NOTE TO USERS

Pre-Osteoblast Response to Three-Dimensional Titanium Scaffolds for Bone Regeneration Applications

Jean-Philippe St-Pierre, B.A.Sc., B.Sc.

Department of Biomedical Engineering
Faculty of Medicine
McGill University, Montréal, QC, Canada

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Master of Engineering

Your file Votre référence
ISBN: 0-494-12651-5
Our file Notre référence
ISBN: 0-494-12651-5

ABSTRACT

Porous metals are often used in orthopaedic load-bearing applications because they promote and direct bone ingrowth while maintaining their mechanical integrity for long periods in physiological environments. However, the influence of microstructural parameters of three-dimensional metallic scaffolds on osteogenic cell response must be studied to improve their design. This study aims to study the biocompatibility and osteoconductivity of novel titanium foams with three pore sizes *in vitro* and to gain knowledge on the mechanisms by which bone is formed within three-dimensional structures. This is achieved through studies of the cell viability of macrophages and adhesion kinetics, proliferation, differentiation, and mineralization of the extracellular matrix by MC3T3-E1 mouse pre-osteoblasts. Results indicate that porous titanium promotes the generation of mineralized extracellular matrix by differentiating pre-osteoblasts and stress the importance of controlling the pore size to allow the impregnation of the structure by tissue.

RÉSUMÉ

Les métaux poreux sont fréquemment utilisés comme implants orthopédiques dans des situations impliquant des charges mécaniques puisqu'ils encouragent et dirigent la croissance osseuse, tout en maintenant leur intégrité mécanique dans un environnement physiologique. Par contre, l'influence des paramètres structuraux de ces supports tridimensionnels sur le comportement de cellules ostéogéniques doit être comprise afin d'optimiser leur design. La présente étude vise à établir la biocompatibilité et la capacité ostéoconductrice de supports de titane poreux caractérisés par trois tailles de pores *in vitro* ainsi qu'à approfondir les mécanismes de formation de l'os à l'intérieur de matrices tridimensionnelles. La viabilité des macrophages, ainsi que la cinétique d'adhésion des préostéoblastes de souris (lignée MC3T3-E1) et leurs processus de prolifération, de différentiation et de minéralisation sur les matériaux de titane poreux ont permis d'atteindre ces objectifs. Les résultats obtenus permettent de conclure que les supports de titane poreux encouragent la formation d'une matrice extracellulaire minéralisée par les cellules osseuses. De plus, il semble important de contrôler la taille des pores des supports métalliques afin de permettre l'imprégnation complète de la structure.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Maryam Tabrizian for her guidance and support throughout my Master's degree. Through her collaboration with IMI-NRC, I have had the opportunity to work on an interesting research project. Moreover, she has permitted me to work autonomously to design, conduct, and analyse experiments, while learning new expertise in scanning electron microscopy and cell culture.

I would also like to express my acknowledgements to Louis-Philippe Lefebvre and Dr. Maxime Gauthier for their guidance and expertise in producing and characterizing porous metallic scaffolds for my research.

Dr. Mary Kaartinen and Dr. Alain Petit were kind in sharing their expertise in cell biology, as was Dr. Hojahollah Vali for his support in analyzing scanning electron microscopy results and suggesting approaches for the study of osteoblast interactions with porous metals. Dr. Satya Prakash kindly granted me access to his laboratory to use his centrifuge and freezer.

I am truly grateful to Mrs. Line Mongeon for her help in the preparation of scanning electron microscopy samples and their subsequent analysis. I am also appreciative of the expertise she taught me in scanning electron microscopy. Furthermore, I would like to acknowledge Dr. Stéphane Ruggeri, Paul-Émile Mongeon, and Shirley Mercier for their work in ensuring that the experimental setup for the fabrication of porous titanium scaffolds was functional.

I would like to express my appreciation to my lab mates for their help and encouragement. In particular, I would like to express my gratitude to Shawn Carrigan for his appreciated advice and support throughout my project and for his help in the preparation of this thesis and all other manuscripts prepared during my degree. I am also grateful for his friendship. I would also like to thank Kim Douglas, Fabrice Burel, and Anna Hillberg for their suggestions and support regarding my project and for their friendship. You have made my stay in Montreal much more enjoyable.

Finally, I would like to express my deepest gratitude to my family for always believing in me and supporting me throughout these years. Je vous remercie sincèrement

pour votre amour et support qui m'ont permis d'affronter les épreuves des dernières années et de grandir de par ces expériences.

Financial Support

The research presented in this thesis was made possible by funding from the Natural Science and Engineering Research Council (NSERC), les Fonds Québécois de la Recherche sur la Nature et les Technologies (FQRNT), the Center for Biorecognition and Biosensors and a McGill University Graduate Studies Fellowship.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

GLOSSARY OF TERMS

A	Titanium foams characterized by the smaller pore size
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
B	Titanium foams characterized by the intermediate pore size
BCA	Bicinchoninic acid
C	Titanium foams characterized by the larger pore size
CBS	Canadian Biomaterials Society
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
EBSS	Earl's balanced salt solution
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EDX	Energy dispersive x-ray analysis
hFOB	human fetal osteoblasts
IMI-NRC	Industrial Materials Institute of the National Research Council Canada
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NiP	Mirror-polished nickel
P	Mirror-polished titanium
PBS	Phosphate buffered saline
PMMA	poly(methyl methacrylate)
RGD	Arginine-Glycine-Aspartic Acid sequence of a protein
RNA	Ribonuclueic acid
RNAse A	Ribonuclease type A
SEM	Scanning electron microscopy
TCP	Tissue culture plate
TGF-β	Transforming growth factor beta
TiA	Titanium foams characterized by the smaller pore size
TiB	Titanium foams characterized by the intermediate pore size

TiC	Titanium foams characterized by the larger pore size
TiP	Mirror-polished titanium

PREFACE

Contribution of Authors

The two manuscripts included in this thesis were written by the student in collaboration with his supervisor, Dr. Maryam Tabrizian. The collection and analysis of results were performed by the student.

In both manuscripts, Dr. Maxime Gauthier and Louis-Philippe Lefebvre, second and third authors respectively, were involved scientifically in modifying the process for the fabrication of titanium foams. They were also responsible for the development of this fabrication process prior to the Master's project presented in this thesis.

CHAPTER 1

Introduction and Research Rationale

More than 35 years ago, a trend toward the replacement of diseased or injured bone structures and joints has led to the development of metallic implants for total joint replacement applications. This treatment approach has produced significant improvements to the quality of life of trauma victims with musculoskeletal injuries, as well as patients with degenerative bone diseases such as osteoarthritis, as supported by the fact that more than 1,250,000 total joint replacements were performed worldwide in 2002 [1]. The most common procedures, knee and hip arthroplasties, were performed at rates of 65 and 47 cases per 100,000 adults, respectively, throughout Canada in 2000 [2]. Nevertheless, metallic implants used for orthopaedic applications still have a limited survivability [3].

One of the challenges involved with the development of appropriate procedures for joint replacements has been the fixation of implants to bone. The importance of implant fixation for the success of the arthroplasty was first demonstrated by Charnley, who obtained impressive clinical results with the use of poly(methyl methacrylate) (PMMA) as cement to fix the femoral component of hip replacements in bone [4]. However, long term clinical studies related to the use of bone cement in orthopaedic implantations have found that this fixation approach has a limited lifetime ranging between 10 and 20 years [5]. It was also demonstrated that the degree of activity of a patient directly affects the longevity of the implant fixation integrity [6]. Hence, this approach yields acceptable results when applied to older patients with sedentary lifestyles, but is inappropriate for younger patients who are more active and place higher demands on their joints.

Following these findings, numerous approaches to implant fixation have been proposed in an attempt to overcome the limitations of bone cement. They all allow to achieve a cementless fixation through modifications of implant surface properties. Examples of these methods include the addition of micro-textures [7] or macroscopic surface features [8]. These systems are based on the knowledge that initial cellular

response of osteoblasts is affected by surface topography. Bioactive coatings, such as hydroxyapatite and calcium phosphate, have also been applied to implant surfaces to promote bone bonding to the material [9]. Another approach to cementless fixation of orthopaedic implants consists of applying a thin porous coating to the surface of the prostheses to obtain mechanical interlocking through bone ingrowth inside the interconnected pores [5].

The need to improve on initial porous metallic coatings has led to the development of processes to produce highly porous metals that provide more volume for bone ingrowth. The osteoconductive properties of these highly porous metals encouraged research toward their application as three-dimensional scaffolds to promote and direct bone ingrowth in load-bearing situations. In fact, poor mechanical properties of polymers and the brittleness of bioactive ceramics limit the applications of these materials to low loading conditions. Among the most interesting applications of porous metals is their use as three-dimensional scaffolds for the regeneration of damaged bone structures in craniofacial reconstruction. As well, porous metals have been investigated for applications as spinal disc cages for the fusion of vertebrae and as dental implants.

Despite extensive efforts directed at the development of processes for the fabrication of highly porous metals, few materials are currently used in clinical settings. Furthermore, our understanding of the impact of microstructural parameters of three-dimensional metallic scaffolds on the adhesion and differentiation of immature osteoblasts is still rather limited. In fact, most cell culture studies report the effects of individual microstructural parameters on two-dimensional surfaces rather than on three-dimensional models. Thus, there is a need to develop metallic foams with controllable microstructures and to gain a better understanding of the interactions between osteoblastic cells and these porous metals, in order to optimize their design.

The research reported in this thesis focuses on cell interactions with novel titanium foams characterized by three pore sizes and produced through a powder metallurgy process developed at the Industrial Materials Institute of the National Research Council Canada (IMI-NRC). As an initial step of this project, the cytocompatibility of titanium foams was investigated through macrophage viability studies. Macrophages were selected for this part of the project because of their sensitivity

to environmental signals. Adhesion of pre-osteoblasts on the scaffolds was then investigated. Cell adhesion provides valuable information on the biocompatibility of biomaterials. Finally, the formation of a bone-like tissue inside the porous structure by differentiating pre-osteoblasts was evaluated.

This three-dimensional cell culture model provides useful qualitative and quantitative insight on the effects of the microstructural parameters of porous metals on the adhesion and differentiation behaviour of pre-osteoblastic cells, towards the formation of a mineralized extracellular matrix. Furthermore, it is a first step in the assessment of the potential of novel titanium foam as a bone engineering scaffold.

References

1. Information found at: http://investor.zimmer.com/industry.cfm.
2. Health Canada. *Arthritis in Canada. An Ongoing Challenge.* **2003**, (Cat.# H39-4/14-2003E).
3. Hench, L. L. *Biomaterials* **1998**, 19, 1419.
4. Charnley, J.; Follacci, F.M.; Hammond, B.T. *J. Bone Joint Surg. Br.* **1968**, 50, 822.
5. Bauer, T.W.; Schils, J. *Skeletal Radiol.* **1999**, 28, 423.
6. Torchia, M.E.; Klassen, R.A.; Bianco, A.J. *J. Bone Joint Surg. Am.* **1996**, 78, 995.
7. Bohm, G.; Lintner, F.; Auterith, A.; Lester, D.K.; Zweymuller, K.A. *Clin. Orthop.* **2001**, 393, 13.
8. Havelin, L.I.; Espehaug, B.; Vollset, S.E.; Engesaeter, L.B. *J. Bone Joint Surg. Br.* **1995**, 77, 11.
9. Palm, L.; Jacobson, S.A.; Ivarsson, I. *J. Arthroplasty.* **2002**, 17, 172.

CHAPTER 2

Thesis Objectives and Outline

2.1 Thesis Hypothesis

It is hypothesized that residuals from the processing chemicals employed in the fabrication of titanium foams do not affect the cytocompatibility of titanium foams because of the careful selection of each product, and that the unique three-dimensional structure of titanium scaffolds promotes the production of bone-like tissue by mouse pre-osteoblasts *in vitro*.

2.2 Thesis Objectives

The primary aim of the research project presented in this thesis is to establish the cytocompatibility of porous titanium produced through a novel powder metallurgy process developed at IMI-NRC with *in vitro* cell culture studies. It also aims to gain insight into the effects of the microstructural characteristics of the three-dimensional scaffolds on the early and long term responses of pre-differentiated osteoblastic cells. Specific objectives of the project are:

1. To produce three types of porous titanium scaffolds with varying pore sizes and to characterize their morphological differences in terms of pore size and density.
2. To ensure that residuals from the process chemicals do not cause cytotoxic effects individually or in combination.
3. To establish the early adhesion kinetics of MC3T3-E1 mouse pre-osteoblasts to porous titanium and to assess their morphological features upon adhesion.
4. To verify that the three-dimensional titanium scaffolds promote the differentiation of MC3T3-E1 pre-osteoblasts and the production of a mineralized extracellular matrix by these differentiated cells.

2.3 Thesis Outline

This thesis is prepared in a manuscript format and is divided in 9 chapters. The literature review is separated in two sections discussing the application of porous metals for hard tissue regeneration (Chapter 3) and reviewing the knowledge on interactions between osteoblasts and metals, as well as their respective experimental investigation tools (Chapter 4). Chapters 5 and 6 present a proceeding published by the Materials Research Society and an article accepted by the peer reviewed journal Biomaterials, respectively. Chapter 7 discusses the relations between the two manuscripts, as well as results from ongoing research, while Chapter 8 draws general conclusions from the results collected during the Master's degree. Chapter 9 proposes directions for the continuation of the research project.

2.4 Concise Description of the Manuscripts

Chapter 5 includes manuscript one, **Adhesion Kinetics of MC3T3-E1 Pre-Osteoblasts to Osteoconductive Porous Titanium Scaffolds**, published in the Materials Research Society Symposium Proceedings Volume 823 (2004). The work presented in this proceeding fulfills objectives 1 and 3 of this thesis. It also presents parts of the results toward the fulfillment of objective 2. Subsequent results to achieve objective 2 are briefly discussed in Chapter 7. This proceeding demonstrates the cellular viability of J774 adherent mouse macrophages in contact with titanium foams with three pore sizes produced through the powder metallurgy process of interest. As well, it shows results of early interactions between pre-osteoblastic cells and the porous materials compared to a two-dimensional mirror-polished titanium reference material. Adhesion kinetics and cell morphology were investigated.

Chapter 6 includes manuscript two, **Three-Dimensional Growth of Differentiating MC3T3-E1 Pre-Osteoblasts on Porous Titanium Scaffolds**, accepted by the periodical Biomaterials. The work presented in this article includes results from objective 1 and fulfills objective 4 of this thesis. The article demonstrates the ability of titanium foams with three different pore sizes to promote pre-osteoblast differentiation towards a mature state that has the ability to produce a mineralized bone-like

extracellular matrix. As with the previous manuscript, results obtained for the titanium foams are compared to a two-dimensional polished titanium reference material.

CHAPTER 3

Porous Metals for Hard Tissue Regeneration

3.1 Biomaterials in Bone Engineering

Over the past 35 years, bone engineering research has focused on the development of strategies to replace diseased and injured hard tissue and joints, with aims to recover lost functionalities, partially or completely, in an attempt to improve the quality of life of patients. Replacement materials for such purposes are typically classified in two categories, transplants and implants [1]. While transplants have been used successfully for bone engineering applications, they have evident limitations. The use of autografts has notably been constrained by availability, second site morbidity, and resorption issues, whereas allografts and xenografts raise ethical, immunological and contamination concerns [1-2].

Limitations associated with transplantation have motivated the development of biomaterials for implantation purposes. Implant materials developed for the replacement of damaged bone structures have generally been successful at improving the quality of life of patients, but all are limited in their survivability [1]. Complications encountered with these systems are generally associated with the maintenance of a stable interface between the material and surrounding tissues, and blood diffusion through that interface. Inadequate mechanical properties and production of wear debris also contribute to the failure of replacement implants [1,3]. These setbacks have recently led to a shift in the focus of research towards the design of advanced biomaterials to assist in partial or complete bone regeneration, to obtain improved implant fixation, or to repair damaged hard tissue structures [1]. Because of the natural regeneration capabilities of bone, the challenge consists of enhancing the rate of bone growth and directing its progression. One approach to this problem consists of designing three-dimensional scaffolds with osteoconductive properties.

3.2 The Need for Porous Metals

Biocompatible polymers and bioactive ceramics have been studied extensively for the fabrication of scaffolds with applications in the regeneration of bone structures [4-5]. These materials generate interest because of their biodegradable or bioresorbable properties and can be modified to achieve control over the degradation or resorption rate. The aim is to match it with the rate at which bone is generated in an attempt to replace the material by newly formed tissue. Nevertheless, polymers have relatively poor mechanical properties and bioactive ceramics are brittle, making them less reliable as constitutional materials. Thus, porous scaffolds made of these types of materials are generally employed for implantation in minimal loading conditions or as scaffolds for the *in vitro* maturation of engineered tissues that can be implanted in the damaged area.

Metals are interesting candidates for the fabrication of porous scaffolds with applications in load bearing situations because they can maintain favourable mechanical properties over extended periods of time in physiological loading environments [6-8]. They are ductile materials characterized by high yield strength and fracture toughness, as well as minimal fatigue behaviour. Thus, the mechanical integrity of three-dimensional metallic supports is superior to that of polymer and ceramic based scaffolds.

The porosity of metallic foams considerably reduces their mechanical properties when compared to bulk metals [9]. Hence, porous metals are not generally used as structural materials for implantation in high load bearing conditions such as those encountered by hip joint replacements, but rather as porous coatings at the surface of dense implants to permit fixation through bone ingrowth. Special care must also be taken to ensure that process conditions for the fabrication of porous metals do not further reduce the mechanical properties of the material. One such example is the need to ensure that the furnace atmosphere is non-oxidizing and does not contain contaminants when sintering titanium, as the metal will react with these contaminants, thereby changing its nature and increasing the brittleness of the material [10].

3.3 Specific Applications of Porous Metals in Bone Engineering

The conventional application of porous metals in bone engineering consists of applying them as coatings on total joint replacement implants for their fixation through bone ingrowth [11].

The success of porous coatings in promoting bone ingrowth, combined with the development of new fabrication processes for the production of metallic materials with high levels of porosity that are more adequate for applications as hard tissue regeneration scaffolds, has opened new avenues for the application of these materials in bone engineering. Notably, their use as bone graft substitutes and for bone augmentation in craniofacial reconstruction has been investigated [12]. Other investigators have proposed the use of porous metals to achieve intervertebral fusion through bone ingrowth as an alternative to fusion cages that require autologous bone grafts [13-15]. Porous metals have also been used as dental implants [16].

3.4 Bone Ingrowth Inside Porous Metals

3.4.1 Mechanism of Bone Ingrowth

The information provided in this section summarizes the highly complex reactions of the body to the implantation of porous metallic scaffolds. Only *in vivo* results are reported in this section, as *in vitro* models for bone genesis inside porous materials are simplified models of the actual mechanism. Nevertheless, *in vitro* models studying the behaviour of osteoblasts on implant materials are often employed to gain knowledge on the effects of specific microstructural parameters of biomaterials on cellular response, as well as the potential of new biomaterials for bone engineering applications because they provide information at the cellular level that is not readily available with present *in vivo* experimental techniques.

The initial healing step after the insertion of a porous metallic implant in bone consists in the formation of a fibrin clot at the interface between the metal and the biological tissue. The fibrin fibres are recruited from the blood coming in contact with the metallic biomaterial and are believed to attach faster on surfaces containing sub-micron scale features [17]. This fibrin network acts as a natural scaffold onto which osteoprogenitor cells and pre-differentiated osteoblasts can adhere, proliferate and

migrate to reach the porous surface of the implant [10]. The necrotic bone fragments produced during the press-fitting step of implantation are usually resorbed by the organism in a very short time span. In a matter of days following the formation of a fibrin clot, cells present at the healing site already produce an extensive extracellular matrix, which is mainly composed of collagen type I. This soft matrix intertwined inside the porous structure can provide reasonable stability and limit micromotion at the interface. It also provides an adequate environment for the differentiation of osteoblasts and subsequent maturation of the extracellular matrix and mineralization process toward the formation of bone in direct apposition with the metallic structure [17]. It is important to specify that initial bone ingrowth does not guarantee the long term stability of the implant because bone is a dynamic organ that responds to mechanical and chemical signals from its environment.

3.4.2 Effect of Implant Micromotion

Among the conditions required for bone ingrowth to occur, some are not related to the material design but rather to the surgical procedure employed. In fact, the importance of obtaining an adequate initial fixation of porous implants, in order to limit micromotion at the interface between the material and bone, is documented [18-19]. It has been shown that small micromotion does not inhibit bone ingrowth, while gross movement promotes the formation of fibrous tissue surrounding the implant without penetrating the porous structure. It is thought that this process is meant to exclude the foreign body from bone. Further studies on this issue identified a range of 28 to 150μm within which the threshold of acceptable micromotion is contained [20]. Thus, porous metals are generally press-fit into the implantation site and loading of the particular area is generally resumed gradually following the surgical procedure.

3.4.3 Promotion of Bone Ingrowth Inside Porous Metals

Various approaches have been proposed for the improvement of the osteoconductive properties of porous metals in an attempt to promote bone ingrowth. The application of a thin ceramic coating at the metal surface renders the surface bioactive, while maintaining the superior mechanical properties of the bulk metallic material [5].

Another way to improve bone ingrowth inside porous metals that has been the subject of an increasing number of investigations consists of incorporating growth factors and other proteins, or bisphosphanate drugs, to the construct [21].

