al.*, 2005). Klokkevold *et al.* (1996) assessed the effect of chitosan on mesenchymal stem cells in terms of osteogenic differentiation and bone formation. Based on this study, 2 mg/ml chitosan created significantly higher number of bone nodules than the control group. However, since the number of cells was not monitored, it is not clear whether chitosan stimulated proliferation of cells and as a result more nodule formation or if it in fact enhanced nodule formation and therefore

osteogenesis directly. To investigate this further, the effect of chitosan on MC3T3-E1 cells was evaluated in chapter 7 in terms of osteogenic differentiation, cell proliferation and viability *in vitro*.

Live/dead staining using Calcein AM and Ethidium homodimer-2 indicated that MC3T3-E1 cells exposed to 2, 0.05 and 0.005 mg chitosan/ml continue to proliferate throughout the study with limited number of dead cells observed. With respect to the osteogenic differentiation of MC3T3-E1 cells, chitosan concentrations of 2 mg/ml and 0.05 mg/ml resulted in significantly higher ALP activity compared to the differentiation medium. These findings support the positive effect of chitosan on osteogenic activity of MC3T3-E1 cells.

The mechanism by which chitosan stimulates osteogenic differentiation is unknown however, due to its N-acetylglucosamine repeating units, chitosan has structural similarity to glycosaminoglycans especially hyaluronic acid (Sandford, 1989) and therefore may imitate their functional activities (Pang *et al.*, 2005). Hyaluronic acid facilitates progenitor cells migration therefore assisting tissue regeneration (Zhu *et al.*, 2006). Also, chitosan has been suggested to increase the migration and differentiation of preosteoblastic progenitor cells (Klokkevold *et al.*, 1996; Malette *et al.*, 1986). Future studies are needed to further understand chitosan's mechanism of action.

Comparing the results of chapter 4 with chapter 7, it is noticed that the osteogenic differentiation of hMSCs was not enhanced when encapsulated with chitosan-GP-HEC (chapter 4) which is contradictory to the results obtained when MC3T3-E1 cells were cultured with 2 and 0.05 mg/ml chitosan concentrations. However, the differences observed could be due to different set-up, different experimental conditions or the fact that chitosan was mixed with GP, HEC and BCP when it was in contact with hMSCs. Nevertheless, since the results obtained with MC3T3-E1 cells are different to those of hMSCs with regard to osteogenic differentiation, this might suggest that the results obtained from MC3T3-E1 cells might not be directly relevant regarding the clinical application of injectable tissue engineered bone. Also, hMSCs are closer to the final application of injectable tissue engineered bone compared to

MC3T3-E1 cells. In any case, even though chitosan-GP-HEC was not enhancing the osteogenic differentiation of hMSCs, at least it was not preventing it which indicates that it is a promising binder for tissue engineered bone.

## 8.1.6 Future Work and General Considerations

Injectable tissue engineered bone is a relatively new concept in the field of bone tissue engineering. It is a promising approach to overcome some of the drawbacks associated with traditional bone tissue engineering techniques since it offers minimally invasive strategy to repair and regenerate bone defects. This technology could be applied to various clinical conditions such as spinal fusion and skull defects. Also, chitosan-GP-HEC binder which surrounds the tissue engineered bone could be used to deliver growth factors to the injury site. In addition, it could offer benefits such as cell protection from harsh haematoma (with high potassium concentration toxic to cells) which is formed in osseous defects after an injury (Street *et al.*, 2000).

Many interesting novel facts were discovered during the course of this thesis with regard to biocompatibility, gelation and degradation of chitosan-GP ± HEC binders as well as survival, osteogenic differentiation and *in vivo* bone formation of cells exposed to chitosan-GP-HEC binders. Nonetheless, more experiments are necessary before injectable tissue engineered bone could be used clinically. For example, in terms of future experiments, injectable tissue engineered bone should be applied in critical size bone defects in animals such as goats so that the success of such approach could be tested in critical size defects. If cell survival is indeed an issue in such critical size defects, then new effective approaches will be necessary to enhance angiogenesis in injectable tissue engineered bone constructs since chitosan-GP-HEC did not have inherent angiogenic properties. Suggestions regarding this matter were made in section 8.1.4. Moreover, hMSCs should be used in addition to gMSCs in preclinical *in vivo* experiments since the application of injectable tissue engineered bone is patient-oriented. For instance, hMSCs could be used in ectopic implantation in nude mice model. Unfortunately, hMSCs cannot be applied in critical size defects in animals such as goats because of immune rejection but they could be used in critical size defects in nude mice or rats. Following successful preclinical tests,

clinical trials using injectable tissue engineered bone would be the next step. If all the necessary regulatory approval is obtained and all the additional requirements are met then with a robust planning scheme it becomes possible to test injectable tissue engineered bone in patients with bone related medical conditions such as spinal fusion or skull defects.