3.5 Requirements for Porous Metals

3.5.1 Type of Metal

The nature of the metal used as a basis for the fabrication of bone engineering scaffolds has major effects on the body response to the implant. Notably, metals are characterized by a wide range of elastic moduli that can be further adjusted to achieve desired properties through alloying. Nevertheless, all types of metals are characterized by an elastic modulus that is considerably higher than that of trabecular bone. Implantation of materials with such high moduli in bone results in stress-shielding, in which case the material prevents the surrounding tissue from receiving stress signals necessary to promote an equilibrium in bone turn-over. Stress-shielding often results in bone resorption in the implant surroundings and eventual loosening of the synthetic material. Highly porous metals can be designed in a manner such that their elastic modulus is comparable to that of the trabecular bone within which they are implanted. This is generally achieved through a judicious choice of base metal and a strict control of the microstructural parameters for the fabrication of porous metals. It is also important to keep in mind the fact that other mechanical properties of bulk metals will be lowered by the fabrication of metallic materials with high levels of porosity. Notably, the yield strength, fracture toughness and fatigue behaviour of porous metals must be maintained at an adequate level for the intended application.

Aside from their mechanical properties, metals selected must fulfill many requirements to be considered biocompatible. Among the most important are the corrosion resistance, which prevents the release of damageable ions and the introduction of mechanical weaknesses in the material, the absence of cytotoxic effects, and finally the promotion of cellular adhesion, proliferation and differentiation [6]. These strict requisites, combined with the fact that procedures by which new biomaterials are approved for implantation are quite extensive, limit the number of metals used for the fabrication of implantable devices. The four main classes of metals used in clinical

implantation for orthopaedic applications are stainless steels, cobalt chrome alloys, tantalum, and titanium and its alloys [22].

Stainless steels have lower corrosion resistance in physiological environments and inadequate mechanical properties compared to other biocompatible metals, but their low cost makes them attractive candidates for applications as temporary implants such as plates and screws for fracture fixations [22]. Cobalt-chromium alloys have better corrosion resistance behaviour than stainless steel and have high hardness properties, making them resistant to wear. Thus, they are generally used for applications such as metal-metal joint replacements in order to minimize the production of wear particles in the synovial fluid [22]. Tantalum has been recently introduced as a biomaterial for the fabrication of bone engineering scaffolds because of its excellent corrosion resistance and overall biocompatibility [9,23]. However, it is more expensive than titanium and its alloys for equivalent overall biocompatibilities.

Titanium and its alloys are considered to be the biomaterials of choice in the fabrication of metallic scaffolds for bone regeneration because of their excellent overall biocompatibility [24-26]. The superior corrosion resistance of titanium over other metals used for implantation contributes to its good response in physiological environments; this property of titanium is particularly important for three-dimensional scaffolds because of the increased surface area. The corrosion resistance of titanium is associated to the spontaneous formation of a dense oxide layer at its surface [25,27]. This oxide layer, composed mainly of TiO_2, is thermodynamically stable and chemically inert and is maintained in physiological environments, contributing to the low ion release levels and high cytocompatibility of titanium. The inertness of the material also contributes to the promotion of appropriate cellular interactions with the material.

3.5.2 Microstructure

Microstructural parameters of porous metals must be controlled for the design of efficient bone engineering scaffolds. It is generally believed that the scaffold needs to mimic the structure of the extracellular matrix in order to promote the desired cellular response in the regeneration of bone. This is achieved by designing scaffolds with high levels of surface topography and roughness. However, it still remains unclear how these

microstructural parameters of implant materials must be organized to influence cellular maturation towards the regeneration of bone.

Early attempts at producing porous metals for applications as implant coatings for fixation purposes focused on materials with low levels of porosity, ranging between 30 and 50% [28-29]. Nevertheless, the production of porous metals with higher porosity levels up to 85% is beneficial because it provides more volume for bone ingrowth, which relates to a higher interfacial strength between the material and surrounding bone. Furthermore, the three-dimensional scaffold requires an interconnected porosity to allow impregnation of the structure with bone, vascularization of the tissue, and diffusion of nutrients.

Perhaps the most studied design parameter of porous metals, the optimal pore size has been the focus of many debates over the years. The study most often cited to that regard, performed by Bobyn et al., identified a range of 50 to 400μm as providing an optimal fixation of the material to bone [30]. The results presented in this study emphasized the difficulty in obtaining mineralized bone matrix inside pores smaller than 50μm, and indicated the presence of fibrous tissue in pores over 400μm. Nevertheless, studies focusing on the effect of an exact pore size on bone ingrowth rather than average pore sizes for a specific type of structure reported bone ingrowth in pores smaller than 50μm [31]. In general, pores must be larger than 150μm for bone reorganization and formation of osteons to occur [32]. Furthermore, larger pores seem to encourage vascularization of the newly formed tissue. As these are important issues to address in order to ensure the formation of healthy bone, larger pore sizes are generally preferred for the fabrication of bone regeneration scaffolds. Studies have notably reported excellent ingrowth in structures with pore sizes larger than 400μm [23,33]. Divergence between the different reports concerning the optimal pore size of porous metals for bone engineering applications might be attributed to the fact that other microstructural parameters of the porous materials used in the studies are not controlled. Notably, the pore shape is a fabrication process dependant parameter that is hard to control. Thus, it is possible that each type of porous metal will have a different optimal pore size because of its unique microstructure.

3.6 Fabrication Processes for Porous Metals

As was briefly discussed in the previous section, two types of porous metals were initially developed for applications as porous coatings on orthopaedic implants. These approaches consisted of sintering metallic beads or fibres to the dense surface of the implant in order to promote a solid-state diffusion and create metallurgical contacts between the coating particles or strings and the bulk material, ensuring the mechanical integrity of the structure [28,29]. A certain control over the porosity level (30 to 50%) and the pore size of these materials has been achieved over the years. As well, evidence of bone growth inside the interconnected structure was reported with various materials including titanium and cobalt chrome alloys [34]. Nevertheless, these first generation porous metals were characterized by a relatively low level of porosity, resulting in limited bone ingrowth levels and interfacial strength. A plasma-sprayed procedure has also been used to produce porous coatings [34]. However, this approach is limited in terms of the range of pore sizes that can be achieved and the control over the microstructure.

Later attempts to produce porous metals that could be used as coatings focused on the development of biomaterials with high levels of porosity (up to 85%) to provide more volume for bone ingrowth, as is beneficial for scaffolding applications in bone engineering. However, the number of highly porous metals available for use in clinical settings is still rather limited. The fabrication processes for highly porous metals that have been approved for implantation in humans include chemical vapour infiltration to deposit tantalum onto vitreous carbon foams [9,15,23,35], self-propagating high-temperature synthesis for the fabrication of porous nitinol from metallic powders [13-14], and conventional powder metallurgy techniques [36]. While these porous metals have been successful at encouraging bone ingrowth both *in vivo* and in clinical trials, a larger range of materials with distinctive and controllable microstructures is required in order to identify the influential parameters of metallic scaffolds and to understand their effects on cellular processes leading to bone ingrowth in an attempt to optimize their design.

Current work toward the fabrication of novel porous metal designs has mainly focused on two approaches. One of these techniques is solid freeform fabrication, which consists of building a ceramic mould through printing of a polymeric binder on ceramic

powder (layer by layer) to hold ceramic particles together, and using this mould to prepare a porous coating that can be implanted as-cast. The ceramic mould can then be eliminated through the decomposition of the binder [37]. This technique has the advantage of an increased control over the microstructure of the scaffold but is limited by the resolution of the printing apparatus.

Powder metallurgy techniques have also been used extensively for the development of porous metal fabrication processes [3,38]. A powder metallurgy process was recently developed at IMI-NRC for the fabrication of titanium foams characterized, by a unique microstructure [39]. This process consists of mixing a titanium powder with a polymeric binder and a foaming agent prior to a three-step thermal treatment. During the initial step of low temperature heat cure, the binder is melted to create a suspension charged with the titanium powder, after which the foaming agent decomposes to release gas that forms the interconnected pores within the scaffold structure. The second phase incorporates the decomposition of the polymeric compound at high temperature. Finally, the remaining titanium particles are sintered to provide the necessary mechanical integrity to the foam. A schematic diagram of the foam fabrication process is shown in Figure 3.1. This process for the fabrication of porous titanium scaffolds is described in more details in Chapters 5 and 6. The flexibility of the process allows for adjustment of the average pore size of the structure and other microstructural parameters. This report presents the results of *in vitro* studies investigating cellular interactions with porous titanium scaffolds fabricated through this procedure.

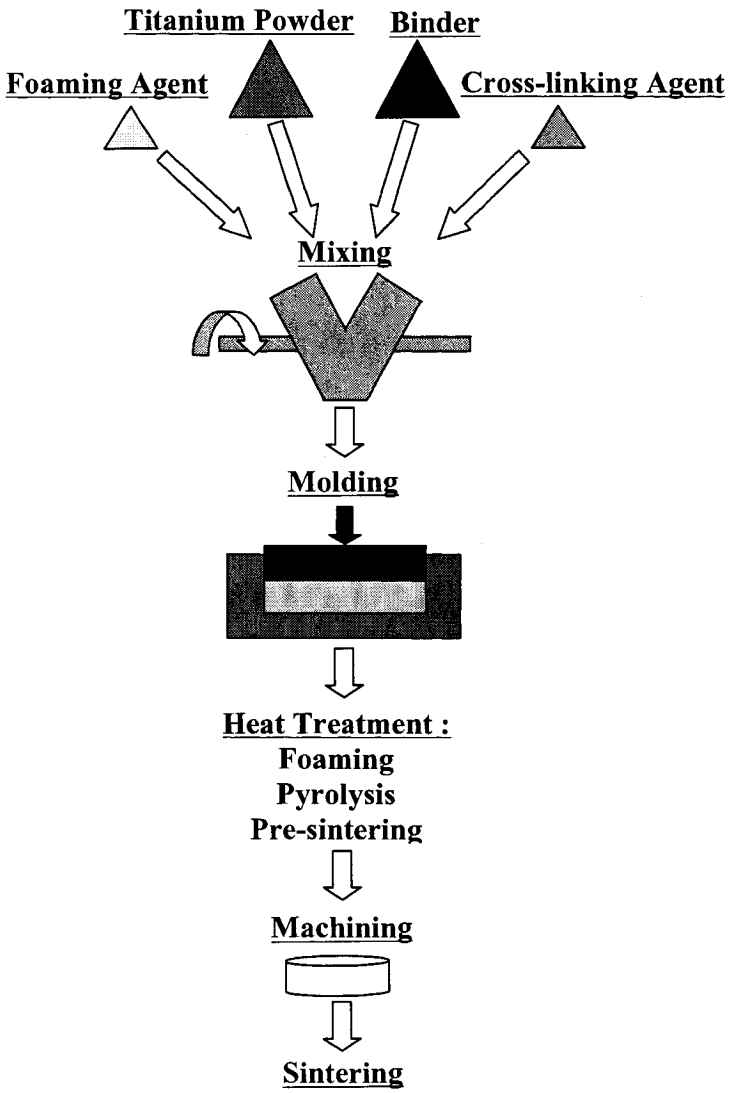


Figure 3.1. Schematic diagram of the modified powder metallurgy process developed at IMI-NRC for the fabrication of titanium foams.

References

1. Hench, L. L. *Biomaterials* **1998**, 19, 1419.
2. Parikh, S. N. *J. Postgrad. Med.* **2002**, 48, 142.
3. Wen, C. E.; Yamada, K.; Shimojima, K.; Chino, Y.; Asahina, T.; Mabuchi, M. *J. Mater. Sci: Mater. Med.* **2002**, 13, 397.
4. Hutmacher, D. W. *Biomaterials* **2000**, 21, 2529.
5. LeGeros, R. Z. *Clin. Orthop.* **2002**, 81.
6. Hanawa, T. *Sci. Technol. Adv. Mater.* **2002**, 3, 289.

7. Niinomi, M. *Mater. Sci. Eng. A* **1998**, 243, 231.
8. Song, Y.; Xu, D. S.; Yang, R.; Li, D.; Wu, W. T.; Guo, Z. X. *Mater. Sci. Eng. A* **1999**, 260, 269.
9. Bobyn, J. D.; Stackpool, G. J.; Hacking, S. A.; Tanzer, M.; Krygier, J. J. *J.Bone Joint Surg. (Br)* **1999**, 81, 907.
10. Pilliar, R. M. *Int. J. Powder Metall.* **1998**, 34, 33.
11. Bauer, T. W.;Schils, J. *Skeletal Radiol.* **1999**, 28, 423.
12. Ayers, R. A.; Simske, S. J.; Bateman, T. A.; Petkus, A.; Sachdeva, R. L.; Gyunter, V. E. *J. Biomed. Mater. Res.* **1999**, 45, 42.
13. Assad, M.; Jarzem, P.; Leroux, M. A.; Coillard, C.; Chernyshov, A. V.; Charette, S.; Rivard, C. H. *J. Biomed. Mater. Res.* **15-2-2003**, 64B, 107.
14. Assad, M.; Chernyshov, A. V.; Jarzem, P.; Leroux, M. A.; Coillard, C.; Charette, S.; Rivard, C. H. *J. Biomed. Mater. Res.* **15-2-2003**, 64B, 121.
15. Sidhu, K. S.; Prochnow, T. D.; Schmitt, P.; Fischgrund, J.; Weisbrode, S.; Herkowitz, H. N. *Spine J.* **2001**, 1, 331.
16. Drummond, J. F.; Dominici, J. T.; Sammon, P. J.; Okazaki, K.; Geissler, R.; Lifland, M. I.; Anderson, S. A.; Renshaw, W. *J. Oral Implantol.* **1995**, 21, 295.
17. Simmons, C. A.; Valiquette, N.; Pilliar, R. M. *J. Biomed. Mater. Res.* **1999**, 47, 127.
18. Cameron, H.; Macnab, I.; Pilliar, R. *S. Afr. J. Surg.* **1972**, 10, 63.
19. Cameron, H. U.; Pilliar, R. M.; Macnab, I. *J. Biomed. Mater. Res.* **1973**, 7, 301.
20. Pilliar, R. M.; Lee, J. M.; Maniatopoulos, C. *Clin. Orthop.* **1986**, 108.
21. Aspenberg, P.; Jeppsson, C.; Wang, J. S.; Bostrom, M. *Bone* **1996**, 19, 499.
22. Breme, H. J.;Helsen, J. A. In *Metals as Biomaterials*; Helsen, J.A.; Breme, H., Ed.; John Wiley & Sons: New York, **1998**, chap. 1.
23. Zardiackas, L. D.; Parsell, D. E.; Dillon, L. D.; Mitchell, D. W.; Nunnery, L. A.; Poggie, R. *J.Biomed.Mater.Res. (Appl.Biomater.)* **2001**, 58, 180.
24. Biehl, V.;Breme, J. *Materialwissenschaft und Werkstofftechnik* **2001**, 32, 137.
25. Eisenbarth, E.; Velten, D.; Schenk-Meuser, K.; Linez, P.; Biehl, V.; Duschner, H.; Breme, J.; Hildebrand, H. *Biomol. Eng* **2002**, 19, 243.
26. Long, M.;Rack, H. J. *Biomaterials* **1998**, 19, 1621.

27. Sul, Y. T.; Johansson, C. B.; Petronis, S.; Krozer, A.; Jeong, Y.; Wennerberg, A.; Albrektsson, T. *Biomaterials* **2002**, 23, 491.
28. Galante, J.; Rostoker, W.; Lueck, R.; Ray, R. D. *J. Bone Joint Surg. Am.* **1971**, 53, 101.
29. Welsh, R. P.; Pilliar, R. M.; Macnab, I. *J. Bone Joint Surg. Am.* **1971**, 53, 963.
30. Bobyn, J. D.; Pilliar, R. M.; Cameron, H. U.; Weatherly, G. C. *Clin. Orthop.* **1980**, 263.
31. Itala, A. I.; Ylanen, H. O.; Ekholm, C.; Karlsson, K. H.; Aro, H. T. *J. Biomed. Mater. Res.* **2001**, 58, 679.
32. Hulbert, S. F.; Young, F. A.; Mathews, R. S.; Klawitter, J. J.; Talbert, C. D.; Stelling, F. H. *J. Biomed. Mater. Res.* **1970**, 4, 433.
33. Kujala, S.; Ryhanen, J.; Danilov, A.; Tuukkanen, J. *Biomaterials* **2003**, 24, 4691.
34. Spector, M. **1982**, 89.
35. Udomkiat, P.; Dorr, L. D.; Wan, Z. *J. Bone Joint Surg. Am.* **2002**, 84-A, 1195.
36. Bloebaum, R. D.; Rhodes, D. M.; Rubman, M. H.; Hofmann, A. A. *Clin. Orthop.* **1991**, 179.
37. Melican, M. C.; Zimmerman, M. C.; Dhillon, M. S.; Ponnambalam, A. R.; Curodeau, A.; Parsons, J. R. *J. Biomed. Mater. Res.* **2001**, 55, 194.
38. Li, J. P.; Li, S. H.; de Groot, K.; Layrolle, P. *Key Eng. Mater.* **2002**, 218-220, 51.
39. Lefebvre, L. P.;Thomas, Y. U.S. Patent No. 6 660 224 (9 December 2003).

CHAPTER 4

Osteoblast-Biomaterials Interactions

4.1 Osteoblast Behaviour on Biomaterials

4.1.1 Osteoblast Adhesion

Cell adhesion is a phenomenon that can be observed naturally during the integration of biomaterials by the body [1]. This initial phase of interactions is critical for pre-differentiated osteoblasts because it influences their proliferation and differentiation behaviour toward mature osteoblasts capable of producing a mineralized extracellular matrix. Studies of cell adhesion phenomena provide essential information on the biocompatibility of the material with which cells interact, making it an important parameter to investigate in the *in vitro* evaluation of a bone engineering biomaterial. Cell adhesion summarizes a series of complex interactions between cells and a substrate upon initial contact. These interactions include the sequential adsorption of proteins to the substrate surface, attachment of cells to specific sequences of these adsorbed proteins, adhesion and spreading [1-2].

Adhesion of cells to a substrate can occur without the preliminary protein adsorption step [2]. This type of adhesion, referred to as passive adhesion, is a non-specific adsorption phenomenon as it can be observed with non-viable cells and even parts of cell membrane. This situation does not occur in physiological conditions, as body fluids coming in contact with implants contain high levels of proteins. The adsorption of proteins on biomaterials following their implantation occurs almost instantaneously. The same phenomenon is observed upon placing a substrate in serum complemented medium for *in vitro* investigations. Cells interact specifically with glycoproteins adsorbed to the surface because they contain an RGD sequence (Arg-Gly-Asp) that is specific to the fixation of cell membrane integrins [1,3-4]. Integrins are composed of α and β subunits. Many forms of these subunits exist, ensuring the adhesion of individual integrin protein to a specific protein adsorbed to the substrate. Some bone extracellular matrix glycoproteins such as fibronectin, vitronectin, laminin, osteopontin and type I collagen can be found in a soluble form in serum [1,5].

Simultaneous to the adsorption of serum proteins to the substrate, cells are brought in close proximity to the surface through a number of long and short range interactions including van der Walls forces and ionic forces [1]. However, the net negative charge of cells at physiological pH, combined with the fact that the surface charge of biomaterials is often negative, creates repulsive forces [2]. Cells appear to use cytoplasmic extensions to bridge the gap with the material. These extensions reflect interactions between transmembrane integrins and the RGD sequences of associated glycoproteins adsorbed to the substrate and indicate the beginning of the adhesion phase. These adhesion sites where integrins react with surface proteins are called focal contacts and maintain the cell membrane within 10 to 15 nm of the substrate [1]. The intracellular portion of focal contact sites is composed of a complex network of proteins including vinculin, paxillin, talin, focal adhesion kinase, and tensin that contribute to the mediation of signals from the integrins to the actin cytoskeleton. Thus, adhesion of cells to a substrate triggers a mechanism that leads to an appropriate reorganization of the cytoskeleton and associated spreading of the cell. This process is highly influenced by the substrate through its control on the adsorption of proteins, as will be discussed in a later section. Thus, the adapted cell shape is a function of the material. Reorganization of actin networks inside the cells is sensed by the nucleus and leads to an adequate phenotype change toward proliferation and differentiation phases. A representation of the cellular system involved in the formation of focal contacts and cytoskeleton organization upon cell adhesion is shown in Figure 4.1.