The studies in this thesis focused on the biological and material aspects of injectable bone tissue engineering as opposed to the mechanical aspects. It is of paramount importance that the cells inside the injectable tissue engineered bone constructs survive and produce bone when implanted in defects. In line with this requirement, the biological and material aspects of such constructs were the subject of investigation in this thesis. Therefore, the potential use for these constructs would be non load-bearing applications such as spinal fusion and skull defects. It should be noted that the current golden standard therapy for bone repair and regeneration (autografts) are applied to the defects in the form of bone chips without having much mechanical properties. Even though these bone chips lack sufficient mechanical properties, they are still the best solution available for bone regeneration. It is therefore debatable how relevant the mechanical properties are with regard to bone defects. Nevertheless, it is a promising solution when tissue engineered bone constructs perform well both biologically and mechanically.

The current tissue engineering strategies for bone repair and regeneration including the approach used in this thesis, involves direct osteogenic differentiation. It should be noted that the natural healing response of the body in case of fracture is also through endochondral bone formation as mentioned in the introduction of this thesis (chapter 1). It is therefore reasonable to combine tissue engineering techniques with natural healing response of the body (endochondral bone formation) when devising strategies to regenerate bone defects in future.

# 8.2 CONCLUDING REMARKS

The studies described in this thesis contributed to further understanding of injectable applications of tissue engineered bone. A promising binder was discovered for

minimally invasive applications of tissue engineered bone with successful *in vitro* and *in vivo* results. Chitosan-GP-HEC was shown to be biocompatible and biodegradable with quick gelation properties at body temperature. Mesenchymal stem cells remained viable when surrounded by the gel and underwent osteogenic differentiation when supplied with the right stimuli. *In vivo* experimentation in mice was another success after bone formation was observed in cell containing groups in the presence or absence of the chitosan-GP-HEC. This further demonstrated the suitability of chitosan-GP-HEC for injectable bone tissue engineering applications.

In addition, chitosan produced positive results regarding enhancement of osteogenic activity and it was shown to be non-angiogenic. Despite all the novel successful results obtained during the course of this thesis, however, more investigations and studies (e.g. evaluation of cell survival in injectable tissue engineered bone implanted in critical size defects) are necessary before we witness clinical applications of injectable tissue engineered bone.

# LIST OF PUBLICATIONS AND CONFERENCE PROCEEDINGS

## PUBLICATIONS

**Ahmadi, R.** & de Bruijn, J.D. 2008, "Biocompatibility and gelation of chitosan-glycerol phosphate hydrogels", *Journal of Biomedical Materials Research Part A*, vol. 86(3), pp. 824-32.

**Ahmadi, R.** & de Bruijn, J.D. 2007, "Chitosan based delivery systems for injectable tissue engineered bone: human mesenchymal stem cell survival, proliferation and osteogenic differentiation", *TERMIS-EU Meeting Abstracts, London, UK, Tissue Engineering*, vol. 13(7), pp. 1655.

**Ahmadi, R.,** Zhou, M., & de Bruijn, J.D. 2005, "The use of thermo-sensitive chitosan as an injectable carrier for bone tissue engineering", *European Cells and Materials*, vol.10, Suppl.2, pp. 61.

**Ahmadi, R.,** Burns, A.J., & de Bruijn, J.D. 2009, "Chitosan-based hydrogels do not induce angiogenesis", *Journal of Tissue Engineering and Regenerative Medicine.*

## Reviewing Experience:

Reviewer for *Tissue Engineering* and *Journal of Tissue Engineering and Regenerative Medicine.*

# CONFERENCE PROCEEDINGS

## Oral Presentations:

Oral presentation at World Biomaterials Congress 2008 entitled "Angiogenesis in chitosan-based injectable systems", Amsterdam, The Netherlands.

2 Oral presentations at European Society for Biomaterials Conference (ESB) 2007 entitled "Chitosan based delivery systems for injectable tissue engineered bone" and "Degradation and gelation behaviour of chitosan based delivery systems", Brighton, United Kingdom.

Oral presentation at Tissue Engineering and Regenerative Medicine International Society (TERMIS) 2007 entitled "Chitosan based delivery systems for injectable tissue engineered bone: human mesenchymal stem cell survival, proliferation and osteogenic differentiation", London, United Kingdom.

Oral presentation at $IOM^3$ Materials Congress 2006 entitled "The use of thermo-sensitive chitosan as an injectable carrier for bone tissue engineering", London, United Kingdom.

## Poster Presentations:

Poster presentation at World Biomaterials Congress 2008 entitled "Osteogenesis of MC3T3-E1 cells is enhanced by chitosan-based polymeric system", Amsterdam, The Netherlands.

Poster presentation at London Technology Network (LTN) 2007 entitled "(Bone) Tissue engineering and adult stem cell technology", London, United Kingdom.

Poster presentation at European Society for Biomaterials Conference (ESB) 2005 entitled "The use of thermo-sensitive chitosan as an injectable carrier for bone tissue engineering", Sorrento, Italy.

Poster presentation at Tissue and Cell Engineering Society (TCES) 2005 entitled "The use of thermo-sensitive chitosan as an injectable carrier for bone tissue engineering", London, United Kingdom.

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