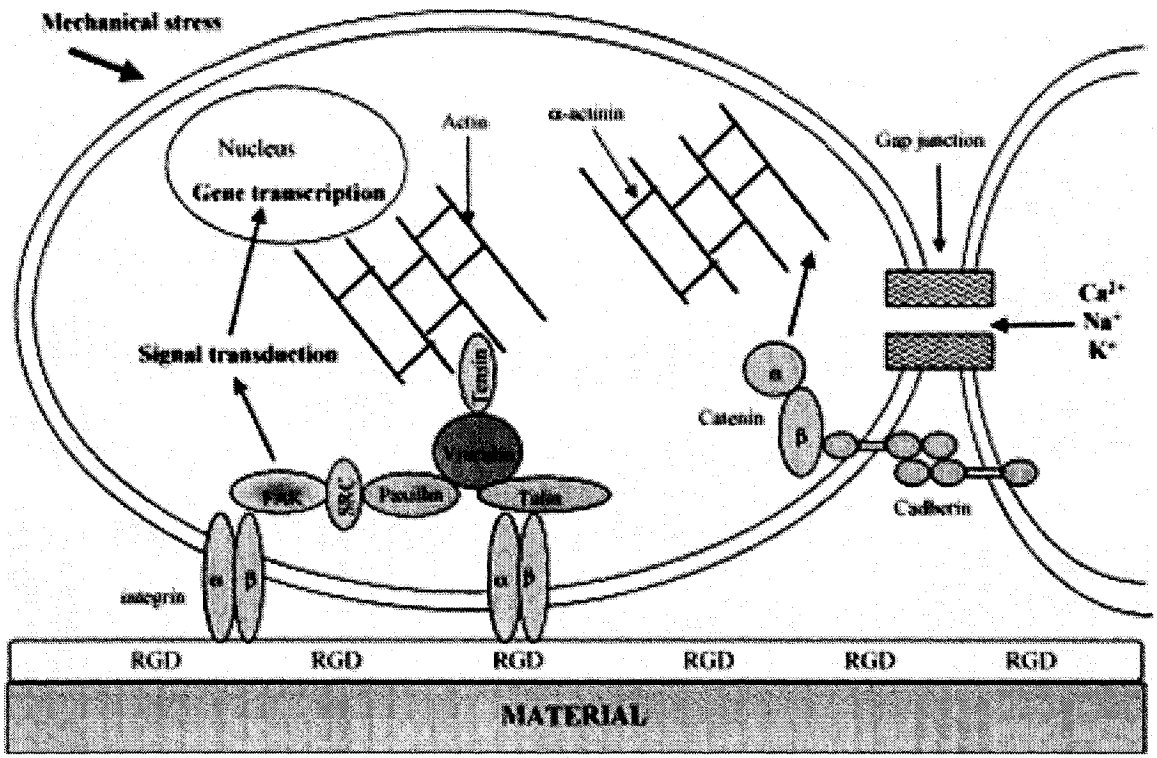


Figure 4.1. Schematic representation of the proteins involved in the formation of focal contacts and the cytoskeletal organization by osteoblasts adhering to biomaterials. (Reprinted from Biomaterials, Vol 23, Anselme K, Osteoblast adhesion on biomaterials, 667-81, © (1999) with permission from Elsevier).

4.1.2 Osteoblast Differentiation

The wound healing process triggered following bone fractures or the insertion of implant materials in bone consists of a series of complex phenomena that require the recruitment of various types of cells to perform specific functions. Notably, osteogenic cells are essential to the regeneration of damaged bone. These cells are generally marrow stromal cells, mesenchymal stem cells, and vascular pericytes [6]. However, pre-differentiated and mature osteoblasts, as well as osteocytes also migrate to the injury site in lesser amounts, in order to contribute to the bone regeneration process. Thus, most osteogenic cells recruited by the body upon implantation of a bone engineering implant must undergo a differentiation process toward a mature osteoblastic stage capable of mineralizing extracellular matrix.

The original theory to describe the process by which osteoprogenitor cells differentiate toward mature osteoblasts proposed a three step process. Briefly, cells follow sequential development stages starting with a proliferation phase, followed by the production of an extracellular matrix that evolves as the cells differentiate, and completed by the mineralization process; each step is characterized by the expression of specific genes [7].

The expression of known differentiation markers seems to indicate that more stages exist in the differentiation sequence and that these stages can overlap more than one of the three theorized developmental stages [7-8]. Cell culture systems used to gain an understanding of the specific steps in the differentiation process can only provide limited information because they often contain multiple cell types other than osteoprogenitor cells that express various levels of markers conventionally used to identify osteoblast differentiation, or because they are heterogeneous in terms of the differentiation stages that osteoprogenitor cells have reached [9]. Recently, the use of poly(A)-polymerase chain reaction techniques has allowed the entire gene expression pattern of single cells or colonies to be followed through time [7-10]. This approach has allowed investigators to separate the differentiation process into seven stages. However, they have not ruled out the possibility that intermediate stages have yet to be identified. A schematic representation of the osteoblast differentiation process is provided in Figure 4.2.

While work remains to be done in identifying proteins that allow to differentiate between cells at various differentiation stages prior to the pre-osteoblast phase, differences between the later type of cells and mature osteoblasts are better understood [8,11]. Notably, pre-osteoblasts are still in the proliferative phase, which is not the case for osteoblasts. Alkaline phosphatase is generally expressed in the pre-differentiated stage, but is up-regulated in more mature cells and its activity is reduced when the mineralization process is under way. The expression of other bone markers following the proliferative phase consist of a peak in osteopontin, followed by bone sialoprotein and osteocalcin, which appears coincidentally with the start of the mineralization process. While alkaline phosphatase is most often used as a differentiation marker for osteoblasts

grown on biomaterials, osteocalcin is the most specific marker to osteoblast differentiation [8,12].

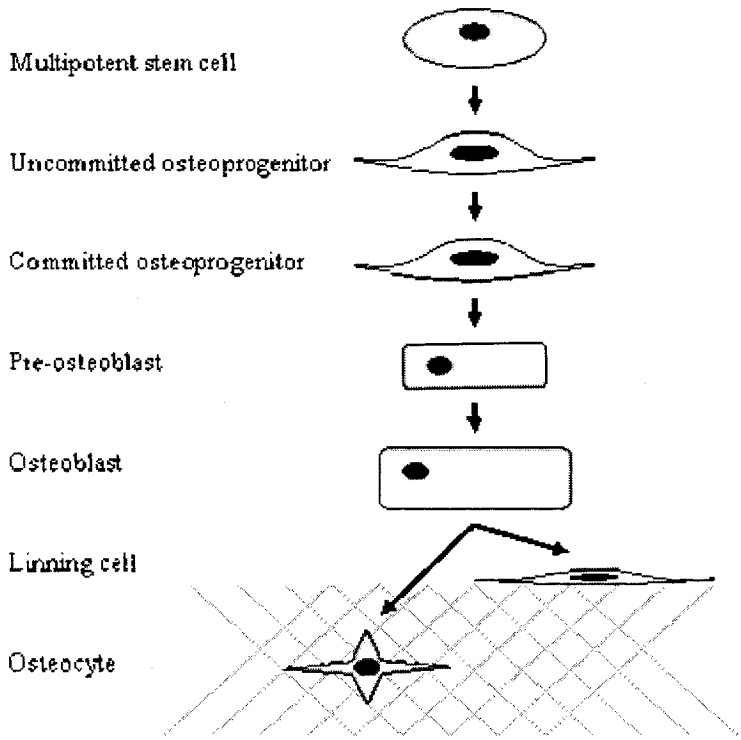


Figure 4.2. Schematic representation of the seven stages osteoblast differentiation process [8].

A small portion of osteoblasts can further differentiate in two types of post-proliferative cells that do not contribute to the formation of mineralized extracellular matrix. Osteocytes are embedded in the matrix and are known to have the capacity to respond to mechanical loading by elevating their expression of insulin-like growth factor-I, osteocalcin, as well as cyclooxygenase [13]. The other differentiation route is toward bone-lining cells, which were proposed to have the potential to be reactivated and provide a readily available source of osteoblasts given specific signals [14].

There is strong evidence that hormones, cytokines, and growth factors have stimulatory or inhibitory effects on the differentiation process of osteoblasts [11]. Thus, their incorporation with scaffolds has been studied in order to improve osteoconductive properties, as discussed in a previous chapter. However, to improve these multifaceted systems, research must focus on gaining knowledge on the required exposure duration to

these molecules, concentration, and stage at which they must be introduced to optimize their effects.

Of more importance is the impact of the extracellular matrix proteins on the differentiation process. It seems that the production of an extensive matrix containing collagen type I is essential to the expression of previously discussed differentiation markers and for the mineralization process to occur [15-16]. These findings corroborate the fact that the quality of the cell adhesion process is of major importance to the promotion of an adequate differentiation toward the formation of a mineralized extracellular matrix. Thus, biomaterials must be designed in order to promote appropriate interactions with cells.

4.2 Microstructural Parameters of Biomaterials Affecting Cell Response

Cells interact with the proteins adsorbed on the surface of a material rather than directly with the substrate. Hence, properties of the material that influence cell response are in fact parameters that modify the protein adsorption process. The nature of proteins adsorbed to a surface has an impact on the cell adhesion process, with the orientation of these proteins being of utmost importance [1,17]. In fact, cell adhesion can only occur if the reactive RGD groups of adsorbed proteins are exposed and in an active conformation to allow interactions with cell integrins.

It is important to note that the surface properties of a material are quite different to its bulk properties [1,18]. Thermodynamic restrictions in the material force unsaturated bonds to the surface, rendering it reactive. This reactive layer is responsible for the formation of an oxide layer at the surface of metals, thus changing the surface chemical composition of the material. Furthermore, an adsorbed contamination layer is generally present at the surface of materials, affecting its surface properties. This phenomenon stresses the importance of employing adequate steps to ensure that a biomaterial is adequately cleaned and that the surface interacting with cells is not contaminated.

Studies of cell behaviour upon interaction with biomaterials discuss the importance of surface properties of a substrate such as its surface chemistry, the material topography and its surface energy, as they influence the cellular response [1,17]. These parameters are often studied through *in vitro* models with two-dimensional surfaces. Such

an experimental setup allows investigations of the individual cell behaviour of a specific cell type without interference by other cells generally recruited to the implantation site in *vivo*, while controlling the microstructural properties of the test material.

An extensive body of literature demonstrates the impact of the material's nature on the number of osteoblasts adhering to the substrate, their morphology, and their capacity to form an organized cytoskeleton and focal contacts [18-23]. Titanium encourages cell adhesion compared to cobalt-chromium-molybdenum and stainless steel alloys commonly used for orthopaedic applications [19-20]. Furthermore, the better adhesion properties exhibited by titanium substrates seem to impact the later interactions of the material with hFOB 1.19 (human fetal osteoblasts), as it was demonstrated that proliferation was delayed on cobalt-chromium-molybdenum and stainless steel compared to titanium, while differentiation markers alkaline phosphatase and osteocalcin were higher on titanium surfaces than the two other biocompatible metals [19].

The effect of surface topography on cell adhesion and differentiation has also been studied extensively for osteoblasts on materials currently used for bone engineering applications [23-31]. Many studies report that the number of cells adhering to a substrate is directly influenced by the material surface roughness [26-28,31]. It also seems that organization of the surface topography can positively influence the number of cells adhering to the substrate [24]. Although, it still remains unclear what type of organization is required to optimize cell response. Cell morphology is also influenced by the surface topography of substrates, as small changes in surface roughness influence the cell's capacity to spread [25,29,31]. More evident morphological changes can be observed for osteoblasts adhered to materials with high levels of surface topography, as they exhibit a polygonal shape with three-dimensional extensions on these structures rather than the flat spindle-like morphology found on less textured materials [23,25]. Such a three-dimensional morphology is also characterized by a less organized cytoskeleton [29] and a different arrangement of focal contacts to reflect the points of contact with the material located at the end of cytoplasmic extensions [24]. Deligianni et al. [26] demonstrated through X-ray photoelectron spectroscopy and radiolabeling that the nature of proteins adsorbed on rough surfaces compared to smooth substrates was more adequate for the promotion of cell adhesion. In fact, they showed that considerably more fibronectin was

adsorbed on rough surfaces, while smooth surfaces encouraged the adsorption of other fetal bovine serum proteins. Furthermore, the rough substrate allowed more total proteins to be adsorbed, probably due to the increased surface area and charge density. It has also been observed that surface topography of metals promotes the modification of the gene expression pattern of osteoblasts toward a more mature stage, as verified by reduced proliferation [29], an increased alkaline phosphatase activity [26,28] and a higher level of mineralization [30].

Surface energy has also been studied extensively as an important parameter influencing cell behaviour on implant materials [32-37]. This parameter is a measure of the wettability of a surface. Thus, it is related to the hydrophilicity of a material. Surface energy is generally influenced by the surface roughness of a material, its charge and the chemical composition of its surface [37]. Associated with the general charge density of the surface, as well as the polarity of that charge, surface energy results in specific surface tension and energy of adhesion to the material [17]. The fact that surface energy affects cellular behaviour confirms the importance of this material property in controlling the protein adsorption process [35]. An increased surface energy impacts osteoblast adhesion positively, while reducing cell spreading [32,37]. As was the case for the other surface parameters affecting cell behaviour, conditions of surface energy improving the ability of osteoblasts to adhere to surfaces have also been associated with a faster shift from proliferation to differentiation as observed through higher alkaline phosphatase activities [34,37].

As explained in Chapter 3, the three-dimensional microstructure of scaffolds influences bone ingrowth *in vivo*. Many studies have reported the use of osteoblastic cell cultures to investigate the osteoconductive and bioactive properties of novel polymeric and ceramic foams [38-41]. However, the effect of three-dimensional microstructural parameters of porous scaffolds on osteoblastic cell behaviour has been scarcely reported in the literature. While it has been demonstrated that adhesion and differentiation of osteoblastic cells are affected by contact with a porous structure in a similar fashion to the effect of increased surface roughness [42], it seems that the pore size does not impact these specific cell behaviour [43]. Rather, the pore size has been shown to affect the mechanisms by which extracellular matrix is laid down, as well as the migration of cells

inside single pores, and the reorganization of the matrix in osteons [44]. Nevertheless, some reports have suggested that the pore shape might also be an important factor in influencing these cell behaviour, as some pore geometries might provide optimal compression and tension conditions permitting the cells to receive and transmit load signals from the material [17]. Even though porous materials have been used for bone engineering applications for over 35 years, the state of knowledge concerning the impact of three-dimensional conditions on cell behaviour does not yet allow the design of optimal porous implant scaffolds.

4.3 Experimental Investigation of Osteoblast Response to Biomaterials

4.3.1 Experimental Methods to Study Osteoblast Adhesion

Osteoblast adhesion to a bone engineering surface is a complex process involving a series of steps at the molecular level. This process is generally studied quantitatively through measures of the number of cells adhering to the surface. These investigations are usually complemented by qualitative assessments of cell morphology and adhesion proteins distribution inside the cells and in their surroundings.

The ideal assay for measurement of the number of adherent cells on a material would have to be simple, short and inexpensive, with a high sensitivity. It is also important to minimize exposure to hazardous compounds [45]. The experimental methods that have been developed to quantify the number of adherent cells each have their advantages and weaknesses. Some of the methods most commonly used to study osteoblasts adhesion to biomaterial's surfaces will be discussed in this section with applications for porous metals in mind.

A direct counting technique often used for the quantification of adherent osteoblasts uses the coulter particle counter [26]. This technique is simple to use, automated and has a good signal-to-noise ratio compared to other direct methods relying on microscopy techniques [46]. Nevertheless, it requires the detachment of adherent cells, which is time consuming, and the use of expensive apparatus. Furthermore, the detachment and subsequent collection of cells is complicated by the use of three-dimensional test materials.

Microscopic techniques are rarely used for cell counting purposes on biomaterials because of the limitations of each technique and the availability of faster indirect techniques. Nevertheless, scanning electron microscopy provides an excellent mean to gain valuable information on adherent osteoblast morphology, which indicates the adequacy of the adhesion process [23]. Epifluorescence microscopes are also used to visualize the distribution of intracellular proteins related to the adhesion process, as well as extracellular matrix [22,24]. However, epifluorescence microscopes are more difficult to use for cells adhered inside three-dimensional scaffolds because the reading at the focal point is blurred by fluorescence at adjacent depts.

Many indirect methods have been developed to measure the number of adherent cells on surfaces. One method that is often used consists of labeling the cells with radioisotopes such as ^{51}Cr prior to the assay [45-47]. Such an approach provides very sensitive results but requires the use of hazardous materials, special safety procedures, and specialized equipment. Furthermore, radiolabeling cells is a time consuming process and radioactive probes are generally expensive and have a short shelf-life. It is also possible to label cells using biotinylation [45,47]. While this technique is not as sensitive as the use of radiolabels, it has the advantage of being easy to use. As cell labeling occurs prior to the seeding procedure for these two techniques, cell viability can be affected by these procedures, thus influencing the results and preventing the methods from being used for long incubation periods [48].

Another approach for the quantification of adherent cells used extensively for osteoblasts attached to bone engineering substrates consists of using enzyme-based systems [45]. These systems have the advantage of a high signal-to-noise ratio due to the possibility to adjust the signal by enzyme catalysis. Furthermore, the detection can generally be obtained through colorimetric or fluorescence measurements. One such example is the use of Calcein-AM as a substrate that can be transformed to fluorescent Calcein through the action of esterases located in the cell cytoplasm [45,48]. Because the hydrolysed product cannot escape the cell membrane, cells must be lysed prior to the measurement.

Another method involves the use of tetrazolium salts such as MTT that can penetrate cells and are transformed to formazan crystals by mitochondrial

dehydrogenases [45,49]. The crystals can be solubilised to form a blue solution of intensity directly proportional to the number of viable cells. Problems with such enzyme based assays relate to the fact that the levels of intracellular enzymes can vary with time. Thus, these tests are not appropriate for comparison of cell numbers on substrates at significantly different time points. Furthermore, factors that could affect enzyme activity such as the pH and the temperature can induce error in the results if not properly controlled.

A last method used to quantify the number of cells adhered to substrates consists of a measure of the DNA content of the bone-like tissue grown *in vitro*. As DNA amounts per cell are consistent for a cell type at every maturation stage, this method allows for comparisons between spaced time points. Generally, these assays consist of binding a fluorescent dye to DNA and can be measured using fluorescence detection apparatus.

4.3.2 Experimental Methods to Study Osteoblasts Differentiation

Various proteins have been identified as differentiation markers for osteoblasts and were discussed previously. Of these, the most often studied in the field of bone engineering are alkaline phosphatase, osteocalcin, and collagen type I. Three different experimental assays are generally used to investigate the presence of these proteins. Alkaline phosphatase is most often studied through its enzymatic activity [26,50-51]. As for osteocalcin, it is an extracellular matrix protein, but a percentage of newly synthesized protein is released in the medium *in vitro* or in the serum *in vivo*, which is proportional to the amount of newly synthesized protein. Thus, osteocalcin content is often measured through enzyme-linked immunosorbent assays of the culture medium [51]. A similar approach can also be employed to measure the amount of collagen type I synthesized. Polymerase chain reaction has also been used to monitor the expression patterns for these differentiation markers [50]. Qualitative assessment of the distribution of these markers in the bone-like tissue grown *in vitro* can be obtained through immunolabeling combined with epifluorescence microscope techniques.

The localization of mineralized regions of the extracellular matrix produced by osteoblasts following differentiation *in vitro* requires the use of stains such as Alizarin

Red and von Kossa. However, these are not adequate for use in highly textured metals because they do not generate enough contrast with the material. To quantify the level of mineralization produced by differentiated osteoblasts, two methods are generally employed. The technique most often used consists of reacting calcium with o-cresolphthalein complexone to form a purple compound that can be assessed with a spectrophotometer. Another technique consists of using atomic absorption spectroscopy to measure the dissolved calcium content. The later technique has better selectivity, does not require time consuming sample preparation, and is quite sensitive [52]. Both methods require that the minerals be dissolved prior to the assay. However, the biochemical approach to measuring calcium content from the bone-like extracellular matrix does not require expensive equipment, as is the case for atomic absorption spectroscopy.

References

1. Anselme, K. *Biomaterials* **2000**, 21, 667.
2. Grinnell, F. *Int. Rev. Cytol.* **1978**, 53, 65.
3. Burridge, K. *Cancer Reviews* **1986**, 4, 18.
4. Grzesik, W. J.;Robey, P. G. *J. Bone Miner. Res.* **1994**, 9, 487.
5. Stanford, C. M.; Solursh, M.; Keller, J. C. *J. Biomed. Mater. Res.* **5-12-1999**, 47, 345.
6. Davies, J. E.;Hosseini M.M. In *Bone Engineering*; Davies, J.E., Ed.; em squared incorporated: Toronto, **2000**, chap. 1.
7. Aubin, J. E. *J. Cell Biochem. Suppl* **1998**, 30-31, 73.
8. Aubin, J. E. *Biochem. Cell Biol.* **1998**, 76, 899.
9. Aubin, J. E.; Liu, F.; Malaval, L.; Gupta, A. K. *Bone* **1995**, 17, 77S.
10. Liu, F.; Malaval, L.; Aubin, J. E. *J. Cell Sci.* **1-5-2003**, 116, 1787.
11. Aubin, J. E. In *Bone Engineering*; Davies, J.E., Ed.; em squared incorporated: Toronto, **2000**, chap. 3.
12. Hauschka, P. V.; Lian, J. B.; Cole, D. E.; Gundberg, C. M. *Physiol Rev.* **1989**, 69, 990.
13. Kawata, A.;Mikuni-Takagaki, Y. *Biochem. Biophys. Res. Commun.* **19-5-1998**, 246, 404.

14. Dobnig, H.;Turner, R. T. *Endocrinology* **1995**, 136, 3632.
15. Jikko, A.; Harris, S. E.; Chen, D.; Mendrick, D. L.; Damsky, C. H. *J. Bone Miner. Res.* **1999**, 14, 1075.
16. Xiao, G.; Cui, Y.; Ducy, P.; Karsenty, G.; Franceschi, R. T. *Mol. Endocrinol.* **1997**, 11, 1103.
17. Boyan, B. D.; Hummert, T. W.; Dean, D. D.; Schwartz, Z. *Biomaterials* **1996**, 17, 137.
18. Okumura, A.; Goto, M.; Goto, T.; Yoshinari, M.; Masuko, S.; Katsuki, T.; Tanaka, T. *Biomaterials* **2001**, 22, 2263.
19. Hendrich, C.; Noth, U.; Stahl, U.; Merklein, F.; Rader, C. P.; Schutze, N.; Thull, R.; Tuan, R. S.; Eulert, J. *Clin. Orthop.* **2002**, 278.
20. Howlett, C. R.; Evans, M. D.; Walsh, W. R.; Johnson, G.; Steele, J. G. *Biomaterials* **1994**, 15, 213.
21. Hunter, A.; Archer, C. W.; Walker, P. S.; Blunn, G. W. *Biomaterials* **1995**, 16, 287.
22. Puleo, D. A.;Bizios, R. *J. Biomed. Mater. Res.* **1992**, 26, 291.
23. Schmidt, C.; Kaspar, D.; Sarkar, M. R.; Claes, L. E.; Ignatius, A. A. *J. Biomed. Mater. Res.* **2002**, 63, 252.
24. Anselme, K.; Bigerelle, M.; Noel, B.; Dufresne, E.; Judas, D.; Iost, A.; Hardouin, P. *J. Biomed. Mater. Res.* **2000**, 49, 155.
25. Degasne, I.; Basle, M. F.; Demais, V.; Hure, G.; Lesourd, M.; Grolleau, B.; Mercier, L.; Chappard, D. *Calcif. Tissue Int.* **1999**, 64, 499.
26. Deligianni, D. D.; Katsala, N.; Ladas, S.; Sotiropoulou, D.; Amedee, J.; Missirlis, Y. F. *Biomaterials* **2001**, 22, 1241.
27. Keller, J. C.; Schneider, G. B.; Stanford, C. M.; Kellogg, B. *Implant. Dent.* **2003**, 12, 175.
28. Korovessis, P. G.;Deligianni, D. D. *J. Spinal Disord. Tech.* **2002**, 15, 175.
29. Linez-Bataillon, P.; Monchau, F.; Bigerelle, M.; Hildebrand, H. F. *Biomol. Eng* **2002**, 19, 133.
30. Schneider, G. B.; Perinpanayagam, H.; Clegg, M.; Zaharias, R.; Seabold, D.; Keller, J.; Stanford, C. *J. Dent. Res.* **2003**, 82, 372.

31. ter Brugge, P. J.; Dieudonne, S.; Jansen, J. A. *J. Biomed. Mater. Res.* **5-9-2002**, 61, 399.
32. Feng, B.; Weng, J.; Yang, B. C.; Qu, S. X.; Zhang, X. D. *Biomaterials* **2003**, 24, 4663.
33. Lampin, M.; Warocquier, C.; Legris, C.; Degrange, M.; Sigot-Luizard, M. F. *J. Biomed. Mater. Res.* **1997**, 36, 99.
34. Liao, H.; Andersson, A. S.; Sutherland, D.; Petronis, S.; Kasemo, B.; Thomsen, P. *Biomaterials* **2003**, 24, 649.
35. Shelton, R. M.; Rasmussen, A. C.; Davies, J. E. *Biomaterials* **1988**, 9, 24.
36. Webb, K.; Hlady, V.; Tresco, P. A. *J. Biomed. Mater. Res.* **5-9-1998**, 41, 422.
37. Zhang, Y. M.; Bataillon-Linez, P.; Huang, P.; Zhao, Y. M.; Han, Y.; Traisnel, M.; Xu, K. W.; Hildebrand, H. F. *J. Biomed. Mater. Res.* **1-2-2004**, 68A, 383.
38. Acil, Y.; Terheyden, H.; Dunsche, A.; Fleiner, B.; Jepsen, S. *J. Biomed. Mater. Res.* **15-9-2000**, 51, 703.
39. Cheung, H. S.;Haak, M. H. *Biomaterials* **1989**, 10, 63.
40. Ferrera, D.; Poggi, S.; Biassoni, C.; Dickson, G. R.; Astigiano, S.; Barbieri, O.; Favre, A.; Franzi, A. T.; Strangio, A.; Federici, A.; Manduca, P. *Bone* **2002**, 30, 718.
41. Zhang, Y.;Zhang, M. *J. Biomed. Mater. Res.* **2002**, 61, 1.
42. Groessner-Schreiber, B.;Tuan, R. S. *J. Cell Sci.* **1992**, 101 (Pt 1), 209.
43. Ishaug-Riley, S. L.; Crane-Kruger, G. M.; Yaszemski, M. J.; Mikos, A. G. *Biomaterials* **1998**, 19, 1405.
44. Frosch, K. H.; Barvencik, F.; Lohmann, C. H.; Viereck, V.; Siggelkow, H.; Breme, J.; Dresing, K.; Sturmer, K. M. *Cells Tissues. Organs* **2002**, 170, 214.
45. Loster, K.;Horstkorte, R. *Micron.* **2000**, 31, 41.
46. An, Y. H.;Friedman R.J. *Journal of Microbiological Methods* **1997**, 30, 141.
47. Mendis, D.; Ginon, I.; Louis, H.; McGregor, J. L.; Poston, R. N. *J. Immunol. Methods* **1-7-2001**, 253, 57.
48. De Clerck, L. S.; Bridts, C. H.; Mertens, A. M.; Moens, M. M.; Stevens, W. J. *J. Immunol. Methods* **3-6-1994**, 172, 115.
49. Mosmann, T. *J. Immunol. Methods* **16-12-1983**, 65, 55.

50. Ehara, A.; Ogata, K.; Imazato, S.; Ebisu, S.; Nakano, T.; Umakoshi, Y. *Biomaterials* **2003**, 24, 831.
51. Schmidt, C.; Ignatius, A. A.; Claes, L. E. *J. Biomed. Mater. Res.* **2001**, 54, 209.
52. Mesquita, R. B.;Rangel, A. O. *Anal. Sci.* **2004**, 20, 1205.

CHAPTER 5

Early Cellular Interactions with Porous Titanium

ADHESION KINETICS OF MC3T3-E1 PRE-OSTEOBLASTS TO OSTEOCONDUCTIVE POROUS TITANIUM SCAFFOLDS

Jean-Philippe St-Pierre[1,2], Maxime Gauthier[2], Louis-Philippe Lefebvre[2], and Maryam Tabrizian[1].

[1] McGill University, Department of Biomedical Engineering, Montreal, Canada.

[2] National Research Council Canada, Industrial Materials Institute, Boucherville, Canada.

5.1 Abstract

Porous metallic scaffolds have recently gained recognition as a promising avenue toward the regeneration of damaged bone structures. Interest in these materials resides in their ability to guide bone growth by presenting a favorable structure for cellular adhesion and three-dimensional proliferation. A powder metallurgy process to fabricate titanium foams with favorable microstructural parameters for applications in bone engineering has recently been developed. This study assesses the potential of this novel material for applications as an osteoconductive scaffold through *in vitro* characterization of early cellular interactions with titanium foams having pore sizes ranging from 167 to 500 μm. The foams exhibit no cytotoxic effects on J774 mouse macrophages while favoring adhesion and proliferation of MC3T3-E1 pre-osteoblasts. Three-dimensional morphology assumed by these cells on porous titanium suggests that the microstructure of the foams is biomimetic.

5.2 Introduction

Porous metals have been employed for more than 30 years in the field of orthopedics as implant coatings to ensure a stable fixation through bone ingrowth. Conventional approaches to the fabrication of such porous coatings include bead and fiber sintering [1,2] and plasma-spray technologies [3]. Recently, a shift has been witnessed in the design of such biomaterials toward the fabrication of highly porous

metals with microstructural parameters that facilitate bone regeneration [4,5]. In addition to conventional applications as porous coatings, these innovative materials have generated interest in related fields such as craniofacial reconstruction. Highly porous titanium foams with a unique and adjustable microstructure produced through a novel powder metallurgy process contribute to this trend [6]. The present study aims to assess the biocompatibility of these titanium foams through cellular viability and adhesion assays.

5.3 Experimental Methods

5.3.1 Scaffold Preparation and Characterization

Titanium scaffolds with three pore sizes, referred to as TiA, TiB and TiC (from smaller to larger pore size) were prepared using a process previously described [7]. Foams were subsequently machined into small discs (12.5 mm diameter and 2 mm thickness) and sintered at 1300°C for 2 hours. The resulting microstructure of these titanium foams is shown in Figure 5.1. Controls were prepared by polishing dense titanium and nickel discs (referred to as TiP and NiP respectively) of similar dimensions to a mirror-finish (0.04 μm) with an automatic polisher (AbraPol, Struers). Before cell culture assays, porous samples and controls were cleaned by 15 minute sonication cycles in soapy water, isopropanol and de-ionized water, with distilled water rinses after each step. Scaffolds were then autoclaved and allowed to dry at 60°C under sterile conditions overnight.

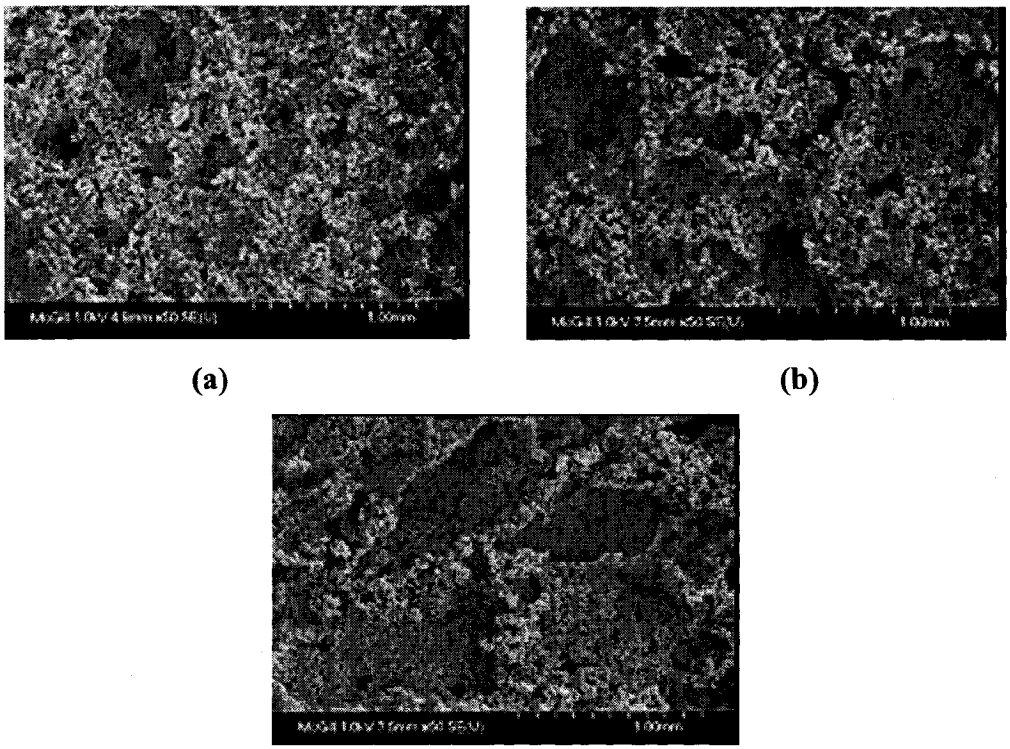


Figure 5.1. Scanning electron microscopy (SEM) images showing the microstructure of titanium foams: (a) TiA (b) TiB (c) TiC.

5.3.2 Cell Culture

J774 mouse macrophages (ATCC, USA) were cultured in RPMI 1640 medium (Gibco BRL, Canada) supplemented with 5% fetal bovine serum (ATCC, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL, Canada). MC3T3-E1 subclone 14 mouse pre-osteoblastic cells (ATCC, USA) were cultured in α-MEM medium (Gibco BRL, Canada) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Both cell lines were grown in T75 cell culture flasks at 37°C in an atmosphere with 100% humidity and 5% CO_2. The complete medium was replaced every 2 to 3 days and confluent cells were subcultured through scraping (J774) or trypsinization (MC3T3-E1).

5.3.3 Cell Viability

Mouse macrophages were seeded in 24-well plates on porous scaffolds and both controls at a concentration of 100,000 cells per millilitre of complete medium and

incubated for 48 hours. Blanks consisted of scaffolds cultured with complete medium in the absence of cells. Following incubation, the medium was replaced and a Vybrant MTT cell proliferation assay (Molecular Probes, USA) was performed. Briefly, a 50 µl aliquot of 12 mM [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) in phosphate buffered solution was added to each well and incubated at 37°C for 4 hours. Formazan crystals formed by cells in contact with the MTT were then dissolved by the addition of 500 µl of sodium dodecyl sulfate-HCl and a subsequent 12 hour incubation. Solution absorbance was read at 570 nm with a spectrophotometer (µQuant, Bio-Tek Instruments). Three replicates were performed for each condition.

5.3.4 Cell Adhesion

Mouse pre-osteoblasts were seeded in 24-well plates on porous scaffolds and both controls (200,000 and 100,000 cells seeded respectively). In addition to blanks for each condition, a third control consisting of empty tissue culture plates (TCP) was also included. After incubations of 30 minutes, 1, 2, 4, or 18 hours, samples were washed three times in Earle's balanced salt solution (EBSS). Quantification of adherent cells was measured through the Vybrant MTT cell proliferation assay described previously. Nine replicates were performed for each type of foam, while 3 replicates were performed for controls.

5.3.5 Cell Morphology

Cell morphology was analyzed by incubating foams and TiP samples with pre-osteoblasts for 18 hours according to the cell adhesion procedure. Adhesion assays were washed three times in EBSS to remove unattached pre-osteoblastic cells and serum proteins. Adhered cells and their extracellular matrix were fixed for 24 hours with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, dehydrated in serial ethanol and amyl acetate baths, and critical point dried. Specimens were sputter-coated with Au-Pd and the cellular morphology was assessed through SEM (FE-SEM S-4700, Hitachi High-Technologies).

5.3.6 *Statistics*

Results are expressed as mean value ± standard deviation. A paired Student t-test was used to identify differences in the data with $p<0.05$ considered significant unless specified otherwise.

5.4 Results and Discussion

5.4.1 *Scaffold characterization*

Table 5.1 presents the pore sizes and porosities of the three types of titanium foam investigated. These results highlight the microstructural differences between each type of foam. SEM observations of the titanium scaffolds up to 2000x magnification did discriminate microstructural surface differences other than pore size between the three conditions.

Table 5.1. Porosity and pore size of the three titanium scaffolds.

Condition	Pore Size (µm)	Porosity[a] (% ± S.D.)
TiA	166.7	73.1 ± 1.6
TiB	425.9	76.6 ± 2.4
TiC	499.6	79.8 ± 2.6

[a] Porosity for each condition is statistically different from the others ($p<0.01$).

5.4.2 *Cell viability*

Figure 5.2 shows the viability of J774 macrophages on titanium foams as a blanked absorbance at 570 nm after 48 hours of incubation. Cellular viability obtained on titanium foams is comparable to that of TiP. In a previous study [8], it was determined that residuals from individual mixture components of the powder metallurgy process affected the nature of titanium surfaces. Nevertheless, these surface modifications did not affect the viability of U-937 human macrophages. Results from the present cellular viability assay demonstrate that the combined effect of the mixture components does not result in cytotoxic effects. As well, cellular viability is not affected significantly by the scaffold pore size but was statistically lower on the negative control (NiP).

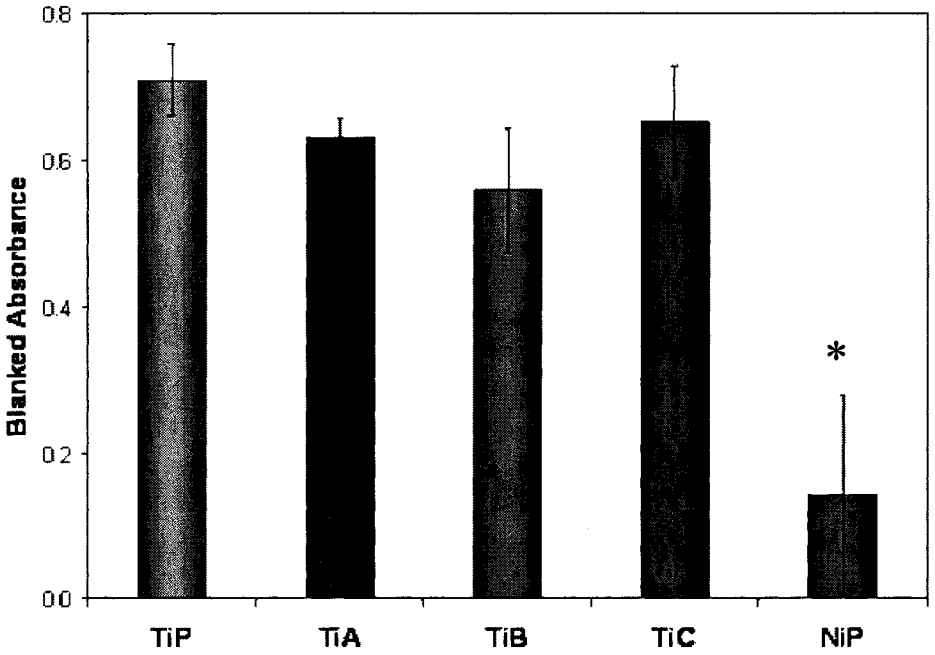
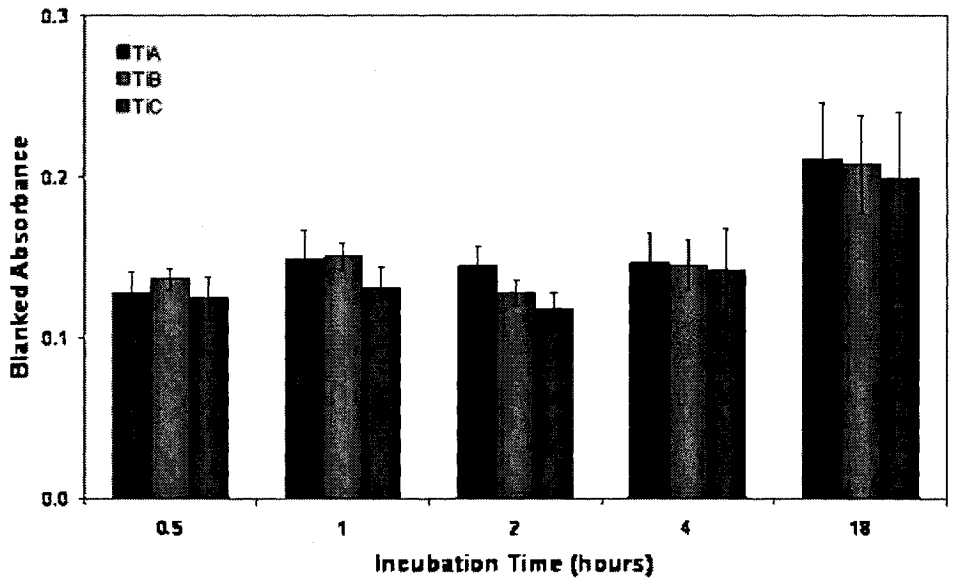


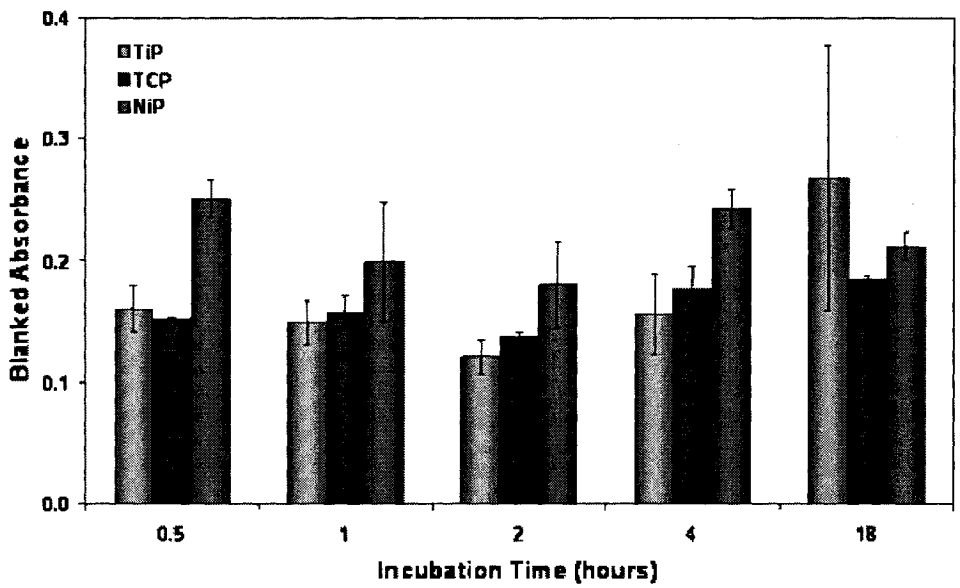
Figure 5.2. Viability of J774 macrophages on titanium foams as a blanked absorbance at 570 nm after 48 hours of incubation. NiP had statistically lower results than the foams and TiP.

5.4.3 Cell Adhesion

Figure 5.3a shows the adhesion kinetics of mouse pre-osteoblasts on titanium scaffolds. The data is presented as a blanked absorbance which is directly proportional to the number of adherent cells (unreported data). Absorbance measurements yielded no significant difference in cell adhesion for the three porous scaffolds. This indicates that, for the range of pore sizes investigated, this parameter did not have an effect on the adhesion kinetics of pre-osteoblastic cells. A decrease in the number of adherent cells on porous scaffolds was observed after 2 hours compared to the 1 hour time point. This trend is statistically significant for TiB and TiC. This effect is not believed to be a cellular response to the material since similar results are observed for TiP and TCP (see Figure 5.3b). This inflection in the adhesion kinetics might be a result of the death of weaker cells due to the trypsination process involved in the seeding procedure. However, more replicates are required for the control samples in order to verify this observation.



(a)



(b)

Figure 5.3. Adhesion kinetics of cells on (a) titanium foams with three different pore sizes and (b) controls. The blanked absorbance is directly proportional to the number of adherent cells.

The number of pre-osteoblasts adhered to porous titanium scaffolds after 18 hours of incubation is significantly higher than for shorter incubation times, indicating the commencement of cell proliferation. The higher proliferation rate observed on TiP, in

comparison with titanium foams, reflects the lower magnitude of surface features on the control, as determined through SEM observations, and is in accordance with the literature [9]. However, the number of viable cells on NiP is lower after 18 hours than 4 hours. This indicates that the smooth surface of the negative control allows for early proliferation but that the material toxicity subsequently affects the cellular viability (Figure 5.3b).

5.4.4 Cell morphology

No morphological differences were identified between pre-osteoblast cells attached to titanium foams of differing pore sizes as the adhesion process is influenced by surface features, such as the topography and chemistry, which do not vary for the conditions investigated [10]. Thus, SEM images of cells adhered on TiA are representative of the morphological features of cells on all scaffold porosities. Attached cells primarily exhibit a polygonal shape with lamellipodia extending in a three-dimensional fashion over the small spaces between titanium particles to anchor themselves and to create cell to cell interactions (Figure 5.4a-b). Similar morphological features have previously been reported for osteoblastic cells adhering to highly textured materials *in vitro* and are believed to promote cellular differentiation [11,12]. It is hypothesized that the dimensions of titanium particles employed in the fabrication of these porous scaffolds provides a desirable surface topography to promote three-dimensional adhesion. In contrast, the morphology of pre-osteoblasts adhered on TiP was mainly spindle-like with long lamellipodia and numerous filopodia (Figure 5.4c-d); isolated cells showed rounded appearance with few or no lamellipodia, indicating a delayed migration due to a lack of signals (Figure 5.4e). As well, cells adhered to polished controls were more flat and spread than on porous scaffolds. This observation can be associated to the respective surface topography of test samples [11]. Although cells do not form a confluent monolayer covering the surface of titanium foams after 18 hours, observations indicate that continuation of the three-dimensional proliferation should lead to monolayer confluency. Such an organization of cells could lead to the formation of a uniform bone matrix.

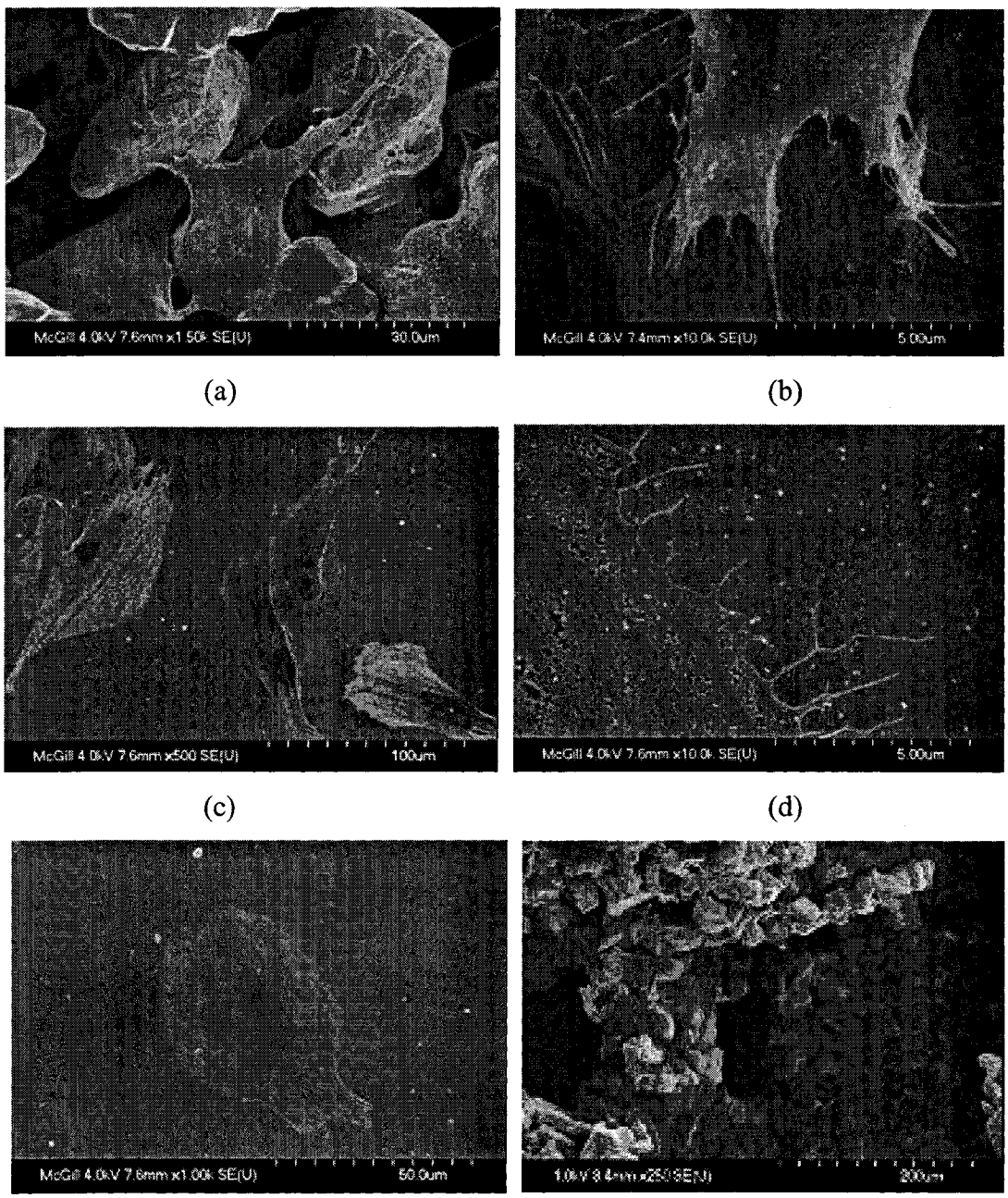


Figure 5.4. SEM images of MC3T3-E1 cells cultured for 18 hours on TiA (a-b) and TiP (c-e). (f) Lower magnification shows a cell monolayer building at the TiA surface (image obtained prior to Au-Pd coating to obtain better contrast between cells (dark gray) and scaffold (light gray)).

5.5 Conclusions

The early mechanisms by which cells adhere on bone regeneration scaffolds and interact with them provide useful insight on the biocompatibility of biomaterials, as well as their potential capabilities to promote osteogenesis. Adhesion kinetics and cellular morphology studies on novel highly porous titanium foams indicate that the foams provide a favorable support for pre-osteoblasts attachment and proliferation. Furthermore, the scaffolds present a biomimetic microstructure that allows cells to assume a three-dimensional morphology, believed to be the basis for cellular differentiation and extracellular matrix mineralization. The potential for such osteoconductive proliferation to ultimately provide a uniform bone matrix on porous titanium scaffolds will be investigated further in future work.

5.6 Acknowledgments

Financial support for this research was provided by the Natural Sciences and Engineering Research Council of Canada and the Fonds de recherche sur la nature et les technologies. The authors are also grateful to Dr. Alain Petit, Prof. Mary Kaartinen, Dr. Stéphane Ruggeri and Lyne Mongeon for their assistance.

5.7 References

1. Welsh, R.P.; Pilliar, R.M.; Macnab, I. *J. Bone Joint Surg. Am.* **1971**, 53, 963.
2. Galante, J.; Rostoker, W.; Lueck, R.; Ray R.D. *J. Bone Joint Surg. Am.* **1971**, 53, 101.
3. Hahn, H.; Palich, W. *J. Biomed. Mater. Res.* **1970,** 4, 571.
4. Bobyn, J.D.; Stackpool, G.J.; Hacking, S.A.; Tanzer, M.; Krygier, J.J. *J. Bone Joint Surg. Br.* **1999**, 81, 907.
5. Curodeau, A.; Sachs, E.; Caldarise, S. *J. Biomed. Mater. Res. (Appl. Biomater.).* **2000**, 53, 525.
6. Lefebvre, L.P.; Thomas, Y. U.S. Patent No. 6 660 224 (9 December 2003).
7. Gauthier, M.; Menini, R.; Bureau, M.N.; So, S.K.V.; Dion, M.J.; Lefebvre, L.P. presented at the 2003 ASM Materials and Processes for Medical Devices Conference, Anaheim, CA, 2003 (to be published in the proceedings).

8. St-Pierre, J.P.; Gauthier, M.; Lefebvre, L.P.; Tabrizian, M. presented at the 2003 Canadian Biomaterials Society, Montreal, QC, 2003 (unpublished).
9. Linez-Bataillon, P.; Monchau, F.; Bigerelle, M.; Hildebrand, H.F. *Biomol. Eng.* **2002**, 19, 133.
10. Anselme, K. *Biomaterials*. **2000**, 21, 661.
11. Schmidt, C.; Kaspar, D.; Sarkar, M.R.; Claes, L.E.; Ignatius, A.A. *J. Biomed. Mater. Res. (Appl. Biomater.)*. **2002**, 63, 252.
12. Yang, Y.; Tian, J.; Deng, L.; Ong, J.L. *Biomaterials*. **2002**, 23, 1383.

CHAPTER 6

Pre-Osteoblast Differentiation on Porous Titanium

THREE-DIMENSIONAL GROWTH OF DIFFERENTIATING MC3T3-E1 PRE-OSTEOBLASTS ON POROUS TITANIUM SCAFFOLDS

Jean-Philippe St-Pierre[a,b], *Maxime Gauthier*[b], *Louis-Philippe Lefebvre*[b], *and Maryam Tabrizian*[a]

[a] *Department of Biomedical Engineering, McGill University, Montreal, Canada*

[b] *Industrial Materials Institute, National Research Council Canada, Boucherville, Canada*

6.1 Abstract

The present work assesses the potential of three-dimensional porous titanium scaffolds produced by a novel powder metallurgy process for applications in bone engineering through *in vitro* experimentation. Mouse MC3T3-E1 pre-osteoblasts were used to investigate the proliferation (DNA content), differentiation (alkaline phosphatase activity and osteocalcin release) and mineralisation (calcium content) processes of cells on titanium scaffolds with average pore sizes ranging from 336 to 557 μm, using polished titanium as reference material. Scanning electron microscopy was employed to qualitatively corroborate the results. Cells proliferate on all materials before reaching a plateau at day 9, with proliferation rates being significantly higher on foams (ranging from 123 to 163 percent per day) than on the reference material (80 percent per day). Alkaline phosphatase activity is also significantly elevated on porous scaffolds following the proliferation stage. However, cells on polished titanium exhibit greater osteocalcin release toward the end of the differentiation process, resulting in earlier mineralisation of the extracellular matrix. Nevertheless, the calcium content is similar on all materials at the end of the experimental period. Average pore size of the porous structures does not have a major effect on cells as determined by the various analyses, affecting only the proliferation stage. Thus, the microstructured titanium scaffolds direct the behaviour of pre-osteoblasts toward a mature state capable of mineralising the extracellular matrix.

Keywords: Osteoblasts; Differentiation; Mineralisation; Three-dimensional scaffolds; Titanium.

6.2 Introduction

Common among engineered implant materials used for the replacement of damaged bone tissue are limited survivability characteristics, with relatively few improvements demonstrated over the past 35 years [1]. Contributing to poor survivability are difficulties associated with the maintenance of a stable interface between the material and surrounding tissues, and poor blood diffusion through that interface. In addition, inadequate mechanical properties and production of wear debris contribute to the failure of replacement materials [1-2]. To address these issues, current attempts in implant design have shifted towards the development of biomaterials incorporating three-dimensional osteoconductive scaffolds for bone regeneration.

Osteoconductive materials guide bone ingrowth by providing cells with a microstructured scaffold that promotes sequential cell maturation. Commencing with the migration of pre-differentiated cells from the host tissue, cells proliferate and differentiate to create bone within the scaffold [3]. Biodegradable and bioresorbable polymers and ceramics have been studied extensively for such purposes [4-6]. These scaffolds generally have adequate properties for implantation in non-loading conditions or in applications as supports for *in vitro* bone maturation before the engineered tissue is implanted. However, the mechanical properties of polymers are insufficient to support bone growth under loading conditions, while ceramics are too brittle [7]. Metallic scaffolds provide an advantageous alternative for these load-bearing applications because they maintain sufficient mechanical strength and fatigue resistance over extended periods *in vivo*. As well, their elastic moduli can be adjusted to match that of trabecular bone to limit stress shielding, thereby preventing bone resorption at the implant interface.

Porous metallic scaffolds are fabricated using a variety of processes to provide a high degree of interconnected porosity to allow bone ingrowth. Fabrication technologies include chemical vapour infiltration to deposit tantalum onto vitreous carbon foams [7-8], solid freeform fabrication [9], self-propagating high-temperature synthesis [10], and

powder metallurgy [2]. While these porous metals have been successful at encouraging bone ingrowth both *in vivo* and in clinical trials, the range of materials and microstructures available is still rather limited.

Recently, a novel powder metallurgy process for the fabrication of titanium foams with a unique microstructure has been developed [11]. This process consists of mixing a titanium powder with a polymeric binder and a foaming agent prior to a three-step thermal treatment. During the initial step of low temperature heat cure, the binder is melted to create a suspension charged with the titanium powder, after which the foaming agent decomposes to release gas that forms the interconnected pores within the scaffold structure. The second phase incorporates the decomposition of the polymeric compound at high temperature. Finally, the remaining titanium particles are sintered to provide the necessary mechanical integrity to the foam. The flexibility of the process allows for adjustment of the average pore size within the range of 50 to 400 μm, the optimal range of pore size for bone ingrowth [12]. Titanium was selected for foam development based on its excellent overall biocompatibility, aided by the spontaneous formation of an oxide layer in both air and physiological environments [13-14]. The shapable character of the material makes it a potential candidate for applications such as craniofacial reconstruction scaffolds.

While characterisation of extracellular matrix (ECM) formation and mineralisation *in vitro* is well established in tissue culture plates [15-17], and for two-dimensional metallic scaffolds [18-20], the behaviour of osteoblasts within three-dimensional titanium scaffolds, and how mineralised ECM is formed, requires further investigation in order to optimise the design of porous scaffolds. The impact of microstructural parameters such as surface topography, nanoscale roughness, and chemical composition on cell behaviour has yet to be understood for three-dimensional materials. The primary aim of this study is to assess the *in vitro* suitability of three-dimensional titanium scaffolds by analysing the differentiation processes of MC3T3-E1 mouse pre-osteoblastic cells and the formation of a mineralised ECM within the porous structure. Greater knowledge on the specifics of *in vitro* ECM formation and mineralisation in a porous metallic scaffold, as compared to conventional two-dimensional models, is also sought. To achieve this objective, cell culture studies on

scaffolds having three different pore sizes are compared to a two-dimensional mirror-polished titanium reference material.

6.3 Materials and methods

6.3.1 Scaffold Preparation

Titanium scaffolds with three pore sizes (A, B, and C from smaller to larger pore size), were prepared according to a novel powder metallurgy process. Briefly, titanium powder (Reading Alloys Inc., USA) was dry-mixed with low density polyethylene, a foaming agent (p,p'-oxybis[benzenesulfonyl hydrazide]), and a cross-linking agent (dicumyl peroxide), all in powdered form. The final pore size of titanium foams was controlled through blend composition. The homogenous mixture was then moulded and heated through a three step thermal treatment that included foaming at 210°C for 2 hours, decomposition of the polymeric binder at 425°C for 4 hours, and a pre-sintering step at 1200°C for 2 hours. Foams were then machined into discs (12.5 mm diameter, 2 mm thickness) and sintered at 1300°C for 2 hours. The density of each disc produced was obtained through gravimetric and volumetric measurements. An average pore size was measured for each of the three foam sizes through analysis of light microscopy images. Porosity and density parameters of titanium scaffolds are provided in Table 6.1. The unique microstructure of titanium foams is depicted in Figure 6.1.

Polished titanium reference materials (referred to as P) were prepared by polishing dense titanium discs (Alfa Aesar, USA), having dimensions similar to the porous scaffolds, with an automatic polisher (Struers, UK) to ensure surface reproducibility. Samples were first wet-ground with 220 grit silicon carbide abrasive papers at 300 rpm with an applied pressure of 200 N. The discs were then polished with 9 and 0.04 μm diamond solutions on appropriate cloths (Struers, UK) at 150 rpm with applied pressures of 175 and 125 N respectively.

Porous samples and polished titanium discs were cleaned through 15 minute sonication cycles in soapy water, isopropanol and de-ionized water, interspersed by rinsing steps in distilled water. Titanium scaffolds were then autoclaved and allowed to dry overnight at 60°C under sterile conditions.

Table 6.1. Microstructural parameters of titanium scaffolds produced through a novel powder metallurgy process.

Scaffold Identification	Density (g/cm^3)	Average Pore Size (μm)
A	1.23 ± 0.09	336.4 ± 48.4
B	1.02 ± 0.11	376.1 ± 49.0
C	0.82 ± 0.19	556.9 ± 196.9

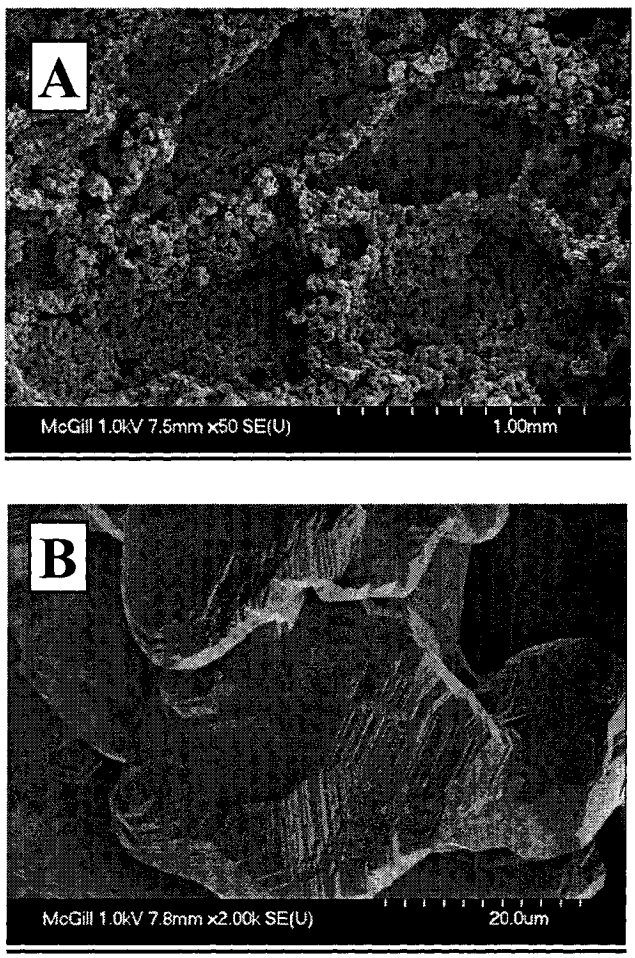


Figure 6.1. Scanning electron micrographs showing the microstructure of titanium scaffold C with an average pore size of 557 μm. (A) The porosity is interconnected and the structure is characterised by a high degree of surface topography. (B) The surface of each titanium particle is characterised by nanoscale surface roughness (Original magnifications 50X and 2000X respectively).

6.3.2 *Cell Culture*

Newborn mouse calvaria-derived MC3T3-E1 subclone 14 pre-osteoblastic cells (ATCC, USA) were cultured in α-MEM medium (Gibco BRL, Canada) supplemented with 10% fetal bovine serum (ATCC, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL, Canada) at 37°C in an atmosphere with 100% humidity and 5% CO_2. The complete medium was replaced every 2 to 3 days and confluent cells were subcultured through trypsinization.

To study cell proliferation, differentiation, and mineralisation of pre-osteoblasts, cells were seeded at a density of 2.0×10^5 cells/well on the porous titanium scaffolds and polished titanium within 24-well plates. Cells were allowed to adhere to the discs for 24 hours prior to samples being transferred to fresh 24-well plates in order to discard cells adhered to the plate rather than titanium. Differentiation was then induced through the addition of 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate to the growth medium; this was considered day 0 of the experiment. Differentiation medium was subsequently replaced every 2 days, with sample analysis or preparation for qualitative assessment on days 0, 1, 5, 9, 13 and 17. As well, differentiation was induced on cells adhered to the tissue culture plate (TCP) as a positive control, with the inclusion of proper negative controls.

6.3.3 *Proliferation*

The number of cells adhering to titanium scaffolds, polished materials, and TCP was measured using a modified version of a CyQUANT cell proliferation assay (Molecular Probes, USA). Briefly, cells were washed twice with PBS before freezing at -80°C until the performance of the assay, at which point cells were thawed at room temperature. Cell lysate was obtained by the addition of 500 μL of 1X CyQUANT cell-lysis buffer to each well followed by a 15 minute incubation at room temperature. An aliquot of the lysate was then mixed with the same quantity of cell-lysis buffer supplemented with 360 mM NaCl, 2 mM EDTA and 1.5 Kunitz/ml DNAse and protease free RNAse A (Worthington Biochemical Corporation, USA) and incubated for 1 hour at room temperature; lysate RNA was digested to ensure that potential fluctuations in RNA

transcription at different stages of cell differentiation would not affect the data. A 100 μL volume of the RNA free solution was then mixed with the same quantity of 2X CyQUANT GR dye in cell-lysis buffer in fluorescence assay microplates and incubated for 5 minutes at room temperature, while shading from ambient light. After the incubation, plates were read with a FL_X 800 microplate fluorescence reader (Bio-Tek Instruments Inc., USA) with a 485/20 excitation filter and a 528/20 emission filter. A standard curve was generated with λ DNA.

6.3.4 Alkaline Phosphatase Activity

The activity of intracellular alkaline phosphatase (ALP) was measured with a commercial phosphatase substrate kit (Pierce, USA). Aliquots of 100 μl of cell lysate were obtained according to the procedure described for proliferation measurements and mixed with an equal amount of 1 mg/ml p-Nitrophenyl Phosphate in 1X Diethanolamine buffer in microplates. Mixtures were then incubated for 30 minutes at 37°C. The reaction was stopped by the addition of 50 μL of 2N NaOH to each well and the resulting optical densities were measured at 405 nm with a μQuant spectrophotometer (Bio-Tek Instruments Inc., USA). Measurements were compared to p-Nitrophenol standards and normalised using the total protein amounts to account for differences in the number of cells on different scaffolds at individual time points.

6.3.5 Osteocalcin Release

The production of osteocalcin by differentiating cells was measured as the release of the ECM protein in the culture medium over a period of 24 hours using a commercial sandwich enzyme-linked immunosorbent assay (Biomedical Technologies, USA). The amount of osteocalcin released in the culture medium is correlated with the synthesis of the protein [21]. Mouse osteocalcin was used to generate a standard curve and results were normalised using the amount of total protein.

6.3.6 *Total Protein*

Cellular protein content was measured with a BCA protein assay kit (Pierce, USA). Briefly, 25 μL aliquots of cell lysate were mixed with 200 μL volumes of BCA working reagent containing cupric sulfate and bicinchoninic acid in microplates and incubated for 30 minutes at 37°C. The resulting optical densities were measured at 562 nm with a μQuant spectrophotometer. Bovine serum albumin was used to generate a standard curve.

6.3.7 *Calcium Content*

Following incubation, cells were washed twice in PBS and incubated for 24 hours in 1N HCl. The calcium content in each solution was then measured with an atomic absorption spectrometer (Perkin Elmer 3110, USA). Two measurements were averaged for each solution. A standard curve was obtained with Ca^{2+} solutions. Results were normalised using total proteins.

6.3.8 *Scanning Electron Microscopy*

Cells and ECM on titanium scaffolds and polished surfaces were washed twice in PBS and fixed with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, dehydrated in serial ethanol and amyl acetate baths, and critical point dried. Specimens were sputter-coated with Au-Pd and imaged with a Hitachi S-4700 field emission scanning electron microscope. Energy dispersive X-ray microanalysis (Oxford Instruments, USA) was also performed on samples prior to the application of the Au-Pd coating to verify the presence of calcium and phosphorus in the ECM produced by differentiated cells.

6.3.9 *Statistics*

Results are expressed as mean ± standard deviation with $n = 5$. Error bars in figures represent standard deviations. Differences between the experimental groups were analysed according to a paired Student's t-test, with $p<0.05$ considered statistically significant. Pore size effects were verified according to a single factor ANOVA.

6.4 Results

6.4.1 Proliferation

The number of MC3T3-E1 mouse pre-osteoblasts attached to scaffolds, reference materials and controls was measured through the cellular DNA content at different time points following the initiation of differentiation. Compared to porous scaffolds, DNA content is significantly higher on TCP at every time point and on polished titanium on days 0 and 1 (Figure 6.2). DNA content is similar for titanium foams A and B at each time point investigated, but is significantly higher for scaffold C. The content increases significantly on all test materials at each time point until day 9, with the exception of day 1 for all porous scaffolds. Following day 9, DNA content remains statistically unchanged on the porous scaffolds, while attached cells are significantly lower, but constant, on both the polished material and the control. In considering the initial 9 day growth phase, proliferation rates measured on each material as the percent increase of DNA content per day indicate significantly higher rates on porous scaffolds than polished titanium and TCP, with an increasing trend in proliferation with smaller pore sizes (Figure 6.3).

6.4.2 Alkaline Phosphatase Activity

Differentiation on the various substrates was characterised using ALP as an early-phase marker. Cells adhering to titanium scaffolds exhibit significantly higher activity of the enzyme than on the polished material and the control on days 9 and 13, with the exception of scaffold A on day 13, though scaffold pore size does not affect the ALP activity of differentiating cells in a uniform fashion (Figure 6.4). ALP kinetics show a significant increase in specific activity of the enzyme on all porous scaffolds at each time point up to day 13, followed by a significant decrease on day 17 for scaffolds A and C. In contrast, the pattern of ALP activity on polished titanium resembles that of TCP controls, which consists of a slow increase over the entire test period, with significantly greater activity on day 17 than on day 5.

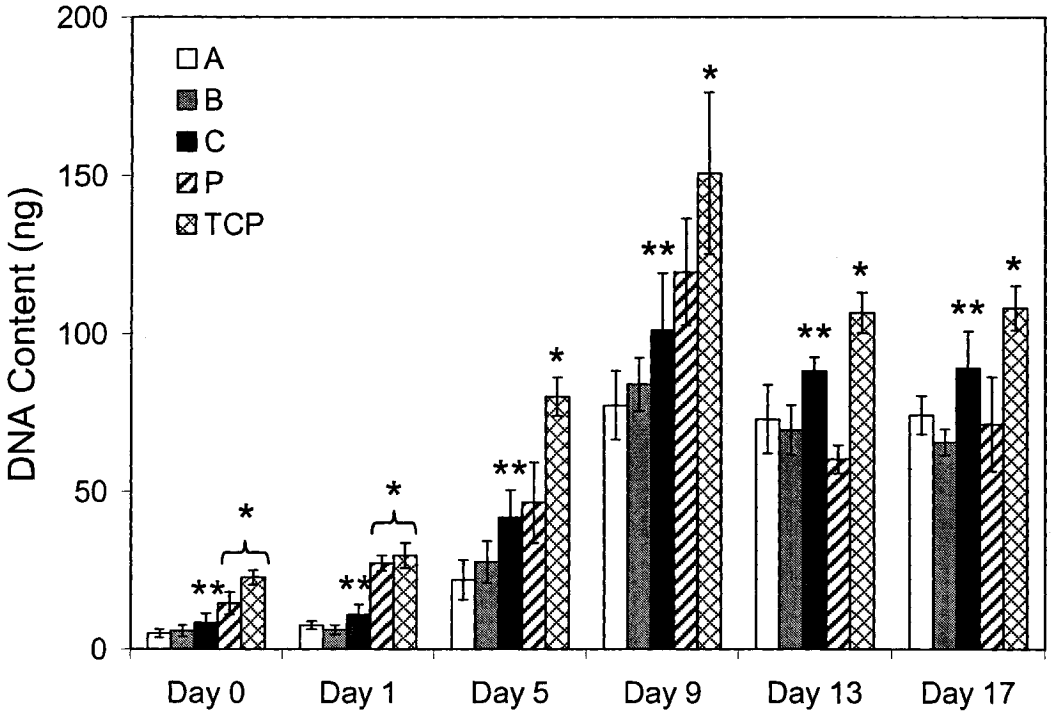


Figure 6.2. DNA content of mouse pre-osteoblasts after 0, 1, 5, 9, 13 and 17 days in culture. Foams (A, B and C with pore sizes of 336, 376 and 557 μm respectively) have significantly lower DNA content than tissue culture plate controls (TCP) at every time point and than polished titanium (P) at days 0 and 1 (* $p<0.05$). Foams with the highest pore size (C) have significantly higher DNA content than other foams (** $p<0.05$).

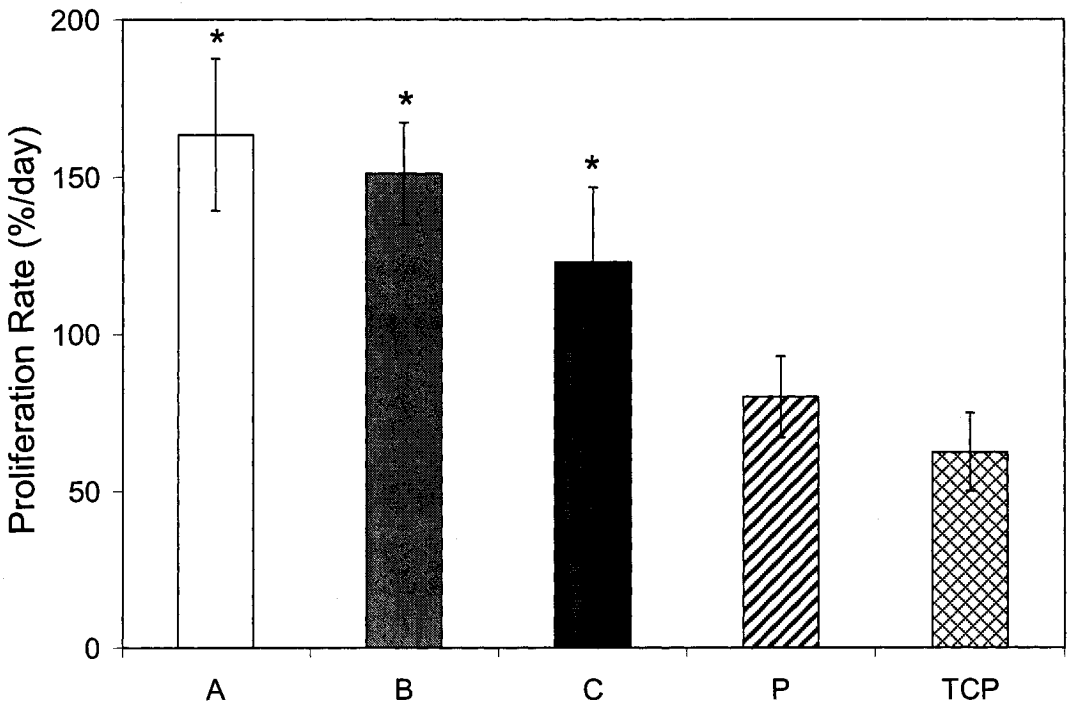


Figure 6.3. Overall proliferation rates of pre-osteoblasts over the initial nine days in culture is significantly higher on porous foams (A, B and C with pore sizes of 336, 376 and 557 μm respectively) than on polished titanium (P) and tissue culture plate controls (TCP) (* $p<0.05$).

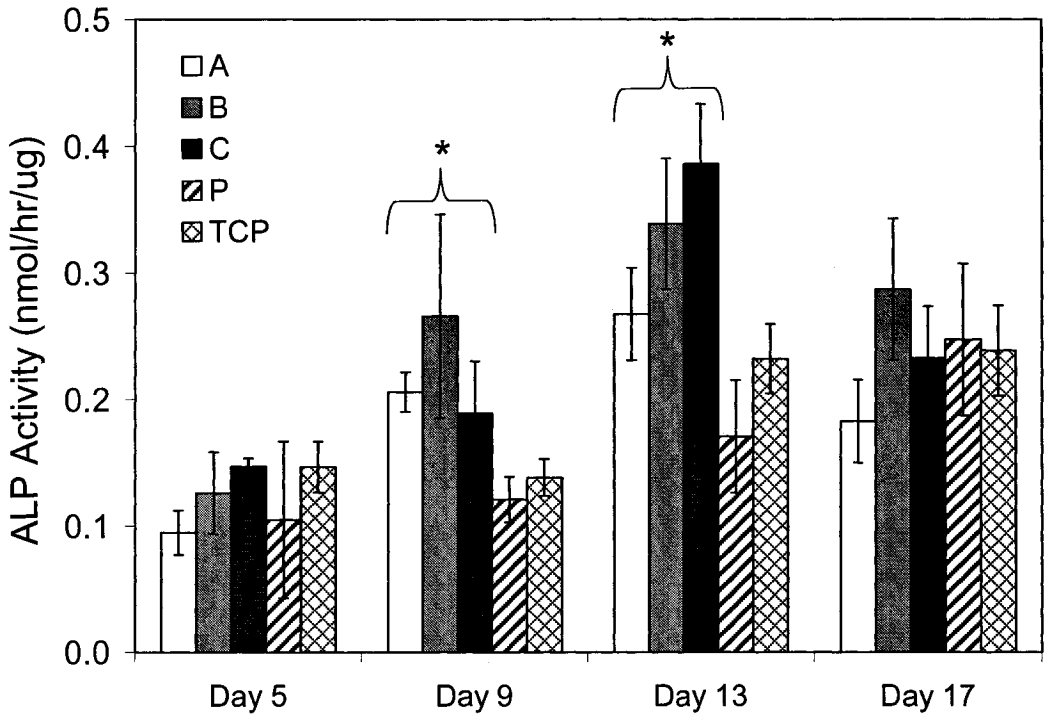


Figure 6.4. Normalised ALP activity of cell lysates from pre-osteoblasts after 5 to 17 days in culture. The specific enzymatic activity is significantly higher on foams than on polished titanium (P) and tissue culture plate controls (TCP) at days 9 and 13, though pore size (A, B and C with pore sizes of 336, 376 and 557 μm respectively) does not affect ALP activity (* $p<0.05$).

6.4.3 Osteocalcin Release

Late stage osteoblast differentiation was measured through osteocalcin release in the culture medium over a 24 hour period. Osteocalcin release is significantly higher on TCP than any other substrate on day 13, while the same is true for polished titanium on day 17, indicating that cells attached to the reference material and control achieve a mature osteoblastic state more rapidly than on porous substrates (Figure 6.5). The pore size of titanium scaffolds does not impact osteocalcin release. Osteocalcin levels increase significantly from day 5 to day 9 on all materials, though a larger rise is observed on day 13. Finally, with the exception of cells grown on scaffold C and TCP, osteocalcin release remains unchanged following day 13.

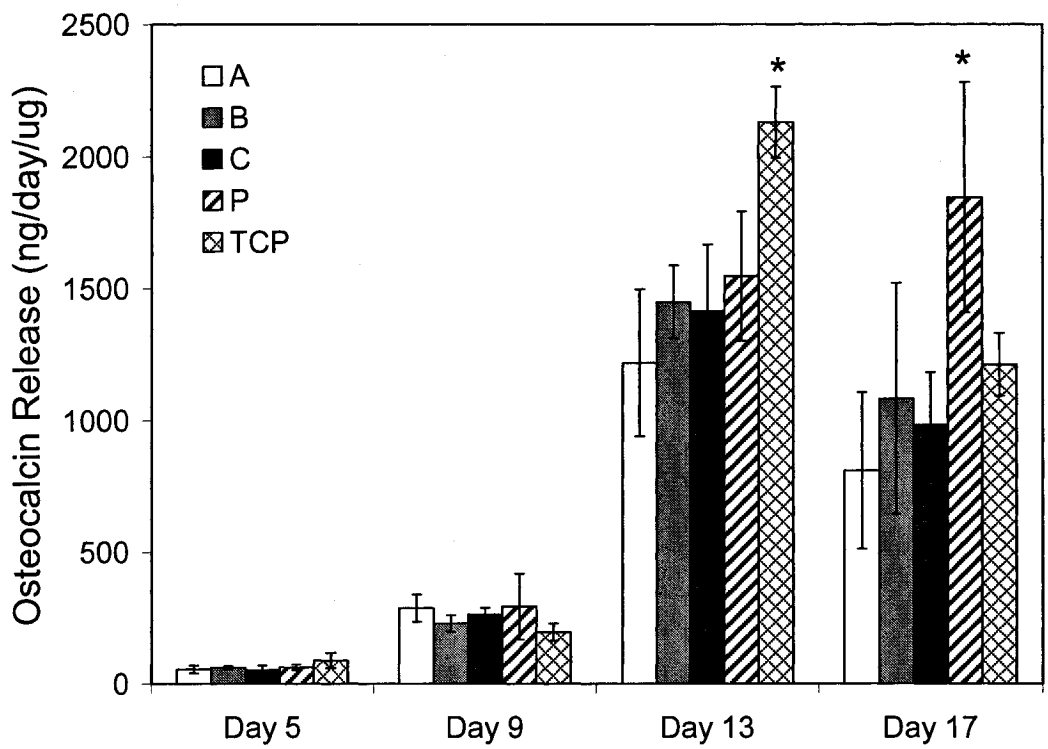


Figure 6.5. Normalised osteocalcin release from cells in medium over 24 hours after 5 to 17 days in culture. Cells grown on tissue culture plates (TCP) on day 13 and on polished titanium (P) on day 17 have significantly higher osteocalcin release than porous scaffolds (* $p<0.05$). Average pore size of titanium foams (A, B and C with pore sizes of 336, 376 and 557 μm respectively) does not affect osteocalcin release.

6.4.4 Calcium Content

Mineralisation of the ECM by differentiated cells was measured through the ECM calcium content. Calcium content for both polished titanium and TCP is significantly higher on day 13 than for porous scaffolds, indicating a lesser degree of mineralisation for osteoblasts grown in porous scaffolds at this time point (Figure 6.6). However, by day 17 there is no significant difference between scaffolds and the polished material, regardless of the pore size. A large standard error resulted from the calcium content analysis on polished titanium on day 13. Macroscopic analysis of these samples confirmed that some replicates had already started mineralising by this time, while cells

on the other substrates had yet to reach this level of maturation; similar observations were made for the samples tested on day 17.

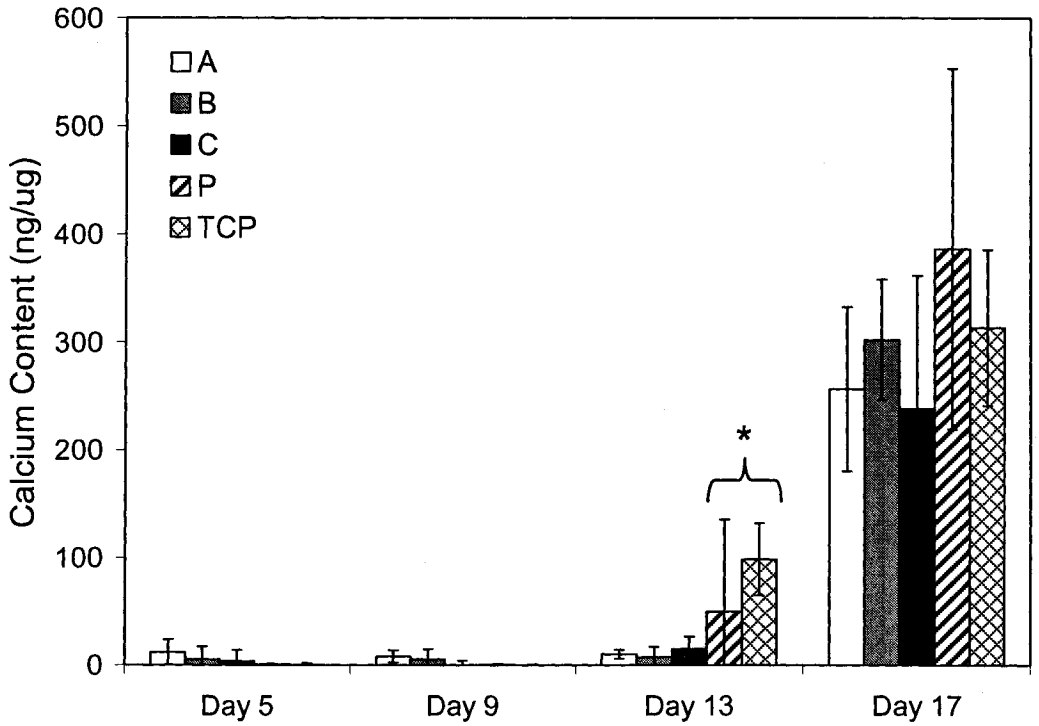


Figure 6.6. Normalised calcium content of the ECM deposited by cells after 5 to 17 days in culture indicate higher mineralisation on polished titanium (P) and tissue culture plate controls (TCP) on day 13, though no difference exists by day 17 regardless of pore size (A, B and C with pore sizes of 336, 376 and 557 μm respectively; * $p<0.05$).

6.4.5 Scanning Electron Microscopy Analysis

Evidence of continued cell proliferation on the titanium scaffolds and polished titanium could be detected up to day 9 through SEM analysis (Figure 6.7). Cells appear to adhere to the scaffold through three-dimensional extensions, gradually filling in pores as proliferation proceeds. Continued progression of ECM development once proliferation is complete is evidenced by densification and mineralisation of the ECM on porous scaffolds, which was verified by EDX to confirm the presence of calcium and phosphorus at day 17. After 5 days, there are already indications of ECM bridging the scaffold pores (Figure 6.7b), with continuing development of the ECM network within the porous

structure at subsequent time points (Figures 6.7c,d). Once the smaller pores are filled with ECM, subsequent growth results in a dense layer of cells covering the porous structure (Figure 6.7d). While no correlations between pore size of titanium foams and growth traits were qualitatively identified, cell growth within larger pores is limited by the ability of cells to bridge the pore with ECM, resulting in less dense, and in some locations, incomplete ECM formation (Figure 6.8). In comparison, cell growth on polished titanium results in more flat, dense, and multi-layered structures with relatively little ECM (data not shown).

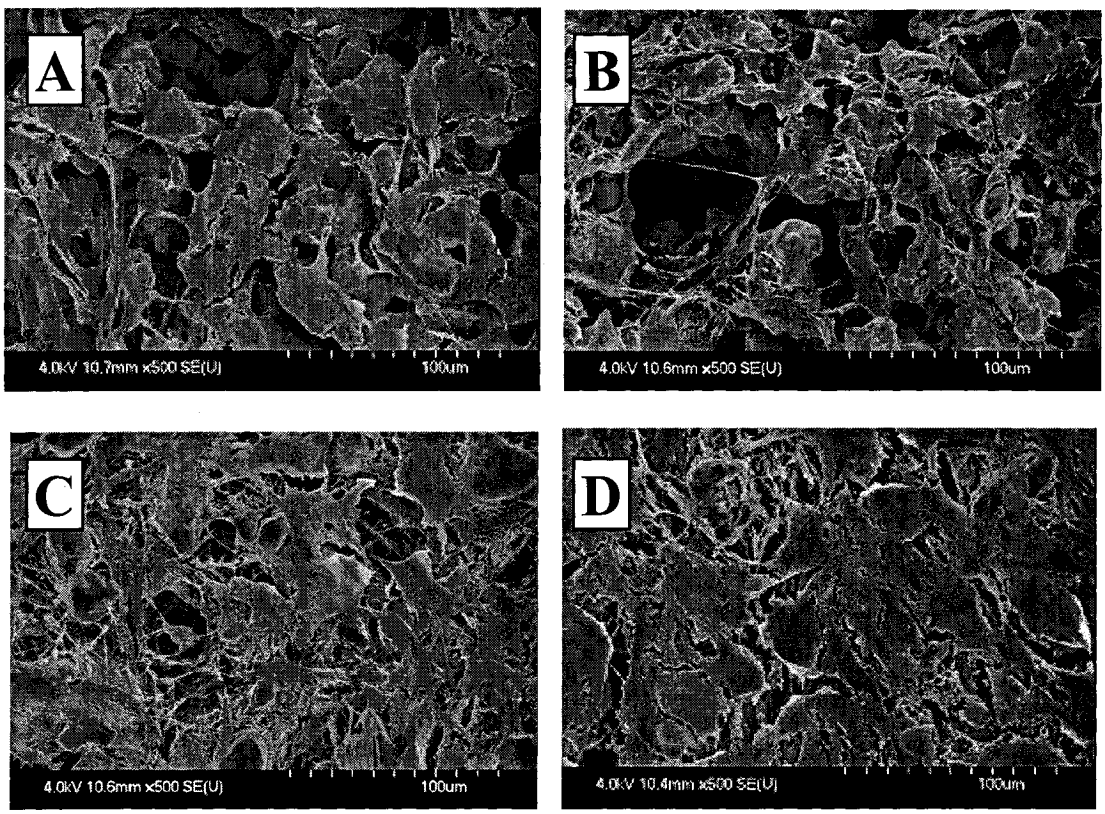


Figure 6.7. Scanning electron micrographs depicting cell proliferation of pre-osteoblasts within scaffold A (336 μm pore size) up to day 9. (A) Day 1. (B) Day 5. (C). Day 9. (D) Day 13 (Original magnification 500X). Observations on days 0 and 17 are comparable to those on days 1 and 13, respectively.

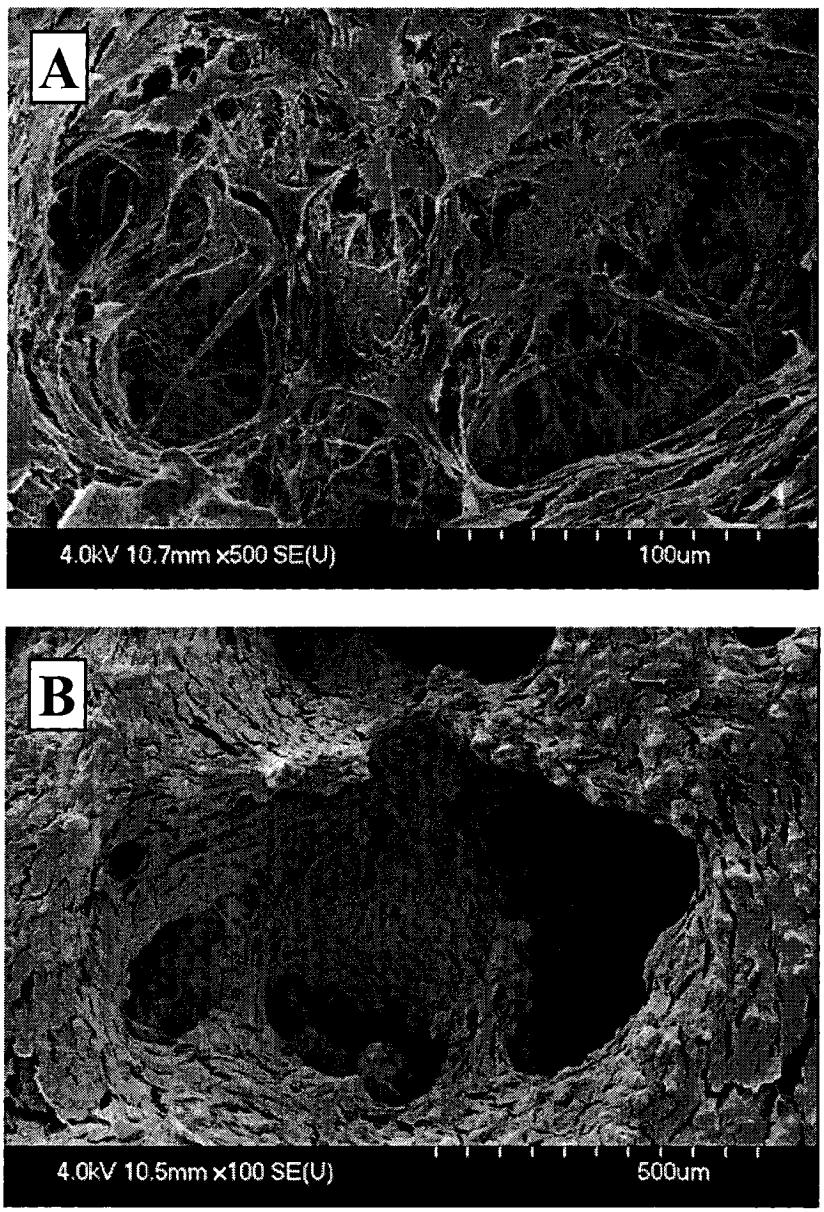


Figure 6.8. (A) Scanning electron micrographs showing the development of extensive ECM networks inside pores of scaffold A (336 μm pore size) after 9 days in culture. (B) Cells grow on the walls of a larger pore inside scaffold A, failing to form a dense ECM network within the pore after 17 days due to its size. This micrograph also provides evidence of cell penetration inside the three-dimensional structure. (Original magnification 100X).

6.5 Discussion

In the present study, the proliferation and differentiation of mouse MC3T3-E1 pre-osteoblasts toward the formation of a mineralised ECM were assessed on porous titanium scaffolds produced through a novel powder metallurgy process. Results obtained with porous scaffolds comprising three different pore sizes were compared to mirror-polished titanium. The clonal mouse pre-osteoblastic MC3T3-E1 cell line was selected for this study due to its high level of differentiation and its ability to form a well mineralised bone-like ECM containing bone markers such as collagen type I, ALP, and osteocalcin, making it an excellent model for differentiation [22-23]. Additionally, it has a distinct reproducibility advantage over primary cell culture systems.

The growth environment provided by the unique microstructure of titanium foams encourages cell proliferation compared to the polished material. The average pore size of titanium scaffolds does impact proliferation, though differentiation and mineralisation processes are not affected. Early-phase cell differentiation activity, as monitored by ALP activity, is also positively impacted by cell interactions with the three-dimensional scaffolds. In contrast, late-phase differentiation release of osteocalcin is lower for cells on titanium scaffolds than on polished titanium, resulting in a delayed transition toward mature osteoblasts capable of mineralising the ECM. Nevertheless, the calcium content on scaffolds was comparable to that obtained on polished surfaces by the end of the incubation period (17 days). SEM analysis reveals greater amounts of ECM laid down by differentiating cells on the porous structures, as compared to polished samples, with the three-dimensional structures providing a more voluminous environment for the deposition of ECM.

The initial cell growth profile, as measured by cellular DNA content on porous titanium scaffolds, exhibits differences compared to polished titanium and TCP (Figure 2). During the initial phases of proliferation, fewer cells adhere to the scaffolds than to the reference material or the control. This can be attributed to the permeability of porous scaffolds, which provides an interconnected structure for cell penetration but allows some cells to attach to the underlying wells rather than the scaffolds upon seeding. By day 9, proliferation peaks for all test materials, as measured by both DNA content (Figure 6.2) and qualitative SEM analysis (Figure 6.7). Though cell counts indicate greater numbers

of cells on polished titanium and TCP, the rates of proliferation measured up to day 9 are significantly greater for porous foams, likely due to the three-dimensional proliferation environment provided by the porous scaffolds (Figure 6.3) [24]. Subsequently, DNA content remains constant on porous scaffolds, resulting in typical sigmoidal curves for all three porosities having a plateau beginning on day 9. However, while polished surfaces and TCP also reach a maximum on day 9, a significant decrease in measured DNA content occurs by day 13. This reduction is consistent with previous reports documenting diminishing DNA content for osteoblasts grown on implant materials [25]. These investigators propose that the dense ECM network produced by differentiating osteoblasts could prevent a complete release of DNA content during cell lysis, thereby lowering proliferation measurements. SEM observations support this justification given the density of the cell layer observed on polished titanium after 13 and 17 days, which is considerably more dense than is observed on day 9 (data not shown) or on porous scaffolds.

The culmination of proliferation on day 9, with an ensuing shift in cell maturation level towards early differentiation stages, is confirmed by a significant increase in ALP activity on porous scaffolds. Following the proliferation stage, the number of cells on titanium scaffolds is comparable to that on polished samples, even though the foams provide substantially higher surface area and volume for cell growth (Figure 6.2). This can be explained by SEM observations indicating that cells are not as densely packed in the ECM network laid down inside pores of the titanium foams than on the surface of polished titanium. However, the greater early-phase differentiation activity on porous scaffolds noted by ALP activity at days 9 and 13 indicates that scaffolds may provide adhered pre-osteoblasts with a more robust foundation to commence differentiation (Figure 6.4). Proliferation and ALP activity were expected to follow trends of lower proliferation and increased ALP production for porous scaffolds, due to their unique microstructure, based on published reports from two-dimensional models linking increasing surface roughness and topography of titanium substrates with the promotion of osteoblasts towards a differentiated phenotype [26-28]. The ALP kinetics obtained in this study are in agreement with the literature, as the enzymatic activity significantly increases on the foams following the proliferation stage, as compared to the polished

material (Figure 6.4). Furthermore, the profile of ALP activity for cells on porous scaffolds is typical of the expression of that enzyme during the early stages of differentiation, as the activity increase is followed by a drop in activity that coincides with the initiation of the mineralisation process [29]. The discrepancy between the noted increased proliferation found on scaffolds and published findings may be attributed to the greater available space for cell growth provided by the porous three-dimensional surface topography.

The increased activity of the early differentiation stage marker (ALP) on titanium foams is not indicative of the late-phase osteocalcin release. While production of the late differentiation stage marker is similar for foams and the polished control up to day 13, significantly higher production results were obtained for cells grown on polished titanium by day 17 (Figure 6.5). The increased production is associated with an earlier shift toward a mature state of osteoblasts capable of mineralising the ECM, as was confirmed by calcium content measurements (Figure 6.6). This result suggests that the two differentiation markers investigated in this study are expressed through independent control pathways, and is consistent with previous observations reported for human osteoblasts grown on various metallic substrates [20]. These investigators attributed this phenomenon to different levels of transforming growth factor-β (TGF- β), which is known to have different patterns of expression by osteoblasts on implant materials of different chemical compositions. This growth factor has been shown to act as an up-regulator of ALP expression by osteoblasts, while down-regulating the expression of osteocalcin [30]. Since surface roughness of metallic implants promotes the release of TGF-β by osteoblasts [28,31], the microstructure typical of titanium foams could promote release relative to cells grown on polished surfaces, thus affecting the expression patterns of differentiation markers.

In the final stage of osteoblast maturation, mineralisation of the ECM, calcium content measurements indicate equivalent performance by three-dimensional scaffolds and the polished samples. The similarity in mineralisation on day 17 is presumed to result from the deposition of a more extensive ECM within the porous structures than on the polished material, as observed through SEM analysis (Figure 6.8a). It is worth noting that macroscopic examinations of the scaffold undersides indicate that ECM production does

not occur through the entire depth of scaffolds over the experimental period, likely due to diffusive limitations on nutrients through the scaffolds and matrix [25,32]. It remains to be determined whether or not the increased amounts of ECM found in scaffolds would promote the deposition of minerals beyond day 17, potentially leading to enhanced bone formation relative to controls in a longer term analysis.

Many investigators have examined the effects of pore size on bone ingrowth in porous metals using *in vivo* models [12,33-34]. While they have identified a range of 50 to 400 μm pore size within which bone ingrowth occurs, no consensus on optimal pore size exists due to the range of materials investigated and their respective microstructural impacts. Similar *in vitro* studies indicate that pore size is an important microstructural parameter to control in the design of three-dimensional osteoconductive scaffolds [35]. In the present study, the average pore size of titanium scaffolds, which included scaffolds with pore sizes greater than 400 μm, was found to significantly affect only the proliferation stage, leaving the differentiation and subsequent mineralisation processes unaffected. Given that the pore size distributions for the three scaffolds differed primarily due to the size of larger pores present, it is possible that differences between the three scaffolds remained indistinguishable to the cells. Though this study demonstrates the suitability of these foams for osteoblast proliferation, differentiation, and mineralisation, additional work is required to differentiate the individual impacts of pore size from microstructural properties. Results from these additional studies would optimise the design of the titanium scaffolds, allowing studies to proceed toward *in vivo* characterisation of these titanium scaffolds to ascertain their suitability for bone tissue regeneration applications.

6.6 Conclusions

The microstructure of porous titanium scaffolds produced through a novel powder metallurgy process promotes MC3T3-E1 pre-osteoblast proliferation and early differentiation compared to polished surfaces. The microstructural properties of foams appear to slow the shift to later stages of cell differentiation toward mature osteoblasts. At the culmination of the investigation period on day 17, cells grown on titanium scaffolds and on polished titanium produced similar levels of ECM mineralisation. These

results indicate that the microstructure of the three-dimensional titanium scaffolds investigated promotes proliferation, differentiation, and mineralisation processes required to characterise the osteoconductive behaviour of porous metallic scaffolds *in vitro*. Furthermore, the average pore size of the structures investigated does not have a major effect on the differentiation and mineralisation processes. Based on quantitative and qualitative analyses, the smaller pore size structure is more favourable than the other two porous structures investigated because of its narrower pore size distribution and fewer large pores, which are more difficult to bridge and fill with ECM. However, cell culture experiments provide a simplified model of the phenomena being studied. A true measure of the osteoconductive utility of these titanium scaffolds requires confirmation of these results through additional characterisation and *in vivo* experimentation.

6.7 Acknowledgments

This study was supported by the Natural Sciences and Engineering Research Council of Canada and les Fonds Québécois de la Recherche sur la Nature et la Technologie. Assistance was kindly provided by Prof Mary Kaartinen and Mme Lyne Mongeon. We would also like to thank M Shawn Carrigan for comments on the manuscript.

6.8 References

1. Hench, L. L. *Biomaterials* **1998**, 19, 1419.
2. Wen, C. E.; Yamada, K.; Shimojima, K.; Chino, Y.; Asahina, T.; Mabuchi, M. *J. Mater. Sci: Mater. Med.* **2002**, 13, 397.
3. Albrektsson, T.;Johansson, C. *Eur. Spine J.* **2001**, 10 Suppl 2, S96.
4. Cheung, H. S.;Haak, M. H. *Biomaterials* **1989**, 10, 63.
5. Hutmacher, D. W. *Biomaterials* **2000**, 21, 2529.
6. Zhang, Y.;Zhang, M. *J. Biomed. Mater. Res.* **2002**, 61, 1.
7. Zardiackas, L. D.; Parsell, D. E.; Dillon, L. D.; Mitchell, D. W.; Nunnery, L. A.; Poggie, R. *J. Biomed. Mater. Res.* **2001**, 58, 180.
8. Bobyn, J. D.; Stackpool, G. J.; Hacking, S. A.; Tanzer, M.; Krygier, J. J. *J. Bone Joint Surg. Br.* **1999**, 81, 907.

9. Melican, M. C.; Zimmerman, M. C.; Dhillon, M. S.; Ponnambalam, A. R.; Curodeau, A.; Parsons, J. R. *J. Biomed. Mater. Res.* **2001**, 55, 194.
10. Assad, M.; Jarzem, P.; Leroux, M. A.; Coillard, C.; Chernyshov, A. V.; Charette, S.; Rivard, C. H. *J. Biomed. Mater. Res.* **15-2-2003**, 64B, 107.
11. Lefebvre, L. P.;Thomas, Y. **2003**,
12. Bobyn, J. D.; Pilliar, R. M.; Cameron, H. U.; Weatherly, G. C. *Clin. Orthop.* **1980**, 263.
13. Breme, H. J.;Helsen, J. A. **1998**, 1.
14. Sul, Y. T.; Johansson, C. B.; Petronis, S.; Krozer, A.; Jeong, Y.; Wennerberg, A.; Albrektsson, T. *Biomaterials* **2002**, 23, 491.
15. Beresford, J. N.; Graves, S. E.; Smoothy, C. A. *Am. J. Med. Genet.* **15-1-1993**, 45, 163.
16. Sowa, H.; Kaji, H.; Yamaguchi, T.; Sugimoto, T.; Chihara, K. *J. Bone Miner. Res.* **2002**, 17, 1190.
17. zur Nieden, N. I.; Kempka, G.; Ahr, H. J. *Differentiation* **2003**, 71, 18.
18. Ahmad, M.; McCarthy, M. B.; Gronowicz, G. *Biomaterials* **1999**, 20, 211.
19. Ku, C. H.; Pioletti, D. P.; Browne, M.; Gregson, P. J. *Biomaterials* **2002**, 23, 1447.
20. Schmidt, C.; Ignatius, A. A.; Claes, L. E. *J. Biomed. Mater. Res.* **2001**, 54, 209.
21. Hauschka, P. V.; Lian, J. B.; Cole, D. E.; Gundberg, C. M. *Physiol Rev.* **1989**, 69, 990.
22. Sudo, H.; Kodama, H. A.; Amagai, Y.; Yamamoto, S.; Kasai, S. *J. Cell Biol.* **1983**, 96, 191.
23. Wang, D.; Christensen, K.; Chawla, K.; Xiao, G.; Krebsbach, P. H.; Franceschi, R. T. *J. Bone Miner. Res.* **1999**, 14, 893.
24. Yang, Y.; Tian, J.; Deng, L.; Ong, J. L. *Biomaterials* **2002**, 23, 1383.
25. Bancroft, G. N.; Sikavitsas, V. I.; van den, D. J.; Sheffield, T. L.; Ambrose, C. G.; Jansen, J. A.; Mikos, A. G. *Proc. Natl. Acad. Sci U. S. A* **1-10-2002**, 99, 12600.
26. Batzer, R.; Liu, Y.; Cochran, D. L.; Szmuckler-Moncler, S.; Dean, D. D.; Boyan, B. D.; Schwartz, Z. *J. Biomed. Mater. Res.* **5-9-1998**, 41, 489.
27. Boyan, B. D.; Lossdorfer, S.; Wang, L.; Zhao, G.; Lohmann, C. H.; Cochran, D. L.; Schwartz, Z. *Eur. Cell Mater.* **24-10-2003**, 6, 22.

28. Lincks, J.; Boyan, B. D.; Blanchard, C. R.; Lohmann, C. H.; Liu, Y.; Cochran, D. L.; Dean, D. D.; Schwartz, Z. *Biomaterials* **1998**, 19, 2219.
29. Ehara, A.; Ogata, K.; Imazato, S.; Ebisu, S.; Nakano, T.; Umakoshi, Y. *Biomaterials* **2003**, 24, 831.
30. Bonewald, L. F.; Kester, M. B.; Schwartz, Z.; Swain, L. D.; Khare, A.; Johnson, T. L.; Leach, R. J.; Boyan, B. D. *J. Biol. Chem.* **5-5-1992**, 267, 8943.
31. Mustafa, K.; Rubinstein, J.; Lopez, B. S.; Arvidson, K. *Clin. Oral Implants. Res.* **2003**, 14, 50.
32. van den Dolder J.; Bancroft, G. N.; Sikavitsas, V. I.; Spauwen, P. H.; Jansen, J. A.; Mikos, A. G. *J. Biomed. Mater. Res.* **1-2-2003**, 64A, 235.
33. Ayers, R. A.; Simske, S. J.; Bateman, T. A.; Petkus, A.; Sachdeva, R. L.; Gyunter, V. E. *J. Biomed. Mater. Res.* **1999**, 45, 42.
34. Itala, A. I.; Ylanen, H. O.; Ekholm, C.; Karlsson, K. H.; Aro, H. T. *J. Biomed. Mater. Res.* **2001**, 58, 679.
35. Frosch, K. H.; Barvencik, F.; Lohmann, C. H.; Viereck, V.; Siggelkow, H.; Breme, J.; Dresing, K.; Sturmer, K. M. *Cells Tissues. Organs* **2002**, 170, 214.

CHAPTER 7

Results and Discussion

Porous metals with high levels of porosity are commonly used as three-dimensional scaffolds to promote and direct bone ingrowth in load-bearing situations. Such applications include fixation of joint replacement implants, craniofacial structures regeneration, spinal fusion, and dental implants. Despite extensive efforts to design processes for the fabrication of these advanced materials, few structures have been used in clinical applications. As well, research on the cell-biomaterial interactions have mainly focused on the impact of surface properties with two-dimensional *in vitro* models. Hence, there is a need to develop novel three-dimensional metallic scaffolds and characterize interactions of osteogenic cells with these materials, in an attempt to optimize their microstructural parameters for a favourable bone regeneration response *in vivo*.

Results discussed in this thesis are aimed at investigating the cellular interactions with novel titanium foams characterized by a unique microstructure with three pore sizes produced through a powder metallurgy process developed recently at IMI-NRC. This study was intended to provide *in vitro* information on the potential of these structures to promote the formation of bone-like tissue. Furthermore, it was intended to gain knowledge on the differences in three-dimensional versus two-dimensional growth and differentiation of pre-osteoblasts, with an emphasis on the effects of pore size.

Prior to the investigation of cell-biomaterial interactions, modifications had to be made to the original fabrication process for the fabrication of titanium foams in order to produce small discs that were of adequate dimensions for cell culture investigations. It was essential to limit machining of the material following the final sintering step, as this distorted the microstructure of foams and would have influenced cellular response. Hence, a pre-sintering step at intermediate temperature was introduced prior to the final sintering stage at high temperature. This step provides foams with sufficient mechanical strength for handling. As the metallurgical links formed between titanium particles during this step are not strong, the foams can be machined with minimal impact on the microstructure. Foams were produced with three pore sizes according to this modified

process and were characterized in terms of their pore sizes and density, which is related to the porosity of the structure.

The three types of foams were found to have significantly different microstructures. However, pore size distribution of the foams was not uniform, resulting in relatively high variability. Nevertheless, smaller pore size foams achieved a better uniformity in pore size distribution due to the addition of higher levels of the cross-linking agent. The conditions to produce the three types of foams were kept constant for the work presented in both manuscripts. Disparity between the pore sizes results reported in the two manuscripts is due to the base used for measurement of averages, the first manuscript (Chapter 5) presents averages based on the number of pores, while the second manuscript (Chapter 6) reports calculations based on the volume of pores.

Initial screening assays were performed to verify the cytocompatibility of the titanium foams. Results indicated that the nature of the titanium surface and its wettability are affected by the processing residuals from the fabrication process (J.P. St-Pierre et al. 23rd CBS meeting, 2003). Nevertheless, these changes caused by individual processing residuals generated on dense titanium discs did not have a significant effect on the viability of U-937 human macrophages after 24 hours of incubation (Figure 7.1), as investigated through a MTT enzyme-based cell viability assay. Cell viability on titanium foams was also investigated with J774 mouse macrophages incubated for 48 hours. From this study, it was concluded that macrophages viability was not significantly different on the three-dimensional titanium scaffolds than on cp-titanium.

Cell adhesion of MC3T3-E1 mouse pre-osteoblasts on the titanium foams was investigated through a study of adhesion kinetics of these cells from 30 minutes to 18 hours following the seeding procedure, as measured through a MTT cell viability assay. As the pattern of adhesion and growth observed on titanium foams was similar to that observed on mirror-polished titanium, it was deduced that foams did not have cytotoxic effects on mouse pre-osteoblasts compared to the reference material, known to be biocompatible.

Cell morphology after 18 hours of incubation demonstrated that pre-osteoblasts exhibit a polygonal shape with three-dimensional extensions on foams because of the material's high level of surface topography. The quality of cell adhesion influences the

morphology exhibited by cells, which in turn influences the phenotype of these cells. The pre-osteoblast morphology observed on porous titanium has been associated with increased differentiation of osteoblasts in previous reports. Thus, it was concluded from these cell adhesion studies that titanium foams provide a proper environment for the adhesion of cells.

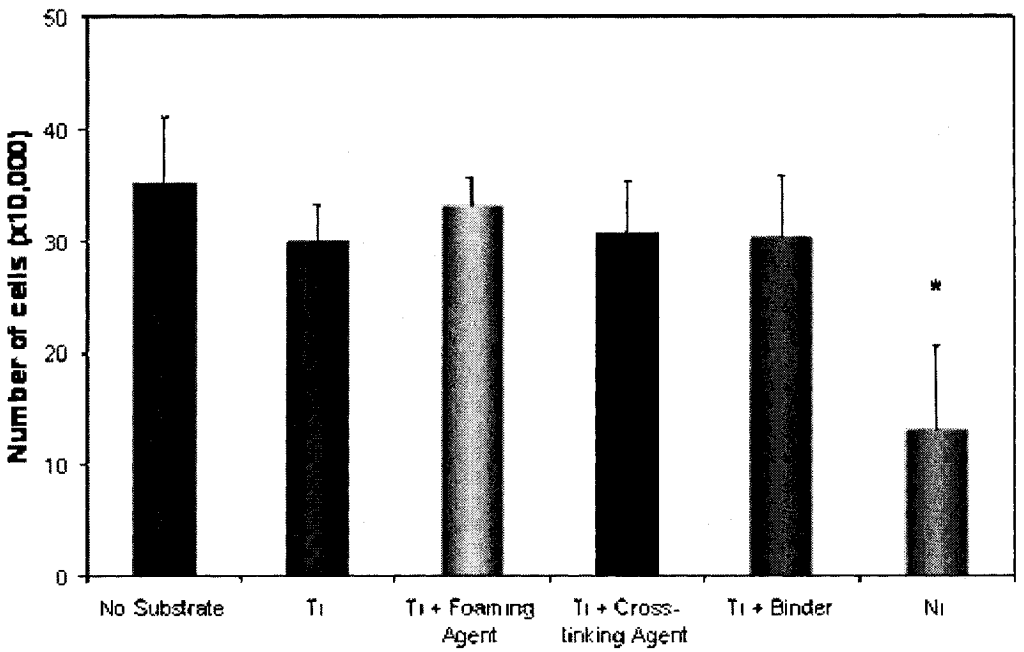
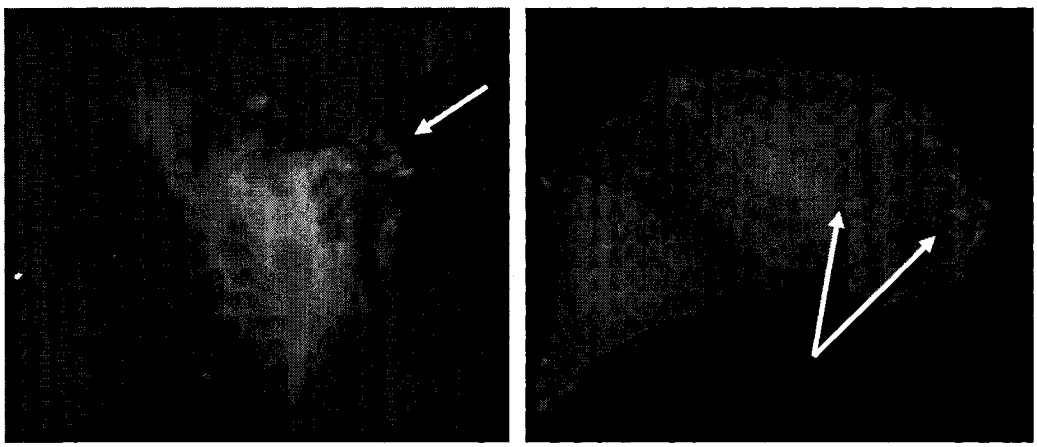


Figure 7.1. Cell viability of U-937 human macrophages after 24 hours of incubation in the presence of residues from the different chemicals employed in the fabrication of titanium foams by powder metallurgy. Viability on titanium discs with residuals is similar to that on titanium controls and on tissue culture plates. The Ni control yielded statistically lower results as compared to other conditions (* $p<0.05$). (J.P. St-Pierre et al. 23rd CBS meeting, 2003)

Ongoing work related to pre-osteoblast adhesion aims to optimize a procedure to visualize focal contacts of pre-osteoblasts adhered to titanium foams. This is achieved through immunological staining of vinculin, a protein that colocalizes with focal contact sites, with subsequent visualization using an epifluorescence microscope. Difficulty in optimizing this procedure and obtaining clear images of the distribution of focal contacts of pre-osteoblasts adhered to titanium foams reside in the three-dimensional structure of

scaffolds. Images are blurred due to out-of-plane fluorescence. Nevertheless, preliminary results have demonstrated focal contact distribution differences between cells adhered to titanium foams and mirror-polished titanium discs. On foams, focal contacts seem to be constrained to the end of cellular extensions, at sites where cells contact titanium particles (Figure 7.2a), while focal contacts can be seen in larger numbers for cells on flat surfaces and are distributed everywhere on cell surfaces (Figure 7.2b). These results are in agreement with the literature and support the fact that cell adhesion mechanisms are different on highly texturized materials such as porous titanium, compared to flat surfaces.



(a) (b)

Figure 7.2. Epifluorescence microscope images of immunological vinculin staining of MC3T3-E1 mouse pre-osteoblasts adhered to (a) porous titanium and (b) mirror-polished titanium. Focal contacts colocalize with vinculin. They can be found at the end of three-dimensional extensions on porous titanium, while they are distributed everywhere on the cell membrane of cells adhered to mirror-polished surfaces (white arrows).

A long term study of the differentiation pattern of mouse pre-osteoblasts adhered to three-dimensional titanium scaffolds, compared to mirror-polished titanium, was also performed. Higher alkaline phosphatase activity on porous titanium following the proliferation stage, confirmed the hypothesis from the cell adhesion study. It seems that the three-dimensional morphology exhibited by pre-osteoblasts on porous titanium is

associated with an early shift toward a more mature differentiation state, compared to the spindle-like and flat shape of these cells on mirror-polished titanium.

It was also shown that differentiation markers are expressed by osteoblasts through independent pathways. Osteocalcin release was reduced on titanium foams compared to the reference material. This reduction of the rate at which osteoblasts differentiate due to the microstructure of titanium foams was confirmed by a delayed shift toward extracellular matrix mineralization. Nevertheless, calcium content was similar on all materials tested by the end of the experimental period, demonstrating that the microstructure of titanium foams promotes cellular differentiation toward the formation of a mineralized bone-like tissue. As cells form extensive extracellular matrix networks inside pores of the foams, compared to a multilayer growth on flat surfaces, it is hypothesized that higher calcium content would have been measured in the extracellular matrix of titanium foams than on mirror-polished titanium after incubation times longer than 17 days.

Pore size of titanium foams did not have a major impact on pre-osteoblast behaviour seeming only to be related to the rate of proliferation of cells in the first 9 days in culture. It is concluded that for the three types of structures studied in this thesis, the average pore size did not have an impact on cell adhesion, differentiation, or mineralization behaviour. Nevertheless, the size of individual pores had an effect on the ability of cells to bridge the pore and fill it with an extracellular matrix network. While conditions are different *in vivo*, this observation provides insight on the reasons for bone ingrowth to occur only in structures with a specific range of pore size.

The studies discussed in this thesis demonstrated that titanium foams produced through a novel powder metallurgy process developed at IMI-NRC are cytocompatible and have osteoconductive properties *in vitro*, as they promote the formation of a mineralized bone-like extracellular matrix by differentiating mouse pre-osteoblasts inside the structure. However, *in vitro* cell culture models are simplified models of the processes by which bone cells promote osteogenesis inside porous metals *in vivo*. Hence, evaluation of the potential of porous titanium for bone engineering applications requires confirmation of the present results through *in vivo* studies of bone ingrowth.

CHAPTER 8

Contribution to the Knowledge and Conclusions

As was stressed throughout the thesis, only a few types of porous metals are currently used in clinical settings for implantation purposes. Thus there is a need to develop three-dimensional metallic scaffolds with unique microstructures, in order to increase the knowledge on the impact of structural parameters on three-dimensional bone ingrowth. The study presented in this report demonstrates the biocompatibility of highly porous three-dimensional titanium scaffolds fabricated through a novel powder metallurgy process, as well as their ability to promote adequate osteoblast interactions. This preliminary *in vitro* study is a first step toward the evaluation of the potential of porous titanium as an alternative to available materials for various applications in bone engineering. Furthermore, this work presents evidences of the differences in the behaviour of osteogenic cells during bone-like tissue formation on two-dimensional surfaces compared to three-dimensional materials. This is important knowledge since extensive research on biomaterials is performed with simplified two-dimensional surfaces and applied to three-dimensional systems. Finally, the importance of controlling the pore size distribution of porous metals in order to eliminate the large pores and ensure an adequate bone-like tissue ingrowth has been demonstrated. The general conclusions derived from this study are:

1- Three types of titanium foams were produced with significantly different microstructures as demonstrated by qualitative observations, density measurements, and average pore size evaluations.

2- Residuals from the process have an effect on the surface properties of titanium. However, these changes do not affect the viability of macrophages when produced individually on titanium discs, or combined as titanium foams.

3- The titanium scaffolds investigated support the adhesion of mouse pre-osteoblastic cells, as well as their subsequent proliferation, and differentiation

toward the formation of a mineralized extracellular matrix within the porous biomaterial.

4- The average pore size of the three-dimensional titanium scaffolds investigated has a minimal effect on the behaviour of differentiating mouse pre-osteoblasts. Nevertheless, the size of individual pores has a major impact on the ability of osteogenic cells to produce an adequate extracellular matrix that fills the structure.

CHAPTER 9

Suggestions for Continuation of the Project

This thesis presents information on the ability of titanium foams to promote the formation of mineralized bone-like extracellular matrix inside pores by differentiating MC3T3-E1 pre-osteoblasts *in vitro*. As well, it provides insight on the processes by which cells achieve this goal in three-dimensional structures. However, further work on the design and biological characterization of porous titanium would contribute toward the optimization of three-dimensional metallic scaffold design for bone engineering applications. Research directions for the continuation of this project are discussed in this chapter.

1. Further characterize cell adhesion interactions with titanium foams.

As discussed in Chapter 7, ongoing work aims to optimize a procedure to visualize cell focal contacts through immuno-fluorescence staining of vinculin. Valuable information on the process by which cells attach to different surfaces has already been gained with this procedure. However, results need to be reproduced with an optimized procedure in order to derive conclusions.

2. Verify the hypotheses formulated from results discussed in Chapter 6.

It was observed in Chapter 6 that alkaline phosphatase and osteocalcin expression are controlled through different pathways. Reports from the literature have suggested that high levels of transforming growth factor-β could be responsible for the up-regulation of alkaline phosphatase and down-regulation of osteocalcin on titanium foams. It would be interesting to verify this hypothesis, while ensuring that the growth factor is expressed at higher levels by cells on titanium foams compared to mirror-polished titanium. The potential of porous titanium for bone engineering applications could also be further assessed through quantification of the amount of extracellular matrix protein collagen type I produced on the different materials tested, as well as by investigating the calcium content after longer incubation periods than those investigated.

3. Gain control on the pore size distribution within porous titanium scaffolds.

As mentioned in Chapter 6, porous titanium scaffolds are characterized by wide pore size distributions. Thus, there is a good probability that some of the pores of titanium scaffolds produced by powder metallurgy are too large to allow osteogenic cells to form extracellular bridges and eventually fill the structure with bone-like tissue (refer to Figure 6.8). Thus, there is a need to gain a better control over the pore size distribution to ensure that bone-like tissue can be produced inside every pore of a given scaffold.

4. Study the effects of other microstructural properties on cell behaviour and bone ingrowth.

The powder metallurgy process developed by IMI-NRC has the advantage that it is quite versatile. The ability to control various microstructural properties of the foams produced, such as the nature of the base material, the topography of pore walls, and the size of openings between pores, should be exploited to gain knowledge on the impact of these parameters on the osteoconductivity of three-dimensional scaffolds. Proper methods of characterization for these properties of porous metals will have to be developed as a part of such a study.

5. Develop a procedure to apply bioactive coatings to titanium foams.

Bioactive ceramics such as hydroxyapatite are known to encourage bone ingrowth compared to titanium. While they are very brittle, they can be used as coatings on metallic implants to improve the surface properties of scaffolds without compromising the mechanical properties of the scaffolds. A sol-gel procedure could be developed to apply such coatings on titanium foams and increase their bone regeneration capabilities. Furthermore, it should be investigated whether such thin coatings can reduce metallic ion release levels, as this is a large concern of three-dimensional metallic scaffolds having high surface area.

6. Verify the bone ingrowth capabilities of titanium foams *in vivo*.

In order to demonstrate the potential of titanium foams produced by a novel powder metallurgy process for applications as three-dimensional scaffolds for bone engineering applications, *in vitro* results must be confirmed by studies of the ability of the material to promote and direct bone ingrowth *in vivo*. Such studies would allow the establishment of specific applications for this material. Through control of the experimental conditions, the potential of porous titanium as a porous coating for orthopaedic implants, a regeneration scaffold for craniofacial applications, a spinal fusion material, and a dental implant could be assessed.

APPENDIX A

Research Compliance Certificates

The research reported in this thesis involved the use of mammalian cells (MC3T3-E1 Subclone 14 mouse pre-osteoblasts, U-937 human macrophages and J774 mouse macrophages from ATCC). A copy of the approved McGill University form for use of biohazardous materials is included in this appendix.

McGill University

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

Projects involving potentially biohazardous materials should not be commenced without approval from the Environmental Safety Office. Submit applications before 1) starting new projects, 2) renewing existing projects, or 3) changing the nature of the biohazardous materials within existing projects.

1. PRINCIPAL INVESTIGATOR: Maryam Tabrizian PHONE: 8129

DEPARTMENT: Biomedical Engineering Rm 313 FAX: 7461

ADDRESS: Lyman Duff Building E-MAIL: maryam.tabrizian@mcgill.ca

PROJECT TITLE:in vitro biocompatibility study of new biomaterials

2. EMERGENCY: Person(s) designated to handle emergencies

Name: Lina Vuch Phone No: work: 398-7398 home:

Name: Pina Sorrini Phone No: work: 398-6736 home:

3. FUNDING SOURCE OR AGENCY (specify): FQRNT, CIHR, NSERC

Grant No.: FQRNT, CIHR, NSERC Beginning date: 2002, 2004, 2001 End date: May 2007, 2005, 2005

4. Indicate if this is

☒ Renewal: procedures previously approved without alterations.

Approval End Date: FQRNT, NSERC (2007, 2005)

☒ New funding source: project previously reviewed and approved under an application to another agency.

Agency: CIHR Approval End Date: 08/05/2005

☐ New project: project not previously reviewed.

☐ Approved project: change in biohazardous materials or procedures.

☐ Work/project involving biohazardous materials in teaching/diagnostics.

CERTIFICATION STATEMENT: The Environmental Safety Office approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in Health Canada's "Laboratory Biosafety Guidelines" and in the "McGill Laboratory Biosafety Manual".

Containment Level (select one): ☐ 1 ☒ 2 ☐ 2 with additional precautions ☐ 3

Principal Investigator or course director: _____ date: 08/07/2004 (day / month / year)

Approved by: Environmental Safety Office: _____ date: 19 07 04 (day / month / year)

Expiry: 31 05 07 (day / month / year)

*as defined in the "McGill Laboratory Biosafety Manual"

5. RESEARCH PERSONNEL: (attach additional sheets if preferred)

Name	Department	Job Title/Classification	Trained in the safe use of biological safety cabinets within the last 3 years? If yes, indicate training date.
Lucie Marcotte	BMED	Research associate	No
Jean-Philippe St-Pierre	BMED	Master student	No
Cyrille Fleury	BMED	Master student	No
Line Mongeon	BMED	Technician	No
Kim Douglas	BMED	PhD student	No
Shahabeddin Faghihi	BMED	PhD student	No

6. Briefly describe:

i) the biohazardous material involved (e.g. bacteria, viruses, human tissues, toxins of biological origin) & designated biosafety risk group

bacteria, cell lines (mammalian)
↳ E. coli (non-pathogenic)

ii) the procedures involving biohazards

cell and bacterial culture

iii) the protocol for decontaminating spills

as requested by McGill - ethanol, detergent, bleach disinfection

7. Does the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) that could increase the hazards?

no

5. RESEARCH PERSONNEL (continued)			
Name	Department	Job Title / Classification	Trained in the safe use of biological safety cabinets within the last 3 years? If yes, indicate training date.
Shawn Carrigan	BMED	PhD student	No
Dariusz Dziong	BMED	Master student	No
Mylène Gravel	BMED	Master student	No
Manuela Mandu	BMED	PhD student	No
Cathy Tkaczyk	BMED	PhD student	No
Anna Hillberg	BMED	Post doctoral fellow	No

. Do the specific procedures to be employed involving genetically engineered organisms have a history of safe use?

~~o~~ N/A

). What precautions will be taken to reduce production of infectious droplets and aerosols?

~~n/a~~ Manipulation of cultures will be carried out in a BSC

10. Will the biohazardous materials in this project expose members of the research team to any risks that might require special training, vaccination or other protective measures? If yes, please explain.

no

11. Will this project produce combined hazardous waste – i.e. radioactive biohazardous waste, biohazardous animal carcasses contaminated with toxic chemicals, etc.? If yes, please explain how disposal will be handled.

no

12. List the biological safety cabinets to be used.

Building	Room No.	Manufacturer	Model No.	Serial No.	Date Certified
Lyman Duff	323	microzone	BK-2-4	801-4534	14/05/04